COMPARISON OF RUMINANT ALPHAHERPESVIRUSES SEROLOGICALLY RELATED TO BOVINE HERPESVIRUS-1

by

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ABSTRACT

Isolates of bovine herpesvirus-1 subtype 1 (BHV-1.1) and bovine herpesvirus-1 subtype 2 (BHV-1.2), the cause of infectious bovine rhinotracheitis-infectious pustular vulvovaginitis were compared with three other distinct ruminant alphaherpesviruses isolated from a goat (CapHV-2), a red deer (CerHV-1) and a reindeer (RanHV-1). Restriction endonuclease profiles produced by BglII differentiated the five ruminant alphaherpesviruses. Amplification of a 468 bp fragment of each of the five alphaherpesviruses by the polymerase chain reaction (PCR) was achieved using 22 bp primers selected from the BHV-1 glycoprotein gI DNA sequence. All the amplified products contained BglI and HinfI cleavage sites. Only RanHV-1 did not contain SmaI or AvaI sites. Serological comparison by ELISA and serum neutralisation (SN) tests using rabbit hyperimmune sera, and cattle, goat, red deer and reindeer convalescent sera revealed a close serological cross-reactivity between all 5 viruses. Analysis of their relationships confirmed that: 1) the two BHV-1 viruses were most closely related, 2) CerHV-1 and CapHV-2 were more closely related to BHV-1 than they were to each other, and 3) RanHV-1 was more related to CerHV-1 than to BHV-1 and CapHV-2. Western blotting using polyclonal sera identified at least seven major proteins common to all the viruses with molecular weights of 130kDa, 108 kDa, 90 kDa, 87 kDa, 74 kDa, 64 kDa and 45 kDa. With a view to identifying epitopes which would allow the differentiation of these viruses, a panel of 14 monoclonal antibodies (Mabs) against CerHV-1 were derived from BALB/c mice previously immunised with gradient purified CerHV-1. Four Mabs which had neutralising activity against CerHV-1 reacted in

Western blots with polypeptides of M_r 68-70 kDa and 74 kDa. The other 10 Mabs reacted in Western blots with polypeptides of M_r 68-70 kDa and/or 64 kDa. Radioimmunoprecipitation (RIP) results indicated that the 74 kDa protein detected in CerHV-1 was analogous to the 74 kDa cleavage product of the gI glycoprotein of BHV-1. The reactivity of each Mab against the five ruminant alphaherpesviruses was tested by ELISA. Two Mabs cross-reacted with all the five ruminant alphaherpesviruses while 10 others showed variable reactivity with the 5 viruses. Two Mabs reacted exclusively with CerHV-1 antigens and these may be suitable for developing a diagnostic test to distinguish antibodies between BHV-1 and CerHV-1 in their natural hosts.

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LIST OF ABBREVIATIONS

Ab	Antibody
Abc	Antibody dependent complement mediated cytolysis
Amp	Ampere
ADCC	Antibody dependent cellular cytoxicity
AGID	Agar gel immunodiffusion
BHV-1	Bovine herpesvirus type 1
BHV-1.1	Bovine herpesvirus type 1.1
BHV-1.2	Bovine herpesvirus type 1.2
BHV-1.3	Bovine herpesvirus type 1.3
BHV-2	Bovine herpesvirus type 2
BHV-6	Bovine herpesvirus type 6
BSA	Bovine serum albumin
°C	Degree(s) centigrade
CapHV-2	Caprine herpesvirus type 2
CBA	Competitive binding assay
CCV	Channel catfish virus
CerHV-1	Cervine herpesvirus type 1
CFA	Freund's complete adjuvant
CHV-1	Canine herpesvirus type 1
CMC	Carboxy methyl cellulose
CO_2	Carbon dioxide
cpe	cytopathic effect
DAB	3,3'diaminobenzidine
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
ds	double stranded
EBTr	Embryonic bovine trachea
EDTA	Ethylene diamine tetra-acetic acid
EHV-1	Equine herpesvirus type 1
EHV-4	Equine herpesvirus type 4
ELISA	Enzyme linked immunosorbent assay
E'199'	Eagle's '199' medium
EM	Electron microscope/electron microscopy
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
g	gram(s)
gB	glycoprotein B
G+C	Guanine plus cytosine
gC	glycoprotein C
gD	glycoprotein D
gE	glycoprotein E
gG	glycoprotein G
gH	glycoprotein H
gI	glycoprotein I
gJ	glycoprotein J
gK	glycoprotein K
gL	glycoprotein L

gM	glycoprotein M
GHV-1	Gallid herpesvirus type 1
gI	glycoprotein I
gII	glycoprotein II
gIII	glycoprotein III
gIV	glycoprotein IV
gp	glycoprotein
h	hour(s)
^3H	tritium
HAT	Hypoxanthine-aminopterin-thymidine
HBSS	Hank's balanced salt solution
HCMV	Human cytomegalovirus
HIV	Human immunodeficiency virus
HGRPT	Hypoxanthine phosphoribosyl transferase
HRP	Horseradish peroxidase
HT	Hypoxanthine thymidine
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
HVC-1	Herpesvirus of cervidae type 1
HVC-2	Herpesvirus of cervidae type 2
IBRV	Infectious bovine rhinotracheitis virus
IBR-IPV	Infectious bovine rhinotracheitis - Infectious pustular vulvovaginitis
ICFA	Freund's incomplete adjuvant
ICTV	International committee for the taxonomy of viruses
IEP	Immunoelectrophoresis
IF	Immunofluorescence
IFAT	Immunofluorescent antibody test
IgG	Immunoglobulin G
i/p	Intraperitoneal
IPVV	Infectious pustular vulvovaginitis virus
IU	International unit(s)
i/v	Intravenous
Kbp	Kilobase pair(s)
kDa	Kilodalton(s)
M	Molar
Mab	Monoclonal antibody
ug	Microgram(s)
ul	Microlitre(s)
um	Micrometre(s)
uCi	Microcurie(s)
MDBK	Madin-Darby bovine kidney cells
MDBP	Major DNA binding protein
ME	2-Mercaptoethanol
min	Minute
mM	Millimole(s)
ml	Millilitre(s)
mm	Millimetre(s)
moi	Multiplicity of infection
Mol.wt.	Molecular weight
MTM	Mixed thymocyte medium

nm	Nanometre(s)
NP40	Nonidet P40
OD_{492}	Optical density at 492 nanometres
OPD	Orthophenylene diamine dihydrochloride
^{32}P	Radioactive phosphorus
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST-20	Phosphate buffered saline with Tween 20
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
pfu	Plaque forming unit(s)
PIC	Eagle's medium without phosphorus
pristane	2,6,10,14-tetra methyl-pentadecane
R	Coefficient of antigenic relationship
RanHV-1	Rangiferine herpesvirus type 1
RE	Restriction endonuclease
RIP	Radioimmunoprecipitation
rpm	Revolutions per minute
SAPU	Scottish antibody production unit
s/c	Subcutaneous/subcutaneously
SDS	Sodium dodecyl sulphate
SHV-1	Suid herpesvirus type 1
SNT	Serum neutralisation test
TBE	Tris Borate Ethylene diamine tetra acetic acid buffer
$TCID_{50}$	50% tissue culture infective dose
TEMED	N,N,N'N'-tetramethyl-ethylenediamine
TK	Thymidine kinase
TMB	3,3'5,5'Tetramethyl benzidine
UK	United Kingdom
USA	United States of America
UV	Ultraviolet light
VN	Virus neutralisation
v/v	volume by volume
VZV	Varicella-zoster virus
w/v	weight by volume
w/w	weight for weight

DECLARATION

I certify that the work presented in this thesis was composed and executed by myself. Whenever collaboration and/or assistance was given by colleagues this has been fully acknowledged.

Japhet Robert Lyaku
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DEDICATION

This thesis is dedicated to my beloved father who passed away during the course of this work.

INTRODUCTION

Bovine herpesvirus type 1 (BHV-1) also known as infectious bovine rhinotracheitis-infectious pustular vulvovaginitis (IBR-IPV) virus is a member of the alphaherpesvirinae subfamily (Roizman *et al*, 1992). It is associated with a wide variety of clinical conditions including respiratory infection, conjunctivitis, vulvovaginitis, abortion and less comonly encephalitis and generalised systemic infection (reviewed by Gibbs and Rweyemamu, 1977a). It is one of the most important agents of bovine respiratory disease either directly or as a predisposing factor for bacterial infection, most commonly *Pasteurella haemolytica* (Yates, 1982).

The species specificity of ruminant alphaherpesviruses has not always been recognised. Antibodies against BHV-1 were widely demonstrated in both domestic and certain wild ruminants (reviewed by Gibbs and Rweyemamu, 1977a; Pastoret *et al*, 1988). It was assumed that ruminants which were seropositive to BHV-1 were either infected or had been in contact with the virus. However, it has been found recently that ruminants other than cattle can become infected with viruses antigenically related to, but distinct from BHV-1 (reviewed by Pastoret *et al*, 1988). At present, four such herpesviruses serologically related to BHV-1 have been isolated from ruminants. Three of these viruses, caprine herpesvirus-2 (CapHV-2) from domestic goats (Mettler *et al*, 1979); cervine herpesvirus-1 (CerHV-1) from red deer (Inglis *et al*, 1983; Ronsholt *et al*, 1987) and rangiferine

herpesvirus-1 (RanHV-1) from reindeer vaginal swabs after dexamethasone treatment (Ek-Kommonen *et al*, 1986) have been isolated in Europe (Figure 1.1). The fourth, a herpesvirus from water buffalo (*Bubalus arnee*) initially typed by serum neutralisation as BHV-1 (St. George and Philpott, 1972; Brake and Studdert, 1985; Bulach and Studdert, 1990) was isolated in Australia. A ruminant species seropositive for BHV-1 may therefore be infected by BHV-1 or one of these or other related herpesviruses. The existence of this close antigenic relationship among ruminant alphaherpesviruses could lead to misinterpretation of both diagnostic tests and epidemiological data. For this reason there is a growing requirement to have more reliable diagnostic tests which can distinguish these viruses both for disease control and animal certification in international trade. It is therefore important to identify the basis of genetic and serological relatedness among the ruminant alphaherpesviruses and particularly to identify the major common and virus-specific glycoproteins and their neutralising antigenic epitopes. Furthermore, the development of monoclonal antibodies capable of discriminating between the different ruminant alphaherpesviruses could lead to reliable immunodiagnostic assays for the comparison of antibodies to alphaherpesviruses in the serum of a wide range of potential hosts.

Figure 1.1. Natural hosts of the five ruminant alpha-herpesviruses studied in this thesis. Cow (top), goats (middle), red deer and reindeer (below, left and right respectively).

CHAPTER 1

REVIEW OF THE LITERATURE

This review of the literature is divided into three parts; firstly a brief review and update of the classification and nomenclature of the family herpesviridae, secondly a review of some of the comparative aspects of the biology of alphaherpesviruses with particular reference to BHV-1 and thirdly a review of alphaherpesvirus glycoproteins.

1.1. CLASSIFICATION AND NOMENCLATURE OF THE HERPESVIRIDAE

The classification and nomenclature of viruses within the family herpesviridae is complex and not yet fully resolved despite the wealth of data on their biology and molecular biology (Porterfield, 1989).

However, in its first report, the International Committee for the Taxonomy of Viruses (ICTV) Herpesvirus Study Group designated Herpesviridae as the name of the herpesvirus family comprising three subfamilies on the basis of their biological properties; the *Alphaherpesvirinae, Betaherpesvirinae* and *Gammaherpesvirinae* and provided guidelines for the classification of herpesviruses into three subfamilies (Roizman *et al*, 1981). The criteria for this classification include host range, reproductive cycle, cytopathology and the characteristics of their latent infection as reviewed in detail by Honess (1984). Recently, the ICTV approved the recommendation of its Herpesvirus Study Group that the members of the family Herpesviridae be classified into the three subfamilies on the basis of biologic properties (Roizman *et al*, 1992). Furthermore, the ICTV also approved the Herpesvirus Study Group proposals to

assign several genera based on DNA sequence homology, similarities in gene and sequence arrangements, and relatedness of important viral proteins demonstrable by immunologic methods (see Table 1.1).

The general properties of the subfamilies are as follows:

Alphaherpesvirinae are characterised by a relatively short replication cycle (hours or days rather than weeks) and a rapid spread in tissue culture with accompanying cytolysis. They are permissive in fibroblasts, and experimental infection of cells from sources other than the natural host can be achieved with most viruses of this subfamily. In their natural hosts they cause mild infections of short duration notably involving the epithelium of the skin or mucosal surfaces, and establish latent infection in their natural hosts, primarily, but not exclusively, in nervous tissue - sensory and autonomic nerve ganglia and the central nervous system (CNS). Herpes simplex virus (HSV) is the type member.

Betaherpesvirinae (the cytomegaloviruses) are characterised by a long replication cycle and slow spread of infection in culture. Development of cytopathic effect (cpe) may take several weeks following low multiplicity of infection (moi) and is typified by giant cell formation. The viruses are notably species-specific both *in vitro* and *in vivo*; fibroblasts of the natural host are usually optimal for virus culture, and infection of cells from other than the natural host can rarely be achieved. *In vivo* infection is systemic and of considerable duration; virus can be isolated from multiple secretions over a period of months or even years. The genomes of betaherpesviruses (180-240

Table 1.1. List of genera within the family Herpesviridae

Subfamily/genus	Viruses	Common name
Alphaherpesvirinae		
Simplexvirus[a]	human herpesvirus 1	herpes simplex virus 1
	human herpesvirus 2	herpes simplex virus 2
	bovine herpesvirus 2	bovine mammillitis
	cercopithecine herpesvirus 1	B virus
Varicellovirus[b]	human herpesvirus 3	varicella-zoster virus
	equid herpesvirus 1	equine abortion virus
	bovine herpesvirus 1	infectious bovine rhinotrachieitis virus
	suid herpesvirus 1	pseudorabies virus
Betaherpesvirinae		
Cytomegalovirus[a]	human herpesvirus 5	human cytomegalovirus
Murontegalovirus[a]	murid herpesvirus 1	murine cytomegalovirus
Roseolovirus[b]	human herpesvirus 6	
	human herpesvirus 7	
Gammaherpesvirinae		
Lymphocryptovirus[a]	human herpesvirus 4	Epstein-Barr virus
	cercopithecine herpesvirus 12	baboon herpesvirus
	cercopithecine herpesvirus 14	African green monkey, EBV like virus
	cercopithecine herpesvirus 15	rhesus monkey EBV like virus
	pongine herpesvirus 1	chimpanzee herpesvirus
	pongine herpesvirus 2	orangutan herpesvirus
	pongine herpesvirus 3	gorilla herpesvirus
Rhadinovirus[a]	saimirine herpesvirus 2	herpesvirus saimiri 2
	alcelaphine herpesvirus 1	virus of malignant catarrhal fever
	murid herpesvirus 2	mouse herpesvirus strain 68

[a]Approved by International Committee on Taxonomy of Viruses
[b]Proposed name for genus
Adapted from Roizman et al, (1992)

kbp) are larger than those of alpha- (125-165 kbp) or gammaherpesviruses (135-172 kbp). Human cytomegalovirus is the type member.

Gammaherpesvirinae are generally termed lymphotropic herpesviruses and are perceived as such because they are associated with lymphoproliferative disease in their natural or experimental hosts. Lymphoid cell lines containing multiple copies of viral DNA can frequently be established from peripheral blood. Lymphocytes in the acute or convalescent phase of infection and in some cases cell lines with similar characteristics can be generated by virus-induced immortalisation of lymphocytes *in vitro*. The viruses are conveniently divided into B-cell tropic (e.g. Epstein Barr Virus (EBV)) or T-cell tropic (e.g. Herpes saimiri) groups but there is no good reason to suppose that these tropisms are mutually exclusive. The term lymphotropic as applied to gammaherpesviruses is somewhat misleading since lymphocytes are generally non-permissive or at best semi-permissive for virus replication and many viruses in the subfamily will productively infect fibroblasts.

The classification of herpesviruses into three subfamilies is based on somewhat subjective and arbitrary criteria, and as a result many herpesviruses are ungrouped and some may have been wrongly classified (Porterfield, 1989). Nevertheless, the grouping has proved useful and its validity has been reinforced by nucleic acid hybridisation and by analysis of nucleotide sequence data both of which have shown that the genetic content and genome organisation of a number of different herpesviruses is generally comparable

with the pre-existing classification, which was primarily based on biological properties (McGeoch *et al*, 1993). There are however some exceptions: Marek's disease virus (Gallid herpesvirus-1 (GHV-1)) and herpesvirus of turkeys (Gallid herpesvirus-2 (GHV-2)), which had been regarded as belonging with the lymphoproliferative *Gammaherpesvirinae.* These two viruses can now be grouped with the *Alphaherpesvirinae* on the basis of limited sequence analyses (Buckmaster *et al*, 1988). The human herpesvirus 6 (HHV-6) was initially thought to be a gammaherpesvirus. However, analysis of its genome structure and studies of nucleotide sequence homology show that it is more closely related to the human cytomegalovirus which is a type member of the *Betaherpesvirinae* than to the primate gammaherpesviruses (Efstathiou *et al*, 1988). The fish herpesvirus channel catfish virus (CCV), had been considered to be a member of the *Alphaherpesvirinae.* However, the complete genome sequence of this virus has now been determined and shows no clear relationship whatever with viruses belonging to the three subfamilies already discussed (Davison, 1992). Thus as proposed by Davison (1992) CCV would probably now be assigned to a separate taxonomic group.

The nomenclature for herpesviruses recommended by the ICTV (Roizman *et al*, 1981) is based on the use of the taxonomic unit to which the natural host belongs followed by the term 'herpesvirus' and a number based on order of discovery. The first two bovine herpesviruses to be described, namely infectious bovine rhinotracheitis-infectious pustular vulvovaginitis (IBR-IPVV) and bovine herpes mammilitis are consistently named bovine

herpesvirus type 1 (BHV-1) and bovine herpesvirus type 2 (BHV-2) respectively.

Comparisons of BHV-1 isolates by restriction endonuclease (RE) profiles of their viral genomes have shown significant differences (Engels *et al*, 1981; Mayfield *et al*, 1983; Misra *et al*, 1983). Four subtypes have been defined from these RE profiles, infectious bovine rhinotracheitis virus (IBRV) as BHV-1 subtype 1.1, infectious pustular vulvovaginitis (IPVV) as BHV-1 subtype 1.2a and 1.2b (Engels *et al*, 1981; Misra, *et al*, 1983; Ludwig, 1984; Metzler *et al*, 1985; Miller *et al*, 1988; Edwards *et al*, 1990; Hamelin *et al*, 1990) and BHV-1 subtype 1.3 (Studdert *et al*, 1981a; Brake and Studdert, 1985). However, the nomenclature of ruminant herpesviruses is still confused as the numerical system of nomenclature recommended by the ICTV is used widely but inconsistently with more recently discovered viruses. Several different names are used to describe herpesviruses of sheep and goats. The domestic goat herpesvirus is named caprine herpesvirus type 2 by Roizman *et al* (1981), BHV-6 by Ludwig (1983) and caprine herpesvirus type 1 by Engels *et al* (1987a) and Roizman *et al* (1992). The sheep herpesvirus associated with sheep pulmonary adenomatosis (Jaagsiekte) is named bovine herpesvirus-5 (Ludwig, 1983), caprine herpesvirus type 1 (Roizman *et al*, 1981) and ovine herpesvirus 1 (Roizman *et al*, 1992).

Lately, since the red deer virus was the first alphaherpesvirus to be identified as a unique cervid herpesvirus, the designation cervid herpesvirus type 1 (CHV-1) was proposed (Ronsholt *et al*, 1987). However, anticipating

that this acronym would further confuse that already used for canine herpesvirus type 1 and caprine herpesvirus type 1; Reid *et al* (1986) proposed herpesvirus of cervidae type 1 (HVC-1). Nettleton *et al* (1987) proposed the acronym CerHV-1 as another alternative for the red deer virus.

Since the publication of the first ICTV Herpesvirus Study Group report in 1981, the ICTV have approved the recommendation of previous Herpesvirus Study Groups to assign several genera which have been updated quite recently (Roizman *et al*, 1992). However, the Herpesvirus Study Group recognised that common names in use prior to the establishment of new rules will continue to be used. It is likely that each of the more than 128 ruminant species in the family bovidae and 42 species in the family cervidae will have at least one alphaherpesvirus (Brake and Studdert, 1985). The nomenclature of the ruminant alphaherpesviruses referred to in this thesis is based on the subfamily from which the natural host belongs followed by the term 'herpesvirus' and a number in accordance with the recommendations by the ICTV (Roizman *et al*, 1981; Roizman *et al*, 1992) (see Figure 1.2 and Table 1.1 and 1.2). The bovine herpesviruses used in this study are referred to as bovine herpesvirus type 1.1 and 1.2 (BHV-1.1 and BHV-1.2). The caprine virus as caprine herpesvirus type 2 (CapHV-2), cervine herpesvirus type 1 (CerHV-1) and rangiferine herpesvirus type 1 (RanHV-1) for the red deer and reindeer herpesviruses respectively. In this thesis the old nomenclature is used for the caprine virus, as since up until the recent ICTV report by Roizman *et al* (1992), caprine herpesvirus type 1 referred to the sheep pulmonary adenomatosis virus which is now called ovine herpesvirus type 1.

Figure 1.2. Classification of the order Artiodactyla showing the relationship between the subfamilies from which ruminant alphaherpesviruses studied in this thesis have been isolated.

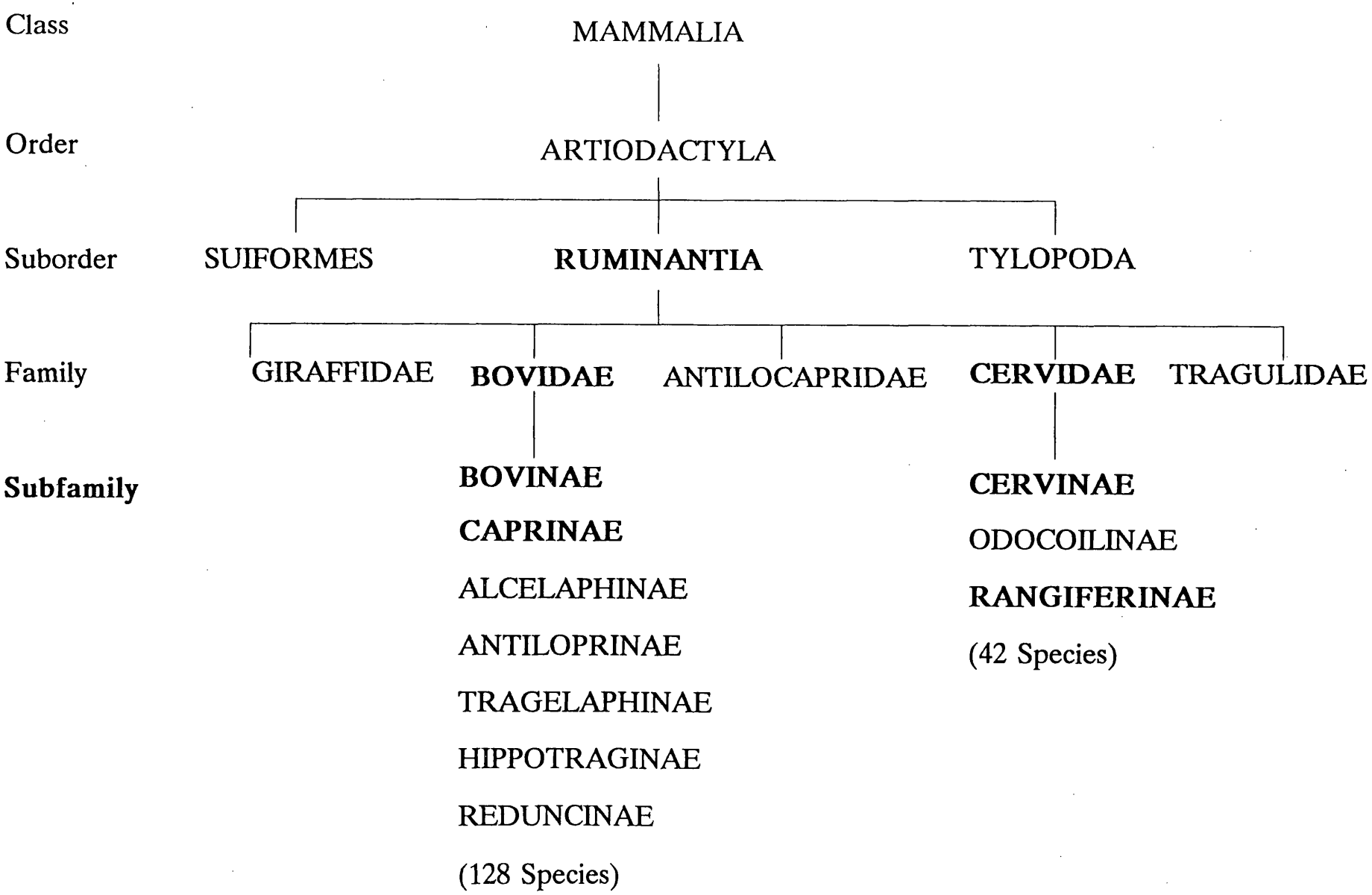

Class
MAMMALIA
Order
ARTIODACTYLA
Suborder
SUIFORMES
RUMINANTIA
TYLOPODA
Family
GIRAFFIDAE
BOVIDAE
ANTILOCAPRIDAE
CERVIDAE
TRAGULIDAE
Subfamily
BOVINAE
CAPRINAE
ALCELAPHINAE
ANTILOPRINAE
TRAGELAPHINAE
HIPPOTRAGINAE
REDUNCINAE
(128 Species)
CERVINAE
ODOCOILINAE
RANGIFERINAE
(42 Species)

Table 1.2. Ruminant alphaherpesviruses and their principle host families and subfamilies within the suborder Ruminantia

ORDER	SUBORDER	FAMILY	SUBFAMILY	PRINCIPAL SPECIES	SPECIES SPECIFIC ALPHAHERPESVIRUS
ARTIODA-CYTLA	RUMINANTIA	BOVIDAE	BOVINAE	Cattle, buffalo	Bovine herpesvirus type 1.1, 1.2a, 1.2b, 1.3 (BHV-1.1, BHV-1.2a BHV-1.2b , BHV-1.3) Bovine herpesvirus type 2 (BHV-2)
			CAPRINAE	Goats	Caprine herpesvirus type 2 (CapHV-2)
		CERVIDAE	CERVINAE	Deer	Cervine herpesvirus type 1 (CerHV-1)
			RANGIFERINAE	Reindeer	Rangiferine herpes virus type 1 (RanHV-1)

Several families and subfamilies within the suborder Ruminantia in which alphaherpesviruses have not been confirmed are omitted (see Figure 1.2).

1.2 COMPARATIVE ASPECTS OF THE BIOLOGY OF ALPHAHERPESVIRUSES.

The alphaherpesvirinae is one of the three subfamilies within the family Herpesviridae. Amongst the members of this subfamily are; Bovine herpesvirus-1 (BHV-1), Bovine mammilitis virus (BHV-2), Suid herpesvirus-1 (SHV-1, Pseudorabies virus, Aujeszky's disease virus), Herpes simplex virus-1 (HSV-1), Herpes simplex virus-2 (HSV-2), Varicella-zoster virus (VZV), Equine herpesvirus-1 (EHV-1), and Caprine herpesvirus-2 (CapHV-2) (Roizman *et al*, 1981).

Other herpesviruses serologically related to BHV-1 have recently been isolated from farmed red deer (*Cervus elaphus*) (Inglis et al, 1983; Nettleton et al, 1986, Ronsholt et al, 1987) and reindeer (*Rangifer tarandus*) (Ek-Kommonen et al, 1986; Rockborn et al, 1990). They were provisionally included in the subfamily alphaherpesvirinae pending further characterisation (Nettleton *et al*, 1986, Ek-Kommonen *et al*, 1986). Evidence from their biological characteristics (Reid *et al*, 1986; Rockborn *et al*, 1990) and genome organisation as shown by restriction endonculease analysis (Vanderplasschen *et al*, 1992) confirms them to be alphaherpesviruses. Both viruses have now been classified as alphaherpesviruses (Roizman *et al*, 1992).

1.2.1 Morphology

All herpesviruses consist of four structural elements; an electron opaque

core containing the double stranded DNA genome, an icosahedral capsid that encloses the core; an electron-dense asymmetrically distributed material abutting the capsid designated the tegument; and an outer membrane or envelope which surrounds the capsid and tegument. Electron microscopy of negatively stained preparations shows that extracellular enveloped virions measure from 120 nm to nearly 300 nm in diameter (Roizman and Furlong, 1974). One possible explanation for this variability is the thickness of the tegument. The major source of variability, however, is the integrity of the envelope. Virions with intact envelopes are impermeable to negative stain and generally retain a quasi-spherical shape while virions with damaged envelopes are permeable to negative stain and have a sunny-side-up egg appearance with an irregular shape and a diameter generally larger than that of an intact virion (Roizman *et al*, 1992).

The icosahedral nucleocapsid within the envelope is about 100 nm to 110 nm in diameter and is composed of 162 hollow capsomeres of which 12 are pentameric and 150 hexameric the latter with a hole running down the long axis (McCombs *et al*, 1971; Fong *et al*, 1973). The envelope contains protrusions or spikes which are more numerous and shorter than those appearing on the surface of many other enveloped viruses (Morgan *et al* 1959; 1968; Spear and Roizman, 1972; Stannard *et al*, 1987). It was thought for many years that the DNA in the core was spooled around a protein plug, since in many electron micrographs it appeared to be a toroid (Furlong *et al*, 1972). However, recently Booy *et al* (1991) have shown using cryoelectron microscopy and image reconstruction, that the DNA forms a uniform dense

ball. The condensed toroidal shapes seen previously may have resulted from the fixation procedures used for preparing specimens (Puvion-Dutilleul *et al*, 1987). The morphology and morphogenesis of herpesviruses has been reviewed by Dargan (1986), Roizman and Sears (1990) and Rixon (1993).

1.2.2. Genome characteristics and organisation

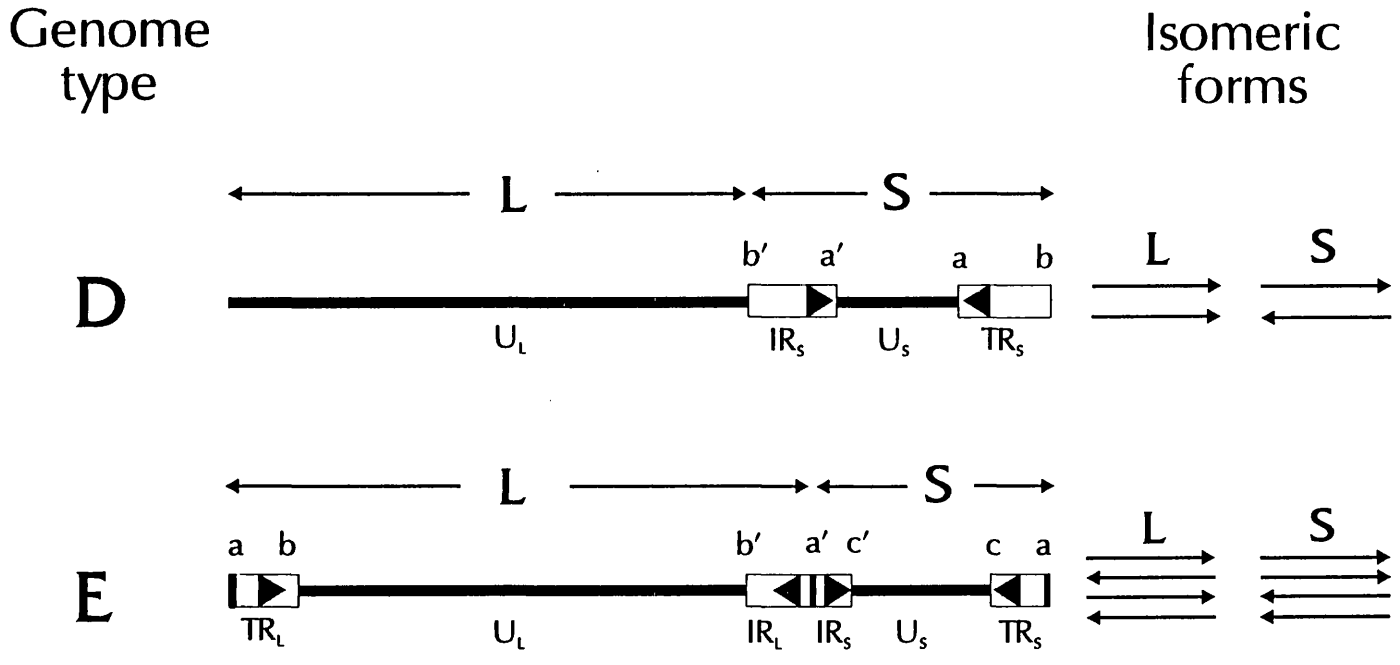
Three mammalian alphaherpesvirus genomes have been sequenced to date: VZV (Davison and Scott, 1986), HSV-1 (McGeoch *et al*, 1985, 1988; Perry and McGeoch, 1988) and EHV-1 (Telford *et al*, 1992). The determination of the total nucleotide sequence of BHV-1 has just been started (Schwyzer and Ackermann, 1992). The linear, double stranded (ds) DNA genomes of the majority of herpesviruses that have been studied in detail have a 3' single nucleotide extension (Roizman, 1990; Roizman and Sears, 1990). Table 1.3 summarises some of the genome characteristics of some of the alphaherpesviruses. The gross arrangement of large scale sequence elements in these genomes is similar, with all possessing a long unique region (U_L) and a short unique region (U_S) bounded by inverted repeats (IR_L, IR_S, TR_L and TR_S). The precise sizes of these elements differ for each virus. The genomes of alphaherpesviruses studied to date belong to either type D or E genome group (Figure 1.3) (Farley *et al*, 1980). Group D genomes are exemplified by those of VZV, SHV-1, BHV-1, EHV-1 and EHV-4 where the sequence at one terminus is repeated in an inverted orientation internally. In these genomes the domain consisting of the stretch of unique sequences flanked by inverted repeats (short or S component) can invert relative to the remaining sequences (long or L component) such that the DNA extracted from virions or infected

Table 1.3. Genome characteristics of some alphaherpesviruses

Virus	Genome type	Size kbp	Mol.Wt kDa	G+C content %	Reference
HSV-1	E	152	96	68	Sheldrick and Berthalot (1975)
HSV-2	E	152	96	69	Gentry and Randall (1973) Graham *et al* (1972)
BHV-2	E	133	88	64	Buchman and Roizman (1978a,b) Martin *et al* (1966)
VZV	D	125	100	46	Dumas *et al* (1980); Davison & Scott (1986)
BHV-1.1	D	137	95	72	Mayfield *et al* (1983); Misra *et al* (1981)
BHV-1.2	D	138	94	72	Misra *et al* (1981)
BHV-1.3	D	140	?	72	Engels *et al* (1986)
CapHV-2	D	137	90	72	Engels *et al* (1987b)
SHV-1	D	140	91	74	Ben-Porat and Kaplan (1985)
EHV-1	D	142	94	57	Soehner *et al* (1965) Whalley *et al* (1981); Cullinane *et al* (1988)
EHV-4	D	148	?	56	Sabine *et al* (1981); Studdert *et al* (1981b); Cullinane *et al* (1988)
FHV-1	D	134	?	46	Rota *et al* (1986)
GHV-1	D	165	?	46	Cebrian *et al* (1982); Fukuchi *et al* (1984); Sugaya *et al* (1990)
CerHV-1	D	141	?	?	Vanderplasschen *et al* (1992)
RanHV-1	D	132	87	?	Vanderplasschen *et al* (1992)

Figure 1.3. Schematic diagram of two alpha- herpesvirus genome types. The basis of their difference is the content and arrangement of unique and redundant nucleotide sequences in populations of mature virus DNA. For each virus L and S indicate 'long' and 'short' unique sequences; a, b, indicate repeated sequences with $a'b'a'c'$ being their complements. Arrows indicate the possible relative orientations of unique sequences represented in the population of mature DNA molecules (two for type D and four for type E)

U_L - unique long, U_S- unique short, TR_L - long terminal repeat, IR_L - long internal repeat, IR_s - short internal repeat, TR_S - short terminal repeat


Genome type
Isomeric forms
L
S
D
b′ a′ a b
U_L
IR_S
U_S
TR_S
L
S
L
S
E
a b
b′ a′ c′ c a
TR_L
U_L
IR_L
IR_S
U_S
TR_S
L
S

cells consists of two equimolar populations differing solely in the orientation of the S compoment relative to the fixed orientation of the L component. Group E viral genomes, exemplified by HSV-1, HSV-2 and BHV-2 have sequences from both termini repeated in an inverted orientation and juxtaposed internally dividing the genomes into two components, L and S each of which consists of unique sequences flanked by inverted repeats. Unlike type D genomes, both components can invert relative to each other and DNA extracted from virions or infected cells consists of four equimolar populations differing in the relative orientation of the two components (see Figure 1.3). The characteristics of the other genome types defined as A, B, C and F are not described here but have been reviewed in detail by Roizman *et al*, 1992.

A direct comparable measure of diversity or unity can be achieved by cross-hybridisation to reveal the degree of nucleotide sequence homology in viruses which appear to be closely related by other criteria (see Table 1.4.). Overall, comparisons of genome organisation between the alphaherpesviruses, HSV-1, HSV-2, VZV, SHV-1 and EHV-1 (Davison and Wilkie, 1983) and between VZV, HSV-1 and BHV-1 (Davison and Scott, 1986; McGeoch *et al*, 1988; Wirth *et al*, 1989) have shown extensive colinearity between genomes of these viruses.

