LARGE GRANULAR LYMPHOCYTE DYSREGULATION IN MALIGNANT CATARRHAL FEVER

by

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DECLARATION

The work presented in this thesis was carried out at the Moredun Research Institute, Edinburgh. The experimental work and the interpretation of the results were carried out by the author. Contributions to the work in this thesis by colleagues are fully acknowledged in the text.

This work has not been nor is currently being submitted for candidature for any other degree.

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ABBREVIATIONS

APC   antigen presenting cell

BCR   B cell receptor

BSA   bovine serum albumin

CI-ELISA   competitive inhibition enzyme-linked immunosorbant assay

CNS   central nervous system

CPE   cytopathic effect

CsA   cyclosporin A

CTL   cytotoxic T lymphocyte

DNA   deoxyribonuleic acid

FACS   fluorescence-activated cell sorting

FITC   fluorescein isothiocyanate

GTP   guanidine triphosphate

IFAT   immunofluorescent antibody test

IL-2   interleukin-2

LAK cell   lymphokine activated killer cell

LGL   large granular lymphocyte

MHC   major histocompatability complex

NK cell   natural killer cell

ORF   open reading frame

PBS   phosphate buffered saline

RNA   ribonucleic acid

RT-PCR   reverse transcriptase polymerase chain reaction

SDS-PAGE   sodium dodecyl-sulphate polyacrylamide gel electrophoresis

TCR   T cell receptor

Tris   Tris(hydroxymethyl)aminomethane

ABSTRACT

Malignant catarrhal fever (MCF) is a fatal, incurable disease of large ruminants. This disease is caused by two related gammaherpesviruses, *Alcelaphine herpesvirus 1* (AlHV-1) and *Ovine herpesvirus 2* (OvHV-2). Lymphoproliferation and infiltration by large granular lymphocytes (LGL) into lymphoid and non-lymphoid organs are characteristic of MCF. LGL cell lines have been established from animals with naturally or experimentally induced MCF. Previous studies have shown these cell lines to be highly proliferative and cytotoxic with the ability to cause disease when inoculated into rabbits. Some cell lines have demonstrated the ability to grow and survive without the need for exogenous IL-2. However, IL-2 mRNA was not detected. The proliferative and cytotoxic LGL phenotype observed in the absence of exogenous IL-2 is associated with virus infection of LGL cells. The characteristics of MCF LGL cell lines are similar to other herpesvirus-infected cell lines, in particular *Herpesvirus saimiri* (HVS).

The role of virus-infected large granular lymphocytes in pathogenesis of MCF is not clear. In the absence of defined viral antigens the main objective of this study was to investigate how MCF viruses interfere with LGL proliferation and cytotoxicity.

In this study, IL-2 independence and a cell surface phenotype of T/ NK cells was demonstrated in most cell lines analysed. The period of survival and growth without exogenous IL-2 was variable but always exceeded that of uninfected control cells. The possible role of IL-15 (a pro-inflammatory cytokine with actions similar to IL-2) in LGL function was investigated. Results showed that IL-15 could generate and maintain the proliferative and cytotoxic phenotype of these cell lines and may be involved in the pathogenesis of MCF.

Virus interference with signal transduction pathways has been demonstrated in other gammaherpesviruses. The hypothesis that the phenotype and function of the LGL cell lines is generated through a similar mechanism was tested. The study showed that the *src* kinases, *lck* and *fyn,* are constitutively activated in LGL cell lines.

The identity of specific virus proteins involved in generating the observed phenotype of the LGL cell lines had not been determined. During the course of this

study, the complete genomic sequence of the AlHV-1 genome was published. Prior to this several open reading frames were detected in AlHV-1 that underwent genomic rearrangement on transition from virulence to attenuation *in vitro*. Thus, a final objective of this study was to determine whether these potential virulence genes are associated with the ability of LGL cell lines to transmit disease. The proteins encoded by these genes (ORF50, A6 and A7) were expressed in *E. coli* as recombinant proteins and used to immunise rabbits. Recombinant virus protein-specific rabbit polyclonal antibodies were generated. Using these reagents, the expression of these proteins in LGL cell lines and AlHV-1-infected monolayer cultures was investigated. The polymerase chain reaction (PCR) was used in parallel experiments to determine the presence of DNA and mRNA encoding these proteins. The results indicated that more complicated rearrangements of the AlHV-1 genome may occur on attenuation of AlHV-1 after extensive passage than has been revealed at present. However, the expression in LGL cells of mRNA encoded by ORF50 and A6, that share sequence homology with the EBV R and Z transactivators, suggests that virus replication occurs in LGL cells.

These results improve the understanding of the MCF viruses and their role in generation of the LGL phenotype.

Chapter 1

Introduction

1.1 Introduction

1.1.1 Malignant catarrhal fever (MCF)

Malignant catarrhal fever is a fatal, incurable disease of large ruminants. Disease-susceptible animals experience high fevers, profuse catarrh and widespread tissue necrosis leading to death (Reid and Buxton, 1989) (Fig 1.1). Proliferation of lymphocytes and infiltration by these cells into lymphoid and non-lymphoid tissues are characteristic of MCF. Whilst normally sporadic, this disease has been reported worldwide in Europe, America, Africa and Southeast Asia and has significant economic impact, particularly in Africa and Asia. Two related gammaherpesviruses *Alcelaphine herpesvirus-1* (AlHV-1) and *Ovine herpesvirus-2* (OvHV-2) are the cause of disease in susceptible animals and are transmitted from asymptomatic 'carrier species'. The carrier species for AlHV-1 are wildebeest (*Connochaetes spp.*) while domestic sheep are carriers for OvHV-2. However, the mechanisms of virus infection, interference with cell function and subsequent destruction of tissues in MCF-affected animals are not understood. Therefore, the study of these aspects of MCF virus infection are important to give us a greater understanding of MCF pathogenesis and the associated host immune response as well as a means to develop specific disease control strategies.

1.1.2 Features of herpesviruses

Herpesviruses have long presented a challenge to the wellbeing of man and animals alike. Some are the direct cause of, or are associated with disease while others merely infect the host without causing any apparent illness. There are more than a hundred different herpesvirus species (Fields *et al*, 1996). They all share common properties such as a double stranded DNA genome, structurally similar virus particles and the ability to persist (in a latent state) within host cells. Differences in genomic organisation, in life cycle and host range as well as the means by which they achieve latency have been used to classify the herpesviruses into three sub-families: the alphaherpesviruses, betaherpesviruses and gammaherpesviruses (Roizman *et al*, 1992).

Fig 1.1: A cow presenting with profuse catarrhal nasal discharge typical of MCF.

The alphaherpesviruses exhibit a variable host range, short life cycle and have the ability to spread rapidly in culture leading to the destruction of infected cells. These viruses establish latency in neuronal cells (sensory ganglia). By contrast, the betaherpesviruses have a long life cycle in a relatively restricted range of host species. Cell to cell spread of these viruses in culture is slow and latency can be maintained in lymphoreticular cells. Lastly, the gammaherpesviruses have a relatively limited host range. In culture, gammaherpesviruses replicate (non-productively) in lymphoblastoid cells though some can also undergo lytic replication in epithelial and fibroblast cells. These viruses are lymphotropic, infecting T or B lymphocytes in which latency can be established. Viruses belonging to the sub-families of herpesviruses are summarised in Table 1.1.

The work presented here will focus on the sub-family of gammaherpesviruses. More specifically, the two viruses that cause MCF: AlHV-1 and OvHV-2. The following literature review will introduce the disease and the viruses involved in more detail. The effect on host cells following viral infection will be discussed with comparisons made to other well-studied gammaherpesviruses.

Table 1.1: Viruses belonging to the family *Herpesviridae*

Subfamily	Viruses	Common name
Alphaherpesvirinae	Human herpesvirus 1	Herpes simplex virus 1 (HSV-1)
	Human herpesvirus 2	Herpes simplex virus 2 (HSV-2)
	Human herpesvirus 3	Varicella-zoster virus (VZV)
	Equine herpesvirus 1	Equine abortion virus
	Bovine herpesvirus 1	Infectious bovine rhinotracheitis virus
Betaherpesvirinae	Human herpesvirus 5	Human cytomegalovirus (HCMV)
	Human herpesvirus 6	
	Human herpesvirus 7	
	Murid herpesvirus 1	Murine cytomegalovirus (MCMV)
Gammaherpesvirinae	Human herpesvirus 4	Epstein-Barr virus (EBV)
	Human herpesvirus 8*	Kaposi's sarcoma-associated virus
	Saimirine herpesvirus 2	Herpesvirus saimiri (HVS)
	Alcelaphine herpesvirus 1	Wildebeest-associated MCF virus
	Ovine herpesvirus 2	Sheep-associated MCF virus
	Equine herpesvirus 2	

Table adapted from Roizman *et al*, 1992. *Schulz, 1998.

1.2 Malignant catarrhal fever (MCF)

1.2.1 Viruses associated with MCF

1.2.1.1 Alcelaphine herpesvirus-1 and -2 (AlHV-1 and -2)

The gammaherpesvirus first classified as *Bovine herpesvirus-3* (BHV-3) by the World Health Organisation (WHO/FAO, 1976) is now better known as *Alcelaphine herpesvirus-1* (AlHV-1) (Reid, 1974; Reid *et al*, 1975; Roizman 1982; Roizman *et al*, 1992) – named after the subfamily *Alcelaphinae* to which the carrier species (wildebeest) belong. The virus was first isolated by Plowright and co-workers (Plowright *et al*, 1960) from the blood of the blue wildebeest (*Connochaetes taurinus*) and later from nasal and ocular secretions of wildebeest calves (Mushi *et al*, 1980a,b).

Virus particles typical of herpesviruses can be detected by electron microscopy in infected cultures (Fig 1.2). These particles consist of an icosahedral nucleocapsid of 100nm with or without a viral envelope. When enveloped, the virions measure 140-220nm in diameter (Plowright *et al*, 1965). Furthermore, the virus was shown to develop in the host cell nucleus and to mature in the cytoplasm (some being found in cytoplasmic vesicles) - a pattern of development typical of herpesviruses (Castro and Daley, 1981). Other characteristics of AlHV-1, such as its ability to induce a cytopathic effect (CPE) in permissive cell cultures, size on filtration and sensitivity to ether and chloroform allow its classification as a herpesvirus (Plowright, 1968, Rweyemamu *et al*, 1974, Plowright *et al*, 1965).

In cattle, and on initial isolation in culture, infectivity is cell-associated but becomes cell-free after extensive passage in tissue culture (Plowright 1965a, Plowright *et al*, 1965, Reid and Rowe 1973, Harkness and Jessett 1981, Russell 1980). In addition, infectivity in rabbits (an experimental model for MCF) is lost after extended passage of the virus in tissue culture (Handley, 1993, Handley *et al* 1995). The two most commonly described isolates of AlHV-1 are called WC11 and C500. The cell-free and extensively passaged virus WC11 was first isolated from blue wildebeest (Plowright *et al*, 1960) and is attenuated in terms of its ability to cause disease in cattle and rabbits. The cell-associated and virulent virus C500 was

first isolated from infected cattle (Plowright *et al*, 1975) and also becomes cell-free and attenuated after extensive passage in bovine turbinate cells (Handley *et al*, 1995).

Virus isolation has traditionally involved the co-culture of cells (e.g. lymphocytes) or oculo-nasal secretions from infected animals with permissive cell monolayer cultures. These latter cell types include fibroblast or epithelial cells such as bovine thyroid cells, kidney cells (Reid and Rowe, 1973), bovine turbinate cells and corneal cells (Mushi *et al*, 1981b), bovine testes cells and adrenal cells as well as rabbit kidney cells (Plowright *et al*, 1960). The cytopathic effect (CPE) observed with cell-associated AlHV-1 is characteristically syncitial (i.e. involving the formation of large multinucleate cells) while cell-free virus produces rounded refractile foci of infected cells.

Genomic Organisation of AlHV-1:

The genomic organisation of AlHV-1 was originally studied by restriction enzyme analysis. The viral genome was shown to consist of a unique region of approximately 110-130kilobases (kb) flanked by tandem repeat sequences of 30kb in total (Bridgen, 1991; Bridgen *et al*, 1989; Ensser *et al*, 1997; Hsu *et al*, 1990a; Shih *et al*, 1988). This type of genomic organisation is reminiscent of other gammaherpesviruses, for example herpesvirus saimiri and herpesvirus ateles (reviewed in Roizman and Baines, 1991). Restriction fragment patterns also showed that Alcelaphine herpesviruses could be split into two groups, Alcelaphine herpesvirus-1 (AlHV-1) and Alcelaphine herpesvirus-2 (AlHV-2). The natural hosts of AlHV-2 are the hartebeest (*Alcelaphus buselaphus*) and topi (*Damaliscus lunatus*) (Heuschele and Fletcher 1984; Seal *et al*, 1989; Shih *et al*, 1989). However, there is no evidence for the occurrence of MCF due to natural transmission of AlHV-2 from host animals to susceptible species. Hybridisation assays using AlHV-1-specific DNA clones as probes suggested that some sequences are shared between AlHV-1 and bovine herpesvirus 4 (BHV-4), a common virus in cattle (Lahijani *et al*, 1995; Shih *et al* 1988). This result correlates with the observed cross-reactivity of sera from AlHV-1 infected animals with BHV-4 in immunofluorescent antibody tests (Rossiter *et al*, 1977, 1980).
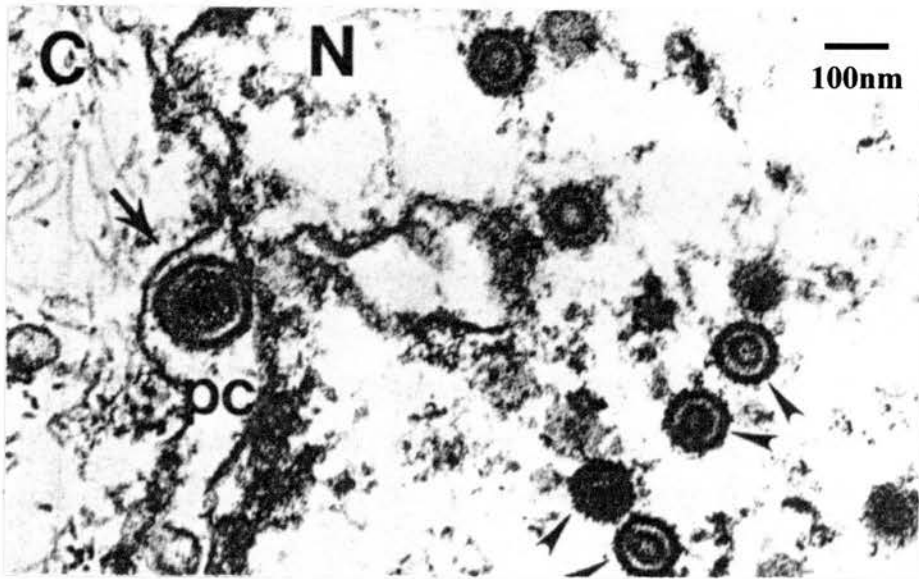
Fig 1.2: Electron micrograph of an AlHV-1-infected bovine embryo kidney cell culture showing the presence of herpesvirus virions (from Castro and Daley, 1982). **N** = nucleus; **C** = cytoplasm; **PC** = perinuclear cisternae; **arrow** = virus particle; **arrowheads** = immature virions.

ORFs specific to AlHV-1

ORFs specific to the γ subfamily

ORFs specific to the β + γ subfamily

ORFs specific to the α + γ subfamily

ORFs common to all herpesviruses

Fig 1.3: Organisation of the AlHV-1 C500 genome. (after Ensser *et al*, 1997).
Open reading frames (ORFs) have been coloured to reflect the conservation of genes between the α, β and γ subfamilies of the Herpesviridae as indicated above. ORFs are numbered according to findings of Ensser *et al* (1997). Arrows indicate direction of transcription. Scale in kilobase pairs. H-DNA represents the variable, non-coding region of the genome.

