Dissertation

MCF PATHOGENESIS: STUDIES ON THE REPLICATION AND TROPISM OF OVINE HERPES VIRUS 2 (OvHV-2) IN PERIPHERAL BLOOD MONONUCLEAR CELLS OF RUMINANTS

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY MATSHEDISO (TSHIDI) L. TSIBANE ENTITLED MCF PATHOGENESIS: STUDIES ON THE REPLICATION AND TROPISM OF OVINE HERPESVIRUS 2 (OvHV-2) IN PERIPHERAL BLOOD MONONUCLEAR CELLS OF RUMINANTS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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ABSTRACT OF DISSERTATION

MCF PATHOGENESIS: STUDIES ON THE REPLICATION AND TROPISM OF OVINE HERPESVIRUS 2 (OvHV-2) IN PERIPHERAL BLOOD MONONUCLEAR CELLS OF RUMINANTS

The pathogenesis of ovine herpesvirus 2 (OvHV-2), the causative agent of malignant catarrhal fever (MCF) is unknown. Based on clinical status, OvHV-2 infected animals can be divided into 4 groups, namely, asymptomatic sheep, subclinically infected, clinically affected, and recovered cattle.

To examine the role of OvHV-2 load and tropism on clinical status, peripheral blood mononuclear cells (PBMCs), obtained from animals in each of the 4 groups were immunomagnetically sorted into $CD2^+$, $CD3^+$, $CD4^+$, $CD8^+$, and $\gamma\delta^+$ T-cell, B-cell, and monocyte subsets. The role of OvHV-2 load and tropism on clinical status was examined by investigating and comparing OvHV-2 DNA copy numbers in total PBMCs and PBMC subsets between and within the 4 groups of animals. Flow-cytometry was used to determine sorted subset percentages within each group to examine the effects of OvHV-2 infection on PBMC subset percentages. PBMC subset percentages from non-infected cattle were additionally examined.

There was no difference in the OvHV-2 tropism between the 4 groups. T-cells were preferentially infected over B-cells and monocytes in all groups. Clinically affected cattle had significantly higher OvHV-2 genome copy numbers in their total PBMCs compared to the other groups. There was no association between OvHV-2 infection status and changes in PBMC subset percentages. Differences in viral DNA copy numbers

within total PBMCs of the 4 groups are suggestive of a role for viral DNA replication on clinical status.

OvHV-2 positive T-cell lymphoblastoid cell lines (LCLs) established from recovered and fatal cases of MCF displayed variable cellular and OvHV-2 replication kinetics. Although all the LCLs harbored predominantly latent OvHV-2 genome, DNase protected and unprotected OvHV-2 genomes were present in the LCL supernatants and all the LCLs supported transcription of messenger RNA to a late viral structural gene. Establishment of an LCL from a recovered case of MCF suggests a persistently latent infection of T-cells within recovered cases.

Treatment of LCLs with several chemicals displayed variable effects on OvHV-2 DNA replication. Dexamethasone treatment may lead to an increase in intracellular viral burden, whereas, acyclovir may successfully decrease OvHV-2 DNA copy numbers. Both of these chemicals may impact supportive MCF therapy.

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DEDICATION

This thesis is dedicated to my parents Reginah and Zacharia Tsibane and, my brothers Sechaba and Mojalefa Tsibane, for their continued support, encouragement and undying love.

TABLE OF CONTENTS

Page

TITLE: **MCF PATHOGENESIS: STUDIES ON THE REPLICATION AND TROPISM OF OVINE HERPESVIRUS 2 (OvHV-2) IN PERIPHERAL BLOOD MONONUCLEAR CELLS OF RUMINANTS**

ABSTRACT.....1
CHAPTER 1 **INTRODUCTION**.....5
CHAPTER 2 **LITERATURE REVIEW**.....9

Introduction.....9
The Herpesviridae Family (Taxonomy, viral structural components and size).....10
Herpesviridae genomic structure and arrangements.....13
Herpesviridae gene organization.....16
Gammaherpesvirinae gene structure and organization.....17
Gammaherpesvirinae host cell gene homologues.....19
MCF clinical signs and histopathological lesions.....21
Proposed MCF pathogenesis.....23
Established OvHV-2 positive lymphoblastoid cell lines.....29
Examined lymphoblastoid cell line and PBMC cell surface markers.....35
A possible role for NK T cells in MCF pathogenesis.....44
Ruminant PBMC subset percentages.....46

CHAPTER 3 **OvHV-2 PREFERENTIALLY INFECTS T-CELLS, ESPECIALLY CD4 AND CD8 POSITIVE T-CELL SUBSETS IN SHEEP AND CATTLE**.....48

Abstract
Introduction
Materials and Methods
Results
Discussion

CHAPTER 4 **ESTABLISHMENT OF OvHV-2 INFECTED LYMPHOBLASTOID CELL LINES FROM PBMC'S OF CLINICAL AND RECOVERED CATTLE WITH MCF**.....67

Abstract
Introduction
Materials and Methods
Results
Discussion

CHAPTER 5 **INDUCTION OR INHIBITION OF OvHV-2 DNA REPLICATION BY CHEMICAL TREATMENT OF OvHV-2 POSITIVE LYMPHOBLASTOID CELL LINES** 88

Abstract
Introduction
Materials and Methods
Results
Discussion

CHAPTER 6 **SUMMARY AND CONCLUSIONS**.....110

APPENDIX.....114

REFERENCES.....122

LIST OF TABLES

Tables		Page
3.1	Detected cell surface markers and monoclonal antibody designation	53
4.1	Examined cell surface markers and monoclonal antibody designation	72
4.2	Source and phenotype of OvHV-2 positive lymphoblastoid cell lines	75
5.1	OvHV-2 positive cell lines and duration in culture	92
5.2	Chemicals / drugs used and their mechanism of action	93
5.3	Lymphoblastoid cell lines and chemicals they were treated with	94
5.4	Summary of categories of effects of treatments on LCLs	101
A-1	Animal Identification: Asymptomatic Sheep	114
A-2	Animal Identification: Subclinically Infected Cattle	114
A-3	Animal Identification: Clinically Affected Cattle	114
A-4	Animal Identification: Recovered Cattle	115
A-5	Necropsy Findings in the Clinically Affected Cattle	115
A-6	Percentage purity of immuno-magnetically sorted PBMC subsets: Asymptomatically Infected Sheep	115
A-7	Percentage purity of immuno-magnetically sorted PBMC subsets: Subclinically Infected Cattle	116
A-8	Percentage purity of immuno-magnetically sorted PBMC subsets: Clinically Affected Cattle	116
A-9	Percentage purity of immuno-magnetically sorted PBMC subsets: Recovered Cattle	116

A-10 Obtained OvHV-2 DNA copies in total PBMCs and PBMC subsets Asymptomatic Sheep.....117

A-11 Percentage purity of immuno-magnetically sorted PBMC subsets: Subclinically Infected Cattle.....117

A-12 Percentage purity of immuno-magnetically sorted PBMC subsets: Clinically Affected Cattle.....117

A-13 Percentage purity of immuno-magnetically sorted PBMC subsets: Recovered Cattle.....118

A-14 Statistical Analysis of OvHV-2 Load and Tropism between the 4 groups of Animals (P-values).....118

A-15 Statistical Analysis of OvHV-2 Tropism in Each Group: Asymptomatic Sheep (Preferentially Infected Subsets).....118

A-16 Statistical Analysis of OvHV-2 Tropism in Each Group: Clinically Affected Cattle (Preferentially Infected Subsets).....119

A-17 Statistical Analysis of OvHV-2 Tropism in Each Group: Recovered Cattle (Preferentially Infected Subsets).....120

A-18 Means and Ranges of Obtained Cattle PBMC subset percentages..... 120

A-19 Comparism of Obtained and Reported PBMC subset percentage means and.....121

LIST OF FIGURES

Figures		Page
2.1	Herpesviridae genomic structure and arrangement	16
2.2	Gammaherpesvirus genome structure and organization	19
2.3	Gammaherpesvirus host gene homologues	20
2.4	Antigen recognition by NK T cells	43
3.1	OvHV-2 load and tropism within whole PBMCs and sorted subsets of asymptomatic sheep, clinically affected and recovered cattle	59
3.2	PBMC subset percentages within non-infected, subclinically infected, clinically affected and recovered cattle	61
4.1	Giemsa stain of MD00 lymphoblstoid cell line	74
4.2	Lymphoblastoid cell line replication kinetics over 5 days	76
4.3	Day 1 – day 5 OvHV-2 cell and supernatant replication kinetics	77
4.4	OvHV-2 copy numbers in DNase treated LCL supernatant	79
4.5	Reverse transcription and cDNA PCR of OvHV-2, ORF 75 and the Bovine GAPDH	80
4.6	Gardella gel and Southren blot of EBV Raji cells and OvHV-2 positive LCLs	81
4.7	Detection of percentage apoptosis within OvHV-2 positive LCLs over a day period	82
5.1	Results of chemical treatment of MD00 cells and supernatant	98
5.2	Results of chemical treatment of TT05 cells and supernatant	99
5.3	Results of chemical treatment of TT08 cells and supernatant	100

ABSTRACT

MCF PATHOGENESIS: STUDIES ON THE REPLICATION AND TROPISM OF OVINE HERPESVIRUS 2 (OvHV-2) IN PERIPHERAL BLOOD MONONUCLEAR CELLS OF RUMINANTS

Malignant catarrhal fever (MCF) is a lymphoproliferative disease of susceptible ruminants, the pathogenesis of which has not been discerned. Ovine herpes virus 2 (OvHV-2) is the causative agent of sheep-associated MCF. Based on the clinical status, OvHV-2 infected animals can be divided into 4 groups, namely, asymptomatically infected sheep, subclinically infected cattle, clinically affected cattle, and recovered cattle.

In order to examine the role of OvHV-2 load and tropism on clinical status, peripheral blood mononuclear cells (PBMCs) were obtained from animals in each of the 4 groups. OvHV-2 load and PBMC tropism was compared between and within each of the 4 groups of infected animals. PBMCs were separated into $CD2^+$, $CD3^+$, $CD4^+$, $CD8^+$, and $\gamma\delta^+$ T-cell, B-cell, and monocyte subsets by immunomagnetic sorting. Total DNA isolated from total PBMCs and the sorted subsets were examined for OvHV-2 DNA by fluorogenic quantitative PCR (qPCR). The role of OvHV-2 load in clinical status amongst the 4 groups was examined by investigating and comparing OvHV-2 copy numbers in total PBMCs. OvHV-2 tropism amongst the 4 groups was examined by investigating and comparing viral copy numbers in the sorted subsets. OvHV-2 subset tropism within individual groups was investigated by comparing viral copy numbers in the different subsets within each group of animals. Flow-cytometry was used to determine sorted subset percentages within each group in order to examine the effects of

OvHV-2 infection on PBMC subset percentages. PBMC subset percentages from non-infected cattle were additionally examined by flow-cytometry. The OvHV-2 infection status in each of the 4 groups and non-infected cattle was confirmed by the MCF competitive inhibition ELISA (CI-ELISA). There was no difference in the OvHV-2 tropism between the 4 groups of animals. Examination of OvHV-2 tropism within groups indicated higher genome copy numbers in T-cells compared to B-cells and monocytes. CD4 and CD8 T-cells had higher genome copy numbers than γδ T-cells in all groups. Clinically affected cattle had significantly higher OvHV-2 genome copy numbers in PBMCs compared to the other infected animal groups. This suggests active viral DNA replication, whereas, undetectable copies within subclinically infected cattle are supportive of limited viral genome replication in latent infection. These data show an association between OvHV-2 genome copy number in PBMCs and clinical disease status in infected animals. No association was found between OvHV-2 infection status and changes in PBMC subset percentages.

To further examine MCF pathogenesis, OvHV-2 positive lymphoblastoid cell lines (LCLs) were established from 3 fatal and 1 recovered case of MCF. The LCLs morphologically resembled large granular lymphocytes and were determined to be CD2+ and CD3+ T-cells by flow-cytometry. Established lymphoblastoid cell lines included exclusive γδ+, CD8+, and mixed γδ+CD8+ phenotypes. Cellular and OvHV-2 replication kinetics were variable between the cell lines. All LCLs were persistently infected with OvHV-2 as demonstrated by qPCR. The LCL established from a recovered case of MCF harbored relatively fewer viral genome copies compared to LCLs from fatal cases. All of the cell lines harbored predominantly latent OvHV-2 genome as demonstrated by circular

DNA on Gardella gel analysis. At peak replication the different LCLs contained between 29 - 140 OvHV-2 genomic equivalents per cell. The source of viral DNA within the LCL supernatant was both DNase protected and unprotected OvHV-2 genome. The ability to establish a T-lymphoblastoid cell line from PBMCs of a recovered case suggests that persistent latent infection may reside in T-cells. The lower number of OvHV-2 genome copies observed in both cells and supernatant of the recovered case suggest that decreasing viral load may be associated with recovery. The ability of the host to control or limit viral DNA replication with a resultant decrease in viral load may contribute to recovery. Presence of DNA replication, messenger RNA to a viral structural gene, and the presence of DNase protected OvHV-2 genome in the LCL supernatant suggests that OvHV-2 LCLs may be able to produce intact virions.

In an attempt to establish an *in vitro* culture system for OvHV-2, LCLs established from fatal cases of MCF were treated with a variety of chemicals. These chemicals have been shown to either induce or inhibit virus DNA replication in cell culture of other gammaherpesviruses. The effects of chemical treatment on OvHV-2 DNA replication were investigated by examining OvHV-2 DNA copy numbers within cells and supernatants of treated and untreated LCLs. The treated LCLs displayed variable responses to similar treatments. None of the chemicals uniformly increased OvHV-2 copies in both cells and supernatant. Some of the chemicals increased intracellular copies without affecting copies in the supernatant. Dexamethasone and acyclovir are chemicals that could have an impact on the treatment of clinical MCF cases. Dexamethasone treatment increased intracellular OvHV-2 copies and may therefore be contraindicated as it increases the intracellular viral burden. Acyclovir decreased OvHV-

2 copies in the cells and supernatant, suggesting that it could be used to inhibit OvHV-2 DNA replication during clinical disease. Optimal doses would, however, have to be optimized in cell culture and animal models.

In summary, our data indicates no differences in OvHV-2 PBMC tropism within the 4 groups of infected animals. Within each group, however, OvHV-2 preferentially infects T-cells over B-cells and monocytes. Viral DNA replication and an increase in viral load may support progression to clinical disease, whereas, a tight regulation on viral replication or latency and relatively lower viral load favors subclinical infection. Host control or limiting of OvHV-2 DNA replication during clinical disease with a resultant relatively lower viral load may support recovery from clinical disease. Recovered cases remain persistently infected with OvHV-2 and T-lymphocytes act as a site of viral latency. Treatment of clinical cases with dexamethasone may be contraindicated as it increases the intracellular viral burden in LCLs. Acyclovir decreases viral genome copies in LCL cultures and may be a potential treatment to inhibit OvHV-2 DNA replication during clinical disease.

CHAPTER 1

INTRODUCTION

Ovine herpesvirus 2 (OvHV-2) is a gamma-2 herpesvirus and the causative agent of sheep associated malignant catarrhal fever (MCF). Gammaherpesviruses are known to cause lymphoproliferative diseases in susceptible hosts. They characteristically infect and cause persistently latent infections in their hosts. Some gammaherpesviruses malignantly transform infected lymphocytes resulting in lymphoid tumors. OvHV-2 causes a generalized lymphoproliferative disease in susceptible ruminants. Many questions abound about the pathogenesis of MCF.

MCF was initially thought to be a uniformly fatal disease, but recent reports indicate that some animals develop subclinical infections and others can recover from clinical disease. Therefore based on the clinical status of infection, OvHV-2 infected animals can be divided into asymptomatically infected sheep, subclinically infected cattle, clinically affected cattle, and recovered cattle. It is not known how or why some animals remain subclinically infected, why others experience a clinical episode, why some animals succumb to clinical disease whereas others recover, especially if animals are kept under the same environmental conditions. The studies reported in this dissertation are aimed at investigating differences in OvHV-2 load and tropism as possible factors contributing to differences in the outcome of infection. Hypotheses for this part of the research were:

- There is a difference in OvHV-2 PBMC tropism between asymptomatically infected sheep, subclinically infected cattle, clinically affected cattle, and recovered cattle.
- OvHV-2 load or burden is different between asymptomatically infected sheep, subclinically infected cattle, clinically affected cattle, and recovered cattle.
- OvHV-2 infection results in differences in PBMC subset percentages within the 4 groups of infected animals.

In order to investigate the above hypotheses, peripheral blood mononuclear cells (PBMCs) were obtained from whole blood of animals in each clinical status group and separated into different cell subsets by immunomagnetic sorting. OvHV-2 genomic equivalents (copy numbers) in total PBMCs and each of the sorted subsets were determined by realtime PCR (qPCR). OvHV-2 copy numbers within total PBMCs gave an indication of the OvHV-2 load within each of the 4 clinical status groups. OvHV-2 genome copy numbers in sorted PBMC subsets gave an indication of the OvHV-2 tropism between and within each of the 4 groups. Sorted PBMC subset percentages in each of the groups were examined by flow cytometry. These subset percentages were correlated with viral load to determine the possible effect of OvHV-2 on PBMC subset percentages.

It is not known if animals that recover from clinical MCF clear their infection or not. It is also not known if these animals recrudesce. The second part of this research was aimed at addressing questions about recovery from clinical MCF, i.e. what happens to the virus during recovery and possible factors that may contribute to recovery. The hypothesis for this study was:

- OvHV-2, like other gammaherpesviruses, results in a persistently latent infection in host lymphocytes, and it should therefore be possible to establish a continuous lymphoblastoid cell line (LCL) from PBMCs of clinical cases and an animal that has recovered from clinical MCF.

To investigate the above hypothesis and to address questions about OvHV-2 infection during recovery, LCLs were established from PBMCs of fatal MCF cases and a recovered case of MCF. Different aspects of OvHV-2 infection such as viral replication kinetics and the type of infection within the established cell lines were examined. Viral replication kinetics within cells and supernatants of the LCLs were examined by qPCR. The type of infection, i.e. whether it is latent or productive (lytic) was examined by Gardella gel which examines the presence of circular viral DNA (latent replication cycle) and linear viral DNA (lytic replication cycle). Transcription of late viral gene messenger RNA was examined by reverse transcriptase PCR. The status of OvHV-2 DNA in culture supernatants was examined by DNAse protection assay to see if it was free DNA or contained within a virion structure.

Results from this study will help determine if OvHV-2 behaves like other gammaherpesviruses, i.e. remains latent in lymphocytes and immortalizes or transforms them such that lymphoblastoid cell lines can be established from PBMCs of these animals after recovery from clinical disease. The results would also help contrast the level and type of OvHV-2 infection within LCLs from recovered and fatal cases of MCF. The generated data are expected shed light on certain aspects of OvHV-2 infection related to recovery from clinical disease.

OvHV-2 has never been progagated in an *in vitro* cell culture system. *In vitro* cell culture systems of other gammaherpesviruses have been developed by inducing virus replication with different chemicals or drugs. In the last part of the research, OvHV-2 infected LCLs were treated with different chemicals or drugs in order to inhibit or induce OvHV-2 DNA replication. The hypothesis was that:

- OvHV-2 DNA replication can be induced or inhibited by treating infected LCLs with a variety of chemicals.

Data generated from this study were expected to document the effects of different chemicals on OvHV-2 replication. Establishment of an in vitro culture system for OvHV-2 would be a significant development for MCF research. Results generated from this study will lead to the successful establishment of an *in vitro* culture system of OvHV-2 that is amenable to *in vitro* manipulation and would be a great tool in MCF pathogenesis studies.

CHAPTER 2

LITERATURE REVIEW

INTRODUCTION

Malignant catarrhal fever (MCF) is a lymphoproliferative disease of ungulates (Plowright, 1990). Affected animals include, cattle, bison, deer, elk and pigs (Albini *et al.*, 2003; Boever & Kurka, 1974; Clark *et al.*, 1972; Plowright *et al.*, 1965; Reid *et al.*, 1979; Ruth *et al.*, 1977). The two epidemiological forms are known as sheep associated (SA-MCF) and wildebeest associated MCF. Two γ_2 herpesviruses, namely, ovine herpesvirus 2 (OvHV-2) and alcelaphine herpesvirus 1 (AlHV-1) are the causative agents of the sheep and the wildebeest associated forms of MCF, respectively (Plowright, 1965). OvHV-2 and AlHV-1 are antigenically related and share about 63% DNA homology (Bridgen & Reid, 1991; Herring *et al.*, 1989; Rossiter, 1981; Rossiter, 1983). Disease caused by the two forms has been shown to be clinically and clinico-pathologically the same (Pierson *et al.*, 1979).

Sheep are thought to be asymptomatic carriers of OvHV-2 and to act as a source of infection for susceptible ruminants. Close contact such as common stable, water, and food troughs between sheep and susceptible hosts was initially thought to be essential for disease transmission (Plowright, 1990). Recent reports, however, indicate that transmission can occur between sheep and cattle as far as 70 metres apart (Powers *et al.*, 2005). It has not been possible to induce SA-MCF by inoculating cattle with tissues from

naturally infected sheep (Plowright, 1990). Recent studies indicate that neonatal lambs, unlike wildebeest calves, do not play an important role in disease transmission. Infected sheep shed virus primarily from the nasal cavity (Li *et al.*, 2001a). OvHV-2 whole virions have been detected in sheep nasal secretions. Their infectivity was also demonstrated in a previously negative animal, confirming nasal secretions as a possible mode of transmission (Li *et al.*, 2004). AlHV-1 and OvHV-2 are classified as herpesviruses, within the subfamily, gammaherpesvirinae (van Regenmortel H., 2005).

THE HERPESVIRIDAE FAMILY:

Taxonomy, viral structural components and sizeTaxonomy:

Herpesviridae are complex, linear double-stranded and enveloped DNA viruses. Virions range in size from 120 – 300nm in size (Roizman B & Furlong D, 1974). Variability in size is mainly due to variability in the thickness of the viral tegument and the state of the envelope (Roizman B & Pellett P., 2001). Taxonomically, the family is divided into three subfamilies, namely, the Alpha, Beta and Gammaherpesviruses. Each subfamily is further divided into different genuses. Alphaherpesvirinae are divided into *Simplexvirus*, *Varicellovirus*, Marek's disease-like viruses, and Infectious laryngotracheitis-like viruses. Betaherpesvirinae are divided into, *Cytomegalovirus, Muromegalovirus, and Roseolovirus.* Gammaherpesvirinae are divided into the *Lymphocryptovirus* and *Rhadinovirus.*

Traditional classification as a herpesvirus was based mainly on viral morphology, giving rise to a very diverse family of organisms with shared characteristic morphology (van Regenmortel H., 2005). Division of the family into the different subfamilies, was

based on broad biological criteria. *Alphaherpesvirinae*, are viruses that exhibit rapid cytopathic effects in *vitro* and establish latency in nervous tissue. *Betaherpesvirinae* show a long reproductive cycle, result in formation of cytomegalic cells, and have a very restricted host-range *in vivo*. *Gammaherpesvirinae*, on the other hand, cause latent infections in lymphocytes and are often associated with lymphoproliferative diseases. Further division of the subfamilies into genuses was based on molecular criteria, mainly the genomic size and structure (Roizman B *et al.*, 1992; Roizman B & Furlong D, 1974).

Recent increase in herpesviridae sequence data has led to taxonomic and phylogenetic classification of virions into distinct lineages based on sequence comparisons or genetic data. These distinct genetic lineages are based on two criteria, namely, the nucleotide or predicted amino acid sequence of conserved herpesviral genes, and the identification of particular genes unique to a viral subset. Genetic content is widely used as a means of taxonomic assignment, however, in the absence of sequence data some virions can be confidently assigned at the genus level based on serological cross-reaction (McGeoch D. *et al.*, 1995; McGeoch D. & Cook S., 1994). The use of genetic data supports and confirms previously used (traditional) taxonomic classification. It has also resulted in the discovery of herpesviruses which cannot be classified within the pre-existing subfamilies or need to be re-classified into new genuses. The genomic sequence of *Ictalurid herpesvirus 1* (Channel catfish virus) only tenuously resembles that of other herpesviruses and forms a distinct genetic lineage. This resulted in classification into a new and unassigned genus, Ictalurid herpes-like viruses. Genetic data also lead to the establishment of 2 new genuses within the *Alphaherpesvirus* subfamily, namely,

Mareks' disease-like viruses and Infectious laryngotracheitis-like viruses (van Regenmortel H., 2005).

Herpesviruses are said to be highly adapted to their hosts with severe disease primarily observed only in the very young, the fetus, the immune suppressed or following infection of an alternative host (van Regenmortel H., 2005).

Viral structure and components:

The structure of herpesviridae is conserved within the family. Virion structural components include the viral core, capsid, tegument, and envelope. The viral core consists of the genome in the form of a torus, defined as a ring-shaped object or chamber (Furlong *et al.*, 1972; Rixon, 1993). The toroid form is suggested to be an artifact of conditions employed in preparing samples, though the model has been firmly established in the literature (Puvion-Dutilleul F. *et al.*, 1987; Rixon, 1993). In some virions, the genome has been observed suspended by protenaceous spindles consisting of fibrils. The fibrils seem to be embedded on the underside of the capsid and appear to pass through the hole of the torus (Roizman B & Pellett P., 2001). The DNA is also said to exist in a liquid crystalline state similar to that suggested for the double-stranded λ and T4 bacteriophages (Lepault J *et al.*, 1987; Rixon, 1993).

The capsid, a protein coat around the genome, consists of 162 capsomers, 12 pentons at the icosahedral vertices, and 150 hexons on the triangular faces and edges. 320 triplexes interconnect the pentons and hexons (White O.D. & Fenner F.J., 1994; Yu *et al.*, 2003). Icosahedral capsids have a 5-fold, 3-fold, and 2-fold symmetry at the vertices, faces, and edges, respectively (Harrison S., 2001). The icosahedral shape is a result of

spontaneous assembly of many identical subunits with specific and well-defined interactions (Harrison S., 2001). The shape allows for the virus to store the maximum volume of viral DNA while minimizing surface area. Capsid formation occurs in the infected cell nucleus. It occurs on a protein scaffold that is later cleaved by a viral protease during maturation (Roizman B & Pellett P., 2001). Three types of capsid morphologies exist during capsid formation, namely types A, B and C. Type A capsids, reveal the least electron opacity by cryo electron microscopy, suggesting empty shells. Type B capsids, also known as intermediate capsids, contain a density core with a diameter of ~600Å. Type C capsids are the most electron opaque with internal densities completely obscuring the boundry of the internal capsid shell suggesting the presence of densely packed double-stranded DNA (Rixon, 1993).

The tegument is poorly defined with no evidence of symmetry. It contains many proteins that vary markedly in abundance and not all of which are required for virion formation (van Regenmortel H., 2005).

GENERAL HERPESVIRIDAE GENOMIC STRUCTURE AND ARRANGEMENTS

The herpesvirus genome is a linear double stranded DNA. Gammaherpesvirus ends are linear with free accessible 5'-phosphate groups that are not protected by a covalently bound protein. Alphaherpesviruses possess a 3' single base extension at the terminus that is said to assist in genome circularization upon cellular entry (Bankier *et al.*, 1985; van Regenmortel H., 2005). During latent infection, the genome occurs in an episomal form (covalently closed and circular), with expression of only a few genes

termed latency-associated genes. Latent genomes retain the ability to replicate and cause disease during reactivation (Roizman B & Pellett P., 2001).