1.2.3. Antigenic relationships

There are numerous reports in the literature indicating the presence of shared or group antigens amongst the herpesviruses. A great variety of

Table 1.4. Nucleotide sequence homology of some alphaherpesviruses by cross-hybridisation

Virus	Nucleotide Sequence homology (%)	Reference
HSV-1, HSV-2	47	Kieff *et al* (1972), Sugino and Kinsbury (1976)
HSV-1, BHV-2	14	Roizman (1982), Sterz *et al* (1973)
HSV-1, SHV-1	8-10	Rand and Ben-Porat (1980)
BHV-1.1, BHV-1.2	95	Misra *et al* (1981)
BHV-1.1, SHV-1	6-8	Bronson *et al* (1972); Bush and Pritchett (1985)

techniques has been used in these studies. However, cross-neutralisation has been suggested by ICTV as the prime serological relationship for qualification of a virus as a member of the herpesvirus genus. Other immunological methods used include immunoelectrophoresis (IEP) (Evans *et al*, 1973) which demonstrated an antigen common to 10 herpesviruses from diverse primary hosts; agar gel immunodiffusion test (AGID) (Killington *et al*, 1977) which identified a common antigen to five viruses, later identified to be the major DNA binding protein by Littler *et al* (1981); immunofluorescence (Ross *et al*, 1972; Blue and Plummer, 1973) and particle agglutination test (Ross *et al*, 1972). Recently, the virus neutralisation test (Nixon *et al.* 1988; Martin *et al* 1990) and ELISA (Nixon *et al*, 1988) have been used to study the antigenic relationship of some ruminant alphaherpesviruses.

Among ruminant alphaherpesviruses. An antigenic relationship exists between BHV-1 and CapHV-2, which is detected as a one-way reactivity i.e. anti CapHV-2 serum reacts weakly with BHV-1 antigen, but anti BHV-1 serum reacts stongly with CapHV-2 antigen (Berrios *et al*, 1975; Mettler et al, 1979; Engels *et al*, 1983; Kao *et al*, 1985; Ackermann *et al*, 1986; Nixon *et al*., 1988; Buddle *et al*, 1990; Martin *et al*, 1990). The main capsid protein (VP4) and the viral glycoprotein gI have been shown to be responsible for this reactivity (Ackermann *et al*, 1986; Friedli and Metzler, 1987; Engels *et al*, 1992). However Ludwig (1983) and Ludwig and Gregersen (1986) found two glycoproteins common to CapHV-2 and BHV-1, with molecular weights of 74kDa and 91kDa to be responsible for the cross-neutralisation between CapHV-2 and BHV-1. Recently Collins *et al* (1993) using a set of Mabs

raised against BHV-1.1 showed that glycoproteins gI and gIV were responsible for the cross-reactivity between BHV-1.1 and BHV-1.3.

CerHV-1 and RanHV-1 have been shown to be antigenically related to BHV-1 (Inglis *et al*, 1983; Ek-Kommonen *et al*, 1986). However, results from Nixon and others (1988) suggested that CerHV-1 and CapHV-2 are more closely related to BHV-1 than they are to each other. The only other virus of widespread importance which may cross-react with BHV-1 is suid herpesvirus-1 (SHV-1). Cross reactivity between suid herpesvirus-1 (SHV-1) and BHV-1 has been reported (Aguilar Setien *et al*, 1980; Bush and Pritchett, 1986; Banks, 1989), but Nelson *et al*, (1972), Hasler and Engels (1986) and Afshar *et al* (1987) failed to demonstrate this cross reactivity by neutralisation and ELISA (Table 1.5).

Within ruminant alphaherpesvirus isolates. There is a wide range of reports often with conflicting results on the serological comparisons of BHV-1 isolates (Gillespie *et al*, 1959; McKercher and Wada, 1964; Buenig and Gratzek, 1967; Bowling *et al*, 1969; House, 1972; Gregersen, 1983; Levings *et al*, 1984; Gregersen *et al*, 1985; Metzler *et al*, 1986; Friedli and Metzler, 1987; Trepanier *et al*, 1988). The weight of evidence is that BHV-1 isolates can not be discriminated by conventional serological methods (Gillespie *et al*, 1959; Gregersen *et al*, 1985; Metzler *et al*, 1986).

Two CapHV-2 strains, one from California (McK/US) (Saito *et al* 1974; Berrios and McKercher, 1975) and one from Switzerland (E/CH) (Engels *et*

Table 1.5. Known serological relationships between some alphaherpesviruses as determined using polyclonal sera

Virus	Method					Reference
	Neutral-isation	Complement fixation	Gel Diffusion	Immunofluo-resence	ELISA	
HSV-1, HSV-2	+	+	+	+	nt	Sterz *et al* (1973) Yeo *et al* (1981)
HSV-1, BHV-2	+	+	+	+	nt	Killington *et al* (1977), Sterz *et al* (1973)
HSV-1, SHV-1	nt	nt	+	+	nt	Killington *et al* (1977), Blue and Plummer (1973)
HSV-1, BHV-1	nt	nt	nt	+	nt	Blue and Plummer (1973)
BHV-1, SHV-1	+	nt	nt	nt	nt	Aguilar Setien *et al* (1980), Banks (1989) Kao *et al* 1985; Ackermann *et al* (1986)
BHV-1, CapHV-2	+	nt	nt	nt	+	Engels *et al* (1983); Nixon *et al* (1988)
BHV-1, CerHV-1	+	nt	nt	nt	+	Nixon *et al* (1988), Inglis *et al* (1983)
BHV-1, RanHV-1	+	nt	nt	nt	nt	Ek-Kommonen *et al* (1986)
CerHV-1, RanHV-1	+	nt	nt	nt	nt	Nettleton *et al* (1987)

* nt = not tested S = serum

al, 1983) have been characterised in some detail. Several other isolates from goats in Norway (Hyllseth *et al*, 1985), Australia (Grewal and Wells, 1986), and New Zealand (Horner *et al*, 1982) are not well characterised. There is no information yet on the serological relatedness among these viruses. However, the clinical and pathological symptoms caused by the Swiss isolate were observed to be similar to those reported for the American strain, although the Swiss isolate did not cause abortion (Waldvogel *et al*, 1981). Goat alphaherpesviruses seem to be widely spread since antibodies have been detected in goat sera in various countries (Kao *et al*, 1985; Koptopoulos, 1992).

Since the first isolations of CerHV-1 (Inglis *et al* 1983; Ronsholt *et al*, 1987) and RanHV-1 (Ek-Kommonen *et al*, 1986) there has been described a new RanHV-1 isolate which is serologically related to BHV-1 (Rockborn *et al*, 1990).

1.2.4. Biology *in vitro*

Alphaherpesviruses are capable of productive cytocidal infections in a wide range of cell tissue types (Honess, 1984).

Host cell range. The host cell range of both BHV-1 and CapHV-2 has been studied extensively (Gibbs & Rweyemamu, 1977a; Kahrs, 1977; Jasty and Chang, 1972; Berrios and McKercher, 1975). BHV-1 multiplies in a wide variety of cell cultures including bovine embryonic kidney, skin and lung, bovine testis, lymph node, thyroid, thymus and kidney, ovine kidney, caprine

kidney, equine and porcine kidney, MDBK and rabbit spleen cell lines. Caprine herpesvirus-2 replicates in cells of ruminant origin including bovine bone marrow, bovine embryonic kidney and lamb embryonic kidney. Bovine embryonic lung cells support replication of virus to high titres (Berrios and McKercher, 1975). Both CerHV-1 and RanHV-1 have been successfully grown in embryonic bovine trachea and turbinate cells (Nettleton *et al*, 1987).

Cytopathology. The cytopathic effect (cpe) of BHV-1 and CapHV-2 in bovine cells is characterised by rounding of the cells (McKercher, 1964; Berrios and McKercher, 1975; Mettler *et al*, 1979). In contrast to BHV-1; CapHV-2 induces a more diffuse cpe with prominent swelling of cells and vacuole formation followed by detachment and clump formation. The two viruses have an eclipse period of 5h followed by an exponential growth phase from 6 to 12 h after infection. CapHV-2 is one of the most rapidly growing herpesviruses known reaching maximum titres 12 h post infection. BHV-1 reaches maximum titres 20h post infection (Engels *et al*, 1983).

The two cervid viruses, CerHV-1 and RanHV-1 show a common cpe, distinct from BHV-1 and CapHV-2. Syncitia formation for CerHV-1 and RanHV-1 is uncommon, and there is a prominent stranding of the infected cells (Ronsholt *et al*, 1987). Plaque formation by BHV-1 isolates is similar, ranging from 3.6-3.9 mm in diameter (Bagust, 1972). The plaques induced by CapHV-2 closely resemble those of BHV-1 by being circular with regular edges, but they are smaller at 1-2 mm in diameter (Stephens and Groman, 1963; Berrios and McKercher, 1975).

Temporal regulation of herpesvirus proteins. Most insight into the temporal regulation of herpesvirus proteins has been gained from studies on HSV. The herpesvirus proteins can be broadly designated as immediate-early (IE), early (E) or late (L) depending on the temporal order of their synthesis and whether their synthesis is dependent upon the successful progression of certain physiological processes in infected cells (Rakusanova *et al*, 1971; Roizman *et al*, 1974). In general the transcription, translation and expression of the herpesvirus polypeptides occur in a temporal cascade (Honess and Roizman, 1974, 1975; Clements *et al*, 1977; Jones and Roizman, 1979), with expression of IE polypeptides being essential for the induction of early genes and functional E proteins subsequently being required for expression of late proteins. Briefly, the role of the different genes in polypeptide synthesis from HSV studies can be described as follows;

Immediate-early genes

IE proteins are first detected about 1h after absorption. Early studies (Honess and Roizman, 1975) suggested that gene expression peaked at about 2-3 hours. However these observations were usually made in the absence of viral protein synthesis or functional Vmw 175 and without using specific antibodies. Where studies have been carried out in the course of a normal infection, high levels of IE polypeptide accumulation have been detected in the middle and late stages of infection. Indeed recent experiments have shown that Vmw 110 steadily accumulates throughout infection with no evidence for repression (Everett and Orr, 1991).

The promoter region of the IE genes, consists of two components, a "TATA" box sequence necessary for basal and induced levels of transcription, and upstream regulatory sequences containing multiple cis-acting elements conferring enhancer function and responsivity to IE regulation (Mackem and Roizman, 1982a, b; Cordingley *et al*, 1983; Preston *et al*, 1984; Kristie and Roizman, 1984; Bzik and Preston, 1986).

All the IE promoters contain GC-rich regions (Sp1 binding sites) and one to three copies in either orientation of the element TAATGARAT (where R is a purine), a cis-acting motif (Mackem and Roizman, 1982c; Whitton *et al*, 1983; Whitton and Clements, 1984; Preston *et al*, 1984; Galloway *et al*, 1984), which has been shown to mediate the stimulation of IE transcription by the major virion tegument protein Vmw 65 (VP16), the product of gene UL48, upstream of the mRNA initiation sites (Post *et al*, 1981; Mackem and Roizman, 1982a; Campbell *et al*, 1984; Preston *et al*, 1984). Vmw65 enhances expression of the immediate-early viral genes (Batterson and Roizman, 1983; Ace *et al* 1988) and also serves an important role in virion morphogenesis (Ace *et al*, 1988; Weinheimer *et al*, 1992).

Vmw65 does not form a direct interaction with DNA (Marsden *et al*, 1987) but instead interacts with one or more cellular factors to form a tertiary complex (IEC) that binds to TAATGARAT (McKnight *et al*, 1987; Preston *et al*, 1988; O'Hare *et al*, 1988; O'Hare, 1993). A cellular component of this complex appears to be an ubiquitous protein with a variety of names NFIII

(Pruijn *et al*, 1986), OBP100 (Baumruker *et al*, 1988; Strum *et al*, 1987), OTF-1 (Gerster and Roeder, 1988), TRF (O'Hare and Goding, 1988) and alpha-H1 (Kristie and Roizman, 1987). This factor was subsequently identified as Oct-1 (O'Hare and Goding, 1988; Stern *et al*, 1989) a transcription factor already implicated in the regulation of a number of cellular genes.

Oct-1 recognizes the TAATGARAT element and also an octomer consensus sequence ATGCAAAT. Although Oct-1 (NFIII) is capable of binding to a range of sequences, there is only efficient formation of the TRF-C (TAATGARAT recognition factor complex) when the TAATGARAT element is present (O'Hare *et al*, 1988; Gerster and Roeder, 1988; ApRhys *et al*, 1989). IEC complex formation facilitates an acidic activating domain, which is located within the carboxy-terminus of Vmw65 to interact with a transcription complex and so amplify gene expression (Dalrymple *et al*, 1985; Triezenberg *et al*, 1988; Sadowski, *et al*, 1988; Cousens *et al*, 1989). Details of the functional role of Vmw65 in viral transcription and expression of IE genes have been reviewed by O'Hare (1993).

Early genes

Synthesis of early genes is initiated after the appearance of immediate-early polypeptides (Honess and Roizman, 1974; Wagner, 1985; Weinheimer and McKnight, 1987; Zhang and Wagner, 1987), since early genes require prior synthesis of IE gene products for their expression (Honess and Roizman, 1974; Clements *et al*, 1977). Different early genes are

expressed with different kinetics, gD although expressed as an early gene, is not maximally produced until the onset of viral DNA replication (Gibson and Spear, 1983; Johnson *et al*, 1986) and as such has been described as a beta-gamma or early-late (EL) gene (Roizman and Batterson, 1985; Wagner, 1985; Harris-Hamilton and Bachenheimer, 1985). This increase in transcription of EL genes does not necessarily show a direct requirement for viral DNA synthesis, as increased expression may be due solely to an increased template copy number (Johnson and Everett, 1986a). As an example, the synthesis of the 65 kilodalton DNA-binding protein of HSV-1, encoded by gene UL42 has been shown to begin prior to viral DNA synthesis and continues to accumulate even late after infection (Goodrich *et al* 1989; Schenk and Ludwig, 1988). It has also further been demonstrated by Goodrich *et al* (1989) that the control of UL42 gene expression is at the level of transcription initiation.

The promoter regions of HSV early genes have been well studied and no viral specific sequences involved in transactivation have been detected. A typical HSV early promoter has been found to contain a "TATA" box element upstream of the transcriptional start site in combination with other elements, including "CCAAT" boxes, GC-rich regions (for Sp1 binding) and G-rich elements (McKnight *et al*, 1985; Everett, 1983; Eisenberg *et al*, 1985; El Kareh *et al*, 1985). Binding of various cellular transcription factors such as the "CCAATT" box binding factor and Sp1 to the promoter of the HSV TK gene has been demonstrated (Jones *et al*, 1985; Graves *et al*, 1986). Although many HSV promoters contain sequences homologous to TATA, CCAAT and

GC-rich elements, there is a great diversity between promoters of the same class (Mackem and Roizman, 1982c; Wagner, 1985). These differences may cause the differential rates of expression described previously for genes of the same class (Honess and Roizman, 1974; O'Hare and Hayward, 1985, Harris-Hamilton and Bachenheimer, 1985).

Late genes

Late gene product accumulation can initially be detected at about 3 hours post absorption, reaching a peak at around 10-16 hours. This is approximately 2 hours after the peak of viral DNA synthesis at 8 hours post absorption (Munk and Sauer, 1964; Roizman, 1969; Wilkie, 1973). The expression of late genes is dependent on the presence of a functional Vmw175 protein (Watson and Clements, 1980) and is also dependent upon the replication of the viral DNA. As demonstrated by experiments with ts DNA-mutants and inhibitors of DNA synthesis (Swanstrom and Wagner, 1974; Honess and Roizman, 1974; Powell *et al*, 1975; Marsden *et al*, 1978; Jones and Roizman, 1979; Holland *et al*, 1980; Conely *et al*, 1981; Pederson *et al*, 1981).

Late genes can be split into two classes; either $gamma_1$ or $gamma_2$, otherwise termed "leaky-late" and "true-late" respectively. "Leaky-lates" are those genes whose expression is detectable in the absence of viral DNA replication, although they require DNA synthesis for maximal expression, whereas "true-late" genes have an absolute requirement for DNA synthesis for expression (Wagner, 1985; Roizman and Batterson, 1985; Johnson *et al*, 1986). There is no operational distinction between "early-late" and

"leaky-late" genes. Examples of "leaky-late" genes are the products of gene UL19, Vmw155 - the major capsid protein and US6(gD). A protein which exhibits "true-late" characteristics is the product of gene US11 (Johnson *et al*, 1986).

Experiments in which an HSV origin of replication was linked to the US11 promoter demonstrated that only the "TATA" box and replication of the host plasmid in cis were required for full expression (Johnson and Everett, 1986a, b). It is unclear what connection exists between the replication of viral DNA and late gene expression, although an increase in template copy number is unlikely to be responsible, as some effect would be detectable on early genes also.

A possible role has been indicated for IE protein Vmw63, where in transfection experiments it has been shown to have very little effect on its own, but can repress the transactivation of IE and E genes by Vmw110 and Vmw175 and augment their transactivation of L genes (Everett, 1986). There is growing evidence that it acts post-transcriptionally (Smith *et al*, 1992) probably by selectively stimulating mRNA 3' processing of late gene poly A sites (McLauchlan *et al*, 1992).

1.2.5. Biology *in vivo*

Epidemiology. The geographical distribution of some of the ruminant alphaherpesvirus infections is shown in Table 1.6. Serological surveys show a

high prevalence of ruminant alphaherpesvirus infections in host populations. Numerous species of ruminants, both wild and domesticated were shown to possess specific antibodies which neutralise bovine herpesvirus 1 (Table 1.7). Although viruses antigenically related but distinct from BHV-1 have been isolated from goats (Saito *et al,* 1974; Mettler *et al,* 1979), red deer (*Cervus elaphus*) (Inglis *et al,* 1983) and reindeer (*Rangifer tarandus)* (Ek-Kommonen *et al.,* 1986) cross-neutralisation tests have suggested that each species is in fact infected with a similar but distinct virus which is specific for that species (Pastoret *et al.,* 1988). Thus goats were stated to be susceptible to BHV-1 virus as a result of serological tests. In fact, this species is most often infected by its own herpesvirus namely CapHV-2 (Ackermann *et al,* 1986; Hasler and Engels, 1986). Experimental infection of goats with BHV-1 produces no more than a mild infection (Wafula *et al,* 1985) and the bovine virus fails to establish a latent infection, indicating a lack of true adaptation (Pirak *et al,* 1983). A similar situation probably exists in the wild ruminants which react serologically to BHV-1. The sensitivity of sheep and cattle to CapHV-2 has been investigated. Berrios *et al* (1975) reported that lambs and calves are not sensitive to infection with the McK/US strain. However, recently Papanastosopoulou *et al* (1991) showed that the virus can cause a mild infection in lambs following an intravenous experimental infection.

Species-specificity has also been demonstrated for cervid viruses in that red deer could be infected with their own virus which remained latent after a primary infection, while experimental infection of deer with BHV-1 failed to

Table 1.6. Epidemiological features of some ruminant alphaherpesviruses

	BHV-1.1	BHV-1.2a, 2b	BHV-1.3	CapHV-2	CerHV-1	RanHV-1
NATURAL HOSTS	Cattle	Cattle	Cattle	Goat	Red deer	Reindeer
	Serological evidence in goats, swine, African wildlife					
GEOGRAPHIC DISTRIBUTION	Worldwide (except Finland and Sweden)		Australia, South America	Europe (not UK) N. America Africa Australia New Zealand	Scotland England Denmark	Finland Sweden

Table 1.7. Free living ruminants known to be susceptible to or showing serological evidence of infection with, BHV-1 and antigenically related viruses

Family	Subfamily	Species
Cervidae	Cervinae	Red deer (*Cervus elpahus)* Eastern wapiti (*Cervus canadensis*)
	Odocoilinae	Roe deer (*Capreolus capreolus*) White-tailed deer (*Odocoileus virginianus*) Mule deer (*Odocoileus heminous*) Elk (*Alces alces*)
	Rangiferinae	Reindeer (*Rangifer tarandus*) Caribou (*Rangifer tarandus caribou*)
Giraffidae	Giraffinae	Giraffe (*Giraffa camelopardalis*)
Antilocapridae		Pronghorn (*Antilocapra americana*)
Bovidae	Tragelaphinae	Eland (*Taurotragus oryx*) Greater kudu (*Tragelaphus strepsiceros*)
	Bovinae	African buffalo (*Syncerus caffer*)
	Alcelaphine	Red hartebeest (*Alcelaphus buselaphus*) Tsessebe (*Damaliscus lunatus*) Blesbok (*Damaliscus dorcas*) White-tailed gnu (*Connochaetes gnou*) Brindled gnu (*Connochaetes taurinus*)

Table 1.7. continued

Family	Subfamily	Species
	Hippotraginae	Roan antelope (*Hippotragus equinus*) Sable antelope (*Hippotragus niger*)
	Reduncinae	Common waterbuck (*Kobus ellipsipyrmnus*) Kob (*Kobus kob*) Lechwe (*Kobus leche*) Southern reedbuck (*Redunca arundinum*) Bohar reedbuck (*Redunca redunca*)
	Antilopinae	Thomson's gazelle (*Gazella thomsoni*) Springbok (*Antidorcas marsupialis*) Impala (*Aepyceros melampus*)
	Caprinae	Chamois (*Rupicapra rupicapra*) Domestic goat (*Capra aegagrus hircus*)

Adapted from Pastoret *et al* (1988)

provide any evidence of replication (Reid *et al,* 1986; Ronsholt *et al.*, 1987). This situation is similar to that in goats, and it is probable that every case of serological conversion in red deer (*Cervus elpahus)* is attributable to the cervid virus. Red deer, therefore, are never likely to be a reservoir of BHV-1 for infecting cattle. In reindeer (*Rangifer tarandus*) early serological surveys indicated serological conversion to BHV-1, suggesting that a situation similar to that in red deer may also exist in reindeer (*Rangifer tarandus*) (Ek-Kommonen *et al,* 1982). However, although the reindeer virus caused mild clinical signs in experimentally-infected cattle, there was no report of the virus causing any symptoms in free-living reindeer (Nettleton *et al,* 1987). RanHV-1 was recently demonstrated by Rockborn and others (1990) to be associated with lesions of the alimentary tract and nose in reindeer. As stated earlier it is evident that ruminant species seropositive for BHV-1 are most likely to be infected with their own alphaherpesvirus which cross react serologically with BHV-1.

The epidemiological features of ruminant alphaherpesvirus infections are probably very similar in Europe and America (Chow and Davis, 1964; Kinyili *et al,* 1979; Kingscote *et al,* 1987), whereas in Africa they are rather more complex judging from the very high prevalence of antibodies to BHV-1 observed in African buffalo (*Syncerus caffer*), eland (*Taurotragus oryx*) and wildebeest (*Connochaetes spp.*) (Table 1.8). The experimental infection of Wildebeest with BHV-1 showed them to be susceptible (Gibbs and Rweyemamu, 1977a). The infection remained confined to the genital tract, and it seems improbable that the infection would spread to other species. In

Table 1.8. Host range of ruminant alphaherpesviruses serologically related to BHV-1

Virus	Host	Continent	Evidence for Infection Antibody (Ab) or Virus Isolation (VI)	Reference
BHV-1	Goats	America Africa Europe	Ab, VI	Mohanty *et al* (1972) Wafula *et al* (1985) Gibbs and Rweyemamu (1977a)
	Cattle	Worldwide (except Finland and Sweden)	Ab, VI	Gibbs and Rweyemamu (1977a)
	Wild ruminants	Africa	Ab, VI	Kaminjolo and Paulsen (1970) Karstad *et al* (1974) Rweyemamu (1970) Hedger and Hamblin (1978)
	Red deer	Europe	Ab	Inglis *et al* (1983) Lawman *et al* (1978) Thiry *et al* (1988a, b)
	Reindeer	Europe, Canada	Ab	Ek-Kommonen *et al* (1986) El-Azhary *et al* (1981)
	Mule deer	N. America	VI	Chow and Davis (1964)

Table 1.8 continued

Virus	Host	Continent	Evidence for Infection Antibody (Ab) or Virus Isolation (VI)	Reference
CerHV-1	Red deer	Europe	Ab, VI	Inglis *et al* (1983) Nettleton *et al* (1986) Thiry *et al* (1988a, b)
RanHV-1	Reindeer	Europe	Ab, VI	Ek-Kommonen *et al* (1986) Rockborn *et al* (1990)
CapHV-2	Goats	Europe (except UK)	Ab, VI	Engels *et al* (1983)
		Australia	Ab, VI	Grewal and Well (1986)
		America	VI	Berrios *et al* (1975) Saito *et al* (1974)
		Africa	Ab	Wafula *et al* (1985)
		Greece	Ab	Koptopoulos *et al* (1988)
		New Zealand	Ab, VI	Horner *et al* (1982)

general, herpesviruses can persist latently in small populations of animals without infection from outside (Pastoret *et al*, 1984; Pastoret *et al.*, 1986; Thiry *et al*, 1986). It therefore seems reasonable to assume that other African species of wild ruminants, serologically positive for BHV-1 are infected with their own alphaherpesviruses.

Clinical syndromes. Ruminant alphaherpesviruses cause infections of veterinary importance in a wide range of animals (Table 1.9). The most common and economically important of ruminant alphaherpesvirus associated diseases is infectious bovine rhinotracheitis (IBR). The disease varies from a mild respiratory disease to a severe infection of the entire respiratory tract. The genital form of the disease, infectious pustular vulvovaginitis (IPV) is also relatively common and the virus is more rarely associated with conjunctivitis, abortion and encephalitis (Porterfield, 1989). Gastroenteritis may occur in adult cattle infected with BHV-1 and is a prominent feature in the generalised disease of neonatal calves, which is often fatal. Genital and respiratory disease are rarely diagnosed in the same herd at the same time. While IBR is uncommon in range cattle it is of major significance in feedlots where primary infection often coincides with transport and introduction to a feedlot of young fully susceptible cattle from diverse sources. BHV-1.3 causes clinical encephalitis, usually without other clinical signs of BHV-1 disease. This isolate has been shown to have different restriction enzyme profiles from BHV-1 and other ruminant herpesviruses (Brake and Studdert, 1985). It was also shown earlier to be antigenically related to but distinguishable from BHV-1 isolates from the respiratory and reproductive

Table 1.9. Diseases caused by alphaherpesviruses in ruminants

Virus	Disease	Reference
BHV-1.1	Infectious bovine rhinotracheitis; enteritis, abortion	Gibbs & Rweyemamu (1977a)
BHV-1.2a, 2b	Infectious pustular vulvo-vaginitis; Infectious balanoposthitis	Ludwig (1983)
BVH-1.3	Encephalitis	Brake and Studdert (1985)
BHV-2	Bovine mammilitis; pseudolumpy skin disease	Gibbs & Rweyemamu (1977b)
CapHV-2	Conjunctivitis, respiratory disease	Berrios & McKercher (1975)
	Abortion and vulvovaginitis	Waldvogel *et al* (1981)
	Enteritis and generalised disease in young kids	Waldvogel *et al* (1981)
CerHV-1	Respiratory disease	Inglis *et al* (1983)
	Ocular disease	Nettleton *et al* (1986)
RanHV-1*	Reproductive disorder?	Ek-Kommonen *et al* (1986)
	Alimentary and respiratory disease	Rockborn *et al* (1990)

*Virus isolated from vaginal swab following dexamethasone treatment; no clinical signs observed.

tracts (Bagust, 1972). In domestic goats CapHV-2 causes rare disease epidemics in kids, in which a frequently fatal disease occurs characterized by severe mucosal lesions of the alimentary and respiratory tracts (Saito *et al*, 1974; Berrios *et al*, 1975). The virus also causes vulvovaginitis (Horner *et al*, 1982; Hyllseth *et al*, 1985; Grewal and Wells, 1986).

In red deer an ocular disease has been associated with cervine herpesvirus type 1 infection (Inglis *et al*, 1983; Nettleton *et al*, 1986). Although the alphaherpesvirus which was isolated originally from the vagina of a reindeer experimentally treated with dexamethasone, was not associated with disease (Ek-Kommonen *et al*, 1986), Rockborn and others (1990) recently demonstrated the virus to be associated with ulcerative and necrotising lesions of the upper alimentary tract and nose.

The evidence to date suggests that alphaherpesvirus infections in both wild and domestic ruminants occur only in their natural hosts and do not cross stably into other species (Nettleton *et al*, 1987).

Pathogenesis. The aspects which have attracted most attention in the pathogenesis of ruminant alphaherpesvirus infection are latency and interaction with the immune system. In BHV-1, following infection by the respiratory or venereal route and virus replication in the mucosa at the portal of entry, a cell-associated viraemia is established and the virus progresses via sensory nerves to sacral or cervical ganglia eventually becoming latent in the trigeminal ganglia (Homan and Easterday, 1980, 1983; Narita *et al*, 1981;

Ackermann *et al* 1982; Ackermann and Wyler, 1984; Rodriguez *et al*, 1984). Restricted latency-associated transcription has been described in cells latently infected with HSV-1, HSV-2, VZV, SHV-1 and BHV-1 (reviewed by Rock, 1993). BHV-1 latency-associated transcripts (LAT's) have been detected in neurons of trigeminal ganglia from latently infected rabbits and cattle (Kutish *et al*, 1990). Earlier Cheung (1989) demonstrated LATs in the trigeminal ganglia of latently infected swine. Other possible sites of latency are in the medulla and cervical spinal cord (Lawrence 1983). Latently infected animals exhibit sporadic shedding of virus and reactivation and shedding can be predictably induced by natural or corticosteroid-induced stress (Sheffy and Davis, 1972; Sheffy and Rodman, 1973; Babiuk and Rouse, 1979; Straub, 1979; Pastoret *et al*, 1978, 1979a,b, 1980b; Thiry *et al*, 1985a,b; Whetstone and Miller, 1989). These features of latent infection have been reproduced in the rabbit (Rock and Reed, 1982) and used to demonstrate that the virus is transcriptionally active in latently infected neurones (Rock *et al*, 1986). Recently Whetstone and Miller (1989) showed that the two BHV-1 virus subtypes can be reactivated proving that two different strains of an alphaherpesvirus can establish latency in the same tissue in the host animal. Live vaccines can also establish latent infections in recipients (Pastoret *et al*, 1980b).

Persistence, reactivation and re-excretion of BHV-1 under natural conditions are well recorded (Studdert *et al*, 1964; Saxegaard, 1966; Bitsch, 1973; Hyland *et al*, 1975; Gregersen and Wagner, 1985). Frequent recurrence of clinical BHV-1 has been reported in closed herds (Hyland *et al*, 1975).

Biological differences have been described in re-excreted virus strains (Pastoret *et al* 1979a,b, 1980a; Homan and Easterday, 1981). However, Castrucci and others (1980) showed that BHV-1 does not undergo significant modifications in its pathogenicity when reactivated from latently infected animals for the first time. Progress has been made in the last few years in describing molecular aspects of viral latency and reactivation. Restricted transcription of the viral genome is seen in latently infected neurones, with latency-associated transcription being antisense and overlapping that from viral immediate early genes (Rock, 1993). Restricted viral transcription in latently infected ganglionic and CNS neurons has been observed with several alphaherpesviruses including HSV-1, HSV-2, VZV, BHV-1 and SHV-1 (Rock 1993). The LATs from BHV-1 localise predominantly to the nucleus, are approximately 0.77 to 1.16 kb in size and map to a region of the genome that overlaps an uncharacterised IE gene present on the complementary strand (Kutish *et al*, 1990). Transcription from the SHV-1 genome in latently infected neurons also appears to be restricted to a single region overlapping an IE gene region (Cheung 1989, Lokensgard *et al*, 1990, Priola *et al*, 1990). In VZV infection unlike the other alphaherpesviruses, satellite cells and perhaps other supporting cells, rather than neurons harbour latent virus. In addition, a much broader pattern of latency associated transcription is observed in latently infected cells; at least five distinct regions of the genome are transcriptionally active during latent infection. The function of latency-associated gene transcription in viral latency has not fully been defined. This question has been approached by examining the behaviour of HSV-1 LAT deletion mutants (viruses with defined deletions in regions encoding LATs or in

promoter/regulatory regions controlling its expression) in animal latency models. Results from a number of laboratories indicate that LAT is not an absolute requirement for establishment or maintenance of latent infection in rabbit or mouse latency models (Javier *et al*, 1988; Ho and Mocarski, 1989; Steiner *et al*, 1989, Leib *et al*, 1989; Block *et al*, 1990; Hill *et al*, 1990; Sedarati *et al*, 1990). However evidence is emerging that LAT may play some role in establishment of latency and viral reactivation *in vivo* and *in vitro*. Sawtell and Thompson (1992) demonstrated that HSV-1 LAT promotes establishment of latent infection in mouse trigeminal ganglia. Although the functional significance of BHV-1 LATs remains largely unknown, the ganglionic events that occur during dexamethasone-induced viral reactivation suggests a role for them in some aspect of viral latency and reactivation. The number of LAT-containing neurons decreased significantly (by 60%) 24 to 48 hours after dexamethasone treatment and returned to normal control values by 72 hours after treatment (Rock *et al*, 1992). Thus, available data suggest that LAT function is not an absolute requirement for viral latency and reactivation under experimental conditions, but rather appears to enhance quantitatively the efficiency of the latency and reactivation cycle. (reviewed by Hill, 1985; Roizman and Sears, 1987; Stevens, 1989 and Rock, 1993).

The mechanisms by which animals recover from alphaherpesvirus infections include cell-mediated as well as humoral immune responses (Rouse and Babiuk, 1978). Virus infected cells may be destroyed by antibody-dependent cellular cytotoxicity (ADCC) (Rouse *et al*, 1975; 1976; Grewal *et al*, 1980; Misra *et al*, 1982) and T cell-mediated cytotoxicity

antibody-dependent complement-mediated (Abc) cytolysis (Babiuk *et al*, 1975). Outside the cells, virions can be neutralised by antibody or lysed by antibody and complement (Babiuk, *et al*, 1975).

1.3. ALPHAHERPESVIRUS GLYCOPROTEINS

The virion envelope of all alphaherpesviruses (and all herpesviruses) contains a number of virus-encoded glycoproteins some of which have been extensively studied (reviewed by Spear 1985, 1993a; Marsden, 1987; Wyler, *et al* 1989; Roizman and Sears, 1990; Mettenleiter, 1991). Many have now been identified and mapped on their respective genomes: 11 in HSV-1 (Marsden, 1987; Roizman and Sears, 1990; Hutchinson *et al*, 1991; Baines and Roizman, 1993; MacLean *et al*, 1993; Spear, 1993a), 5 in VZV (Davison *et al*, 1986; Kinchington *et al*, 1986, 1990; Ling *et al* 1991), 5 in BHV-1 (Marshall *et al*, 1986; van Drunen Littel-van den Hurk and Babiuk, 1986a; Meyer *et al*; 1991), 7 in SHV-1 (Hampl *et al*, 1984; Lukacs *et al*, 1985; Rea *et al*, 1985; Klupp and Mettenleiter, 1991; Klupp *et al*, 1992), 9 in EHV-1 (Allen and Yeargan, 1987; Audonnet *et al*, 1990; Elton *et al*, 1991; Crabb *et al*, 1992; Telford *et al*, 1992; Whittaker, *et al*, 1992) and 9 in EHV-4 (Cullinane *et al*, 1988; Riggio *et al*, 1989; Nicolson and Onions, 1990; Nicolson *et al*, 1990; Crabb *et al*, 1992; Cullinane *et al*, 1993) There is at present very little or no information on the glycoproteins of the caprine and the two cervid alphaherpesviruses. The role of herpesvirus glycoproteins in their primary interaction with the host continues to be a subject of intense interest as some of them are continuously being shown to play important roles in the pathogenicity of these viruses. At present 6 complete herpesvirus

genome DNA sequences including the 3 mammalian alphaherpesviruses described in section 1.2.2 have been published. The other three herpesviruses are Epstein-Barr virus (EBV) (Baer *et al*, 1984) HCMV (Chee *et al*, 1990) and Channel catfish virus (CCV) (Davison, 1992). At least four other herpesvirus sequences are nearing completion (e.g. HSV-2 (McGeoch; personal communication, 1993); EHV-2 (Telford and Davison; personal communication, 1993); human herpesvirus 6 (Martin *et al* 1991); herpesvirus saimiri-2 (Albrecht *et al*, 1992)). Following the publication of the genome sequences of the above named viruses, there has been an accumulation of numerous reports of sequence and functional homologies among genes and proteins of different members of the herpesvirus family. As the complete nucleotide sequences of the many herpesviruses become available, these homologies will be confirmed and extended.

1.3.1. Nomenclature

Until very recently there has been no common nomenclature of individual herpesvirus glycoproteins. Over the last few years, workers studying the glycoproteins of herpesviruses have used different nomenclatures for each individual virus. This plethora of names has been confusing, particularly to those outside the field. However, the availability of increasing numbers of monospecific antisera, monoclonal antibodies, rapidly accumulating DNA sequence data and development of predictive methods to identify possible glycoproteins from analysis of DNA sequences (Perlman and Halvorson, 1983; McGeoch 1985, 1990; Hull and McGeoch, 1989; Griffin, 1991) has led to the adoption of common nomenclatures for the glycoproteins of individual

herpesviruses including HSV (Spear, 1976), BHV-1 (Misra *et al*, 1981; van Drunen Littel van den Hurk and Babiuk, 1986a), SHV-1 (Hampl *et al* 1984), VZV (Davison *et al*, 1986) and EHV-1 (Whittaker *et al*, 1992). Considerable progress is being made in identifying analogous and or homologous glycoproteins in different herpesviruses (Marsden, 1987; Owen and Mittal, 1988; Mittal and field, 1980; Sheppard and May, 1989; Audonnet *et al*, 1990; Zhang and Leader, 1990; Nicolson *et al*, 1990; Flowers *et al*, 1991; Whittaker *et al*; 1991, 1992; Carpenter and Misra, 1991; Ross and Binns, 1991; Mettenleiter 1991; Litwin *et al*, 1992; La Boissiere *et al*, 1992; Telford *et al* 1992; Scott *et al*, 1993). Despite the increasing wealth of information on the homologies of the herpesvirus glycoproteins there is very little evidence of efforts being made to adopt a commmon nomenclature. To date only gH is used to denote the homologous glycoproteins in the family Herpesviridae. Since the bulk of the knowledge about alphaherpesvirus glycoproteins is derived from the extensive studies on the prototype herpesvirus, HSV-1, a common nomenclature of alphaherpesvirus glycoproteins could be adopted on the basis of their respective homologies with HSV-1 glycoproteins as recently proposed by Whittaker and Coworkers (1992).