H-DNA

A1 A2 A3 A4 3 6 7 8 9 A5 10 11 17 18 19 20 21

0 10 20 30 40

22 23 24 25 26 27 29B 31 29A 35 37 39 41 46 A7 53
30 32 33 34 36 38 40 42 43 44 45 47 48 50 A6 A8 52

40 50 60 70 80

66 67A 69
54 55 56 57 58 59 60 61 62 63 64 65 67 68 73

80 90 100 110 120

73 75 A9 A10 H-DNA repeats

120 130 140 150 160

kbp

10
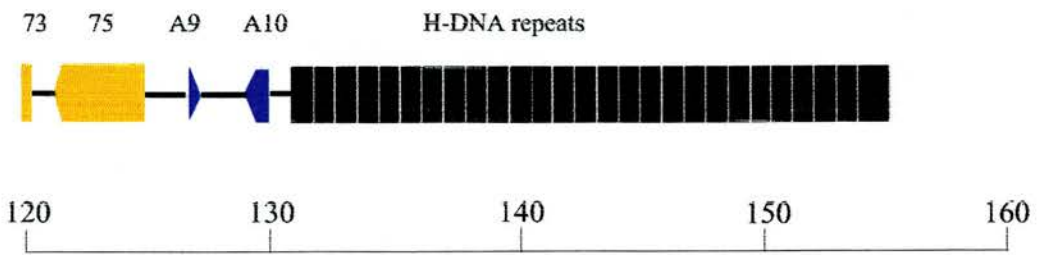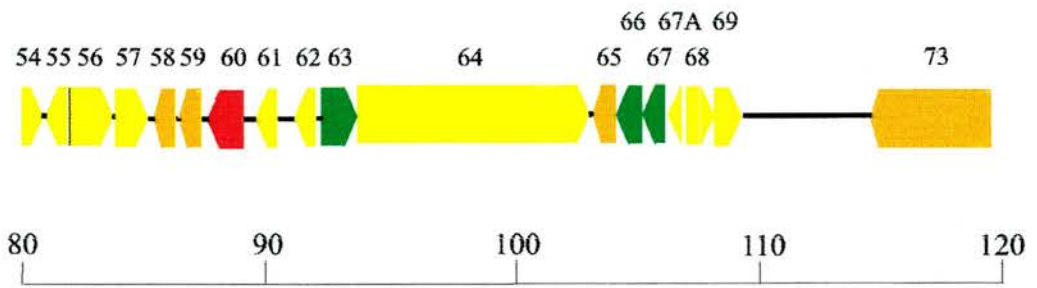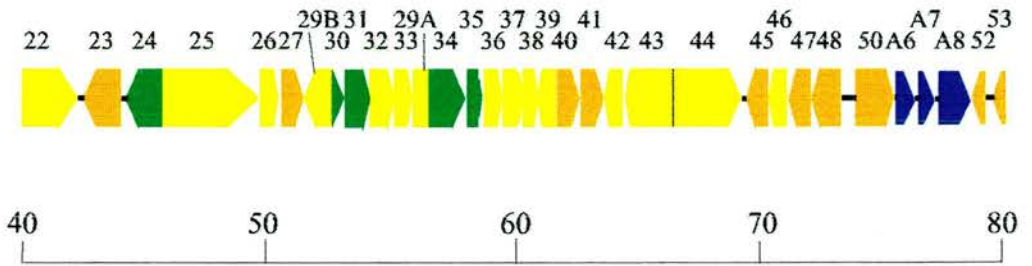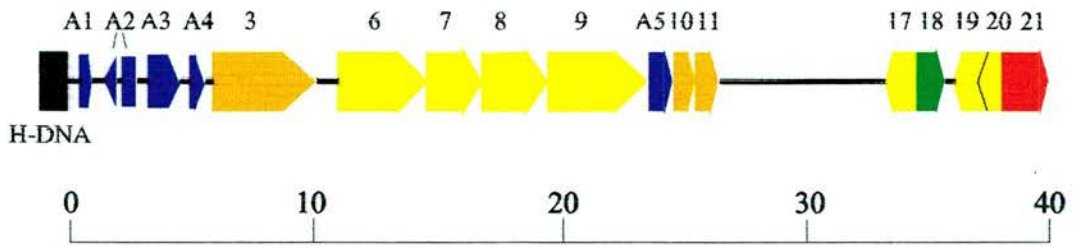
During the course of this project, Ensser *et al* (1997) published the whole genomic sequence for virulent AlHV-1 strain C500 (Fig 1.3). This work confirmed that AlHV-1 is a gammaherpesvirus based on genomic organisation. Open reading frames (ORFs) of interest with a potential immunomodulatory role were identified by comparison with DNA and amino acid sequences of known immunomodulatory proteins. ORF A3 was first identified by Ensser and Fleckenstein (1995) as a semaphorin homologue - a protein also expressed by poxviruses with a possible role in immunosuppression (Kolodkin *et al*, 1993). ORF A5 and A9 are homologous to seven transmembrane, G-protein-coupled receptors (GCRs) and Bcl-2 respectively. Both these proteins may allow virus evasion of the immune system and are discussed in more detail in Chapter 1.3. An earlier restriction enzyme analysis was also performed on the high passage attenuated C500 virus and compared to the low passage virulent C500 virus (Handley, 1993; Handley *et al*, 1995). This analysis revealed a rearrangement in the viral genome following attenuation. Several ORFs were identified of which two encoded proteins that were thought to be involved in the virulence to attenuation switch. These are now known to be proteins encoded by ORF 50 and ORF A7 of Ensser's (1997) sequence. These two ORFs have colinear homologues in equine herpesvirus-2 (EHV-2), herpesvirus saimiri (HVS), human herpesvirus-8 (HHV-8) and Epstein-Barr virus (EBV) (where they encode proteins involved in virus reactivation and entry, respectively). This will be discussed in more detail in Chapter 1.3.

1.2.1.2 Ovine herpesvirus 2 (OvHV - 2)

While OvHV-2 virions have not yet been isolated (due to the lack of a permissive system for virus growth), there is substantial evidence for its presence in the carrier species (sheep) as well as in MCF susceptible animals (Chapter 1.2.2.2). Propagation of lymphoblastoid cell lines derived from diseased animals has been achieved. Some of these cells can transmit the disease to deer and rabbits (Berrie *et al* 1984; Cook and Splitter 1988; Reid *et al*, 1985, 1986).

While the genomic sequence of OvHV-2 has only partially been determined, molecular studies of the lymphoblastoid cell lines have been informative. A genomic OvHV-2 DNA fragment has been identified with homology to an AlHV-1 WC11

DNA clone located at the left terminal region of the genome (Bridgen and Reid 1991). This fragment was cloned, sequenced and used to develop an OvHV-2-specific PCR to detect virus DNA in sheep, MCF-affected cattle and lymphoblastoid cell lines (Baxter *et al*, 1993; Li *et al*, 1995).

1.2.1.3 Involvement of other viruses in MCF

Serological studies have indicated the presence of antibodies specific for AlHV-1 in species of Bovidae other than wildebeest and sheep. These studies suggest that these species are infected with and are carriers for, the same or similar gammaherpesviruses (Table 1.2).

In vitro, viruses have been recovered from topi (*Damaliscus korrigum*) (Mushi *et al*, 1981a), red hartebeest (*Alcelaphus buselaphus*) (Reid and Rowe, 1973) - designated alcelaphine herpesvirus 2 (AlHV-2) - and the roan antelope (*Hippotragus equinus*) (Heuschele and Fletcher, 1984) - designated hippotragine herpesvirus 1 (HipHV-1). Experimental transmission of AlHV-2 from red hartebeest to cattle and rabbits and HipHV-1 from roan antelope to rabbits produced MCF-like syndromes (Reid and Bridgen, 1991; Reid and Rowe, 1973). Molecular studies of AlHV-2 and HipHV-1 have confirmed that these are related to but distinct from AlHV-1 (Reid and Bridgen, 1991; Seal *et al*, 1989; Shih *et al*, 1989). However, there is no epidemiological evidence indicating that these viruses naturally spread to susceptible animals and cause MCF.

Table 1.2: Carrier species in the family _Bovidae_ in which antibody reactive with AlHV-1 or virus similar to AlHV-1 has been isolated.

Subfamily	Species	Common name	Virus
Alcelaphinae	_Connochaetes spp_	Wildebeest	Alcelaphine herpesvirus 1 (AlHV-1)[1]
	Alcelaphus buselaphus	Red hartebeest	Alcelaphine herpesvirus 2 (AlHV-2)[2]
	Damaliscus korrigum	Topi	Alcelaphine herpesvirus 2 (AlHV-2)[3]
	D. dorcas phillipsi	Blesbok	
Hippotraginae	_Oryx spp_	Oryx	
	Hippotragus equinus	Roan antelope	Hippotragine herpesvirus 1 (HipHV-1)[2]
	H. niger	Sable antelope	
Caprinae	_Ovis spp_	Sheep	Ovine herpesvirus 2 (OvHV-2)[1]
	Capra spp	Goat	Ovine herpesvirus 2 (OvHV-2)?[4]
Ovibovidae	_Ovibos moschatus_	Musk ox	

[1]Viruses associated with natural and experimental MCF.

[2]Viruses associated with experimental MCF only. Natural transmission not observed.

[3]No evidence for involvement in natural or experimental MCF.

References for the above are cited in the text (Chapter 1.2.1.3).

[4]Detected by serology (Heuschele and Fletcher, 1984) and OHV-2-specific PCR (Wiyono _et al_, 1994). Virus not yet isolated.

1.2.2 Epidemiology

Malignant catarrhal fever was first reported in Europe by Goetz in 1930. At that time, the aetiology of the disease was obscure though the presence of sheep in the vicinity of susceptible ruminants was associated with the occurrence of disease. In South Africa a similar syndrome was described which could be transmitted from wildebeest (*Connochaetes gnu*) to susceptible animals (Mettam, 1923). MCF-susceptible animals include domestic cattle, water buffalo, Bali cattle, many species of deer and other exotic ruminant species in zoological collections (Table 1.2) (Reid and Buxton 1989). Identification of the 'carrier species' led to the naming of two forms of MCF: wildebeest-associated MCF (WA-MCF) and sheep-associated MCF (SA-MCF). The epidemiology of these two forms of MCF will be discussed in the following sections.

1.2.2.1 Wildebeest-associated MCF (WA-MCF) - also known as 'snot sickness' or 'snotsiekte'

Mettam (1923) showed that WA-MCF could be directly transmitted to cattle using blood from the black wildebeest (*Connochaetes gnou*). The blue wildebeest (*Connochaetes taurinus*) was also implicated as a carrier species for the causal agent of MCF (Daubney and Hudson, 1936). In 1960, the causal agent of WA-MCF was identified as a virus (Plowright *et al*, 1960). The virus, now referred to as *Alcelaphine herpesvirus-1* (AlHV-1), was characterised as a herpesvirus (Plowright *et al*, 1965) (see Chapter 1.2.1.1).

Infection of natural hosts / carrier species

In wildebeest (*C. taurinus*), the carrier species, infection with AlHV-1 is found throughout the population. Most wildebeest calves become infected during the first 3 months of life with 90% of calves between the ages of 4 to 12 months having antibody to AlHV-1 (Plowright, 1965a, 1965b, 1967). In adult wildebeest, neutralising antibody to AlHV-1 is present and very little virus can be detected (Plowright, 1967). The appearance of viraemia following pregnancy suggests that in adult wildebeest AlHV-1 is latent but can be reactivated. Stress, malnutrition and

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administration of corticosteroids to adult wildebeest can also lead to reactivation and isolation of virus (Plowright 1965a, b, Rweyemamu *et al*, 1974). The detection of virus in a proportion of foetuses demonstrated the possibility of vertical transmission (Plowright, 1965a, 1965b). However, in the majority of cases, wildebeest calves acquire the virus as neonates while maternal antibodies are still present with no evidence of any clinical response following infection (Mushi *et al*, 1981b). The major source of AlHV-1 infection in wildebeest is thought to be 6-8 week old wildebeest calves as cell-free virus can be detected in both nasal and ocular secretions (Rweyemamu *et al* 1974; Mushi *et al* 1980a). The spread of AlHV-1 by aerosol would explain the efficiency of transmission within the wildebeest population.

Studies indicated that AlHV-1 infection of wildebeest calves leads to a primary lytic viral replication (probably in the mucosal epithelium) and virus shedding (Rweyemamu *et al* 1974; Mushi *et al* 1980a, 1980b). The presence of a mucosal immune response in calves was demonstrated by the presence of neutralising IgA antibodies (Rurangirwa *et al*, 1982). The production of neutralising IgA may drive the virus from productive replication to latency. This may explain the cessation of virus shedding at 3 months of age and the appearance of latent and persistent virus within the adult wildebeest population.

Transmission to susceptible species

The seasonal occurrence of MCF was related to the coincidence between the calving season of wildebeest and the appearance of MCF in cattle. Under laboratory conditions, Plowright (1965b) demonstrated the association between wildebeest calves and the occurrence of MCF by housing wildebeest calves and cattle together. The presence of viraemic wildebeest calves up to 3 months of age was associated with the occurrence of MCF in cattle. Cattle did not contract MCF in the presence of wildebeest calves over the age of 3 months. The route of transmission of AlHV-1 from wildebeest to cattle through the spread of virus in wildebeest foetal fluids and afterbirths was suggested by anecdotal evidence from the Masai people of East Africa. This theory was tested, but no virus could be detected in foetal membranes or fluids (Rossiter *et al*, 1983). In addition, Rossiter showed that AlHV-1 (in culture

medium) could be inactivated by strong ultraviolet light under pasture conditions. These experiments implied that, in open pasture, survival of the virus in oculo-nasal secretions or afterbirth is unlikely due to rapid inactivation. However, this has not been proven and conditions in culture medium cannot be directly compared to those in secretory fluid. Therefore, the detection of cell-free virus in nasal and ocular secretions of wildebeest calves (Rweyemamu *et al* 1974; Mushi *et al* 1980a) is further evidence that wildebeest calves are the source of infection for cattle. Cases of MCF have been reported outwith the wildebeest calving season (Barnard *et al*, 1989) but are likely to be a consequence of long incubation periods following infection of cattle (Reid and Buxton, 1989).

Infection of susceptible species

In contrast to wildebeest, the infection of cattle by AlHV-1 does not lead to contagious spread within the remaining herd. The lack of natural transmission from cattle is thought to be due to the cell-associated nature of the virus in cattle (Mushi and Rurangirwa, 1981a). It has been suggested that in cattle virus replication is repressed or incomplete as detection of virus antigen or DNA in MCF-affected tissue is low (Bridgen *et al*, 1992). Also, when tested for reactivity to AlHV-1 envelope glycoproteins, wildebeest sera consistently detected 6 polypeptides in contrast to the 3 polypeptides recognised by sera from AlHV-1-infected cattle (Herring *et al*, 1989). This suggests that all viral transcripts are not expressed in cattle and may explain the lack of natural transmission from cattle. In a small number of cases, congenital transmission of AlHV-1 from MCF-recovered cows to their calves has been demonstrated with a proportion of those calves developing MCF (Plowright *et al*, 1972). This suggests that in animals that recover from MCF, infection with AlHV-1 is persistent and latent where subsequent pregnancy may reactivate the virus leading to infection of the foetus. The recovered animal is also immune to subsequent challenge with virus. Reactivation and recurrence of MCF in recovered deer have also been achieved following administration of immunosuppressants (Heuschele *et al*, 1985). However, in most cases, infection of susceptible animals with AlHV-1 leads to disease and death.

1.2.2.2 Sheep-associated MCF (SA-MCF)

Of the two forms of MCF, the SA-MCF form appears to have a wider range of susceptible species including domestic cattle, water buffalo, Bali cattle and pigs (Piercy, 1954; Løken *et al*, 1998; Rossiter *et al*, 1989; Wiyono *et al*, 1994). Several species of farmed deer also seem to be highly susceptible (Denholm and Westbury, 1982; Heuschele *et al*, 1985; Reid *et al*, 1987; Wyand *et al*, 1971). Though normally sporadic where only one or two animals in the herd are affected, more severe outbreaks have been reported thus demonstrating the importance of studying this second form of MCF (Reid and Buxton, 1989).

Infection of natural/ carrier species

As the name suggests, sheep are the carrier species for this form of MCF. The body of evidence supports this claim. Firstly, cases of SA-MCF often coincide with the sheep lambing season (Reid *et al*, 1984). Furthermore, cattle have also developed MCF following the introduction (to the same premises) of sheep obtained from farms where cases of MCF have occurred (Kock and Neitz, 1950, Piercy 1954). While direct contact between sheep and susceptible species is not always readily established (Hoffman *et al*, 1984, Reid *et al*, 1987) the presence of antibodies reactive with AlHV-1 was demonstrated in the majority of sheep sera tested and in cattle affected by SA-MCF (Rossiter 1981, 1983). Therefore, a virus was present in all sheep tested and in cattle affected by SA-MCF that is antigenically similar to AlHV-1. This virus is now referred to as *Ovine herpesvirus 2* (OvHV-2).

Further serological and molecular evidence demonstrated the relationship of OvHV-2 to AlHV-1. For example, Li and others developed a competitive inhibition enzyme-linked immunosorbent assay (CI-ELISA) specific for AlHV-1 that also detects OvHV-2 (Li *et al*, 1994) but not other common herpesviruses of cattle. Using this assay MCF virus-specific antibody was detected in some clinically affected cattle, deer and bison as well as in asymptomatic sheep (Li *et al*, 1995). Furthermore, DNA from OvHV-2-infected cattle and deer cell lines and lymphoid cells of sheep hybridised with AlHV-1 DNA clones (Bridgen *et al*, 1989; Bridgen and Reid, 1991; Baxter *et al*, 1993). An OvHV-2 genomic DNA fragment with homology to an AlHV-1 DNA clone which contained sequences with homology to

the 140 and 160kDa tegument proteins of the gammaherpesviruses Epstein-Barr virus (EBV) and herpesvirus saimiri (HVS) respectively (Bridgen and Reid 1991) formed the basis of a polymerase chain reaction (PCR)-based assay for specifically identifying the SA-MCF virus.

OvHV-2 virions have not been isolated. However, using the PCR and serological assays mentioned above, the epidemiology of OvHV-2 infection has been studied. The pattern of infection in lambs is similar to that of wildebeest calves infected with AlHV-1. The source of OvHV-2 infection is thought to be lambs up to 3 months of age (Li *et al*, 1995) where the presence of viral DNA has been demonstrated in nasal and ocular secretions as well as mucosal epithelia (Baxter *et al*, 1997). Some *in utero* infection has also been noted (H. Reid, personal communication) and the presence of virus in colostrum suggests an additional source of infection for lambs (Li *et al*, 1995). PCR tests showed that the majority (>95%) of sheep tested (n = 26) were infected with virus by the age of one year and that B cells are a site of latent infection (Baxter *et al*, 1997, Li *et al*, 1998). While reactivation might theoretically occur in immunosuppressed sheep, no studies to look for herpesvirus particles in such animals have been performed.

Transmission and infection of susceptible species

The source of OvHV-2 infection is likely to include contaminated secretions from lambs. Aerosol transmission of OvHV-2 appears to be just as efficient as that of AlHV-1 with cases of SA-MCF occurring when cattle and sheep have been separated by up to 100 meters (Reid *et al*, 1986).

As with AlHV-1 infection, infection of cattle by OvHV-2 does not lead to spread of MCF to other cattle and healthy individuals in MCF-affected herds do not have virus-specific antibody (Reid *et al*, 1986). The reasons for an individual's susceptibility to MCF is unknown and has not been associated with the presence of prior illness or other potential predisposing factors.