The entire linear viral genome, referred to as the M-genome, is divided into two regions, the H-DNA and L-DNA. The two regions classified based on their rate of sedimentation during isopyknic centrifugation. The H-DNA is heavier repetitive DNA found at the termini of the genome, has a very high G-C content (~80%), and makes up one third of the M-genome. The L-DNA is lighter and makes up the unique coding region of the genome with a relatively lower G-C content of about ~30%. H-DNA may also be found interspersed within the L-DNA region, dividing the genome into unique long and short regions. The L-DNA is not infectious without the H-DNA indicating that the latter is required for viral replication. Absence of a TATA box or a polyadenylation site within the H-DNA sequence shows lack of transcription or translation from the H-DNA during viral replication. For *Herpes virus saimiri* (HVS), terminal repeats are required for viral entry into the lytic cycle and for maintenance of the episomal DNA in latently infected cells (White *et al.*, 2003). H-DNA is required for very few replicative functions, although it makes up about one-third of the genome. This suggests an evolutionary trait where certain gene function was lost but a minimum genome size requirement is maintained.

Herpesviridae genomes can be divided into 6 different groups, designated A-F (Fig.2.1), based on the location and arrangement of their terminal repeats (A.J.davison & D.J.McGeoch, 1995; Roizman B & Pellett P., 2001).

Group A: Consists of a large sequence of repeats from one terminus that is directly repeated on the other end, e.g. channel catfish herpesvirus.

Group B: The terminal sequence is repeated directly numerous times at each end and the total number of repeats may vary at each end, e.g. Herpes virus saimiri (HVS).

Group C: The stretch of direct terminal repeats is smaller (<100bp), but there may be other directly repeated but unrelated sequences (>100bp) that subdivide the L-DNA or unique region into several well-delineated stretches, e.g. Epstein Barr virus (EBV).

Group D: The repeat sequence from one terminus is repeated in an inverse orientation, internally. This divides the unique region into a unique-long/ large (U_L) and short/ small (U_S) sequence. The unique-short sequence, flanked by inverted repeats, can invert relative to the unique-long sequence. Such an inversion in infected cells results in two equimolar genomic populations differing solely on the position of the unique-short sequence, e.g. Varicella zoster virus (VZV).

Group E: The terminal repeats from both ends are repeated internally in an inverted manner and juxtaposed to each other. The unique region is therefore divided into two regions, unique long and short, each flanked by inverted repeats. Both unique regions (long and short) can invert relative to each other resulting in 4 equimolar populations in infected cells that differ in the position of the unique regions relative to each other, e.g. Herpes simplex virus (HSV) and Human cytomegalo virus (HCMV).

Group F: In this final group, the terminal sequences are not identical and are neither repeated in a direct or an inverted manner, e.g. Tupaia herpesvirus.

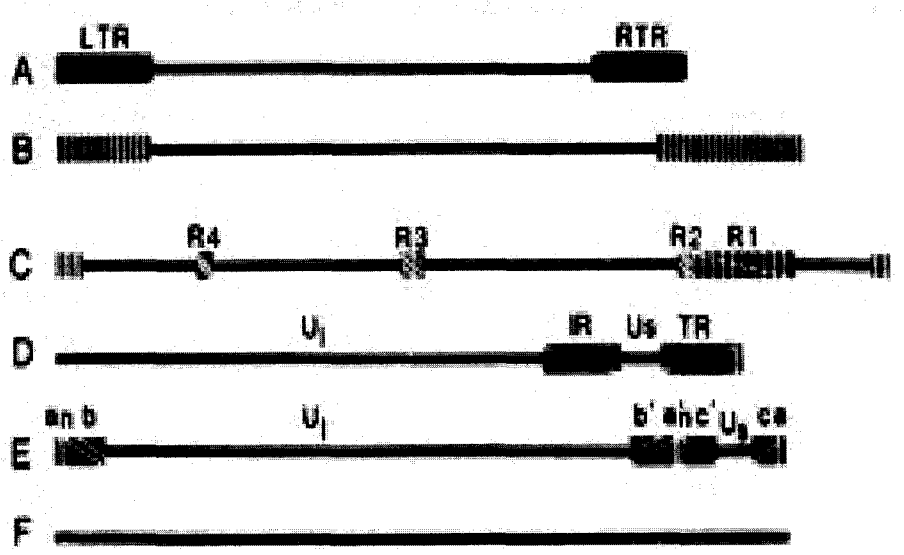


Fig. 2.1: 6 groups of herpesviridae genomes based on the genomic organization and location of the different repeats (A.J.davison & D.J.McGeoch, 1995; Roizman B & Pellett P., 2001)

HERPESVIRIDAE GENE ORGANIZATION AND FUNCTION

Though widely divergent in sequence, herpesviridae genes are conserved at the protein function level. Herpesviridae genes are grouped into the following 5 categories (A.J.davison & D.J.McGeoch, 1995).

1) Genes that code for proteins that control the replicative cycle. This includes proteins that regulate transcription resulting in coordinated gene expression or proteins that modulate processes in infected cells to facilitate viral replication, i.e. shutting down host cell macromolecular synthesis in favor of viral replication.
2) Genes that encode proteins essential for the DNA replicative machinery, i.e. DNA polymerases, the helicase-primase complex, etc.
3) Enzymes involved in the DNA or nucleotide metabolism, i.e. thymidine kinase, uracil-DNA glycosylase, DHFR, etc.
4) Genes that encode virion structural proteins.

5) Genes encoding proteins involved in viral pathogenesis or latency, the absence of which does not affect viral replication or growth. This category also includes viral proteins that modulate the host's immune system.

There are about 40 conserved core genes within the family that are found throughout each of the above gene function groups (A.J.davison & D.J.McGeoch, 1995). The core genes are grouped into 7 core gene blocks within the genome. They exist in various permutations of order and orientation throughout the family. Gene block arrangement and order are conserved at the subfamily level.

GAMMAHERPESVIRIDAE GENE STRUCTURE AND ORGANIZATION

Gammaherpesviridae are divided into two genera, the Lymphocrypto and Rhadinoviruses. The term rhadinovirus, Greek for 'fragile', was coined from the extreme intragenomic heterogeneity in the GC content of the L and H-DNA's, supposedly resulting in fragmentation during density centrifugation (Roizman B. *et al.*, 1981). AlHV-1 and HVS, both rhadinoviruses, have a 46.17% and 34.5% GC content in the L-DNA and a 71.83% and 70.8% content in the H-DNA, respectively (Albrecht *et al.*, 1992; Coulter, 2001). The L-DNA contains different open reading frames (ORFs) that are tightly packed and evenly arranged amongst the two DNA strands. An uncommon characteristic of both AlHV-1 and Equine herpesvirus 2 (EHV-2), is presence of a non-coding area in a non-repetitive region of the genome (Coulter, 2001).

The DNA sequence can be divided into regions based on sequences that are either conserved within the whole family, the subfamily, genus, or are variable and specific to the virus of interest. Genes conserved within the gammaherpesvirus subfamily can be

divided into blocks I-V (Fig. 2.2). Genes conserved throughout the whole family (core genes) can be found within conserved blocks I, II and IV. They code for proteins involved in nucleotide metabolism, DNA replication, virion structural proteins, proteins involved in viral DNA packaging, modification of the infected cell, and in enabling the virus envelope to fuse with the cell membrane (Elliot kieff & Alan B.Rickinson, 2001). Blocks III and V contain genes that are only conserved within the gammaherpesvirus subfamily. These genes are thought to impart special gammaherpesvirus characteristics within the subfamily. The conserved gene blocks are interspersed within variable regions. These variable regions encode genes that are conserved within some but not all members of a subfamily and genes that are unique to a specific virion. Though it shares DNA homology, collinearity, lymphoproliferative syndromes, and induced disease with some of the other rhadinoviruses, AlHV-1 is thought to use different kinds of unique genes to bring about the lymphoproliferation and cell transformation in culture (Ensser *et al.*, 1997). Unique genes are prefixed with the first letter of the virion, i.e. K and A in Kaposis sarcoma virus and AlHV-1, respectively (Coulter & Storz, 1979).

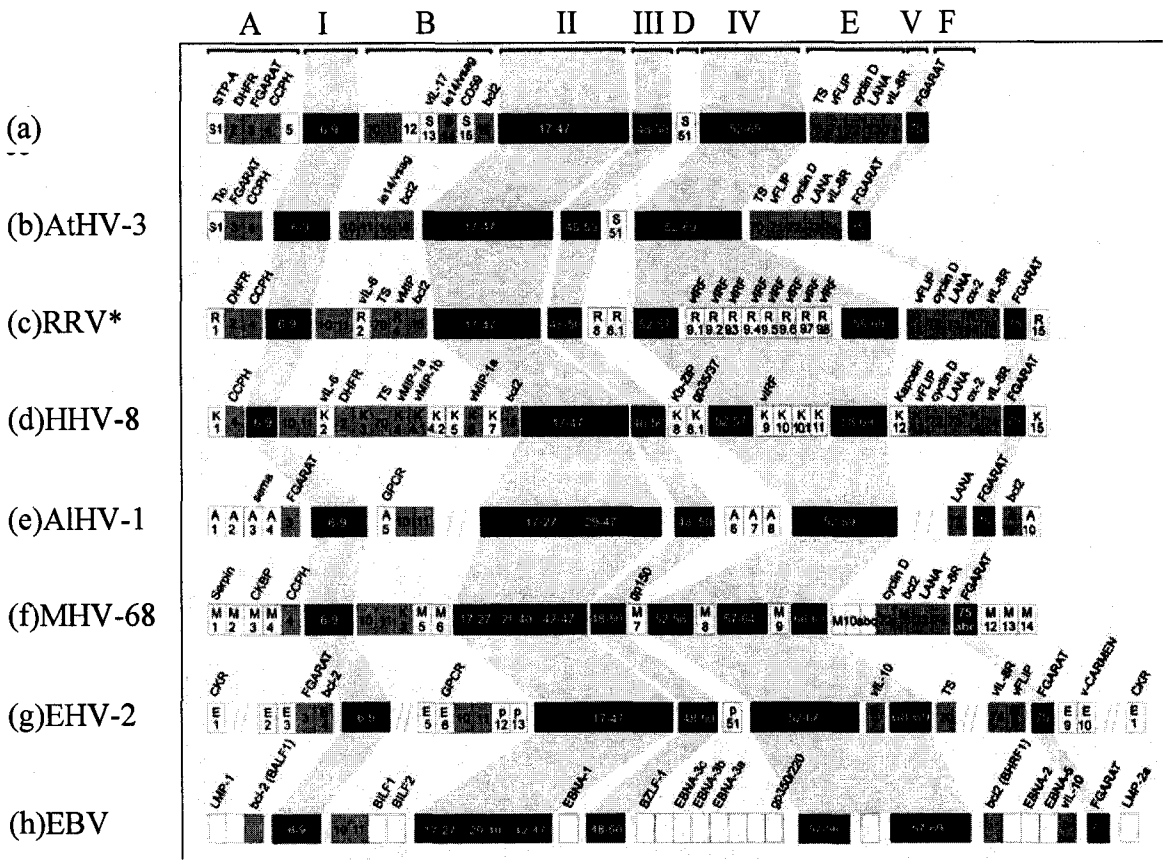


Fig 2.2: Genomic structure and organization of gammaherpesvirus genomes (Coulter, 2001).

GAMMAHERPESVIRIDAE HOST CELL GENE HOMOLOGUES

Gammaherpesviruses, especially rhadinoviruses, contain genes that are homologous to host cell genes (Fig. 2.3). These genes usually lack introns, indicating capture from host mRNA. The genomic area near terminal repeats seems to be a hot spot for host gene homologue integration, possibly assisted by the process of genomic replication that occurs in these areas. The left-hand end of many gammaherpesviruses is considered important in virus pathogenesis and oncogenesis due to the presence of these host cell homologues or genes that cause transformation of host cells (Coulter, 2001).

These cellular homologues are usually an added advantage to survival of the virion and usually code for proteins that interfere with the immune system, enzymes involved in nucleotide metabolism, and for putative regulators of cell growth. Viral homologues that modulate the immune system include complement control protein homologue (CCPH), HVS-ORF 14,-which is homologous to mouse mammary tumor virus superantigen, and vCD59- viral CD 59 homologue. Homologous genes for nucleotide metabolism include dihydrofolate reductase (DHFR) and thymidylate synthase (TS). Regulators of cell growth or apoptosis homologues include cyclin-viral cyclin D homologue and viral *bcl2* homologue. Cellular homologues to genes that are related to cytokine or cytokine transduction include vIL-6, the CC chemokine family's macrophage inflammatory proteins alpha and beta (MIP1α and MIPβ), interferon responsive factor (IRF), vIL-8R-viral IL-8 receptor, vIL-17, G-coupled receptor alpha and beta (gcrα/β), and semaphorin.

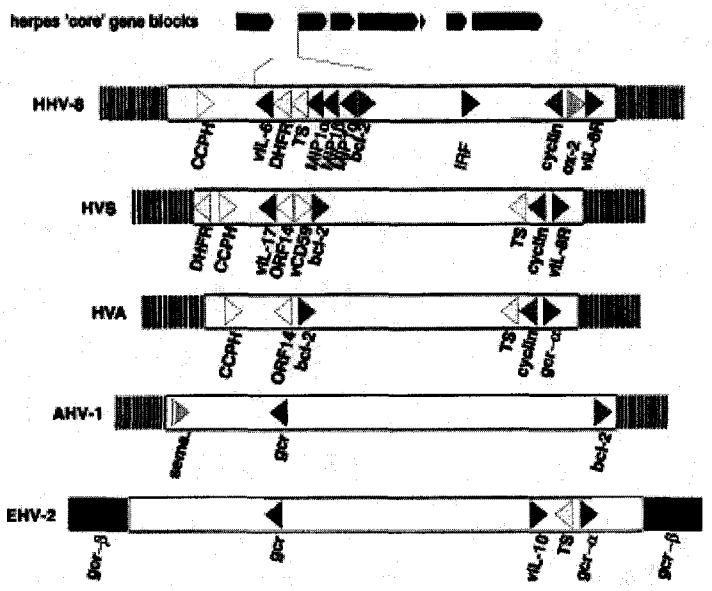


Fig 2.3: Cellular gene homologues found in the different gammaherpesvirus genomes (Neipel F. *et al.*, 1997).

MCF CLINICAL SIGNS AND HISTOPATHOLOGICAL LESIONS

Disease caused by both OvHV-2 and AlHV-1 have been shown to be clinically and clinico-pathologically the same (Pierson *et al.*, 1979). Four clinical forms of the disease have been described; the per-acute, head-and-eye, intestinal, and mild form. An additional chronic form of the disease has also been mentioned (Gotze R., 1930; Plowright, 1990). There is not always a clear-cut distinction between the different forms. Pyrexia and lymphadenopathy are common amongst the different forms.

The per-acute form, commonly observed in deer and bison is characterized by fever and acute death without other signs. The intestinal form is characterized by persistent pyrexia, enlarged lymph nodes and loose watery feces up to the time of death. The diarrhea might be bloody. The mild form of the disease has been described in some publications as an experimental phenomenon of the disease that might be missed. It may vary in presentation from an acute-disease like syndrome, to transient pyrexia with minimal nasal catarrhal. The temperature rarely exceeds 40.5°C. There is lymphadenopathy and there might be vague urticaria-like skin lesions. The disease course may last for 24 hours or up to several weeks.

The head-and-eye form is the most commonly reported form of disease. It is characterozed by lacrimation, blepharospasms, corneal opacity, muco-purulent ocular and nasal discharge. The animal may become dyspneic. There is hyperemia of the muzzle and nasal passages, erosions and ulcerations of the muzzle and oral mucosa, and salivation that becomes more ropy. There might also be lameness and diarrhea. These animals usually succumb about 3 days after the onset of clinical signs (Pierson *et al.*, 1978; Plowright, 1990).

Underlying histopathological lesions involve 3 major components, namely,lymphoid hyperplasia, epithelial lesions, and vascular lesions. A generalized lymphoid infiltration and hyperplasia is observed throughout the body. Vascular lesions involve perivascular cuffing with lymphoid infiltration and necrosis of the tunica media and tunica intima (necrotizing vasculitis). Thrombosis is reported to be very rare. Epithelial changes include marked sub and intra-epithelial infiltration with lymphocytes. Affected epithelia shows necrosis and sloughing. Underlying blood vessels are usually involved with the lymphoid proliferation and infiltration but seldom show thrombotic change. Very rare occurrence or absence of thrombosis disproves the suggestion that epithelial lesions may be due to vascular damage or compromise (Liggitt *et al.*, 1978). The severity of epithelial degenerative and necrotic changes seems related to the severity of lymphoid accumulation in the epithelium and lamina propria suggesting that the degenerative changes are more due to the infiltrating lymphocytes than vascular damage (Liggitt *et al.*, 1978). There is also marked necrosis and destruction of small lymphocytes in lymphopoeitic organs. The generalized lymphoid infiltration and proliferation is by large lymphoblastoid cells (Plowright, 1990). No herpesviral antigens or virion structures are detectable in tissues of infected hosts (Liggitt *et al.*, 1978). Immunohistochemistry demonstrated CD8+ T-cells as the predominant infiltrating cells within vascular brain lesions of cattle and bison. The majority of these cells were found to be positive for OvHV-2 DNA by in situ PCR (Simon *et al.*, 2003).

PROPOSED MCF PATHOGENESIS

Although Plowright alluded to some animals surviving clinical disease (Plowright, 1964; Plowright, 1990), MCF has always been considered a fatal infection with high mortality and resultant death 3-7 days after the onset of clinical signs (Plowright *et al.*, 1960; Plowright, 1990; Plowright & Rossiter, 1975; Selman *et al.*, 1974). Recent studies, however, report recovered and sub-clinically infected cases (Li *et al.*, 1995; Li *et al.*, 1996; O'Toole *et al.*, 2002; O'Toole *et al.*, 1995; O'Toole *et al.*, 1997; Penny, 1998; Schultheiss *et al.*, 1998).

Recovery of some animals from a clinical episode or failure to show disease after experimental inoculation was suggested to be due to exposure to either a different strain of the virus or partially resistant animals due to previous exposure to the virus (Rweyemamu *et al.*, 1976). More and more published reports indicate that within a group of exposed animals, some will develop disease and others will not (Berezowski *et al.*, 2005). Recent data also indicates that some animals do recover from acute disease without previous exposure or partial resistance (O'Toole *et al.*, 1997). It remains intriguing why and how some animals seem to be more susceptible than others or how some recover from an acute episode and others do not. Based on the above, OvHV-2 infected animals can therefore be divided into the following 4 groups:

Group 1: Sheep that become asymptomatically infected at an early age (~ 3-6 months) (Li *et al.*, 1995) and remain latently infected throughout life. These animals do not show any clinical signs of infection and act as a reservoir of infection for susceptible ruminants, i.e. cattle, bison, deer and elk.

Group 2: Includes cattle that remain sub-clinically infected after contact with asymptomatically infected reservoirs (sheep). These animals either have antibodies to MCF viruses demonstrated my competitive inhibition ELISA or test positive for OvHV-2 DNA by PCR. However, the infection does not progress to a clinical phase. Some animals have been shown to intermittently test positive for the presence of OvHV-2 DNA for over a year after infection (Powers *et al.*, 2005). It is not known if these animals ever progress to a clinical disease.

Group 3: Includes cattle that develop a clinical and fatal case of MCF from contact with asymptomatically infected sheep.

Group 4: Includes cattle that experience a clinical episode of MCF followed by recovery. These animals continue to test positive for OvHV-2 by PCR and or ELISA. Some animals may develop chronic corneal opacity or go blind in one or both eyes, due to the brief clinical episode. It is also not known if these animals ever revert to a clinical episode of MCF. Corticosteroid treatment has been incriminated in both remission or recrudescence of clinical disease (Heuschele *et al.*, 1985; Milne & Reid, 1990) Recovery and chronic disease are suggested to be a significant part of the clinical spectrum of MCF, which might occur with more frequency than originally thought (O'Toole *et al.*, 1995; O'Toole *et al.*, 1997).

Sheep have traditionally been thought to be asymptomatic carriers of OvHV-2 and to play the same epidemiological role as wildebeest in Africa. The terms, latent carriers (Pierson *et al.*, 1973) and sub-clinical (Li *et al.*, 1995) infection, have been used to define infection in sheep. There have been reports, some unsubstantiated, suggesting occurrence of clinical MCF in sheep (Buxton *et al.*, 1985; Schmitz & Grumbein, 1981).

A recent study reported the development of MCF-like disease in sheep after aerosol inoculation of nasal secretions containing high doses (~$3.7X10^9$ copies) of OvHV-2 (Li *et al.*, 2005). Implications of occurrence of clinical disease in sheep on the pathogenesis and epidemiology of MCF are unknown.

The pathogenesis of MCF that results in lymphoproliferation, lymphocyte infiltration, necrotic vasculitis, and epithelial necrosis remains a mystery. Ultrastructural studies of the lesions have not demonstrated viral structures within the tissue lesions (Liggitt & DeMartini, 1980). Immunoflourescence and *in situ* hybridization of lesions have failed to detect both viral antigens and nucleic acids (Edington *et al.*, 1979; Rossiter, 1985; Rossiter, 1980). CD8+ T-lymphocytes are the predominant cells associated with the vascular lesions (Ellis *et al.*, 1992; Nakajima *et al.*, 1994; Nakajima *et al.*, 1992). In situ PCR and immunohistochemical studies of bovine and bison brain lesions, indicated a predominance of CD8+ T-lymphocytes and the majority of these cells were positive for OvHV-2 DNA (Simon *et al.*, 2003). There is no evidence that endothelial cells within the vascular lesions harbor OvHV-2 DNA. It is also not known if the epithelial cells within the epithelial lesions are positive for viral DNA. The lymphoid infiltration could indeed be due to a lymphocyte dysregulation (Rossiter, 1985) or it may be due to lymphocytes, mainly cytotoxic CD8 cells, responding to infected host epithelial cells. Most importantly in the brain lesions, the infiltrating CD8+ T-cells have been shown to be infected or harbor viral DNA (Simon *et al.*, 2003). This suggests that a selective process might be involved in the lymphoid infiltration that occurs, resulting in tissue infiltration only by infected CD8+ T-cells. Though it is not known if epithelial cells within the lesion are infected, endothelial cells within the vascular lesions have not been shown to harbor viral

DNA (Simon *et al.*, 2003). The mechanism involved might be a dysregulation of the infected CD8 T-cells and a cell mediated immune reaction gone wrong with resultant destruction and necrosis of both the vascular endothelium and mucosal epithelia.

There are more questions than answers as far as MCF disease pathogenesis is concerned. Some of these include the following:

- What are the reasons or factors involved in varied clinical outcomes after infection?
- Long incubation periods between exposure and development of disease have been reported. What happens to the virus during the incubation period?
- What is the cause of the lymphoproliferation?
- What causes the damage to epithelial surfaces?
- What is the 'trigger' in the host or virus responsible for clinical disease, recovery, or sub-clinical infection?
- What host factors contribute to development of clinical disease in some animals, but not others, especially if the different animals are kept under the same conditions?
- Do sub-clinically infected and chronic recovered animals ever recrudesce?
- Which cells within the PBMC population are infected?
- Does viral titer play a role in disease pathogenesis?
- Does the phenotype of an infected cell (PBMC) play a role in disease pathogenesis and outcome of infection?

Possible reasons for differences in outcome of infection:

- Possibility of natural and unnatural hosts. Herpesviruses have evolved closely with their natural hosts such that they cause little, if any, disease in their natural hosts except during immune suppression. HVS and HVA do not cause disease in their

natural hosts the squirrel and spider monkeys, respectively. However, they do cause lymphoproliferative disease in other new world monkeys auc as tamarins, marmosets, and owl monkeys. Similar to the above mentioned examples, sheep might be natural hosts of OvHV-2 resulting in asymptomatic infections as opposed to clinical disease as is observed in some cattle. In a recent report, an MCF-like disease was induced in sheep under unnatural or experimental conditions (Li *et al.*, 2005)

- Differences in viral titer or copy numbers in the different groups of MCF-affected animals.
- Predominant state of the virus in the host, latent versus productive infection. Also tying in with host protective mechanisms and the ability to maintain a tight regulation on viral replication and or gene expression.
- It is a possibility that animals that test positive for OvHV-2 by PCR, but do not show any clinical signs might be harboring the infection in an episomal form as in the case of host B-cells that harbor latent EBV, or in latently infected neural ganglia in HSV1, or HSV 2 or varicella zoster. OvHV-2 might remain latent with resultant inapparent disease or might go into a lytic cycle with resultant clinical disease. Attempts to transmit MCF to hamsters, using cell suspensions from positive animals that had extended survival times failed (O'Toole *et al.*, 1997). The above statements assume that lytic viral replication is partly responsible for the resultant lympho-proliferation. At this point, the exact cause of the pathology that occur as a result of clinical MCF is not known. The suggested immune dysregulation or auto-immune disease has not been proven conclusively.

- Host's immune system. The host immune system is thought to play an important role as far as gammaherpesvirus infections are concerned. Infections in immuno-competent individuals usually remain latent. Occurrence of clinical signs (viral associated malignancies) due to EBV and Kaposis Sarcoma are usually associated with immuno-compromised hosts or are usually a suggestion of an underlying immuno deficiency (Moore & Chang, 2001; Rickinson & Kieff, 2001). High AlHV-1 antibody titers did not seem to protect immunized animals from challenge with a virulent strain or the development of disease following exposure to wildebeest (Plowright *et al.*, 1975). This together with failure of detection of viral antigens in ultra-structural studies of the lesions may be an indication that direct viral antigens and the host immune system plays minimal importance in actual development of clinical disease.
- Viral gene rearrangements may render one viral strain more pathogenic than the next.
- The presence of more than one viral subgroup or strains with varying pathogenic properties and sequence diversity. HVS is classified into subgroups, A, B and C. Subgroup B is less oncogenic than subgroup C, which has the strongest oncogenic properties. Tamarins are susceptible to all subgroups. Subgroup B viruses were not able to cause disease in adult common marmosets, whereas, subgroup C, parental strain C488, causes acute peripheral T-cell lymphoma within only a few weeks in common marmosets or cottontop tamarins. Sheep might not be susceptible as a natural host to any OvHV-2 subgroups but the presence of different subgroups may cause disease with varying outcomes in susceptible hosts, including cattle (ArminEnsser & Bernhard Fleckenstein, 2005).

- Evasion of the immune system. Herpesviridae, especially gammaherpesviridae have evolved and adopted different mechanisms to successfully evade detection by the host immune system. Some mechanisms involve maintaining the virion in a latent state, whereas, some involve the use of captured host gene homologues, especially those affecting the host's immune system.
- Genome methylation may play a role in disease outcome of the different groups of animals. Methylation of some promoters in the viral genome may suppress expression of specific genes. This could maintain the viral episome in a quiescent form, as opposed to actively replicating or expressing genes, i.e. virulence genes with resultant unfavorable outcome (Coulter, 2001).

ESTABLISHED OvHV-2 POSITIVE LYMPHOBLASTOID CELL LINES

LCL Characterization:

Lymphoblastoid cell lines can be established from peripheral blood mononuclear cells (PBMCs) and tissues of animals infected with most gammaherpesviruses. Epstein Barr virus (EBV), the type species for lymphocryptoviruses, results in the establishment of latently infected B-lymphoblastoid cell lines. Herpesvirus saimiri (HVS), the type species for the rhadinoviruses, results in the establishment of T-lymphoblastoid cell lines.

Such T-lymphoblastoid cell lines (LCLs) are IL-2 dependent and can be established from clinical cases of sheep associated malignant catarrhal fever (SA-MCF) (Reid *et al.*, 1983). They are characterized as T-cell lines with NK-cell activity, and a cytotoxicity that is MHC I unrestricted (Cook & Splitter, 1988; Reid *et al.*, 1983). The cell lines range in phenotype from either $CD4^+$, $CD8^+$, or $\gamma\delta^+$ (gamma-delta), to a mixed

$CD4^+CD8^+$ phenotype (Burrells & Reid, 1991). Only LCLs established from an MCF case in deer were gamma delta positive. Some cell lines were infectious to rabbits, though no viral structures could be demonstrated by electron microscopy (Reid *et al.*, 1983). As few as 10^2 cells established from a clinical case in a rabbit were required to propagate disease. Infection and subsequent dysfunction of large granular lymphocytes were suggested to play a central role in the pathogenesis due to lack of electron microscopic evidence of the virus. They are thought to be anergic cells that might be activated, giving rise to characteristic lesions of MCF (Reid *et al.*, 1983; Schock *et al.*, 1998). Histopathologically, large lymphocytes are observed in the tissue infiltration and perivascular cuffing that occurs in clinical cases, whereas, small lymphocytes within lymphoid tissues appear necrotic (Liggitt *et al.*, 1978; Pierson *et al.*, 1978).