The nomenclature of BHV-1 glycoproteins was initially introduced by Misra *et al* (1981) and modified by van Drunen-Littel van den Hurk *et al* (1984) and van Drunen Littel van den Hurk and Babiuk (1985). However, van Drunen Littel van den Hurk and Babiuk (1986a) proposed a new nomenclature based on roman numerals which is now being adopted by many other workers (see Table 1.10). The major BHV-1 glycoproteins under this new nomenclature are: gI a complex of three glycoproteins with apparent molecular weights of 130 kDa/74 kDa/55

kDa; gII (108 kDa), gIII (97 kDa) which also occurs as a dimer with an apparent molecular weight of 180 kDa; and gIV (71 kDa) also occurring as a dimer of 140 kDa. The amino acid sequences of BHV-1 gI, gII, gIII and gIV are homologous to those of HSV gB, gE, gC and gD respectively (Babiuk *et al*, 1987; Wyler *et al*, 1989; Fitzpatrick *et al*, 1989; Misra *et al*, 1988; Tikoo *et al*, 1990; Whitbeck *et al*, 1988).

1.3.2. Homologous alphaherpesvirus glycoproteins

The adoption of common nomenclatures for glycoproteins of individual herpesviruses has increasingly made possible comparative identification of homologous or analogous glycoproteins in different alphaherpesviruses (see Table 1.11). The 11 HSV-1 glycoproteins identified and characterised in detail are designated gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL (reviewed by Marsden, 1987 and Roizman and Sears, 1990; Hutchinson, *et al* 1992; Spear, 1993a) and gM (Baines and Roizman, 1993; MacLean *et al*, 1993). Glycoproteins H and L are considered as a complex gH/gL since each, in the absence of the other is incorrectly processed (Fuller and Lee, 1992; Hutchinson *et al*, 1992). Homologous glycoproteins are found in other alphaherpesviruses; the corresponding homologues in some of the other alphaherpesviruses are listed in Table 1.11. In HSV, glycoproteins gB, gD, gH, gK and gL appear to be essential for virus replication since virus mutants deficient in any one of these glycoproteins are unable to replicate (Cai *et al*, 1988; Ligas and Johnson, 1988; Fuller *et al* 1989; Hutchinson *et al*, 1992; Fuller and Lee, 1992; reviewed by Spear, 1993a). Essential glycoproteins homologous to gB, gD and gH have been characterized in BHV-1 (gI, gIV and

Table 1.10 Classification and nomenclature of BHV-1 glycoproteins

New names				
Consensus nomenclature[a]	Proposed nomenclature[b]	Previous nomenclature[c]	Properties	HSV glycoprotein homologue[d]
gI (130kDa/74kDa/55 kDa complex)	gIa (130kDa) gIb (74kDa) gIc (55 kDa) pgIa (117kDa) pgIb (62kDa)	GVP6 GVP11a GVP16 pGVP6 pGVP11a	Derived from one primary precursor pI (105kDa). GVP6 is probably the uncleaved counterpart of GVP11a and GVP16 which are linked by disulphide bridges. Also found as a multimer, previously designated GVP1 (Mr >330kDa) Misra *et al* (1981). Contains N linked complex and high mannose type oligosaccharides.	gB
gII (108kDa)	gII (108kDa) pgII (100kDa)	GVP7 pGVP7	Derived from primary precursor pII (90kDa). A monomer containing N-linked complex and high mannose type oligosaccharides	gE
gIII (91kDa also exists as a 180kDa dimer)	gIII (91kDa)	GVP9	Derived from primary precursor pIII (61kDa) . A monomer but also found as a dimer previously designated GVP3 (180kDa). Contains N-linked type and O-linked type oligosaccharides.	gC
gIV (71kDa also exists as a 140kDa dimer)	gIV (71kDa) pgIV (63kDa)	GVP11b pGP11b	Derived from primary precurson pIV (58kDa). A monomer containing N-linked complex type and O-linked oligosaccharides.	gD

Table 1.10 continued

References

[a]van Drunen Littel-van den Hurk and Babiuk (1986a); Zamb (1987), Hughes *et al* , (1988); Whitbeck *et al* (1988); Misra *et al* (1988); Tikoo *et al* (1990, 1993); Chase *et al* (1990), Liang *et al* (1991, 1993); Okazaki *et al* (1991); Dubuisson *et al* (1992); Kopp and Mettenleiter (1992).

[b]van Drunen Littel-van den Hurk and Babiuk (1986a)

[c]Misra *et al* (1981); van Drunen Littel-van den Hurk *et al* (1984), van Drunen Littel-van den Hurk and Babiuk (1985).

[d]Babiuk *et al* (1987); Wyler *et al* (1989); Fitzpatrick *et al* (1989); Misra *et al* (1988); Whitbeck *et al* (1988); Tikoo *et al* (1990).

Table 1.11. Homologous alphaherpesvirus glycoproteins

HSV-1[a]	HSV-2[b]	VZV[c]	BHV-1[d]	SHV-1[e]	EHV-1[f]	EHV-4[g]
gB-1	gB-2	gpII	gI	gII	gp14	gp14
gC-1	gC-2	gpV	gIII	gIII	gp13	gp13
gD-1	gD-2	ni	gIV	gp50	gD	gD
gE-1	gE-2	gpI	gII	gI	gE	gE
gG-1	gG-2			gX		gG
gH-1	gH-2	gpIII	gH	gH	gH	gH
gI-1	gI-2	gpIV		gp63	gI	gI
gJ-1	gJ-2					
gK-1						
gL-1						
gM-1						

References

(references are to papers identifying homology and to previous sequence papers; they are not exhaustive)
ni - no homologue identified as there is no HSV-1 gene counterpart in VZV (Davison and Scott, 1986)

[a,b,c,d,f] Kit *et al* (1983), Longnecker and Roizman (1986) reviewed by Marsden (1987); Honess*et al*, (1989); Roizman and Sears (1990); Hutchinson*et al* (1992); Telford *et al* (1992); Spear (1993), Baines and Roizman, 1993; MacLean*et al*, 1993
[a,b,d] Fitzpatrick *et al* (1988), Bandyopadhyay *et al* (1990), Bello *et al* (1987)
[c] Davison and Scott (1986), Davison and McGeoch (1986), Davison and Taylor 91987)
[d] van Drunen Littel-van den Hurk and Babiuk (1986a); Zamb (1987); Whitbeck *et al* (1988); Misra *et al* (1988); Meyer *et al* (1991
[e] Ben Porat *et al* (1983); Rae *et al* (1985); Robbins *et al* (1986); McGeoch *et al* (1987); Mettenleiter (1991); Kimman *et al* (1992a, b); Pensaert *et al* (1992)
[f,g] Allen and Yeargan, (1987); Audonnet *et al* (1990); Nicolson *et al* (1990); Telford *et al* (1992); Whittaker *et al* (1992).

gH) (Lawrence *et al*, 1986; Misra *et al*, 1988; Whitbeck *et al*, 1988; Chase *et al*, 1990; Blewett and Misra, 1991; Liang, *et al*, 1991; Meyer *et al*, 1991; Misra and Blewett, 1991; Fehler *et al*, 1992), SHV-1 (gII, gp50 and gH) (Hampl *et al*, 1984; Lukacs *et al*, 1985; Mettenleiter *et al*, 1986; Petrovskis *et al*, 1986a; Robbins, *et al*, 1987; Klupp and Mettenleiter, 1991; Peeters *et al*, 1992; Pensaert *et al*, 1992; Karger and Mettenleiter, 1993). Glycoproteins gC, gE, gG, gI, gJ and gM of HSV are dispensable for viral growth in tissue culture (Longnecker and Roizman, 1987; Baines and Roizman, 1991; Spear, 1993b) as are glycoproteins gIII, gI, gX and gp63 for SHV-1 (Robbins *et al*, 1986; Petrovskis *et al*, 1986b; Zuckermann *et al*, 1989; Mettenleiter, 1991; Kimman *et al*, 1992b), glycoprotein gIII for BHV-1 (Liang *et al*, 1991, 1992) and probably VZV gpV (Kichington *et al*, 1990; Ling *et al*, 1991).

Although glycoproteins homologous to the essential HSV gB, gD and gH have been identified in VZV, EHV-1 and EHV-4 no similar functional studies have been reported in these viruses except for EHV-1 in which gD has been recently shown to play a major role in penetration of cells (Whittaker *et al*, 1992). However, although HSV gD homologues have been identified in the other alphaherpesviruses as listed in Table 1.11, VZV is the only alphaherpesvirus at this time to have been shown not to encode a glycoprotein gD homologue (Davison and Wilkie, 1983; Davison and Scott, 1986).

1.3.3. Genetic locations of homologous glycoproteins

A tremendous increase in the wealth of information is being derived from DNA sequence data highlighting the genomic organisation of most of the

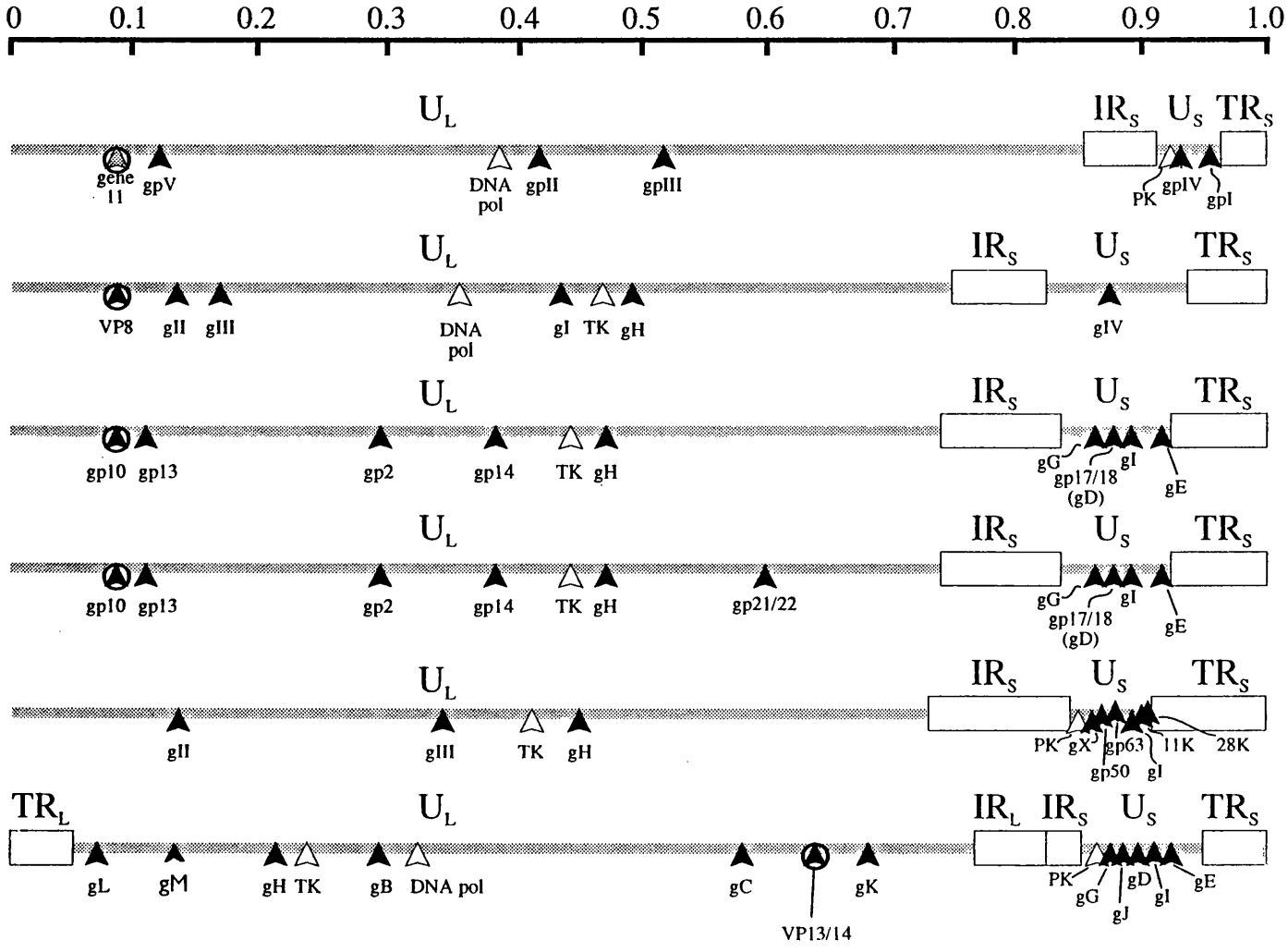
members of the alphaherpesvirus group. Comparisons of genome organisations between five alphaherpesviruses, HSV-1, HSV-2, VZV, SHV-1 and EHV-1 has shown extensive genome colinearity between the viruses (Davison and Wilkie, 1983). Varicella-zoster virus (VZV) has also been shown to have a gene arrangement collinear to those of BHV-1 and HSV-1 (Wirth *et al*, 1989; McGeoch, *et al*, 1988; Davison and Scott, 1986). The colinear arrangement within related regions indicates that the viruses share a common gene arrangement especially within those regions encoding a stretch of homologous proteins. However, because colinear arrangements are not maintained throughout entire genomes, homologous glycoproteins of several alphaherpesviruses including HSV, BHV-1, SHV-1, VZV, EHV-1 and EHV-4 show both similarities and differences in their map locations (Roizman *et al*, 1984; Rea *et al*, 1985; Ackermann *et al*, 1986; Gompels and Minson, 1986; Keller *et al*, 1986; Lawrence *et al*, 1986; Petrovskis *et al*, 1986b; Wathen and Wathen, 1986; Allen and Yeargan, 1987; Marsden, 1987; Misra *et al*, 1988; Whitbeck *et al*, 1988; Wirth *et al*, 1989; Zamb, 1987; McGeoch *et al*, 1988; Davison and Scott, 1986; McGeoch, 1990; Yalamanchili and O'Challaghan, 1990; Yalamanchili *et al*, 1990; Simmard *et al*, 1990; Zhang and Leader, 1990; Nicolson *et al*, 1990; Ling *et al*, 1991; Mettenleiter, 1991; Flowers and O'Callaghan, 1992; Telford *et al*, 1992; Whittaker *et al*, 1992; Spear, 1993a). Map locations of the genes encoding some of the homologous glycoproteins in these alphaherpesviruses are shown in Figure 1.4.

1.3.4. The structure of essential glycoproteins

Alphaherpesvirus glycoproteins have characteristics typical of a

transmembrane glycoprotein (Pellet *et al*, 1985). These characteristics include an N-terminal hydrophobic signal sequence, a hydrophobic region close to the carboxy terminus which may function as a transmembrane anchor sequence (membrane-spanning domain) and a hydrophilic external surface region with potential N-linked glycosylation sites. By comparing the amino acid sequences of HSV gB homologues from 13 different herpesviruses, Borchers and her coworkers (1991) were able to show several regions of domains conserved in the closely related sequences, including a four amino acid motif, 10 cysteine residues, several proline residues and some potential N-glycosylation sites suggesting that the secondary and tertiary structures of these gBs were similar. Like HSV-1 gB, the predicted BHV-1 gI amino acid sequence exhibits two broad hydrophobic regions likely to represent an amino-terminal signal sequence and a transmembrane anchor domain (Whitbeck *et al*, 1988). Additional features shared with HSV-1 gB include 6 potential N-linked glycosylation sites, four of which are colinear. One additional feature of BHV-1 gI extracellular domain is the presence of a 22-amino acid region for which there is no counterpart in HSV gB. A similar situation has been noted for the gB homologues of VZV (Keller *et al*, 1986), SHV-1 (Robbins *et al*, 1987), EHV-1 (Whalley *et al*, 1989) and EHV-4 (Riggio *et al*, 1989). This region of nonsimilarity represents a post-translational protease cleavage site (Borchers *et al*, 1991; Blewett and Misra, 1991). All the above gB homologues with the exception of HSV gB, exist in their mature form largely as cleaved, disulfide-linked heterodimers (van Drunen Littel-van den Hurk and Babiuk, 1986a; Grose *et al*, 1984; Lukacs *et al*, 1985; Meredith *et al*, 1988; Sullivan, *et al*, 1989). HSV gB

Figure 1.4. Locations of the genes encoding alphaherpesvirus glycoproteins and, where known, DNA pol, TK, PK and the homologues for a major HSV tegument protein VP13/14 The six alphaherpesvirus genomes are represented in their prototype orientations. The genes encoding glycoproteins are shown with a solid arrowhead whilst those encoding the enzymes are shown with an open arrowhead. The HSV tegument protein and its homologues are shown by ◮ where the protein product has been identified and ▲ where it has not.



Virus	Genome size	Genome type
VZV	125kbp	D
BHV-1	137kbp	D
EHV-4	144kbp	D
EHV-1	145kbp	D
SHV-1	150kbp	D
HSV-1	152kbp	E
HSV-2	152kbp	E

exists as an uncleaved dimer (Eberle and Courtney, 1982; Classon-Welsh and Spear, 1987; Chapsal and Pereira, 1988). Recently Blewett and Misra (1991) demonstrated that cleavage of BHV-1 gI is not essential for its function and that the uncleaved BHV-1 gI could complement the function of HSV-gB, whereas Rauh and her coworkers (1991) showed that SHV-1 mutants lacking the essential glycoprotein gII could be complemented by BVH-1 gI. However, whether these structural differences are related to differences in their biological functions, remains to be determined.

Many HSV gD homologues including BHV-1 gIV (Whitbeck *et al*, 1988; Tikoo *et al*, 1990); SHV-1 gp50 (Petrovskis *et al*, 1986a), EHV-1 gD (gp18/gp60) (Audonnet *et al*, 1990) and EHV-4 gD (gp 17/18) (Cullinane *et al*, 1993) share regions of significant amino acid sequence similarities which are confined to the central regions. These regions of sequence similarities contain conserved cysteine residues located within the central portion of the surface-exposed domain (Flowers and O'Callaghan, 1992).

EHV-1 gD contains 12 cysteine residues, while SHV-1 gp50 and HSV-1 and -2 gD each contain 7 residues (Watson *et al*, 1982; Watson, 1983; McGeoch *et al*, 1985; Petrovskis *et al*, 1986a). Studies of HSV-1 gD have indicated that 6 cysteines form intramolecular disfulfide bonds that are essential for the structural integrity of the protein (Wilcox *et al*, 1985; Long *et al*, 1990). Despite these similarities differences do exist: for example, whilst HSV-1 gD (Cohen *et al*, 1983; Serafini-Cessi *et al*, 1988) and BHV-1 gIV (van Drunen Littel-van den Hurk and Babiuk, 1985) are both N- and

O-glycosylated, SHV-1 gp50 is glycosylated by O-linked carbohydrate only (Petrovskis *et al*, 1986a), whereas EHV-1 and EHV-4 gD are glycosylated by mainly N-linked oligosaccharides (Whittaker *et al*, 1992; Cullinane *et al*, 1993).

A comparison of identities of amino acid sequences between related glycoproteins are summarised in Table 1.12. In addition comparisons of amino acid sequences of other related glycoproteins revealed an overall homology of 21% between EHV-1 gD and SHV-1 gp50, and 18% between EHV-1 gD and HSV-1 gD (Flowers *et al*, 1991). EHV-4 gD shares 83% sequence homology with EHV-1 gD, 38% with BHV-1 gIV, 34% with SHV-1 gp50, 25% with HSV-1 gD and 24% with HSV-2 gD (Cullinane *et al*, 1993). The sizes of these gD homologues are very similar: 394 residues in HSV-1 gD, 393 in HSV-2 gD, 402 in SHV-1 gp50 (McGeoch, 1990), 402 in EHV-1 gD (Audonnet *et al*, 1990), 402 in EHV-4 gD (Cullinane *et al*, 1993) and 417 residues in BHV-1 gIV (Tikoo *et al*, 1990).

Although HSV-1 gH appears to be only a relatively minor component of the virion envelope, it is essential to the viral replicative cycle (Desai *et al*, 1988). Glycoprotein gH homologous proteins have been described in all herpesviruses examined so far including alpha, beta and gammaherpesvirinae (Gompels and Minson, 1986; Keller *et al*, 1987; Cranage *et al*, 1988; Gompels *et al*, 1988a; Heineman *et al*, 1988; Josephs *et al*, 1991; Klupp and Mettenleiter, 1991; Meyer *et al*, 1991; Nicolson *et al*, 1990; Pachl *et al*, 1989; Klupp *et al*, 1992) and they constitute the second most highly

Table 1.12. Identity of amino acid sequences between related alphaherpesvirus glycoproteins*

Gene family		HSV-1	HSV-2	SHV-1
gB	HSV-2	85.6		
	SHV-1	50.4	50.7	
	BHV-1	49.4	48.1	60.8
gC	HSV-2	69.0		
	SHV-1	22.1	17.5	
	BHV-1	18.0	21.0	24.6
gD	HSV-2	82.2		
	SHV-1	10.9	10.9	
	BHV-1	11.4	14.8	32.8
gH	SHV-1	27.4		

*Identities were determined after alignment of sequences from PALIGN programme of PCGENE. Adapted from Spear (1993b).

conserved group of herpesvirus glycoproteins, surpassed only by the gB homologues (Fuller *et al*, 1989). The gH homologues and the gB homologues have been shown to possess all the characteristics of membrane glycoproteins including an N-terminal hydrophobic region which contains a signal sequence, a C-terminal region which serves as a transmembrane anchor domain and potential sites for N-linked glycosylation (Gompels *et al*, 1988a). With the exception of SHV-1 gH which is only 686 amino acids in length (Klupp *et al*, 1992) the gH's of alphaherpesviruses tend to be larger than their beta- and gamma herpesvirus counterparts, ranging from 838 (HSV-1) to 882 (BHV-1) as compared to 706 to 743 for the EBV, HVS and HCMV gH's (Nicolson *et al*, 1990). It has also been shown by Nicolson and coworkers (1990) that the C-terminal region of the protein is clearly conserved to a greater degree than the N-terminal.

Several features emerged from a comparison of the amino acid sequences of gH proteins of alpha-, beta- and gammaherpesviruses by Gompels *et al* (1988a,b) and Cranage *et al* (1988). First, there was great diversity of sequences in the N-terminal region of the protein. Second, they possessed an unusually short cytoplasmic domain: 14 to 15 amino acids in alphaherpesviruses and seven or eight amino acids in beta- and gammaherpesviruses. Third, four conserved cysteine residues lying within conserved local sequences were found at similar positions relative to the putative transmembrane domain. Out of the 10 cysteine residues in SHV-1 gH, 7 are found at colinear positions in BHV-1 gH, VZV gpV, EHV-4 gH, and HCMV gH, 6 in EBV gH and HVS gH; but only 4 in HSV-1 gH. Fourth,

a single C-terminal N-glycosylation site is conserved throughout herpesvirus gH proteins (Klupp and Mettenleiter, 1991). Fifth, considerable variation exists in potential N-linked glycosylation sites among the herpesviruses; eleven have been shown in the VZV, EHV-1 and EHV-4 gHs, eight in HVS, seven in HSV-1, six in HCMV, five in EBV, three in SHV-1 and six in BHV-1 (Keller *et al*, 1987; Robertson *et al*, 1991; Nicolson *et al*, 1990; Gompels *et al*, 1988a; Gompels and Minson, 1986; Cranage *et al*, 1988; Baer *et al*, 1984; Klupp and Mettenleiter, 1991; Meyer *et al*, 1991). All the conserved features imply some degree of similarity of both the secondary and tertiary structure of gH from different herpesviruses.

1.3.5. Functions and Activities

Herpesvirus glycoproteins that are present in the virus envelope are known to mediate several important steps in virus-host cell interactions (reviewed by Spear, 1985, 1993a; Mettenleiter, 1991). They are involved in virus adsorption to target cells (Schreurs *et al*, 1988; Mettenleiter *et al*, 1990; Sawitzky *et al*, 1990; Herold *et al*, 1991; Okazaki *et al*, 1991; Liang *et al*, 1991, 1992, 1993; Karger and Mettenleiter, 1993), are essential for penetration (De Luca *et al*, 1982; Highlander *et al*, 1987; Cai *et al*, 1988; Ligas and Johnson, 1988; Fuller *et al*, 1989; Rauh and Mettenleiter, 1991; Peeters *et al*, 1992; Fehler *et al*, 1992; Whittaker *et al*, 1992; Hutchinson *et al*, 1993) and also influence virus release from infected cells (Schreurs *et al*, 1988; Whealy et al, 1988; Mettenleiter *et al*, 1987; Zsak *et al*, 1989; van Drunen-Littel van Den Hurk *et al*, 1989). Most notably, all essential herpesvirus glycoproteins analysed so far are involved in fusion events between either virus envelope

and cellular cytoplasmic membrane or between cytoplasmic membranes of infected and adjacent uninfected cells (Manservigi *et al*, 1977; Chase *et al*, 1989; Fitzpatrick *et al*, 1990a; Tikoo *et al*, 1990; Peeters *et al*, 1992). By virtue of their involvement in the first steps of virus infection, glycoproteins are important in determining whether virions are able to enter target cells and initiate a productive infection. Glycoproteins of herpesviruses are also major targets of the host's immune response both at the humoral and cellular level (Glorioso *et al*, 1984; Spear, 1985, 1993a,b; Bishop *et al*, 1986; Zarling *et al*, 1986; Blacklaws *et al*, 1987; Johnson and Feenstra, 1987; Rosenthal *et al*, 1987; Van Oirschot *et al*, 1990; Zuckermann *et al*, 1990; Kimman *et al*, 1992a). Among the 11 HSV glycoproteins identified to date; gB, gD, gH and probably gK and gL appear essential for virus replication, since virus mutants deficient in any of these glycoproteins are unable to replicate (Cai *et al*, 1988; Ligas and Johnson, 1988; Fuller *et al*, 1989; Hutchinson *et al*, 1991; Spear, 1993b). Table 1.13 summarises the various viral activities affected by deletions of genes encoding homologous glycoproteins in selected alphaherpesviruses.

Essential glycoproteins of other alphaherpesviruses homologous to HSV gB, gD and gH have also been characterised (see Table 1.11). Whereas a functional analysis of HSV and SHV-1 glycoproteins is well advanced, studies aimed at elucidating the functions those of the other alphaherpesviruses including BHV-1, EHV-1, EHV-4 and VZV glycoproteins have only recently been started. The functions of the essential glycoproteins gB, gD and gH of HSV-1 and of their homologues in SHV-1 (gII, gp50 and gH) have been

Table 1.13. Homologous glycoproteins encoded by selected alphaherpesviruses and effects of gene deletions on various viral activities

Gene family	Name of glycoprotein			Deletion of genes causes loss of: Viral propagation *in vitro* *			Virion infectivity+			Cell fusion			Other properties of glycoprotein		
	HSV	PRV	BHV-1	HSV	PRV	BHV-1	HSV	PRV	BHV-1	HSV	PRV	BHV-1	HSV	PRV	BHV-1
gB	gB	gII	gI	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Binds heparin; syn gene[s]	-	-
gC	gC	gIII	gIII	No	No	No	Part	Part	Part	No	No	No	Binds heparin, C3b & C3bi[c]	Binds heparin	Binds heparin
gD	gD	gp50	gIV	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	-	Mediates inter-ference; syn gene	Mediates inter-ference	Mediates inter-ference
gH	gH	gH	gH	Yes	Yes	-	Yes	Yes	-	-	Yes	-	Forms heterodimer with gL	-	-
gL	gL	gL	-	-	-	-	-	-	-	-	-	-	Forms heterodimer with gH; syn gene	-	-
gK	gK	-	-	-	-	-	-	-	-	-	-	-	syn gene	-	-

Table 1.13 continued

Gene family	Name of glycoprotein			Deletion of genes causes loss of: Viral propagation *in vitro**			Virion infectivity+			Cell fusion			Other properties of glycoprotein		
	HSV	PRV	BHV-1	HSV	PRV	BHV-1	HSV	PRV	BHV-1	HSV	PRV	BHV-1	HSV	PRV	BHV-1
gE	gE	gI	-	No	No	-	-	-	-	No	-	-	Binds IgG-Fc; forms hetero-dimer with gI	Forms hetero-dimer with gp63	-
gI	gI	gp63	-	No	No	-	-	-	-	-	-	-	Forms hetero-dimer with gE	Forms hetero-dimer with gI	-
gG	gG	gX	-	No	No	-	-	-	-	-	-	-	-	Secreted	-
gJ	gJ	NA	-	No	NA	-	-	NA	-	-	NA	-	-	NA	-

*Yes, the mutant cannot be propagated in cultured cells, except on a cell line that expresses the missing gene and thus the lethal defect;
No, the mutant can be propagated in ordinary cultured cells although it might be at a selective disadvantage compared to wild-type virus
+An entry is made here only if specific infectivity of mutant virions has been assessed
- No entry means that the information is not yet available
[s]Syn genes of HSV are those that can acquire non-lethal mutations that enhance virus-induced cell fusion
[c]Components of the complement pathway
Partial means that the specific infectivity of virions is reduced but not enough to prevent propagation of virus
NA - not applicable
Adapted from Spear (1993b)

studied extensively in the past few years (Little *et al*, 1981; Noble *et al*, 1983; Fuller and Spear, 1987; Highlander *et al*, 1987; Cai *et al*, 1988; Cohen *et al*, 1988; Desai *et al*, 1988; Ligas and Johnson, 1988; Johnson and Ligas, 1988; Fuller and Spear, 1989; Foa-Tomasi *et al*, 1991; DeLuca *et al*, 1982; Hampl *et al*, 1984; Robbins *et al*, 1987; Klupp and Mettenleiter, 1991; Liang *et al*, 1991; Rauh *et al*, 1991; Kimman *et al*, 1992a, b; Kopp and Mettenleiter, 1992; Peeters *et al*, 1992). Several of these studies showed that virus particles that were treated with neutralising monoclonal antibodies, or virus mutants that lacked one of these glycoproteins were unable to enter target cells despite their ability to adsorb to these cells. Furthermore, overexpression in mammalian cells of gB, gD and gH of HSV-1 and their homologues indicated that these proteins were also involved in cell fusion by acting as viral attachment proteins (VAPs) mediating specific interactions with receptors on the surfaces of permissive cells (Campadelli-Fiume *et al*, 1988a, b, 1990; Johnson *et al*, 1988; Manservigi *et al*, 1988; Petrovskis *et al*, 1988; Johnson and Spear, 1989; Fitzpatrick *et al*, 1990a; Tikoo *et al*, 1990; Liang *et al*, 1991; Fehler *et al*, 1992; Forrester *et al*, 1992; Klupp *et al*, 1992; Peeters *et al*, 1992). There is also evidence which indicates that these glycoproteins are essential for the penetration of the virus particle into the target cell and that they are involved in the spread of the virus by fusion of infected cells with non-infected cells (Tikoo *et al*, 1990; Liang *et al*, 1991; Peeters *et al*, 1992, reviewed by Spear 1993b). Of the non-essential glycoproteins, the HSV-1 gC homologous glycoproteins, gIII (BHV-1), gIII (SHV-1) all bind to a cellular heparin-like receptor, thereby mediating adsorption of the virus to target cells (Mettenleiter, *et al*, 1990; Herold *et al*, 1991; Okazaki *et al*, 1991; Sawitzky

et al, 1990). Recently, Liang and his coworkers (1993) identified a cluster of 5 heparin-binding sites located between amino acids 129 and 310 in BHV-1 gIII and between amino acids 90 and 275 in SHV-1 gIII as the sites involved in the virus attachment process. In initial studies aimed at analysing whether homologous glycoproteins also function in a heterologous viral background, the non-essential SHV-1 gIII gene was replaced by the gene encoding its HSV-1 homologue, gC. Although expression in infected cells of gC from the recombinant SHV-1 genome could be shown, expression levels were low and mature gC could not be found in recombinant SHV-1 virions. In addition, complementation of defects associated with the lack of gIII was not observed (Whealy *et al*, 1989). However soon afterwards, Chase and coworkers (1990) showed that gD-1 homologues shared a common function by demonstrating the ability of bovine cells expressing BHV-1 gIV to partially resist infection by BHV-1, HSV-1 and SHV-1. Previously, cell lines constitutively expressing SHV-1 gp50 had been shown to be able to interfere not only with SHV-1 replication but also, and even more efficiently, with HSV-1 replication (Petrovskis *et al*, 1988) suggesting that both proteins were at least in part functionally similar. Recently Rauh *et al* (1991) showed that gI (BHV-1) is able to complement functionally the defect in an SHV-1 mutant lacking the homologous essential glycoprotein gII. Kopp and Mettenleiter (1992) demonstrated a stable rescue of a glycoprotein gII deletion mutant of SHV-1 by the homologous gI glycoprotein of BHV-1. Their results conclusively demonstrated that gI (BHV-1) in an SHV-1 background can execute all essential functions normally provided by glycoprotein gII of SHV-1. Overall, such findings indicate that essential homologous glycoproteins can function in

a heterologous herpesvirus background and that substitution of glycoproteins by their homologues from a different herpesvirus can influence viral growth characteristics *in vitro* and probably *in vivo*. Thus, there is growing evidence that herpesviral glycoproteins exhibiting sequence homology also share functional properties.

1.3.6. Involvement of glycoproteins in the host immune response

The immune response to HSV infection involves several host mechanisms. These include natural resistance (natural killer cells and interferon) (reviewed by Lopez, 1985; Nash and Cambouropoulos, 1993) and adaptive humoral and cell-mediated responses (Norrild, 1985; Nash *et al*, 1985, Nash and Cambouropoulos, 1993). The immune response to other alphaherpesvirus infections involve similar host immune mechanisms (Rouse and Babiuk, 1978; Gerber *et al*, 1978; Ben-Porat *et al*, 1986). Viral glycoproteins are major targets for these host immune mechanisms (Vestergaard and Norrild, 1979; Glorioso *et al*, 1984; Marshall *et al*, 1986, Ben-Porat *et al*, 1986; Israel *et al*, 1988). However, other viral components including immediate early and nucleocapsid proteins have been identified as targets for the host's immune response (Van Oirschot *et al*, 1988; Eloit *et al*, 1991; Banks *et al*, 1991).

Although the nature and role of natural resistance is still a poorly defined area, there is evidence implicating natural killer (NK) cells in resistance to HSV (reviewed by Lopez, 1985; Nash and Cambouropoulos, 1993). Experiments by Bishop *et al* (1986) suggested that human cells with NK activity possess clonal specificity for HSV-1 infected targets involving

determinants on HSV-1 gB and gC.

The humoral immune response against herpesviral infections involves two major mechanisms. These include antibody-dependent cell cytotoxicity (ADCC), which is effective against target cells *in vitro* early in the infectious cycle, and neutralising antibodies which are active against cell-free virions preventing spread to the nervous system and in the blood stream (reviewed by Kohl, 1991). Earlier studies with HSV-1 demonstrated that monospecific antibodies against each of the glycoproteins gB, gC, gD and gE were able to neutralise virus (Powell *et al*, 1974; Spear, 1975; Vestergaard and Norrild, 1979; Dix *et al*, 1981) and mediate complement dependent cytolysis of virus infected cells (Norrild *et al*, 1979). BHV-1 glycoproteins gI, gIII and gIV have all been shown to stimulate production of neutralising antibodies in mice, rabbits and cattle, and they all serve as targets for antibody-dependent complement-mediated lysis of virus-infected cells (Collins *et al*, 1984; van Drunen Littel-van den Hurk *et al*, 1984; van Drunen Littel-van den Hurk and Babiuk, 1985; van Drunen Littel-van Den Hurk and Babiuk, 1986b; Marshall *et al*, 1986; Okazaki *et al*, 1986; Trepanier *et al*, 1986; Israel *et al*, 1988; van Drunen Littel-van den Hurk *et al*, 1991). Antibody binding assays indicate that pigs infected with SHV-1 produce antibodies against gI, gII, gX, gp50, immediate early and nucleocapsid proteins (McGinley and Platt, 1988, 1989; Van Oirschot *et al*, 1988; Cheung, 1990; Eloit *et al*, 1988, 1991; Kimman *et al*, 1992a).

Cell-mediated immunity is an important part of the host's reponse to HSV

infection and is required for the clearance of virus from the sites of infection (reviewed by Nash and Wildy, 1983; Nash *et al*, 1985; Nash and Cambouropoulos, 1993). As pointed out by Nash and Cambouropoulos (1993) whereas CD4 T cells can initiate rapid clearance of infectious virus from epidermal surfaces probably by recruiting and arming macrophages; CD8 T cells from HSV-infected mice or humans have the ability to recognise proteins produced at different stages of the infectious cycle, particularly, the immediate early (IE) gene products which are major antigens recognised in association with MHC class I. Similarly it was previously demonstrated by Fitzpatrick *et al* (1988) that cytotoxic T lymphocytes induced by BHV-1 were directed against murine cells expressing gI or gIII. Recently Denis *et al* (1993) identified both gIII and gIV as target antigens for the BHV-1-specific cytotoxic T lymphocytes CD4 and CD8. In SHV-1 infections, gIII has also been identified as a target antigen for cytotoxic T cells in pigs and mice (Zuckermann *et al*, 1990). Furthermore, it has been observed that non-neutralising antibodies to glycoproteins gB, gC, gD, gE and gG passively confer protection against a lethal HSV challenge showing that these glycoproteins function as targets for antibody-dependent cell-mediated cytotoxicity (Balachandran *et al*, 1982; Schrier *et al*, 1983). A similar observation has also been reported for SHV-1, where Mabs directed against gII, gIII and gp50 passively protected mice and pigs against a lethal SHV-1 infection (Eloit *et al*, 1988; Van Oirschot *et al*, 1990).