1.2.3 Clinical symptoms and pathological features of MCF

Malignant catarrhal fever is recognised in three forms: (1) head and eye, (2) intestinal and (3) neurological forms (Goetz, 1930). The disease is associated with variable but characteristic pathological indicators. It is proposed that inappropriate responses of susceptible species to viral infection may contribute to the pathological changes observed (Reid and Buxton, 1989).

The following sections will review the clinical symptoms followed by a description of the pathological signs. These features will be discussed with respect to MCF caused by either AlHV-1 or OvHV-2, as many of the features are common to both forms of the disease.

1.2.3.1 Clinical symptoms associated with MCF

The pathology and symptoms associated with MCF have been most thoroughly studied in cattle. The symptoms appear following a variable incubation period of a few weeks to six months. In experimental transmission studies, the variation in incubation period is a function of the virus inoculum used and the route of infection (Mushi and Wafula 1983; Pierson et al, 1973; Plowright, 1968). The symptoms may vary according to the course of the disease and are grouped into four forms: peracute, acute, subacute or chronic.

Most commonly, an animal will first appear to have a decreased appetite accompanied by a raised temperature (41-42°C) and pulse rate (Daubney and Hudson 1936; Liggitt et al, 1978; Reid et al, 1979; Selman et al, 1974). Watery secretions from the eye nose and mouth progresses to a profuse and coloured muco-purulent discharge (Selman et al, 1978). At this stage, a progressive corneal opacity is commonly observed where both eyes are affected. Photophobia and swelling of the eyelids are also found. The muzzle, initially dry and hot, may crack and bleed with time (Selman et al, 1978). The nares are often occluded with catarrhal discharge and in severe cases can block the nasal passages entirely. Superficial lymph node enlargement can be palpated prior to the onset of pyrexia (Daubney and Hudson 1936; Selman et al, 1978) and swelling of the joints can occur. Hyperaemia also occurs (after the onset of pyrexia) in the oral mucosa accompanied by focal erosion

on the lips, tongue, hard palate and buccal papillae. Symptoms, involving the nervous system, have also been reported i.e. the neurological form of MCF. These include hyperaesthesia, depression, muscular tremors, lack of co-ordination, circling and nystagmus (small involuntary movements of the eyes) (Clark *et al*, 1970; Daubney and Hudson 1936; Selman *et al* 1978). These symptoms are consistent with the acute and subacute forms of MCF - also known as the 'head and eye' form. Constipation is a common symptom and often followed by haemorrhagic diarrhoea until death. However, this is more often seen in deer where the course of the disease is peracute.

The symptoms of peracute cases are less severe and less common. For example, corneal opacity and enlargement of the superficial lymph nodes are not marked and erosion of the oral mucosa is limited to the buccal papillae. In the peracute form, death can occur within one to three days of onset of pyrexia. The disease in deer is commonly associated with acute diarrhoea and dysentery (Orr and MacKintosh 1987). The urine may contain blood and albumin. This set of clinical features is also known as the 'intestinal form' of MCF and can last for up to nine days before death. At the other extreme, death can occur without any prior clinical signs (Reid *et al*, 1987; Wyand *et al*, 1971).

In chronic cases, pyrexia can sometimes be short-lived and corneal symptoms can extend to erosion and ulceration (Daubney and Hudson 1936). In general, symptoms observed in chronic cases tend to be severe and/or more widespread than those described for the acute and subacute cases of MCF. In a recently described form of cutaneous MCF, lesions on the back, neck and flanks were found (Holliman *et al*, 1994; Michel, 1994) as a feature of a chronic case of MCF. Secondary bacterial infections following the appearance of skin lesions, fever and enlarged subcutaneous lymph nodes were also observed.

A mild form of MCF has also been reported where the symptoms are few and the animals survive. This form was first observed in experimental transmission studies (Goetz, 1930). More recently, cases have been reported where cattle and deer have recovered from naturally transmitted MCF. In the cases involving cattle, treatment with steroids following the appearance of clinical signs may have contributed to the recovery (Milne and Reid 1990; Penny, 1998). However, the

timing of steroid administration may affect the outcome of the disease. For example, the application of steroids to a sika deer (*Cervus nippon*) showing signs of MCF did not improve the symptoms and the deer continued to develop symptoms typical of chronic MCF leading to death (Wilson *et al*, 1983). In another case where recovery was observed, the disease was re-activated following steroid treatment and chronic symptoms returned (Heuschele *et al*, 1985). Other cases of recovery have been reported (Mushi and Rurangirwa 1981b; Plowright, 1981) but the true frequency of natural recovery is difficult to determine due to the involvement of medical intervention and the potential for misdiagnosis.

1.2.3.2 Macroscopic lesions

The diagnosis of MCF has traditionally only been possible after post-mortem examination. Certain macroscopic lesions are characteristic of MCF.

In all forms of MCF, enlargement, hyperplasia and oedema of the peripheral lymph nodes are common. In addition, the spleen is often grossly swollen, particularly in AlHV-1-induced MCF. In deer and chronic cattle cases where necrosis has occurred, the lymph nodes and spleen can appear smaller than normal (Plowright, 1953; Reid *et al*, 1979). Haemorrhagic areas in the retropharyngeal lymph nodes accompany necrosis (Denholm and Westbury 1982; Oliver *et al*, 1983; Selman *et al*, 1974, 1978).

Mucosal surfaces are affected to a greater or lesser degree. In the buccal cavity, pharynx, soft palate, tongue, oesophagus and fore-stomach, patches of necrosis can be found (Daubney and Hudson 1936; Selman *et al*, 1978). The intestine is usually severely affected with haemorrhages and erosions. Deer seem to be particularly susceptible to these changes with additional oedema of the intestinal wall and mucosal surfaces of the respiratory system. Pathology can sometimes be seen in the genital tract but is usually confined to erosion of the vaginal mucosa (Denholm and Westbury 1982; Oliver *et al*, 1983; Reid *et al*, 1979, 1987; Wyand *et al*, 1971).

Enlargement of the liver (Daubney and Hudson 1936) and spleen is found in the 'intestinal' and 'head and eye' form of MCF. This is unlike the peracute form in which the liver pathology does not appear to be involved. Cystitis is also apparent in

the two more severe forms. Additionally, raised white foci can be observed on the kidney in the 'head and eye' form of MCF (Goetz 1930). Other macroscopic lesions are found in the synovial membranes (appearing swollen) and the brain (with excessive and cloudy cerebro-spinal fluid) (Daubney and Hudson 1936; Liggitt et al, 1978).

1.2.3.3 Microscopic lesions

As discussed above, macroscopic lesions can be highly variable and dependent on affected species. In contrast, microscopic lesions are much more consistent. They can be divided into four groups: hyperplasia and necrosis in lymphoid organs, interstitial accumulation of lymphocytes in non-lymphoid organs, vasculitis and epithelial degeneration.

Hyperplasia involves the spleen and lymph nodes. In the spleen, hyperplasia is limited to the periarteriolar lymphoid sheaths (PALS). Very little follicular development is observed. The severity of the lesion increases with duration of illness. In some cases, only a few clusters of lymphoid cells remain in the PALS (Reid et al, 1979). Hyperplasia in lymph nodes is concentrated in the paracortical region. This involves the expansion in numbers of lymphoblastoid cells (Selman et al, 1974,1978). The cortex may show signs of necrosis and haemorrhage but hyperplasia is not as severe as in the paracortex (Denholm and Westbury, 1982). Again, follicular and germinal centre development is rare (Selman et al, 1978). Macrophages and lymphoblasts occupy most of the medullary region (Selman et al, 1974; Wyand et al, 1971).

On histopathological examination, interstitial lymphoid cell accumulation of non-lymphoid organs is widespread. Organs involved include the liver, renal cortex, trachea, urinary bladder, pancreas, lachrymal and salivary glands and the cardiac and skeletal muscles. This type of lesion in the cornea is very characteristic of MCF (Berkman et al, 1960; Liggitt et al, 1978; Liggitt and De Martini 1980b; Reid et al, 1979). Lesions in the brain are also consistently present (Liggitt et al, 1978; Reid et al 1979). The types of cells involved in these accumulations have been identified as lymphocytes and lymphoblasts. Neutrophils can be detected in severely ulcerated

corneas. Macrophages have also been detected but are much rarer (Edington *et al*, 1979; Liggitt and De Martini 1980a,b; Patel and Edington 1980, 1981).

Vasculitis (affecting arteries, arterioles, veins and venules) and epithelial degeneration are characteristic lesions of MCF (Berkman *et al*, 1960; Daubney and Hudson 1936). Again, the severity of lesions is related to the duration of illness. The organs commonly affected are the eyes, liver, kidneys, lungs, oral epithelium, brain and gastrointestinal tract. Accumulations of mononuclear cells are consistently associated with the appearance of these lesions and may be important in the pathogenesis of MCF (see Chapter 1.2.3).

1.2.3.4 Pathological features induced by AlHV-1 and OvHV-2 in rabbits

In experimentally infected rabbits, some differences in AlHV-1 and OvHV-2-induced pathology have been observed. OvHV-2 infection in rabbits is associated with enlargement of the mesenteric and submandibular lymph nodes, appendix and spleen (Buxton and Reid 1980; Buxton *et al*, 1984; Westbury and Denholm 1982). Epithelial lesions and diarrhoea are present but are much less severe than in ruminant MCF. Dysentery, lesions of the urinary bladder and lymphoid infiltration of kidneys are uncommon. Arteritis is also uncommon but can sometimes be found in the lungs (Buxton *et al*, 1984).

The lymph nodes in rabbits affected with AlHV-1-induced MCF are similar to those in OvHV-2-infected rabbits. However, the spleen and submandibular lymph nodes tend to be even larger than in rabbits infected with OvHV-2. Conversely, the mesenteric lymph node is smaller by comparison. In AlHV-1 infection frequent occurrence of arteritis, degeneration of the thymus and lymphoid infiltration in the kidneys has been observed (Edington *et al*, 1979).

1.2.4 Pathogenesis and immunopathology

1.2.4.1 Experimental transmission of MCF

The mechanisms underlying the pathogenesis of MCF are still not fully understood. Experimental transmission studies have been useful for the study of

MCF pathogenesis. Transmission of MCF to cattle and laboratory animals has been achieved through the transfer of whole blood or lymphoid cell suspensions from cases of MCF and are described in the following section.

Using blood from cases of MCF in cattle, infection of healthy cattle with AlHV-1 has been followed. Viraemia was detectable 8-17 days after inoculation and the first sign of clinical disease (pyrexia) was noted 3-15 days thereafter. Clinical disease as described in the previous section ensued over the next 5-8 days leading to death. During clinical disease, virus was detectable first in the lymphoid organs (e.g. lymph nodes, spleen) then disseminated to the non-lymphoid organs (e.g. lungs, kidney, liver, ocular and nasal epithelium). The average incubation period (ie. time taken to develop pyrexia) was 22 days (Plowright, 1968). The average incubation period in experimental infections using OvHV-2 was similar to that seen with experimental AlHV-1 infection (Piercy 1952a, b).

Transmission of MCF to laboratory animals such as rabbits, hamsters, guinea pigs and rats using AlHV-1 and to rabbits and hamsters using OvHV-2 have been successful (Buxton *et al* 1988; Jacoby *et al*, 1988; Kalunda *et al*, 1981; Reid *et al*, 1986). Rabbits in particular are good models for the disease as the pathology seen is similar to that seen in ruminant MCF, with an incubation period of 10-14 days (Piercy, 1955; Rossiter, 1981). The site of primary replication and the initial cell type targeted by AlHV-1 *in vivo* is unknown although the presence of cell-associated virus in nasal and ocular secretions of infected cattle would suggest that the mucosal epithelia are the target for primary infection (Mushi and Rurangirwa, 1981a). Studies in rabbits inoculated intravenously with lymph node cells from a rabbit infected with AlHV-1 demonstrated that the spleen was the first lymphoid organ to show signs of virus infection and productive replication (Edington and Patel, 1981). Virus infection in the spleen was detected four days after inoculation. Twelve days after inoculation virus spread to other lymphoid tissue with the onset of pyrexia. Infected cells were identified by indirect immunofluorescence as medium sized lymphocytes. Viral antigens were detected in the nucleus and cytoplasm of these cells. However, these modes of transmission are not the natural route of infection. The presence of cell-free virus in wildebeest oculo-nasal secretions and OvHV-2 DNA in sheep nasal secretions suggests that the natural route of infection could be

intranasal. Animals inoculated by the intranasal route with cell-free AlHV-1 from wildebeest secretions experienced shorter incubation periods compared to the intravenous route of infection (Mushi and Wafula, 1983). The longer half-life of the virus *in vitro* at 32°C compared to 37°C (Harkness and Jessett, 1981) may also account for the shorter incubation periods recorded in intranasal compared to intravenous inoculation.

Antibody responses to AlHV-1 and OvHV-2 infection have also been studied. The immunofluorescent antibody test has been widely used in serological studies. Using this method, the presence of antibody was demonstrated in cattle and rabbits experimentally infected with AlHV-1. Antibody was detected approximately one week before pyrexia with titres increasing until death (Rossiter, 1982a). However, this form of serological test is limited by cross-reactivity with the common bovine herpesviruses, BHV-2 and BHV-4 (Rossiter *et al*, 1980). Using the virus neutralisation assay, neutralising antibody was detected in all wildebeest tested. In experimental transmission studies, neutralising antibody was detected in 100% of MCF-recovered cattle but only in a proportion of fatally affected cattle (Plowright, 1965b, 1968). In Rossiter's (1982a) study of AlHV-1 infection in rabbits and cattle, neutralising antibody was also detected in rabbits shortly before the onset of pyrexia. However, in experimentally infected cattle neutralising antibody to AlHV-1 was not detected. These results suggest that in wildebeest and recovered cattle, productive virus replication - involving expression of late transcripts - has occurred prior to the virus becoming latent. The presence of neutralising antibody in lower proportions of fatally infected cattle suggests that in these animals the virus does not express the late transcripts to which neutralising antibody is directed. This supports the idea that virus replication is repressed or incomplete in these animals. It should be noted however, that presence of neutralising antibodies in cattle or rabbits does not protect the animals from MCF (Barnard, 1984; Mirangi, 1991; Plowright et al, 1975; Rossiter et al, 1989).

1.2.4.2 Pathogenesis of MCF

The main features of MCF pathology can be summarised as follows: lymphocyte hyperplasia in lymphoid and non-lymphoid organs, epithelial

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degeneration and vasculitis. In MCF-affected tissue no specific viral cytopathology is observed (Edington *et al*, 1979; Rossiter 1985) though cells from such tissue display infectivity despite little viral antigen having been detected (Rossiter, 1980).

Several hypotheses for the pathogenesis of MCF have been proposed. These include virus-induced cytopathic effects or virus-induced transformation of infected cells. Other theories suggest a role for the host immune system such as immune complex formation, virus-mediated stimulation of autoimmunity, and cell-mediated immune dysfunction. These hypotheses are reviewed in the following paragraphs.

Virus-induced cytopathic effect is well documented for many viruses (Fields *et al*, 1996). Indeed, the isolation of AlHV-1 *in vitro* depends on the appearance of a cytopathic effect in cell monolayers. Therefore, it was suggested that the necrotic lesions seen in MCF could be the result of virus-mediated cytolysis (Liggitt and De Martini 1980a). Other authors have suggested that virus-induced lymphocyte transformation is responsible for the lymphoproliferation (Denholm and Westbury 1982; Edington *et al*, 1979; Heuschele *et al*, 1985; Hunt and Billups 1979). However, these hypotheses are not compatible with the lack of viral antigen or the low numbers of virus-infected cells ($1:10^4$) detected in tissue (Bridgen *et al*, 1992). Therefore, as virus-mediated cytopathic effects and virus-mediated transformation are not obvious in MCF pathogenesis the role of the immune system must be considered.

Immune complex formation is responsible for the pathogenesis of rheumatoid arthritis, serum sickness and glomerulonephritis (Roitt *et al*, 1998). The involvement of such a mechanism might explain the widespread vasculitis typical of MCF pathology. Patel and Edington (1982) were able to detect deposition of immune complexes in infected bovine tissue. However, the specificity of the antibodies involved in these complexes could not be determined. A number of other studies have failed to detect immune complexes in cattle, rabbit and deer (Liggitt and DeMartini, 1980a,b; Rossiter, 1980,1981; Mushi and Rurangirwa, 1981c). Once again, the paucity of viral antigen in MCF-affected tissue indicates that immune complex-mediated pathogenesis is not likely.

The similarity of lesions in cattle with MCF to lesions seen in contact hypersensitivity, graft rejection and graft-versus-host disease implicated the

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involvement of the host immune system in MCF pathogenesis. The presence of lymphoid infiltration in MCF lesions is consistent with this theory (Liggit and DeMartini, 1980b). However, none of these hypotheses can explain the lymphoid hyperplasia that occurs prior to tissue destruction and death.

The proliferation of lymphocytes in lymphoid tissues of MCF-affected animals is generally restricted to the T-cell-dependent areas and the preservation of tissue architecture in these tissues suggested a hyperplastic rather than a neoplastic event (Buxton *et al*, 1984). In experimentally infected rabbits, hyperplasia - which can be inhibited by cyclosporin-A (Cs-A) *in vivo* - appears to take place soon after infection and is accompanied by a relative decrease in the number of B cells (Buxton *et al*, 1984). Thus, virus-induced transformation of lymphocytes in MCF pathology seems unlikely. Evidence for the *in vivo* involvement of immunopathology in OvHV-2 infection includes studies of infiltrating lymphocytes in vascular and epithelial lesions. Immunohistochemistry showed the majority of lymphoid cells at these sites to be $CD2^+/CD8^+/CD6^+$ (Ellis *et al* 1992, Nakajima *et al* 1992). Most infiltrating cells had the morphology of large lymphoblastoid cells. Proliferation of $CD8^+$ T cells was responsible for the typical hyperplasia of MCF seen in lymphoid organs (Nakajima *et al* 1992, Schock and Reid, 1996).