Factors that determine the phenotype of the LCLs in culture are unknown. In addition it is unknown how the LCL phenotype may be connected to or might contribute to the pathogenesis. Infection with OvHV-2 and the development of clinical disease is associated with lymphoproliferation and tissue infiltration (Plowright, 1990). Infection also allows for cells to survive in culture or be immortalized. The lymphoproliferation, the trigger of which is unknown, only occurs in clinical cases and not in both sheep and subclinical cases. Established LCLs from MCF clinical cases might help provide answers about the MCF pathogenesis *in vivo*.

Mechanisms involved in LCL immortalization / transformation:

Mechanisms involved in LCL immortalization by OvHV-2 *in vitro* might play a role in the lymphoproliferation that occurs *in vivo*. Lck and Fyn kinase, both src kinases, are constitutively activated in bovine and rabbit IL-2 independent large granular

lymphocytes infected with OvHV-2 and AlHV-1, respectively. Src kinases are crucial for initial activation of T-cells via several cell receptors, including the T-cell receptor and CD2 (Swa *et al.*, 2001).

Other gammaherpesviruses (HVS and EBV) also cause immortalization or transformation of lymphocytes in culture. Some of the mechanisms involved in immortalization or transformation have been thoroughly studied. Herpesvirus saimiri (HVS), the prototype of the *rhadinoviruses*, is not pathogenic in its natural host, the squirrel monkey, even after immunosuppression. About 80% of the natural host population is positive. Virus persists in T-cells of the natural host for a lifetime and can be obtained from at least one in 10^6 cells by co-cultivation with permissive monolayer cells (Mittrucker *et al.*, 1992). In contrast with the benign nature of the virus in the natural host, HVS has a high oncogenic potential in other New World primates. Susceptible animals usually die from rapidly progressing neoplasia within a couple of weeks after infection. Cell lines can be established from infected animals. They initially produce virus in 1-10% of cells but eventually loose this ability to produce virus after prolonged culture (Schirm *et al.*, 1984).

HVS-transformed marmoset cell lines had a $CD2^+$, $CD8^+$, $CD4^-$ and $CD56^+$ phenotype (Kiyotaki *et al.*, 1986).. It is difficult to clone or establish and maintain these LCLs at low density, an indication that cell to cell contact (auto-stimulation) is necessary for continuous growth (Schock *et al.*, 1998). Binding of the CD2 molecule to its natural ligand (IL-2) creates the critical stimulating signal in the activation dependent growth (Mittrucker *et al.*, 1992). HVS subgroup C strains transform human T-cells in culture. These cells respond to cell to cell contact leading to IL-2 dependent autocrine growth.

The left most region of HVS was found to be highly variable amongst the different strains, namely, group A, B and C. This region was suggested to be directly involved in oncogenesis. Subgroups A and B have a single gene within the hypervariable region, namely, StpA (Saimiri transformation protein) and StpB, respectively. Subgroup C, however, has two genes in the same loci, namely, StpC and Tip (two in one protein), both of which are required for transformation (Duboise *et al.*, 1998). The transforming potential of StpC exceeds that of StpA, whereas StpB shows no transforming ability (Choi *et al.*, 2000; Jung *et al.*, 1991). The Stp transforming effects are thought to be either via association with Ras, via the activation of the Ras-mediated signaling or via their ability to activate NF-kB. Tip was shown to bind to and become phosphorylated by Lck, a Src-family protein kinase that plays an essential role in T-cell signaling (Tsygankov, 2005). Therefore viral oncogenes, autostimulation (cell to cell contact), and ligand binding to the CD2 receptor contribute to the growth and proliferation of transformed cells in culture.

State (form) of the virus in cultured LCLs:

Despite thorough studies, virus particles or proteins have not been detected in lesions of MCF clinical cases (Liggitt & DeMartini, 1980). Viral particles have not been detected in all but one reported OvHV-2 positive lymphoblastoid cell lines (Reid *et al.*, 1983; Rosbottom *et al.*, 2002). The majority of cells in lymphoblastoid cell lines are thought to be latently infected, with a very few cells productively infected. Latent virus is in an episomal (nonintegrated, covalently circular) form, with productive virus in a linear from.

EBV (Epstein Barr virus) DNA could be separated from high molecular weight DNA of latently infected Raji cells by sedimentation (Tanaka & Nonoyama, 1974). Based on the sedimentation, the latent EBV genome was thought to be a nonintegrated, covalently closed circular form (Adams & Lindahl, 1975). Raji cells were infected at a rate of 50-60 latent EBV genomic equivalents per cell (Nonoyama & Pagano, 1973). High multiplicity of non integrated, covalently closed, circular viral DNA molecules were also reported in HVS transformed cell lines (Schirm *et al.*, 1984). These forms (episomal, non-integrated, covalently closed and circular), were visualized by electron microscopy following partial denaturation mapping of HVS (Herpesvirus saimiri) and HVA (Herpesvirus ateles) in transformed tumor and lymphoid cell lines. Large deletions were observed in the L-DNA (unique region) of the viral genomes indicating that certain parts of the genome are not required for persistence or maintenance of the episomal forms (Kaschka-Dierich *et al.*, 1982). In another report, substantial rearrangements and large deletions in the HVS genome of a transformed lymphoblastoid cell line were observed. About 73% of the genome was dispensable for maintenance of the viral episome (non-integrated, covalently circular form) in culture (Schirm *et al.*, 1984).

Transformation or immortalization by gammaherpesvirus can yield productive or non-productive cell lines. Though predominantly latently infected, productive cell lines can be induced to a productive/lytic state, whereas, non productive cell lines cannot (Ben-Sasson & Klein, 1981). Defective viral genomes with deletion or loss of parts of the L-DNA sequence were observed in some HVS transformed non producer cell lines. No hotspots were reported for rearrangements, deletions or inversions of HVS genome in transformed cell lines (Fleckenstein *et al.*, 1977).

Like in other gamma herpesviruses, OvHV-2 nonintegrated covalently circular (episomes) and linear forms were detected in LCLs established from bovine and rabbit lymphocytes, respectively. The two different genomic forms, namely, episomal (non-integrated, covalently circular) and linear are thought to indicate non productive (latent) and productive (lytic) infectious states, respectively (Rosbottom *et al.*, 2002). Viral genomic equivalents of either the linear or episomal OvHV-2 DNA in culture have not yet been examined.

Alcelaphine herpesvirus 1 (AlHV-1) genomic rearrangements in culture are associated with a loss of virulence in susceptible hosts. The virus transitions in culture from a cell associated virulent (CAV), to a cell free attenuated (CFA) form (Handley *et al.*, 1995). Like in HVS, the changes in the genome involve rearrangements and deletions with truncations of specific ORFs. None of the observed changes were consistent, but they were found to routinely involve translocation of a similar region of DNA from around the center of the DNA to areas either next to or in between the terminal repeats on either end of the genome (Wright *et al.*, 2003).

Existence of an OvHV-2 variant was suggested after discovery of a 2 base substitution within the *Rsa I* and *Bmy I* restriction sites of ORF 75. The nucleotide substitution resulted in an amino acid change from lysine to threonine and failure to be digested by the above mentioned restriction enzymes (Masters *et al.*, 2003). Unlike AlHV-1, OvHV-2 cannot be cultured productively *in vitro*. Research is hampered by the lack of reagents and a consistent failure to isolate the virus and define a productive cell line system (Rosbottom *et al.*, 2002).

EXAMINED LCL AND PBMC CELL SURFACE MARKERS

CD2 cell surface marker:

The CD2 cell surface marker is a 75 kDa β subunit of the IL-2 receptor and an adhesion molecule for signal transduction. It is expressed on the surface of T-cells and NK-cells but not on B-cells (Goldsby R *et al.*, 2000). Bovine CD2 (BoCD2) is expressed by the majority of CD4 and CD8 positive T-cells in the peripheral circulation including $WC1^-$ γδ (gamma delta) T-cells. The marker is not expressed on $WC1^+$ γδ T-cells, B-cells, and monocytes (Naessens & Howard, 1993).

CD3 cell surface marker:

The CD3 molecule is the signal transduction element of the T-cell receptor that is present on T-cells (T-helper and T-cytotoxic) but not on NK-cells. It is expressed on both αβ and γδ T-cells (Naessens & Howard, 1993).

Gamma delta (γδ) cell surface marker:

The ruminant γδ T-cell population is very large and complex compared to the human and mouse populations. The γδ T-cell population is even larger in neonatal animals where γδ T-cells are the predominant fraction of the T-cell population (Hein & Mackay, 1991). In addition, ruminant $CD4^-CD8^-$ $\gamma\delta^+$ T-cells express a 220KD surface antigen referred to as the WC1 molecule. The WC1 molecule is also referred to as T19 and has been thoroughly studied in sheep. The WC1 cDNA clone was shown to encode an integral membrane protein with an extra-cellular domain consisting of 11 scavenger receptor cysteine-rich-repeats, making it part of the scavenger receptor cysteine-rich

family. This family of transmembrane proteins share homology with CD5 and CD6 molecules (Wijngaard *et al.*, 1992). The BoCD6 molecule, together with its human homologue, is suggested to be involved in transduction of activation signals across the T-cell membrane. Stimulation of the CD6 molecule results in *in vitro* proliferation of peripheral blood mononuclear cells (Letesson & Bensaid, 1991).

The molecule was termed WC1 for workshop cluster 1, due to lack of an obvious human homologue (Morrison & Davis, 1991). Recently, however, the presence of WC-1 like sequences has been reported in genomes of all mammals tested including mice and humans (Wijngaard *et al.*, 1992). Phenotypically, the ruminant γδ cell population can be divided into two groups, namely, a WC1 (T19 in sheep) positive and a WC1 negative population. Additionally, different functional subsets (WC1.1 or WC1.2), have been reported based on the form of the WC1 molecule. These two subsets differ according to interferon (INF) production, their proliferative capacity to specific cytokines, and representation within the total PBMC population. Minor differences in their intracytoplasmic tail sequences are thought to affect signaling (Rogers *et al.*, 2005). Gamma delta cells have an unusual distribution in lymphnodes. Unlike αβ T-cells, which re-circulate from the peripheral circulation to the lymphnodes, γδ T-cells home in epithelial surfaces, followed by re-circulating back to the lymph nodes via lymph (Hein & Mackay, 1991). Though their function is unknown, they are suggested to protect epithelial surfaces despite their high percentages in peripheral circulation (Haas *et al.*, 1993; Hein & Mackay, 1991). γδ T cells reportedly lack, CD4, CD8 and CD6 markers, with $WC1^+$ cells additionally lacking a CD2 marker (Clevers *et al.*, 1990; Naessens & Howard, 1993). The TcR1-N24 monoclonal antibody used in this study, detects both

WC1 positive and negative cells, essentially the majority, if not all of the γδ T-cells in circulation (Davis *et al.*, 1996).

CD4 and CD8 cell surface markers:

The CD4 and CD8 molecules are expressed on the cell surface of αβ T-cells as coreceptors to the T cell receptor (Howard *et al.*, 1991; Letesson & Bensaid, 1991).

B-cell cell surface marker:

Mature B-cells express membrane bound IgD and IgM on their cell surface. The surface Ig molecules have a short cytoplasmic tail which is associated with disulfide-linked heterodimer Ig-α/Ig-β intracellular signaling molecules. The B-cell receptor is therefore divided into a ligand binding surface immunoglobulin molecule and the signal-transducing Ig-α/Ig-β heterodimer (Goldsby R *et al.*, 2000).

Monocytes/Macrophages cell surface marker:

The CD14 marker is expressed on the surface of macrophages and monocytes (Sager *et al.*, 1998). It is a receptor for endotoxin (lipopolysaccharide / LPS) and activates monocytes to release cytokines (TNF / tumor necrosis factor) and upregulate adhesion molecules when LPS is bound (Goldsby R *et al.*, 2000).

NK cell marker:

NK cells are part of a small population of lymphocytes known as null cells. They do not express any of the surface molecules that would identify them as either B or T-cells. Antigen binding receptors are not synthesized and expressed leading to their lack of immunologic specificity and memory. NK cells are large granular lymphocytes which constitute about 5-10% of lymphocytes in human peripheral blood. Specific NK-cell markers for ruminants have not existed for a long time. A recent discovery of an NK-specific cell marker (NKp46) in cattle has been reported (Storset *et al.*, 2004). NKp46 is an activating receptor expressed exclusively on NK cells of primates and rodents. It is a type I transmembrane glycoprotein with two extra-cellular C-type Ig-like domains and is involved in cell-mediated lysis of several targets including antigen presenting cells (Falco *et al.*, 1999; Pessino *et al.*, 1998; Sivori *et al.*, 1999; Spaggiari *et al.*, 2001). The recently reported NK-cells in cattle comprise about 1-10% of mononuclear cells in the peripheral circulation. These large granular lymphocytes are $NKp46^+$ and the majority are $CD2^+$ with a variable fraction of $CD8^+$ cells. These cells were negative for the TCR1, CD3, CD4, B-cell, WC1, and granulocyte markers, suggesting they were not a T-cell or B-cell subset. They also demonstrated spontaneous cytotoxic activity which was inhibited by blocking the NKp46 marker (Storset *et al.*, 2004).

NK T cells:

NK T cells are a distinct and functionally important T-cell lineage. They are sometimes referred to as a specialized type of NK cell (Godfrey *et al.*, 2005; Hammond *et al.*, 1999). They represent a specialized T-cell lineage characterized by a limited T-cell

repertoire and an antigen experienced phenotype capable of rapid secretion of large amounts of IFN-gamma and IL-4 (Van, I *et al.*, 2005). NK T cells display characteristics of both T-cell and NK-cells. Phenotypically, they are large granular lymphocytes with an ability to kill altered host cells (Goldsby R *et al.*, 2000). The CD16 surface molecule is expressed with a variety of other NK cell receptors. They also express T-cell receptors, which react with a CD1 (an MHC-like molecule) instead of an MHC I or MHC II molecules. The majority of these cells, but not all, are CD1d restricted. Antigens recognized in a CD1 restricted manner were shown to be lipids and glycolipids rather than peptides (Goldsby R *et al.*, 2000).

Activated NK T-cells rapidly secrete large amounts of regulatory cytokines such as IFN-gamma and IL-4. They are able to support either humoral (antibody production) or cell mediated responses (cytotoxic T-cells). These cells are viewed by some immunologists as a type of rapid response system that has evolved to provide early help, while the conventional T-helper response is still developing (Goldsby R *et al.*, 2000).

NKT 1 and NKT 2 subsets are described based on their diverse immune responses (regulation of autoimmune disease, immune response to infections, and prevention of tumor metastasis), and production of antagonistic and regulatory cytokines (IL-4 and IFN-gamma). NKT1 cells express a higher level of CD49b, an alpha-2 integrin, with no expression of CD69, an early activation surface protein. Upon activation, NKT1 cells are characterized by high secretion of IFN-gamma, IL-2, and a low secretion of IL-4. NKT-2 cells express CD69 but are negative for or express very low CD49b. They produce a high level of IL-4, with very low levels of both IFN-gamma and IL-2 (Stenstrom *et al.*, 2004). They (NKT cells) are phenotypically, functionally and

developmentally heterogeneous. The three distinct subsets, $CD4^+$, DN (double negative) and $CD8^+$ are differentially distributed in a tissue-specific manner. The double negative population may be $\gamma\delta^+$ NK T cells. CD1-restricted $\gamma\delta^+CD4^-CD8^-$ T-cells that are able to lyse tumor cells have been identified (Hammond *et al.*, 1999).

NK1 T-cells may play a role in the pathogenesis of MCF. Lymphocytes observed in tissue lesions are large lymphocutes. The smaller lymphocytes observed in lymphoid tissues appear necrotic. LCLs developed from clinical cases are seen as large granular lymphocytes with NK-cell activity and are MHCI unrestricted. They express CD2, CD3 antigens and the different T-cell co-receptors such as CD4, CD8, or gamma delta. Regular NK cells do not express T-cell receptors, whereas, regular lymphocytes do not appear large and granular, and do not express NK cell markers. The infiltrating lymphocytes in MCF, together with the established cell lines may be NKT cells. Mucosal damage in MCF is histopathologically associated with infiltrating lymphocytes and not vascular occlusion or damage by the infiltrating cells. The damage could be due to exocytosis and release of lysosomal contents by infiltrating, anergic NKT cells. The trigger for the lymphoproliferation and tissue infiltration is unknown and may be due to the presence of the virus or expression of certain viral genes. Failure to demonstrate viral structures or proteins within lesions or infected cells has consistently been reported in the literature. Identification of the LCLs or infiltrating lymphocytes in MCF tissue lesions as NK or NKT cells could be accomplished by evaluating them for the presence of the recently characterized bovine NK cell marker (NKp46).

The NK T-cell CD1 molecule:

NK T cells are CD1 restricted. The CD1 molecule is functionally and structurally related to MHC I and MHC II molecules (Porcelli *et al.*, 1989). Though their overall structure resembles each other, there are key differences in the antigen binding site of the CD1 and MHC molecules. The CD1 antigen binding groove extends deeply into the inner core of the protein and is lined with hydrophobic amino acids that are well suited for binding amphipathic antigens (Van, I *et al.*, 2005).

The CD1 molecules are widely expressed on cells of hematopoietic origin, especially 'professional' antigen presenting cells like dendritic cells, cortical thymocytes, and splenic B-cells (Bendelac *et al.*, 1997; Goldsby R *et al.*, 2000). The molecules are non-polymorphic transmembrane glycoproteins encoded by linked genes outside the major histocompatability complex (MHC) (Porcelli & Modlin, 1999). They are divided into group I and group II based on sequence homology. Group I consists of CD1a, CD1b, CD1c and CD1e isoforms that are present in humans but not mice. Group II consists of the CD1d isoform and is found in both humans and mice (Kang & Cresswell, 2002).

Three bovine CD1 subgroups have been identified, namely, BoCD1w1, BoCD1w2 and BoCD1w3. These markers are expressed on immature cortical thymocytes, some B-cell populations, monocyte/macrophages, and on dendritic cells. Some clusters within the BoCD1w2 subgroup seem to be homologous to the human CD1b molecules (Howard *et al.*, 1993). All mammalian genomes examined to date encode CD1 genes (Calabi & Milstein, 1986; Dascher & Brenner, 2003; Porcelli, 1995).

Intracellular trafficking of the human CD1d molecule is regulated by association with MHC class II molecules. Subsets of the CD1d molecule were found to be associated

with MHC II molecules on both the cell surface and in the late endosomal/lysosomal compartments. The interaction of the two molecules is initiated in the endoplasmic reticulum with the MHC II invariant chain complexes and is maintained throughout the MHC II trafficking pathway (Kang & Cresswell, 2002).

α-galactosylceramide (α-GalCer) is composed of a combination of a sugar head group and lipid tails.. This molecule acts as a potent stimulator of NKT cells (Godfrey *et al.*, 2005; Koch *et al.*, 2005; Zajonc *et al.*, 2005). Interaction of the CD1d molecule with antigens explains the specificity of NKT cells for particular glycosphingolipid antigens. The ceramide bases (lipid tails) fit snugly in the hydrophobic pockets of the CD1d molecule on the APC, whereas the galactosyl (sugar head) group is exposed to TCR recognition by NKT cells. Antigen recognition specificity is linked with the NKT cell's expression of a very limited TCR repertoire, consisting of an invariant TCRα chain and a restricted, but not invariant, TCRβ repertoire. High conservation of the CD1d mediated antigen recognition pathway enable NKT cells to recognize the same glycosphingolipid antigen (α-GalCer) in the context of mouse or human CD1d and vice versa (Godfrey *et al.*, 2004). Conformational changes (breathing) occur in the heavy chain of the CD1d molecule during transition from a non-loaded to an antigen loaded form (fig 2.4) (Koch *et al.*, 2005). This is likened to the 'Venus Fly trap' model of classical MHC class I loading in which the open conformation of the empty MHC class I allows the α1 and α2 helices to close around the peptide antigen. (Godfrey *et al.*, 2005; Voyle *et al.*, 2003). The NKT cell TCR, has been reported to show a degree of plasticity, which is unexpected in the binding site based on the diversity of antigens, such as α-GalCer, β-GalCer (Parekh *et al.*, 2004), isoglobotrihexosylceramide (Zhou *et al.*, 2004) and α-glycuronosylceramide

(Kinjo *et al.*, 2005; Wu *et al.*, 2005), that are recognized by the binding site (Godfrey *et al.*, 2005).

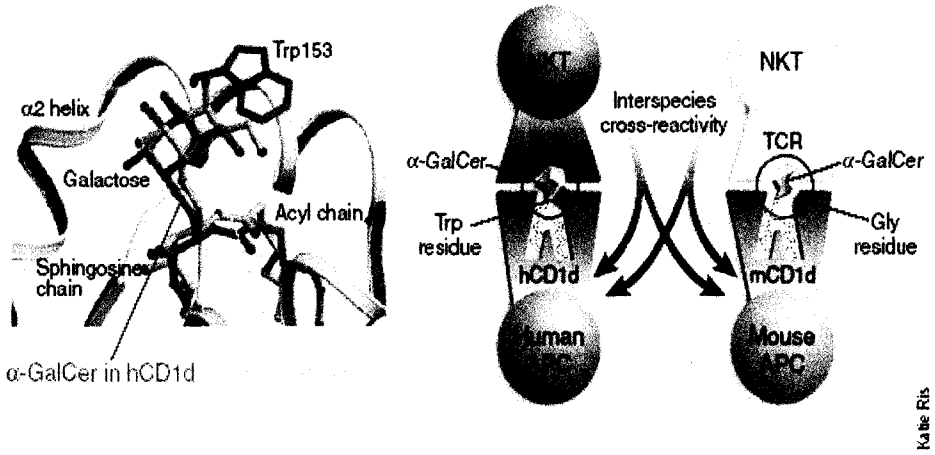


Fig 2.4: Conserved antigen recognition by NKT cells despite species polymorphism in the presentation of α-GalCer by human and mouse CD1d. The conformation of α-GalCer is in cyan when presented by human CD1d (hCD1d) and in gold when in complex with mouse CD1d (mCD1d). The α-GalCer molecules are superimposed and the single CD1 molecule is in gray. The exposed positioning of the critically important galactose head group between the α-helices of CD1 makes it potentially available for TCR interaction. In contrast the sphingosine and acyl lipid tails of the α-GalCer descend into the CD1d cleft. The orientation of the galactose head group differs for human versus mouse CD1d, mainly because of the presence of a bulky tryptophan side chain in the human CD1 (Trp153, green), in contrast to the glycine residue (no side chain) in mouse CD1d. Given the reciprocal cross-reactivity between mouse and human NKT cells with CD1d-α-GalCer complexes from both species, this variability in the orientation of galactose head group (encircled at right; Trp residue in green) suggests there is greater degree of plasticity in NKT TCR recognition than previously believed. APC, Antigen presenting cell (Godfrey *et al.*, 2005).

A possible role for NK T cells in MCF pathogenesis:

NKT cells are considered enigmatic T-lymphocyte subsets that do not play by the rules of traditional lymphocytes. Based on the secreted cytokines, NKT subsets might be pro or anti-inflammatory, promoting or suppressing a cell mediated immune response which may or may not be beneficial to the host (Godfrey *et al.*, 2005). MCF pathogenesis has been suggested to be due to a dysregulated immune response (Liggitt *et al.*, 1978; Reid *et al.*, 1983). Large granular T lymphocytes with cytotoxicity to both primary cultures and cell lines have been propagated from MCF cases. The cells were able to propagate MCF to other animals. Infection and subsequent dysfunction of these large granular lymphocytes was suggested to play a crucial role in the MCF pathogenesis. The cytotoxicity was determined to be in an MHC class I unrestricted way (Cook & Splitter, 1988; Reid *et al.*, 1983). NKT cells have been linked to the plaque formation associated with atherosclerosis and are thought to exacerbate some types of autoimmunity (Godfrey *et al.*, 2005). Atherosclerotic plaques are noticed in histopathologic lesions of chronic MCF cases and it has also been suggested to be an auto-immune disease (Liggitt & DeMartini, 1980; O'Toole *et al.*, 1997). MCF pathogenesis could possibly be due to a dysregulation of Lymphocytes, NK or NK T-cells, or a combination of all three cell types. I would like to suggest a possible role for mainly the NK T-cells in the MCF pathogenesis, based on the following:

1. Morphology: Both NK and NKT cells are said to be large granular lymphocytes. Lymphoblastoid cell lines (LCLs) established from MCF cases are shown to be large granular lymphocytes. Ultrastructural studies of MCF lesions demonstrate tissue

infiltration by large lymphocytes with necrosis of smaller lymphocytes in lymphoid tissues.

2. NKT cells, also referred to as a specialized lineage of the T-cells, express a CD2 marker, a TCR, co-receptor (CD4, CD8 or gamma-delta), together with an NK-cell marker. Recently characterized bovine NK cells were NKp46+, CD2+, CD8+ (variable), TCR-, CD3-, CD4-, WC1- and CD14-. NK T-cells, however, express CD2, CD3, TCR, and an NK cell marker. In summary, regular NK cells are large and granular, exhibit NK cell activity, but do not express a T-cell receptor. Regular T lymphocytes are not large and granular, do not exhibit NK cell activity, nor do they express NK-cell surface markers. NKT cells express properties of both NK cells and T lymphocytes and appear as large granular lymphocytes with NK cell activity expressing both NK cell and T-cell surface markers. Involvement of NKT cells in MCF pathogenesis can be confirmed or ruled out by incubating involved cells (from established LCLs or within lesions) with both T-cell and the recently characterized bovine NK cell marker.
3. Ultra-structural and phenotypic analysis of lesions demonstrates the presence of, large lymphocytes that are predominantly CD8+ T lymphocytes. Established LCLs from clinical cases yield different T-cell subsets of large granular lymphocytes with NK cell activity. The cytotoxicity is said to be MHC unrestricted. NK T cells express both gamma-delta and alpha-beta T-cell receptors with the different coreceptors. There is a possibility that cells within the MCF lesions and established LCLs could be NKT cells. Incubating the cells with both the T-cell and NK cell marker would help confirm or rule out this hypothesis.

4. Large granular lymphocytes, with cytotoxicity to both primary cultures and cell lines have been established from MCF cases. The cytotoxicity was determined to be MHC class I unrestricted. NKT cells are non MHC but CD1 restricted. Functionally, the LCLs were characterized as natural killer cells, although, monoclonal antibodies classified them as T-cells.
5. NKT cells can reportedly prevent or cause a pathologic immune mediated response.
6. MCF has been suggested to be an immune mediated disease due to dysregulation of cells of the immune system or anergic lymphocytes.
7. NKT cells have been linked to the plaque formation associated with atherosclerosis and are thought to exacerbate some types of autoimmunity. Atherosclerotic plaques are observed in vascular lesions of recovered MCF cases.
8. Mucosal lesions (ulcerations) in MCF cases are shown to be related to lymphoid infiltration and not blood vessel occlusion. It is possible that the infiltrating lymphoid cells are dysregulated NK T cells. Exocytosis of their lysosomal granules with resultant damage to adjacent tissues could be responsible for the observed lesions in association with infiltrating lymphocytes.

RUMINANT PBMC SUBSET PERCENTAGES

Ruminant B and T lymphocyte percentages vary with the individual animal and age, and generally constitute between 10-30 and 50-70% of PBMCs, respectively (Morrison *et al.*, 1988). In sheep, mean percentages and ranges of major lymphocyte subtypes were reported as follows; B-cells (29.6%, 11-50), γδ T-cells (36.6%, 22-68), $CD4^+$ T-cells (14.1%, 8-22), and $CD8^+$ T-cells (12%, 4-22) (Smith *et al.*, 1994). The

percentage of γδ T-cells in sheep is reportedly highest after birth and accounts for 60% of the total T-cell population (Hein *et al.*, 1990). This is followed by a steady decline to about 5-10% in older animals of about 5-8 years (Clevers *et al.*, 1990). Bovine monocytes make up 5-20% of the total PBMC population (Goddeeris *et al.*, 1986). The newly characterized bovine NK cell marker (NKp46) detected between 1-10% NK cells in the general circulation (Storset *et al.*, 2004).