1.3.7. Antigenic structure of the major glycoproteins of ruminant alphaherpesviruses

Knowledge of both the physical and antigenic structure of ruminant alphaherpesvirus glycoproteins is limited. However some progress has been made on the three major BHV-1 glycoproteins i.e. gI, gIII and gIV. The putative functions of each of glycoproteins gI, gIII and gIV have been correlated to corresponding epitopic structures by mapping antigenic domains recognised by virus-neutralising Mabs (Marshall *et al*, 1988). Seven different epitopes on gI defined as I, II, III, IVa, IVb, IVc and V as well as eight epitopes on gIV defined as Ia, Ib, II, IIIa, IIIb, IIIc, IIId and IV have been mapped using two panels of monoclonal antibodies in competition binding assays (van Drunen Littel-van den Hurk *et al*, 1985; Hughes *et al*, 1988). Nine epitopes defined as Ia, Ib, II, III, IV, V, VI, VII, VIII have also been mapped on gIII (van Drunen Littel-van den Hurk *et al*, 1985). Further analysis suggests that both the antigenic and immunogenic structure of the eight epitopes on gIV, seven of which are neutralising, are carbohydrate independent (van Drunen Littel-van den Hurk *et al*, 1990). In contrast, three of the carbohydrate-dependent epitopes on gI were shown to correspond to neutralising epitopes IVa, IVb, and IVc (van Drunen Littel van den Hurk *et al*, 1985). Two of these epitopes IVa and IVb are also involved in infected-cell lysis. Monoclonal antibodies to these epitopes exhibit reduced binding to gI after carbohydrate removal, indicating that both antigenicity and immunogenicity of epitopes IVa, IVb and IVc are carbohydrate-dependent in contrast with the carbohydrate independence of epitopes I, II, III and V. Epitope IV have been mapped to the amino terminus of gI between residues

68 and 119 (Fitzpatrick *et al* 1990b). This region contains a potential N-linked glycosylation site at position 105 of the gI sequence, further suggesting carbohydrate dependence of this epitope. It has also been shown that domains gI-IV and gIV-III have attachment functions, while domains gI-III, gI-V and gIV-I are involved in penetration (Hughes *et al*, 1988; Fitzpatrick *et al*, 1990b). Recently Tikoo *et al* (1993) demonstrated that gIV protein mutants lacking N-glycans at residue 102 (gN2) or residues 41 and 102 (gN1N2) showed altered reactivity with conformation-dependent gIV-specific monoclonal antibodies. This change affects the formation of the discontinuous epitopes and reduces the ability to induce a potent neutralising antibody response in mice. Okazaki *et al* (1991) previously showed that an anti-BHV-1 monoclonal antibody which recognises an antigenic epitope between amino acid residue 114 and 144 could block the binding of BHV-1 gIII to heparin. Recently, Liang *et al* (1993) demonstrated that a cellular heparin-like moiety with five heparin-binding sites clustered between amino acids 129 and 310 in BHV-1 gIII plays an essential role for BHV-1 gIII-mediated virus attachment.

Differences in the antigenic structure of the major glycoproteins gI, gIII and gIV of BHV-1.1 and the neurovirulent BHV-1.3 were demonstrated recently by Collins *et al* (1993) using a panel of Mabs prepared against the BHV-1.1 glycoproteins. The antigenic differences could, for some epitopes be correlated with biological function. One Mab which neutralised BHV-1.1 but not BHV-1.3 was shown to react with a gI epitope, IVb, present on BHV-1.1 but not BHV-1.3.

1.4 AIM OF THE STUDY

The major aim of this work was to compare the serological relationships among five ruminant alphaherpesviruses. By raising a panel of monoclonal antibodies against one of them i.e. the cervine herpesvirus-1 (CerHV-1) and using polyclonal serum against bovine herpesvirus type 1 (BHV-1), cervine herpesvirus type 1 (CerHV-1), rangiferine herpesvirus type 1 (RanHV-1) and caprine herpesvirus type 2 (CapHV-2) it was intended to distinguish and differentiate by western blotting and radioimmunoprecipitation infections by these viruses. The monoclonal antibodies were then used to identify common and virus specific antigenic determinants. The monoclonal antibodies which were able to differentiate the ruminant alphaherpesviruses were assessed for their suitability and potential in developing a sensitive and reliable diagnostic assay.

CHAPTER 2

GENERAL MATERIALS AND METHODS

This chapter describes the general techniques used in the study. All specific materials and methods are described in the appropriate chapters.

2.1. Cell Culture and Virus Stocks

2.1.1. Cells

Embryonic bovine trachea (EBTr) cells were established as a semicontinuous cell line at the Moredun Research Institute from explant cultures of tracheal rings from an abattoir foetus aged between 4-6 months. After establishment of sufficient cell outgrowth from the explant cultures, cells were trypsinised and expanded. A master stock was established free of contaminating viruses and mycoplasma at passage level 6 and cells were used between passage levels 6 and 14. The embryonic bovine trachea (EBTr) cells were used for the propagation of virus, and for the preparation of lysates of both infected and non-infected cells. The cells were grown in Eagle's '199' medium (E'199') (Northumbria Biologicals Ltd) containing 10% heat inactivated foetal bovine serum (FBS) (Advanced Protein Products Ltd), 100 IU/ml penicillin, 100 ug/ml streptomycin, and 1mM L-Glutamine (British Drug House, (BDH)).

2.1.2. Virus stocks

The ruminant alphaherpesviruses examined in this study and their histories

are listed in Table 2.1. All viruses except Bovine herpesvirus 1.2 had already been plaque purified three times. BHV-1.2 was also plaque purified three times before being used as stock virus. Master stocks of all five viruses were established by growing them in EBTr cells. Working stocks for these viruses were prepared from the masterstocks and stored at -70°C until required.

2.1.3. Plaque purification of BHV-1.2 virus

Confluent monolayers of EBTr cells in twelve-well plastic plates (Linbro-Flow Labs) were washed three times with warm Hank's balanced salt solution (HBSS). Ten parallel ten-fold serial dilutions of virus were made and each dilution (10^2 to 10^7 pfu) was inoculated on to two wells (200 ul/well). After 1 hour of absorption at 37°C, the virus inoculum was removed and 2 mls of overlay medium containing 1.5% carboxy methyl cellulose (CMC) was added to each well. The plates were incubated at 37°C in 5% CO_2 for 2 to 3 days. Plates were examined and wells containing a single plaque were chosen. Media and cells around the plaque were collected using a thin tipped capillary pipette. The virus was resuspended into 1 ml of Eagle's medium (E'199') containing 100 IU/ml penicillin, 100 ug/ml streptomycin, 50 IU/ml polymixin, 5 ug/ml fungizone and 50 IU/ml aerosporin. This procedure was repeated three times. Thereafter the virus was propagated in a 25 cm^2 flask (Flow Labs) and then a 75 cm^2 flask (Flow Labs). After harvesting and clarifying the media at 1000g for 15 minutes at 4°C, the viral supernatant was aliquoted into 4 ml amounts and stored at -70°C as the masterstock virus.

Table 2.1. Ruminant alphaherpesvirus stocks

Virus strain or isolate	Species	Isolation Site	Country	Year	Reference
*BHV-1.1 (6660)	Bovine	Respiratory tract	Scotland	1978	Nettleton and Sharp (1980)
+BHV-1.2 (Aberdeen)	Bovine	Ocular-respiratory tract	Scotland	1962	Dawson *et al* (1962)
*CerHV-1	Red deer	Ocular-respiratory tract	Scotland	1982	Inglis *et al* (1983)
++RanHV-1	Reindeer	Genital tract	Finland	1985	Ek-Kommonen *et al* (1986)
**CapHV-2 (E/CH)	Goat	Respiratory tract	Switzerland	1981	Engels *et al* (1983)

*Viruses isolated at Moredun - BHV-1.1 masterstock passage level 7
- CerHV-1 masterstock passage level 7

+Virus supplied by Dr. Margaret Lucas, MAFF Central Veterinary Laboratory, Weybridge. Virus used at passage level 20

++Virus supplied by Dr. Ek-Kommonen, National Veterinary Institute, Helsinki, Finland. Virus passage level not known

**Virus supplied by Dr. Steven Edwards, MAFF Central Veterinary Laboratory, Weybridge. Virus passage level not known

2.1.4. Plaque assay

The stock viruses were all quantified by a plaque titration assay. Two mls of EBTr cell suspension containing approximately 2 x 10^5 cells/ml were added to all wells in twelve-well plastic plates (Linbro, Flow Labs Ltd) and incubated at 37°C in 5% CO_2 for 24-48 hrs until confluent monolayers were formed. Ten-fold serial dilutions from 10^{-1} to 10^{-10} were carried out for each virus stock to be titrated. All wells were washed three times with warm Hank's balanced salt solution before two wells were each inoculated with 200 ul of each virus dilution. The virus inoculum was removed after 1 hour of absorption at 37°C in 5% CO_2. Two mls of overlay medium containing E'199' with 1.5% carboxy methyl cellulose (CMC) was added to each well. The plates were then incubated for 2 to 3 days at 37°C in 5% CO_2. The cells were fixed for 1 hour at room temperature with a 10% formol saline solution. The cells were stained for 10 minutes with 0.1% crystal violet in 20% ethanol. The plates were washed thoroughly with water and plaques were counted and quantified as plaque forming units per ml (pfu/ml).

2.1.5. Virus production

Monolayers of EBTr cells in 225 cm^2 plastic-flasks (Corning, UK) were washed three times with Hanks balanced salt solution and then inoculated with virus at a multiplicity of infection (moi) between 0.1 and 10 pfu/cell depending on the experiment being undertaken. After absorption for 1 hour at 37°C the inoculum was withdrawn and 100 ml maintenance medium (E'199') supplemented with 2% heat inactivated foetal bovine serum (FBS) (Advanced

Protein Products Ltd), 100 IU/ml penicillin, 100 ug/ml streptomycin, and 1 mM L-glutamine was added and incubated at 37°C until infection was complete as judged by the development of maximum cytopathic effect (cpe). At this time, 2-4 days, more than 90% of the cells were round, enlarged and refractile under the microscope. The medium was harvested, clarified by centrifugation at 7800g for 15 minutes at 4°C in a JA21 Beckman centrifuge and the supernatant virus was stored in 10ml aliquots at -70°C. Control EBTr cells were mock-infected with Hanks balanced salt solution and treated similarly.

2.1.6. Purification of virus

Clarified supernatant containing virus was pelleted at 40,000g for 45 minutes at 4°C in a JA21 Beckman centrifuge. The viral pellet was resuspended in 1 ml of phosphate buffered saline (PBS) (173 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4) and stored at -70°C until required. A 10% to 25% w/w ficoll 400 (Pharmacia) step gradient was prepared by weighing out 2g ficoll 400 and 18g 1 x TNE buffer (1MNaCl, 100 mM Trisma, 10 mM EDTA, 200 ml distilled water, pH 7.5) for 10% and 5g ficoll 400 and 15g 1 x TNE buffer for 25%. The resuspended viral pellets were thawed, mixed thoroughly, loaded onto the step gradient and centrifuged at 95,000g for 1 hour at 4°C in an SW40 rotor on an LS2 or LS5 Beckman ultracentrifuge. The visible virus band at the 10-25% ficoll interface was removed by a pipette, resuspended in 1 ml of 1 x TNE buffer and mixed well by inversion. The virus was then pelleted at 95,000g for 40 minutes in an

SW40 rotor at 4°C on an LS2 or LS5 Beckman ultracentrifuge. The virus was finally resuspended in 1 ml PBS and stored at -70°C until required.

2.1.7. Preparation of labelled cell extracts

Monolayers of EBTr cells were prepared by seeding 25 cm^2 flasks (Flow Labs Ltd) with approximately 1 x 10^5 cells/ml (a total of 1 x 10^6 cells) in 10 ml of E'199' medium supplemented with 10% FBS, 100 IU/ml penicillin, 100 ug/ml streptomycin and 1mM L-glutamine. When confluent, the monolayers were mock-infected or infected with 20 pfu/cell. Virus was allowed to absorb at 37°C for 1 hr. The inoculum was removed and 10 mls of E'199' medium containing 2% FBS, 100 IU/ml penicillin, 100ug/ml streptomycin, 1 mM glutamine and one tenth the normal concentration of methionine or one fifth the normal concentration of glucose was added to both mock infected and infected flasks and incubated at 37°C for 6 hrs. The medium was removed and replaced with 10 ml of the same medium immediately followed by addition of 15 uCi/ml of D-[6-^{3}H]-glucosamine (Amersham) to all flasks and reincubated at 37°C for a further 10-14 hours.

2.1.8. Harvesting of labelled cell extracts

After 16-24 hrs of incubation, the medium was removed and 5 ml/flask of sample buffer containing 50 mM Tris.HCl, pH 6.8, 2% SDS, 10% glycerol, 200 mM 2-mercaptoethanol, and 0.05 (w/v) bromophenol blue was added to both mock infected and infected cells and harvested. Samples were heated at 95°C for 5 minutes aliquoted in 1 ml amounts and stored at -70°C until

required.

2.2. Preparation of antisera

2.2.1. Rabbit antisera against each virus

Each of the gradient purified viruses was diluted in PBS and emulsified in an equal volume of Freund's complete or incomplete adjuvant (Difco Labs, USA) as indicated in Table 2.2. Five rabbits, previously tested and found to be free from antibody to all five viruses, were each given one immunisation subcutaneously (s/c) with one of the five gradient purified viruses at different concentrations in Freund's complete adjuvant (CFA) (Table 2.2), followed by a booster immunisation in Freund's incomplete adjuvant (ICFA) subcutaneously 3 weeks later. After six weeks, they received another booster immunisation of the virus in PBS intravenously (i/v). They were then all bled twice, 10 and 17 days respectively after the final booster immunisation. All sera were heat inactivated at 56°C for 30 min, aliquoted in 0.5 ml amounts and stored at -20°C.

2.2.2. Red deer antisera against CerHV-1

Two adult female red deer aged between 2 and 3 years were bled before each was inoculated intranasally with 10 ml of CerHV-1 in PBS containing 5 x 10^7 pfu/ml. The animals were bled twice, 3 and 6 weeks post-infection. All sera were heat inactivated at 56°C for 30 minutes and stored at -20°C.

Table 2.2. Schedule for immunisation of rabbits for the production of polyclonal hyperimmune sera against the five ruminant alphaherpesviruses

Rabbit Identification Number	Date of immunisation	Viral immunogen/ route of inoculation	Volume (ml) inoculated	Titre of virus in immunogen pfu/ml
1887	12/7/90	1.0ml BHV-1.1 in CFA s/c	1.0	2.25×10^8
	10/8/90	1.0ml BHV-1.1 in ICFA, s/c	1.0	2.25×10^8
	25/9/90	1.0ml BHV-1.1 in PBS, i/v	1.0	2.25×10^8
1826	12/7/90	1.0ml BHV-1.2 in CFA, s/c	1.0	7.5×10^8
	10/8/90	1.0ml BHV-1.2 in ICFA, s/c	1.0	7.5×10^8
	25/9/90	1.0ml BHV-1.2 in PBS, i/v	1.0	7.5×10^8
1827	10/8/90	1.0ml RanHV-1 in CFA, s/c	1.0	3.25×10^8
	30/8/90	1.0ml RanHV-1 in ICFA, s/c	1.0	3.25×10^8
	15/10/90	1.0ml RanHV-1 in PBS, i/v	1.0	3.25×10^8
1884	11/9/90	1.0ml CapHV-2 in CFA, s/c	1.0	7.5×10^7
	1/10/90	1.0ml CapHV-2 in ICFA, s/c	1.0	7.5×10^7
	12/11/90	1.0ml CapHV-2 in PBS, i/v	1.0	7.5×10^7
1882	11/9/90	1.0ml CerHV-1 in CFA, s/c	1.0	4.75×10^7
	1/10/90	1.0ml CerHV-1 in ICFA, s/c	1.0	4.75×10^7
	12/11/90	1.0ml CerHV-1 in PBS, i/v	1.0	4.75×10^7

Note: s/c - subcutaneous
i/v - intravenous
CFA - Freund's complete adjuvant
ICFA - Freund's incomplete adjuvant

2.2.3. Field sera against the five viruses

Field sera from host species of each virus were obtained from different sources. Cervine (red deer) (Nettleton *et al* 1986) anti-CerHV-1, bovine (cattle) anti-BHV-1.1 (Lyaku *et al*, 1990) and BHV-1.2 sera were obtained from the Moredun Research Institute. Goat anti-CapHV-2 (Waldvogel *et al*, 1981) sera were gifts from Dr. M. Engels of the University of Zurich, Switzerland while reindeer anti-RanHV-1 (Ek-Kommonen *et al*, 1986) sera were kindly supplied by Professor B. Hyllseth of the Norwegian College of Veterinary Medicine, Oslo, Norway.

2.3. Serological techniques

2.3.1. Serum neutralisation test (SNT)

Microneutralisation tests using 100 $TCID_{50}$ of virus/well were employed to test ascitic fluid or serum for evidence of antibody. The SNT was carried out as described elsewhere (Bitsch, 1978). Briefly two-fold serial dilutions of ascitic fluid or heat inactivated serum mixed with an equal volume of virus (25 ul) containing 100 $TCID_{50}$ of virus/well were incubated for 24 hours at 37°C in a CO_2 incubator. At the end of incubation, 50 ul of EBTr cells in E'199' medium were added to the test wells, and the microtitre plates were further incubated for 3-4 days at 37°C in a CO_2 incubator after which wells were examined for the presence of viral cpe. Antibody titres were calculated by the Spearman-Karber method, and judged to be the highest dilution resulting in a 50% reduction of viral cpe relative to a virus control.

2.3.2. Enzyme-linked immunosorbent assay (ELISA)

Preparation of the ELISA antigen

The ELISA antigen for each virus was prepared from EBTr infected or mock-infected cells. After 3 days infection, media was retrieved, clarified in a JA21 Beckman centrifuge at 7800g at 4°C for 15 minutes. The supernatant was removed and the cell pellets added back to the flasks. One ml of 0.5% Nonidet P40 (NP40) in PBS was pipetted into each flask, and the flasks placed at 4°C for 1 hour (tilting every 15 minutes to remove attached cells). The detergent-treated cells were recovered and clarified in a Micro Centaur microcentrifuge (MSE, UK) at 13,400 g for 5 minutes at room temperature. The pooled supernatant was aliquoted into 200 ul amounts and stored at -70°C (Lyaku *et al*, 1990).

The assay

To perform the assay, flat bottomed 96-well polyvinyl plates (Cooke M129A Dynatech Laboratories, Billinghurst, Sussex) were coated overnight at 4°C with 100 ul/well of the viral antigen at an appropriate dilution (1/100 predetermined by checkerboard titration) in 50 mM carbonate-bicarbonate buffer, pH 9.6. The wells were coated alternately in rows with positive and control antigens respectively. The wells in column one were not coated and were used as a blank in each plate. Fifty microlitres of each sample was tested undiluted (hybridoma supernatants) or at a single dilution as described in the text (polyclonal sera and ascitic fluid) and the plates incubated for 2 hours at room temperature in a humid box. After washing with PBS containing 0.05%

Tween 20 (PBST-20), 50 ul of the appropriately diluted horseradish-peroxidase (HRP) conjugate (Scottish Antibody Production Unit (SAPU), Lanarkshire) was added to all wells except those in column one, and incubated for 1 hour at room temperature. After the final washing step with PBST-20, 100 ul of 40 mg orthophenylene diamine (OPD) (Sigma) per 100 ml of citrate phosphate buffer pH 5 containing 10 ul of 30% hydrogen peroxide (H_20_2) was added to each well. The reaction was stopped after 30 minutes of incubation at 37°C in the dark by addition of 25 ul/well of 2.5M sulphuric acid (H_2SO_4). The optical density (OD) was read at 492 nm wavelength (against substrate as blank) in a multichannel recorder (Titertek Multiskan Flow Laboratories) interfaced to a BBC computer and Epson FX-80 printer (Acorn Computer Ltd, Cambridge, UK) using a Titertek Multiskan interface (Flow Laboratories) and a 3187 serial interface type 312B (Flow Laboratories).

2.4 Other techniques

2.4.1. Total protein estimation by the Pierce Method.

Total protein estimation of the gradient purified stock virus and the ELISA antigen from infected and mock-infected EBTr cell lysates was determined by the Pierce method using a BSA protein assay reagent kit from Pierce and Warriner (UK) Ltd. Solutions containing a known concentration of bovine serum albumin (BSA) were used as standards. Results were read at 550 nm expressed as ug/ml and printed out from a computer interfaced to a Titertek Multiskan Plus.

Figure 2.1. BHV-1.1 virus particles viewed under the electron microscope. The virus particles were obtained from the supernatant of infected EBTr cells and purified through a ficoll gradient. They were stained with phosphotungstic acid and visualised by electron microscopy. Arrow indicates virion and capsid.
Total magnification x 75000

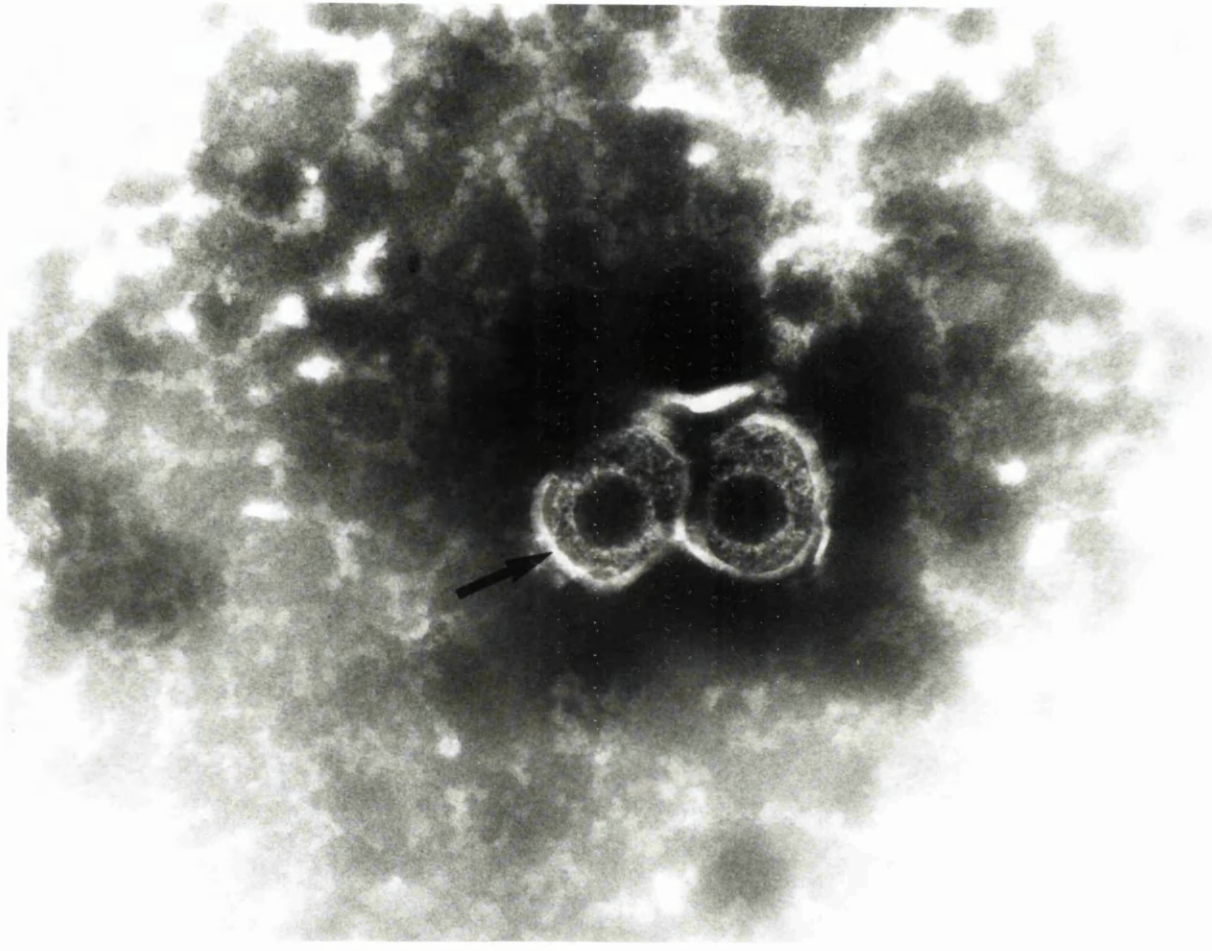
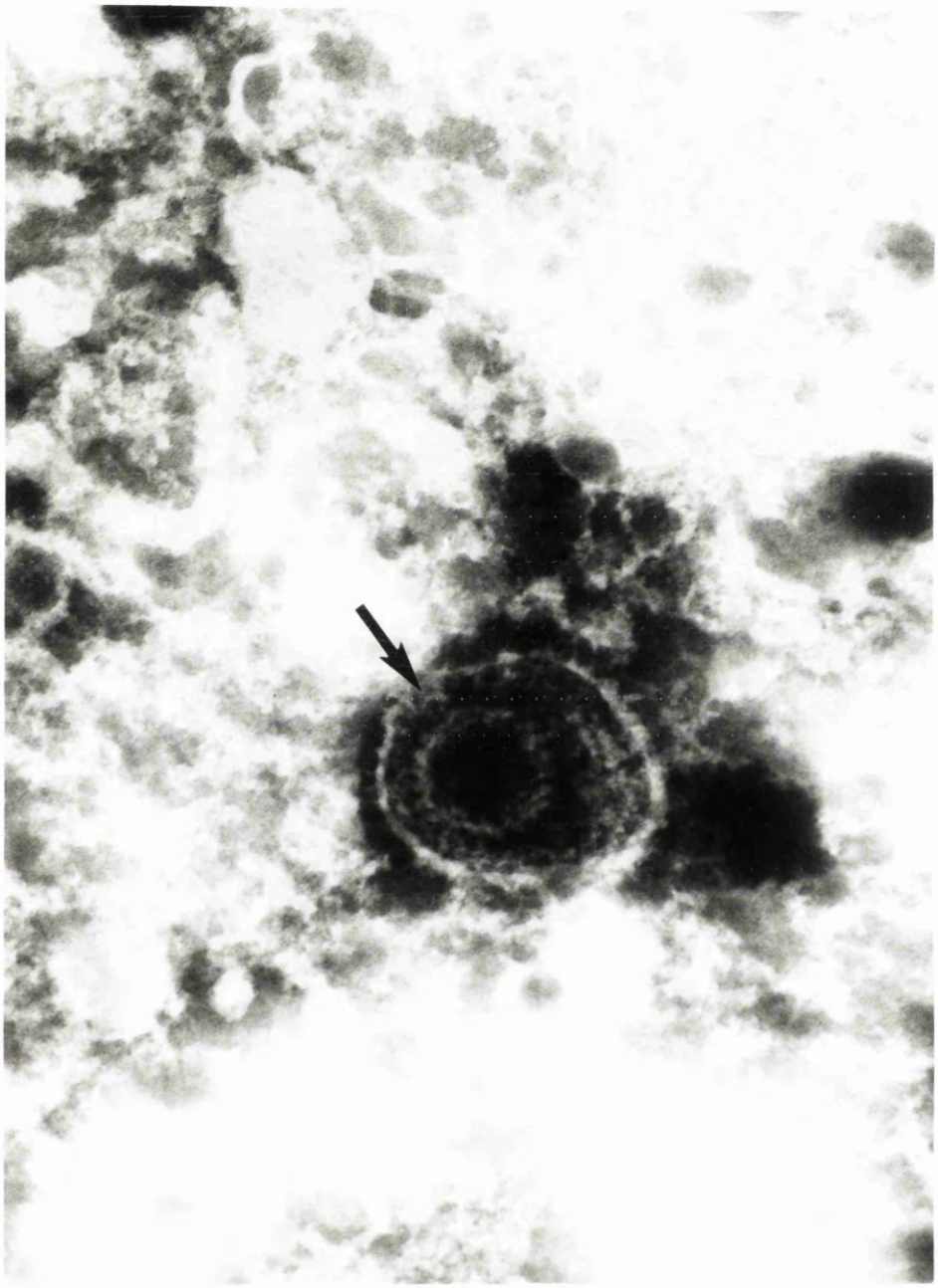


Figure 2.2. CerHV-1 virus particles viewed under the electron microscope. The virus particles were obtained from the supernatants of infected EBTr cells, and purified through a ficoll gradient. They were stained with phosphotungstic acid and visualised by electron micrscopy. Arrow indicates virion and capsid.
Total magnification x 120000



2.4.2. Electron Microscopy

The gradient purified viruses were resuspended in a drop of distilled water and applied to a formvar/carbon coated grid. Grids were negatively stained with 1% potassium phosphotungstic acid, pH 7.0 and examined in a Siemens Elmiskop 1A electron microscope. Electron micrographs for BHV-1.1 and CerHV-1 were prepared by Mrs. L. Inglis of the Electron Microscopy Department, Moredun Institute (see Figure 2.1 and 2.2).

2.4.3. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out in 10% discontinuous vertical slab gels (18 cm x 16 cm) (Laemmli, 1970) under reducing conditions as described by Herring and Sharp (1984). Briefly, stocks of 30% acrylamide (Bio-rad) were prepared in distilled water where the ratio of acrylamide to the cross-linking agent (N,N'-methylene bisacrylamide) (Bio-rad) was 28.5:1.5. After filtering through Whatman No. 1 filter paper, the solutions were used to prepare resolving gels containing 10% acrylamide in 250 mM Tris.HCl, pH 8.8 and 0.1% SDS, and stacking gels consisting of 3% acrylamide in 122 mM Tris.HCl pH 6.8, 0.1% SDS. The gel solutions were polymerised chemically by the addition of 0.025 percent by volume of mM TEMED (Bio-Rad) and ammonium persulphate (Bio-Rad). Following polymerisation of the resolving gel (approximately 1 hr), a stacking gel was added on top with a teflon comb to form the sample wells. The stacking gel was allowed to set (approximately 20 mins) and the comb removed gently. Before addition to the gel, protein samples were boiled at 95°C for 5 min in sample buffer containing 50 mM

Tris.HCl, pH 6.8, 2% SDS, 10% glycerol, 200 mM 2-mercapto-ethanol (BDH) and 0.05% (w/v) bromophenol blue (Sigma). Electrophoresis was carried out in a buffer containing 250 mM Tris.HCl pH 8.8, 192 mM glycine (BDH) and 0.1% SDS at 40 mA overnight at room temperature. Following electrophoresis, gels with the resolved proteins were then transferred onto nitrocellulose (Schleicher and Schuell) or fixed in methanol:acetic acid:water (50:7:50) containing 0.2% Commassie brilliant blue R250 (Sigma) followed by destaining in 5% methanol and 7% acetic acid. For radiolabelled samples, gels were dried under vacuum onto Whatman 182 filter paper and autoradiographed with Kodak X-omat S film at -70°C.

2.4.4. Western blotting

The electrophoretically separated proteins were transferred to a 200 mm x 200 mm 0.2um, nitrocellulose sheet (Schleicher and Schuell) in a buffer containing 250 mM Tris.HCl, pH 8.3, 192 mM glycine and 20% (v/v) methanol using electroblotting equipment (Bio-rad) as described by Herring and Sharp (1984). Following transfer the nitrocellulose was stained with Ponceau red (Sigma) for 10 minutes. The Ponceau red was poured off and the nitrocellulose membrane washed in distilled water until red-stained protein bands were observed. The positions of the standards and the area of protein transfer were marked and the strip with the molecular weight markers was cut-off.

The nitrocelluose strips were then incubated in blocking solution

containing PBS, 0.05% Tween 20 and 10% horse serum. All washes using a buffer containing PBS and 0.05% Tween 20 were performed 3 times for 5 min at room temperature and all incubation periods were for 1 h at 37°C. Both steps were carried out with gentle mixing. After blocking, the nitrocellulose was cut into 5 mm wide strips and each strip was incubated in a predetermined dilution of 1:200 of each monoclonal antibody (Mab) (ascites) or 1:100 of polyclonal sera. The nitrocellulose-bound immune complexes were reacted with HRP-labelled sheep anti-mouse, goat anti-rabbit or rabbit anti-bovine IgG (SAPU). The strips were further washed and incubated for 20 min in the dark at room temperature with 4 mg of 3, 3'diaminobenzidine (DAB) (Sigma) in 100 ml 0.05M Tris.HCl containing 10ul of 30% H_2O_2. The reaction was stopped by washing the strips in tap water, they were then blotted and air dried. The apparent molecular weights of nitrocellulose-bound proteins or immune-complexes detected were calculated from their mobility in the gels by extrapolation from a standard curve obtained by plotting mobilities of the marker proteins against their known molecular weights on a semi- logarithmic scale (Weber and Osborn, 1969).

2.4.5. Radioimmunoprecipitation (RIP)

Radioimmunoprecipitation (RIP) of ^{3}H-glucosamine labelled virus was done by acid elution. Briefly, 50 ul of Mab (ascites diluted 1/10) was added to 20 ul of ^{3}H-glucosamine labelled virus infected cell homogenate, mixed with 5 ul of sheep anti-mouse IgG and incubated at 4°C overnight. One hundred microlitres of a 10% *Staphylococcus aureus* suspension was added and mixed

at 4°C for 30 minutes. The mixture was washed twice in 3 ml of STN buffer (0.15M NaCl, 0.01M Tris pH 7.4, 0.25% Nonidet P40) by spinning at 1000g for 10 minutes at 10°C. After the last wash, as much fluid as possible was removed from the pellet. Two hundred microlitres of 0.2M glycine pH 2.5 was added and mixed on ice for 30 minutes. The mixture was centrifuged at 1300g for 15 minutes at 10°C from which 100ul of the supernatant was collected and mixed with 50 ul of sample buffer [30% stacking buffer (0.015M SDS, 0.5M Tris pH 6.8), 30% glycerol, 15% 2-mercaptoethanol and 6% SDS]. The samples were heated at 95°C for 10 min and electrophoresis carried out on 10% acrylamide gels in a buffer containing 250 mM Tris.HCl, pH 8.8, 192 mM glycine and 0.1% SDS at 10 mA overnight at room temperature. The gels were fixed in methanol:acetic acid:water (50:7:50) for 1 hour, destained in 5% methanol and 7% acetic acid, followed by soaking in EnHance (Dupont, UK) for 1 hour and washing twice in tap water each for 20 minutes. The gels were dried under vacuum onto Whatman 182 filter paper in a Biorad gel dryer for 2 hours and exposed to Kodak X-omat S film for autoradiography at -70°C.

2.4.6. Restriction endonuclease profiles

Restriction endonuclease profiles for the five viruses including equine herpesvirus-1 (EHV-1) and herpes simplex virus-1 strain syn 17^+ as controls were obtained as described by Lonsdale (1979) with some modifications. Twenty-four well plates (Corning) were seeded with 500ul per well containing approximately 1 x 10^6 cells per ml in E'199' medium. The plates were

incubated at 37°C overnight. Two wells with the monolayers were infected with each of the five viruses (Table 2.1) at a moi of 10. Two additional wells were infected with EHV-1 given by Dr. E. Telford and two further wells with HSV-1 strain 17^+ given by Dr. J. Harland. These two viruses were included to provide control viral DNA known restriction endonuclease profiles for the two enzymes used. The plates were incubated at 37°C for 1h. The medium was removed and the wells were washed twice each with 800ul of phosphate free Eagle's medium (PIC). Four hundred and fifty microlitres of PIC were added to each well and the plate incubated at 37°C for 2h. Fifty microlitres of PIC containing 10 uCi ^{32}P in orthophosphoric acid (Amersham UK) was added to each well and the plate incubated at 37°C for 2-3 days. The viruses were harvested after a complete cpe by adding 500 ul of 5% SDS solution per well followed by incubation at 37°C for 5 min. The cells and the medium were transferred into 15ml Sarsted tubes to which 1ml of saturated phenol (Phenol mixed at 2:1 v/v) with saturation buffer (75mM NaCl, 50mM EDTA, 10mM Tris, pH 7.5) was added. The tubes were inverted gently several times, left to stand for 10 min at room temperature. The top aqueous layer from each tube was removed using sawn-off blue tips and transferred into round bottomed glass test tubes. Two ml of ethanol (Analar, UK) was added to each tube, covered with Nescofilm and inverted gently several times to precipitate the DNA followed by centrifugation at 900g for 10 min at room temperature. The supernatants were poured-off, the tubes inverted and left to dry at 37°C for 15min. The viral DNAs were redissolved in 200ul of water containing 50ug/ml of RNAse A (Sigma) (previously boiled for 10 min) for 2h at 37°C in

a shaking water bath. Twenty microlitres of each viral DNA was digested for 16h at 37°C with 5-10 units of restriction endonuclease BglII using reaction conditions recommended by the manufacturer (Bethesda Research Laboratories (BRL), Gibco, Paisley, UK). The reaction was stopped by the addition of 5ul of RE stop solution (0.025M EDTA, 40% sucrose, 0.22% bromophenol blue) followed by electrophoresis through a 0.5% horizontal agarose gel (25 x 20 x 0.5 cm) in Tris-Borate (TBE) buffer (0.89 Tris/HCl, 0.89M Boric Acid, 0.02M EDTA, pH 8.3) at 60 volts. The gel was dried onto a glass plate in an oven at 95°C for 3h. The dry gel was exposed onto X-ray K-Oxomat S film for 16-20h at room temperature for autoradiography.