Lymphoblastoid cell lines can be generated *in vitro* with a similar phenotype to the infiltrating cells observed *in vivo*. That cell lines can be propagated using lymphoid cell suspensions from MCF-affected tissue supports the idea that these cells may play a role in MCF pathogenesis. They have the morphology of large granular lymphocytes (LGLs) and can transmit MCF to rabbits (Reid *et al*, 1989). LGLs generated from OvHV-2 infected animals have the phenotype of $CD2^+/CD8^+/TCR\alpha\beta^+$ T cells (Burrells and Reid, 1991; Cook and Splitter, 1988; Schock *et al*, 1998) although $CD4^+/TCR\alpha\beta^+$ and $CD4^-/CD8^-/TCR\gamma\delta^+$ cell lines have also been observed. Functionally, these cells displayed major histocompatablilty complex (MHC)-unrestricted, non-antigen specific cytotoxic activity that is common to both natural killer (NK) cells and lymphokine activated killer (LAK) cells (Cook and Splitter, 1988, Reid *et al*, 1989b). LGL cell lines derived from MCF-affected animals grow in culture in the presence of exogenous interleukin-2 (IL-2) and/or feeder cell monolayers. Some of these lymphoblastoid LGL cell lines were able to

grow in the absence of exogenous IL-2 for longer periods than uninfected control cells (Reid *et al*, 1989b). Lymphoblastoid LGL cell lines established from rabbits experimentally infected with AlHV-1 or OvHV-2 displayed a similar morphology, phenotype and function (Reid *et al*, 1983, Schock and Reid, 1996).

Therefore, the hypothesis that best fits the pathology observed was suggested by Reid and Buxton (1989) who proposed that lymphocyte dysregulation through inappropriate cytokine stimulation leads to the production of large granular lymphocytes (LGL) with NK/LAK cell characteristics which ultimately causes tissue destruction. The target cell for virus infection is thought to be the LGL where viral DNA, if not virions, has been detected (Bridgen *et al*, 1989; Bridgen and Reid, 1991; Baxter *et al*, 1993). LGL derived cytokines could therefore be responsible for the T cell proliferation observed *in vivo*. In support of this hypothesis is the detection in OvHV-2-infected LGL cells of mRNA for the cytokines, IL-4, IL-10, INF-γ and TNF-α but not for IL-1β or IL-2 (Schock *et al*, 1998).

1.2.5 Diagnosis and prevention

1.2.5.1 The development of diagnostic tests for MCF

Historically, firm diagnosis of MCF has only been possible using post-mortem histopathological examinations. The wide range and severity of lesions make diagnosis a difficult task. However, the presence of generalised vasculitis, cuffing in the CNS, hyperplasia of lymph nodes and lymphoid infiltration of the kidney, urinary and gall bladder is considered sufficient for a firm diagnosis. This does not rule out the possibility of misdiagnosis. Theileriosis, trypanosomiasis and rinderpest are among a number of diseases that have been confused with MCF (Plowright, 1981; Reid and Buxton 1989).

Virus isolation as a means of diagnosis is only possible for AlHV-1-induced MCF. Recovery of virus from viable lymphoid or buffy coat cells is possible on co-culture with permissive cell monolayers. The development of a typical syncitial CPE in 10 to 20 days is a good indicator but specificity still needs to be confirmed by indirect immunofluorescence or immunoperoxidase assays (Ferris *et al*, 1976;

Rossiter, 1981). However, these tests are dependent on the specificity of the sera involved, which are from carrier or MCF-susceptible animals.

Diagnosis by indirect immunofluorescence using sera from diseased animals has been hampered by low titres or non-specificity (Rossiter *et al*, 1977). The specificity is tested by reactivity to AlHV-1 antigens expressed in virus-infected monolayer cells. While this test has been used to diagnose WA-MCF (AlHV-1 infection), the use of the test in SA-MCF is dependent on antigenic relationship between AlHV-1 and OvHV-2 (Herring *et al*, 1989). However, antigenic relationship to other known herpesviruses (particularly BHV-4) has been observed (Plowright 1967; Reid *et al*, 1975; Rossiter, 1981, 1983; Rossiter and Jessett 1980; Rossiter *et al*, 1977, 1978, 1980) therefore the results of all serological tests using whole sera from animals with MCF must be interpreted with care. Recently, Li *et al* (1994) developed a competitive inhibition enzyme-linked immunosorbent assay (CI-ELISA) that could be useful for diagnosis of SA-MCF. This test relies on the inhibition (by the test sera) of a monoclonal antibody with specificity for a glycoprotein in AlHV-1, which is not cross-reactive with BHV1, BHV2 or BHV4 proteins but is cross-reactive with OvHV-2.

With the emergence of new molecular techniques, the specificity and sensitivity of diagnostic tests has improved. The molecular characterisation of the AlHV-1 genome and partial characterisation of OvHV-2 has allowed specific DNA probes to be developed. *In situ* hybridisation using an AlHV-1-specific *Hind*II fragment encoding the major terminal non-coding repeat sequence allowed the detection of this virus in tissue and lymphocyte cultures derived from rabbits with experimental MCF (Bridgen *et al*, 1992). The sensitivity of this test was thought to be a hundred fold higher compared to indirect immunofluorescence using whole sera from animals with MCF. The result nevertheless confirmed previous findings that in tissues from animals with MCF a very low number of cells were infected with AlHV-1.

The development of polymerase chain reaction (PCR)-based techniques has made a major impact on the diagnosis of MCF. This method uses virus specific DNA sequences to amplify viral DNA and is much quicker than virus isolation. It has the added advantage of being able to detect and amplify viral sequences that are

present at very low concentrations in tissue samples. Diagnosis is now possible during the clinical stage of the disease. PCR-based assays have been developed for detecting AlHV-1 DNA of open reading frame 21 (ORF21) with homology to the EBV ORF, BXLF1 (encoding thymidine kinase) and a WC11 clone with homology to parts of the sequence for EBV major capsid proteins encoded by the open reading frames, BDLF1 and BcLF1 (Hsu et al, 1990b; Katz et al, 1991; Michel, 1993; Tham et al, 1994). For diagnosis of OvHV-2 infection, DNA sequences homologous to the 140kd and 160kd tegument proteins of EBV and HVS respectively are used for PCR-based assays (Baxter et al, 1993). The PCR has allowed early detection of OvHV-2 in Indonesian ruminants and should lead to a better understanding of the epidemiology of MCF in Southeast Asia (Wiyono et al, 1994). The first case of MCF (caused by OvHV-2) in pigs, verified by PCR, was recently reported (Løken et al, 1998). The accuracy of PCR as a diagnostic test was confirmed in a field validation study (Müller-Doblies et al, 1998). PCR showed more than 94% agreement with hisopatholgical diagnosis (which is still regarded as the definitive test for MCF). CI-ELISA showed a similar degree of specificity but had a much lower level of sensitivity compared to PCR.

1.2.5.2 Prevention and control of MCF

Many attempts have been made to immunise animals against the MCF viruses and have concentrated on the development of a protective antibody response. However, the presence of neutralising antibody (at high titres) does not protect cattle or rabbits against a challenge with cell-associated virus (Barnard, 1984; Mirangi, 1991; Mirangi and Rossiter 1991; Piercy, 1954; Plowright et al, 1975; Reid and Rowe, 1973; Rossiter et al 1989). Where immunisation has been successful in rabbits using live kidney cells from rabbits infected with the cell-free AlHV-1 virus WC11, protection against the cell-associated AlHV-1 C500-infected rabbit spleen cells was short-lived (Rossiter, 1982b). It is presumed that in cases where animals have recovered from MCF, protective immunity must be cell-mediated rather than humoral (Heuschele et al, 1985; Plowright et al, 1972). No vaccine has yet been developed for the prevention of MCF. Therefore, the control of MCF still relies on

the separation of carrier and susceptible species during the lambing/calving season (Reid and Buxton, 1989).

The inability of infected and diseased animals to eliminate the MCF viruses may be due to the expression of viral genes that interfere with the host immune response either directly or indirectly. Viral interference of the host cell responses will be discussed with respect to other well-studied viruses in the following section.

1.3 Herpesvirus modulation of host cell function

To ensure survival and replication in host cells, viruses must first escape the normal immune and anti-viral responses of the host.

1.3.1 Antiviral immune responses

The first line of defence against a virus infection is the innate immune system. On infection, the infected cell produces interferons (IFNα/β/γ) and tumour necrosis factor (TNF), both cytokines with antiviral activity. Anti-viral activity includes the blocking of protein translation, degradation of viral RNA and induction of host cell apoptosis or programmed cell death (Billiau 1996; Roitt *et al*, 1998). Apoptosis triggered by virus infection can be mediated by the induction of intracellular proteins such as Bax or via death receptors such as Fas and tumour necrosis factor receptor-1 (TNFR-1) (Boyd *et al*, 1995; Sato *et al*, 1994; Thome *et al*, 1997). Another effect of interferons, specifically IFNγ, is the activation of natural killer (NK) cells and macrophages and the triggering of the adaptive immune system (Billiau 1996; Heise and Virgin 1995).

The principle contribution of B cells in the adaptive immune response to virus infection is the production of virus-specific antibody. Neutralising antibody can block the binding and entry of free virions into host cells. Antibody, in conjunction with NK cells, can mediate antibody-dependent cell cytotoxicity (ADCC) against virus infected cells. This is facilitated by the production of perforin - a cytolytic protein - released from the cytoplasmic granules of NK cells (Doherty 1993). Antibody can also mediate the activation of the complement system - a series of proteins that bind to the cell membrane, leading to the formation of the membrane

attack complex (MAC) and cell lysis. The combination of antibody and complement can sometimes act to opsonise the virus or virus-infected cell, making phagocytosis possible (Sissons and Oldstone 1980).

The anti-viral immunity mediated by T cells can be direct and indirect. The CD4$^+$ T helper (Th) cells have an indirect role by recruiting and activating macrophages to the site of virus infection by the production of cytokines. For example, the production of cytokines such as IL-2, IFNγ and TNFβ produced by Th1-type cells enhance monocyte activation as well as CD8$^+$ cytotoxic cells (CTLs). CTLs - major effector cells in anti-viral immunity - recognise virus antigen presented by major histocompatability complex (MHC-I) on virus-infected cells (Doherty *et al*, 1992; Ramsay 1993). The regulation of all these responses is controlled by the cytokine network.

1.3.1.1 Control of the immune response

The cytokine network includes a large group of soluble proteins that regulate immune and inflammatory responses. They can co-ordinate the clearance of virus by stimulating effector cells and control their responses to ensure minimal damage to host cells. Within this group of cytokines are the chemokines that are known for their proinflammatory and chemoattractant properties. The effects of cytokines and chemokines are controlled by the expression of different families of cell surface receptors. For example, IL-2 and IL-15 are stimulators of T cell proliferation. IL-2 is a cytokine produced mainly by T cells while IL-15 is produced by monocytes, dendritic cells and fibroblasts (Carson *et al*, 1995; Doherty *et al,* 1996; Grabstein *et al*, 1994; Jonuleit *et al*, 1997). The response of cells to IL-2 is controlled by the presence of the IL-2 receptor (IL-2R). This receptor is a heterotrimer consisting of the α, β and γ subunits (Smith, 1988). IL-15 shares many of its properties with IL-2 due to its use of the common subunits IL-2Rβ and IL-2Rγ (Carson *et al*, 1994; Giri *et al*, 1994; Grabstein *et al*, 1994) . The additional expression of the α subunit of the IL-15 receptor (IL-15Rα) increases the affinity of the receptor for IL-15 just as the induction of the IL-2Rα subunit on activated cells increases the affinity of IL-2 for its receptor (Anderson *et al*, 1995; Giri *et al*, 1995a,b; Kondo *et al*, 1986; Robb,

1986; Takeshita *et al*, 1992). Another family of receptors is used by chemokines including MCP-1 (macrophage chemotactic protein) and IL-8. Chemokine receptors are G-protein-coupled receptors (GCRs) and have a characteristic structure with seven transmembrane domains. The specificity of these receptors is determined by the N-terminal extracellular domain (Murphy, 1994; Gayle *et al*, 1993).

The specificity of the anti-viral immune response is determined by the engagement of a virus antigen with the antigen receptors of B cells and T cells via presentation of antigen by antigen presenting cells (APC). The nature of the immune response is also controlled by the engagement of cytokine receptors and co-stimulatory molecules on APCs and T cells such as CD2, CD4, CD8, CD28 and the IL-2 receptor on T cells (Roitt *et al*, 1998). The result of the stimulation of these extracellular proteins is the induction of intracellular proteins that transmit messages to the nucleus (i.e. signal transduction). These signalling pathways can intersect with each other creating a complex biochemical network within the cytoplasmic space. The outcome is the transcription of genes and the generation of an appropriate response. This process is fundamental to the responses generated in innate and adaptive immunity (Crabtree and Clipstone, 1994).

The proteins involved in the signalling pathways - i.e. signal transduction molecules - include kinases (which phosphorylate proteins on tyrosine, serine or threonine residues), phosphatases (which de-phosphorlate proteins) and several adaptor molecules (including guanosine triphosphate (GTP)/ guanosine diphosphate (GDP) exchange factors). The interaction of cell surface receptors with signalling molecules and the resultant cascade of protein phosphorylation and de-phosphorylation is the basis of signal transduction (Crabtree and Clipstone, 1994). For example, p56 *lck* - a T cell-specific signalling molecule (belonging to the *src* family of tyrosine kinases) - is activated in the early stages of T cell activation via the T cell receptor. This then leads to the activation of downstream signal transduction molecules (Fig 1.4). The events following cytokine receptor engagement is similar though associated with a different family of signal transduction molecules - i.e. the JAKs (Janus kinases) and STATs (signal transducers and activators of transcription) (Heim, 1996).

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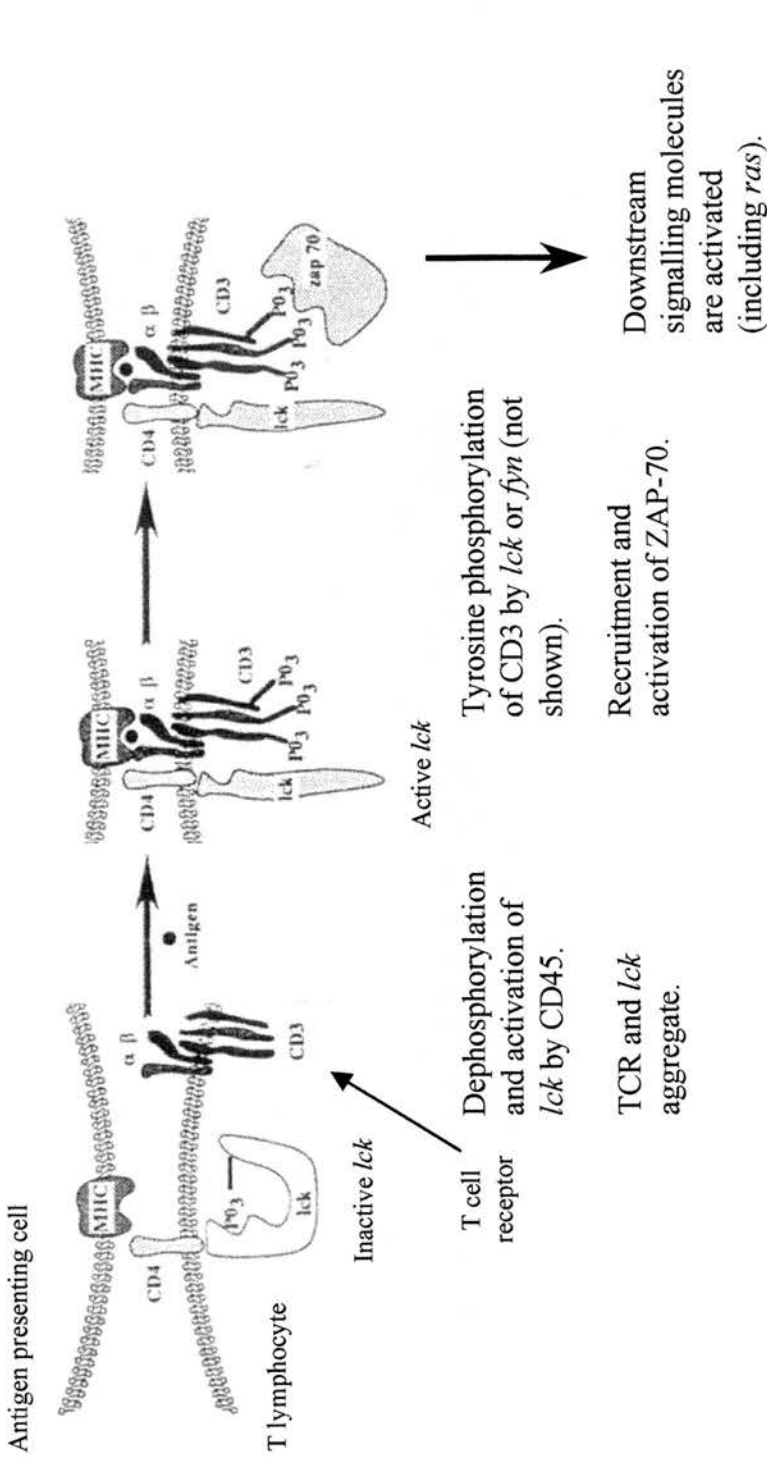
Fig 1.4: Molecular interactions at the cell surface leading to initiation of T cell activation. Signal transduction molecules of the *src* kinase family associated with the T cell receptor (TCR) and CD4 are shown in a series of phosphorylation events following antigen-mediated stimulation through the TCR. (from Crabtree and Clipstone, 1994). Note: PO₃ indicates phosphate group.