CHAPTER 3

OvHV-2 PREFERENTIALLY INFECTS CD4 AND CD8 POSITIVE T-CELL SUBSETS IN SHEEP AND CATTLE

ABSTRACT

The pathogenesis of OvHV-2 infection is not well discerned. OvHV-2 infection of total PBMCs and PBMC subsets was evaluated in order to examine the role of viral load and tropism on clinical status. Whole blood was collected from asymptomatically infected sheep (n=5), subclinically infected cattle (n=5), clinically affected cattle (n=8) and recovered cattle (n=3). Peripheral blood mononuclear cells were magnetically sorted into $CD2^+$, $CD3^+$, $CD4^+$, $CD8^+$, and $\gamma\delta^+$ T-cells, B-cells, and monocytes. OvHV-2 genomic equivalents in total PBMCs and sorted PBMC subsets were examined by realtime qPCR. PBMCs were additionally obtained from non-infected cattle (n=4). Flow cytometry was used to analyze $CD2^+$, $CD3^+$, $CD4^+$, $CD8^+$, and $\gamma\delta^+$ T-cell, B-cell, and monocyte PBMC subset percentages from OvHV-2 infected and non-infected animals.

OvHV-2 DNA was below the limits of detection within total PBMCs and PBMC subsets of subclinically infected cattle. There was no difference in OvHV-2 tropism between the 4 groups of animals. OvHV-2 copy numbers within PBMCs of clinically affected cattle were significantly greater than in all the other groups. Genome copy numbers within subsets of clinically affected cattle were uniformly greater than in subsets

of subclinically infected cattle. There were no statistical differences in viral copy numbers between asymptomatic sheep and recovered cattle, or between subclinically infected and recovered cattle. CD4+ and CD8+ T-cells in all groups were preferentially infected over B-cells and monocytes. Gamma delta T-cells had the lowest viral copy numbers of all T-cells in all animal groups. Observed means and ranges of subset percentages in non-infected and OvHV-2 infected cattle were comparable to those reported in the literature.

In summary, differences in OvHV-2 tropism between asymptomatic sheep, subclinically infected cattle, clinically affected cattle, and recovered cattle were not observed. Higher viral load within clinically affected cases is suggestive of active viral genome replication. $CD4^+$ and $CD8^+$ T-cells were preferentially infected over γδ T-cells, B-cells, and monocytes. There was no association between OvHV-2 infection and changes in PBMC subset percentages.

INTRODUCTION

Malignant catarrhal fever (MCF) is a lympho-proliferative disease of susceptible ruminants (Plowright, 1990). There are two epidemiological forms of MCF, sheep associated (SA-MCF) and wildebeest associated MCF. Two γ_2 herpesviruses, ovine herpesvirus 2 (OvHV-2) and alcelaphine herpesvirus 1 (AlHV-1), are the causative agents of sheep and wildebeest associated forms of MCF, respectively (Plowright, 1965). The disease caused by both forms has been shown to be clinically and clinico-pathologically the same (Pierson *et al.*, 1979). A productive cell culture system for the *in*

vitro propagation of OvHV-2 has not been developed (Cook & Splitter, 1988). OvHV-2 infection or previous exposure can be detected by PCR (Baxter *et al.*, 1993) and competitive inhibition ELISA (CI-ELISA) (Li *et al.*, 1994; Li *et al.*, 2001b). A definitive diagnosis of MCF is confirmed by histopathology.

MCF was initially thought to be a uniformly fatal disease of cattle (Plowright, 1990). Recent reports, however, describe cattle that remain subclinically infected over prolonged periods of time (Li *et al.*, 2001b; O'Toole *et al.*, 2002; Powers *et al.*, 2005) and others that recover from clinical disease (Michel & Aspeling, 1994; Milne & Reid, 1990; O'Toole *et al.*, 1995; O'Toole *et al.*, 1997). Therefore, OvHV-2 infected animals can be divided into 4 groups based on the clinical status; 1) asymptomatic sheep, 2) subclinically infected cattle, 3) clinically affected cattle, and 4) recovered cattle. Sheep (group 1) are asymptomatically infected and act as a source of infection for susceptible ruminants (Collins *et al.*, 2000; Dunowska *et al.*, 2001). Seroprevalence in domestic sheep is reportedly as high as 99% in certain geographic areas in the United States (Li *et al.*, 1995). Although laboratory induced clinical disease has been reported in sheep (Li *et al.*, 2005), naturally occurring disease is yet to be confirmed. Subclinically infected cattle (group 2) test positive for OvHV-2 by PCR and or ELISA with no signs of clinical disease, nor history of recovery from previous MCF (Li *et al.*, 2001b; O'Toole *et al.*, 2002; Powers *et al.*, 2005). Clinically affected cattle (group 3) experience an overt clinical episode that is often fatal. Recovered cattle (group 4) experience a clinical episode followed by recovery. Recovered cattle remain OvHV-2 positive for extended periods of time and may harbor chronic lesions of MCF (Michel & Aspeling, 1994; Milne & Reid, 1990; O'Toole *et al.*, 1995; O'Toole *et al.*, 1997).

OvHV-2 copy numbers (genomic equivalents) in PBMCs and tropism in sorted PBMC subsets within the 4 animal groups have not been examined. Proliferating and infiltrating cells have been phenotyped within bovine vascular and epithelial lesions, but not in the general circulation. CD8+ T-cells are the predominant infiltrating cell type observed in tissues of clinically affected cattle (Nakajima *et al.*, 1992). Macrophages, CD4+, and rarely γδ+ T-cells are also present (Nakajima *et al.*, 1994). In another study, immunohistochemistry and in situ PCR showed the majority of infiltrating cells to be CD8+ T-cells and a large number of these cells to be infected with OvHV-2 (Simon *et al.*, 2003). Tissue lesions also contained macrophages but no CD4+ or B-lymphocytes. OvHV-2 was detected exclusively in B-cells and a number of epithelial tissues within sheep (Baxter *et al.*, 1997). An infection cycle in sheep bearing similarities to Epstein-Barr virus (EBV) and mouse herpesvirus-68 (MHV 68) was therefore suggested. We hypothesized that OvHV-2 infects T-cell subsets in PBMCs of susceptible ruminants, unlike the reported exclusive B-cell infection in sheep PBMCs.

Lymphocyte subset percentages in PBMCs of healthy ruminants and those with lymphotropic infections such as enzootic bovine leukosis (EBL) caused by bovine leukemia virus (BLV) have been extensively reported (Hein & Mackay, 1991; Mackay *et al.*, 1988; O'Reilly *et al.*, 1991; Smith *et al.*, 1994; Waters *et al.*, 1995; Wu *et al.*, 1999; Wyatt *et al.*, 1999). PBMC subset percentages within different groups of OvHV-2 infected animals have not been examined. We also hypothesized that OvHV-2 infection results in a difference in PBMC subset percentages within the different groups of infected animals. The objective of this study was to quantify the level of OvHV-2 DNA copies within PBMCs and evaluate the viral tropism within total PBMCs and sorted PBMC

subsets of asymptomatic sheep, subclinically infected cattle, clinically affected cattle, and recovered cattle. In addition, PBMC subset percentages within the 4 groups of infected animals were examined and compared with clinically normal non-infected cattle.

MATERIALS AND METHODS

Samples:

EDTA whole blood was collected from 4 groups of OvHV-2 infected animals including asymptomatic sheep (n=5), subclinically infected cattle (n=5; 14-18 months old), clinically affected cattle (n=8; 3 years or younger) and recovered cattle (n=3; 2 years or younger). The OvHV-2 infected cattle were female Holsteins obtained from a dairy with a history of cases of MCF. Whole blood was also collected from non-infected adult Holstein cattle (n=4) from another dairy that had no exposure to sheep or history of clinical MCF. The OvHV-2 status of non-infected animals was confirmed by negative CI-ELISA. The sub-clinically infected cattle were identified in a previous study and tested positive for OvHV-2 by PCR or CI-ELISA (Powers *et al.*, 2005). They were clinically normal and had no history of previous disease consistent with MCF. Clinically affected cattle were experiencing a clinical episode at the time of sampling. Recovered cattle experienced a confirmed clinical episode of MCF followed by recovery. Blood samples from recovered cattle were obtained at least 1 month after the initial MCF episode.

Monoclonal antibodies and magnetic beads:

The primary monoclonal antibodies (Table 3.1) (VMRD-Veterinary Medical Research and Development) were cross-reactive between sheep and cattle, except for CD3, CD4, γδ, and B-cell markers. Ovine CD3 marker was not examined. The secondary antibody was an anti-mouse IgG:FITC conjugate (Serotec). Magnetic Anti-FITC beads and MS Columns (Miltenyi Biotec) were used for the immunomagnetic sorting.

TABLE 3.1: Detected cell surface markers and Monoclonal antibody designation.

Surface Marker	Species	Antibody Designation
CD2: pan T-cells	Bovine and sheep	MUC2A
CD3: pan T-cells	Bovine	MM1A
CD4: T helper cells	Bovine	CACT138A
CD4: T helper cells	Sheep	GC1A
CD8: T-cytotoxic cells	Bovine and sheep	CACT80C
γδ: gamma-delta T-cells	Bovine	GB21A
γδ: gamma-delta T-cells	Sheep	CACT26A
B-cells	Bovine	LCT27A
B-cells	Sheep	BIg501E
CD14:Macrophages/Monocytes	Bovine and sheep	CAM36A

Immunomagnetic PBMC cell sorting:

PBMCs were isolated by ficoll density gradient from whole blood samples. The isolated PBMCs were sorted into CD2+, CD3+, CD4+, CD8+ and γδ+ T-cells, B-cells and monocytes. PBMC aliquots were incubated individually with different primary antibodies (Table1), followed by a secondary FITC conjugated antibody (Serotec), for 30 minutes each at 4°C. The cells were then washed twice with FACs buffer. PBMCs were further incubated with anti-FITC beads (Miltenyi Biotec) for 30 minutes at 4°C. Incubated PBMCs were run through an MS column fitted on an octomax magnetic separation stand (Miltenyi Biotec). Positive cells bound to the MS column were eluted with FACs buffer

for further processing. Purity of the magnetically sorted PBMC subsets was evaluated by flow cytometry. DNA was isolated from each of the sorted subsets (DNeasy kit, Qiagen). Isolated DNA was examined for OvHV-2 DNA by the OvHV-2 specific realtime PCR (qPCR) (Hussy *et al.*, 2001).

PBMC subset percentages:

PBMC subset percentages from non-infected cattle and the four groups of infected animals were determined by flow cytometry. PBMC subset percentages were determined for CD2+, CD3+, CD4+, CD8+, and γδ+ T-cells, B-cells, and monocytes. Isolated PBMCs were incubated with primary monoclonal antibodies (Table 3.1), followed by incubation with an FITC conjugated secondary monoclonal antibody (Serotec) at 4°C for 30 minutes each. Incubations were followed by two washes with FACs buffer. Subset percentages were evaluated by flow cytometry. Flow cytometric data were analyzed by SUMMIT™ OFFLINE (Cytomation).

OvHV-2 qPCR:

OvHV-2 genome copies from total PBMCs and PBMC subsets were quantitated by realtime PCR. Total DNA was isolated from total PBMCs and sorted PBMC subsets. (DNeasy kit, Qiagen). The qPCR employed the same primers and probe as previously reported (Hussy *et al.*, 2001) with a slight modification to the reaction conditions. Both primers and probe were included in the reaction mixture at 150 nM and the reaction cycle was modified to; Cycle1: 50 degrees – 2 minutes; Cycle 2: Step1: 95 degrees – 15 sec; Step 2: 60 degrees - 1min. Data was acquired during step 2 on the Bio Rad iCycler. The

OvHV-2 ORF 75 PCR fragment (430bp) (Baxter *et al.*, 1993), cloned into pGEMTeasy was used as the qPCR standard.

MCF direct competitive inhibition ELISA (CI-ELISA):

The direct competitive inhibition ELISA (CI-ELISA) has been described (Li *et al.*, 1994; Li *et al.*, 2001b). The assay detects antibodies to MCF viruses in ruminants and was performed at the Washington Animal Disease Diagnostic Laboratory (WADDL).

Statistical analysis:

PBMC subset percentages and OvHV-2 DNA equivalents (copy numbers) between the different animal groups were analyzed with the non-parametric one way procedure and Wilcoxon scores (rank sums) were obtained for the groups. These analyses were then followed with the Wilcoxon two-sample test and the level of significance was determined based on a two-sided test ($P \leq 0.05$). Differences in OvHV-2 tropism within each of the 4 animal groups were analyzed with the Friedman's test. The Distribution-Free, Two-sided, all treatments multiple comparisons based on the Friedman Rank Sums general configuration was used to control for overall error rate. The level of significance was determined at $P \leq 0.05$.

RESULTS

OvHV-2 ELISA:

All asymptomatic sheep, subclinically infected cattle, clinically affected cattle, and recovered cattle were MCF CI-ELISA positive, confirming current or prior exposure to OvHV-2. All non-infected cattle were MCF CI-ELISA negative.

Magnetic sorting and OvHV-2 qPCR: quality control

All magnetically sorted subsets attained a purity of > 90% by flow cytometry, with a majority of the subsets between 95-99% pure (Appendix; Table A6 - A9). The OvHV-2 specific qPCR had a sensitivity of at least 2.7 copies per mix and an efficiency of > 90% on cell assays.

Comparison of OvHV-2 load and tropism between the 4 animal groups:

OvHV-2 genome copy numbers in total PBMCs and PBMC subsets of the 4 groups were compared and statistically evaluated in order to examine OvHV-2 load and tropism between asymptomatic sheep, subclinically infected cattle, clinically affected cattle, and recovered cattle (Fig 3.1, Appendix; Table A10- A14). OvHV-2 tropism was determined based on detection or non-detection of OvHV-2 DNA in PBMCs and PBMC subsets of the 4 groups. Viral load was determined by examining OvHV-2 genomic equivalents in PBMCs and PBMC subsets of the 4 groups.

PBMCs: OvHV-2 DNA was detected within total PBMCs of all groups except sub-clinically infected cattle. Viral DNA copy numbers in total PBMCs from clinically affected cattle were significantly greater than in sheep ($P < 0.05$), recovered ($P < 0.05$), and subclinically infected cattle ($P < 0.05$).

CD2: OvHV-2 DNA was detected in $CD2^+$ cells from all groups except subclinically infected cattle. OvHV-2 DNA copy numbers within clinically affected cattle were significantly greater than in subclinically infected cattle ($P < 0.05$) and recovered cattle (P

< 0.05). Viral genome copies in sheep were significantly greater than in subclinically infected cattle ($P < 0.05$).

CD3: CD3 was not examined in sheep. OvHV-2 DNA was detected in $CD3^+$ cells from clinically affected and recovered cattle, but not subclinically infected cattle. OvHV-2 genome copies in $CD3^+$ cells from clinically affected cattle were significantly greater than in subclinically infected cattle ($P < 0.05$).

CD4: OvHV-2 DNA was detected in $CD4^+$ cells from all animal groups except subclinically infected cattle. OvHV-2 genome copies were greater in clinically affected cattle than in both subclinically infected and recovered cattle ($P < 0.05$).

CD8: OvHV-2 DNA was detected in $CD8^+$ cells from all animal groups except subclinically infected cattle. OvHV-2 genome copies in $CD8^+$ cells from sheep were greater than subclinically infected cattle ($P < 0.05$). $CD8^+$ cell OvHV-2 copies within clinically affected cattle were also greater than in subclinically infected cattle ($P < 0.05$). There were no differences between the other groups of animals.

γδ: OvHV-2 DNA was detected in γδ-T cells from all animal groups except subclinically infected cattle. γδ-T cell OvHV-2 genome copies were significantly greater in clinically affected cattle than in both sheep and subclinically infected cattle ($P < 0.05$). There were no statistical differences between the other groups.

B-cells: OvHV-2 DNA was only detected within B-cells of clinically affected cattle. Detected OvHV-2 genome copies within clinically affected cattle were significantly greater than in sheep and subclinically infected cattle ($P< 0.05$).

Monocytes: OvHV-2 DNA was detected in monocytes from clinically affected and recovered cattle, but not in sheep and subclinically infected cattle monocytes. OvHV-2

genomic copies in monocytes from clinically affected cattle were significantly greater than sheep and subclinically infected cattle ($P < 0.05$).

OvHV-2 tropism of PBMC subsets within each animal group:

OvHV-2 copy numbers within sorted subsets of each group were compared to each other and statistically evaluated in order to determine which PBMC subsets were preferentially infected in asymptomatic sheep, subclinically infected cattle, clinically affected cattle, and recovered cattle (Fig 3.1, Appendix; Table A15- A17).

Asymptomatic Sheep: OvHV-2 DNA was detected in all examined sheep PBMC subsets except B-cells and monocytes. The sheep CD3 surface marker was not examined. Viral genomic copy numbers in CD2+ (pan T) and CD8+ T-cells were significantly greater than in B-cells and monocytes ($P < 0.05$). OvHV-2 genomic copy numbers in CD8+ T-cells were also significantly greater than in γδ T-cells ($P< 0.05$). There were no statistically significant differences between all the other subsets. OvHV-2 preferentially infected T-cells, specifically CD8+ T-cells over both B-cells and monocytes in PBMC subsets of sheep.

Subclinically infected cattle: OvHV-2 genomic copies were below the level of detection in all the PBMC subsets examined.

Clinically affected cattle: OvHV-2 DNA was detected in all PBMC subsets of clinically affected cattle. Viral genome copies within CD2+ ($P < 0.001$) and CD3+ ($P < 0.05$) pan T-cells were significantly greater than viral genome copies in B-cells and monocytes. OvHV-2 genome copies in CD4+ ($P < 0.05$) and CD8+ ($P < 0.001$) T-cells were significantly greater than in both B-cells and monocytes. Viral genome copies within

CD8+ T-cells were also greater than in γδ T-cells ($P < 0.05$). T-cells, specifically CD4+ and CD8+ were preferentially infected over B-cells and monocytes. γδ T-cells were the least infected subset of all the T-cells.

Recovered cattle: OvHV-2 DNA was detected in all examined PBMC subsets of recovered cattle except B-cells. Viral genome copies in CD2+ T-cells were significantly greater than in B-cells ($P < 0.05$). Copies in CD8+ T-cells were significantly greater than in both B-cells and monocytes ($P < 0.05$). Within recovered cases, CD2+ T-cells were preferentially infected over B-cells. CD8+ T-cells were preferentially infected over B-cells and monocytes.

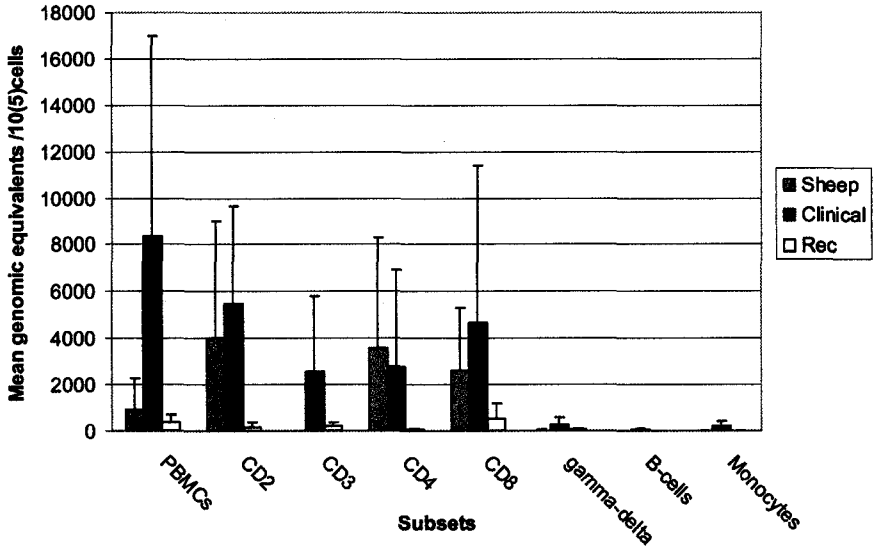


Figure 3.1: OvHV-2 load (genome copies) and tropism within total PBMCs and PBMC subsets of asymptomatic sheep, clinically affected cattle, and recovered cattle. OvHV-2 DNA was undetectable in PBMCs and PBMC subsets of subclinically infected cattle (not included in the figure). Clinically affected cattle had significantly higher OvHV-2 genome copies in their total PBMCs. T-cells were preferentially infected over B-cells and monocytes in all groups. γδ T-cells harbored significantly less OvHV-2 genome copies than other T-cells. Error bars represent the standard deviation within groups.

PBMC subset percentages:

In order to examine whether OvHV-2 affects relative percentage of each PBMC subset, flow-cytometry was used to analyze subset percentages within non-infected cattle, subclinically infected cattle, clinically affected cattle, and recovered cattle (Appendix; Table A-18). The examined subset percentages included CD2+, CD3+, CD4+, CD8+, γδ+ T-cells, B-cells, and monocytes. Mean PBMC subset percentages within the 4 groups were compared and statistically evaluated (Fig 3.2). Percentage of CD2+ T-cells within clinically affected cattle was significantly lower than in recovered cattle ($P < 0.05$). Percentage B-cells within clinically affected cattle were significantly lower than in non-infected ($P < 0.05$), subclinically infected ($P < 0.05$), and recovered cattle ($P < 0.05$). Percentage of monocytes within clinically affected cattle was significantly higher than within non-infected and subclinically infected cattle ($P < 0.05$). However, mean subset percentages and ranges from examined non infected and OvHV-2 infected cattle were comparable to those reported in the literature (Appendix, Table 3.19) (Hein & Mackay, 1991; Mackay *et al.*, 1988; O'Reilly *et al.*, 1991; Smith *et al.*, 1994; Waters *et al.*, 1995; Wu *et al.*, 1999; Wyatt *et al.*, 1999).

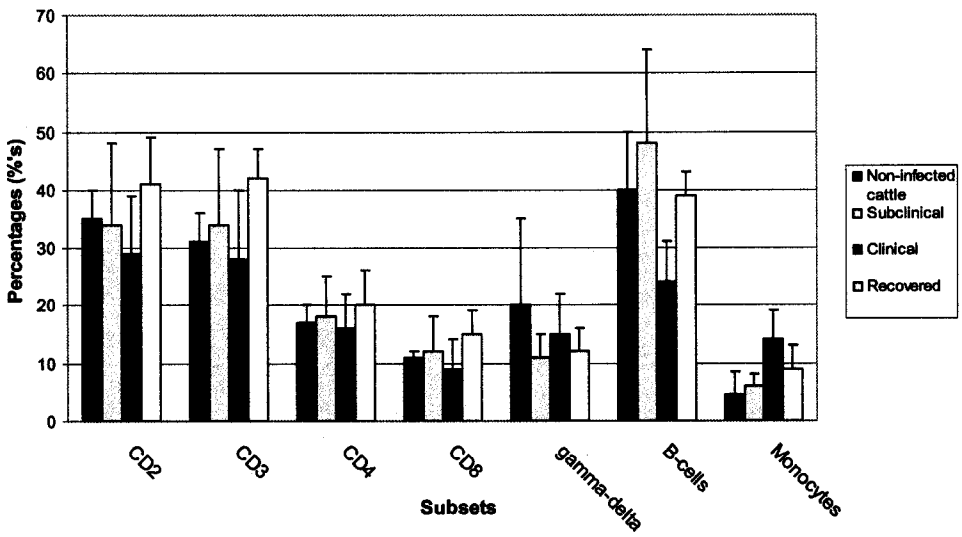


Figure 3.2: Flow-cytometric examination of PBMC subset percentages within non-infected, subclinically infected, clinically affected, and recovered cattle. Observed means and ranges of subset percentages within all 4 groups were comparable to those reported in the literature. Error bars represent the standard deviation within groups.

DISCUSSION

The results show no difference in OvHV-2 tropism between asymptomatic sheep subclinically infected cattle, clinically affected cattle, and recovered cattle. This is in contrast with a previous report of an exclusive B-cell infection in PBMC subsets of asymptomatic sheep (Baxter *et al.*, 1997). OvHV-2 infection was below the level of detection within B-cells and monocytes of asymptomatic sheep. Infection was also below the level of detection within total PBMCs and PBMC subsets of subclinically infected cattle. Subclinically infected cattle in our study were reported in a previous study (Powers *et al.*, 2005). Previous exposure to OvHV-2 in subclinically infected cattle was confirmed by the direct CI-ELISA. Latent gammaherpesvirus infections are associated with a drastic drop in viral load and the number of infected cells. Latency within HHV-8

infection is characterized by intermittent viremia and very difficult detection of viral DNA by PCR (Harrington, Jr. *et al.*, 1996). Intermittent detection of OvHV-2 DNA in subclinically infected cattle suggesting fluctuating viral loads has been reported (Powers *et al.*, 2005). Our data can not distinguish between clearance of infection or establishment of a latent OvHV-2 infection within subclinically infected cattle.

Clinically affected cattle were the only group with a detectable B-cell infection. These infected B-cells had the lowest viral copy numbers of all PBMC subsets within clinically affected cattle. The OvHV-2 genomic copies associated with B-cell roughly represented 0.97% of total OvHV-2 DNA in PBMCs, whereas CD2+ T-cells represented 99% of the infection within clinically affected cattle. This low level of OvHV-2 DNA within B-cells could be accounted for by contamination of B-cell subsets by infected T-cells. If this was indeed contamination, it curiously only occurred within B-cells of clinically affected cattle but not of asymptomatic sheep and recovered cattle. Very low levels of T-cell infection have been reported in EBV (Kikuta *et al.*, 1988) and HHV8 (Harrington, Jr. *et al.*, 1996; Kikuta *et al.*, 1997) infections, both of which were initially reported to be exclusively B-cell tropic (Ambroziak *et al.*, 1995). Lymphoblastoid cell lines could not be established from PBMCs with EBV infected T-cells and T-cells were thought to be abortively infected *in vitro* (Kikuta *et al.*, 1988). This unusual infection of T-cells was linked to clinically affected syndromes in both EBV and HHV-8 (Kikuta *et al.*, 1988; Kikuta *et al.*, 1997). At this point, we are unable to dismiss detection of infection within B-cells of clinically affected cattle solely as contamination.

OvHV-2 load within PBMCs of clinically affected cattle was significantly greater than within all the other groups. OvHV-2 genome copies within PBMC subsets of

clinically affected cases were uniformly and significantly greater than those within subclinically infected cattle. Viral genome copies in total PBMCs, CD2+ pan T-cells and CD4+ T-cells of clinically affected cattle were greater than those in similar subsets within recovered cattle. The relatively higher viral copy numbers within PBMCs of clinically affected cattle are suggestive of active viral DNA replication, with subsequent increase in the viral genome load. Viral genome copies in total PBMCs and certain PBMC subsets of recovered cattle where relatively less than in clinically affected cattle. This is suggestive of an initial high viral load resulting clinical disease followed by reduction in viral DNA replication in association with recovery from clinical disease. The factors associated with or responsible for the decrease in viral genome copies in recovered cattle are unknown at this point.

It is significant that differences in viral DNA copies within clinically affected versus recovered cattle were observed given that 8 clinical and only 3 recovered cattle were examined. Examination of more recovered cattle may reveal differences in viral loads within the other PBMC subsets examined.

Undetectable viral DNA copies within subclinically infected cattle are consistent with a tight regulation on viral replication as is commonly seen during latency in gammaherpesvirus infections. Undetectable viral DNA copies in total PBMCs and PBMC subsets of subclinically infected cattle may also be suggestive of clearance of infection by the host. This would however not be consistent with gammaherpesvirus pathogenesis which involves establishment of life long latency in lymphocytes after the initial infection. Detection of viral DNA in total PBMCs and PBMC subsets of recovered cases refutes the idea of viral clearance but supports a decrease in viral DNA genome

copies after recovery. The form of infection (lytic or latent) in infected PBMCs of recovered cattle is unknown at this point.