2.4.7. Polymerase Chain Reaction (PCR)

The BHV-1 gI gene (Whitbeck *et al* 1988) 468bp fragment was selected for amplification of the 5 viral DNAs by PCR. As primers the synthetic oligonucleotides P_1-5'-CACGGACCTGGTGGACAAGAAG-3' (positions 624-645 and P_2-5'-CTACCGTCACGTGCTGTGTACG-3' (positions 1070-1091) were used (Vilcek, 1993). PCR was carried out according to the following protocol: Five 100ul eppendorf were used, where into each, 9ul of distilled water, 2.5ul of 50% glycerol (diluted with water at 1:1 vol/vol), 2.5ul 10x reaction buffer (Boehringer-Mannheim), 2ul P_1 (50pmol), 2ul P_2 (50pmol), 3ul of one of the 5 viral DNAs (phenol-chloroform extracted as in section 2.4.6) was added. The water phase in each eppendorf tube was covered with 40ul paraffin oil. The mixture was heated at 100°C for 6 min, then chilled in ice for 5 min. Two microlitres of 2.5mM dNTP (Pharamacia),

and 2ul (1 unit) Taq polymerase (Boehringer-Mannheim) were added to each eppendorf. Thirty five amplification cycles were carried out in a Perkin-Elmer-Cetus DNA themal cycler at 95°C for 1 min, 56°C for 1 min, 72°C for 1 min. Twenty ul of each of the five amplification products were analysed by electrophoresis in a 2% agarose gel (Tris-acetate-EDTA buffer, 110V for 45 min) and detected by staining with ethidium bromide (Maniatis *et al*, 1982). Restriction endonuclease cleavage of each of the five amplification products was carried out using four enzymes; BglI, HinfI, SmaI and AvaI (Boehringer-Mannheim). Briefly, 18.5ul of distilled water, 3ul amplified DNA, 2.5ul 10x reaction buffer (Boehringer-Mannheim) and 1ul (10 units) enzyme were added into a 50ul eppendorf and incubated in a water bath at 37°C for 1h. The mixture was analysed by electrophoresis in 10% SDS-PAGE gels (see section 2.4.3) at 100V for 90 min and detected by silver staining as described by Herring and Sharp (1984).

2.5. Production of CerHV-1 monoclonal antibodies

Mouse monoclonal antibodies specific for cervine herpesvirus-1 (CerHV-1) were raised using the following immunisation schedule and fusion protocol.

2.5.1. Immunisation schedule

A total of ten 5-6 weeks old Balb/c mice which were to become spleen cell donors were each immunised intra-peritoneally (IP) with 200 ul containing 2 x 10^9 pfu/ml of gradient purified cervine herpesvirus-1 mixed with an equal

volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan, USA). Three weeks later, the mice were each given a second i/p injection of 200 ul containing 2 x 10^9 pfu/ml of the same virus mixed with an equal volume of Freund's incomplete adjuvant (Difco Laboratories, Detroit, Michigan, USA). Eight to fourteen months later, and three days before a fusion was performed, a final 200 ul containing 2 x 10^9 pfu/ml dose of the same virus in PBS was given to one mouse intravenously.

2.5.2. Fusion Protocol

Three days after the final "booster" dose the mouse was killed by anaesthetic overdose and blood collected from an incised axilla. At the same time, one non-immunised mouse was killed in the same way and blood collected similarly. The spleens were removed aseptically and each one placed into a universal bottle containing RPMI medium with 10% FBS. The two spleens from the different mice were treated similarly but separately in two sterile petri dishes. Using a 0.45mm-gauge needle, a total of 10 ml of medium was injected into several sites on each spleen forcing the cells out into the petri dishes until the whole capsule appeared almost transparent. Myeloma cells of line NSO/1 derived from NSI/1-Ag4.1 (Galfre and Milstein, 1981) in continuous culture at the Moredun Institute with a viability above 95% (checked by staining with Nigrosin) were harvested. Aliquots with viable counts less than 95% were discarded. The cells were centrifuged for seven minutes at 500g and both the spleen and myeloma cell pellets were washed once in RPMI medium containing 10% FBS and once in serum-free

RPMI medium. The cell pellets were finally resuspended each in 10 ml of serum-free RPMI medium. Aliquots of both spleen cell suspensions (100 ul) were diluted with an equal volume of 0.2% Nigrosin stain, and the live unstained cells were counted in a cell counting chamber (Modified-Fuchs Rosenthal Counting Chamber).

Samples of at least 5 x 10^6 cells of both immunised spleen and myeloma cells were set aside as controls. Aliquots of immunised spleen cells were mixed with myeloma cells at a ratio of 10:1 and centrifuged at 500g for seven minutes. The supernatant was drained completely and all traces of fluid removed. Fusion was carried out by slowly adding one ml of 50% warm Polyethylene glycol (PEG) 1500 (BDH) over a period of one minute while gently mixing the cell pellets by rotation. Mixing of the cell pellets was continued for a further minute and one ml of serum free RPMI medium was added over one minute. Nine ml more of serum-free RPMI medium was added over a period of 3-5 minutes and the mixture was centrifuged at 500g for five minutes. The supernatant was discarded and the pellet was gently tapped to resuspend it before being added to 100 ml of RPMI medium containing 20% FBS, and double concentrate HAT. Non-immunised (normal) spleen cells to give a final concentration of 2 x 10^5 cells per ml were then added to the mixture. One hundred microlitres of the cell suspension was added to each of the central sixty wells of 96-well flat-bottom microtitre plates (Nunc, Denmark). Spleen cells from the immunised, non-immunised mice and unfused myeloma cells were also plated out separately as controls. The

spleen cells were included to monitor the secretion of antibody and the unfused myeloma cells served to monitor the efficiency of the aminopterin at inhibiting their growth. Plates were maintained in a humidified incubator at 37°C in 5% CO_2. One day following fusion, plates were inspected to ensure freedom from bacterial contamination. One week after fusion, plates were fed with 100 ul of fresh RPMI with 15% FBS containing 2% hypoxanthine thymidine (HT) and then re-incubated for a further seven days. Supernatants from growing hybridomas were tested for specific antibody and positive hybridomas which covered more than half of the bottom of the well were subcultured for cloning.

2.5.3. Growth stimulating factors.

Following the fusion, the density of cells plated in each well is so low that cells do not receive sufficient growth stimulating factors. In order to overcome this shortfall, normal mouse spleen or "feeder" cells or mixed thymocyte medium (MTM) were added to the culture medium to act as cell growth promoters as cells secrete growth stimulating factors into the culture medium when the cell density is high.

2.5.4. Screening of hybridomas for specific antibody.

Secretion of specific antibodies against CerHV-1 by hybridomas in the culture medium of each well was screened by ELISA as described earlier (section 2.3.2). Briefly 50 ul of the culture medium was added to each of two wells on a 96-well plate coated with either EBTr cells infected with CerHV-1

or mock-infected EBTr cells. The plates were incubated for 2 hours at room temperature. After washing twice with PBST-20 50 ul of sheep anti-mouse IgG HRP conjugate (SAPU) at a predetermined dilution (1:1200) was added to each well. Subsequent procedures were as described in the ELISA. Mouse sera, reactive or non-reactive with CerHV-1 infected cells were used in each test as controls. The antibody level was estimated by the optical density (OD_{492}) value after subtraction of the OD_{492} given by the same culture medium on the control antigen.

2.5.5. Subcloning of positive hybridomas.

Hybridoma cells from antibody positive wells were cloned three times by the limiting dilution method to establish their stability and to avoid antibody secreting hybridoma cells being overgrown by non-antibody secreting hybridoma cells. Subcloning was performed when the antibody secreting hybridoma cells covered more than half of the bottom of the well. Cells from one well, harvested in 200ul of medium were diluted in 1.8 ml RPMI with 10% FBS. RPMI media containing 10% FBS (100ul) was added to each well of 96-well flat-bottomed plates. A 100 ul aliquot of the diluted cells was added to each well of the top row and double diluted downwards discarding the last 100 ul. The remaining cell suspension was placed onto two wells of a four-well plate (Costar, Cambridge, England) containing 0.5ml of RPMI medium with 10% FBS. The plates were then covered and incubated at 37°C in an atmosphere of 5% CO_2.

2.5.6. Storage of hybridoma cells

Hybridoma cells which were secreting specific antibody were frozen down at every stage of the cloning procedure. By the the end of the third cloning when almost all wells became positive cell lines were expanded slowly by transfer to 4-well plates (Flow Labs Inc, USA) then to 25cm^2 flasks (Gibco Ltd, Scotland). When the hybridoma cells had grown in the flask, the cells were removed and pelleted by centrifugation at 500g for 5 min and the culture medium collected and stored at 4°C. The cell pellet was resuspended in a small volume of supernatant before addition of 1 ml of ice cold freezing medium containing 10% dimethylsulphoxide (DMSO) (Sigma) in RPMI containing 50% FBS and dispensed in 1.8 ml screw-capped cell culture vials. The vials were wrapped in cotton wool, placed in a box covered with cotton wool and held at -70°C overnight. The vials were then transferred to storage in liquid nitrogen.

2.5.7. Recovery of frozen hybridoma cells from liquid nitrogen storage

Frozen vials were removed from liquid nitrogen storage, and their contents thawed quickly in a waterbath at 37°C. When thawed, the cell suspension was taken out from the vial with a pasteur pipette and transferred to a centrifuge tube containing 20 ml of cold serum-free RPMI medium. The cells were pelleted by centrifugation at 500g for five minutes, the pellet resuspended in a flask with 10 ml RPMI medium containing 20% FBS and then incubated at 37°C in an atmosphere of 5% CO_2.

2.5.8. Determination of immunoglobulin isotype

The immunoglobulin isotype and light chain component of each of the CerHV-1 specific monoclonal antibody preparations was determined on cell culture supernatants by a commercial mouse monoclonal antibody isotyping kit (Amersham, UK) following the manufacturers instructions.

2.5.9. Production of ascitic fluid

A total of five male or female BALB/c mice aged between 10-18 weeks were injected intraperitoneally each with 0.5ml of 2, 6, 10, 14 tetramethylpentadecane (pristane) (Sigma). Eight to fifteen days later, 2.5 x 10^7 hybridoma cells which had been cloned three times were centrifuged at 500g for seven minutes at room temperature. The supernatant was removed, the pellet resuspended in 1.5 ml of PBS and 0.3 ml containing 5 x 10^6 cells was injected intraperitoneally into each of the pristane-primed mice which developed ascites two to three weeks later. The mice were sacrificed when their abdomen exhibited visible signs of swelling. Ascitic fluid was then tapped from the peritoneal cavity with 1.25 mm gauge needles. The ascitic fluid was clarified by centrifugation at 1000g at 4°C for 15 minutes to pellet any cells and the oily layer aspirated from the surface of the fluid. The ascitic fluid was aliquoted in small amounts and stored at -20°C.

2.5.10. Purification and isolation of IgG from ascitic fluid.

Ascitic fluid for each Mab was clarified by centrifugation in the MSE Micro Centaur at 11,600g for 10 min. The supernatant was carefully removed

and immediately applied to a column of Protein G Sepharose (Pharmacia) (approximately 5 ml volume in 1 cm diameter column) pre-equilibrated with 1 x PBS at approximately 18 ml/hr at 4°C. Bound IgG was recovered by eluting from the column with 0.1M glycine/HCl, pH 2.7. Fractions containing IgG were neutralised by addition of 0.6ml of 1M Tris.HCl to pH 7.0, pooled and then dialysed against PBS and concentrated in Sartorius collection bags (Sartorius Ltd). Protein concentration was measured at 280 nm and the purified IgG aliquoted in 0.5 ml amounts.

2.5.11. Conjugation of IgG with horseradish-peroxidase (HRP)

The conjugation of purified IgG of each Mab and rabbit anti-deer IgG was done by the periodate method as described by Wilson and Nakane (1978) using sodium meta-periodate ($NaIO_4$) (Sigma) and horseradish peroxidase (HRP) (Boehringer, Mannheim). Bovine serum albumin (Miles Laboratories) was added to a final concentration of 10 mg/ml, and aliquots were quickly frozen and stored at -20°C until required. Conjugations were kindly performed by Mr. A. Dawson.

2.6. Reagents and Solutions used

Unless stated otherwise all reagents were obtained from BDH Chemicals Ltd, Poole, Dorset or from Sigma (London) Ltd and were of analytical grade or better. Recipes of the solutions used in this study are given below:

2.6.1. ELISA STOCK REAGENTS

1. SODIUM CARBONATE (0.2M)

Sodium carbonate(anhydrous) (Na_2CO_3)
21.198 g make up to 1 litre with deionised water.
Place on stirrer to dissolve. Store at 4°C.

2. SODIUM HYDROGEN CARBONATE (0.2M)

Sodium hydrogen carbonate ($NaHCO_3$)
16.80 g make up to 1 litre with deionised water.
Place on stirrer to dissolve. Store at 4°C.

3. PHOSPHATE BUFFER 0.5M

Potassium dihydrogen orthophosphate (KH_2PO_4)
Di-potassium hydrogen orthophosphate(anhydrous)
also known as potassium phosphate K_2HPO_4

KH_2PO_4 12.63 g (0.093M)
K_2HPO_4 70.66 g (0.406M)

Deionised water to 1 litre.
Place on stirrer to dissolve. Store at 4°C.

4. EDTA 0.1M

Ethylenediaminetetraacetic acid (Disodium salt,Dihydrate).
(Na_2EDTA)
3.72 g make up to 100 mls with deionised water
Place on a stirrer to dissolve. Store at 4°C.

5. TWEEN 20 [10% SOLUTION]

Polyoxyethylene sorbitan monolaurate
(Tween 20)
100 mls make up to 1 litre with deionised water
Place on a stirrer to dissolve. Store at 4°C

2-hydroxypropane 1,2,3 -tricarboxylic acid-1-water
($C_3H_4(OH)(COOH)_3, H_2O$)

21.01 g make up to 1 litre with deionised water.
Place on a stirrer to dissolve. Store at 4°C

7. DI-SODIUM HYDROGEN ORTHOPHOSPHATE 0.2M

Di-sodium hydrogen orthophosphate (anhydrous)
(Na_2HPO_4)

28.39 g make up to 1 litre with deionised water
Place on a stirrer to dissolve. Store at 4°C

8. SULPHURIC ACID 2.5M

Sulphuric acid (concentrated) (H_2SO_4)

133 ml make up to 1 litre with deionised water
Store at room temperature

2.6.2. ELISA WORKING SOLUTIONS

1. COATING BUFFER - (CARBONATE/BICARBONATE 50 mM)

		(50 ml)
0.2 M Na_2CO_3	79 ml	3.95 ml
0.2 M $NaHCO_3$	171 ml	8.55 ml
Deionised water	750 ml	37.5 ml

pH to 9.6 (with 0.2M $NaHCO_3$)

2. WASH FLUID [PBST]

A) X10 PBS:-

Sodium Chloride (NaCl)	400g	800g
Potassium Chloride (KCl)	10g	20g
di-Sodium Hydrogen Orthophosphate (Na_2HPO_4)	57.5g	115g
Potassium diHydrogen Orthophosphate (KH_2PO_4)	10g	20g
Dissolve in Distilled Water	5000ml	10000ml

Mix well.
Store in refrigerator.

B) PBST:-

Dilute x10 PBS to x1 with distilled water.
Add TWEEN 20 at 0.05%.

3. SERUM & CONJUGATE DILUENT:-

Sodium Chloride (NaCl)	29 g	2.9g
0.5M Phosphate buffer	20 ml	2.0g
10% Tween 20	5 ml	0.5ml
0.1M EDTA	10 ml	1.0ml
Deionised water	make up to 1 litre	100 ml
pH to 7.2 (with 0.2M NaOH)		

* On day of test add

Ovalbumen (albumin, chicken egg)	5 g/litre

The ovalbumen is unstable in solution and has a deleterious effect on the solution if stored. The bulk diluent is therefore made up without albumen and stored at 4°C. When adding the ovalbumen make a paste with a small amount of solution then gradually add the rest. This is then filtered through No.1 Whatman (7.0cm) filter paper using a Buchner funnel (pre-wet filter paper with deionised water).

2.6.3. SDS-PAGE REAGENTS AND SOLUTIONS

1. 30% Acrylamide stock solution

Acrylamide	30g
N,N, Methylene bisacrylamide	0.8g
add distilled water	100 ml

2. 2x Laemmli stacking gel buffer, pH 6.8

Trisma base	6.06g
SDS	0.4g
add distilled water to	200ml

3. 4x Laemmli resolving gel buffer, pH 8.8

Trisma base	36.32g
SDS	0.8g
add distilled water to	200ml

4. Laemmli electrode buffer, pH 8.3

Tris 7-9	15.14g
Glycine	72.09g
SDS	5.0g
add distilled water to	5000ml

5. Sample buffer, pH 6.8

Glycerol	20g
SDS	4g
Bromophenol blue	2mg

Mercaptoethanol	5ml
Tris-HCl (1M)	25 ml
add distilled water to	100 ml

6. 3% polyacrylamide stacking gel

30% Acrylamide solution	2.4ml
2x Laemmli stacking gel buffer	12.0ml
Distilled water	9.6ml
10% Ammonium persulphate (APS)	150ul
Temed	20ul

7. 10% polyacrylamide resolving gel

30% Acrylamide solution	21.3ml
4x Laemmli resolving gel buffer	16.0ml
Distilled water	25.6ml
Sucrose	3.2g
10% Ammonium persulphate (APS)	150ul
Temed	20ul

Both the polyacrylamide stacking and resolving gels were degassed under vaccuum before polymerisation.

2.6.4. WESTERN BLOTTING REAGENTS AND SOLUTIONS

1. Electroblotting buffer, pH8.3

Tris 7-9	120g
Glycine	57.65g
Methanol	1000ml
add distilled water to	5000ml

2. Phosphate buffered saline (PBS) (x10)

Sodium chloride	400g
Potassium chloride	10g
di-sodium orthophosphate	57.5g
Potassium dihydrogen orthophosphate	10g
add distilled water to	5000ml

3. Washing fluid, pH7.2

1 x PBS	5000ml
Tween 20	25ml
di-Sodium ethylene diamine tetra acetic acid (EDTA)	1.85g
Sodium chloride	102.25g

CHAPTER 3

GENETIC DIFFERENCES AND SEROLOGICAL RELATIONSHIP AMONG THE FIVE RUMINANT ALPHAHERPESVIRUSES

3.1. Introduction

Since the introduction of restriction endonuclase (RE) analysis into herpes virology, the method has become widely used to differentiate between HSV-1 and HSV-2 (Wilkie *et al*, 1974; Hayward, *et al*, 1975; Lonsdale, 1979; Ueno *et al*, 1982; Sakaoka *et al*, 1987), and to discriminate isolates of other alphaherepsviruses including VZV (Hondo *et al*, 1987), SHV-1 (Nishimori *et al*, 1987), BHV-1 (Engels *et al*, 1981; 1987a; Misra *et al*, 1983; Brake and Studdert, 1985; Metzler *et al*, 1985; Osorio *et al*, 1985; Seal *et al*, 1985; Honda *et al*, 1989; Bulach and Studdert, 1990; Edwards *et al*, 1990; Hamelin *et al*, 1990; Miller *et al*, 1991), EHV-1 (Allen *et al*, 1983), feline herpesvirus-1 (FHV-1) (Grail and Harbour, 1990) and canine herpesvirus-1 (CHV-1) (Xuan *et al*, 1990). Comparison of common restriction sites between closely related viruses has been used to suggest evolutionary relationships between them (Brake and Studdert, 1985; Bulach and Studdert, 1990; Rimstad *et al*, 1992).

More recently the polymerase chain reaction (PCR) has allowed amplification of specific tracts of DNA from chosen viruses for more exacting comparisons. Banks (1993) used the PCR to discriminate between very closely related strains of SHV-1. Vilcek (1993) also showed that it is possible

to amplify a known BHV-1 DNA sequence by PCR. It was of interest to see if PCR using BHV-1 primers could be used to amplify DNA sequences of other alphaherpesviruses i.e. CapHV-2, RanHV-1 and CerHV-1 known to be antigenically related to BHV-1 (Nixon *et al*, 1988; Martin *et al*, 1990; Rimstad *et al*, 1992). Despite reports on the existence of antigenic relationships among the five ruminant alphaherpesviruses; only a few studies are available on the extent and identity of the proteins responsible for their relationships. The main capsid protein (VP4) and gI have been shown to be responsible for cross-reactivity between BHV-1 and CapHV-2 (Ackermann *et al*, 1986; Friedli and Metzler, 1987). Recently, Collins *et al* (1993) showed glycoproteins gI and gIV to be responsible for the cross-reactivity between BHV-1.1 and BHV-1.3.

This chapter reports experiments to confirm that the five viruses were different from each other by: 1) a comparison of the DNA profiles by RE, 2) the use of PCR for the amplification of DNA sequences of the five alphaherpesviruses using 22 bp primers selected from the published sequence of the immunologically important BHV-1 gI glycoprotein gene (HSV-1 gB homologue) (Whitbeck *et al*, 1988) followed by RE digestion of the amplified fragments. The chapter also describes an investigation of the extent of the serological relationships among the five ruminant alphaherpesviruses using ELISA, SNT and Western blotting. Rabbit hyperimmune sera, cattle, goat, red deer and reindeer convalescent field sera were used to investigate the quantitative cross-reactivity among these viruses.

3.2 Materials and Methods

3.2.1. Viruses and Cells

The plaque purified viruses examined in this study and their histories are listed in Table 2.1. A semicontinuous primary cell line of embryonic bovine trachea (EBTr) cells was used for virus growth as previously described (Section 2.1.1. and 2.1.5.). (Nettleton *et al*, 1983). The viruses were purified down a 10% to 25% Ficoll step gradient as described in Chapter 2.

3.2.2. Antibodies

Rabbit hyperimmune sera: Positive rabbit sera against each of the five viruses was prepared as previously described in Chapter 2. The negative sera were pre-inoculation sera from each rabbit.

Cattle and red deer field sera: the 40 cattle and 40 red deer field sera were samples submitted to the Moredun Research Institute for diagnostic analysis from different parts of Scotland. All sera were heat inactivated at 56°C for 30 min and stored in 1 ml aliquots at -20°C.

Cattle, goat, red deer and reindeer convalescent field sera: Field convalescent sera against BHV-1, CapHV-2, CerHV-1 and RanHV-1 were from cattle, goat, red deer and reindeer respectively. These were used in Western blotting and had been obtained from different sources as described in Chapter 2 (section 2.2).

3.2.3. Restriction endonuclease (RE) profiles and PCR

The materials and methods used for restriction endonuclease (RE) profiles and amplification of DNA sequences of the five ruminant alphaherpesviruses by PCR are described in detail in Chapter 2 (sections 2.4.6 and 2.4.7).

3.2.4. ELISA

Infected and non-infected cell lysates were used as the viral and control antigens respectively. The ELISA procedure is described in Chapter 2 (section 2.3.2). The horseradish peroxidase (HRP)-conjugates used were; goat anti-rabbit IgG conjugate (Sigma), rabbit anti-deer IgG (a gift from Dr. Steven Edwards, Weybridge) purified by DEAE cellulose ion exchange chromatography and conjugated by the periodate method, rabbit anti-bovine IgG conjugate and donkey anti-sheep/goat IgG conjugate (SAPU, Scotland). All conjugates and antigens were used at optimal predetermined dilutions. The corrected optical densities (OD_{492}) were obtained from the ELISA assays by taking the mean of duplicate wells containing infected cell lysate as antigen after subtracting the mean OD_{492} of duplicate wells containing a non-infected cell lysate control antigen.

Antigenic relationships were expressed by calculating the coefficient of antigenic similarity (R) (Archetti and Horsfall, 1950; Hubalek, 1982) according to the formula:

$$R = 100 \times \sqrt{\frac{\text{titre of strain I with antiserum II} \times \text{titre of strain II with antiserum I}}{\text{titre of strain I with antiserum I} \times \text{titre of strain II with antiserum II}}}$$

An R value ≤ 25 represents four-fold differences in titres between homologous and heterologous antisera indicating significant antigenic differences that are greater than would be expected from variations in the assay (Hubalek, 1982). An R value <5 has been used to assign viruses to different serotypes (Archetti and Horsfall, 1950).

3.2.5. Serum Neutralisation Test (SNT)

The serum neutralisation test was done as described in Chapter 2 (section 2.3.1) to determine the neutralising activity of rabbit homologous and/or heterologous serum against each of the five ruminant alphaherpesviruses (Table 2.1).

3.2.6. SDS-PAGE and Western blotting

SDS-PAGE was carried out in 10% discontinuous slab gels (Laemmli, 1970) under reducing conditions as described in detail in Chapter 2. Briefly, 200 ul of each of the alphaherpesvirus antigens was loaded in a 15cm central well of a gel flanked on each side with control antigen (50 ul) containing proteins of mock-infected cells and molecular weight marker standards. Electrophoresis was carried out overnight followed by electroblotting using a technique described by Burnette (1981) with modifications described by

Herring and Sharp (1984). The blot was cut into 5 mm wide strips and non-specific binding sites were blocked by PBS-containing Tween-20 for 60 min at room temperature. The strips were then probed with a 1:100 dilution of each of the field antisera from cattle, goat, red deer and reindeer, and 1:100 dilution of each of the homologous and heterologous rabbit hyperimmune sera prepared against each virus. After thorough washing, the nitrocellulose-bound immune complexes were reacted with a 1:1500 dilution of rabbit anti-bovine or goat anti-rabbit HRP conjugates for 1h at room temperature. The strips were finally incubated for 20 minutes in the dark at room temperature with 4 mg of 3,3'diaminobenzidine (DAB) (Sigma) in 100 ml of 0.05M Tris/HCl containing 10ul of 30% H_2O_2. The reaction was stopped by washing the strips in tap water.

3.2.7 Statistical analysis

Regression, correlation coefficient analysis, student paired t-test, and analysis of variance (ANOVA) were used to measure the relationships in ELISA OD_{492} between the five viruses.

3.3 Results

3.3.1. Restriction endonuclease (RE) profiles of viral DNA

Restriction endonuclease digestion of the five viral DNAs was carried out to confirm that the viruses which were being used were different from each other. The restriction endonuclease profiles of the five ruminant alphaherpesviruses and the two controls, EHV-1 and HSV-1 strain 17^+ are

shown in figure 3.1. Although the first two tracks appeared faint (especially track 2, which was amplified by photography) and HSV-1 DNA appears only partially digested (track 6) which could have been due to insufficient enzyme being used for digestion; there is a clear indication that the viruses used were different from each other.

3.3.2. Amplification of DNA sequences of five alphaherpesviruses by PCR

The polymerase chain reaction (PCR) was used to amplify a 468 bp fragment of each of the five alphaherpesviruses using 22 bp synthetic oligomers selected from the BHV-1 gI gene sequences as primers (Figure 3.2). All five amplified products were readily detectable on a 2.0% agarose gel following optimisation of the PCR reaction conditions. Successful amplification was achieved only with a combination of glycerol in the PCR buffer and an initial denaturation temperature of 96°C.

3.3.3. Specificity of amplification

Amplification with primers gI-P_1 and gI-P_2 produced the predicted 468 bp fragment of each of the five alphaherpesviruses (Figure 3.2). Since the polymerase chain reaction often produces non-specific amplification products (Saiki *et al*, 1988), it was necessary to confirm their specificity by restriction endonuclease digestion. Digestion of the amplification products with the four enzymes, BglI, HinfI, SmaI and AvaI produced the BHV-1.1 fragments predicted from the gI gene sequences (Figure 3.3). The three amplification

Figure 3.1. Electrophoretic patterns of BglII restriction enzyme digests of ^{32}P labelled DNA from BHV-1.1 (lane 1), BHV-1.2 (lane 2), CapHV-2 (lane 3), CerHV-1 (lane 4), RanHV-1 (lane 5), HSV-1 strain 17^{+} (lane 6) and EHV-1 (lane 7). The samples were run in adjacent wells of the same gel: the tracks have been separated to allow labelling of the fragments in individual wells.

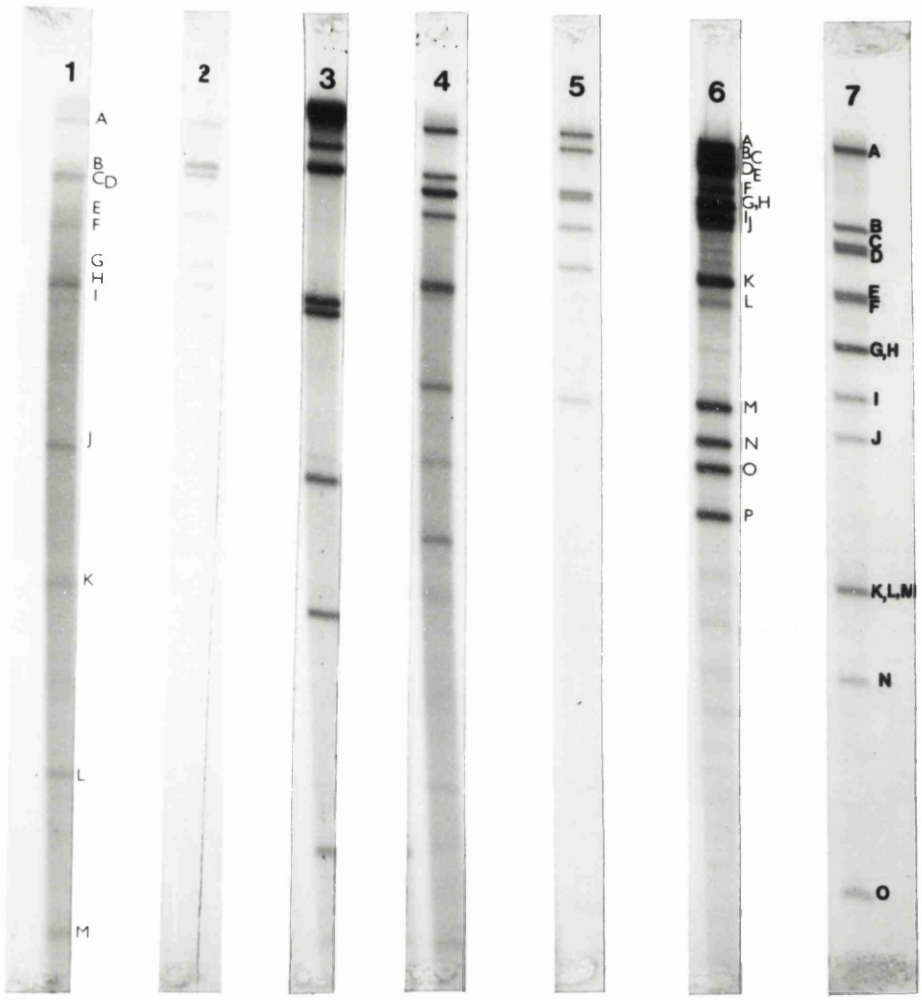

1
A
B
C
D
E
F
G
H
I
J
K
L
M
2
3
4
5
6
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
7
A
B
C
D
E
F
G,H
I
J
K,L,M
N
O

Figure 3.2. PCR amplification products of gI sequences for the five alphaherpesviruses analysed on a 2% agarose gel containing ethidium bromide. Amplified product from BHV-1.1 (lane 1), BHV-1.2 (lane 2), Molecular weight markers (M_r) (lane 3), RanHV-1 (lane 4), CapHV-2 (lane 5), CerHV-1 (lane 6), Bovine viral diarrhoea virus (BVD) cDNA control (lane 7). The DNA bands were visualised by ultraviolet light.

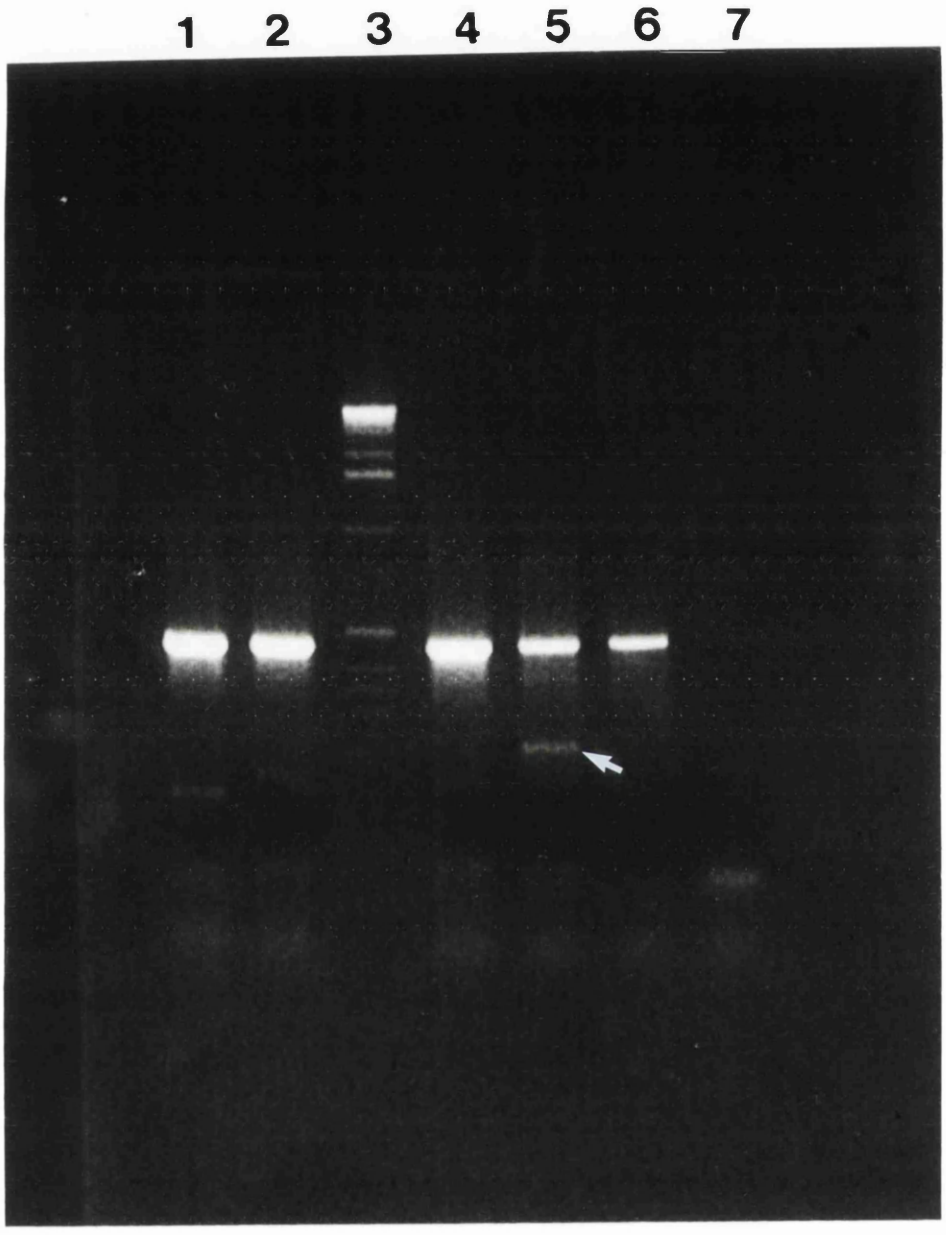
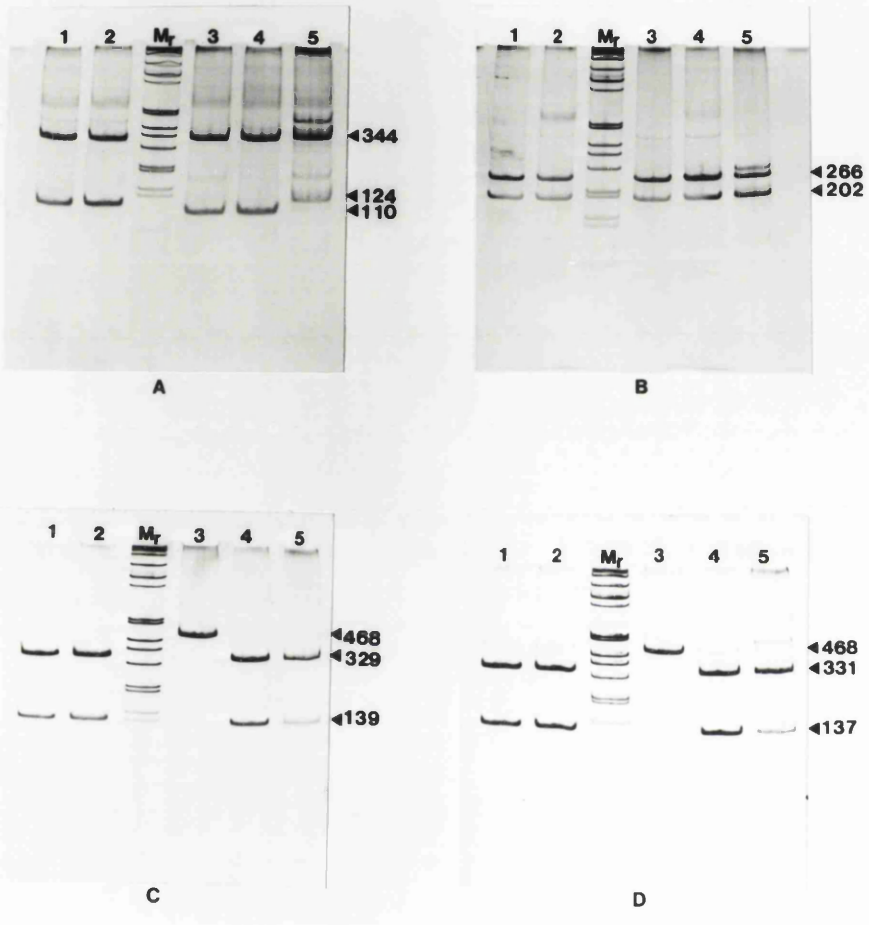

1
2
3
4
5
6
7

Figure 3.3. PCR specificity of the amplified products for the five alphaherpesviruses digested with restriction enzymes BglI (A), HinfI (B), SmaI (C) and AvaI (D) analysed by 10% SDS-PAGE and visualised by silver staining. Digested amplification products from BHV-1.1 (lane 1), BHV-1.2 (lane 2), RanHV-1 (lane 3), CerHV-1 (lane 4), CapHV-2 (lane 5) and Molecular weight markers (Mr).