1.3.2 Immune evasion mechanisms adopted by herpesviruses

Viruses have developed a variety of strategies that enable them to evade the anti-viral immune responses. The infection and persistence of viruses in a host cell will sometimes change the behaviour of the infected host cell. In other cases, the immune environment in which the virus exists can be altered to aid virus survival. The mechanisms used by the MCF viruses to modulate host cell behaviour are unclear. Therefore, the following sections will review the mechanisms used by other herpesviruses focussing on the related gammaherpesviruses herpesvirus saimiri (HVS) and Epstein-Barr virus (EBV) in particular (summarised in Table1.3). As for all gammaherpesviruses, HVS and EBV are both lymphotropic viruses that transform T cells and B cells respectively and are associated with lymphoproliferative disease (Masucci and Ernberg, 1994; Meinl et al, 1995). Studying the mechanisms employed by these and other herpesviruses to deregulate host cell function may give valuable insights into the mechanisms utilised by the MCF viruses.

Cell transformation is a major mechanism by which viruses modulate host cell behaviour. Many viruses encode proteins that can modulate the growth of an infected cell, which drives the expansion of host cells and therefore the persistence of viral genome. Viruses such as Kaposi's sarcoma herpesviruses (KSHV or HHV-8) (Boshoff and Weiss, 1998), human T cell leukaemia virus (HTLV-1) (Feuer et al, 1993) and EBV (Ring, 1994) exhibit transforming activity. The transforming ability of EBV has been associated with two groups of proteins: the EBNAs (EBV nuclear antigens) and the LMPs (latent membrane proteins) (Ring, 1994). Transformation can be achieved by viral interaction with normal cellular activation pathways (as exemplified by the HVS proteins, Tip and STP -see next section). Another mechanism of transformation is virus-induced evasion of host cell apoptosis as demonstrated in HTLV-1-infected T cells and EBV-infected B cells (Copeland et al, 1994; Gregory et al, 1991). The interaction of Bax (the intracellular protein induced during apoptosis) with the anti-apoptotic protein Bcl-2 inhibits apoptosis (Sato et al, 1994). Some viruses may inhibit host cell apoptosis by exploiting the anti-apoptotic activity of Bcl-2. For example, a Bcl-2 homologue is encoded by HHV-8 and cellular Bcl-2 is induced by LMP-1 of EBV (Cheng et al, 1997; Henderson et al, 1991; Sarid et al, 1997). Viral proteins that inhibit the signaling complex of death

receptors can also inhibit apoptosis. For example, HHV8 and equine herpesvirus 2 (EHV2) encode proteins with structural homology to death effector domains or caspase recruitment domains - both important in mediating protein interactions of signaling molecules associated with death receptors (Bertin *et al*, 1997; Thome *et al*, 1997, 1999). Therefore, the presence of a Bcl-2 homologue encoded by ORF A9 of AlHV-1 might suggest that an anti-apoptotic mechanism may also be important for virus survival and persistence (Ensser *et al*, 1997).

Interference with antibody and complement function (one of the earliest host defence mechanisms against pathogens) allows viruses to evade the host's immune system. Several viral proteins have been shown to exhibit antibody or complement binding activity. Examples include the glycoproteins gC, gE and gI of herpes simplex viruses (HSV-1 and HSV-2), gI of varicella zoster virus (VZV) and protein m138 of murine cytomegalovirus (MCMV) (Bell *et al*, 1990; Hung *et al*, 1992; Litwin *et al*, 1992; Thäle *et al*, 1994). Gammaherpesviruses including HVS have also been shown to encode proteins with homology to cellular complement regulatory proteins and will be discussed in the next section. Interestingly, EBV also inhibits complement activation despite the absence of viral homologues to complement control proteins (Mold *et al*, 1988). Therefore, viruses that interfere with host defence mechanisms at an early stage may have a greater chance of survival and thence the ability to replicate or establish latency.

The evasion of innate immune responses is clearly important for virus survival and replication. However, the long-term persistence of viruses within the host also depends on successful evasion of adaptive, cell-mediated immune responses. Proteins with major histocompatability complex (MHC) class II - binding activity have been noted in HVS (ORF14) and EBV (BZLF2) whilst proteins with MHC class I homology are present in MCMV (m144) and HCMV (UL18) (Davis-Poynter and Farrell, 1996). The herpes simplex virus (HSV) protein, IE12 also binds to and down-regulates MHC class I expression (York *et al*, 1994). These viral proteins may aid immune evasion by inhibiting viral recognition and may be an immunosuppressive mechanism.

Viruses have been able to bypass cell-mediated immunity by disrupting the cytokine network by the production of viral proteins that mimic cytokines or viral

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homologues to cytokine receptors and chemokine receptors that block cytokine and chemokine function respectively. Cytokine homologues have been demonstrated in the herpesviruses EBV (BCRF1 = IL-10) and EHV-2 (ORF E7 = IL-10) (Moore *et al*, 1990; Telford *et al*, 1995; Vieira *et al*, 1991). Cellular IL-10 inhibits the inflammatory response mediated by macrophages and is involved in the regulation of helper T cell (Th) responses. IL-10 shifts the immune response towards a Th2 response, which includes enhancement of B cell responses and inhibition of CTL, and NK responses (normally controlled by Th1 cells). Thus, viral IL-10 may aid virus survival by inhibiting the normal immune responses to virus infection controlled by Th1-derived cytokines (Moore *et al*, 1993). Viral cytokine receptor and chemokine receptor homologues have also been found in poxviruses (McFadden *et al*, 1995) and herpesviruses. Examples in herpesviruses include HCMV, MCMV, human herpesvirus 6 and 7 (HHV-6 and 7), EHV-2, EBV and HHV8 (Cesarman *et al*, 1996; Chee *et al*, 1990; Davis-Poynter *et al*, 1997; Gompels *et al*, 1995; Nicholas 1996; Telford *et al*, 1995). The function of these homologues has not been determined but it has been speculated that they might play a role in tissue tropism. This is illustrated by the chemokine receptor homologue in MCMV encoded by ORF M33. The deletion of this ORF leads to the loss of the ability to replicate in the salivary gland, a major site of replication and persistence (Davis-Poynter *et al*, 1997). Alternatively, viral chemokine receptors may decrease the concentration of chemokines at the site of infection thereby decreasing chemotaxis of immune effector cells to the area. Conversely, the expression of viral chemokine receptors on infected cells may modify the chemotactic nature of the infected cell thus assisting in the dissemination of the virus to different tissues. The predicted seven transmembrane structure of the protein encoded by ORF A5 of AlHV-1 is suggestive of a viral chemokine receptor. However, the function of ORF A5 has yet to be determined

The co-evolution of viruses with their hosts has generated viral proteins that can interrupt signalling pathways and alter cell function to benefit virus replication and persistence. The importance of this mechanism is demonstrated by the range of viruses that encode protein kinase binding proteins. The polyomavirus middle T antigen and the human T-cell leukaemia virus (HTLV-1) protein TAX have been

shown to interact with protein tyrosine kinases (PTKs) (Courtneidge *et al*, 1991; Sinclair and Farrell, 1995). The best examples within herpesviruses come from studies of EBV and HVS. The EBV latency protein LMP2A is constitutively phosphorylated and associates with the tyrosine kinases, *lyn* and *syk*, in B cells. A reduction in *lyn* activity was also observed in EBV-transformed cells and LMP-2A blocked reactivation of the virus (Miller *et al*, 1994, 1995). Thus, LMP-2A acts as a factor for the maintenance of latency. The association of altered cellular kinase activity with virus infection has also been noted in HVS-transformed cells and will be discussed in more detail in the next section.

1.3.3 Herpesvirus saimiri (HVS) - mediated host cell modulation

The gammaherpesvirus, herpesvirus saimiri (HVS) has attracted attention from those studying MCF. HVS epidemiology is summarised by Falk *et al* (1972). HVS infects the majority of squirrel monkeys without apparent disease. The transmission of this virus to marmosets induces a lymphoproliferative disease with similar pathological features to MCF. In addition, the disease cannot be transmitted between marmosets and the virus infects T cells which can be propagated *in vitro* as LGL cell lines. The characteristics of HVS-transformed T cells have some similarities to LGL cell lines derived from cases of MCF. HVS-transformed cells have a variable dependency on exogenous IL-2 and a requirement for high cell densities to achieve optimum growth (Meinl *et al*, 1995). HVS-transformed T cells exhibit a hypersensitivity to CD2-mediated stimulation (Mittrücker *et al*, 1992). Given such similarities, it is possible to speculate that the diseases induced by HVS and the MCF viruses could share some common elements of pathogenesis.

Extensive study of HVS shows that this virus uses a wide range of mechanisms for immune modulation that have also been demonstrated in other herpesviruses. For example, the HVS proteins encoded by ORF 04 and 15 exhibit strong homology (up to 69% amino acid identity) to cellular proteins involved in complement regulation, i.e. CD55 and CD59 respectively (Albrecht and Fleckenstein, 1992). CD55 (or decay accelerating factor - DAF) inhibits C3 convertase while CD59 inhibits C9 activation and subsequent assembly of the membrane attack complex (MAC). Both viral proteins show functional as well as

sequence homology and cells transfected with these proteins were protected from complement mediated cytolysis (Fodor *et al*, 1995; Rother *et al*, 1994).

HVS also encodes a secreted protein in ORF 14 that exhibits MHC II binding activity and elicits non-specific T cell expansion (Yao *et al*, 1996) which may lead to the diversion of the immune system away from virus-specific responses. Other HVS proteins display homology to Bcl-2 (Nava *et al*, 1997), to the cytokine IL-17 (Knappe *et al*, 1998; Rouvier *et al*, 1993; Yao *et al*, 1995) and to a chemokine receptor, IL-8R (Nicholas *et al*, 1992). HVS also induces the expression of a cellular homologue to IL-10 in transformed T cells (Knappe *et al*, 2000).

The transforming ability of HVS was mapped to genes within the left terminal region of the unique DNA (Jung *et al*, 1991). The variability of this region is the basis for the classification of HVS into three subgroups - A, B and C. Transformation of susceptible monkey T cells can be achieved by all three subgroups but only subgroup C strains have demonstrated this activity in human T cells (Biesinger *et al*, 1992). Two proteins encoded in this area of the HVS genome are associated with transforming activity. They are the HVS transforming protein (STP) and the tyrosine kinase interacting protein (Tip) - both are products of a bicistronic mRNA encoded by ORF 1. Both these proteins are required for transformation of human T cells (Medveczky *et al*, 1993). Interestingly, the closely related herpesvirus ateles also encodes a protein (Tio) with homology to Tip and STP (Albrecht *et al*, 1999; Albrecht, 2000), which may also explain its ability to infect and transform T cells.

The characteristics of HVS-transformed cells may be attributed to the affinity of STP and Tip for *lck* as it is involved in T cell receptor, CD2, CD4 and IL-2 receptor-mediated signalling (Biesinger *et al*, 1990, 1995; Jung *et al*, 1995a; Lund *et al*, 1996). The constitutive activation of, and interaction with, STAT-1 and STAT-3 (Heim, 1996) by Tip is also dependent on *lck* and the presence of the *lck*-binding domain on Tip (Lund *et al*, 1997,1999). Some authors have reported the upregulation of *lck* activity (Guo *et al*, 1997; Noraz *et al*, 1998; Wiese *et al*, 1996). This would support the role of Tip (in co-operation with STP) as a transformation factor (Duboise *et al*, 1998a). The finding that Tip and STP mediate IL-2 independence in HVS-transformed T cells also supports this hypothesis (Lund *et al*,

1996; Medveczky *et al*, 1993). Meanwhile, others have reported the downregulation of *lck* kinase activity (Duboise *et al*, 1998b; Jung *et al*, 1995b). If this is the case, then Tip may act in a similar way to LMP-2A of EBV by blocking virus reactivation and maintaining latency.

Herpesvirus saimiri transforming protein (STP) is encoded by the two subgroups A and C. The STP of subgroup C (STP-C488) forms a complex with a downstream signalling molecule called p21-*ras*. Subsequent activation of *ras* by STP-C488 is associated with cell transformation (Jung and Desrosiers, 1995). Meanwhile, the STP of subgroup A (STP-A11) may induce transformation by a different mechanism. It was shown that interaction with *c-src* via an SH2 binding domain allows binding of STP-A11 to *lck* and another T cell-specific tyrosine kinase, *fyn* (Lee *et al*, 1997). Interaction with *lck* is clearly an important mechanism as other viruses (HIV and myxomavirus) also modulate *lck*-mediated signalling (Barry *et al*, 1995; Greenway *et al*, 1995). Finally, viral enhancement of CD2 mediated stimulation (e.g. via its ligand CD58 on neighbouring cells) could induce autocrine growth of transformed cells (Mittrücker *et al*, 1992) and may explain the requirement for high cell densities to achieve optimum growth.

These examples illustrate that while the whole sequence of a viral protein may have little homology to cellular proteins, the presence of small sequence motifs with homology to cellular binding domains may be enough for viral proteins to disrupt normal cell functions. The mechanisms of HVS-mediated host cell modulation have similarities to other herpesviruses including EBV. However, comparison of the HVS and AlHV-1 genomes illustrates significant differences in that homologous proteins to HVS transforming proteins, cytokines and complement regulatory proteins are not found in AlHV-1. Therefore, it is likely that AlHV-1 encodes unique effector proteins that can generate similar functions to that seen in HVS-transformed cell lines. The viral immunomodulatory proteins mentioned here are summarised in Table 1.3.

Table 1.3: Herpesvirus immunomodulatory proteins/genes (continues on next page)

Gene / Protein	Function (homology)	Virus
EBNAs/ LMPs	Cell transformation	Epstein-Barr virus (EBV)
Tip/ STP	"	Herpesvirus saimiri (HVS)
BHRF1	(Bcl-2 homology)	EBV
ORF16	"	HVS
ORF16	"	Human herpesvirus8 (HHV8)
ORFE8	"	Equine herpesvirus2 (EHV2)
gE/gI	Antibody binding	Herpes simplex virus1/2 (HSV1/2)
gI	"	Varicella zoster virus (VZV)
M138	"	Murine cytomegalovirus (MCMV)
gC	Complement binding /regulation	HSV1/2
ORF4 (*CD55*), ORF15 (*CD59*)	"	HVS
BZLF2	MHC-II binding	EBV
ORF14	"	HVS
UL18	(MHC-I homology)	Human cytomegalovirus (HCMV)
M144	"	MCMV
IE12	MHC-I binding	HSV

For references see text (sections 1.3.2 and 1.3.3). EBNAs - Epstein-Barr virus nuclear antigens. LMPs - latent membrane proteins.
Note: viral proteins are indicated in italic and homologies are indicated within parenthesis.

Table 1.3 Herpesvirus immunomodulatory proteins/genes (continued)

Gene / Protein	Function (homology)	Virus
BCRF1 (*IL-10*)	(Cytokine homology)	EBV
ORFE7 (*IL-10*)	"	EHV2
ORF13 (*IL-17*)	(Chemokine homology)	HVS
ORFK4 (*MIP-1α*), ORFK4.1 (*MIP-1β, MCP*), ORFK6 (*MIP-1α*)	"	HHV8
US27, US28, UL33, UL78	(Chemokine receptor homology)	HCMV
M33, M78	"	MCMV
U12/ U15	"	Human herpesvirus6/7 (HHV6/7)
BILF1	"	EBV
ORF74 (*IL-8R*)	"	HVS
ORF74, ORFE1, ORFE6	"	EHV2
ORF74	"	HHV8
LMP2A	Protein kinase binding	EBV
Tip/ STP	"	HVS
Tio	"	Herpesvirus ateles (AtHV)

1.4 Objectives and strategies

In the absence of defined viral virulence proteins, the primary objective of this project was to investigate how the MCF viruses (AlHV-1 and OvHV-2) interfere with proliferation and cytotoxicity of large granular lymphocyte (LGL) cell lines derived from MCF-affected cattle and rabbits.

1.4.1 Specific objectives:

To characterise the effect of virus infection on host cells by comparing phenotype and functional differences between infected LGL cell lines and control (uninfected) cells (Chapter 3). This work aimed to build on the previous observation that IL-2 mRNA was not expressed in OvHV-2-infected cattle cell lines (Schock, 1996). The study will extend these findings by asking whether virus-mediated dysregulation of the cytokine network or interference with signal transduction pathways is responsible for the phenotype of the cell lines. In light of the IL-2-independence of some LGL cell lines and the similarities of MCF viruses to other gammaherpesviruses, two objectives were proposed.

- Firstly, the work in this study examined the possible role of the cytokine IL-15 in the maintenance of LGL phenotype and function. IL-15 is functionally similar to IL-2 and stimulates the proliferation and cytotoxicity of T cells. The ability of IL-15 to maintain the phenotype of the LGL and its involvement in an autocrine or paracrine loop was tested by growth response assays, immunocytochemistry and RT-PCR (Chapter 3).

- Secondly, as viral interference with signal transduction pathways has been demonstrated in other gammaherpesviruses the work presented here aimed to determine if MCF virus infection altered the activation of some T cell-specific protein kinases. An indication of the involvement of signal transduction pathways in general was tested in pilot studies and investigated the effect of biochemical inhibitors of signal transduction on IL-2-independent growth. In

addition, the presence of tyrosine phosphorylated proteins in infected and uninfected control cells were compared. Specific kinase activity of immunoprecipitated protein kinases was then assessed by phosphotransferase assays measuring the kinase-mediated transfer of radioactively labelled phosphate to a substrate (Chapter 4).

LGL cell lines were obtained from cattle naturally infected with OvHV-2 and rabbits experimentally infected with AlHV-1 or OvHV-2. In order to determine the effect of virus proteins on LGL phenotype and function (and to remove stimulus due to IL-2), the cells in these experiments were cultured in the absence of exogenous IL-2 before use.