This data suggests that establishment of active viral DNA replication, or the lack thereof, in infected PBMCs, may be an important deciding factor in whether infection remains subclinical, progresses to a clinical disease, or whether recovery from clinical disease occurs. Within subclinically infected cattle, viral or host control mechanisms may be able to tightly regulate OvHV-2 DNA replication such that it does not occur or occurs at a very low level and viral DNA remains below the level of detection by the qPCR. Clinical disease may result due to the ability of the virus to replicate its DNA with a resultant increase in OvHV-2 genome copies or load. Recovered cattle may experience viral DNA replication and a high viral load during the clinical episode, but be able to control or successfully reduce viral genome copy numbers resulting in recovery from clinical disease. Host or viral factors associated with recovery from clinical disease are unknown at this point. Factors associated with the switch from latent to productive infection and vice versa need to be investigated further. The immune system plays an important role in the development and control of gammaherpesvirus infections (Speck & Virgin, 1999; Sunil-Chandra *et al.*, 1994). The role of the immune system and additional factors such as viral gene expression in development of MCF needs to be examined. There was no difference in viral genome copy numbers between asymptomatic sheep and recovered cattle subsets. There was also no difference in viral genome copies between recovered and subclinically infected cattle.

Examination of infected subsets within each of the 4 groups revealed a preferential T-cell infection compared to B-cells and monocytes. Gamma delta T-cells

had the lowest viral copy numbers of all T-cells. CD4+ and CD8+ T-cells were preferentially infected over gamma-delta T-cells. Phenotypic analysis of infiltrating and proliferating cells within MCF vascular and epithelial lesions showed gamma-delta T-cells to be rarely involved compared to other T-cells (Nakajima *et al.*, 1994). Our data also suggests a limited role for gamma-delta T-cells in OvHV-2 infection. This seems somewhat contradictory as pure gamma-delta positive lymphoblastoid T-cells can be established from PBMCs of clinically affected cattle (Data in chapter 4).

The comparison of PBMC subset percentages between non infected and infected cattle suggests a decrease in B-cell percentages within clinically affected cattle. This finding would be especially relevant if a B-cell infection could be confirmed within clinically affected cattle. However, means and ranges of subset percentages obtained from asymptomatic sheep, non-infected cattle, subclinically infected cattle, clinically affected cattle, and recovered cattle were comparable to means and ranges of PBMC subset percentages reported in the literature (Hein & Mackay, 1991; Mackay *et al.*, 1988; O'Reilly *et al.*, 1991; Smith *et al.*, 1994; Waters *et al.*, 1995; Wu *et al.*, 1999; Wyatt *et al.*, 1999). Ruminant subset percentages vary according to age, sex, breed, nutrition, stress, environment, etc (Smith *et al.*, 1994). The mean and range of B-cell subset percentages within clinically affected cattle were also comparable to those reported in the literature and the purported decrease in B-cell percentages is probably of little physiologic consequence.

In summary, there was no difference in OvHV-2 tropism between asymptomatic sheep, subclinically infected cattle, clinically affected cattle, and recovered cattle. Our data is suggestive of an active viral DNA replication and higher viral DNA loads in

PBMCs of clinically affected cattle compared to asymptomatically infected sheep, subclinically infected cattle, and recovered cattle. OvHV-2 genome copy numbers may play an role in determining whether infection progresses from subclinical to clinical disease and whether recovery from clinical disease occurs. OvHV-2 showed a greater tropism for T-cells, specifically CD4+ and CD8+ T-cells in the 4 groups of animals. An association between OvHV-2 infection and changes in PBMC subset percentages could not be demonstrated. The contribution of other factors like viral gene expression and the host's immune response to infection status needs further investigation.

CHAPTER 4

ESTABLISHMENT OF OvHV-2 INFECTED LYMPHOBLASTOID CELL LINES FROM PBMCs OF CLINICAL AND RECOVERED CATTLE WITH MCF

ABSTRACT

Continuous cell lines were established from peripheral blood mononuclear cells (PBMCs) of 3 fatal and 1 recovered case of MCF in order to further examine the pathogenesis of malignant catarrhal fever (MCF). Morphologically, the cell lines resembled large granular lymphocytes and were all positive for OvHV-2 DNA. Phenotypically, the lymphoblastoid cell lines (LCLs) expressed CD2 and CD3 pan T-cell markers. Flow-cytometric analysis further characterized the cell lines as either exclusively γδ+, CD8+, or mixed γδ and CD8+ T-cell lines. Cellular and viral replication kinetics were variable between the LCLs. Ovine herpesvirus 2 (OvHV-2) DNA was identified in both cells and supernatant of all cell lines. Viral DNA copies increased over time indicating active DNA replication. Cells and supernatant from the recovered case LCL harbored fewer viral copies than LCLs from fatal cases. At peak replication in cell culture, the 4 cell lines harbored 66, 59, 140 and 29 OvHV-2 genomic equivalents/cell. The source of OvHV-2 DNA in the supernatant was both DNase protected and unprotected OvHV-2 DNA. DNase protected OvHV-2 DNA copies made up between 0.03 and 0.3% of the total OvHV-2 DNA copies in the supernatant. All the LCLs supported transcription of message for ORF 75, a late structural viral protein. Gardella

gel analysis identified predominantly circular OvHV-2 DNA consistent with latent infection in the cells. A high level of apoptosis was detected in all the cell lines.

In summary, OvHV-2 infected lymphoblastoid cell lines can be established from PBMCs of both clinically affected and recovered cases of MCF in cattle. Successful establishment of a cell line from a recovered case suggests persistent OvHV-2 infection after recovery. T-cells may be a site of viral latency within recovered animals. OvHV-2 actively replicates its DNA and transcribes message for a late structural protein within infected LCLs. DNase protected viral genome can be found in the supernatant.

INTRODUCTION

Malignant catarrhal fever (MCF) is a lymphoproliferative disease of susceptible ruminants (Plowright, 1990). Ovine herpesvirus 2 (OvHV-2), a γ2 herpesvirus, is the causative agent of sheep associated MCF (Buxton *et al.*, 1984; Reid *et al.*, 1984). The disease was initially thought to be a uniformly fatal disease. Recently however, recovered (Michel & Aspeling, 1994; Milne & Reid, 1990; O'Toole *et al.*, 1995; O'Toole *et al.*, 1997), and subclinically infected (Li *et al.*, 2001b; O'Toole *et al.*, 2002; Powers *et al.*, 2005) animals have been reported.

Lymphoblastoid cell lines (LCLs) have been established from tissues and cerebrospinal fluid of animals with MCF (Baxter *et al.*, 1997; Burrells & Reid, 1991; Cook & Splitter, 1988; Reid *et al.*, 1983; Schock *et al.*, 1998; Swa *et al.*, 2001). Establishment of LCLs from peripheral blood mononuclear cells (PBMCs) of MCF cases has not been reported. Examination of LCLs from PBMCs may contribute to improved

understanding of the effects OvHV-2 infection has on lymphocytes and the pathogenesis of MCF.

Previously reported LCLs obtained from MCF cases were morphologically characterized as large granular lymphocytes and shown to have NK cell activity or indiscriminate non-MHC-linked cytotoxicity (Baxter *et al.*, 1997; Burrells & Reid, 1991; Cook & Splitter, 1988; Reid *et al.*, 1983; Schock *et al.*, 1998; Swa *et al.*, 2001). Infection and subsequent dysfunction of these large granular lymphocytes is suggested to account for the extensive lymphoproliferation and tissue damage that occurs during MCF (Reid *et al.*, 1983). Phenotypically, exclusive CD8+, CD4+, gamma delta+, and mixed CD4+CD8+ LCLs have been established from tissues of clinically affected cases (Burrells & Reid, 1991). In the absence of exogenous IL-2, LCL growth and proliferation is due to constitutive activation of small tyrosine kinases, Lck and Fyn kinase, which are crucial for the initial activation of T-cells via several surface receptors (Swa *et al.*, 2001)

Attempts to propagate OvHV-2 in cell culture remain unsuccessful (Cook & Splitter, 1988). Lymphoblastoid cell lines are a source of OvHV-2 DNA and are an important tool in the study of MCF pathogenesis. Established cell lines have played a critical role in understanding the pathogenesis of related gammaherpesviruses such as Epstein-Barr virus (EBV). The examination of established cell lines identified EBV as the cause of Burkitt's lymphoma (BL) and infectious mononucleosis (IM) (Diehl *et al.*, 1968; Henle *et al.*, 1968; Niederman *et al.*, 1968). B-cell lines can be established from patients with acute and chronic cases of infectious mononucleosis up to 20 years after recovery (Diehl *et al.*, 1968).

An OvHV-2 positive cell line has never been established from a recovered case of MCF. Such a cell line would provide insight on OvHV-2 infection during recovery. Raji cells, an EBV cell line from a case of Burkitt's lymphoma contains 50-60 viral genomic equivalents per cell in a latent form (Adams & Lindahl, 1975; Nonoyama & Pagano, 1973). This latent EBV genome exists as a covalently closed circular (CCC) form within Raji cells (Adams & Lindahl, 1975). The number of OvHV-2 genomic equivalents (copy numbers) within LCLs is unknown. A predominantly latent and an abortive productive OvHV-2 infection were reported in LCLs from bovine and rabbit cases of clinically affected MCF, respectively (Rosbottom *et al.*, 2002). OvHV-2 positive LCLs obtained from PBMCs of clinical or recovered cases of MCF have not been examined.

Our hypothesis is that OvHV-2 infected lymphoblastoid cell lines can be established from PBMCs of cattle with clinical MCF and recovered cattle. Establishment of an OvHV-2 infected LCL from a recovered case of MCF would help confirm the establishment of persistent latent infection in lymphocytes of recovered cattle. The objective of this study was to establish LCLs from PBMCs of clinical and recovered cases of MCF and characterize the cell phenotype, cell replication kinetics, and cell apoptosis as well as quantify OvHV-2 DNA copies in cells and supernatant. Results of our study should aid in further understanding OvHV-2 infection during active disease and recovery.

MATERIALS AND METHODS

Sampling: Whole blood was collected from 3 fatal and 1 recovered case of MCF in Holstein cattle that were examined at the Colorado State University James L. Voss

Veterinary Teaching Hospital. Clinical cases exhibited signs of MCF during sampling and later succumbed to the disease. The recovered case experienced a brief episode of MCF, followed by recovery over a 2 week period, and was returned to the originating dairy. Whole blood was obtained from the recovered case 3 months after the clinical episode. All of the sampled animals tested positive for OvHV-2 by PCR and the MCF direct competitive inhibition ELISA (CI-ELISA) (Li *et al.*, 2001b).

Cell Lines: Cell lines from the recovered and fatal cases of MCF were established in the following manner. PBMCs were harvested from whole blood by a ficoll-hypaque gradient (specific gravity 1.077 g/ml). Harvested PBMCs were washed twice in Hank's buffered salt solution (HBSS) and resuspended at a density of $5x10^5$ cells/ml in RPMI 1640 medium supplemented with 20% Fetal bovine serum (FBS), L-glutamine (2mM), penicillin (100U/ml), streptomycin (100ug/ml), interleukin-2 (10 units/ 20 ml) and 2-mercaptoethanol ($5\ X10^{-5}$ M). Cells were cultured at 37 degrees C and 5% CO_2. Cell lines were split every 5-7 days at a ratio of 1:1.

Morphological Characterization: Cytospin preparations of $1x10^6$ cells were fixed in cold methanol and stained with the Giemsa stain. Stained cytospins were examined with light microscopy.

Phenotypic Analysis: Cells were incubated with a variety of bovine primary monoclonal antibodies for lymphocyte surface markers (VMRD, Table 4.1), followed by a secondary FITC conjugated antibody (Serotec). All incubations were at 4°C for 30 minutes and were followed by two washes in FACs buffer. Expressed cell surface markers were analyzed by flow cytometry. Flow cytometric data was analyzed by SUMMIT™ OFFLINE (Cytomation).

TABLE 4.1: Examined cell surface markers and monoclonal antibody designation.

Surface Marker	Cell Type	Antibody designation
CD2	pan T-cells	MUC2A
CD3	pan T-cells	MM1A
CD4	T helper cells	CACT138A
CD8	Cytotoxic T-cells	CACT80C
γδ	gamma-delta T-cells	GB21A
Cell surface IgG	B-cells	LCT27A
CD14	Macrophages/ monocytes	CAM36A

LCL Replication Kinetics: LCL replication kinetics were determined by the trypan blue exclusion assay. Cells from each of the cell lines were seeded at 5 x 10^5 cells/ml on day 0, followed by daily viable cell counts from day 1 to 5 of culture.

OvHV-2 Replication Kinetics: An aliquot of culture media was collected from cell lines on day 1 to day 5 after culture. The cell lines were centrifuged at 1000 RPM for 5 minutes and supernatant and cells were separated and saved separately at -20°C until DNA isolation. DNA was isolated from saved aliquots of cells and supernatants (DNeasy Tissue kit, Qiagen). DNA isolated from cells and supernatant was examined for OvHV-2 DNA by qPCR. The OvHV-2 specific qPCR procedure employed the same primers and probes as previously described (Hussy *et al.*, 2001) with a slight modification to the reaction conditions. Both primers and probe were included in the reaction mix at 150nM and the reaction cycle was modified to; Cycle1: 50 degrees-2 minutes; Cycle 2: Step1: 95 degrees-15 sec; Step 2: 60degrees-1 min. Data was acquired during step 2 on the Bio Rad iCycler. An OvHV-2, ORF 75, PCR fragment (430bp), cloned into pGEMTeasy was used as a qPCR standard.

ORF 75 Message Expression: Total RNA was isolated from each of the LCLs by the standard Trizol RNA isolation method. The RNA was isolated on day 5 of culture. The isolated RNA was treated with DNase to remove contaminating DNA. 500ng of RNA was reverse transcribed with the one-step RT-PCR kit (Qiagen). The cDNA was examined for message to the OvHV-2, 238bp, ORF 75 fragment by hemi-nested PCR (Baxter *et al.*, 1993). The cDNA was also examined for a 500bp bovine GAPDH fragment to normalize for RNA input (Wahl *et al.*, 2004).

DNAse Protection Assay: Day 1, 3 and 5 aliquots of the collected supernatants above, were additionally treated with DNase (Fermentas). Effective DNase treatment was confirmed with the regular OvHV-2 PCR. Successful DNase treatment was followed by DNA isolation from 200ul of supernatant by the standard DNA isolation protocol (DNeasy tissue kit, Qiagen). The isolated DNA was examined for OvHV-2 DNA by the OvHV-2 specific qPCR as above.

Gardella Gel Analysis: Gardella gel analysis allows the separation and detection of both circular and linear OvHV-2 DNA in the cells. The horizontal Gardella gel was performed as previously reported (Gardella *et al.*, 1984). 10^7 cells from each cell line were loaded on the gel. Bovine turbinate cells were used as negative controls. EBV-Raji cells were used as a positive control for circular herpesviral DNA. Cell samples contained greater than 95% viable cells as determined by trypan blue staining. Viral specific DNA sequences were detected with DIG labeled OvHV-2 and EBV specific probes during the Southern blot. The DIG-labeled OvHV-2 probe was 422 bp in size and based on an ORF 75 PCR (Baxter *et al.*, 1993). The DIG labeled EBV probe was 387 bp long and based on

the EBNA-1 PCR by (Ambinder *et al.*, 1990). The OvHV-2 and EBV fragments were labeled with a PCR DIG Probe Synthesis Kit (Roche Applied Science).

Apoptosis Studies: The annexin V kit (R&D Biosystems) was used to examine the percentage of cells undergoing apoptosis from day 1 to day 5 of culture. 5 x 10^5 cells from each cell line were incubated with reagents from the kit on a daily basis. The percentage of cells undergoing apoptosis was detected by flow cytometry. Our percentages were based on cells that only took up annexin and not both annexin and propidium iodide (PI).

RESULTS

Morphological Analysis of MD00 LCLs: The morphology of MD00 LCLs examined with Giemsa stain were large vacuolated lymphocytes with prominent nuclei and nucleoli. Mitotic figures were also observed (Fig 4.1).

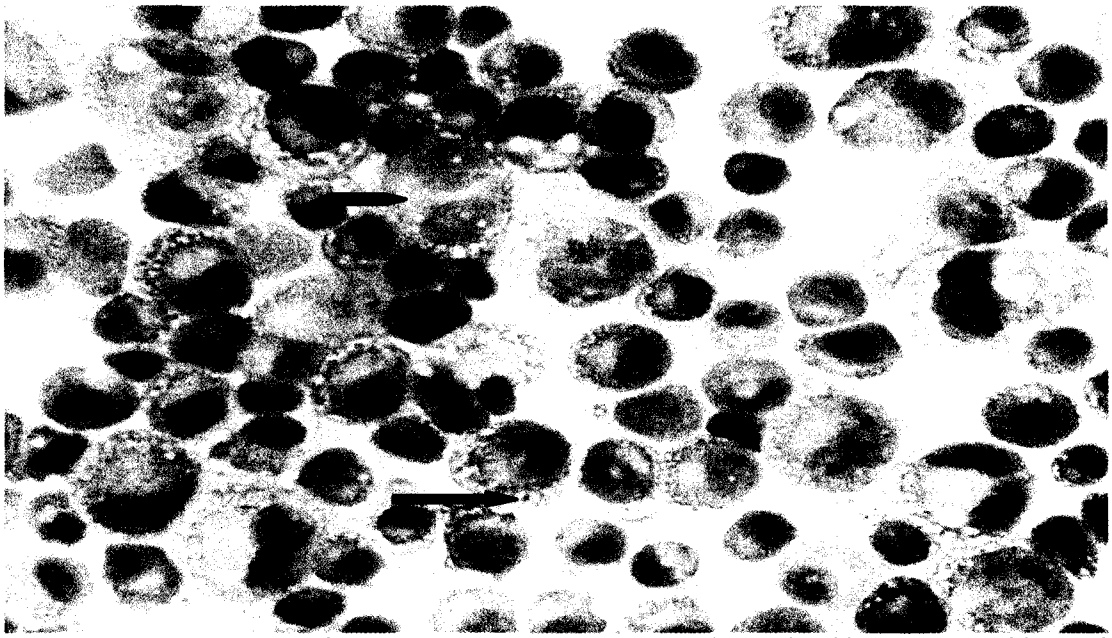


Figure 4.1: A Giemsa stain of MD00 LCLs. Stain shows large and vacuolated lymphocytes with prominent nuclei and nucleoli. Arrow head depicts mitotic figures. Arrow depicts cytoplasmic vacuoles.

T-Cell Phenotype of LCLs: Surface marker expression on the cells was examined by flow cytometry (Table 4.2). All the cell lines were CD2 and CD3 positive T-cells. Additionally, the MD00 cell line was exclusively gamma delta positive. Both the TT08 and CR10 cell lines were exclusively CD8 positive, whereas the TT05 cell line was a mixed CD8 and gamma delta positive lymphoblastoid cell line (LCL).

Table 4.2: Source and phenotype of OvHV-2 positive lymphoblastoid cell lines (LCLs)

LCLs (Time period in culture)	Source	Phenotype	IL-2 dependency
MD00 (6 years)*	Fatal MCF, adult cow	$\gamma\delta^+$	IL-2 independent
TT05 (2 years)*	Fatal MCF, 1 year old heifer	Mixed $\gamma\delta^+$ and $CD8^+$	IL2-dependent
TT08 (1.5 years)*	Fatal MCF 6 month old heifer	$CD8^+$	IL-2 dependent
CR 10 (1 year)*	Recovered MCF, 2 year old cow	$CD8^+$	IL-2 dependent

* indicates the length of time the cells have been in continuous culture.

Cellular Replication Kinetics: Cellular replication kinetics were examined by enumeration in culture using the trypan blue exclusion assay to assess viability (Fig 2). Cells from each of the 4 cell lines were cultured at $5x10^5$ cells/ ml on day 0. Viable cells were counted daily over the 5 day culture period. Cellular replication kinetics varied between the 4 cell lines. There was a slight decrease in the cell concentration for the MD00 and TT05 cells after 24 hours of culture. MD00 cells concentration remained steady and did not double over the 5 day culture period. After a brief decline, TT05 cells exhibited a doubling time of > 72 hours, and tripled in 96 hours followed by a plateau. TT08 and CR10 cells exhibited a doubling time of 48 hours. TT08 cell numbers then

plateaued, whereas CR10 cells more than quadrupled in 72 hours followed by a steep decline in cell concentration over the remainder of the culture period.

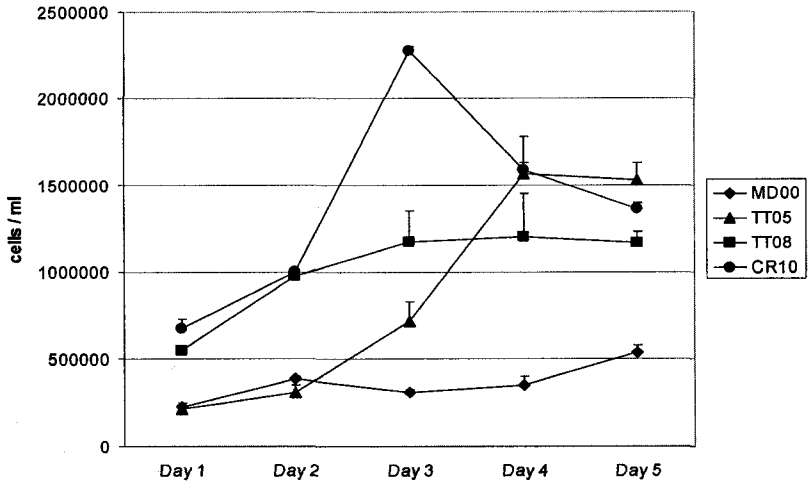


Fig 4.2: Cell line replication kinetics over 5 days in culture. Viable cells were counted daily with the trypan blue exclusion assay. MD00 (♦) exhibited a steady growth and did not double. TT05 (▲) doubled in > 72 hours, tripled in 96 hours followed by a plateau. TT08 (■) doubled in 48 hours followed by a plateau. CR10(●) cells doubled in 48 hours, more than quadrupled in 72 hours followed by a rapid decline. Error bars represent the standard deviation of two counts.

OvHV-2 Replication Kinetics: OvHV-2 DNA replication kinetics were determined by examining viral genome copy numbers (genomic equivalents) within the cells and their supernatants over a period of 5 days by qPCR (Fig 4.3a and 4.3b). Viral DNA copy numbers were variable between the different cell lines. Cellular OvHV-2 genome copies in cell lines from fatal cases (MD00, TT05 and TT08) were relatively higher than those from the recovered case (CR10). The supernatant from the recovered case also had a lower concentration of OvHV-2 DNA copies than in supernatants from fatal cases. Over a period of 5 days, cellular viral DNA copies peaked at day 4 within all the cell lines, followed by a decline in copy numbers at day 5. At peak (day 4), cell lines MD00, TT05, TT08, and CR10 had 66, 59, 140 and 29 viral DNA copies per cell, respectively. Cell line

TT08 had more than double the cellular viral DNA copies at peak compared to the other cell lines. MD00 cells showed a decrease in viral DNA copies between day 2 and 3, followed by a peak and decline on days 4 and 5.

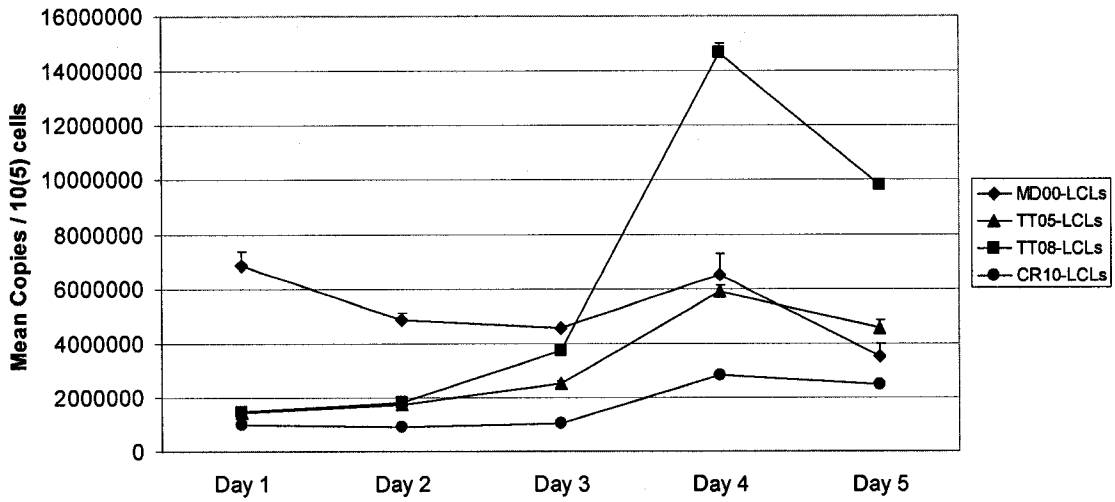


Fig 4.3(a): D1-D5 OvHV-2 replication kinetics in cells of LCLs by qPCR. Cellular OvHV-2 genome copy numbers over a 5 day period were assessed per 10^5 eukaryotic cells. Cell lines established from fatal cases, MD00 (♦), TT05 (▲) and TT08 (■) harbored more viral copies than CR10 (●) established from a recovered case of MCF. Viral copies peaked at day 4 in all cell lines. Peak copy numbers (genomic equivalents) in MD00, TT05, TT08 and CR10 were 66, 59, 140 and 29, respectively. Error bars represent the standard deviation of three replicates.

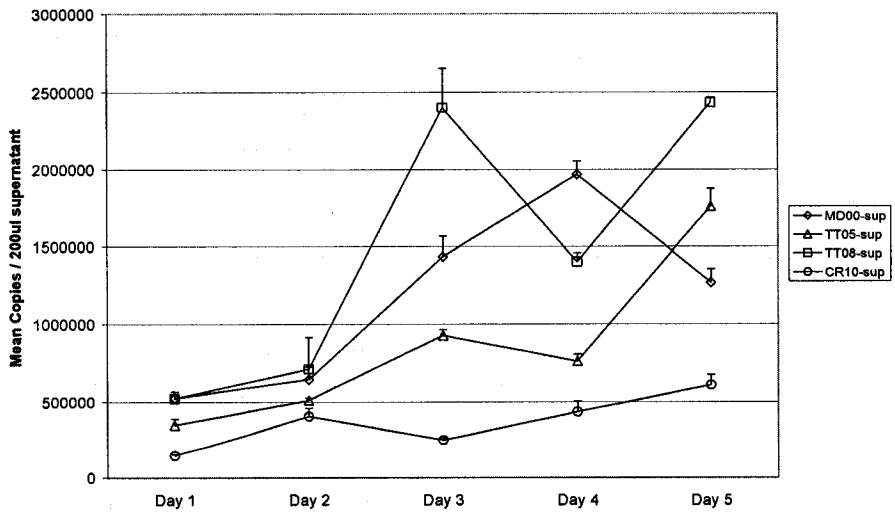


Fig 4.3(b): OvHV-2 DNA concentration in LCL supernatants examined by qPCR on days 1 thourgh 5 of culture starting with $5x10^5$ LCLs. Viral genome copy numbers over a 5 day period were assessed per 200ul of supernatant. Viral DNA copies were relatively lower in the supernatants compared to cells (Fig 4.3a). Supernatant of LCLs from fatal cases, MD00 (◊), TT05 (Δ) and TT08 (□) harbored relatively higher viral copies than CR10 (○) established from a recovered case of MCF. Error bars represent the standard deviation of three replicates.