1
2
Mr
3
4
5
344
124
110
A
1
2
Mr
3
4
5
266
202
B
1
2
Mr
3
4
5
468
329
139
C
1
2
Mr
3
4
5
468
331
137
D

products (panel B) for BHV-1.2, CerHV-1 and RanHV-1 contained the same fragments following digestion with HinfI as were generated from the BHV-1.1 fragment. Two additional bands were evident in CapHV-2 (panels A, B, track 5) : that above the 344 bp fragment (panel A and B) most probably represents undigested 468 bp DNA whilst that above the 124 bp fragment (panel A) and above the 266 bp fragment (panel B) is probably the minor product of amplification arrowed (Fig. 3.2, track 5). However, the sizes of the other two major bands (344bp and 124 bp) corresponded to those of BHV-1.1, BHV-1.2. CerHV-1 and RanHV-1 contained slightly different BglI restriction sites from those of the other three (panel A), while BHV-1.1, BHV-1.2, RanHV-1, CerHV-1 and CapHV-2 contained an identical HinfI site and BHV-1.1, BHV-1.2, CerHV-1 and CapHV-1 contained SmaI (panel C) and AvaI (panel D) restriction sites. The RanHV-1 amplification product did not contain SmaI and AvaI restriction sites (panels C and D, lanes 3). The results indicated that the five viruses could be placed into three groups: the first containing BHV-1.1, BHV-1.2 and CapHV-2, the second and third comprised RanHV-1 and CerHV-1 respectively. Six other enzymes (HpaI, HindIII, PstI, DdeI, BglII and PvuI) were used to try to discriminate between the three viruses in group one but none cut within the fragment.

3.3.4. Analysis of antigenic relationships by ELISA and SNT.

Initially, optimum conditions were established by checkerboard titrations using the five viral antigens ranging from a dilution of 1/10 to 1/3200, and conjugate dilutions from 1/100 to 1/102400. Optimal results with specific

binding were achieved with antigen dilutions of 1/100 for all viruses and the three conjugates at a dilution of 1/1500. Using the rabbit hyperimmune sera against each virus comparison of the homologous and heterologous reactions revealed considerable cross-reactivity (Fig. 3.4A-3.4E). The reaction of each antiserum was strongest against the virus used for immunisation of the rabbits with the exception of the two BHV-1 viruses which were very similar (Fig 3.4B and 3.4C). The titre of each rabbit hyperimmune polyclonal serum with each virus in the ELISA and SNT are given in Table 3.1A and 3.1B respectively. In two cases there was an indication of a one way reactivity with the heterologous viruses. Sera raised against RanHV-1 and CapHV-2 virus react strongly with lysates of cells infected with the two BHV-1 viruses in the ELISA, whereas sera raised against the 2 BHV-1 viruses reacted weakly with RanHV-1 and CapHV-2 in both the ELISA and SNT. As shown in Table 3.1C the coefficients of antigenic similarity were calculated in order to compare the similarity of the five viruses to each other using the ELISA titres. The values obtained for each pair of viruses indicate that (1) the two bovine herpesviruses are most closely related, (2) the cervine and caprine herpesviruses appear to be more closely related to the two bovine herpesviruses than they were to each other and (3) the rangiferine virus appeared to be more closely related to the cervine virus than to the bovine or caprine viruses.

When convalescent field sera were used the cognate anti-species conjugate generally gave the best results, although there was cross-reactivity with the

Figure 3.4A-E. Titration of rabbit homologous and heterologous polyclonal sera raised against five ruminant alphaherpesviruses against lysates of cells infected with each of the five viruses. Antigens: A- caprine herpesvirus-2 (CapHV-2) B- bovine herpesvirus-1.1 (BHV-1.1), C- bovine herpesvirus-1.2 (BHV-1.2), D- cervine herpesvirus (CerHV-1), and E- rangiferine herpesvirus-1 (RanHV-1). Results from the five sera from rabbits immunised with: BHV-1.1 (△), BHV-1.2, (○) CapHV-2 (▽), CerHV-1 (▲) and RanHV-1 (●). Optical density values (OD_{492nm}) represent the positive viral antigen minus the respective negative control antigen. Each optical density value represents the average of two replicates.

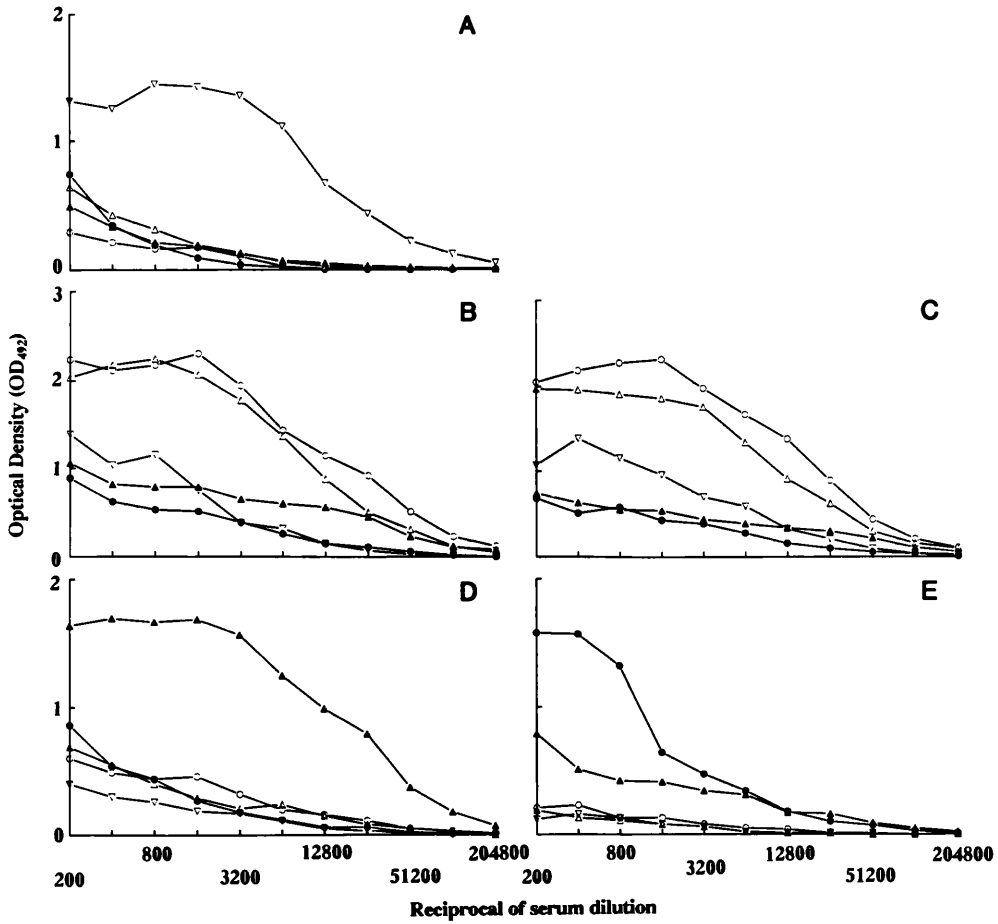

A
B
C
D
E
Optical Density (OD_{492})
2
1
0
3
2
1
0
200
800
3200
12800
51200
204800
Reciprocal of serum dilution

TABLE 3.1A Antibody titres[a] of rabbit homologous and heterologous polyclonal antisera with the five ruminant alphaherpesviruses

Polyclonal Antisera Raised against	Antigens				
	BHV-1.1	BHV-1.2	CapHV-2	CerHV-1	RanHV-1
BHV-1.1	102400	102400	3200	12800	800
BHV-1.2	204800	204800	3200	25600	1600
CapHV-2	12800	51200	102400	6400	800
CerHV-1	102400	102400	3200	102400	25600
RanHV-1	25600	25600	1600	6400	25600

[a]ELISA titre expressed as the reciprocal of the highest serum dilution giving an OD_{492} of ≥ 0.1
Homologous polyclonal antisera are underlined

TABLE 3.1B. Serum neutralisation antibody titres of rabbit homologous and heterologous antisera with the five ruminant alphaherpesviruses

Polyclonal Antisera Raised against	Antigens				
	BHV-1.1	BHV-1.2	CapHV-2	CerHV-1	RanHV-1
BHV-1.1	90	256	11	3	<2
BHV-1.2	64	360	8	6	2
CapHV-2	3	3	1024	11	8
CerHV-1	45	16	45	2048	32
RanHV-1	32	45	32	90	128

TABLE 3.1C Antigenic relationships among 5 ruminant alphaherpesviruses by ELISA (R values)

Virus	BHV-1.1	BHV-1.2	CapHV-2	CerHV-1	RanHV-1
BHV-1.1	100				
BHV-1.2	100	100			
CapHV-2	6	9	100		
CerHV-1	35	35	4	100	
RanHV-1	9	9	2	25	100

TABLE 3.2 **Comparison of three different conjugates with 1/50 dilutions of one field bovine serum and one red deer serum against five ruminant alphaherpesviruses**

	OPTICAL DENSITY (OD_{492})									
	Bovine sera (Q3842)					Deer Sera (Q523)				
Conjugates	BHV-1.1	BHV-1.2	CapHV-2	CerHV-1	RanHV-1	BHV-1.1	BHV-1.2	CapHV-2	CerHV-1	RanHV-1
Rabbit anti-bovine HRP at 1:1500	2.36	2.39	1.05	1.64	1.23	1.52	1.38	0.35	1.74	0.64
Donkey anti-ovine HRP at 1:1500	0.67	0.56	0.08	0.21	0.18	0.46	0.44	0.06	0.56	0.19
Rabbit anti-cervine HRP at 1:1500	1.19	0.99	0.17	0.40	0.31	1.61	1.32	0.41	1.66	0.81

three anti-ruminant IgG conjugates and especially between the bovine and red deer conjugates which detected deer sera equally well (Table 3.2). The analysis of pairs of OD_{492} readings among the five viruses for each bovine and red deer field serum (Table 3.3a and 3.4a) indicate significant correlations between the OD_{492} values for all viruses (Table 3.3b and 3.4b) respectively. In each case the paired t-test revealed significant differences ($P \leq 0.001$) between the OD_{492} of each pair of viruses except between those of the two BHV-1 viruses which were not significantly different ($P > 0.05$).

Figure 3.5A to 3.5C and 3.5D to 3.5F show the results of viral antigen titrations against 3 bovine and 3 red deer field sera respectively (Table 3.5a and 3.5b). In each case it appears that the relative reactivities of the different sera with antigen are largely independent of the antigen concentration but reflect their antigenic similarity.

3.3.5. Analysis of viral antigens by Western blotting

The immune reactions of the viral antigens of all five viruses with both homologous and heterologous rabbit hyperimmune sera and convalescent antisera from cattle, goat, red deer and reindeer were analysed by Western blotting. Figure 3.6 shows the polypeptides which were recognized from cells infected with each of the five viruses by the polyclonal sera. The convalescent sera hardly recognised any proteins from any viral-infected cells. However several weak bands with apparent molecular weights of 200, 68-70, 45 kDa in CapHV-2; 87-90, 68-74, 55, 50, 46, 42 kDa in CerHV-1 and 180,

TABLE 3.3a ELISA reactivity of 40 field bovine sera against five ruminant alphaherpesviruses

Serum No.	Sample Identification Number	OD_{492} produced against antigens					
		BHV-1.1	BHV-1.2	CapHV-2	CerHV-1	RanHV-1	Bovine viral diarrhoea virus (BVD) (control)
1	Q3937	2.36	2.27	0.38	0.81	0.50	0.00
2	Q3911	2.38	2.31	0.52	1.50	0.60	0.00
3	Q3910/4	2.34	2.31	0.51	1.47	1.19	0.00
4	Q3910/3	2.30	2.25	0.46	0.74	0.49	0.00
5	Q3910/2	2.25	2.27	0.41	0.60	0.36	0.01
6	Q3910/1	2.28	2.13	0.34	0.61	0.61	0.01
7	Q3877	2.38	2.35	0.68	1.06	0.90	0.00
8	Q3865/2	2.39	2.38	0.37	0.96	0.50	0.02
9	Q3858/5	2.42	2.40	0.27	0.64	0.57	0.00
10	Q3842	2.42	2.38	0.67	1.33	0.87	0.00
11	Q3825/3	2.11	2.13	0.32	0.80	0.54	0.00
12	Q3825/1	1.37	1.35	0.17	0.37	0.26	0.02
13	Q3814/1	2.24	2.15	0.48	0.82	0.50	0.01
14	Q3811/6	0.94	1.04	0.12	0.25	0.12	0.00
15	Q3811/5	1.23	1.22	0.11	0.34	0.17	0.01
16	Q3811/4	1.39	1.38	0.10	0.34	0.08	0.01
17	Q3777/5	0.65	0.57	0.05	0.15	0.09	0.01
18	Q3777/4	2.00	1.98	0.32	0.66	0.43	0.02
19	Q3761/8	2.21	1.90	0.62	1.31	0.80	0.00
20	Q3761/7	2.12	2.05	0.40	0.83	0.54	0.02
21	Q3761/6	2.09	1.88	0.50	1.01	0.76	0.02
22	Q3761/5	1.85	2.13	0.56	1.19	0.92	0.00
23	Q3761/4	2.19	2.09	0.40	1.12	0.72	0.00
24	Q3761/3	1.92	2.26	0.47	1.11	0.85	0.02
25	Q3761/2	2.33	2.29	0.52	0.88	0.68	0.00
26	Q3761/1	2.29	2.29	0.69	1.06	0.70	0.00
27	Q3747	1.80	1.87	0.16	0.65	0.42	0.00
28	Q3746	1.85	1.76	0.23	0.56	0.46	0.00
29	Q3744	1.90	1.64	0.12	0.53	0.33	0.01
30	Q3741/2	1.13	1.25	0.12	0.28	0.17	0.01
31	Q3741/1	2.12	2.25	0.37	0.79	0.74	0.01
32	Q3740/14	1.16	1.17	0.07	0.31	0.15	0.01
33	Q3740/13	2.21	2.17	0.29	0.63	0.41	0.02
34	Q3740/12	0.74	0.84	0.08	0.20	0.06	0.01
35	Q3740/11	0.94	1.07	0.08	0.32	0.09	0.01
36	Q3740/10	1.58	1.45	0.15	0.39	0.25	0.00
37	Q3740/7	1.78	1.87	0.30	0.66	0.43	0.00
38	Q3740/6	2.30	2.19	0.59	1.21	0.95	0.00
39	Q3740/9	2.41	2.40	0.64	1.40	1.09	0.00
40	Q3740/8	2.35	2.23	0.38	0.82	0.66	0.00

TABLE 3.3b. Correlation coefficients of OD_{492} readings of 40 field bovine sera as determined by ELISA with pairs of ruminant alphaherpesviruses.

Virus	BHV-1.1	BHV-1.2	CapHV-2	CerHV-1	RanHV-1
BHV-1.1	1.000				
BHV-1.2	0.972	1.000			
CapHV-2	0.799	0.809	1.000		
CerHV-1	0.779	0.788	0.902	1.000	
RanHV-1	0.774	0.796	0.883	0.920	1.000

TABLE 3.4a ELISA reactivity of 40 field red deer sera against five ruminant alphaherpesviruses.

Serum No.	Sample Identification Number	OD_{492} produced against antigens					
		BHV-1.1	BHV-1.2	CapHV-2	CerHV-1	RanHV-1	Bovine Viral Diarrhoea Virus (BVD) (control)
1	Q521	1.77	1.67	0.56	1.74	0.75	0.00
2	Q522	1.63	1.54	0.42	1.46	0.72	0.00
3	Q523	1.96	1.97	0.75	1.96	1.23	0.01
4	Q524	1.90	1.76	0.58	1.78	1.08	0.00
5	Q527	1.76	1.66	0.44	1.45	1.01	0.01
6	Q528	1.92	1.85	0.78	1.96	1.21	0.00
7	Q529	1.93	1.87	0.80	2.00	1.40	0.01
8	Q530	0.64	0.75	0.04	1.01	0.19	0.00
9	Q531	1.55	1.58	0.33	1.71	0.56	0.00
10	Q532	2.10	2.06	0.92	2.08	1.46	0.00
11	Q534	0.06	0.24	0.00	0.09	0.00	0.00
12	Q535	2.11	2.08	0.80	2.04	1.42	0.00
13	Q536	1.48	1.45	0.42	1.67	0.70	0.01
14	Q537	2.09	2.08	1.15	2.13	1.62	0.00
15	Q538	1.74	1.51	0.39	1.66	0.91	0.00
16	Q540	1.93	1.90	0.63	1.96	1.41	0.00
17	Q541	2.21	2.19	0.81	2.02	1.40	0.00
18	Q542	1.82	1.79	0.60	1.95	0.89	0.00
19	Q543	2.04	2.05	0.76	2.03	1.23	0.00
20	Q544	2.05	2.17	0.91	2.07	1.59	0.00
21	Q545	1.84	1.82	0.65	1.90	1.01	0.00
22	Q546	1.60	1.58	0.51	1.80	0.99	0.00
23	W547	1.64	1.40	0.33	1.64	0.72	0.00
24	Q548	1.86	1.83	0.45	1.82	0.87	0.00
25	Q549	1.68	1.68	0.75	1.61	1.10	0.00
26	Q550	1.77	1.69	0.84	1.81	1.32	0.00
27	Q551	1.85	1.74	0.63	1.94	0.82	0.00
28	Q553	1.73	1.77	0.79	1.98	1.31	0.00
29	Q554	1.18	1.11	0.31	1.36	0.68	0.01
30	Q555	1.70	1.59	0.45	1.82	0.94	0.00
31	Q556	2.34	2.32	0.95	2.27	1.47	0.00
32	Q557	1.86	1.69	0.37	1.84	0.62	0.00
33	Q558	2.19	2.12	0.94	2.16	1.44	0.00
34	Q559	0.91	0.90	0.12	1.28	0.17	0.00
35	Q560	2.25	2.20	0.80	2.21	1.39	0.00
36	Q561	1.56	1.55	0.35	1.68	0.84	0.00
37	Q562	1.95	1.97	0.76	1.92	1.39	0.00
38	Q563	2.06	1.97	0.80	1.93	1.47	0.00
39	Q552	0.05	0.19	0.03	0.15	0.00	0.00
40	Q564	2.03	2.06	0.58	2.17	1.01	0.00

TABLE 3.4b. Correlation coefficients of OD_{492} readings of 40 field deer sera as determined by ELISA with pairs of ruminant alphaherpesviruses.

Virus	BHV-1.1	BHV-1.2	CapHV-2	CerHV-1	RanHV-2
BHV-1.1	1.000				
BHV-1.2	0.988	1.000			
CapHV-2	0.842	0.871	1.000		
CerHV-1	0.964	0.966	0.815	1.000	
RanHV-1	0.877	0.898	0.951	0.837	1.000

Figure 3.5A-F. Titration of ruminant alphaherpesvirus antigens: BHV-1.1 (△), BHV-1.2 (○), CapHV-1 (▽), CerHV-1 and (▲) RanHV-1 (●) against three different field bovine (A-C) and red deer (D-F) sera by ELISA. Optical density values (OD_{492nm}) represent the positive viral antigen minus the respective negative control antigen. Each optical density value represents the average of two replicates.

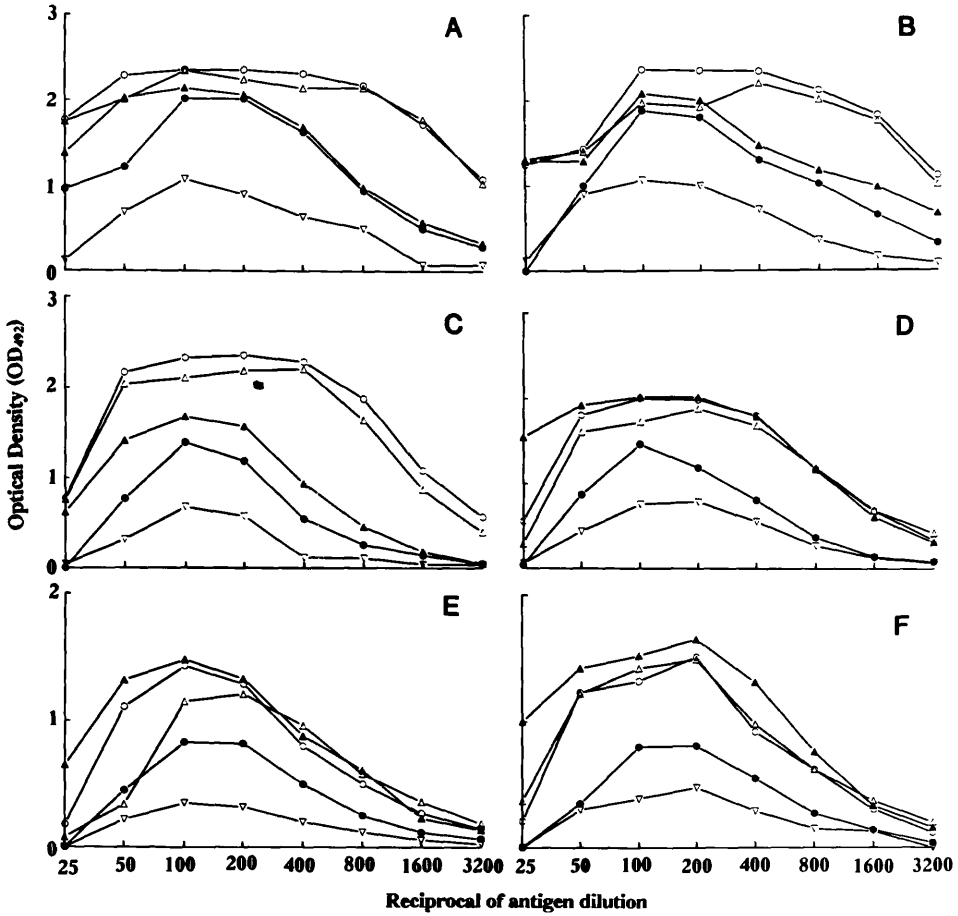

A
B
C
D
E
F
Optical Density (OD_{492})
Reciprocal of antigen dilution
25 50 100 200 400 800 1600 3200

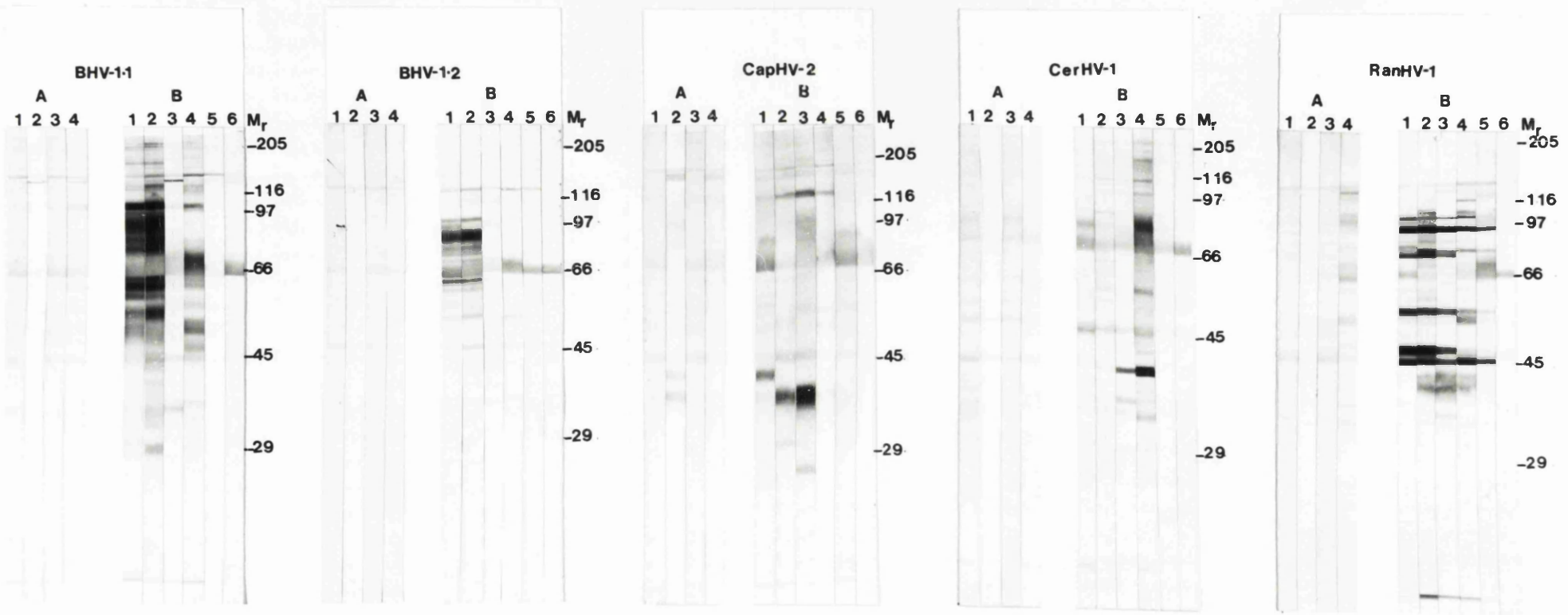
TABLE 3.5a. Titration of 5 ruminant alphaherpesvirus antigens against 3 bovine field sera (Q3910, Q3842, Q3865) by ELISA

Reciprocal of Antigen Dilution	Antigens OD_{492} Bovine sera (diluted at 1:50); rabbit anti-bovine HRP at 1:1500														
	BHV-1.1			BVH-1.2			CapHV-2			CerHV-1			RanHV-1		
	Q3910	Q3842	Q3865	Q3910	Q3842	Q3865	Q3910	Q3842	Q3865	Q3910	Q3842	Q3865	Q3910	Q3842	Q3865
25	1.75	1.31	0.76	1.77	1.25	0.77	0.12	0.12	0.04	1.38	1.29	0.62	0.96	0.00	0.00
50	2.01	1.40	2.04	2.29	1.43	2.17	0.69	0.90	0.32	2.03	1.29	1.41	1.22	1.00	0.77
100	2.35	1.97	2.11	2.36	2.36	2.33	1.08	1.07	0.68	2.15	2.08	1.68	2.02	1.88	1.39
200	2.25	1.92	2.19	2.36	2.35	2.36	0.90	1.00	0.58	2.07	2.00	1.57	2.02	1.80	1.19
400	2.15	2.20	2.21	2.32	2.34	2.29	0.64	0.72	0.12	1.69	1.47	0.94	1.63	1.30	0.55
800	2.15	2.01	1.64	2.18	2.12	1.88	0.49	0.36	0.11	0.98	1.80	0.46	0.94	1.02	0.26
1600	1.78	1.76	0.87	1.72	1.83	1.08	0.07	0.17	0.04	0.57	0.98	0.18	0.49	0.65	0.14
3200	1.03	1.01	0.40	1.80	1.12	0.57	0.07	0.09	0.04	0.32	0.67	0.05	0.28	0.32	0.05

TABLE 3.5b. Titration of 5 ruminant alphaherpesvirus antigens against 3 red deer field sera (Q521, Q523, Q524) by ELISA.

Reciprocal of Antigen Dilution	Antigens OD_{492} Deer sera (diluted at 1:50); rabbit anti-deer HRP at 1:1500														
	BHV-1.1			BVH-1.2			CapHV-2			CerHV-1			RanHV-1		
	Q521	Q523	Q524	Q521	Q523	Q524	Q521	Q523	Q524	Q521	Q523	Q524	Q521	Q523	Q524
25	0.37	0.29	0.08	0.21	0.56	0.18	0.00	0.06	0.00	1.00	1.54	0.65	0.00	0.04	0.00
50	1.22	1.60	0.34	1.23	1.80	1.11	0.30	0.44	0.22	1.42	1.92	1.32	0.35	0.87	0.45
100	1.42	1.72	1.15	1.32	2.00	1.43	0.39	0.76	0.35	1.52	2.02	1.48	0.80	1.46	0.83
200	1.49	1.88	1.21	1.51	1.99	1.29	0.48	0.79	0.32	1.65	2.02	1.33	0.81	1.19	0.82
400	0.98	1.68	0.96	0.92	1.80	0.80	0.29	0.56	0.20	1.31	1.79	0.88	0.55	0.81	0.50
800	0.62	1.19	0.58	0.62	1.17	0.50	0.15	0.27	0.12	0.76	1.17	0.61	0.17	0.37	0.25
1600	0.37	0.69	0.36	0.30	0.68	0.27	0.13	0.06	0.06	0.33	0.61	0.23	0.16	0.14	0.12
3200	0.20	0.42	0.19	0.12	0.34	0.15	0.00	0.07	0.03	0.16	0.31	0.14	0.04	0.08	0.07

Figure 3.6. Western blot of cattle (A_1), goat (A_2), red deer (A_3), reindeer (A_4) convalescent field sera and rabbit hyperimmune sera against BHV-1.1 (B_1), BHV-1.2 (B_2), CapHV-2 (B_3), CerHV-1 (B_4), RanHV-1 (B_5) and normal rabbit sera (B_6). Each serum was reacted with detergent extracts (NP-40) of BHV-1.1, BHV-1.2, CapHV-2, CerHV-1 and RanHV-1 infected cell antigens resolved by 10% SDS-PAGE under reducing conditions and transferred to the nitrocellulose strips shown on each of the five panels of 10 strips. M_r are molecular weight markers in kilodaltons (kDa).


BHV-1·1
BHV-1·2
CapHV-2
CerHV-1
RanHV-1
A
B
1 2 3 4
1 2 3 4 5 6
Mr
205
116
97
66
45
29

90, 46 kDa in RanHV-1 were recognized by the anti-BHV-1 serum. The caprine convalescent serum weakly recognized polypeptides of 130 kDa in the two BHV-1 viruses, polypeptides 200 kDa, a 180 kDa doublet, 97 kDa, 43 kDa and 38 kDa in CapHV-2, and hardly recognized any proteins in CerHV-1 or RanHV-1 viruses. The cervine and reindeer convalescent sera weakly recognized polypeptides in BHV-1.1 and BHV-1.2 infected cells with molecular weights ranging from 130 kDa to 45 kDa. The CerHV-1 sera reacted weakly with polypeptides in cells infected with its homologous virus and RanHV-1 while the RanHV-1 sera showed a stronger reactivity with its homologous virus and with more proteins than will all heterologous viruses.

The rabbit hyperimmune sera were in general far more reactive than convalescent sera. A summary of the molecular weights of recognised polypeptides is presented in Table 3.6, but a few general points may be made. The two anti BHV-1 hyperimmune sera reacted with more BHV-1.1 than BHV-1.2 polypeptides. The spectrum of polypeptides from BHV-1.1 infected cells recognised by both anti-BHV-1.1 and BHV-1.2 sera was very similar as was the spectrum from BHV-1.2 infected cells recognised by both anti-BHV-1.1 and BHV-1.2 infected cells. Few BHV-1.1 and BHV-1.2 polypeptides were recognized by the CapHV-2 and RanHV-1 rabbit hyperimmune sera. The CerHV-1 rabbit hyperimmune sera recognised more BHV-1.1 than BHV-1.2 polypeptides. In contrast, there were more CapHV-2 and RanHV-1 polypeptides recognized by the two BHV-1 hyperimmune sera. Whereas RanHV-1 hyperimmune sera showed a poor recognition of CerHV-1

TABLE 3.6. Summary of antigenic reactivity of ruminant alphaherpesvirus polypeptide antigens against their homologous rabbit hyperimmune sera as visualised by Western blotting (see Figure 3.6).

Virus	Approximate molecular weights (kDa)
BHV-1.1	205, 180, 130, 116, 97-108, 87-90, 74-77, 64-66, 55, 45, 43, 30, 28
BHV-1.2	120, 108, 87-90, 74-77, 66-70, 65, 64, 63, 55, 46
CapHV-2	200, 180, 130, 108, 97, 90-66, 55, 46, 40, 33, 28
CerHV-1	205, 180, 130, 120, 108, 100, 90-70, 65, 55, 46, 42, 40, 33
RanHV-1	130, 112, 97-108, 74, 70-64, 46

polypeptides, more RanHV-1 polypeptides were recognized by the CerHV-1 hyperimmune sera. Finally, there was marked and strong recognition of the RanHV-1 polypeptides by all the four heterologous hyperimmune sera.

3.4. Discussion

3.4.1. Restriction endonuclease profiles

The restriction endonuclease profiles with BglII of the five ruminant alphaherpesviruses indicated that the viruses were different from each other. The reasons for the poor radiolabelling of the two BHV-1 viruses was not immediately apparent. Perhaps the virus titre was lower than expected. Harvesting of the viruses at different times after labelling would possibly have helped to improve the amount of radiolabelled DNA in these samples.

3.4.2. Analysis of viral DNA by PCR

Polymerase chain reaction (PCR) was used to amplify a 468 bp fragment of the five ruminant alphaherpesviruses using 22 bp oligomers from the BHV-1 gI gene sequences (Whitbeck *et al*, 1988) as primers. A successful amplification was achieved after optimising the PCR reaction conditions. Particularly important were inclusion of glycerol in the PCR buffer and initial denaturation of the viral DNA's at an elevated temperature of 96°C as suggested by Vilcek (1993) perhaps necessary because of a high G + C content. For several of the PCR reaction conditions, slight deviations from the optimal values can result in reduced or undetectable amplification (Saiki *et al*, 1988). Restriction enzyme digestion was used to verify the specificity of the

amplified fragments since PCR often produces non-specific amplification products that may cross-hybridize with the desired product or may coincidentally be of the same size as the expected fragment (Saiki *et al*, 1988). The results indicated that the five alphaherpesviruses examined contained gI sequences that were amplified and that at least three different viruses were present. Additional enzymes were used in an attempt to discriminate between BHV-1.1, BHV-1.2 and CapHV-2 but none cut the amplified products. This was perhaps not surprising as it is probable that the PCR fragments for these three viruses contained sequences with similar restriction sites for many of the enzymes used, as the BHV-1 gI gene selected for amplification (the HSV gB gene homologue) is known to be highly conserved amongst the herpesviruses (Hammerschmidt *et al*, 1988; Borchers *et al*, 1991; Griffin, 1991). Although the PCR fragment analysis failed to unambigously establish the difference in the five viruses, together with the RE profiles of whole viral genomes, it provided evidence to indicate that all five viruses were different from each other.

3.4.3. Antigenic relationships by ELISA and SNT

The present study reveals evidence of a wide cross-reactivity as shown by both heterologous rabbit polyclonal and field sera on each of the five alphaherpesviruses. The results indicate antigenic similarity among these viruses. Indeed, ELISAs and SNT performed in these studies clearly show that a close serological relationship exists between the bovine, caprine, cervine and reindeer alphaherpesviruses. The results suggest that (1) the two bovine

herpesviruses are most closely related, (2) the cervine and caprine viruses are serologically more related to the closely related BHV-1 viruses than they are to each other and (3) the rangiferine herpesvirus appeared to be more closely related to the cervine virus than to the bovine or caprine virus. Rimstad *et al* (1992), Nixon *et al* (1988) and Martin *et al* (1990) obtained serological results compatible with the present study. Furthermore, the results indicate that the rangiferine virus is more closely related to the cervine virus than to the caprine virus. The existence of the cross-reactivities among these viruses in the present data supports the previous views by Nettleton and others (1986) that the widespread evidence of antibodies to CerHV-1 in wild and farmed red deer in Britain (overall prevalence 29%) is likely to be due to infection with CerHV-1 rather than BHV-1. In addition, while most antigens displayed cross-reactivities between pairs of viruses, a number of one-way reactivities between heterologous viruses were also noted. The latter phenomenon was observed between the two BHV-1 viruses and the caprine virus and also between the BHV-1 viruses and the rangiferine virus (RanHV-1). One-way reactivity between BHV-1 and CapHV-2 was previously reported by others (Ackermann *et al*, 1986; Friedli and Metzler, 1987). Recently, Rimstad *et al*, (1992) reported a one-way reactivity between BHV-1 and RanHV-1. The results indicated that a weak one-way reactivity in one or other direction could not have been a function of the sensitivity of the test system used but rather a reflection of their antigenic similarities. Furthermore, it has been shown that at least some of the cross-reacting antibodies bind to the viral proteins VP4 and gI (Ackermann *et al*, 1986; Metzler *et al*, 1985).

Whether the one-way reactivities observed between species examined in this study were really due to epitopes of the same polypeptide merits further investigation, particularly with Western blotting and epitope mapping by competitive ELISAs with monoclonal antibodies.