During the course of this study, the genomic DNA sequence of AlHV-1 was published (Ensser *et al*, 1997). Prior to this publication, a region of the AlHV-1 genome had been characterised that underwent rearrangement upon passage of the virus in culture. This rearrangement was associated with loss of virulence (Handley, 1993: Handley *et al*, 1995). Three proteins encoded in this region were of particular interest as potential virulence factors. Given this information a further objective was:

- To produce the three putative virulence proteins (encoded in ORF50, A6 and A7) in an *E coli* expression system and to produce specific rabbit polyclonal antibodies (Chapter 5). Using these antibodies the expression of the proteins was determined (by immunofluorescence) in virus infected bovine turbinate cells and LGL cell lines. Expression at protein level was compared to expression at the mRNA level (determined by RT-PCR). The involvement of these proteins in the ability LGL cell lines to transmit disease was thus tested (Chapter 6).

Chapter 2

Materials and methods

2.1 Animals and viruses

2.1.1 Viruses

AIHV-1:

The WC11 strain of AlHV-1, first isolated from blue wildebeest, has been extensively passaged in fibroblast cultures and can be harvested as a cell-free virus (Plowright, 1968; Plowright *et al*, 1960). WC11 is attenuated in terms of its ability to induce disease in susceptible animals. The C500 strain was first isolated from an infected ox (Plowright *et al*, 1975). Throughout short-term passage (up to pass no. 5) in bovine turbinate (BT) cultures this virus is cell associated and becomes cell-free after extensive passage. C500 (at low passage) is fully virulent in terms of its ability to induce disease in susceptible animals while C500 (at high passage) is attenuated. 'Virulence' and 'attenuation' -as defined above- was determined using 10^4 PFU (plaque forming units) of low pass C500 or 5×10^7 TCID$_{50}$ (50% tissue culture infective virus dose) of high pass C500 as inoculum in rabbits. Rabbits inoculated with the stated doses of low pass C500 virus died of MCF while those inoculated with high pass C500 virus survived without apparent disease. These doses represent an excess of virus in the high pass C500 inoculum compared to the low pass C500 inoculum thus reducing the possibility that 'attenuation' of high pass C500 was due to lower virus dose (H. Reid, personal communication).

For infection of rabbits, the virulent C500 strain was used and came in the form of spleen and/or lymph node cell suspensions from rabbits with AlHV-1-induced MCF. Cell suspensions were stored in liquid nitrogen in Iscove's modification of Dulbecco's medium (IMDM) (Gibco BRL, Paisley, UK) supplemented with 40% foetal bovine serum (FBS) and 10% dimethyl sulphoxide (DMSO) until required. For the generation of rabbit LGL cell lines, the virulent C500 strain was maintained by repeated passage in rabbits as pathogenicity is lost on repeated culture in BT cells. The WC11 and C500 isolates at low passage (pass no 4 or 5) and high passage (pass no. >350) were also used to infect bovine turbinate (BT) cells. Details for the culture of virus in BT cells or LGL cell lines are described in Chapter 2.2 ('cell culture').

OvHV-2:

OvHV-2 has never been isolated as a free virus but viral DNA can be detected in large granular lymphocyte (LGL) cell lines derived from naturally and experimentally infected animals (i.e. cattle and rabbits).

Infection of rabbits with OvHV-2 was achieved using lymph-node/ spleen cell suspensions from rabbits infected with OvHV-2-induced MCF. These cell suspensions were handled and stored in the same manner as the C500-infected cell suspensions described above. OvHV-2-infected rabbit LGLs were also generated in the same manner as AlHV-1-infected cell lines.

2.1.2 Rabbits

Specific pathogen-free 3-12 month old male and female New Zealand white rabbits were inoculated intravenously with pathogenic AlHV-1 or OvHV-2 in lymphocytes as described above. The inoculum consisted of approximately 10^7 spleen or lymph node cells from rabbits with MCF, which contained approximately 10^4 plaque forming units (infective doses) of virus. The rectal temperature was recorded each day and the animals killed after 2 days of febrile response ($>40^{\circ}$C). LGL cell lines were established from these rabbits (Table 2.1).

For the preparation of polyclonal antibodies, the rabbits were immunised intramuscularly with an emulsion of complete then incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI) mixed with equal parts antigen and administered four weeks apart. Four weeks later an intravenous boost of antigen with no adjuvant was given. Serum was tested two weeks later for the presence of specific antibody. The nature and concentration of antigen and the reactivity of serum are described in Chapter 5. Animals were killed using CO_2 when the serum was collected for antibody purification (Chapter 2.4.7).

Table 2.1: MCF large granular lymphocyte (LGL) cell lines used in this study.

Cell line (species)	Date in culture	Virus (strain ref. no.)
BJ652 (rabbit)	16.11.88	AlHV-1,C500 (BJ190)
BJ926 (")	02.04.93	AlHV-1,C500 (1303a)
BJ1001 (")	31.01.94	AlHV-1,C500 (1330)
BJ1263 (")	17.11.95	AlHV-1,C500 (1365)
BJ1859 (")	16.10.98	AlHV-1,C500 (BJ1803)
BJ1860 (")	19.10.98	AlHV-1,C500 (BJ1803)
BJ1265 (")	28.11.95	Indonesian OvHV-2 (1362)
BJ1857 (")	15.10.98	European OvHV-2 (BJ667)
BJ1035 (cattle)	23.06.94	European OvHV-2 (94/139)
BJ1044 (")	04.07.94	European OvHV-2 (94/154)
BJ1104 (")	01.12.94	European OvHV-2 (94/369)
BJ1196 (")	24.07.95	European OvHV-2 (95/320)

2.1.3 Cattle

Selected clinical cases of bovine MCF referred by the Scottish Agricultural College Veterinary Services and practising veterinary surgeons were transported to the Moredun Research Institute. After post-mortem examination, affected tissues were removed aseptically. Single cell suspensions were prepared from spleen, retropharyngeal, prescapular, prefemoral and mesenteric lymph nodes (Chapter 2.2.3). The established bovine (OvHV-2-infected) MCF LGL cell lines used in this study (Table 2.1) were derived from these tissues.

2.2 Cell culture

2.2.1 Media

For wash medium, 500ml of sterile Hank's balanced saline solution (HBSS) was supplemented with 5% (v/v) foetal bovine serum (FBS), 200U/ml penicillin, 200μg/ml streptomycin (Gibco BRL, Paisley, UK) and 10U/ml heparin (Sigma-Aldrich, Gillingham, UK).

Routine culture medium was Iscove's modified Dulbecco's medium (IMDM). A 500ml bottle of IMDM was supplemented with 10% (v/v) foetal bovine serum, 200U/ml penicillin, 200μg/ml streptomycin, 10μM 2-mercaptoethanol and 0.5μg/ml fungizone (Gibco BRL, Paisley, UK). Foetal bovine serum was free of bovine diarrhoea virus (BVDV) and batch selected to ensure optimum growth of MCF virus-infected LGL cell lines.

2.2.2 Cell culture conditions

Cells were incubated in 25cm^2 flasks (Costar UK Ltd, High Wycombe, UK), 24-well cell culture plates or 96-well flat-bottomed cell culture plates (Costar UK Ltd, High Wycombe, UK) in a humidified atmosphere of 5% CO_2 at 37°C.

2.2.3 Preparation of control cells

2.2.3.1 Cattle and rabbit peripheral blood mononuclear cells (PBMC)

Cattle PBMCs were prepared from fresh heparinised peripheral blood. The buffy coat was collected after centrifugation of 10mls whole blood at 1400 x g for 30 minutes. This fraction was then diluted 1:1.5 with sterile phosphate buffered saline (PBS) and layered onto Lymphoprep (Nycomed, Oslo, Norway) in an equal volume and centrifuged at 1400 x g for 30 minutes. The interface layer of mononuclear cells was collected and washed twice in HBSS wash medium. These cells were used immediately as 'resting' cell controls without further culture. Alternatively, cells were counted in a haemocytometer and re-suspended in IMDM at a density of 2×10^6 cells/ml. To stimulate the cells, the T cell mitogen concanavalin A (conA) (ICN Biochemicals, Cleveland, OH) was added at 5µg/ml in IMDM to the cell cultures in 24-well plates. After 72 hours in conA, cells were washed in IMDM and maintained in IMDM at a density of 5×10^5/ml with 20U/ml human recombinant interleukin-2 (hrIL-2) (Eurocetus UK Ltd, Harefield, UK). The lymphoblasts that developed were used in experiments as control cells. These cells were also phenotyped by flow cytometry (as described in Chapter 2.3.1)

Rabbit spleen, mesenteric or popliteal lymph nodes were removed aseptically. The tissue was chopped finely with scissors and passed through a stainless steel sieve. After one wash and re-suspension in wash medium (HBSS) the cells were layered onto 'Lymphoprep', mononuclear cells collected and stimulated with conA as described above to generate lymphoblasts.

2.2.3.2 Rabbit and mouse adherent cells

For the detection of IL-15 in rabbit and cattle MCF LGL cell lines (Chapter 3) rabbit adherent cells or mouse fibroblast cells (LTK⁻) were chosen as positive controls.

Rabbit monocytes (contained in the adherent cell population) were isolated from lung and peritoneal lavage samples. Briefly, crude cell suspensions were washed in IMDM (without serum) at 4°C and resuspended to a cell density of 10^6/ml. Cells (1ml) were plated out on coverslips in a 24-well plate. 10% FBS was added to

the cultures and the cells were incubated at 37°C with 5% CO_2 overnight to allow the attachment of adherent cells to the coverslips. Non-adherent cells were removed by 2-3 washes in IMDM. The cultures were then stimulated with 10μg/ml bacterial lipopolysaccharide (LPS) (Sigma Aldrich, Gillingham, UK) for 18-24 hours. The coverslips were then washed in PBS and fixed in 50: 50 methanol: acetone for 5-10 minutes. Once dried the coverslips were stored at -20°C until ready for use.

Mouse LTK⁻ cells (Spandidos and Wilkie, 1983) were prepared in a similar fashion. An aliquot of LTK⁻ cells stored in liquid nitrogen were thawed and cultured at 5×10^6 cells/ml in IMDM + 10% FBS on coverslips and fixed as above.

2.2.4 Maintenance of MCF LGL cell lines

Maintenance of MCF LGL cell lines (which grow in suspension) was based on methods previously determined by Reid *et al* (1989b). All MCF LGL cell lines were grown in 25cm² flasks or 24-well plates and maintained in IMDM. Half of the medium was replaced with fresh medium once a week and if required the cultures were split (when cell densities reached 2×10^6/ml or more). Cell lines were maintained in 350U/ml of hrIL-2 (Eurocetus UK Ltd, Harefield, UK) for optimum growth. Approximately a week prior to use, cells were transferred to medium containing 20U/ml of hrIL-2 to maximise any viral effect on cell phenotype and minimise the effect of hrIL-2 on the cells. However, for the IL-15 (Chapter 3) and kinase (Chapter 4) studies, cell lines were deprived of hrIL-2 to establish short or medium-term IL-2-independent cell cultures. For these cultures, cells were set up at 1×10^6/ml after washing in culture medium. Half the total culture volume of medium was replenished with fresh medium approximately once a week. Cultures were readjusted to 10^6 cells/ml when required.

2.2.5 Infection of bovine turbinate (BT) cells with AlHV-1

BT cells between pass no. 5-20 were seeded at a density of 10^5cells/ml in 1ml IMDM + 10% FCS on coverslips (21 x 6mm) and incubated at 37°C until monolayers were formed. A 200μl aliquot containing 2×10^4 BT cells infected with cell-associated AlHV-1 C500 (low pass) (i.e. pass no. 5) was used to infect the BT

cell monolayers until 50 - 70% of the monolayer showed cytopathic effect (CPE). For the infection of BT cell monolayers with cell-free C500 (high pass) (pass no. 340 or higher) a 200µl aliquot of a 1:4 dilution of culture supernatants from an C500-infected BT cell culture (showing maximum CPE) was added to the monolayers. For the infection of BT cells the titre of virus in this inoculum is not routinely quantified in this laboratory as cultures are fixed when similar levels of CPE have been achieved.

For the infection of BT cells with cell-free WC11 (purified virus), 4mls of a liquid nitrogen stock of WC11 was added to a 5mls suspension of BT cells (1.5×10^7 cells) in IMDM. The titre of WC11 was between 10^{-5} - 10^{-6} TCID$_{50}$/ml. The mixture was incubated and shaken several times at 37°C for 60mins to allow adsorption to proceed. The suspension was diluted to 10^5 cells/ml and FCS added to a final concentration of 10%. These cells were allowed to form monolayers on coverslips as before until 50-70% CPE was reached.

At this stage, coverslips were harvested and fixed in cold acetone for 5-10 minutes and stored at -20°C with silica gel until required. Once thawed the coverslips were mounted onto glass slides with a permanent mountant for easy handling.

2.2.6 Determination of cell growth and viability

50µl samples of cells were mixed with an equal volume of 0.1% (w/v) nigrosine (Merck Ltd, Lutterworth, UK). Dead cells were determined by the incorporation of nigrosine into the cells. Live and dead cells were counted in a haemocytometer. The density of live cells and percentage viability (i.e. number of live cells / number of live + dead cells) were calculated.

2.3 Cell phenotype analysis

2.3.1 Flow cytometry (FACS analysis)

FACS (or fluorescence-activated cell sorting) analysis allows the detection of cell surface antigens using fluorescently-labelled antibodies. The FACScan can distinguish between cell types according to cell size and density and can measure the intensity of fluorescent signal from each cell analysed. Furthermore, rapid analysis of large numbers of cells can be achieved. Therefore, the expression of a particular antigen within a population of cells can be determined by this method.

Cells were washed and re-suspended in FACS wash buffer (Earls medium with 2% foetal bovine serum and 0.1% sodium azide) to a density of 5 x 10^5/ml. In round-bottomed 96-well plates (Gibco BRL, Paisley, UK), cells were incubated with 50µl of the desired primary antibody (for details see Chapter 3) prepared in FACS wash buffer for 30 minutes (to 2 hours) on ice. The cells were centrifuged at 250 x g for 5 minutes and the supernatant removed. The pellets were washed twice in FACS wash buffer. The fluorescein isothiocynate (FITC) conjugated secondary antibody (rabbit-anti-mouse 1:50) (Dako Ltd, Ely, UK) was diluted in FACS wash buffer, added to the cells and mixed thoroughly. This mixture was incubated on ice for 30 minutes in the dark. After a further two washes the cells were fixed in 1% paraformaldehyde in wash buffer and transferred to FACS tubes (Becton Dickinson, Mountain View, CA). Analysis was performed on 10000 cells with a Becton Dickinson FACScan (Mountain View, CA). Lymphocytes and blast cell populations were distinguished on the basis of the forward scatter/ side scatter profile. The FITC green fluorescence (FL-1) on a logarithmic scale was plotted against cell numbers.

2.3.2 Immunofluorescence antibody test (IFAT)

IFAT is similar to FACS analysis in that it detects the expression of cellular antigens using fluorescently labelled antibodies. In this case, the labelling took place on fixed immobilised cells as opposed to cells in suspension. This technique has an added advantage to FACS analysis in that sub-cellular localisation of antigens may

be determined. This technique was used for the detection of viral antigens in MCF LGL cell lines and in MCF virus-infected BT cells.

Cell suspensions (at a density of 5×10^5/ml) were cytocentrifuged onto slides and fixed in cold acetone for 5-10 minutes. In a moist box, 50-75µl 1:20 blocking serum in PBS was added and slides incubated at 37°C for 30 minutes. The type of blocking serum was determined by the species from which the secondary antibodies were derived. After two washes in PBS, excess moisture was removed and the same volume of the desired primary antibody was added (for details on antibodies used see Chapter 3 and 6). This process was repeated for the FITC-conjugated secondary antibody (1:40) (Dako Ltd, Ely, UK and SAPU, Carluke, UK). The slides were mounted with Citifluor (UKC Chem. Lab., Canterbury, UK) and viewed under a UV microscope.

2.3.3 Determination of cell morphology by Leishman's staining

Cell suspensions (at a density of 5×10^5/ml) were cytocentrifuged onto slides and cell morphology was revealed by staining the cells with Leishman's stain. For this, slides were incubated in undiluted Leishman's stain for 2 minutes followed by an incubation of 10 minutes in Leishman's stain diluted 1:2 with water. After rinsing in water and once dry the slides were mounted with Coverbond mounting media (American Scientific Products, McGraw Park, IL.). The cells were then examined by light microscopy

2.3.4 BLT (benzyloxycarbonyl-L-lysine thiobenzyl) esterase assay

The BLT esterase assay was used to identify cells expressing the cytotoxicity-associated enzyme, BLT esterase – a tryptase. This assay reveals the presence of the serine protease (tryptase) in cytoplasmic vesicles or granules. It is detected as a yellow stain that can be seen in cells by microscopy or measured in lysed cells or cell-free supernatants by absorbance at 412nm.

BLT esterase detection by cytochemistry:

Cytocentrifuge preparations were made from a cell sample of each cell line and cells were fixed with cold acetone for 5 -10 minutes. The assay was performed by

incubation of slides in BLT buffer (4.2mg BLT ester in 50ml, 0.2M Tris pH8.1 + 0.02% Fast Blue) at 37°C for 30 minutes. After thorough washing of slides the cells were counter-stained with haematoxylin for 2mins and observed under a light microscope. BLT esterase is identified by a yellow stain in the cytoplasmic vesicles or granules.