DNAse Protection Assay (Fig 4.4): In order to examine the source of OvHV-2 DNA in cell line supernatants, DNA was isolated from day 1, 3, and 5-DNase treated supernatants and examined for OvHV-2 DNA by qPCR. The effectiveness of the DNase treatment was confirmed by standard hemi-nested OvHV-2 PCR and OvHV-2 DNA could not be detected in DNase treated supernatants prior to protease treatment and further DNA isolation. DNase protected OvHV-2 DNA was detected in the supernatants of all cell lines. Viral DNA copy concentration was significantly lower than in DNase untreated supernatants. DNase protected viral DNA copies made up 0.06% (MD00), 0.3% (TT05), 0.03% (TT08) and 0.3% (CR 10) of the total OvHV-2 DNA copies in DNase untreated supernatants. Viral copy numbers within all supernatants, except TT08 peaked at day 5. Unlike in the other cell lines, viral copies in TT08 supernatant peaked on day 3 with a decline by day 5. TT05 supernatant had the highest OvHV-2 DNA concentration after DNase treatment whereas TT08 supernatant displayed the highest viral DNA concentration before DNase treatment. CR10 supernatant which had the lowest viral DNA concentration before DNase treatment, displayed more viral copies than MD00 and TT08 after DNase treatment.

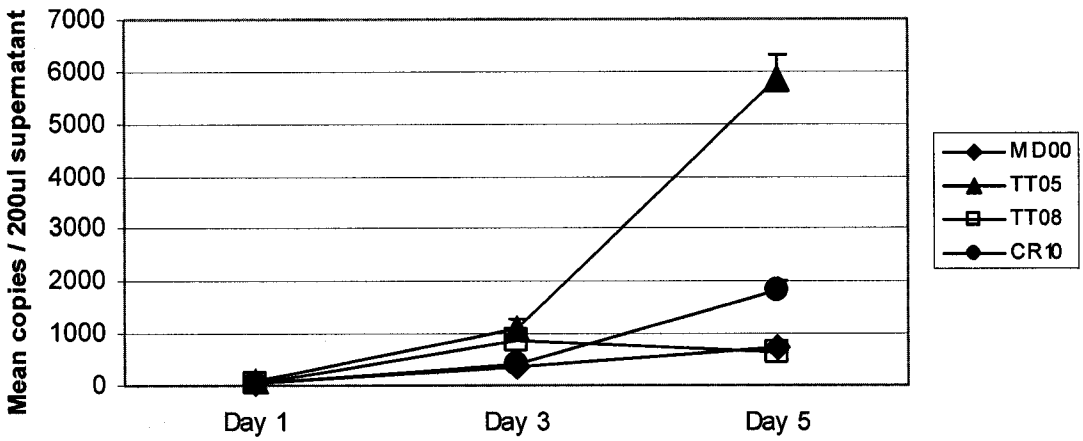


Fig 4.4: OvHV-2 DNA concentration in DNase treated cell line supernatants on days 1, 3, and 5. There was a decrease in viral DNA copy concentration within supernatants after DNase treatment. Copy numbers peaked on day 5 in cell lines MD00 (♦), TT05 (▲) and CR10 (●) and on day 3 in TT08 (□). Day 5 copy number concentration in CR10 (from recovered case) was higher than in both MD00 and TT08 (established from fatal cases of MCF). DNase protected OvHV-2 genome in LCLs represented about 0.06% (MD00), 0.3% (TT05), 0.03% (TT08) and 0.3% (CR10) of total the total OvHV-2 genomes in the supernatant. Error bars represent the standard deviation of 3 replicates.

Detection of ORF 75 Message in Cell Lines: ORF 75 is suggested to code for FGRAT, an OvHV-2 structural protein. Presence of ORF 75 message indicates late gene expression and suggests late protein synthesis and possible production of whole virions (Rosbottom *et al.*, 2002). The presence of ORF 75 message within LCLs was examined by reverse transcription to cDNA followed by the OvHV-2 PCR. ORF 75 messenger RNA was detected in all cell lines (Fig 4.5-A). There was no DNA contamination of the examined RNA as indicated by absence of an amplicon in controls without reverse transcriptase. Equal amounts of RNA loading from the different cell lines were examined in the RT-PCR as confirmed by the bovine GAPDH RT-PCR (Fig 4-5-B)

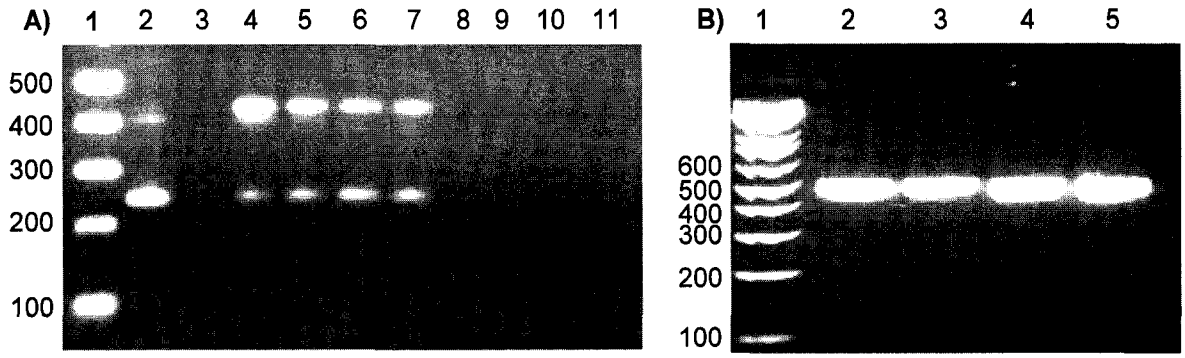


Fig 4.5: A) Reverse transcription and a heminested PCR of the OvHV-2 ORF 75 fragment. Lanes: 1) 1kb plus ladder, 2) Positive control, 3) No template control, 4) MD00 cDNA, 5) TT05 cDNA, 6) TT08 cDNA, 7) CR10 cDNA, 8) MD00 cDNA no RT control, 9) TT05 cDNA no RT control, 10) TT08 cDNA no RT control, 11) CR10 cDNA no RT control. A 238 bp ORF 75 hemi-nested fragment was amplified in all the cells (4-7). A 422 bp fragment was carried over from the primary reaction (2, 4-7). There was no DNA contamination of the RNA (8-11). B) Amplification of a bovine specific 500bp GAPDH fragment using reverse transcribed cDNA from figure A) Lanes: 1) 1kb plus ladder, 2) MD00 cDNA 3) TT05 cDNA 4) TT08 cDNA 5) CR10 cDNA. The figure indicates that equal amounts of RNA were loaded for the reverse transcription and subsequent PCR reactions.

Gardella Gel of the LCLs (Fig 4.6)**:** The presence of circular and linear DNA in the established LCLs was examined by Gardella gel analysis (Gardella *et al.*, 1984). Latently infected cells harbor covalently closed circular (CCC) viral genome, whereas productively infected cells harbor a linear viral genome which migrates faster than the CCC form on the gel (Gardella *et al.*, 1984; Rosbottom *et al.*, 2002). Raji cells which harbor a latent form of EBV were used as a positive control. An EBV specific CCC band was detected within the Raji cells. An OvHV-2 specific CCC was detected in cell lines established from fatal cases of MCF (MD00, TT05, and TT08). A faint CCC band was also detected within CR10. The EBV and OvHV-2 probes did not cross-react and identified CCC bands for EBV and all four OvHV-2 LCLs. Bovine turbinate cells were used as a negative control and no OvHV-2 specific band was detected from these cells. Linear OvHV-2 DNA (productive infection) was not detected in any of the cell lines examined.

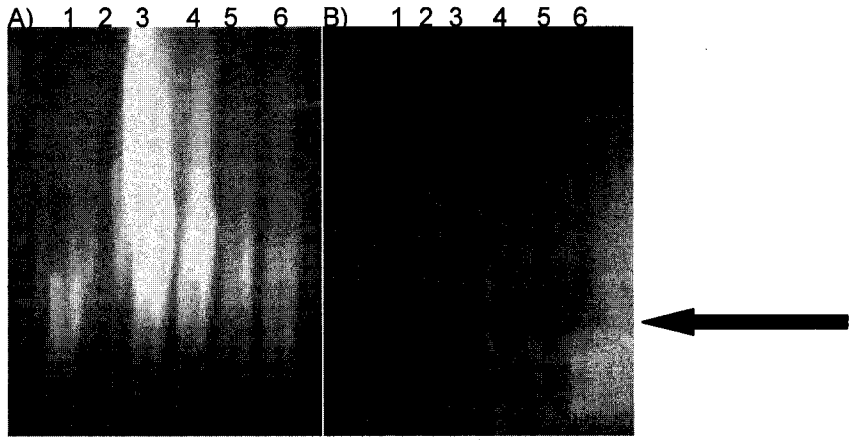


Fig 4.6: Gardella gel (A) and Southren blot (B) analysis of EBV positive Raji cells (lane 1) and OvHV-2 positive MD00 (lane 3), TT05 (lane 4), TT08 (lane 5) and CR10 (lane 6) cell lines. Bovine turbinate (lane 2) cells were included as a non infected control. An EBV-specific covalently closed circular (CCC) fragment was detected within Raji cells (b-lane1). An OvHV-2 specific CCC fragment was detected within MD00 (b-lane3), TT05 (b-lane 4), TT08 (b-lane 5) and CR 10 (b-lane 6). The OvHV-2, CCC fragment within CR10 was faint compared to the other cell lines. No viral fragment was detected within the bovine turbinate cells (b-lane 2). Arrow indicates covalently closed circular viral DNA.

Apoptosis Studies: Cells were stained with the annexin V kit on a daily basis for 5 days and apoptosis was examined by flow cytometry. The annexin V kit can differentiate apoptotic and necrotic cells. Early apoptotic cells stain only with annexin, whereas, necrotic and late apoptotic cells stain with both annexin and propidium iodide (PI). The percentagesof apoptotic cells detected represent early apoptotic cells which stained with annexin but did not take up PI. Apoptotic cells were detected in all the cell lines over a period of 5 days. The percentage of apoptosic cells was the highest within the gamma delta positive MD00 cell line and ranged between 50 and 70%. It ranged between 17 and 50% in TT05 cells, between 40 and 50% within TT08, and 35 and 50% within CR10 cells. The percentage of apoptosic cells within all the cell lines showed a decline over a period of 5 days.

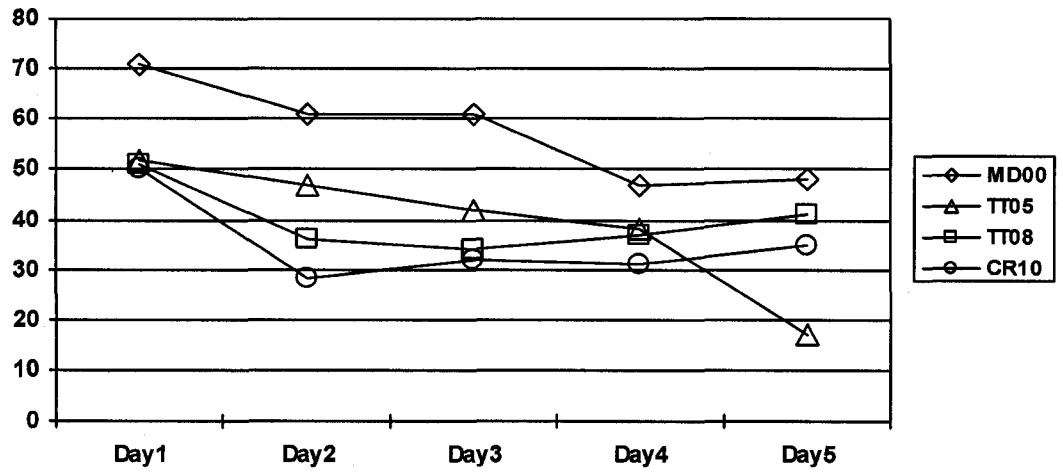


Figure 4.7: Detection of mean percentage apoptosis in cell lines over a 5 day period. Cells were stained with the annexin V kit and apoptosis detected by flow cytometry. MD00 (◊) had the highest percentage of early apoptotic cells. Percentage of early apoptotic cells ranged from 70-50% (MD00), 50- 17% (TT05 (Δ), 40-50% (TT08 (□) and 50-35% (CR10 (○) over a period of 5 days in culture.

DISCUSSION

Lymphoblastoid cell lines (LCLs) from clinical cases of MCF have been reported in the literature (Burrells & Reid, 1991; Cook & Splitter, 1988; Reid *et al.*, 1983; Schock *et al.*, 1998; Swa *et al.*, 2001). Establishment of OvHV-2 positive LCLs from PBMCs as opposed to tissues of clinical and recovered cases of MCF has not been reported. This is also the first report of establishment of an OvHV-2 positive LCL from a recovered case of MCF.

Gammaherpesviruses characteristically cause persistentl latent infections in lymphocytes. EBV results in a life long, persistently latent infection within B-cells of patients with infectious mononucleosis (IM) (Diehl *et al.*, 1968; Kanegane *et al.*, 2002; Niederman *et al.*, 1968; Rickinson *et al.*, 1974). B-cell lines can be established from PBMCs of patients with chronic IM for up to 20 years after recovery from acute IM (Diehl *et al.*, 1968). Recent reports indicate that animals can recover from a clinical

episode of MCF (Michel & Aspeling, 1994; Milne & Reid, 1990; O'Toole *et al.*, 1995; O'Toole *et al.*, 1997). It is however unknown if recovered cases clear infection or remain latently infected. Our ability to establish a lymphoblastoid cell line from PBMCs of a recovered case suggests development of a latent OvHV-2 infection within T-lymphocytes. The phenotype and detection of OvHV-2 DNA within established LCLs suggests T-lymphocytes as a site of OvHV-2 latency after recovery. This is consistent with the current concept that gammaherpesviruses cause lifelong infections by establishing latency within host lymphocytes (Epstein & Achong, 1977; Rickinson *et al.*, 1974). EBV *in vivo* infection is a complex mixture of latent, reactivated, transforming, or replicative types of infection. Our data suggests latency within T-cells as one of the features of OvHV-2 *in vivo* infection. Additional features of OvHV-2 infection need to be examined. MCF recrudescence has not been reported in recovered cases. The suggested lifelong infection and latency in infected T-cells raises questions about possible recrudescence or the role played by OvHV-2 latency and the immune system in the pathogenesis of MCF.

The mechanism of OvHV-2 positive lymphoblastoid cell line outgrowth in culture is currently unknown. EBV cell lines from PBMCs of chronic IM carriers were shown to arise by a different mechanism from that of Burkitt's lymphoma cell lines. Cell lines from chronic IM carriers arose by a two step mechanism that involves induction of lytic EBV replication in latently infected B-cells followed by infection and transformation of other cells in culture. Burkitt's lymphoma cell lines arise by outgrowth of *in vivo* malignantly transformed cells (Rickinson *et al.*, 1974).

Our LCLs were morphologically large granular lymphocytes and phenotypically T-cells, similar to previously reported OvHV-2 positive cell lines derived from tissues of MCF cases (Burrells & Reid, 1991; Cook & Splitter, 1988; Reid *et al.*, 1983; Schock *et al.*, 1998; Swa *et al.*, 2001). The phenotypes varied from exclusive gamma-delta+ and CD8+ to a mixed gamma-delta and CD8+ T-cell line. At this point, the factors that determine which T-cell subsets develop into LCLs remain unknown.

OvHV-2 positive LCLs have similarities to NK T-cells based on their large granular lymphocyte morphology and their reported non MHC-linked NK cell activity. NK T-cells are thought to be part of the innate immune system and to provide a rapid response while the conventional T-helper response is still developing (Goldsby R *et al.*, 2000). NK T-cells display markers for both T-cells and NK cells on their surface. Their T-cell markers can include CD4, CD8 and gamma delta T-cell markers. Activated NK T-cells rapidly secrete large amounts of regulatory cytokines including IFN-gamma and IL-4. NK T-cells may support either humoral (antibody production) or cell mediated (cytotoxic T-cell) immune response. Infection and dysregulation of large granular lymphocytes is thought to play an important role in MCF pathogenesis (Reid *et al.*, 1983). The recently characterized, bovine NK cell marker should help further characterize established LCLs (Storset *et al.*, 2004).

Our lymphoblastoid cell lines displayed variable replication kinetics. The MD00 cells that have been continuously cultured for more than 6 years exhibit a steady growth without doubling in numbers. Earlier passages of this cell line displayed a doubling time of 48 hours. TT05 and TT08 cells in continuous culture for 2 and 1.5 years, respectively, display exponential growth followed by a plateau. Unlike the other cell lines, CR10 cells,

established from a recovered case and in continuous culture for a year, more than quadrupled in numbers followed by a rapid decline. The length of time in culture and availability of nutrients in the media may affect the cellular replication kinetics. CR10 cells which are relatively 'younger' than the other cell lines have more capacity to grow, with a rapid depletion of nutrients in the media resulting in rapid decline in cell numbers. There does not seem to be a correlation between cellular and OvHV-2 DNA replication kinetics.

All of the cell lines established from PBMCs displayed an increase in intracellular OvHV-2 DNA after 4 days in culture. The increase indicates active viral DNA replication within cells. At peak OvHV-2 DNA copy numbers, MD00, TT05, TT08 and CR10 cell lines contained 66, 59, 140 and 29 OvHV-2 genomic equivalents (copy numbers)/cell, respectively. EBV copy numbers vary from 1 to more than 100 copies depending on the cell line (Nonoyama & Pagano, 1973; zur & Schulte-Holthausen, 1970). The CR10 cells, derived from a recovered case of MCF had relatively fewer OvHV-2 DNA copies in both cells and supernatant compared to cell lines from fatal cases of MCF. OvHV-2 load may play a role in recovery.

The OvHV-2 DNA in LCL supernatants was shown to be both DNase protected and unprotected DNA. DNase protected OvHV-2 DNA accounted for between 0.03 and 0.3% of the total OvHV-2 DNA in the supernatant. It is not known if the DNase protected OvHV-2 DNA were contained in whole, enveloped virions obtained from budding, or from encapsidated virions released during cell death. Encapsidated, but non-enveloped immature, and non-infectious AlHV-1 particles could be found in both the nucleus and cytoplasm of infected cells (Castro & Daley, 1982). These immature forms would be

DNase protected and released into the supernatant during cell death. Cells and supernatant from MD00 cells failed to cause clinical MCF when injected into rabbits (data not shown).

ORF 75 is believed to be a late productive cycle gene and transcription of its message would therefore be an indicator of productive cycle gene expression (Rosbottom *et al.*, 2002). Detection of ORF 75 messenger RNA within all of the cell lines examined suggests a productive cycle of gene expression occurs to some extent within all of the cell lines. The Gardella gel assay separates latent (covalently closed circular) viral DNA from productive (linear) viral DNA. Our results show a predominance of latent (covalently closed and circular) OvHV-2 DNA and no detectable productive (linear) viral genome within all the cell lines. The Gardella gel also confirmed the presence of fewer OvHV-2 copies within the recovered case LCL versus those in LCLs from fatal cases. However, though only predominant latent infection was demonstrable by the Gardella gel, presence of a productive OvHV-2 infection within all the cell lines is supported by the following; detection of active viral DNA replication, presence of late productive gene messenger RNA, and DNase protected viral DNA in the supernatants. It is possible that only a few cells within each of the cell lines are productively infected. The Gardella gel assay may not be sensitive enough to detect the few linear OvHV-2 DNA copies present.

A high level of apoptosis was detected within all the cell lines. The significance of this finding is uncertain. Lymphocytes die by apoptosis *in vivo* and the majority of the cells in culture could be dying by apoptosis. However, cells within the culture were more than 80% viable by trypan blue exclusion and (propidium iodide) PI stain. The anti human annexin antibody used in the study, could possibly bind non-specifically to an

unrelated surface marker in bovine cell resulting in a falsely positive high level of apoptosis. The role of apoptosis in the MCF pathogenesis requires further investigation.

In summary, this is the first report of establishment of OvHV-2 positive cell lines from PBMCs of both clinical and recovered cases of MCF. All of the cell lines were of a T-cell phenotype. This study also reports the first establishment of an OvHV-2 positive cell line from a recovered case of MCF. Persistent OvHV-2 infection with latency in T-cells of recovered cases is suggested by this finding. Cell lines from recovered cases harbor less viral DNA per cell than cell lines from fatal cases of MCF. OvHV-2 positive cell lines undergo active viral DNA replication, late gene expression, and DNase protected OvHV-2 DNA can be detected in cell line supernatants. However, only circular OvHV-2 DNA associated with non-productive viral infection could be identified in the LCLs.

CHAPTER 5

CHEMICAL INDUCTION OR INHIBITION OF OvHV-2 DNA REPLICATION IN OvHV-2 POSITIVE LYMPHOBLASTOID CELL LINES

ABSTRACT

Ovine herpesvirus 2 (OvHV-2), the causative agent of sheep associated malignant catarrhal fever (MCF), has never been isolated and a productive in vitro culture system for the virus does not exist. In order to develop such a system, 3 OvHV-2 positive lymphoblastoid cell lines (LCLs) were treated with a variety of chemicals over a period of 5 days. The chemicals used were either inducers or inhibitors of viral DNA replication in other gammaherpesvirus systems. The effects of treatment on OvHV-2 DNA replication were evaluated by examining viral DNA copy numbers in cells and supernatants of treated and untreated LCLs by real-time PCR (qPCR). The three OvHV-2 cell lines displayed varied responses to similar treatments. Some of the chemicals did not affect viral copy numbers and therefore had no effect on OvHV-2 DNA replication. Some chemicals significantly increased viral DNA copy numbers in either cells or supernatant. None of the chemicals resulted in a uniform increase in viral copies in both cells and supernatant of individual cell lines. Some of the chemicals decreased viral DNA copy numbers in either cells or supernatant, whereas, some chemicals uniformly decreased viral DNA copies in cells and supernatant of individual cell lines.

Dexamethasone and acyclovir treatments increased and decreased intracellular viral DNA copy numbers, respectively. Acyclovir additionally decreased viral DNA copies in the LCL supernatant. Both of these drugs may have an impact on the treatment of clinical MCF cases. Dexamethasone, which is occasionally used in supportive treatment of MCF cases, may be contraindicated as it may increase the intracellular viral burden. Alternatively, acyclovir may be used successfully to lower OvHV-2 load during clinical disease.

In summary, our study suggests a variety of chemicals or drugs that may successfully induce or inhibit OvHV-2 DNA replication. These drugs can be investigated further and may eventually lead to the development of an OvHV-2 in vitro productive system, which would greatly enhance MCF pathogenesis studies. Dexamethasone and acyclovir may have a direct impact on treatment of clinical MCF. The effects of both drugs need further investigation both in cell culture and MCF animal models.

INTRODUCTION

Ovine herpes virus 2 (OvHV-2) is the causative agent of sheep-associated malignant catarrhal fever (MCF) (Buxton *et al.*, 1984; Reid *et al.*, 1984), a lympho-proliferative disease of susceptible ruminants (Plowright, 1990). OvHV-2 has never been isolated and a productive *in vitro* culture system for the virus does not exist (Cook & Splitter, 1988) Similar to other gammaherpesviruses, lymphoblastoid cell lines can be established from tissues and peripheral blood mononuclear cells (PBMCs) of clinical cases (Burrells & Reid, 1991; Cook & Splitter, 1988; Reid *et al.*, 1983; Schock *et al.*,

1998; Swa *et al.*, 2001). An OvHV-2 *in vitro* culture system that is amenable to further manipulation would be an invaluable tool in the study of MCF pathogenesis.

A variety of chemicals have successfully been used to develop *in vitro* culture systems for other gammaherpesviruses, such as EBV (Epstein Barr virus) (Luka *et al.*, 1979) and HHV-8 (human herpesvirus 8 / Kaposi's sarcoma herpesvirus) (Renne *et al.*, 1996). In culture, the EBV genome within cell lines exists in a latent, covalently closed circular (CCC) form (Adams & Lindahl, 1975). The different EBV cell lines can be classified as either producer or non producer cell lines. The majority of cells within producer cell lines contain the latent EBV genome, with about 2-10% of cells undergoing spontaneous induction and production of whole virions. Non producer cell lines, however, are latently infected without spontaneous induction (Luka *et al.*, 1979). Deletions in the EBV genome within Raji cells, a non producer cell line, are thought to contribute to the inability of Raji cells to produce EBV either spontaneously or upon induction (Polack *et al.*, 1984). Chemical treatments lead to an increase in the level of induction within producer cell lines. Only early viral genes were induced in non producer cell lines, whereas both early and late viral genes were induced in producer cell lines (Fresen *et al.*, 1978; zur *et al.*, 1978a).

OvHV-2 positive cell lines used in this study were developed from fatal cases of MCF and have been in continuous culture over a prolonged period of time. These cell lines were shown to be predominantly latently infected with OvHV-2 and to release DNase protected OvHV-2 genomes into their supernatants. It is not known if the DNase protected DNA is contained in fully intact infectious virions or non-infectious abortive

virions. There are no published reports regarding different chemicals that can affect the OvHV-2 DNA replication in lymphoblastoid cell lines.

In this study, we hypothesized that OvHV-2 DNA replication can be induced or inhibited by treatment with a variety of chemicals. The objective of the study was to induce or inhibit OvHV-2 DNA replication by treating the LCLs with different chemicals which have been shown to either induce or inhibit other gammaherpesviral DNA replication. The effects of chemical treatment on OvHV-2 DNA replication was evaluated by examining viral DNA copy numbers within cells and culture media supernatants of chemically treated and untreated LCLs by qPCR. This study will provide information on the different chemicals that may have an *in vitro* and possibly *in vivo* effect on the OvHV-2 DNA replication. Results from our study could also serve as an important foundation that could lead to the successful development of an *in vitro* culture system for OvHV-2.

MATERIALS AND METHODS

Cell Lines: Three OvHV-2 positive cells lines (MD00, TT05 and TT08), were established from PBMC's of fatal cases of malignant catarrhal fever (MCF) and maintained in culture over a prolonged period of time (Table 5.1). The cell lines were maintained in RPMI 1640, 20% FBS, 2mM L-glutamine, 100U/ml penicillin, 100ug/ml streptomycin and 5x10(-5) M 2-β-mercaptoethanol. Interleukin-2 (10 units / 20 ml) was also added to culture media for the TT05 and TT08 cell lines but the MD00 cell line was IL-2 independent. Cells were cultured in 5% CO_2 at 37°C and subcultured at a ratio of 1:1 every 5-7 days.

Chemical Treatments: The LCLs were treated with different chemicals (Table 5.2 and 5.3) and incubated at 37°C in 5% CO_2 for 5 days. Variable concentrations of the different chemicals were tested on 10^6 cells of each LCL (Table 5.3). On day 5 of treatment, cells and supernatant were harvested and stored at -20°C until further processing. Cells were washed once in PBS before storage. DNA was harvested from stored cells and supernatants (DNeasy kit, Qiagen). OvHV-2 genomic equivalents within the isolated DNA were determined using the OvHV-2 qPCR.

OvHV-2 qPCR: The OvHV-2 qPCR employed the same primers and probes as previously described (Hussy *et al.*, 2001) with a slight modification to the reaction conditions. Both primers and probe were included in the reaction mixture at 150nM and the reaction cycle was modified to; Cycle1: 50 degrees-2 minutes; Cycle 2: Step1: 95 degrees-15 sec; Step 2: 60 degrees-1 min. Data was acquired during step 2 on the Bio Rad iCycler. The OvHV-2 ORF 75, PCR fragment (430bp), cloned into pGEMTeasy was used as the qPCR standard.

Statistical analysis: Experiments were performed in quadruplicate or triplicate and repeated at least once. Data was statistically analyzed with the two tail, Student's T test, two samples assuming equal variance. The level of significance was determined at $P < 0.05$.

Table 5.1: OVHV-2 positive lymphoblastoid cell lines and duration in culture.

LCLs	Time Period in Culture	IL-2 Dependency
MD00	6 years	IL-2 independent
TT05	2 years	IL-2-dependent
TT08	1.5 years	IL-2 dependent

TABLE 5.2: Chemicals / drugs used and their mechanism of action and or effects.