3.4.4. Antigenic relationships by Western blotting

Analysis of rabbit polyclonal and host field sera for the five ruminant alphaherpesvirus antibodies in Western blots revealed a wide range of cross-reacting antibodies. While a marked strong reactivity of each polyclonal serum against the homologous virus was observable, there was also a marked variation of reactivity of each polyclonal serum against the heterologous virus. Whereas the two BHV-1 polyclonal sera recognized more of CapHV-2 and RanHV-1 proteins, far fewer of the two BHV-1 proteins were recognized by either CapHV-2 or RanHV-1 polyclonal sera. This result suggests a one-way reactivity which has also been reported by others (Ackerman *et al*, 1986; Friedli and Metzler, 1987; Rimstad *et al*, 1992). The cross-reacting antibodies were mainly directed against glycoproteins with molecular weights 130 kDa, 95 kDa, 68-74 kDa, 55 kDa and 45 kDa (figure 3.6). The 130 kDa, 74 kDa and 55 kDa proteins have been previously identified to be components of glycoprotein gI in BHV-1 (van Drunen *et al* 1986). These results support the recent findings of Engels *et al* (1992) and Koptopoulos (1992), that the cross-reacting antibodies were mainly induced by glycoprotein gI (HSV gB homologue), the major capsid protein and several non-structural proteins,

whereas glycoproteins gIII and gIV (HSV gC and gD homologues respectively) were found to induce virus-specific antibodies (Ackermann *et al*, 1986; Friedli and Metzler, 1987). In contrast, Collins *et al* (1993) recently showed glycoproteins gIII and gIV to be responsible for the cross-reactivity between BHV-1.1 and BHV-1.3. The reactivities of cattle, goat, red deer and reindeer field sera with BHV-1, CapHV-2, CerHV-1 and RanHV-1 antigens in the Western blots confirms the viral origins of these antigens. As glycoprotein gI is known to be the most conserved within the whole herpesvirus group (Hammerschmidt *et al*, 1988) cross-reacting antibodies were to be expected. HSV gC homologues have been shown to exhibit the most virus- and strain-specific properties (Ben-Porat *et al*, 1986; Friedli and Metzler 1987; Allen and Coogle, 1988).

CHAPTER 4

PRODUCTION AND CHARACTERISATION OF MONOCLONAL ANTIBODIES AGAINST CERVINE HERPESVIRUS TYPE 1

4.1. Introduction

Antibodies have been used widely in the laboratory diagnosis of a wide range of diseases caused by viruses as well as in detailed investigations of virus structure. However, serological assays have always had problems of interpretation, reproducibility and standardisation, resulting partly from the unavoidable heterogeneity of the antibodies in the test. Since Kohler and Milstein (1975) introduced the hybridoma technology for generating monoclonal antibodies (Mab), which overcomes these limitations, both the application and usefulness of immunoassays has greatly expanded. This technology has also significantly improved the quality and reliability of serological assays. It has also made identification and analysis of viral proteins easier.

The general procedure and practice which has now become established is the use of a myeloma cell line, which grows in culture but is deficient in the enzyme hypoxanthine phosphoribosyl transferase (HGPRT), as a fusion partner with spleen cells of previously immunised animals. After fusion, cells are grown in a selective culture medium supplemented with hypoxanthine, aminopterin and thymidine (HAT). In this medium the main pathway of

cellular DNA synthesis is blocked by aminopterin, while in addition, the alternative or "salvage" pathway cannot be carried out in the myeloma cells because of their deficiency in HGPRT. Thus only the hybrids between the myeloma and spleen cells survive in the HAT medium where the spleen cells supply functional HGPRT enzyme and the myeloma cells supply the ability to grow in tissue culture. The hybrid cell lines or hybridomas grow continuously in culture. These hybridomas are thereafter screened for their secretion of specific antibody. Several workers have produced Mabs against BHV-1 (Collins *et al*, 1984; Metzler *et al*, 1985; Chang *et al*, 1986; Fitzpatrick *et al*, 1990b; Shen *et al*, 1991; Baranowski *et al*, 1993.). There is no description of production of Mabs against the other ruminant alphaherpesviruses.

This chapter describes the production and characterisation of monoclonal antibodies against one of the five ruminant alphaherpesviruses namely CerHV-1.

4.2. Materials and Methods

The materials and methods for the production of monoclonal antibodies against CerHV-1 are described in detail in Chapter 2 (section 2.5).

4.2.1. Serum Neutralisation Test (SNT)

Microneutralisation tests were carried out as described in Chapter 2 to determine the neutralising activity of each Mab against cervine herpesvirus-1 (CerHV-1).

4.2.2. SDS-PAGE and Western blotting

SDS-PAGE of each of the five alphaherpesviruses and Western blotting of all Mabs to determine their protein specificities were carried out as previously described in Chapter 2.

4.2.3. Indirect Fluorescent Antibody Test (IFAT) for CerHV-1 Mabs.

Embryonic bovine tracheal cells (EBTr) at 2 x 10^5 cells per ml were grown in E'199' medium on coverslips in 10 ml tubes and incubated at 37°C until monolayered. The tubes and coverslips were washed twice in warm Hanks balanced salt solution. Duplicate tubes were either mock-infected or infected each with 0.2 ml (m.o.i. of 10) of CerHV-1, and incubated at 37°C for 1 hour. One ml of E'199' medium containing 2% foetal bovine serum (FBS), 100 IU/ml penicillin, 100 ug/ml streptomycin and 1 mM glutamine were added and incubated at 37°C for 4, 8, 12, 16, 20 and 24 hours (for a time course appearance of proteins recognised by the Mabs). All coverslips were washed twice in warm PBS and placed on labelled blotting paper to air dry. The coverslips were then fixed in neat cold acetone for 10 minutes and 0.1 ml of each Mab at a previously determined appropriate dilution was added to each coverslip and incubated at 37°C for 30 minutes in a moist atmosphere. All coverslips were washed three times in warm PBS for 10 minutes using fresh PBS each time. Coverslips were allowed to air dry and 0.1 ml of sheep anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) at a predetermined dilution was added and incubated at 37°C for 30 minutes in a

moist atmosphere. Coverslips were washed three times in warm PBS for 10 minutes, allowed to air dry and then mounted on slides using phosphate buffered glycerol (pH 8.2) before being examined under a Leitz Ortholux 2 ultraviolet microscope.

4.3. RESULTS

4.3.1. Characterisation of hybridomas

The screening ELISA method was simple, sensitive and capable of testing more than two hundred hybridoma cell culture supernatant samples at a time. Out of the five fusions performed, only the first and last were successful. The second, third and fourth fusions failed to produce any positive hybridomas possibly due to a toxic batch of PEG being used. A total of 515 wells containing HAT resistant hybridomas were obtained from the two successful fusions from which 32 wells were shown to contain hybridomas producing anti-CerHV-1 specific antibody (Table 4.1). Of these 32 hybridomas selected for analysis, 14 clones were propagated successfully with the remainder growing poorly or being lost due to overgrowth with non-secreting hybridomas during subcloning. The 14 hybridomas were successfully subcloned three times by the limiting dilution method using initially RPMI 1640 medium with 20% MTM as a growth-supporting factor. The hybridomas were weaned off MTM in the second subcloning. MTM was also not used during their expansion in 25 cm^2 flasks. Both the duration of production and amount of ascitic fluid in mice injected with the hybrid cells appeared to be a growth characteristic of each individual clone. Abdominal

TABLE 4.1. A summary of five fusions and hybridomas produced.

Fusion number	No. of myeloma cells used	No. of immunised spleen cells used	No. of wells with HAT-resistant hybridomas	No. of positive hybridomas established	No. of monoclonal antibodies obtained[a]
1	1.90×10^7	1.01×10^8	351	18	6
2	2.80×10^7	2.92×10^8	0	0	0
3	2.40×10^6	2.38×10^7	0	0	0
4	2.50×10^7	2.56×10^8	12	0	0
5	2.05×10^7	2.05×10^8	164	14	8

[a]Cloned three times by limiting dilution

TABLE 4.2. Immunoglobulin isotype of antibodies secreted by hybridomas

Mab designation	[a]Isotype IgG1	IgG2a	IgG2b
2G8		+	
4D7			+
4F10			+
4G8	+		
6C10	+		
8B11	+		
1G7		+	
2C3		+	
3B6		+	
3C6		+	
3D9		+	
3E5		+	
5C2		+	
6E9		+	

[a]All are K light chains

swelling developed between 2-3 weeks after i/p inoculation. All of the 14 selected Mabs were of the IgG class with K light chains. Three of the 6 Mabs obtained in the first fusion were IgG1 (4G8, 6C10 and 8B11), 1 IgG2a (2G8) and 2 IgG2b (4D7 and 4F10). All of the 8 Mabs from the second successful fusion were of the IgG2a isotype (Table 4.2). The 14 Mabs were titrated in parallel in an ELISA to construct binding affinity curves, where all of them were observed to have good binding affinities. However the three IgG1 Mabs (4G8, 6C10 and 8B11) showed higher and similar ELISA binding affinities (Figure 4.1 and Table 4.3).

4.3.2. Quantification and conjugation of Mabs to HRP

Although ascites can be used directly in tests or assays without any purification, it was decided to purify Mabs for conjugation to HRP as a standard reagent for further experimental work to avoid possible complications caused by unknown reactivities of other, non Ig, HRP-conjugated ascitic proteins. The immunoglobulin content of ascitic fluid from 9 Mabs which had each produced 2-4 mls of ascites per mouse were quantified (Table 4.4) and conjugated to HRP.

4.3.3. Protein specificities of the monoclonal antibodies in Western blots.

The protein specificities of the Mabs were determined by Western blotting. The resolved CerHV-1 proteins on the nitrocellulose strips were probed with each of the Mabs present in the appropriately diluted ascitic fluid. A notable variation in the reactivity of the 14 Mabs with polypeptides from

Figure 4.1. Binding affinity curves of the 14 CerHV-1 Mabs (4G8, 8B11, 6C10, 4D7, 1G7, 4F10, 3E5, 2G8, 2C3, 5C2, 6E9, 3C6, 3B6 and 3D9) (ascites) against homologous virus in an indirect ELISA.

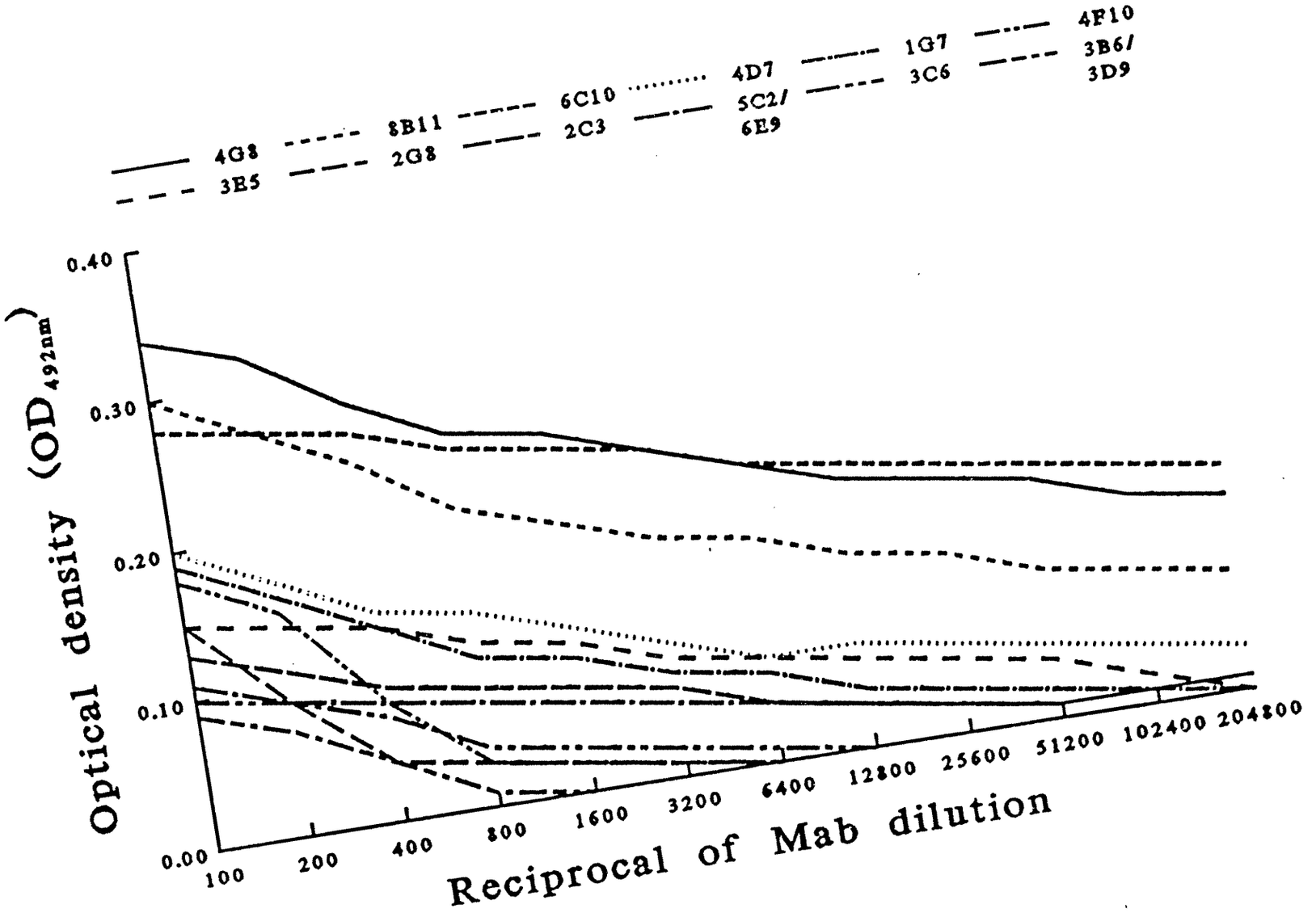

4G8
3B5
8B11
2G8
6C10
2C3
4D7
5C2/
6E9
1G7
3C6
4F10
3B6/
3D9
Optical density (OD_{492nm})
0.40
0.30
0.20
0.10
0.00
100
200
400
800
1600
3200
6400
12800
25600
51200
102400
204800
Reciprocal of Mab dilution

Table 4.3. Binding characteristics of mabs (ascites) to CerHV-1 in an indirect ELISA

Reciprocal of Mab Dilution	Mabs OD_{492}													
	4G8	8B11	6C10	4D7	1G7	4F10	3E5	2G8	2C3	5C2	6E9	3C6	3B6	3D9
100	0.34	0.30	0.28	0.20	0.19	0.18	0.15	0.15	0.13	0.11	0.10	0.10	0.09	0.09
200	0.32	0.27	0.27	0.17	0.16	0.15	0.14	0.09	0.11	0.09	0.09	0.09	0.07	0.07
400	0.28	0.24	0.26	0.14	0.13	0.08	0.13	0.04	0.09	0.08	0.08	0.07	0.04	0.04
800	0.25	0.20	0.24	0.13	0.10	0.03	0.11	0.03	0.08	0.07	0.07	0.04	0.01	0.01
1600	0.24	0.18	0.23	0.11	0.09	0.02	0.10	0.02	0.07	0.06	0.06	0.03	0.00	0.00
3200	0.22	0.16	0.22	0.09	0.07	0.01	0.08	0.01	0.06	0.05	0.05	0.02	0.00	0.00
6400	0.20	0.15	0.20	0.07	0.06	0.00	0.07	0.00	0.04	0.04	0.04	0.01	0.00	0.00
12800	0.18	0.13	0.19	0.07	0.04	0.00	0.06	0.00	0.03	0.03	0.03	0.00	0.00	0.00
25600	0.17	0.12	0.18	0.06	0.03	0.00	0.05	0.00	0.02	0.02	0.02	0.00	0.00	0.00
51200	0.26	0.10	0.17	0.05	0.02	0.00	0.04	0.00	0.01	0.01	0.01	0.00	0.00	0.00
10240	0.14	0.09	0.16	0.04	0.01	0.00	0.02	0.00	0.01	0.01	0.01	0.00	0.00	0.00
20480	0.13	0.08	0.15	0.03	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.00	0.00	0.00

TABLE 4.4. Purification of ascitic fluid containing some of the monoclonal antibodies showing the amount of immunoglobulin recovered per ml of ascitic fluid

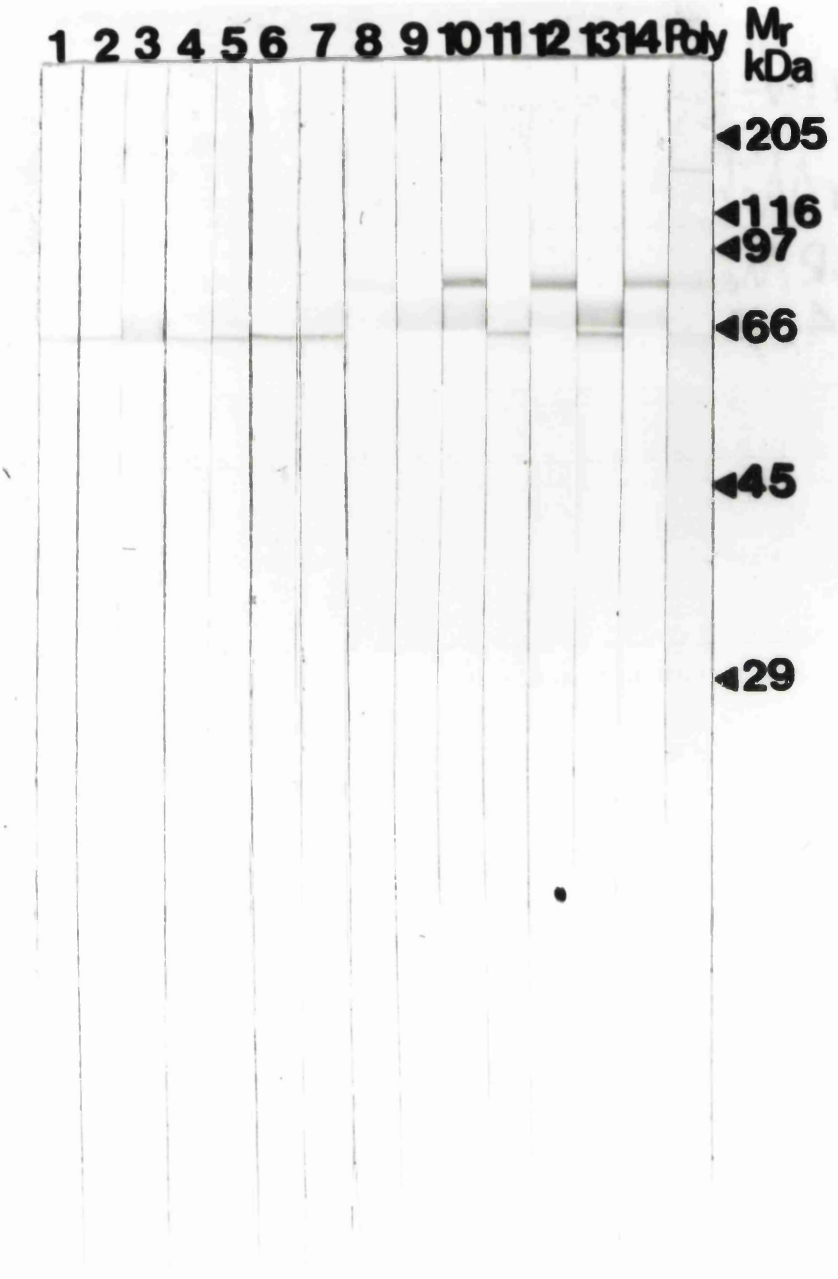
Mab designation	Istotype	Amount of immunoglobulin per ml of ascites
1G7	IgG2a	3.40 mg
3B6	IgG2a	1.42 mg
3C6	IgG2a	1.08 mg
3D9	IgG2a	3.92 mg
4D7	IgG2b	6.48 mg
4F10	IgG2b	5.90 mg
4G8	IgG1	3.53 mg
6C10	IgG1	2.23 mg
8B11	IgG1	3.34 mg

CerHV-1-infected cell extracts under reducing conditions (Figure 4.2) was observed. Of these 14 Mabs, 4 Mabs (1G7 -lane 1, 2C3 - lane 2, 3B6 - lane 4, 3D9 - lane 6) reacted with the 64kDa polypeptide while 3 Mabs (3E5 - lane 7, 5C2 - lane 11, 6D9 - lane 13) reacted with both the 64 kDa polypeptide and diffusely with a 68-70kDa polypeptide. Mab 2G8 (lane 3) reacted with a 64-69 kDa polypeptide. Mab 4F10 (lane 9) showed a diffuse reaction with a 68-70 kDa polypeptide. Mab 3C6 (lane 5) reacted with the 64 kDa polypeptide and also appeared to react faintly with one of 74kDa. The remaining 4 Mabs (4D7 - lane 8, 4G8 - lane 10, 6C10 - lane 12 and 8B11 - lane 14) reacted with the 74kDa polypeptide and a diffuse 68-70 kDa band. Mouse anti-CerHV-1 polyclonal serum which was included as a control in the Western blots reacted weakly with 130kDa, 74kDa and 64 kDa polypeptides of the same size as those recognised by the CerHV-1 Mabs. None of the Mabs or the mouse anti-CerHV-1 polyclonal serum recognized any bands in extracts of mock-infected cells.

4.3.4. Reactivity of Mabs in SNT and ELISA.

The Mabs were also characterised in terms of their reactivity in SNT and ELISA. The Mabs which were directed against the 64 kDa, 64-69 kDa, 68-70 proteins showed no neutralising activity against CerHV-1, whereas the four Mabs which were directed against the 74 kDa and 68-70 kDa had similar neutralising activity with titres $\geq$1:256. The Mabs showed varying optical density (OD_{492}) values in the ELISA where two Mabs (2C3, and 5C2) had the lowest OD_{492} values of $\geq$0.1 to 0.19, eight Mabs (1G7, 3C6, 3D9, 3E5, 4F10,

Figure 4.2. Western blot of CerHV-1 Mabs with a detergent extract of CerHV-1 infected cells electrophoresed through a 10% SDS-polyacrylamide gel under reducing conditions and transferred to nitrocellulose. Mabs tested were 1G7, 2C3, 2G8, 3B6, 3C6, 3D9, 3E5, 4D7, 4F10, 4G8, 5C2, 6C10, 6E9 and 8B11 (lanes 1 to 14) and mouse anti-CerHV-1 serum (Poly). M_r are molecular weight markers.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 Poly
Mr kDa
205
116
97
66
45
29

4G8, 6C10 and 8B11) had OD_{492} values of ≥0.3 and the remaining four Mabs (2G8, 3B6, 6E9 and 4D7) had intermediate OD_{492} values of ≥0.2 to 0.29 (Table 4.5).

4.3.5. Immunofluorescence staining of Mabs in IFAT.

EBTr cells grown on coverslips and infected with CerHV-1 at a moi of 0.1 pfu/cell were fixed at 20h or at 0, 6, 12, 18 and 24h post infection for the time course experiment. The cultures on the coverslips fixed at 20h post infection were probed with all 14 Mabs. The coverslips were fixed at 20h post infection so as to provide some uninfected cells among the infected cell to act as negative controls. For the time course experiment, eight selected Mabs representing the two Mab groups were used to probe the fixed cultures to study both their patterns of IF staining and time course appearance of the proteins recognized by these Mabs.

The results obtained in these tests are summarised in Tables 4.6 and 4.7 and the typical IF appearance is shown in a single photograph in Fig. 4.3. The bulk of photographs for the IF staining were not obtained because of an unrecognised fault in the camera. Mabs which reacted with the 64 kDa protein showed predominantly a weak diffuse fluorescence which was distributed on the surface of infected cells. Only 4F10 showed in addition a diffuse cytoplasmic fluorescence, while no fluorescence was observed with Mab 2G8. The Mabs which recognized the 74 kDa and 68-70 kDa proteins had a more intense fluorescence distributed diffusely both on the surface of

TABLE 4.5. Monoclonal antibodies to CerHV-1; neutralising activity reactivity in an ELISA test and Western blots

Mab designation	Isotype[a]	SNT[b]	ELISA test[c]	Polypeptide specificity in Western blots (kDa)
1G7	IgG2a	<2	+++	64
2C3	IgG2a	8	+	64
3B6	IgG2a	<2	++	64
3C6	IgG2a	<2	+++	64, 74
3D9	IgG2a	<2	+++	64
3E5	IgG2a	8	+++	64, 68-70
5C2	IgG2a	<2	+	64, 68-70
6E9	IgG2a	<2	++	64, 68-70
2G8	IgG2a	8	++	64-69
4F10	IgG2b	<2	+++	68-70
4D7	IgG2b	>256	++	68-70, 74
4G8	IgG1	256	+++	68-70, 74
6C10	IgG1	256	+++	68-70, 74
8B11	IgG1	256	+++	68-70, 74

[a]All are K light chains
[b]Titres expressed as the reciprocal of the highest serum dilution resulting in a 50% reduction of plaques relative to a virus control
[c]OD_{492} +≥0.1; ++≥0.2; +++ ≥0.3

TABLE 4.6. Immunofluorescence staining of acetone-fixed CerHV-1 infected cells using 14 Mabs

Protein specificity (kDa)	Mab	IF in fixed CerHV-1 infected cells		
		Intensity	Distribution	Pattern
	1G7	+	S	D
	2C3	+	S	D
	2G8	-	-	-
64 kDa	3B6	+	S	D
	3C6	+	S	D
	3D9	+	S	D
	3E5	+	S	D
	4F10	++	S, C	D
	5C2	+	S	D
	6E9	+	S	D
74 kDa	4D7	++	S,C	D
	4G8	++	S,C	D
	6C10	++	S,C	D
	8B11	++	S,C	D

+ is a subjective indication of the degree of fluorescence intensity
S - surface
C - cytoplasm
D - diffuse

TABLE 4.7. Time course appearance of the 64kDa and 74kDa proteins as shown by the IF staining of CerHV-1 infected cells with 8 selected Mabs.

Protein Mab	64 kDa				74 kDa				Poly-clonal
Time (hrs)	1G7	3B6	3D9	3E5	4D7	4G8	6C10	8B11	
0	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	+
12	-	-	-	-	-	-	-	+	++
16	+	+	+	+	+	+	+	++	+++
20	++	+ +	+ +	+ +	+	+ +	+ +	++	+++
24	+++	+++	+++	++ +	++	+ +++	++ +	+ ++	+++

Note + is a subjective indication of the degree of fluorescence

Figure 4.3. Typical immunofluorescent staining pattern of the CerHV-1 Mabs (4F10, 4D7, 4G8, 6C10, 8B11) in an indirect immunofluorescent test (IFAT) at 20h post infection. Infected cells appear fluorescent among a dark background of non-infected cells.

infected cells and within the cytoplasm (Table 4.6).

The appearance of the two proteins in the time course experiment was shown to be from 12hr for the 74 kDa and 68-70 kDa proteins and 16 hrs for the 64 kDa, 64-69, 68-70 kDa proteins. There was an indication of an increase in amount of these proteins with time as shown by the increased intensity in fluorescence (Table 4.7).

4.4. DISCUSSION

4.4.1. Production of Mabs

The low proportion of successful fusions (2 out of 6) which were obtained in this study highlights the inherent problems associated with monoclonal antibody production. Tedder, Yao and Anderson (1982) and others emphasise how small variations in the technique, such as the use of different batches of PEG and/or the use of feeder cell layers, can enhance or decrease the number of antibody positive clones. Of 32 positive hybridomas only 14 were successfully cloned three times. Several clones (1G7, 2C3, 2G8, 5C2) grew very slowly and yielded less ascitic fluid with lower immunoglobulin content per ml as compared to the rest which grew faster yielding more ascitic fluid. These differences appeared to be a growth characteristic of each individual clone.

4.4.2. Isotypes

Isotype determination of the Mabs was important because it affected their

behaviour and choice of reagents in subsequent experiments. Knowledge of their isotypes was particularly relevant when choosing the detection systems for Western blotting and during purification of Mabs from ascitic fluids.

All 14 clones were identified as single isotypes lending support for the adequacy and efficiency of the subcloning which was carried out and their stability.

4.4.3. Protein specificity and reactivity of Mabs in ELISA and IFAT.

Mabs produced by four hybridomas (4D7, 4G8, 6C10 and 8B11) had similar high neutralising titres (Table 4.5) and binding characteristics (Figure 4.1 and Table 4.3). Three of them were of the IgG1 and one was of the IgG2b isotype. The IgG2b Mab had a higher neutralisation titre than those of the IgG1 isotype. The lack of neutralising capability of the other Mabs could have been due to the stringent conditions of 100 $TCID_{50}$ of the virus used. Mabs representing all isotypes of IgG which had complement-requiring or enhanced neutralising activities have been reported against other alphaherpesviruses (Collins *et al*, 1984; Highlander *et al*, 1987; Horimoto *et al*, 1990; Okazaki *et al*, 1986; Shen *et al*, 1991).

Of the ruminant alphaherpesvirus structural proteins, data from BHV-1 studies have shown four major glycoproteins to be present on the virus envelope and plasma membrane of BHV-1 infected cells (van Drunen Littel-van den Hurk *et al*, 1984; Collins *et al*, Marshall *et al*, 1986; Hughes *et*

al, 1988; Baranowski *et al*, 1993). These are: gI, a 130kDa disulphide-linked 74/55kDa heterodimer; gII, a 108kDa glycoprotein; gIII a 180kDa/91kDa dimeric glycoprotein and gIV, a 140kDa/71kDa dimeric glycoprotein. It has been shown further that gI, gIII and gIV all stimulate the production of neutralising antibodies in mice, rabbits and cattle and they all serve as targets for antibody-dependent complement-mediated lysis of virus-infected cells (Collins *et al*, 1984; van Drunen Littel-van den Hurk *et al*, 1984; van Drunen Littel-van den Hurk and Babiuk, 1985; Marshall *et al*, 1986; Okazaki *et al*, 1986; Trepanier *et al*, 1986; Israel *et al*, 1988; van Drunen Littel-van den Hurk *et al*, 1990). These glycoproteins are homologues of the HSV glycoproteins gB, gC and gD respectively. Glycoproteins gI and gIV have also been shown to be the primary targets for neutralising and protective antibodies (Babiuk *et al*, 1987). Four Mabs (1G7, 2C3, 3B6, 3D9) recognized a cell-associated glycoprotein of 64kDa perhaps similar to that reported earlier for BHV-1 by Metzler *et al* (1985). The reactivity of Mab 3E5, 5C2, 6E9 with the 64 kDa and a 68-70 kDa band was quite apparent as was the reactivity of 4F10 to the diffuse 68-70 kDa protein band. The reactivity of one Mab to more than one protein has also been observed by other workers (Collins *et al* 1984). Whether the multiple glycoproteins recognized by these Mabs were related was not investigated. The multiple reactivity is unlikely to be due to antibodies or two or more specificities since the hybridoma cell lines had been cloned. The IF staining patterns produced by the Mabs and their protein specificities bear similarities to those shown elsewhere for BHV-1 (Shen *et al*, 1991). The four Mabs for the 74kDa and 68-70 kDa proteins gave

an even diffuse distribution on the cell surface and cytoplasm, whereas there was an almost exclusively diffuse surface staining with the 64kDa Mabs.

CHAPTER 5

THE USE OF MONOCLONAL ANTIBODIES IN THE ANALYSIS OF RUMINANT ALPHAHERPESVIRUS ANTIGENS

5.1. Introduction

There is an increasing awareness of the difficulties experienced in distinguishing the ruminant alphaherpesviruses due to their close serological relationships. The viruses are related structurally and antigenically; infection with one virus induces to varying extents, antibodies reactive with other viruses in this group (Pastoret *et al*, 1988). The five viruses cannot easily be differentiated by tests which rely on the detection of antibody using polyclonal sera. However, monoclonal antibodies directed against the virus specific antigens like BHV-1.1, gIII or gIV (Friedli and Metzler, 1987; Collins *et al*, 1993) have enabled differentiation of BHV-1.1 from BHV-1.3 or SHV-1.

To facilitate the differential diagnosis of the five ruminant alphaherpesvirus antigens or infections the reactivity of the panel of Mabs produced in the present study against each of the five viruses was investigated. The information about their reactivity was then used to select Mabs with the potential for differentiating these viruses from each other. Several Mabs were selected to investigate whether the two CerHV-1 proteins, with molecular weights of 64kDa and 74kDa, to determine if these proteins were glycosylated

and to see if they were homologues of known BHV-1 proteins.

5.2. Materials and Methods

5.2.1. Cells and viruses

The five viruses (Table 2.1, Chapter 2) were grown in a semi- continuous cell line of embryonic trachea cells (EBTr) as described in Chapter 2.

5.2.2. Antibodies

All 14 Mabs were used to determine their reactivity to the 5 ruminant alphaherpesviruses by ELISA. Three Mabs directed against the 64kDa (3B6, 3C6, 4F10) and the 4 Mabs against the 74kDa protein (4D7, 4G8, 6C10, 8B11) were selected to try to define epitopes on the two proteins and to identify analogous/homologous proteins in BHV-1.1. A bovine viral diarrhoea (BVD) Mab VPM22 was included as a control.

5.2.3. ELISA with Mabs against the five viruses

The ELISA procedures described in Chapter 2 with some minor modifications were used to determine the reactivity of each Mab with each of the five alphaherpesviruses as described below. Briefly, infected and non-infected cell lysates of each alphaherpesvirus (Table 2.1, Chapter 2) were used as the viral and control antigens respectively for coating a 96-well polyvinyl plate (Cooke M129A Dynatech Labs) overnight at 4°C. Wells in column one were used as a blank in each plate. A two-fold dilution of each

Mab (ascitic fluid) was done in rows for each plate and incubated for 2 hours at room temperature in a humid chamber. Appropriately diluted sheep anti-mouse IgG conjugated to HRP was added to all wells except those in column one, and incubated for 1hr at room temperature. Orthophenylene diamine (OPD) (Sigma) in citrate buffer, pH 5.0 was added as substrate. The reaction was stopped by addition of 2.5M H_2SO_4 and the optical density was read at 492nm.

5.2.4. Radioimmunoprecipitation of CerHV-1 and BHV-1.1 antigens.

Radiolabelled antigens were prepared and the radioimmunoprecipitation procedure was performed as described in Chapter 2. [^{3}H]-glucosamine-labelled CerHV-1 and BHV-1.1 antigens were each immunoprecipitated with Mabs 3B6, 3C6 and 4F10 (for 64kDa) and Mabs 4D7, 4G8, 6C10, 8B11 (for 74kDa). The immune complexes were analysed by SDS-PAGE and autoradiography as described previously.

5.2.5. Competitive binding assay (CBA)

A modified enzyme-linked immunosorbent assay (ELISA) described previously (Lyaku *et al,* 1990) was carried out for two groups of Mabs; 3B6, 3C6 and 4F10 (specific for 64kDa) and 4D7, 4G8, 6C10 and 8B11 (specific for 74kDa). Bovine viral diarrhoea (BVD) Mab VPM22 was used as a control. From a starting dilution of 1:100, 100ul of serial two-fold dilutions of each Mab were added to columns of wells of a flat-bottomed microelisa plate

coated with CerHV-1 viral antigen at a dilution of 1:100 (determined previously by checkerboard titration) and incubated at 37°C for 1 hour. After three washings with PBST-20, 100 ul of horseradish peroxidase (HRP)-labelled Mab at a predetermined dilution of 1:100 which gave optimum OD_{492} was added to each well containing the competing Mab. The conjugated Mab was also added to control wells without the competing Mab. After another three washes with PBST-20, 100ul of OPD as substrate solution containing 10ul of 30% H_2O_2 was added to all wells and incubated at 37°C for 20 minutes in the dark. The reaction was stopped with 2.5M H_2SO_4 solution and the OD_{492} was determined. The amount of competitive binding inhibition was estimated from the OD_{492} in the presence or absence of unlabelled competing Mabs.

5.3. Results

5.3.1. Reactivity of the Mabs against the five alphaherpesviruses

The reactivity of each Mab with the five ruminant alphaherpesviruses is shown in Table 5.1. The Mabs show a wide range of serological reactivity indicating their potential for the differentiation of these viruses. Particularly noteworthy are two Mabs (3D9 and 3E5) which cross-reacted with all five ruminant alphaherpesviruses; two Mabs (4D7 and 8B11) reacted exclusively with CerHV-1; 3 Mabs (2G8, 4F10 and 4G8) reacted with all viruses except BHV-1.1 and one Mab (6E9) which reacted with all viruses except RanHV-1. Caution must be exercised in the interpretation of this table as although 4

TABLE 5.1. ELISA reactivity patterns of the 14 Mabs with the ruminant alphaherpesviruses in the ELISA

Mab designation	Isotype	Ruminant alphaherpesvirus antigens				
		BHV-1.1	BHV-1.2	CapHV-2	RanHV-1	CerHV-1
3D9	IgG2a	++	+	+	+	+++
3E5	IgG2a	++	+++	+	+	+++
2G8	IgG2a	-	+	+	+	++
4F10	IgG2b	-	+	+	++	+++
4G8	IgG1	-	+	++	++	+++
3C6	IgG2a	+	-	++	+	+++
2C3	IgG2a	++	-	-	++	+
3B6	IgG2a	+	-	-	++	+
6E9	IgG2a	++	+	+	-	++
1G7	IgG2a	+++	+	-	-	+++
5C2	IgG2a	+	-	-	-	+
6C10	IgG1	+	-	-	-	+++
4D7	IgG2b	-	-	-	-	++
8B11	IgG1	-	-	-	-	+++

- negative result OD_{492} <0.1

OD_{492} + ≥0.1, ++ ≥ 0.2, +++ ≥ 0.3

categories of reactivity have been defined (+++, ++, + and -) adjacent categories, might not be significantly different i.e. +++ might not be different from ++.