BLT esterase quantification in cell lysates:

In the IL-15 dose-response assays (see Chapter 3.2.7), 50µl of culture supernatant and cell lysate (from 10% of the total population of cells per well) were taken to measure the BLT esterase content. To each sample, 200µl of 0.3mM BLT ester, 0.3mM dithio-bis-2-nitrobenzoic acid in PBS pH7.2 was added (at saturating substrate concentration as described by Haig *et al*, 1996). Following an incubation of 1 hour at 37°C in 97-well flat-bottomed plates, the reaction was stopped by the addition of 5µl, 5mM 'Pefabloc' (Boehringer Mannheim, Mannheim, Germany). BLT esterase levels were quantified by absorbance at 412nm on an ELISA plate reader.

2.3.5 Immunocytochemistry

Immunocytochemistry was performed on cytocentrifuge preparations of fixed cells for the detection of IL-15-producing cells (see Chapter 3).

After an initial wash in PBS/0.05%Tween 80 the cells were incubated in 1:50 normal horse serum and normal rabbit serum in PBS/high salt buffer (0.5M NaCl in PBS) for 30mins at 25°C to block non-specific antibody binding sites. Normal goat IgG was used as negative control. Cells were incubated for 30 minutes at 25°C in goat anti-human IL-15 (Santa Cruz Biotechnology, Santa Cruz, CA), specific for a carboxy terminal peptide of the human IL-15 precursor, diluted in PBS/high salt over a range of concentrations (for details see Chapter 3.2). Following two 10 minute washes in PBS/Tween80, the cells were incubated as before in a biotinylated secondary antibody diluted in PBS/high salt. A Vectastain ABC-alkaline phosphatase kit and Vector Red substrate (Vector Laboratories, Peterborough, UK) was used to detect bound antibodies.

2.4 Protein analysis

2.4.1 Activation of cells, preparation of cell lysates and immunoprecipitates

5×10^6 or 10^7 LGL cells (grown with or without hrIL-2) were washed twice in serum-free medium (IMDM) then allowed to rest for 5 minutes at 37°C. For maximal stimulation of T cells different concentrations of concanavalin A (conA) (a T cell mitogen) ± TPA (a phorbol ester) were added for varying lengths of time at 37°C in 1ml serum-free medium (for details see Chapter 4). Stimulation was stopped by rapid removal of medium from the cell pellets and then placing the tubes on ice. For immunoprecipitations, 1ml cold lysis buffer (1% Triton X-100 or IGEPAL-CA630, 10mM Tris pH7.2, 150mM NaCl, 1mM activated Na_3VO_4, 1mM NaF) containing protease inhibitors ('Complete^{TM}', Boehringer Mannheim, Mannheim, Germany) was added to cell pellets and mixed by vortexing for 10 seconds. Cell lysates to be used for the detection of proteins in whole lysates were lysed in 50-100µl of lysis buffer. The protease inhibitors came in the form of a tablet that is dissolved in 25mls of lysis buffer prior to use. This tablet contained an mixture of serine, cysteine and metalloprotease inhibitors (the manufacturers were unwilling to specify the identity of the inhibitors used in this tablet). After 20 minutes on ice the samples were vortexed and passed through a 26G needle and returned to ice. Another 20 minutes later cellular debris was removed from the lysates by centrifugation at 12000 x g for 15 minutes. Protein concentrations in the 50-100µl lysates as determined by the 'Pierce' assay (Chapter 2.4.8) were between 10-14mg/ml and in the 1ml lysates between 1-2mg/ml.

To minimise immunoprecipitation of non-specific proteins, each cell lysate was cleared by adding an agarose-IgG complex made from 10µl normal whole serum (from the same species as the precipitating antibody) added to 20µl 'protein A/G-PLUS-agarose' (Santa Cruz Biotechnology, Santa Cruz, CA). This mixture was incubated at 4°C for 30minutes and the IgG-agarose complexes removed by centrifugation at 12000 x g for 30s. Specific proteins were immunoprecipitated from the cleared lysate using specific antibodies at the concentrations shown in Chapter 4. After a 1hr incubation at 4°C, all primary antibody-antigen complexes were

precipitated by the addition of 20μl 'protein A/G-PLUS-agarose' (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour or overnight at 4°C with constant agitation. The resulting protein-agarose complexes were washed three times by repeated resuspension in lysis buffer and centrifugation at 10000 x g for 30seconds. Agarose pellets were either boiled in reducing Laemmli sample buffer (for use in Western blot analysis - Chapter 2.4.4) or used in phosphotransferase assays (Chapter 2.4.5).

2.4.2 Sodium dodecylsulphate - polyacrylamide gel electrophoresis (SDS-PAGE)

The separation of proteins was performed on the Mini Protean II system (BIO-RAD, Hemel Hempstead, UK) by the method described by Laemmli (1979). Briefly, samples (whether whole cell lysates or immunoprecipitated material) were boiled in reducing Laemmli sample buffer (62.5mM Tris-HCl pH7.8, 10% w/v sucrose, 2% w/v SDS, 0.001% w/v bromophenol blue, 5% v/v β−mercaptoethanol prior to loading onto gels. Depending on the molecular weight of the protein of interest, proteins were separated on a 10%, 12% or 15% acrylamide gel made in separating gel buffer (0.375M Tris-HCl pH8.8, 1% w/v SDS, 0.1% v/v TEMED, 0.1% w/v ammonium persulphate (APS)) at 200V for 45-50mins in tank buffer (25mM Tris, 200mM Glycine, 5mM SDS). The separating gel was overlaid with a 4% acrylamide stacking gel made in stacking gel buffer (0.15M Tris-HCl pH6.8, 0.5% (w/v) SDS, 0.2% (v/v) TEMED, 0.2% (w/v) APS) at all times.

Separation of proteins by Serva Blue-SDS-PAGE:

This method of SDS-PAGE was used for the separation and purification of recombinant proteins (see Chapter 5). The use of Serva Blue (a dye that binds to proteins) in the upper tank buffer allows protein bands to be visualised in the gel during separation and without using harsh fixatives as in Coomassie blue staining (see Chapter 2.4.3).

Samples in non-reducing Laemmli sample buffer (62.5mM Tris-HCl pH7.8, 10% sucrose, 2% SDS, 0.001% bromophenol blue) were loaded into a single large

well on a BIORAD Mini Protean II system using a thick comb (1.25mm). Up to 400µl total volume was loaded per gel. The upper tank was filled with cathode buffer (3.08g/l Tris, 14.4g/l glycine, 0.05% SDS, 24mg/l Serva Blue - Sigma Aldrich, Gillingham, UK) and the lower tank filled with tank buffer as for a traditional SDS-PAGE. Molecular weight standards (BioRad, Hemel Hempstead, UK) were loaded in a small well at the end of the gel to help with identification of proteins. Once identified, the relevant protein band was excised from the gel and stored at -70°C until required.

2.4.3 Protein detection in SDS-PAGE gels

Proteins in SDS-PAGE gels were detected by Coomassie brilliant blue solution (Sigma Aldrich, Gillingham, UK) or silver staining. For Coomassie staining of proteins, gels were incubated in Coomassie Blue solution for 10 minutes, with agitation. Non-specific staining was removed by several washes in de-stain (30% (v/v) methanol, 12% (v/v) acetic acid). For a more sensitive detection method, silver staining was used. Firstly, the proteins in the gel were fixed with silver stain 1st fix (50% v/v methanol, 10% v/v acetic acid) for 30 minutes followed by 15 minutes in silver stain 2nd fix (5% v/v methanol, 7% v/v acetic acid). After a 10 minute wash in distilled water (DW) the proteins were reduced in dithiotreitol (DTT, 5µg/ml) for 15 minutes and washed again in DW. The gel was stained with 0.1% silver nitrate solution for 20 minutes and rinsed in DW several times. Proteins were developed in silver stain developer (0.3M sodium carbonate, 0.02% w/v formaldehyde). To stop the reaction citric acid powder was added (to a final concentration of 0.1M) to 100mls of developer.

2.4.4 Western blot analysis

Proteins were transferred to a nitro-cellulose membrane (Schleicher & Schuell, Germany) by semi-dry western blot. The gel and membrane were sandwiched between several sheets of filter paper soaked in transfer buffer (25mM Tris, 200mM glycine, 5mM SDS) and then placed in the western blot apparatus. Transfer took place at 70mA per gel for 3 hours. The membrane was blocked with

5% non-fat milk (Marvel) in wash buffer (WB) (0.5M NaCl, 0.5% (v/v) Tween 80 in PBS) for 30 minutes to 1hour. The membranes were incubated in the desired primary antibody for 1 hour then HRP-conjugated secondary antibodies (Dako Ltd, Ely, UK) diluted 1:2000 in wash buffer for 1 hour. Between the blocking, primary and secondary antibody stages, thorough washing in 20-30mls of wash buffer was performed for at least 30 minutes with constant agitation. All membranes were given a brief rinse in PBS prior to detection of HRP by enhanced chemiluminescence - ECL (Amersham International, Little Chalfont, UK) or TMB stabilised substrate (Promega UK Ltd, Southampton, UK).

For the detection of primary antibodies via an alkaline phosphatase amplification system, a 1:2000 dilution of goat anti-mouse IgG conjugated to alkaline phosphatase (Upstate Biotechnology Inc (UBI), Lake Placid, NY) was prepared in wash buffer. Membranes were incubated in this mixture for 1 hour. After a wash in wash buffer for at least 30 minutes followed by several rinses in PBS, the membrane was developed in alkaline phosphatase substrate solution (1% nitro-blue tetrazolium (NBT), 1% bromochloroinodyl phosphate (BCIP) - of stock solution as supplied by UBI - in 100mM Tris pH 9.5, 100mM NaCl, 5mM $MgCl_2$) at room temperature until bands were visible (approximately 10 - 30 minutes). The enzyme reaction was terminated by washing the membrane in distilled water.

2.4.5 Determination of protein kinase activity

Phosphotransferase assay for protein kinases (as used in Chapter 4):

Protein kinases were immunoprecipitated as described in Chapter 2.4.1 and using specific antibodies at the concentrations shown in Chapter 4. The precipitated samples were washed three times in lysis buffer then twice in kinase buffer (20mM Tris/HCl pH7.5, 10mM $MgCl_2$, 5mM $MnCl_2$) prior to use in the phosphotransferase assay.

The activation of kinases was assayed by the transfer of radioactively labelled phosphate groups in $[\gamma\text{-}^{32}P]$-ATP (Amersham International, Little Chalfont, UK) to the *src* kinase substrate, acid-treated enolase (rabbit muscle enolase, Sigma Aldrich, Gillingham, UK). For acid treatment, the enolase was resuspended to $10\mu g/\mu l$ in

enolase buffer (50mM HEPES pH7.0, 1mM DTT, 1mM MgCl$_2$) and incubated on ice for 30-60 minutes. Prior to use, an equal volume of 50mM acetic acid was added and the mixture was incubated at 30°C for 5 minutes then neutralised with 20mM HEPES.

Protein-agarose pellets were resuspended in 25µl kinase buffer containing 25µM ATP, 320µCi/ml [γ-^{32}P]-ATP and 400µg/ml acid-treated enolase. The mixture was incubated at 30°C for 10 minutes. Samples were mixed with reducing Laemmli sample buffer (62.5mM Tris-HCl pH7.8, 10% sucrose, 2% SDS, 62.5mM Tris-HCl pH7.8, 10% sucrose, 2% SDS, 0.001% bromophenol blue, 5% β–mercaptoethanol) for a further 20 minutes at room temperature then boiled for 5 minutes before proteins were separated by SDS-PAGE using 10% or 12% acrylamide gels (Chapter2.4.2). To detect the molecular weight markers and to check for protein loading the acrylamide gel was stained with Coomassie brilliant blue (Chapter 2.4.3). The gel was then dried and exposed to X-ray film for between 2-16 hours, depending on the incorporation of isotope. Kinase activity was detected as a band of γ-^{32}P phosphorylated substrate (enolase) at 48kDa. To quantify the incorporation of γ-^{32}P into enolase, enolase bands were excised from the gel and radioactivity measured by β-scintillation counting on a Tri-Carb liquid scintillation analyzer (Packard, Meriden, CT.) Incorporation of radioisotope into enolase was calculated for each sample as follows:

c.p.m. (enolase + kinase) - c.p.m. (enolase without IP kinase)

[note: IP = immunoprecipitated, c.p.m. = counts per minute]

2.4.6 Electroelution of recombinant proteins

Recombinant proteins separated by Coomassie Blue SDS-PAGE (see Chapter 2.4.3) can be collected from acrylamide gels by application of a current through the excised protein bands. First, gels containing the excised protein band were roughly chopped and placed in the cathode (-ve/ upper) chamber of a BIORAD electroelution unit. Both chambers and the tank unit were filled with tank buffer (25mM Tris, 200mM Glycine, 5mM SDS). A current of 50mA was applied for approximately 30

minutes or until all the blue dye (protein) had moved from the upper to the lower chamber. The electroeluted material was collected, washed in Vivaspin concentrators (with a 10kDa cut-off membrane) (Vivascience, Lincoln, UK) with PBS and stored at 4°C.

2.4.7 Affinity purification of rabbit polyclonal sera on a protein A-sepharose column

Whole serum was purified on a protein A-sepharose column by FPLC (fast performance liquid chromatography) (Pharmacia^{TM}).

The serum was first diluted 1:2 in PBS followed by filtration through a 0.45μm filter. A small 3-5ml column of protein A- sepharose was packed and washed thoroughly with equilibration buffer (50mM Tris, 150mM NaCl, 0.02% azide, pH8.4). Several bed volumes of elution buffer (100mM glycine, 150mM NaCl, 0.02% azide, pH3) and equilibration buffer were passed over the column to remove any material bound to the column. After a final wash in equilibration buffer, 0.5-1ml of filtered serum was loaded onto the column. The flow through (unbound proteins) was collected. At this stage the O.D.$_{280}$ was monitored for all fractions collected to detect proteins. The column was washed through with equilibration buffer until the O.D.$_{280}$ returned to baseline and all unbound proteins were eluted. IgG bound to the column was removed using elution buffer passed through the column at a rate of 1ml/min. Eluted samples were detected by the rise in O.D.$_{280}$, the relevant fractions collected and immediately neutralised with 1M Tris, pH10. Samples were either tested for reactivity against the original antigen or aliquoted and stored (in PBS/0.05% azide, pH7.5) at -20°C for future use. Finally, the column was cleaned and washed with several cycles of equilibration and elution buffer followed by a final wash in PBS/0.02% azide, pH7.5. This column was stored at 4°C until required.

2.4.8 Determination of protein concentration by the 'Pierce BCA assay'

The reaction of protein with copper ions and bicinchoninic acid (BCA) – the two reagents in this assay- produces a change in colour that can be measured by

absorbance at 540nm (Smith *et al*, 1985). The method used was as described in the manufacturer's protocol (Pierce, Rockford, IL). Briefly, 10μl of the test sample was placed in duplicate wells of a 96-well flat-bottomed plate. Bovine serum albumin (BSA) was also placed in duplicate wells and prepared in the same buffer as the test sample over a concentration range of 0.05 - 2.0 mg/ml. To each well 200μl of the Pierce reaction mix (one part 4% w/v $CuSO_4$ in 50 parts BCA solution) was added and mixed thoroughly. The plate was wrapped in film and incubated at 37°C for 30 minutes. Colour change was detected by absorbance at 540nm. The standard curve of BSA concentration against OD_{540} was used to determine the concentration of the test sample.

2.5 Molecular biology techniques

2.5.1 Preparation of total cellular RNA

Cells from control and MCF virus-infected cell cultures were pelleted and the medium was removed. The cell pellets (containing up to 10^7 cells) were homogenised in 5ml of RNA extraction buffer (23.6g of guanidine isothiocyanate (GTC), 0.36ml β-mercaptoethanol, 5ml of 250mM sodium citrate pH7, 2.5ml of 10% w/v N-lauroylsarcosine, 47ml of water) using the technique described by Chirgwin *et al* (1979). Half a milliliter of 2M sodium acetate pH 4.8 were added followed by 5ml of water-saturated phenol and 1ml of chloroform: isoamyl alcohol (24:1) and the mixture shaken vigorously for 5 minutes. After 10 minutes on ice, the solution was centrifuged at 12000 x g for 30 minutes at 4°C. The aqueous phase containing the RNA was removed and the RNA precipitated by the addition of 5ml of isopropanol. The precipitate was pelleted and dissolved in 0.5ml RNA extraction buffer. 0.5ml of isopropanol was added and the mixture was incubated at -70°C for 30 minutes. The resulting precipitate was then pelleted, washed in 70% ethanol and resuspended in 100 μl of autoclaved water.

Quantification of RNA:

Nucleic acid was quantified by use of a spectrophotometer. DNA and RNA have characteristic UV spectra, the peak of absorbance is at 260 nm, while the absorbance at 280mn is a measure of the amount of contaminating protein which is present (Sambrook *et al* 1989). The absorbance at 260nm gives a measure of the concentration of the nucleic acid sample. An absorbance of 1 ODU_{260} corresponds to a concentration of 40 µg/ml for RNA. The ratio OD_{260}/OD_{280} is approximately 2.0 for pure RNA samples.

2.5.2 Analysis of gene expression by reverse transcription polymerase chain reaction (RT-PCR)

First strand cDNA was prepared from total cellular RNA using Expand^{TM} reverse transcriptase (Boehringer Mannheim, Mannheim, Germany). Specific sequences within the cDNA were then amplified using the polymerase chain reaction.