Chemical	Effects and Mechanism of Action
5-Azacytidine (AZA)	A cytosine nucleoside analogue that demethylates CpG dinucleotides in promoters allowing for gene expression to occur (Jones & Taylor, 1980). It causes lytic induction in some but not all EBV positive lymphoblastoid cell lines (Ben-Sasson & Klein, 1981; Feng *et al.*, 2004).
Acyclovir [9-(2-Hydroxyethoxymethyl) guanine] OR Acycloguanosine	A guanosine nucleoside analogue that selectively inhibits herpesviral DNA replication through inhibition of the viral DNA polymerase. Incorporation into the viral DNA also results in chain termination (Allaudeen *et al.*, 1982; Elion *et al.*, 1977).
n-Butyrate	A naturally occurring fatty acid that induces erythroid differentiation and is commonly used to form cyclic AMP derivatives (Leder & Leder, 1975). It caused induction of EBV in producer and non producer cell lines (Luka *et al.*, 1979).
Cyclic AMP (cAMP)	A second messenger involved in the regulation of a wide variety of cell functions (Kobayashi *et al.*, 1999).
Cisplatinum	A chemotherapeutic agent that induces lytic infection in EBV-positive epithelial cell tumors, but not lymphoblastoid cell lines (LCLs) (Feng *et al.*, 2002).
Dexamethasone	A glucocorticosteroid and an immunosuppressant. Glucocorticoids regulate the transcription of a variety of cellular and viral genes (Beato, 1989). It has been shown to induce EBV early antigens (Schuster *et al.*, 1991) and DNA replication (Daibata *et al.*, 2005).
Doxorubicin	A chemotherapeutic agent that induces lytic EBV infection in lymphoblastoid cell lines (LCLs), through activation of the two immediate early genes, BZLF1 and BRLF1 (Feng *et al.*, 2004)
Epinephrine	A catecholamine that stimulates adenyl cyclase activity (Makman, 1970).
12-O-Tetradecanoylphorbol-13-acetate (TPA)	A cocarcinogen and a tumour promoter (Hecker, 1968; Hecker, 1976; Makman, 1970). It induced early and late EBV antigens in EBV non producer and producer cell lines, respectively (zur *et al.*, 1978b).
Valproic acid (2-propylpentanoic acid)	An eight carbon, branched chain, fatty acid that has considerable structural similarities to butyric acid. It induced lytic gene expression in HHV-8 infected BCBL-1 cells (Shaw *et al.*, 2001).
5-Fluorouracil	A chemotherapeutic agent that induces lytic infection in EBV-positive epithelial cell tumors, but not LCLs (Feng *et al.*, 2002; Feng *et al.*, 2004).

TABLE 5.3: Lymphoblastoid cell lines and chemicals that they were treated with.

MD00	TT05	TT08
Azacytidine (5ug/200ul)*	Azacytidine (5ug/200ul)*	Azacytidine (5ug/200ul)*
Acyclovir (1,10 & 50ug/200ul)*	N/D	N/D
Butyrate (3mM)*	N/D	N/D
cAMP (0.3, 3 & 10ug/200ul)*	N/D	N/D
Cisplatinum (1ug/200ul)*	Cisplatinum (1ug/200ul)*	Cisplatinum (1ug/200ul)*
Dexamethazone (0.5, 1 & 5ug/200ul)*	N/D	N/D
Doxorubicin (0.2uM)*	Doxorubicin (0.2uM)*	Doxorubicin (0.2uM)*
Epinephrine (0.5, 1 & 5ug/200ul)*	N/D	N/D
TPA (10, 20 & 50ng/200ul)*	N/D	N/D
Valproic acid (3mM)*	Valproic acid (3mM)*	Valproic acid (3mM)*
5-Fluorouracil (5ug/200ul)*	5-Fluorouracil (5ug/200ul)*	5-Fluorouracil (5ug/200ul)*

*Chemical / drug concentrations examined per 10^6 cells
N/D-not done

RESULTS

In order to induce or inhibit OvHV-2 DNA replication, 3 OvHV-2 infected cell lines (MD00, TT05 and TT08) were treated with a variety of chemicals (Tables 5.2 and 5.3) and incubated for 5 days. Following incubation DNA was isolated from cells and supernatant. The MD00 cell line was treated with all of the chemicals. TT05 and TT08 were additionally treated with some of the chemicals (Table 5.3). The effects of chemical or drug treatment on OvHV-2 DNA replication were determined by examining OvHV-2 copy numbers in cells and supernatants of treated LCLs. Similar treatments displayed different effects on different cell lines (Table 5.4).

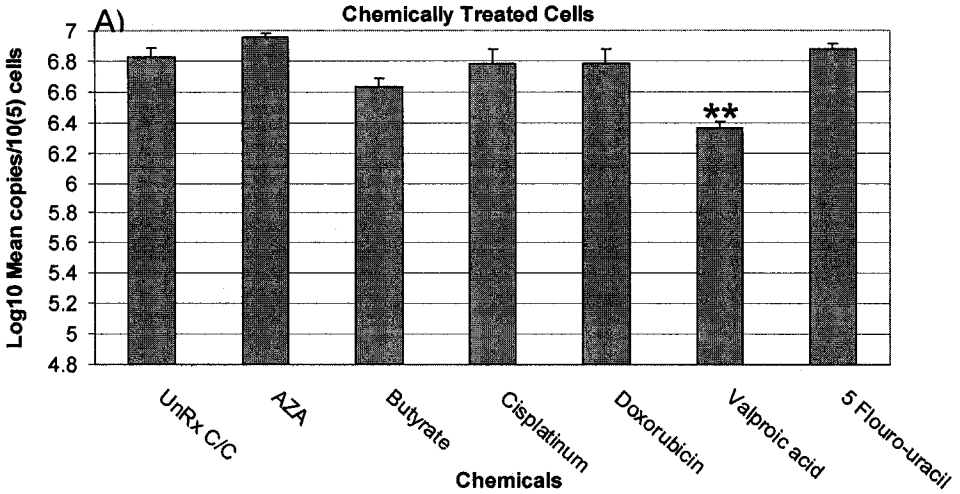
MD00 (Fig 5.1 and Table 5.4): AZA, butyrate, and TPA had no significant effect on OvHV-2 DNA copy numbers. Cisplatinum, Doxorubicin and 5Fluoro-uracil significantly increased OvHV-2 copies in the supernatant ($P < 0.001$). Valproic acid decreased viral DNA copy numbers in the cells ($P \leq 0.001$) but increased genome copy numbers within

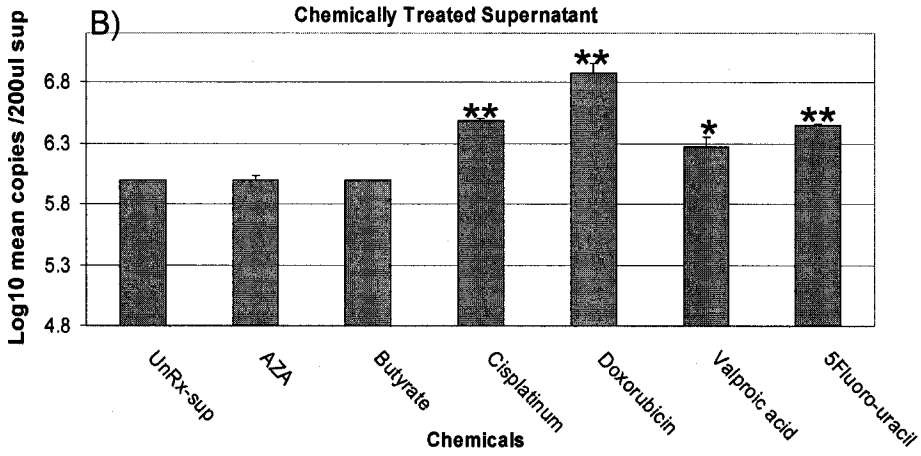
the supernatant ($P < 0.05$). Higher concentration of cAMP decreased viral genome copy numbers within the cells ($P < 0.001$) and increased viral copy numbers in the supernatant ($P < 0.05$) or decreased viral DNA copy numbers within both the cells and supernatant ($P \leq 0.001$). Dexamethasone increased OvHV-2 copy numbers within the cells ($P < 0.05$) at all of the concentrations tested, but not in the supernatant. The lowest concentration of epinephrine slightly increased viral copy numbers within the cells ($P < 0.05$). Higher epinephrine concentrations had no effect on copy numbers. Acyclovir resulted in a decrease in OvHV-2 copy numbers in either just the cells or both cells and supernatant ($P < 0.001$).

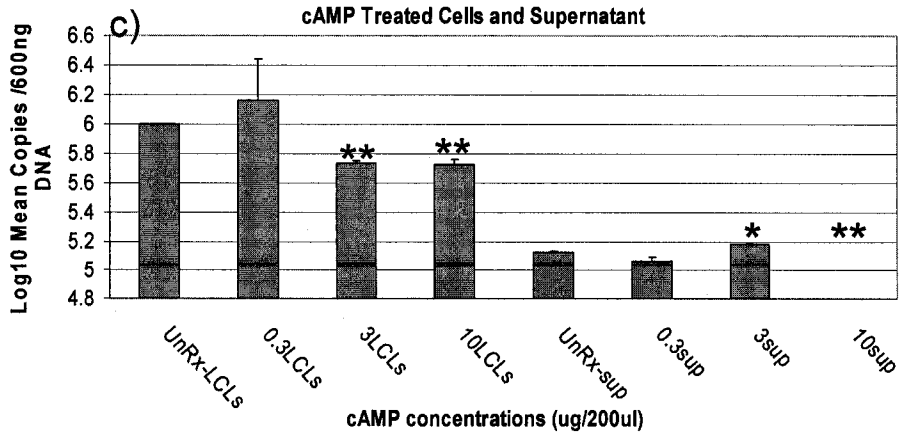
TT05 (Fig 5.2 and Table 5.4): AZA had no effect on OvHV-2 DNA copies. Cisplatinum significantly decreased viral copies in the cells ($P < 0.001$), but increased them in the supernatant ($P < 0.05$). Doxorubicin significantly decreased viral copies in the cells ($P < 0.05$) but had no effect on viral copy number in the supernatant. Valproic acid and 5FU did not affect viral copies in the cells but significantly increased copies in the supernatant ($P < 0.001$).

TT08 (Figure 5.3 and Table 4): AZA, cisplatinum, and 5-FU had no effect on viral copies in both cells and supernatant. Doxorubicin significantly increased viral copies in the cells ($P < 0.05$) and decreased them in the supernatant ($P < 0.001$). Valproic acid significantly increased OvHV-2 copies in the cells ($P < 0.05$) but not in the supernatant.

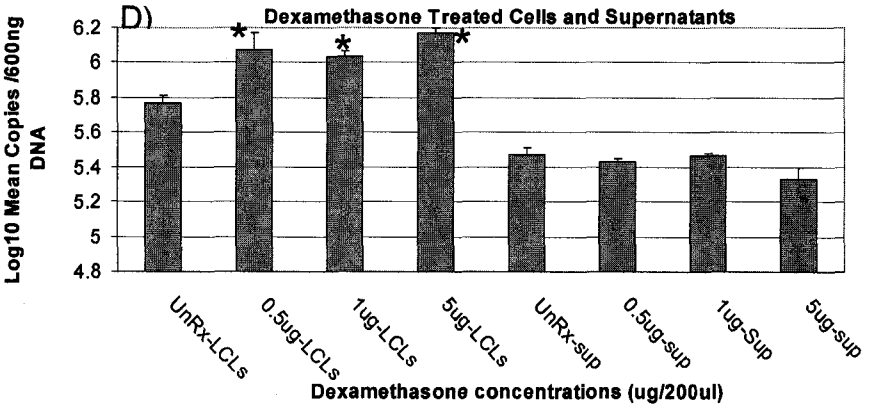
RESULTS:
CHEMICALLY TREATED MD00 CELLS AND SUPERNATANT

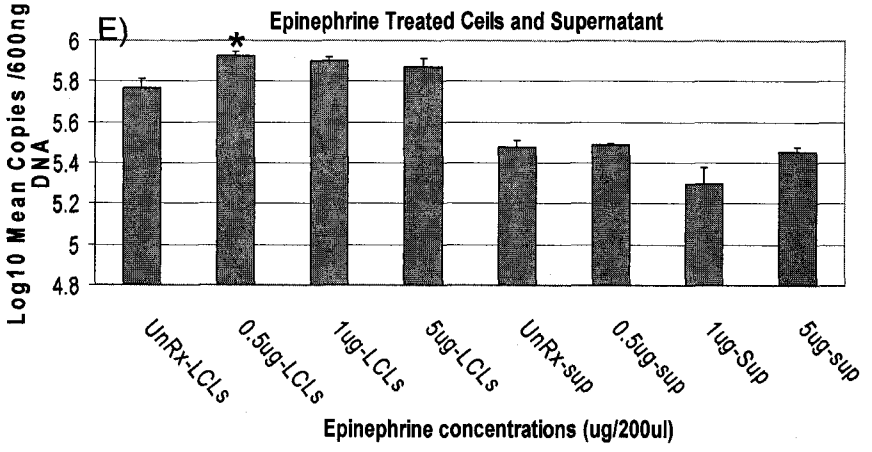


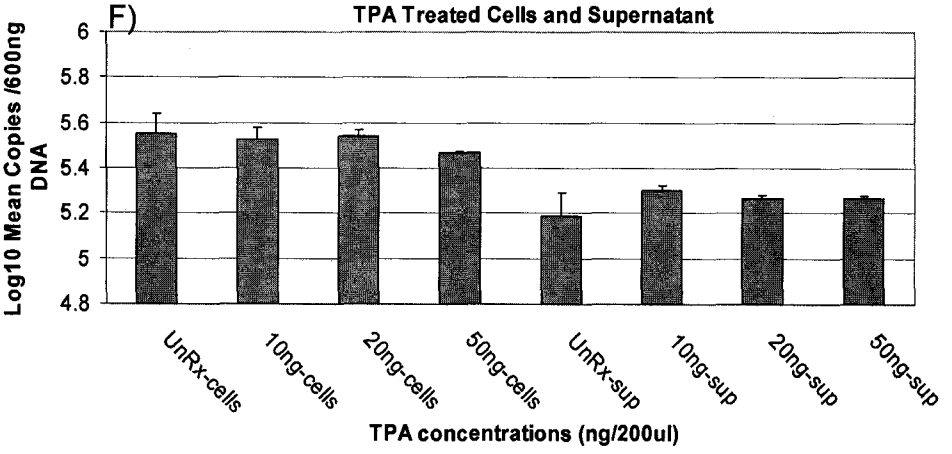




CHEMICALLY TREATED MD00 CELLS AND SUPERNATANT







CHEMICALLY TREATED MD00 CELLS AND SUPERNATANT

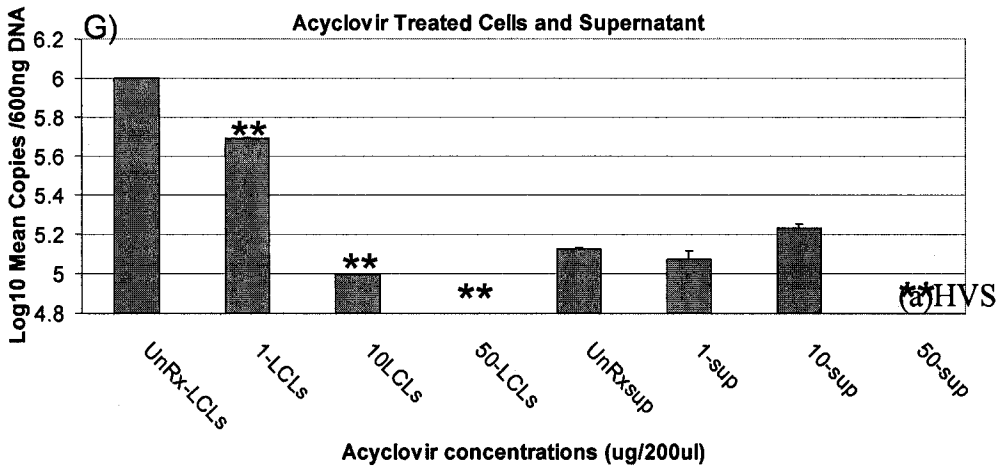


Fig 5.1 (A-G): Effects of chemical (Table 5.3) treatment on OvHV-2 copy numbers in MD00 cells and supernatant after 5 days of treatment. Varying concentrations of some of the chemicals, i.e. cAMP, dexamethasone, epinephrine and acyclovir, were examined (C-G). *P < 0.05; **P < 0.001. AZA, butyrate, and TPA had no effect on OvHV-2 DNA copy numbers in either cells or supernatant. Cisplatinum, doxorubicin and 5-fluorouracil did not affect intracellular copy numbers but significantly increased viral DNA copies in the supernatant. Dexamethasone and epinephrine significantly increased intracellular viral DNA copies without affecting copies in the supernatant. Valproic acid and cAMP significantly decreased intracellular viral DNA copies but significantly increased DNA copy numbers in the supernatant. Acyclovir significantly decreased OvHV-2 copies in both the cells and supernatant.

CHEMICALLY TREATED TT05 CELLS AND SUPERNATANT

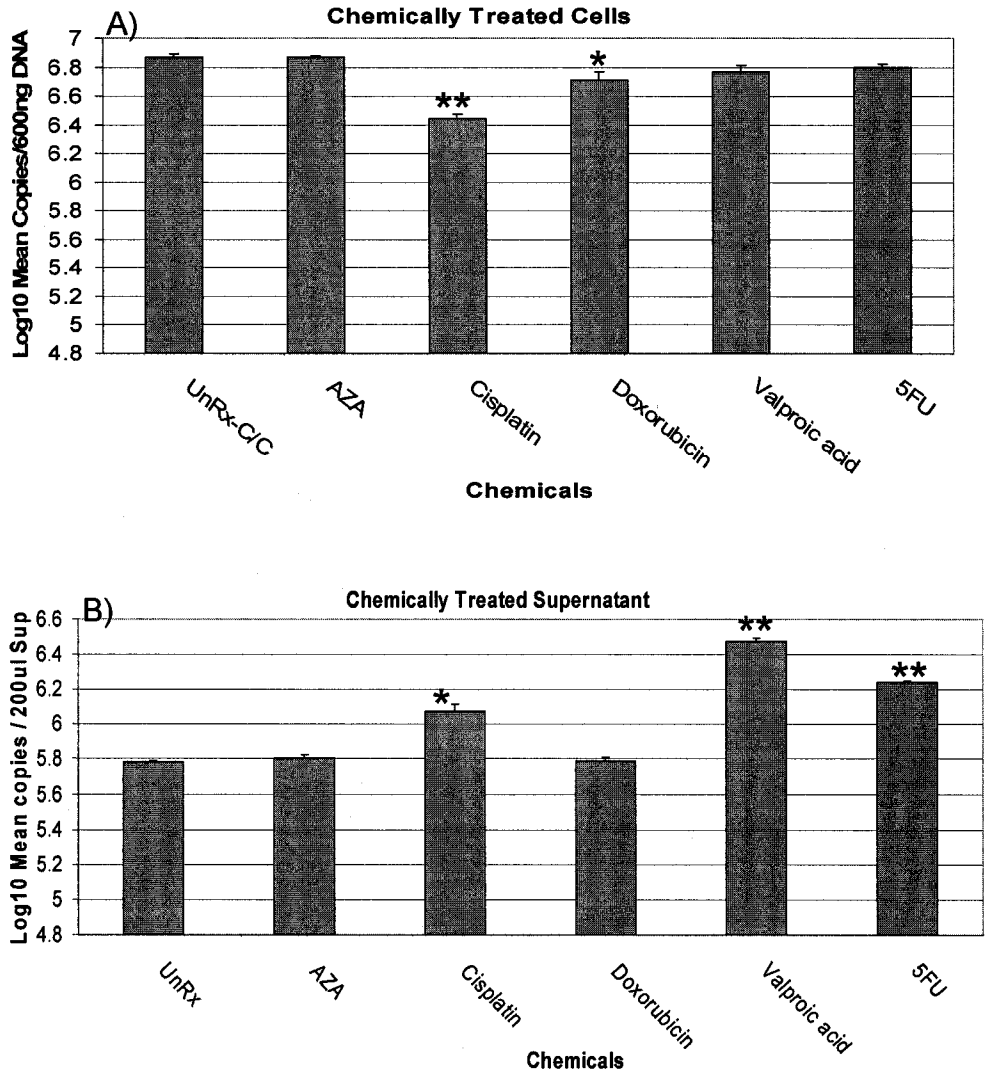


Fig 5.2 (A-B): Effects of chemical (Table 5.3) treatment on OvHV-2 copy numbers in TT05 cells (A) and supernatant (B) after 5 days of treatment. *P < 0.05; **P < 0.001 AZA did not affect OvHV-2 copy numbers in either the cells or supernatant. Valproic acid and 5FU had no effect on intracellular viral DNA copies but significantly increased copies within the supernatant. Doxorubicin significantly decreased intracellular viral DNA copies without affecting copy numbers in the supernatant. Cisplatinum significantly decreased intracellular viral DNA copies but did not affect viral copies in the supernatant.

CHEMICALLY TREATED TT08 CELLS AND SUPERNATANT

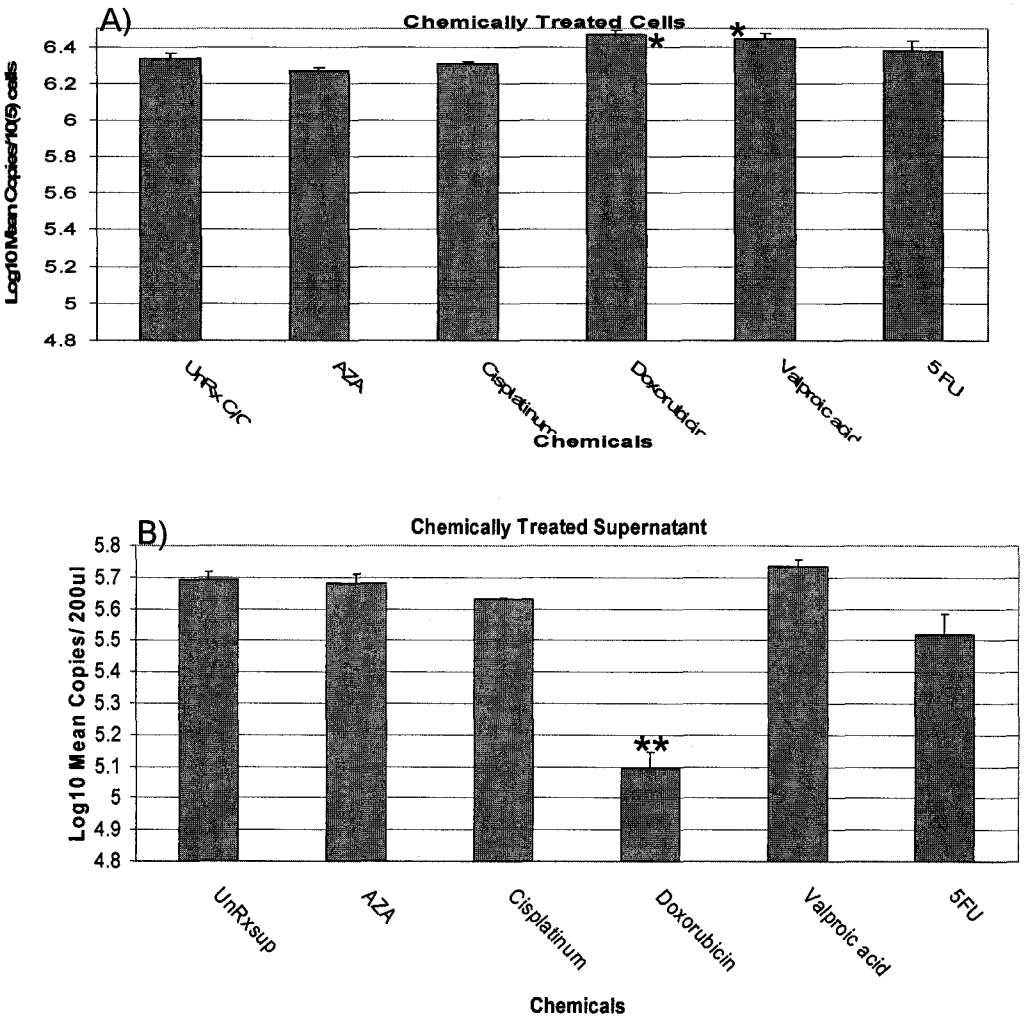


Fig 5.3 (A-B): Effects of chemical (Table 5.3) treatments on OvHV-2 copy numbers in TT08 cells (A) and supernatant (B) after 5 days of treatment. *P < 0.05; **P < 0.001 AZA, cisplatinum and 5FU had no effect on OvHV-2 DNA copy numbers in both the cells and supernatant. Valproic acid significantly increased intracellular copies but did not affect copy numbers in the supernatant. Doxorubicin significantly increased intracellular copies but significantly decreased copies in the supernatant.

TABLE 5.4: Summary of categories of effects of treatments on LCLs:

	Treated cell lines and chemicals / drugs inducing the effects		
Different effects of treatment	**MD00**	**TT05**	**TT08**
1)No effect on viral copy numbers in both cells and supernatant	AZA, Butyrate, TPA, Epinephrine (1 and 5ug)	AZA	AZA, cisplatinum, 5FU
2)No effect on intracellular copies but increased copies in the supernatant	Cisplatinum, Doxorubicin, 5FU	Valproic acid, 5FU	None
3) No effect on intracellular copy numbers but decreased copies in the supernatant	None	None	None
4)Increased viral copies in both cells and supernatant	None	None	None
5) Increased viral copies in the cells without affecting copies in the supernatant	Dexamethasone, Epinephrine (0.5ug)	None	Valproic acid
6) Increased intracellular copies but decreased copies in the supernatant	None	None	Doxorubicin
7)Decreased intracellular copies without affecting copies in the supernatant	Acyclovir	Doxorubicin	None
8)Decreased intracellular copies but increased copies in the supernatant	Valproic acid, cAMP	Cisplatinum	None
9) Decreased viral copies in both cells and supernatant	cAMP, acyclovir	None	None

DISCUSSION

Established OvHV-2 infected lymphoblastoid cell lines (LCLs) were treated with a variety of chemicals with the aim of inducing or inhibiting viral DNA replication. The effect of chemical treatment on OvHV-2 DNA replication was detected as a change in viral genomic equivalents (copy numbers) in cells or supernatant of treated versus untreated samples. MD00 LCL was treated with a majority of the chemicals. Two other

cell lines, TT05 and TT08, were additionally treated with some of the chemicals. The cell lines reacted differently to treatment with similar chemicals. This suggests that conclusions about the effect of chemical treatment on DNA replication of one OvHV-2 positive LCL cannot be extended to other LCLs without confirmation. EBV cell lines were also shown to react differently to similar chemical treatments (Feng *et al.*, 2002); Feng *et al.*, 2004). The chemical concentrations examined were similar to those used to induce DNA replication in other gammaherpesviruses. A variety of concentrations were examined for chemicals not reported in the gammaherpesvirus literature.

Some of the chemicals which have been reported to induce other gammaherpesviruses had no effect on OvHV-2 copies in both the cells and supernatant. This apparent lack of effect on viral DNA replication could be due to a variety of factors including:

1) The examined chemical concentration might not be effective on OvHV-2 LCLs, though proven effective on other gammaherpesvirus cell lines.

2) Treated cells might not be susceptible to the mechanism of action of the chemical. For example, 5-azacytidine (AZA), demethylates CpG dinucleotides within promoters allowing for gene expression and DNA replication to occur (Jones & Taylor, 1980). DNA methylation is involved in the control of gene expression (Christman *et al.*, 1977) and initiation of transcription and gene expression is often associated with demethylation in the vicinity of promoters (Chen *et al.*, 2001). AZA has been shown to induce lytic EBV replication in some (Ben-Sasson & Klein, 1981) but not all EBV positive lymphoblastoid cell lines (Feng *et al.*, 2004). Therefore, if the OvHV-2 genome is not methylated or if

gene expression is not controlled through methylation, AZA would not effectively induce viral gene expression or DNA replication.