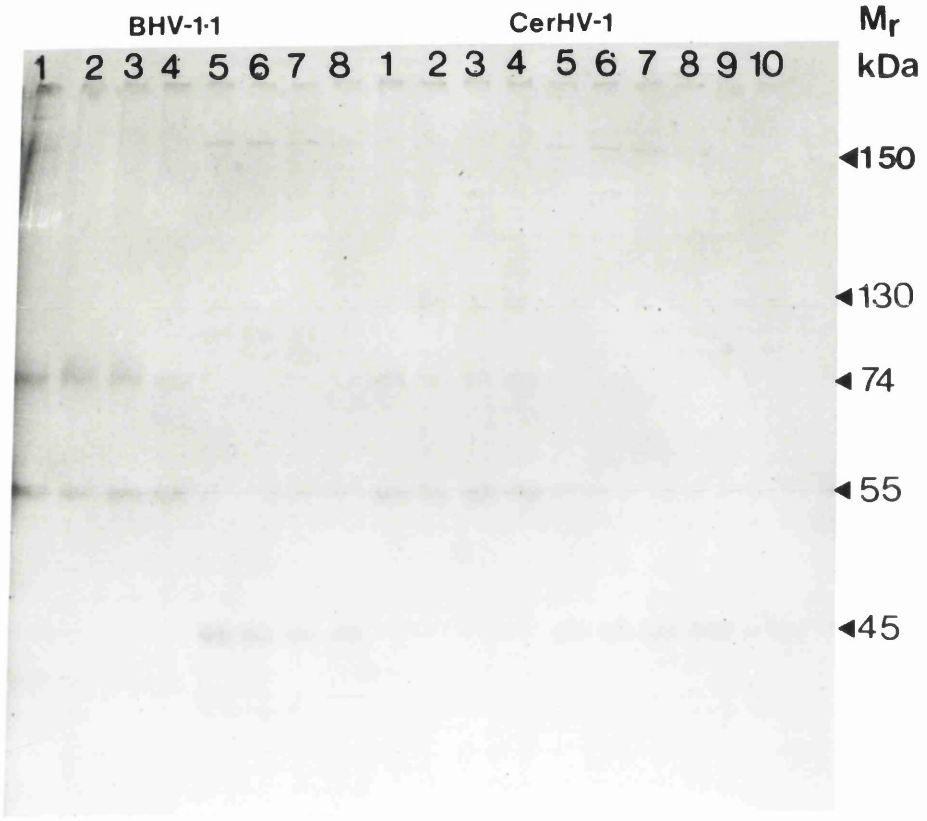
5.3.2. Comparison of antigenically related proteins of CerHV-1 and BHV-1.1

The two groups of selected Mabs which reacted against the 64kDa (3B6, 3C6, and 3D9, 4F10) and 74kDa protein (4D7, 4G8, 6C10, 8B11) in the Western blotting experiments both showed comparable immunoprecipitation patterns with the two viruses (Figure 5.1). The four Mabs (4D7, 4G8, 6C10, 8B11) precipitated two glycoproteins with molecular weights of 74kDa and 55kDa in both viruses. A 130kDa glycoprotein was also faintly precipitated from CerHV-1 infected cells by three (4G8, 6C10, 8B11) of the four Mabs directed against the 74 kDa glycoprotein. The four Mabs specific for the 64kDa protein precipitated two glycoproteins with molecular weights of 150kDa and 45kDa.

5.3.3. Competitive binding assays (CBA)

Initially, checkerboard titrations were performed to determine the optimal conditions for competitive ELISA's where maximal dilutions of both CerHV-1 antigen and HRP-conjugated Mabs which gave OD_{492} readings of about 1.0 in the absence of competitor Mabs. The optimum dilutions were established to be 1:100 for CerHV-1 antigen, 1:50 for 3B6, 3C6, 4F10 and

Figure 5.1. Autoradiograph of immunoprecipitation of [^{3}H] glucosamine labelled BHV-1.1 (left) and CerHV-1 (right) viral proteins with CerHV-1 monoclonal antibodies: 4D7 (lane 1), 4G8 (lane 2), 6C10 (lane 3), 8B11 (lane 4) 3B6 (lane 5), 3C6 (lane 6), 3D9 (lane 7), 4F10 (lane 8). Lane 9 and 10 are BHV-1.1 and CerHV-1 mock infected cell lysates immunoprecipitated with a mixture of all the eight Mabs such that the concentration of each was the same as when used individually. The track labelled M_r shows molecular weight marker positions.


BHV-1·1
CerHV-1
Mr
kDa
1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8 9 10
150
130
74
55
45

4G8, 1:100 for 6C10 and 1:400 for 8B11 HRP-conjugated Mabs. Mabs directed against the same protein were tested in two-way competition inhibition assays. Competition assays with HRP-conjugated Mabs directed against the 64kDa glycoprotein are shown in Table 5.2 and Figure 5.2. Two Mabs (3B6 and 3C6) showed similar reciprocal competitive inhibition reactions and thus may be assigned to one overlapping epitope on the 64 kDa protein. Mab 4F10 reacted differently with the two competitor Mabs (3B6 and 3C6) showing only partial inhibition. However a similar reciprocal competition inhibition was observed when 4F10 was used as the competitor Mab.

Competition assays for the 74kDa glycoprotein are shown in Table 5.3 and Figure 5.3. Unlike the experiments with the Mabs against the 64kDa glycoprotein, there was no clear evidence for competitive inhibition between any of the Mabs against the 74kDa glycoprotein. The main problem was the absence of self-competition. Most likely this was due to Mab 4D7 being insufficiently concentrated while Mab's 4G8, 6C10 and 8B11 were not diluted enough. Unfortunately, there was insufficient time to optimise the antibody dilutions for the anti-74kDa glycoprotein antibodies.

5.4. Discussion

5.4.1. Reactivity of the Mabs against the five viruses in the ELISA

The ability of monoclonal antibodies (Mabs) to detect minor antigenic

TABLE 5.2. Competitive binding assay of Mabs directed against a CerHV-1 64kDa glycoprotein.

Competitor Mabs	Dilution of Competitor Mab	OD_{492} Conjugated Mabs 3B6	3C6	4F10	VPM22 (BVD Mab)
	100	0.18	0.10	0.40	0.00
	200	0.18	0.12	0.42	0.00
3B6	400	0.19	0.12	0.57	0.01
	800	0.21	0.18	0.60	0.02
	1600	0.24	0.26	0.64	0.01
	3200	0.15	0.23	0.72	0.01
	6400	0.25	0.32	0.80	0.03
	12800	0.29	0.38	0.84	0.02
	*	0.81	0.84	0.67	0.01
	100	0.18	0.08	0.42	0.02
	200	0.20	0.08	0.46	0.02
	400	0.20	0.13	0.56	0.01
3C6	800	0.24	0.15	0.61	0.01
	1600	0.19	0.26	0.64	0.02
	3200	0.24	0.26	0.71	0.02
	6400	0.26	0.40	0.80	0.00
	12800	0.33	0.47	0.80	0.00
	*	0.85	0.84	0.62	0.01
	100	0.10	0.02	0.04	0.01
	200	0.12	0.05	0.05	0.01
	400	0.12	0.05	0.06	0.01
4F10	800	0.16	0.07	0.08	0.01
	1600	0.22	0.15	0.15	0.03
	3200	0.23	0.19	0.18	0.03
	6400	0.30	0.29	0.20	0.01
	12800	0.35	0.42	0.30	0.01
	*	0.79	0.81	0.68	0.01

Note *OD_{492} of conjugated Mabs in the absence of competitor Mabs.

Figure 5.2. Competitive binding assays (CBA) for 64kDa protein. Competitor Mabs 3B6 (above), 3C6 (middle) and 4F10 (below) with conjugated Mabs 3B6 (●), 3C6 (■) and 4F10 (▲).

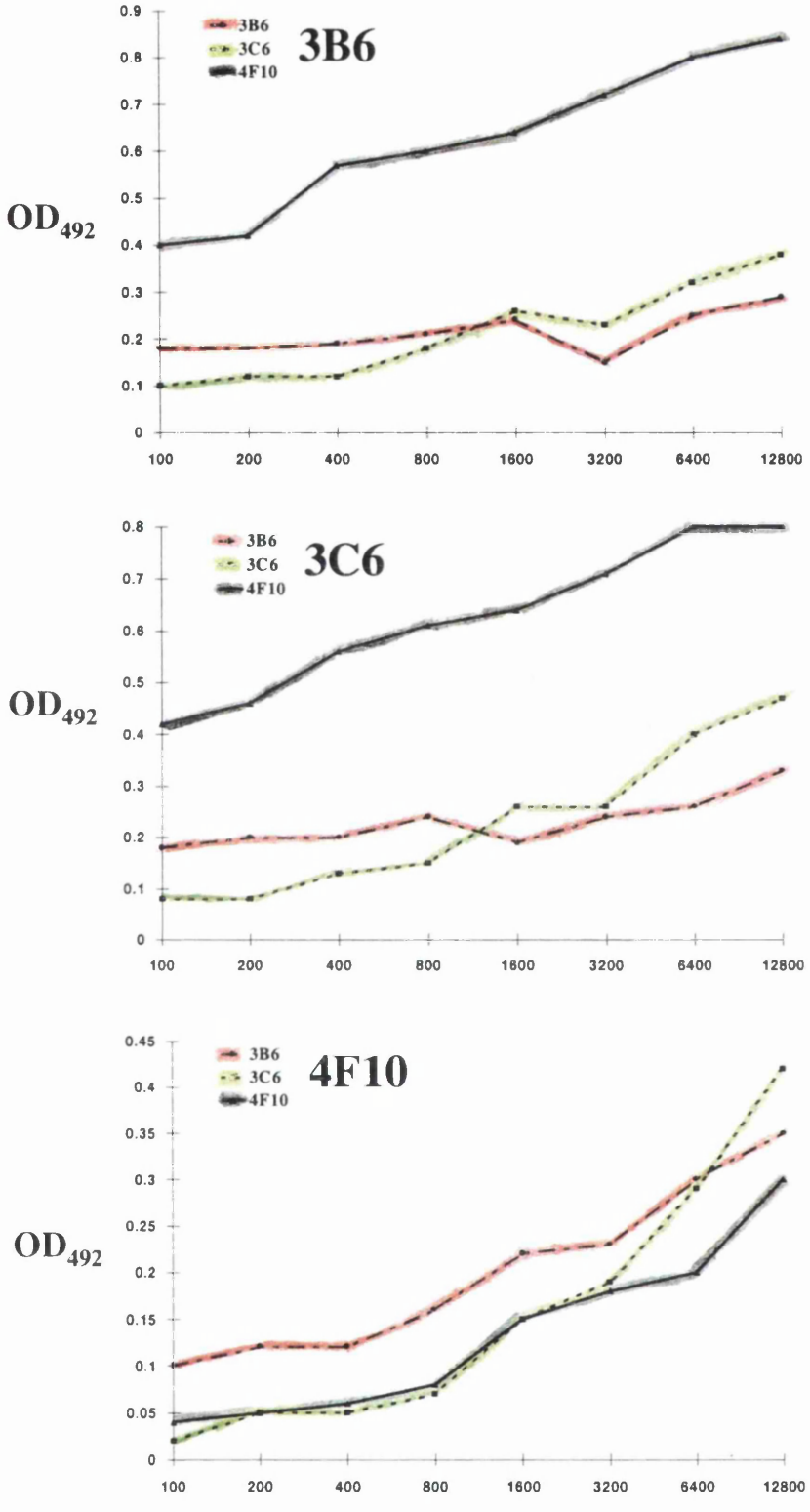
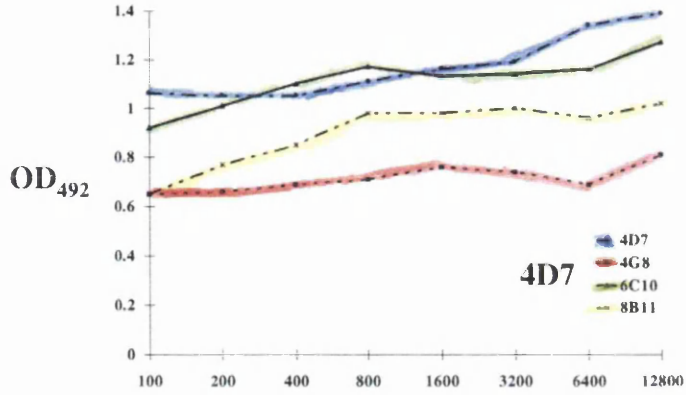

3B6
3C6
4F10
3B6
OD492
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
100
200
400
800
1600
3200
6400
12800
3B6
3C6
4F10
3C6
OD492
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
100
200
400
800
1600
3200
6400
12800
3B6
3C6
4F10
4F10
OD492
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
100
200
400
800
1600
3200
6400
12800
Reciprocal of dilution of Competitor Mab

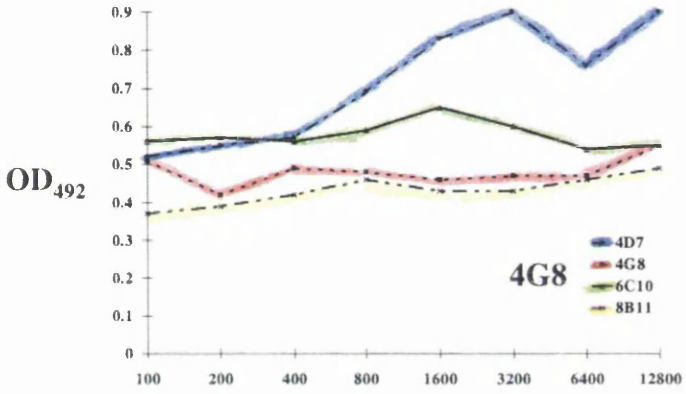
TABLE 5.3. Competitive binding assay of monoclonal antibodies directed against a CerHV-1 74kDa glycoprotein

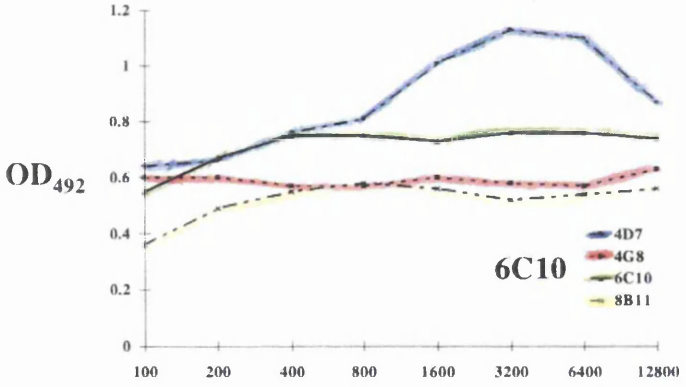
Competitor Mabs	Dilution of Competitor Mab	OD_{492} Conjugated Mabs 4D7	4G8	6C10	8B11	VPM22 (BVD Mab)
	100	1.06	0.65	0.92	0.65	0.00
	200	1.05	0.66	1.01	0.77	0.00
	400	1.05	0.69	1.10	0.85	0.00
4D7	800	1.11	0.71	1.17	0.98	0.01
	1600	1.16	0.76	1.13	0.98	0.00
	3200	1.19	0.74	1.14	1.00	0.01
	6400	1.34	0.69	1.16	0.96	0.01
	12800	1.39	0.81	1.27	1.02	0.01
	*	1.31	1.14	2.12	1.78	0.01
	100	0.52	0.51	0.56	0.37	0.03
	200	0.55	0.42	0.57	0.39	0.01
	400	0.57	0.49	0.56	0.42	0.03
4G8	800	0.69	0.48	0.59	0.46	0.02
	1600	0.83	0.46	0.65	0.43	0.02
	3200	0.90	0.47	0.60	0.43	0.03
	6400	0.76	0.47	0.54	0.46	0.03
	12800	0.90	0.55	0.55	0.49	0.02
	*	1.50	1.14	2.28	1.94	0.03
	100	0.64	0.60	0.55	0.36	0.00
	200	0.66	0.60	0.67	0.49	0.01
	400	0.76	0.57	0.75	0.55	0.01
6C10	800	0.81	0.57	0.75	0.58	0.01
	1600	1.01	0.60	0.73	0.56	0.01
	3200	1.13	0.58	0.76	0.52	0.01
	6400	1.10	0.57	0.76	0.54	0.01
	12800	0.87	0.63	0.74	0.56	0.00
	*	1.30	1.11	2.34	1.99	0.00
	100	0.76	0.67	0.89	0.57	0.01
	200	0.84	0.67	1.03	0.73	0.02
	400	0.91	0.66	0.99	0.68	0.02
8B11	800	0.95	0.75	1.01	0.77	0.02
	1600	1.10	0.69	0.96	0.79	0.02
	3200	1.37	0.68	1.03	0.77	0.03
	6400	1.22	0.76	1.03	0.76	0.01
	12800	1.77	0.78	1.04	0.84	0.01
	*	1.52	1.08	2.10	1.80	0.01

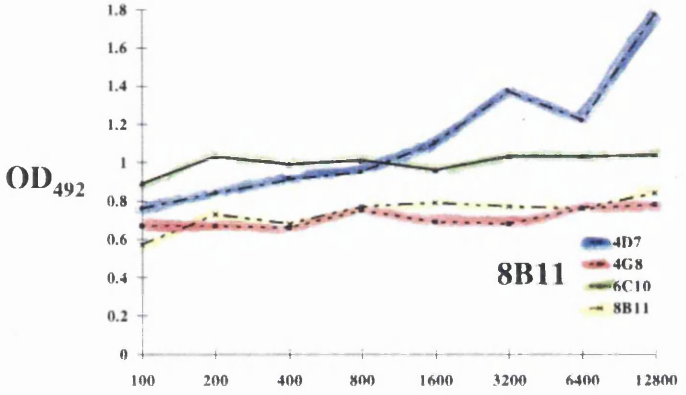
Note *OD_{492} of conjugated Mabs in the absence of competitor Mabs

Figure 5.3. Competitive binding assays (CBA) for 74kDa protein. Competitor Mabs 4D7 (above), 4G8 (2nd middle) 6C10 (3rd middle) and 8B11 (below) with conjugate Mabs 4D7 (●), 4G8 (■) 6C10 (▲) and 8B11 (×).









Reciprocal of dilution of Competitor Mab

differences has facilitated the differentiation of serologically closely related viruses or even variants of the same strain of virus (Prabhakar *et al*, 1982). The different reactivities of the Mabs developed in this study support this view (Table 5.1). Two Mabs (3D9 and 3E5) cross-reacted with all five ruminant alphaherpesviruses, confirming their close antigenic relationship reported previously by others (Nixon *et al*, 1988; Martin *et al*, 1990) and more recently during the course of this study by Rimstad *et al* (1992). These Mabs could be used to establish the common viral protein antigens which form the basis of their close serological relationship. Two Mabs (4D7 and 8B11) reacted exclusively with CerHV-1 and could be used to differentiate this virus from the other four viruses. While on one hand a group of 3 Mabs (2G8, 4F10, and 4G8) failed to react with BHV-1.1, 5 other Mabs (2C3, 3CB6, 3C6, 5C2 and 6C10) did not react with BHV-1.2. The differential reactivity of these eight Mabs supports the existence of antigenic differences between the BHV-1.1 and BHV-1.2 isolates. This is in agreement with others (Metzler *et al*, 1985; 1986) who showed that the two BHV-1 types could be differentiated from each other by the discriminating reactivity of one of their Mabs or by differences in the mobilities of virus induced-polypeptides in SDS-polyacrylamide gels. Recently, Collins *et al*, (1993) demonstrated that Mabs directed against gIII of BHV-1.1 could be used to discriminate BHV-1.1 from BHV-1.3.

5.4.2. Radioimmunoprecipitation of CerHV-1 and BHV-1.1 antigens

The four Mabs 4D7, 4G8, 6C10 and 8B11 all immunoprecipitate 74kDa and 55 kDa glycoproteins from both BHV-1.1 and CerHV-1 infected cells. The two glycoproteins i.e. 74kDa and 55kDa have been previously identified to be the uncleaved components of the 130kDa heterodimer glycoprotein in BHV-1 (Misra *et al*, 1981) which is gI according to the nomenclature proposed by van Drunen Littel van den Hurk *et al* (1984). This glycoprotein has been identified to be a homologue of gB in HSV (Whitbeck *et al*, 1988). According to Ludwig (1983) and Ludwig and Gregersen (1986), a similar glycoprotein of 74kDa was shown to be responsible for the cross-neutralization between CapHV-2 and BHV-1. These studies identify for the first time the CerHV-1 gB homologue.

Three Mabs (4G8, 6C10 and 8B11) additionally precipitated a 130 kDa glycoprotein in CerHV-1. Mab 4D7 however failed to precipitate this protein in CerHV-1. All four Mabs failed to precipitate a similar glycoprotein in BHV-1.1. It is likely that the 130kDa, 74kDa and 55 kDa glycoproteins are related and that processing occurs at a different rate in CerHV-1 infected cells than in BHV-1.1 infected cells. The nature of the precursor-product relationship has not been examined but one possible scheme would involve cleavage of the 130 kDa glycoprotein to yield the 74kDa glycoprotein which in turn is cleaved to yield the 55kDa glycoprotein. The reactive epitope would like within the 55 kDa glycoprotein. An alternative explanation would be that

the 74kDa glycoprotein physically associates with other (130kDa and 55kDa) glycoproteins. The observation that only the 74kDa glycoprotein is seen on western blots supports this latter view. The observation that Mab 4D7 does not precipitate the 130kDa glycoprotein suggests it might recognise a different epitope on the 74kDa glycoprotein from that seen by Mabs 4G8, 6C10 and 8B11.

The Mabs (3B6, 3C6, 3D0 and 4F10) reactive with the 64kDa glycoprotein by Western blotting immunoprecipitated two proteins with molecular weights of 150kDa and 45kDa. The failure of these antibodies to imunoprecipitate a 64kDa glycoprotein is not clear but perhaps it might be a stable end product with the 150kDa and 45kDa glycoproteins being precursor forms. There is a precedent for such a scheme in BHV-1 where a 64kDa glycoprotein was shown to have precursor 148kDa and 42kDa proteins (Friedli and Metzler, 1987). This possibility could be tested with pulse-chase experiments.

5.4.3. Analysis of epitopes on the 64kDa and 74kDa glycoproteins

Competition between a Mab and a Mab conjugate in CBA indicates that the epitopes that are recognized by the two Mabs overlap or are adjacent on the antigen (Stone and Nowinsky, 1980), especially for reciprocally competing Mabs. Non-reciprocal competition may occur due to the affinity and avidity of the two Mabs differing greatly; where the binding of one Mab

induces a change in the conformation that alters the binding site of the other Mab. The binding of one Mab may sterically hinder the binding of the other Mab; or the conjugating of one Mab to peroxidase may alter the ability of the other Mab to bind. The results for the 64kDa glycoprotein showed reciprocal competition suggesting that Mabs (4F10, 3C6 and 3B6) identified a single or overlapping epitope(s). Although the reactivities of these three Mabs show three different patterns with the five viruses (Table 5.1) this does not imply that the Mabs identify different epitopes since the degree of reactivity with the BHV-1.1, BHV-1.2, CapHV-2 and RanHV-1 differ by only one category for any particular virus which might not be significantly different (Section 5.3.1).

CHAPTER 6

GENERAL DISCUSSION

The major aim of the work described in this thesis was to compare the serological relationships among the five ruminant alphaherpesviruses. The five viruses used in this study were shown to be different from each other by the restriction endonuclease profiles as described in Chapter 3. Antigenic relationships were analysed by the use of polyclonal sera and monoclonal antibodies as described in Chapters 3, 4 and 5 respectively. This chapter brings together findings obtained from this study and those of others to discuss the following: First, the basis of the close serological relationships which exist among these viruses as determined by the viral proteins recognised by both polyclonal sera and monoclonal antibodies. Second, the implications of these serological relationships for epidemiological data, diagnostic tests and development of vaccines. Third, the use of monoclonal antibodies as tools in developing sensitive and reliable diagnostic tests which are capable of differentiating infections caused by these ruminant alphaherpesviruses. Fourth, the importance of understanding the genetic structure, composition and the possible evolutionary relationships which exist among these viruses which could be used to form a clear and objective herpesvirus classification.

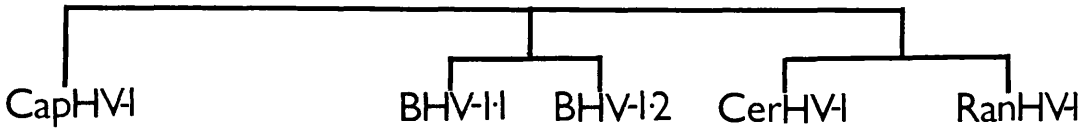
6.1. Serological relationships and their implication for epidemiological data and diagnostic tests.

The existence of close serological relationships among the five ruminant

alphaherpesviruses was established by ELISA, SNT and Western blotting (Chapter 3). Until recently there have been few studies on the serological relationships among these viruses. Nixon *et al*, (1988) and Martin *et al* (1990) reported the serological relationships between BHV-1, CapHV-2 and CerHV-1. While work on this thesis was proceeding Rimstad *et al* (1992) published a report containing preliminary findings on the serological relationships between BHV-1, CapHV-2 and RanHV-1. The present study reports for the first time an extensive comparison and analysis of the antigenic relationships among this group of five ruminant alphaherpesviruses. The evidence in this study supports and confirms all the findings in the above mentioned reports, namely that: 1) the two BHV-1 viruses are the most closely related, 2) CerHV-1, CapHV-2 and RanHV-1 are more closely related to the two BHV-1 viruses than they are to each other, 3) the CerHV-1 is more related to the BHV-1 viruses than to CapHV-2 or RanHV-1, and 4) RanHV-1 is more related to CerHV-1 than to the two BHV-1 and CapHV-2 viruses (see Figure 6.1.). The extent and degree of serological cross-reactivities reported in the present work, supports the view that ruminants other than cattle can indeed be seropositive to a virus antigenically related to but distinct from BHV-1. The data presented here supports the view that antibodies to CerHV-1 in wild and farmed red deer in Britain (overall prevalence 29%) is likely to be due to infection with CerHV-1 rather than BHV-1.

The available evidence suggests that alphaherpesvirus infections in both wild and domestic ruminants occur only in their natural hosts and do not cross stably into other species (Brake and Studdert, 1985; Pastoret *et al*, 1988). However,

Figure 6.1. A schematic diagram of antigenic relationships among the five ruminant alphaherpesviruses.


CapHV-1
BHV-1·1
BHV-1·2
CerHV-1
RanHV-1

experimental latent infections of a species different from that of the natural host have been demonstrated, for example, BHV-1 infections in domestic goats (Pirak *et al,* 1983); and red deer (Reid *et al,* 1986). The close serological relationship observed in this study among these viruses precludes the use of polyclonal antibodies in distinguishing, for example, CerHV-1 from BHV-1 infection in red deer.

These findings highlight the potential of misinterpretation of both diagnostic tests and epidemiological data. As pointed out earlier by Nixon and others (1988), the best results for diagnostic serology is likely to be achieved by using an appropriate virus isolate. Considerations should also be given in the development and use of appropriate test protocols to avoid possible misinterpretation of results where tests are being done for the purposes of national or international disease control or animal certification. Thus, if the objective is to control the spread of CerHV-1 in red deer or CapHV-2 in goats, it should be mandatory to use cognate antigen for diagnosis.

6.2. Prospects for the use of monoclonal antibodies in the development of reliable differential diagnostic tests.

The work reported in this thesis has shown clearly the potential of monoclonal antibodies for differentiation of the serologically closely related ruminant alphaherpesviruses (Chapter 4 and 5). The Mabs showed a wide range of reactivity, with two Mabs (4D7 and 8B11) being specific for CerHV-1. A group of 3 Mabs (2G8, 4F10, and 4G8) failed to react with BHV-1.1 while 5 other Mabs

(2C3, 3B6, 3C6, 5C2 and 6C10) did not react with BHV-1.2. Two Mabs (3D9 and 3E5) reacted with all the five ruminant alphaherpesviruses. Therefore the use of such Mabs could facilitate the differentiation of these alphaherpesviruses. The Mabs 4D7 and 8B11 (Table 5.1) which reacted exclusively with CerHV-1 could potentially be used to develop a test which could differentiate CerHV-1 from the other four serologically related alphaherpesviruses although this potential has yet to be realised. Kit and others (1992) have described a blocking ELISA for distinguishing BHV-1.1 infected animals from those vaccinated with a gene-deleted marker vaccine. For their ELISA, undiluted test sera were used to block the binding of an anti-IBR virus gIII monoclonal antibody HRP-conjugate to gIII antigen. A similar sensitive anti-pseudorabies virus glycoprotein gIII blocking ELISA for distinguishing between pseudorabies-infected and vaccinated pigs has also been reported (Kit *et al,* 1990). It can therefore be argued that monoclonal antibodies directed against virus specific antigens like gIII and gIV (Ben-Porat *et al,* 1986; Friedli and Metzler, 1987; Allen and Coogle, 1988) could be more advantageous and reliable for developing differential diagnostic tests for the ruminant alphaherpesviruses (van Drunen Littel-van den Hurk *et al*, 1990). An alternative approach could be the use of anti-idiotypes (antibodies to specific Mabs) as "network" antigens (Kholer *et al,* 1989) in direct ELISAs. The potential of using anti-idiotypes lies in their ability to biologically mimic the epitope(s) by their bearing the internal image of a particular antigen (against which the Mab(s) are directed). Recently anti-idiotypes capable of inducing neutralising antibodies to BHV-1 gIV and gI have been developed (Srikumaran *et al,* 1990; Hariharan *et al,* 1991). Their work not only promises a new avenue of

using anti-idiotypes as antigens in diagnostic tests, but also as potential immunogens against these viruses.

Another approach would be synthetic peptides mimicking defined epitopes. In particular, synthetic peptides can be used as specific antigens in diagnostic ELISA test systems for the detection of antibody in sera (Gnann *et al,* 1987; Marsden *et al,* 1992). As pointed out by Norrby and others (1987) and Gnann and coworkers (1987), one of the major advantages of peptide tests has been shown to be an easy and fast distinction between virus subtype infections e.g. diagnosis of human immunodeficiency virus HIV-1/HIV-2 infections. Oligopeptides represent mostly sequential epitopes of limited size. Thus, their use in diagnostic test systems require methods for the definition of those dominant antigenic sites that are recognised by the immune response and accessible to antibody binding. In this case, identification and characterisation of antigenic sites is a pre-requisite for the successful use of a peptide as antigen for antibody detection in sera. The smallest epitopes for antibody recognition have been described as four to six amino acids (Tang *et al,* 1988); generally peptides of about 12-20 amino acids are used. As recently reported by Marsden *et al* (1992), branched peptides which consist of a small two-fold bifurcating, polylysine core onto which the peptide of interest is synthesised are capable of detecting lower amounts of antibodies than could be detected with monomeric peptides. The physical aspects of branched peptides which might be important for the observed increase in sensitivity include the following. First, they might bind more readily to the microtitre wells than monomeric peptides. Second,

multivalent binding between antibody and branched peptide would result in a considerable increase in stability, compared to simple monovalent binding of monomeric peptides (Tam and Zavala, 1989). Third, to be immunoreactive monomeric peptides have to both bind to the plastic well and remain available for binding to the antibody, whereas one or more arms of a branched peptide could bind to the plastic and leave the others free to bind to the antibody. It is possible that in binding to the plastic, physical constraints are imposed on a monomeric peptide to prevent it from interacting freely with the antibody. The large savings potentially available when using branched peptides for peptide-based serodiagnostic kits and their greater sensitivity leads to the suggestion that they might become the reagents of choice.

6.3. Identification and characterisation of antigens and implications for vaccine development.

Identification and characterisation of the envelope glycoproteins of the ruminant alphaherpesviruses is required to understand their relative immunological significance in the naturally-infected natural hosts. This is a necessary pre-requisite to the development of new effective vaccines for these important pathogens particularly BHV-1 (Lupton and Reed, 1990). It has been shown that herpesvirus glycoproteins play important roles in pathogenicity, particularly in adsorption, penetration, and in the mediation of cell-to-cell spread of virus (Cai *et al*, 1988; Ligas and Johnson, 1988). These glycoproteins are known to be among the targets for the host's humoral and cell mediated immune responses (reviewed by Spear, 1990, 1993a). In the

present study, ELISA, SNT and Western blotting indicated that the five viruses possess many shared as well as unique antigenic determinants. These findings support previous reports on the existence of the wide serological relationships among the ruminant alphaherpesviruses (Ludwig, 1983; Buddle *et al*, 1990; Engels *et al*, 1992). As for all investigated herpesviruses, it could be shown that only a few glycoproteins are of importance for the immune response of the host against virions and infected cells. To date, three major BHV-1 envelope glycoproteins namely gI, gIII and gIV have been shown to be involved in virus neutralisation (van Drunen Littel-van den Hurk and Babiuk 1986b; Marshall *et al*, 1988). In addition, genes for these three glycoproteins have been identified and sequenced (Zamb, 1987) and the gene products were found to induce high levels of antibody in cattle which could neutralise virus and participate in antibody-dependent cell cytotoxicity of BHV-1 infected cells (Collins, *et al* 1993). Animals immunised with these glycoproteins were fully protected from disease (Babiuk *et al*, 1987). Israel *et al* (1992) have recently shown that an intranasal vaccination with BHV-1 gI, expressed from recombinant baculovirus-infected insect cells, can induce protective antibodies in the nasal secretion of cattle.

Since control of BHV-1 is based largely on vaccination, and the safety of the commonly used live attenuated vaccines is continuously being questioned due to latency and consequent reactivation (Pastoret *et al*, 1980; Whetstone *et al*, 1989) development of subunit vaccines consisting of any one or a combination of these immunodominant glycoproteins could be considered as a

viable alternative.

6.4. Genetic structure, composition, possible evolutionary relationships and herpesvirus classification.

The taxonomy of ruminant alphaherpesviruses as for the whole family herpesviridae is based on their antigenic properties and the restriction enzyme analysis of their genomes. Initially the classification of herpesviruses into subfamilies was based on simple measurements or observations e.g. cytopathology or growth time that could be readily determined in the majority of laboratories. Most herpesviruses assigned to the three subfamilies would have been assigned now to the same subfamilies on the basis of the wider range of more objective criteria currently available. Indeed with a few exceptions, the criteria proposed in 1981 yielded a scheme of relationships which mirrored that since determined by DNA sequence homology, gene organisation and composition. However, with the ever accumulating information based on the herpesvirus genomes, the exceptions are considered to be highly significant in that they indicate the criteria used to align viruses to the three subfamilies to have been unsatisfactory for some viruses.

The key exceptions are the gallid herpesvirus 1 (GHV-1, Marek's disease herpesvirus) and herpesvirus of turkeys classified in 1981 as members of Gammaherpesvirinae, the human herpesvirus-6 (HHV-6) which was not known at that time and the fish herpesvirus, channel catfish virus. The classification of GHV-1 and the herpesvirus of turkeys as a members of the Gammaherpesvirinae

reflected the observation that the virus caused tumour-like growths in chickens, the tumours were of lymphoid type and the viral genomes were present and expressed in cells contained in those tumours. Current data indicate that their genome arrangement resembles that of the Alphaherpesvirinae rather than that of Gammaherpesvirinae (Buckmaster *et al,* 1988). The complete genome sequence of the fish herpesvirus, channel catfish virus (CCV) shows no clear relationship whatever with the viruses of the three herpesvirus subfamilies thus Davison (1992) proposed that it should be assigned to a separate taxonomic group. Recently the ICTV proposed several objective criteria for the classification of herpesvirus subfamilies (Roizman *et al,* 1992). These criteria include (i) conservation of genes and gene clusters (e.g. DNA polymerase, glycoproteins B, C and H, the major single-stranded DNA binding protein, major capsid protein); (ii) the arrangement of gene clusters relative to each other; (iii) the arrangement of the terminal sequences involved in packaging of the viral genome; and (iv) the presence and distribution of nucleotides that are subject to methylation (Davison and Taylor, 1986; Deiss *et al,* 1986). The imaginative application of the polymerase chain reaction (PCR) will enhance and readily speed up the realisation of the aforementioned objective criteria for the comparative analysis and classification of the herpesviruses. As the results in this thesis have indicated; it is possible to amplify a small section of heterologous alphaherpesvirus DNA by using synthetic primers selected from known gene sequences of one of the closely related herpesviruses.

The objectives of herpesvirus taxonomy should be firstly to provide a

quantitative measure of the relatedness of the different herpesviruses to each other from which one can infer an evolutionary tree. Secondly, it should serve the utilitarian function of assisting those involved in the isolation of new viruses to predict biologic properties in the same fashion that biochemical and morphologic properties identify bacteria.

The major aims of this study were firstly to compare and analyse the serological relationships among the five ruminant alphaherpesviruses. The results in this present study show the existence of close serological relationships among these viruses (Lyaku *et al,* 1992a). Secondly, the potential for distinction and differentiation of these five viruses was suggested by the reactivity of a panel of the 14 Mabs (Lyaku *et al* 1992b). The information obtained in this thesis shows the potential ability of Mabs in differentiating closely related viruses. Further work is however required to unravel the nature of the relationships which exist among these viruses, to exploit for diagnostic purposes the Mabs described and to produce more Mabs for developing sensitive and reliable differential diagnostic tests.

6.5. Future work

The information provided by findings in this thesis opens several avenues for future work. Firstly, the two Mabs (4D7 and 8B11) directed against gI and specific for CerHV-1 should be further investigated to see if they can form the basis of a discriminatory Mab capture based ELISA. The two Mabs (4D7 and 8B11) could also be used for affinity purification of gI

which could in turn be used to raise more Mabs for both physical and functional epitope mapping of this glycoprotein. Secondly, the genes responsible for the expression of CerHV-1 gI, gIII and gIV might be identified by PCR and nucleotide sequencing of the viral DNA. The nucleotide sequences of these genes could then be used to specify overlapping synthetic peptides which could be synthesised and used for the screening of sera to serodiagnostic tests capable of distinguishing the five ruminant alphaherpesviruses. Recombinant glycoprotein gIII antigens could also be produced by cloning the respective fragment containing gIII gene into a suitable expression system (eg Baculovirus). The possibility of using such antigens in the development of diagnostic tests could also be explored. This approach has already been used for the serological distinction of vaccinated from wild type infections in SHV-1 and BHV-1.1 (Van Oirschot *et al*, 1988, 1990; Kit *et al*, 1992). As well as this approach, data from HSV (Lee *et al* 1985, 1986) and recently from EHV-4 (Crabb *et al*, 1992) strongly indicate that fine discriminatory serological tests can be developed using glycoprotein G to detect HSV-1 or HSV-2 and EHV-1 or EHV-4 type-specific antibodies respectively. This was only possible after the accumulation of detailed knowledge of the molecular biology of HSV and EHV. Further detailed analysis of the ruminant alphaherpesviruses will be necessary before such elegant discriminatory tests can be developed.

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