3) The chemical could be cytotoxic or induce apoptosis within LCLs before a noticeable effect on viral DNA replication. Higher doses of sodium butyrate induced apoptosis in HHV-8 infected BCBL-1 cells prior to assembly and secretion of enveloped virions (Yu *et al.*, 1999)

4) Cells and supernatant might have been harvested before the chemical showed its effect on viral DNA replication. Induction of EBV gene expression was shown to occur as early as 6-8 hours after chemical treatment (Ben-Sasson & Klein, 1981). Cells and supernatants in our experiment were harvested on day 5 of treatment.

5) If the LCL examined is a non producer cell line or the virus is unable to replicate its DNA, chemical induction will induce early gene expression without progression to DNA replication and detectable change in viral genomic equivalents. EBV genomic rearrangements and deletions in Raji cells are thought to contribute to their inability to produce EBV spontaneously and upon induction (Polack *et al.*, 1984). Genomic rearrangements have been reported in AlHV-1 (Wright *et al.*, 2003) and other gammaherpesvirus cell lines in vitro (Kouzarides *et al.*, 1987). LCLs examined in this study have been continuously in culture for 1-6 years and OvHV-2 genomic alterations could have occurred *in vitro*.

Some of the chemicals did not affect OvHV-2 copy numbers in the cells, but significantly increased viral copies in the supernatant (Table 5.4). The mechanism involved in increased supernatant viral DNA is unclear. Chemicals that caused this effect are chemotherapeutic agents and will be toxic to host cells (Feng *et al.*, 2002; Feng *et al.*,

2004). Cell cytotoxicity would be followed by cell death and release of OvHV-2 DNA into the supernatant, hence the increase in viral DNA within the supernatant. Cell viability measurements and DNase protection assay could give an indication of the level of cytotoxicity and the source of viral signal in the supernatant, respectively. If the increase in viral DNA within the supernatant was due to an increase in viral replication, there would be an accompanying increase in intracellular viral copies. Chemotherapeutic agents like cisplatinum, 5FU and doxorubicin have been shown to increase early and late EBV antigens within cell lines (Feng *et al.*, 2002; Feng *et al.*, 2004). Therefore a true increase in OvHV-2 copies as a result of treatment with chemotherapeutic drugs cannot yet be discounted. These drugs were used in lytic induction therapy for EBV infected B-cell lymphomas. The therapy entails chemically inducing EBV lytic replication in latently infected tumor cells, followed by treatment with gancyclovir and chemotherapeutic agents. Induction of EBV and treatment with ganciclovir is said to increase susceptibility of tumor cells to chemotherapeutic agents (Feng *et al.*, 2002; Feng *et al.*, 2004). These chemotherapeutic agents could be applicable for *in vitro* induction of OvHV-2 replication in LCLs and help develop an *in vitro* productive model of the virus. None of the chemicals examined uniformly increased OvHV-2 copies in both the cells and supernatant (Table 5.4).

Some of the chemicals examined increased intracellular OvHV-2 copies without an accompanying increase in the supernatant (Table 4). This effect might be due to induction of early viral gene expression and DNA replication by the treatment chemicals, not necessarily followed by release of whole virions into the supernatant. At this point,

the definite cause of the increase is unknown and additional experiments are required to confirm or rule out different possibilities.

Dexamethasone was one of the drug treatments associated with an increase in intracellular viral DNA copies (Table 5.4). All doses examined increased intracellular OvHV-2 copies. Dexamethasone has also been reported to induce both early and lytic EBV antigens (Daibata *et al.*, 2005; Schuster *et al.*, 1991). Results of dexamethasone treatment are of particular interest as corticosteroids are sometimes included in supportive therapy of clinical MCF cases. Though corticosteroids may bring about immunosuppression, our results suggest they undesirably increase the intracellular OvHV-2 DNA load, possibly exacerbating clinical disease. There are conflicting reports about the effectiveness of corticosteroid treatment on clinical MCF. Our data suggests cautious use of corticosteroids during supportive therapy. However, if the lesions of MCF are primarily immunologically mediated (Liggitt *et al.*, 1978; Reid *et al.*, 1983), the beneficial effects of corticosteroids on lesion development may supercede the detrimental effects on viral replication.

Some chemical treatments decreased intracellular OvHV-2 copies, but significantly increased copies in the supernatant (Table 5.4). Any increase in total viral copies would have to be through an increase in the level of DNA replication. Since viral replication can only occur intra-cellularly, a true increase or trigger of viral DNA replication would be accompanied by an increase in intracellular viral copies. If the viral replication cycle is complete, there would also be an increase in release of whole virions into the supernatant. There would therefore be an increase in viral copies in both cells and supernatant. Our data supports possible toxicity of the drug to host cells, with cell

necrosis, followed by release of unprotected viral DNA into the supernatant. cAMP demonstrated its toxicity in the following manner; the lowest concentration had no effect on both intra and extra cellular viral copy numbers. A higher dose decreased the intracellular viral copies but increased copy numbers in the supernatant, whereas, the highest dose was toxic enough to decrease viral copies in both cell and supernatant. The above is more compatible with progressive toxicity on host cells as the dose increases. Further experiments are required to confirm or rule out the above, i.e. viability cell counts would indicate direct effects of the drugs or chemicals on cell viability. DNase treatment of the supernatant would indicate whether viral DNA within the supernatant is DNase protected or not. Effects of lower and non toxic concentrations of the drug should also be examined.

Some treatments, i.e. acyclovir, significantly decreased OvHV-2 copy numbers in both cells and supernatant (Table 5.4). Acyclovir [9-(2-Hydroxyethoxymethyl) guanine], a synthetic acyclic nucleoside, is a guanine derivative with an acyclic side chain, 2-hydroxyethoxymethyl at position 9. Acyclovir selectively inhibits alpha herpesviral DNA replication (Elion, 1993). This selective antiviral action is due to acyclovir's selective phosphorylation by the herpesviral thymidine kinase, to mono, di and triphosphates. Phosphorylated acyclovir inhibits viral DNA polymerase 10-30 times more than cellular DNA polymerase. Viral DNA replication is also inhibited through chain termination due to incorporation of the phosphorylated acyclovir (acycloguanosine) into the viral DNA (Allaudeen *et al.*, 1982; Elion, 1993). Though highly selective, in vitro inhibition of host DNA polymerases by acyclovir has also been reported. In our study, all doses (1, 10 and 50ug / 200ul) of acyclovir examined significantly decreased intracellular OvHV-2 copy

numbers. This effect may be through both inhibition of viral DNA replication and toxicity to the cells. At this point, the exact mechanism of action of acyclovir on OvHV-2 DNA replication is unknown. The two lower doses (1 and 10ug / 200ul) significantly decreased intracellular viral copies but did not affect copy numbers in the supernatant. If cytotoxicity alone played a large role in the observed decrease, there should have been a corresponding increase in viral DNA within the supernatant. Based on its mechanism of action, acyclovir treatment would have an effect on intracellular, but not cell free virus. The highest dose examined resulted in a decrease in viral copy numbers within both the cells and supernatant. The dose might have been high enough to decrease intracellular viral copies and subsequently affect release of virus into the supernatant. Acyclovir has also been reported to inhibit Epstein-Barr virus (EBV) replication. It significantly reduced EBV copy numbers in the EBV-producer cell line, P3HR-1, but did not affect the number of latent genomes within Raji cells, an EBV non producer cell line. The few genomes remaining after acyclovir treatment of P3HR-1 cells were shown to be covalently closed circular or latent EBV genomes. Acyclovir is therefore effective on productive but not latent viral DNA. This lack of effect is thought to be due to the control of latent DNA genomes by cellular control mechanisms. Acyclovir was also shown to affect viral DNA replication and not early antigen synthesis (Colby *et al.*, 1980). Inhibition of EBV DNA polymerase was not the primary mechanism responsible for acyclovir's inhibition of EBV DNA replication (Allaudeen *et al.*, 1982). The mechanism of action involved in OvHV-2 inhibition by acyclovir is unknown. It would be interesting to examine the effects of acyclovir on the other OvHV-2 positive cell lines.

Our data hints to the effects of certain chemicals on OvHV-2 DNA replication. It can therefore be used as a baseline and improved upon in the process of developing a productive *in vitro* system for OvHV-2 replication. Further experiments are required to confirm the reported effects of the chemical treatment on the cell lines. Additional studies could expand our understanding on three different levels, namely, DNA, RNA, and protein levels. The DNA level would confirm effects of treatment on DNA replication. Examined RNA would indicate the effects on transcription and gene expression, whereas, viral protein production would confirm presence of whole virions after treatment. The additional experiments that can be performed include but are not limited to the following:

1) Viability cell counts during chemical treatment to determine the effects of treatment on cell viability.
2) On the DNA level, Gardella gel analysis of treated and untreated cells to show a change or shift in latent (circular) to productive (linear) viral genome after chemical treatment.
3) On the RNA level, reverse transcription or northern blot of total RNA to indicate a change in the level of identified early or late viral message. Techniques such as microarrays which are expensive and not yet available or specifically customized for OvHV-2 research, would give an idea of which viral genes are affected by chemical treatment.
4) Flow-cytometry or immuno-fluorescent assays can confirm the presence, absence, or increase of identified viral proteins before and after chemical treatment.
5) DNase protection assays and electron microscopy could confirm presence or absence of whole virions before and after chemical treatment.

In conclusion, the suggested effects of chemical treatments on the three cell lines need to be followed up with additional experiments. Most interestingly were the effects of dexamethasone and acyclovir treatments on viral DNA copy numbers. Both of these drugs may have implications on the treatment of clinical MCF. Treatment with dexamethasone may be contraindicated as our data suggests a corresponding increase in intracellular viral burden. Acyclovir may inhibit viral replication and lower the overall viral burden during clinical disease. However, safe and effective doses would have to be optimized in both cell culture and animal models before use in clinical MCF cases.

CHAPTER 6

SUMMARY AND CONCLUSIONS

The studies reported in this dissertation addressed questions about the role of the OvHV-2 tropism and load in the clinical status of infection within asymptomatic sheep, subclinically infected cattle, clinically affected cattle, and recovered cattle. There was no difference in OvHV-2 tropism amongst the 4 groups. OvHV-2 preferentially infected T-cells over B-cells and monocytes in all the groups. OvHV-2 DNA replication and an increase in viral load may result in progression of infection to clinical disease, whereas, latency or a tight regulation on viral DNA replication and load may result in a subclinical infection. Results indicated that OvHV-2 infection did not affect PBMC subset percentages in any of the 4 groups.

The ability to establish a T-lymphoblastoid cell line from PBMCs of a recovered case of MCF suggests a persistently latent OvHV-2 infection after recovery and T-cells as the site of latency. A relatively lower OvHV-2 load in LCLs from the recovered case compared to those from fatal cases of MCF, is consistent with the ability of the host to control and reduce the OvHV-2 load during a clinical episode possibly leading to recovery. Established OvHV-2 positive lymphoblastoid cell lines from recovered and fatal cases of MCF were shown to transcribe message for late structural protein and release DNase protected viral genome into the supernatant.

Supportive treatment of clinical MCF with dexamethasone may be contra-indicated as it increased the intracellular viral burden, although there may be other reasons to use this drug in treatment of MCF cases. Acyclovir may be successfully used to inhibit OvHV-2 DNA replication during clinical MCF. However, determination of effective and optimal doses requires further investigation.

Results from this study demonstrate that OvHV-2 infection shares similarities with other gammaherpesvirus infections. Herpesvirus Saimiri (HVS) and Herpesvirus Ateles (HVA), also gammaherpesviruses, result in asymptomatic infection in their natural hosts, namely, squirrel and spider monkeys, respectively, but will cause lymphoproliferative disease in susceptible hosts like tamarins, marmosets and owl monkeys. OvHV-2 results in an asymptomatic infection in sheep, considered that natural host, but will cause lymphoproliferative disease in susceptible ruminants. Factors associated with asymptomatic infection in the natural host versus clinical disease in other hosts are unknown. Viral tropism studies demonstrate that OvHV-2 preferentially infects T-lymphocytes in the different hosts, allowing for establishment of T-lymphoblastoid cell lines from tissues and PBMCs of clinical cases. Following an acute episode of infectious mononucleosis (IM), EBV copy numbers in host PBMCs are severely reduced and the virus goes latent in B-cells. EBV infected B-cell lines can be established from infectious mononucleosis (IM) cases up to 20 years after acute IM. The severe reduction in copy numbers and latency with very limited, if any, viral gene expression are some of the ways EBV evades the host immune system. The ability to establish a persistently infected T-lymphoblastoid cell line from PBMCs of a recovered case of MCF suggests that OvHV-2 may also cause a lifelong and persistently latent infection in recovered animals and that

T-cells act as a site of latency. Interestingly, subclinically infected cattle were positive for OvHV-2 by ELISA but viral copies remained undetectable by qPCR. The data suggests a latent and tightly regulated OvHV-2 infection with a severe reduction in the viral load exist within subclinically infected animals. This may also be a mechanism employed by OvHV-2 to avoid the host immune system. Clearance of viral infection remains a possibility in subclinically infected cattle. This however, would not be consistent with gammaherpesvirus infections and pathogenesis. Gammaherpesviruses result in a life long, persistently latent infection within host lymphocytes after infection. At this point it is unknown if LCLs can be successfully established from PBMCs of subclinically infected cattle. Lymphoblastoid cell lines from other gammaherpesviruses, i.e. EBV, were predominantly latently infected and were either spontaneous producers or non producers of virus. Results from this study demonstrate that OvHV-2 positive cell lines are also predominantly latently infected with OvHV-2. Detection of messenger RNA to a late structural gene and presence of DNase protected viral DNA in the supernatant are suggestive of a spontaneous induction in the different OvHV-2 positive LCLs.

Data presented in this dissertation addressed some important questions about the MCF pathogenesis within subclinically infected, clinically affected and recovered cattle. However, there are still some unanswered questions about differences in the OvHV-2 infection within the 4 groups of animals. For instance, the role of the host immune system and viral gene expression in clinical status needs further investigation. It is not known whether progression from a subclinical infection to clinical disease occurs or whether recrudescence occurs in recovered cases. Factors associated with possible disease progression or recrudescence also need further investigation. Establishment of an OvHV-

2 in vitro culture system and animal models for experimental infection and disease would be a significant development in the study of the MCF pathogenesis. Chemicals that may affect OvHV-2 DNA replication and possibly lead to development of an in vitro culture system need further investigation.

APPENDIX:

Table A-1: Animal Identification: Asymptomatic Sheep

Asymptomatic Sheep		
Animals	**ID**	**Date Examined**
1	2988	10-8-04
2	510	10-11-04
3	Suffolf (No Tag)	10-13-04
4	Red # 43	10-18-04
5	Brown Navajo / Jacobs	10-27-04

Table A-2: Animal Identification: Subclinically Infected Cattle

Subclinically Infected Cattle		
Animals	**Hirsch ID**	**Date Rxamined**
1	553	5-7-04
2	712	5-14-04
3	1536	4-18-05
4	1718	4-19-05
5	1457	4-20-05

Table A-3: Animal Identification: Clinically Affected Cattle

Clinical cases / Clinically Affected Catlle			
Animals	**Hirsch ID**	**VTH ID**	**Date Examined**
1	2762	815786	10-03-03
2	2735	815904	10-15-03
3	2822	816599	02-05-04
4	1304	817527	5-26-4
5	1438	819164	1-10-05
6	2047	819335	1-31-05
7	2809	819710	3-31-05
8	1247	819736	4-4-05

Table A-4: Necropsy Findings in the Clinically Affected Cattle

Animals	VTH ID	Necropsy Findings
1	815786	Eyes – bilateral corneal opacity Oral mucosa – 1 to 3mm sharply dermacated mucosal ulcerations Bladder – multiple, focal, 1 to 2 mm dark red circular areas withi the mucosa
2	815904	Recovered from clinical disease
3	816599	Head – bilateral corneal opacity Gastrointestinal tract – oral ulcerations Bladder – hematuria, multifocal haemorrhages Lymph nodes – generalized lymphadenopathy
4	817527	Head – bilateral corneal opacity, right corneal ulceration. Gastrointestinal tract – ulcerations in the rumen, reticulum, omasum, abomasums and interstines. Bladder – ulcerations. Lymph nodes – lymphoid hyperplasia
5	819164	Recovered from clinical disease
6	819335	Head – bilateral corneal opacity and oculonasal discharge. Tracheal mucosa covered with thick yellow mucinous exudate, ulcerative lesions. Lymph nodes – lymphoid hyperplasia
7	819710	Head – bilateral corneal opacity, severe scleral injection. Gastrointestinal tract – ulcerative lesions, diarrhea Bladder – multifocal ulcerations, haemorrhagic cystitis
8	819736	Recovered from clinical disease

Table A-5: Animal Identification: Recovered Cattle

Recovered Cattle			
Animal	**Hirsch ID**	**VTH ID**	**Date Examined**
	2735		4-7-05
	1438		4-8-05
	1247		5-26-05

Table A-6: Percentage purity of immuno-magnetically sorted PBMC subsets: Asymptomatically Infected Sheep

	Examined sheep				
Subsets	**1**	**2**	**3**	**4**	**5**
CD2	95%	94%	96%	97.2%	98%
CD4	95%	95%	96%	96%	97%
CD8	93%	90%	92%	96.5%	97%
Yd	95%	97%	97.4%	96%	94%
B-cells	90%	99%	99%	97.5%	98.5%
Mon	94%	92%	92%	95%	94%

Table A-7: Percentage purity of immuno-magnetically sorted PBMC subsets: Subclinically Infected Cattle

	Examined Subclinically Infected Cattle				
Subsets	**1**	**2**	**3**	**4**	**5**
CD2	97%	99%	99%	97%	96%
CD3	98%	99%	98%	98%	97%
CD4	98%	97%	97%	97%	94%
CD8	97%	98%	94%	95%	93%
Gamma-delta	93%	94%	92%	95%	93%
B-cells	91%	99.7%	99%	91%	98%
Monocytes	99%	91%	94%	99%	91%

Table A-8: Percentage purity of immuno-magnetically sorted PBMC subsets: Clinically Affected Cattle

	Examined Clinically Affected Cattle							
Subsets	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
CD2	97%	98%	98%	99%	99%	98%	95%	98%
CD3	98%	99%	99%	99%	98%	97%	92%	99%
CD4	96%	97%	97%	99%	98%	98%	95%	99%
CD8	95%	97%	94%	98%	96%	97%	92%	97%
Gamma-delta	94%	93%	93%	96%	92%	97%	94%	94%
B-cells	99%	99%	98%	99%	99%	98%	99%	99%
Monocytes	91%	94%	96%	99%	90%	95%	97%	93%

Table A-9: Percentage purity of immuno-magnetically sorted PBMC subsets: Recovered Cattle

	Examined Recovered Cattle		
Subsets	**1**	**2**	**3**
CD2	99%	99%	97%
CD3	99%	98%	99%
CD4	98%	98%	99%
CD8	99%	97%	98%
Gamma-delta	97%	95%	97%
B-cells	99%	99%	99%
Monocytes	92%	90%	91%

Table A-10: Obtained OvHV-2 DNA copies in Total PBMCs and PBMC subsets: Asymptomatic Sheep

	Obtained Mean OvHV-2 Copies / 10(5) cells					Mean OvHV-2 copies / 10(5) cells
Subsets	**1**	**2**	**3**	**4**	**5**	
PBMCs	-	2 x 10(3)	-	40	2.7x10(3)	948
CD2	46	8.9 x 10(3)	74	953	1x10(4)	3994.6
CD4	-	9.1 x 10(3)	-	388	8.4x10(3)	3578
CD8	118	6.6 x 10(3)	90	3.7x10(3)	2.5x10(3)	3577.6
Yd	-	-	-	-	118	2601.6
B-cells	-	-	-	-	-	23.6
Mon	-	-	-	-	-	0

Table A-11: Obtained OvHV-2 DNA copies in Total PBMCs and PBMC subsets: Subclinically Infected Cattle

	Obtained Mean OvHV-2 Copies / 10(5) cells					Mean OvHV-2 copies / 10(5) cells
Subsets	**1**	**2**	**3**	**4**	**5**	
PBMCs	-	-	-	-	-	-
CD2	-	-	-	-	-	-
CD3	-	-	-	-	-	-
CD4	-	-	-	-	-	-
CD8	-	-	-	-	-	-
Gamma delta	-	-	-	-	-	-
B-cells	-	-	-	-	-	-
Monocytes	-	-	-	-	-	-
PBMCs	-	-	-	-	-	-

Table A-12: Obtained OvHV-2 DNA copies in Total PBMCs and PBMC subsets: Clinically Affected Cattle

	Obtained Mean OvHV-2 Copies / 10(5) cells								Mean OvHV-2 copies / 10(5) cells
Subsets	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	
PBMCs	3131	9	1.5x10(4)	5.2x10(3)	1.3x10(4)	949	2.5X10(4)	4.3x10(3)	8324
CD2	4200	863	1.1x10(4)	9.3x10(3)	6.4x10(3)	55	9.5X10(3)	2.3X10(3)	5452
CD3	2084	63	521	1.4x10(3)	5.4x10(3)	76	9.3X10(3)	1.6X10(3)	2556
CD4	5040	103	1.2x10(4)	3.6x10(3)	1.2x10(3)	5	44	102	2764
CD8	889	295	1.4x10(3)	5.4x10(3)	2x10(4)	89	2X10(3)	7.2X10(3)	4659
Gamma delta	241	162	251	492	966	25	47	49	279
B-cells	23	80	88	39	-	7	185	-	53
Mon	128	40	155	550	455	-	25	280	204

Table A-13: Obtained OvHV-2 DNA copies in Total PBMCs and PBMC subsets: Recovered Cattle

	Obtained Mean OvHV-2 Copies / 10(5) cells			**Mean OvHV-2 copies / 10(5) cells**
Subsets	**1**	**2**	**3**	
PBMCs	-	377	723	366.6667
CD2	-	371	159	176.6667
CD3	-	191	382	191
CD4	-	100	-	33.33333
CD8	-	312	1.3x10(3)	537.3333
Gamma delta	-	38	100	46
B-cells	-	-	-	0
Monocytes	-	30	-	10

Table A-14: Statistical Analysis of OvHV-2 Load and Tropism between the 4 groups of Animals (P-values)

	P-values (S-Significant OR N-Not Significant)					
Subsets	**Sheep vs Clinical**	**Sheep vs Recovered**	**Sheep vs Subclinical**	**Clinical vs Recovered**	**Clinical vs Subclinical**	**Recovered vs Subclinical**
PBMCs	0.03(S)	1(N)	0.2 (N)	0.05(S)	0.001(S)	0.1 (N)
CD2	0.5 (N)	0.4 (N)	0.008 (S)	0.05(S)	0.002 (S)	0.1 (N)
CD3	-	-	-	0.1 (N)	0.002 (S)	0.1 (N)
CD4	0.8 (N)	0.3 (N)	0.2 (N)	0.04 (S)	0.002 (S)	0.4 (N)
CD8	0.8 (N)	0.4 (N)	0.008 (S)	0.2 (N)	0.002 (S)	0.1 (N)
Gamma-delta	0.009 (S)	0.5 (N)	1(N)	0.1 (N)	0.002 (S)	0.1 (N)
B-cells	0.3 (N)	1(N)	1(N)	0.1(N)	0.03 (S)	1 (N)
Monocytes	0.008 (S)	0.4 (N)	1(N)	0.08 (N)	0.008 (S)	0.4 (N)

Table A-15: Statistical Analysis of OvHV-2 Tropism in Each Group: Asymptomatic Sheep (Preferentially Infected Subsets)

Compared Subsets	**P-values**	**Significant (S) OR NoT (N)**
CD2vs CD4	0.3	**N**
CD2vs CD8	1	**N**
CD2vs gamma-delta	0.009	**S**
CD2vs B-cells	0.003	**S**
CD2vs Monocytes	0.003	**S**
CD4vs CD8	0.3	**N**
CD4vs gamma-delta	0.2	**N**
CD4vs B-cells	0.09	**N**
CD4vs Monocytes	0.09	**N**

CD8vs Gamma-delta	0.008	S
CD8vs B-cells	0.003	S
CD8vs Monocytes	0.003	S
Gamma delta vs B-cells	0.8	N
Gamma delta vs Monocytes	0.8	N
Monocytes vs B-cells	1	N

Table A-16: Statistical Analysis of OvHV-2 Tropism in Each Group: Asymptomatic Sheep: Clinically Affected Cattle (Preferentially Infected Subsets)

Compared Subsets	P-values	Significant (S) OR NoT (N)
CD2 vs CD3	0.1	N
CD2vs CD4	0.07	N
CD2vs CD8	0.8	N
CD2vs gamma-delta	0.003	S
CD2vs B-cells	0.00003	S
CD2vs Monnocytes	0.00003	S
CD3 vs CD4	0.8	N
CD3 vs CD8	0.3	N
CD3 vs Gamma-delta	0.2	N
CD3 vs B-cells	0.01	S
CD3 vs Monocytes	0.02	S
CD4vs CD8	0.2	N
CD4vs gamma-delta	0.3	N
CD4vs B-cells	0.03	S
CD4vs Monocytes	0.04	S
CD8vs Gamma-delta	0.01	S
CD8vs B-cells	0.00007	S
CD8vs Monocytes	0.0002	S
Gamma delta vs B-cells	0.3	N
Gamma delta vs Monocytes	0.3	N
Monocytes vs B-cells	0.95	N

Table A-17: Statistical Analysis of OvHV-2 Tropism in Each Group: Asymptomatic Sheep: Recovered Cattle (Preferentially Infected Subsets)

Compared Subsets	P-values	Significant (S) OR NoT (N)
CD2vs CD3	0.9	N
CD2vs CD4	0.2	N
CD2vs CD8	0.9	N
CD2vs gamma-delta	0.3	N
CD2vs B-cells	0.04	S
CD2vs Monnocytes	0.07	N
CD3 vs CD4	0.3	N
CD3 vs CD8	0.7	N
CD3 vs Gamma-delta	0.4	N
CD3 vs B-cells	0.07	N
CD3 vs Monocytes	0.1	N
CD4vs CD8	0.1	N
CD4vs gamma-delta	0.9	N
CD4vs B-cells	0.6	N
CD4vs Monocytes	0.7	N
CD8vs Gamma-delta	0.2	N
CD8vs B-cells	0.01	S
CD8vs Monocytes	0.03	S
Gamma delta vs B-cells	0.4	N
Gamma-delta vs Monocytes	0.6	N
B-cells vs Monocytes	0.9	N

Table A-18: Means and Ranges of Obtained Cattle PBMC subsets percentages

Subsets	Non infected Cattle	Clinically Affected Cattle	Recovered Cattle	Subclinically Infected Cattle
CD2	35(31-40)	29 (19-48)	41(35-50)	34(15-49)
CD3	31(26-37)	28 (12-41)	42(37-46)	34(15-51)
CD4	17(13-20)	16 (9-28)	20(14-25)	18(9-25)
CD8	11(10-13)	9 (4-20)	15(12-20)	12(6-21)
γδ	20(10-43)	15 (8-23)	12(8-16)	11(4-16)
B-cells	40(31-50)	24 (16-34)	39(34-42)	48(23-65)
Monocytes/ macrophages	4.5(2-11)	14 (8-21)	9(7-13)	6(4-9)

Table A-19: Comparism of Obtained and Reported PBMC subset Percentage Means and Ranges

PBMC Subsets	Non infected Cattle	Subclinically Infected Cattle	Clinically Affected Cattle	Recovered Cattle	Reported Means and Ranges
CD2	35(31-40)	34(15-49)	29 (19-48)	41(35-50)	46%
CD3	31(26-37)	34(15-51)	28 (12-41)	42(37-46)	12-63%
CD4	17(13-20)	18(9-25)	16 (9-28)	20(14-25)	12-30%
CD8	11(10-13)	12(6-21)	9 (4-20)	15(12-20)	8-17%
γδ	20(10-43)	11(4-16)	15 (8-23)	12(8-16)	15-50%
B-cells	40(31-50)	48(23-65)	24 (16-34)	39(34-42)	6-32%
Monocytes/ macrophages	4.5(2-11)	6(4-9)	14 (8-21)	9(7-13)	5-20%

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