2.5.2.1 cDNA synthesis

Total cellular RNA (5 µg) and 2 µl (1µg) of primer (random hexamers - Promega UK Ltd, Southampton, UK) was placed in a tube and made up to a total volume of 10 µl with sterile distilled water. To remove the secondary structure and allow annealing of the primer to cellular RNA the mixture was heated to 65°C for 10 minutes then allowed to cool on ice. The following were then added in the order indicated, 4µl of 5x Expand^{TM} RT buffer (250mM TRIS-HCl, 25mM $MgCl_2$, 200mM KCl, 2.5% Tween 20, pH 8.3250mM TRIS-HCl, 25mM $MgCl_2$, 200mM KCl, 2.5% Tween 20, pH 8.3), 2µl 100mM DTT, 2µl 10mM dNTPs, 0.5 µl of RNase inhibitor (Promega UK Ltd, Southampton, UK) and 1µl (50 units) of Expand^{TM} reverse transcriptase (RT) - a final volume of 20µl. The mixture was carefully mixed and incubated for 10 minutes at 30°C followed by 45 minutes at 42°C. The reaction was stopped by placing on ice. The sample was then ready for PCR amplification or stored at -20°C for later use.

2.5.2.2 Polymerase chain reaction (PCR)

PCR amplification was performed using the Expand™ long template PCR system (Boehringer Mannheim, Mannheim, Germany). The reaction was set up in a total volume of 50 μl as follows, 3μl cDNA from the 20μl RT reaction, 5μl 10x PCR buffer (Boehringer PCR buffer 3 containing 22.5mM $MgCl_2$ and detergents, pH9.2), 1μl of each primer at 2μM, 2.5μl 10mM dNTPs, 37μl water and 0.75μl Taq DNA polymerase mix (Boehringer Mannheim, Mannheim, Germany). 30 μl of mineral oil was added and the tubes placed in a Hybaid Omnigene thermal cycler.

PCR reactions involved one cycle of denaturation at 93°C for 2 minutes. A further 10 cycles consisted of denaturation at 93°C for 10 seconds, followed by 30 seconds at an annealing temperature calculated to be 10°C below the melting temperature (T_m) of the two oligonucleotide primers, followed by 5 minutes elongation at 68°C. The next 15 cycles involved the same conditions for denaturation and annealing. For higher yields, the elongation step was increased by 20 seconds for each subsequent cycle. A final cycle with a prolonged elongation step of 7 minutes was performed.

Eight microliter samples of the amplified products were analysed on agarose gels and their identity confirmed by digestion at internal restriction sites or by Southern blot and hybridisation to specific probes.

2.5.2.3 Agarose gel electrophoresis of PCR products

DNA fragments resulting from PCR amplification were analysed by conventional agarose gel electrophoresis. Agarose concentration was 1 % w/v and gel dimensions were either 10x7 cms or 20x20 cms. Electrophoresis buffer was TBE (45mM Tris/borate, 1mM EDTA pH7.3) and gels were run at 75 volts for one hour (10x7cm gels) or at 15V overnight (20x20cm gels). Ethidium bromide was added to a final concentration of 0.5μg/ml and the DNA fragments were visualised by their fluorescence under UV illumination. A permanent record was obtained by photographing the gels on heat sensitive paper (Mitsubishi video copy processor, UVP). The size of the DNA fragments was determined by comparison with known standards (1kb ladder - Promega UK Ltd, Southampton, UK).

2.5.2.4 Southern blot analysis

After electrophoresis in agarose gels, DNA was transferred to Hybond-N membranes (Amersham International, Little Chalfont, UK), using a modification of the crush blot method of Smith and Summers (1980). The DNA was denatured in 0.5 M NaOH/1.5 M NaCl for 30 minutes. The gel was transferred to a 1M ammonium acetate/0.02M NaOH solution. After 1 hour, the gel was removed and a piece of Hybond-N, cut slightly bigger than the gel, was placed on top of the gel. Three pieces of 3MM filter paper were cut to the same size as the gel, soaked in the ammonium acetate and placed on top of the membrane. A 10 cm thickness of paper towel was positioned on top of the filter paper and finally a weight was placed on top of the towels. The blot was left overnight for the DNA transfer to take place by capillary action. The next day, the membrane was air-dried and the DNA fixed to the Hybond membrane in a Hybaid UV cross-linker at 70,000 joules/cm^2.

Hybridisation of labelled internal probe for IL-15 to immobilised DNA:

An internal IL-15 cDNA probe representing residues 231-270 based on the sequence of bovine IL-15 was labelled with digoxygenin (DIG). DIG labelling and subsequent hybridisation followed the manufacturer's protocol (Boehringer Mannheim, Mannheim, Germany). The hybridisation fluid was 5xSSC (750mM NaCl, 75mM sodium citrate) containing 0.1% w/v blocking reagent (Boehringer Mannheim) and 0.02% SDS. Following hybridisation, the membranes were washed to remove unbound probe. The membranes were washed at high stringency with 0.1xSSC and 0.1% SDS at 65°C.

Detection of bound DIG labelled probe for IL-15:

DIG labelled probe that had hybridised to an immobilised target was detected immunochemically using a commercial kit (Boehringer Mannheim, Mannheim, Germany). Non-specific binding was blocked by incubating the membrane for 30 minutes in Tris buffered saline (TBS) (100mM Tris/HCl, 150mM NaCl) containing 0.5% (w/v) blocking reagent (Boehringer Mannheim). The membranes were then transferred to TBS containing a 1:5000 v/v dilution of an anti-DIG alkaline phosphatase conjugate for 60 minutes. Unbound conjugate was removed by two 15

minute washes in TBS. The filters were then equilibrated in 100mM Tris/HCl, 150mM NaCl, 50mM MgCl$_2$, pH 9.5. The enzyme substrates were then added, 45µl NTB (75 mg/ml nitroblue tetrazolium salt in dimethylformamide) and 35µl X-phosphate (50mg/ml in 5-bromo-4-chloro-3-indolyl phosphate toludinium salt in dimethylformamide). The filters were left in the dark for a few hours to allow the purple colour to develop fully. The reaction was stopped by washing in Tris/EDTA (TE) buffer (10mM Tris pH 7.5, 1mM EDTA).

2.5.3 Expression of recombinant AlHV-1 proteins in *E. coli*

2.5.3.1 Amplification of AlHV-1 ORF 50, A6 and A7 and ligation into pTrXFus.

For the expression of AlHV-1 proteins in *E.coli* (see Chapter 5) the ThioFusion^{TM} Expression System (Invitrogen, Groningen, Netherlands) was used. The vector pTrXFus contains the following restriction sites - Kpn I, Sma I, BamH I, Xba I, Acc I, Sal I, Pst I. The primers used are detailed in Chapter 5.

In order to ligate the PCR products to the pTrXFus vector DNA and the insert DNA were first digested with two restriction enzymes (see Chapter 5). The restriction enzymes were purchased from Boehringer Mannheim (Mannheim, Germany) and used with the accompanying buffers following the manufacturer's instructions. The final concentration of each enzyme was 5units/µg of DNA, which represents an excess of enzyme. The enzymes made up 10% of the total reaction volume to remove any damaging effects of glycerol on enzyme activity. Reaction volume was adjusted to 20 µl and digestions were incubated at 37°C for 1hr. Approximately 100ng of vector or insert DNA was used for each reaction. The digests were extracted using phenol/chloroform and precipitated using ethanol. The digested DNA was then resuspended in O.1x TE buffer (10mM Tris pH 7.5, 1mM EDTA) to a concentration of 100ng/µl.

Ligation of DNA was performed using T4 DNA ligase. The enzyme catalyses the formation of a phosphodiester bond between a 5' phosphate group and a 3' hydroxyl group resulting in the covalent linking of two DNA molecules that have compatible ends produced by restriction enzyme digestion. Ligation reactions were

set up in 20 µl total volume containing 100ng of vector, insert DNA at an insert : vector ratio of 4 : 1, 2 µl of 10x DNA ligase buffer (660mM Tris/HCl, 50mM $MgCl_2$, 10mM DTT, 10mM ATP, pH 7.5) and 2 units of T4 ligase (Boehringer Mannheim, Mannheim, Germany). Reactions were incubated at 37°C for 2 hours or at 12°C overnight.

2.5.3.2 Preparation of competent *E. coli*

E. coli, strain JM109, (Yannitch-Perron *et al* 1985) were streaked on LB plates and incubated overnight at 37° C. A single colony was picked and used to inoculate 100 ml of LB broth. The bacteria were allowed to grow to an OD_{600} of 0.5 to 0.6 when the cells were transferred to pre-chilled tubes and incubated on ice for 10 min. The bacteria were then centrifuged in a Beckman rotor J2-21 at 4000 x g for 10 min. The media was decanted and the cells resuspended in ice cold 0.1M $CaCl_2$ and then placed on ice for 30 min. The bacteria were pelleted again at 4000 x g for 10 min and then resuspended in a volume of ice cold 0.1M$CaCl_2$ containing 15% (w/v) glycerol corresponding to 1/ 50 of the original volume of medium. The bacteria could then be used directly or stored at -70° C. *E. coli*, strain G1724 (Invitrogen, Groningen, Netherlands) was prepared in a similar manner but was grown on RM plates or broth. Table 2.2 shows the recipes for media used for the growth and induction of *E.coli* in recombinant protein expression.

2.5.3.3 Transformation of competent *E. coli* with plasmid DNA

The *E. coli* strain JM109 was used as a standard strain for the expression of high yields of recombinant proteins. The G1724 strain was supplied with the vector pTrXFus. This expression system is induced by the addition of tryptophan and was chosen for its ability to express high yields of soluble proteins.

A 50µl aliquot of the frozen stock of competent bacterial cells (G1724 for thioredoxin recombinants and JM109 for GST recombinants) was thawed on ice. The ligation reaction (approximately 50ng of DNA) was added and the mixture held on ice for 30 minutes, after which it was heat-shocked at 42°C for 2 minutes. The transformed G1724 cells were spread on low tryptophan RMG-agar (Invitrogen,

Groningen, Netherlands) plates containing 100µg/ml ampicillin and 0.5% glucose then incubated at 30°C, overnight. The low tryptophan agar inhibits induction of protein expression. The transformed JM109 cells were spread on LB plates containing 50µg/ml ampicillin and incubated at 37°C overnight.

2.5.3.4 Selection and growth of ampicillin resistant colonies

One ampicillin resistant colony was inoculated into 1ml RM medium (Invitrogen) (for G1724 cells) or LB-broth (for JM109 cells) containing 100µg/ml or 50µg/ml ampicillin respectively. G1724 cells were incubated at 30°C as growth of G1724/pTrX transformed bacteria at >30oC leads to uncontrolled expression, which may cause plasmid rearrangements and deletions. Transformed JM109 cells were grown at the standard temperature of 37°C. Cells were incubated overnight with constant agitation.

Table 2.2: Media used for growth and induction of *E. coli*

Medium	Recipe
LB Broth	1% (w/v) Tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl.
L-agar	As LB plus 15g/l Difco agar.
RM Media	16.7mM Na_2HPO_4, 22mM KH_2PO_4, 8.5mM NaCl, 18.7mM NH_4Cl, 1mM $MgCl_2$, 0.2% (w/v) Casamino acids.
RMG-agar	As RM plus 2% Casamino acids, 1mM $MgCl_2$, 1.5% agar.
Induction Media	As RM plus 0.5% glucose.

All media were sterilised by autoclaving for 15 minutes at $1.05kg/cm^2$. Ampicillin was added to the media after autoclaving. Reagents were supplied by Invitrogen, Groningen, Netherlands.

2.5.3.5 Induction of protein synthesis

50µl of the overnight culture of G1724 cells were added to 10mls of induction medium (Invitrogen) containing 100µg/ml ampicillin and 0.5% glucose. Similarly, JM109 cells were added to 10mls of L-broth containing 100µg/ml ampicillin. Again, G1724 cells were incubated at 30°C while JM109 cells were grown at 37oC, with agitation, for approximately 3 hours, until OD_{600} of 0.3 - 0.5 was reached. Thioredoxin fusion proteins were induced by addition of 100µg/ml (final concentration) tryptophan while GST fusion proteins were induced by the addition of 100µg/ml (final concentration) IPTG (isopropyl-1-thio-β-D-galactoside). Incubation at 37°C proceeded for 4 hours. Once induction was complete cells were centrifuged and lysed for collection of proteins. Alternatively, cell pellets were stored at -20°C until required.

2.5.3.6 Harvesting recombinant fusion proteins from *E. coli*

Cell pellets were resuspended in 1ml 20mM Tris pH8/ 2.5mM EDTA. To this, 100µg/ml lysozyme, 0.1% Triton X-100 and 5µg/ml leupeptin (a protease inhibitor) was added and the mixture incubated at 30°C for 30 minutes. $MgCl_2$ was then added to a final concentration of 8mM. DNA and RNA were removed from the lysate by the addition of 50U DNaseI (Boehringer Mannheim, Mannheim, Germany) and RNase to a final concentration of 10µg/ml then allowed to incubate on ice for 30 minutes. Soluble and insoluble proteins were separated by centrifugation at 13000 x g for 10 minutes. The insoluble pellet was resuspended in 100µl reducing Laemmli sample buffer (62.5mM Tris-HCl pH7.8, 10% sucrose, 2% SDS, 0.001% bromophenol blue, 5% β–mercaptoethanol). An equal volume of 2x reducing Laemmli sample buffer was added to 10µl of the supernatant containing the soluble proteins. A total of 20µl supernatant samples and 10µl pellet samples were analysed by SDS-PAGE (see Chapter 2.4.2) and the gel stained with Coomassie blue to detect the presence of recombinant proteins. Recombinant proteins were identified by Western blotting analysis (see Chapter 2.4.4) using an anti-thioredoxin (Invitrogen, Groningen, Netherlands) or an anti-GST antibody (Amersham, Little Chalfont, UK).

2.6 Statistical Analysis

Data generated for Figs 3.6 and 3.7 were analysed by analysis of variance (ANOVA) using the data analysis software, 'Minitab'. ANOVA was felt to be the most appropriate method of statistical analysis and is very similar to the Student's t-test. ANOVA has the advantage over the Student's t-test in that the risk of spurious results arising from multiple testing is removed. This outweighed any potential problem with the use of ANOVA - the most serious of these being the assumption of constant variance in all subgroups within the analysis. However, any heterogeneity of variance would be unlikely to affect the results of these analyses seriously.

2.7 Frequently used reagents

Coomassie blue de-stain	30% (v/v) methanol, 12% (v/v) acetic acid
10X DNA ligase buffer	660mM Tris/HCl, 50mM $MgCl_2$, 10mM DTT, 10mM ATP, pH 7.5
Elution buffer	100mM glycine, 150mM NaCl, 0.02% azide, pH3
Equilibration buffer	50mM Tris, 150mM NaCl, 0.02% azide, pH8.4
Expand[TM] RT buffer	250mM TRIS-HCl, 25mM $MgCl_2$, 200mM KCl, 2.5% Tween 20, pH 8.3
Laemmli sample buffer	62.5mM Tris-HCl pH7.8, 10% sucrose, 2% SDS, 0.001% bromophenol blue ± 5% β–mercaptoethanol
Lysis buffer	1% Triton X-100 or IGEPAL-CA630, 10mM Tris pH7.2, 150mM NaCl, 1mM activated Na_3VO_4[*], 1mM NaF
PBS	150mM NaCl, 200mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4

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[*]For the maximal inhibition of protein tyrosine phosphatases by Na_3VO_4, the stock solution needs to be activated prior to use. The initial solution was adjusted to pH10.0 (and appears yellow) then boiled until it turns colourless. These steps were repeated until the solution remains colourless at pH10.0. This procedure depolymerises the vanadate, converting it into a more potent inhibitor (Gordon, 1991).

PBS/high salt	0.5M NaCl in PBS
RNA Extraction buffer	23.6g of guanidine isothiocyanate (GIT), 0.36ml β-mercaptoethanol, 5ml of 250mM sodium citrate pH7, 2.5ml of 10% w/v N-lauroylsarcosine, 47ml water
Separating gel buffer	0.375M Tris-HCl pH8.8, 1% SDS, 0.1% (v/v) TEMED, 0.1% (w/v) ammonium persulphate (APS)
Silver stain 1st fix	50% (v/v) methanol, 10% (v/v) acetic acid
Silver stain 2nd fix	5% (v/v) methanol, 7% (v/v) acetic acid
Silver stain developer	0.3M sodium carbonate, 0.02% (w/v) formaldehyde
5xSSC	750mM NaCl, 75mM sodium citrate
Stacking gel buffer	0.15M Tris-HCl pH6.8, 0.5% (w/v) SDS, 0.2% (v/v) TEMED, 0.2% (w/v) APS
Tank buffer	25mM Tris, 200mM Glycine, 5mM SDS
TBE buffer	45mM Tris/borate, 1mM EDTA pH7.3
TBS	100mM Tris/HCl, 150mM NaCl
TE buffer	10mM Tris pH 7.5, 1mM EDTA
Transfer buffer	50mM Tris, 40mM Glycine, 1mM SDS
Wash buffer (WB)	0.5M NaCl, 0.5% (v/v) Tween 80 in PBS

Chapter 3

Phenotypic characterisation of MCF virus-infected LGL cell lines: response to IL-15

3.1 Introduction

Lymphocyte cell lines have been generated in tissue culture from the tissues of animals naturally and experimentally infected with MCF viruses. These cell lines can be maintained for months or even years and display the morphology of large granular lymphocytes (LGLs) (Fig 3.1). Previous phenotypic analyses of these cell lines demonstrated that they exhibited a T cell or NK cell phenotype. In addition, these LGLs were shown to have cytotoxic activity towards a variety of primary and established monolayer cell cultures. This cytotoxicity was non-MHC restricted and non-antigen specific (Burrells and Reid 1991; Cook and Splitter, 1988; Reid *et al*, 1983, 1989; Schock and Reid, 1996; Schock *et al*, 1998). Furthermore, it was shown that high cell densities and occasionally feeder monolayer cells (fibroblasts) were also important for the maintenance of the LGLs in tissue culture (Reid *et al*, 1989). For optimum growth, exogenous IL-2 was routinely added to cultures. These cells were described as lymphokine activated killer (LAK) cells and expressed IL-2Rα (Cook and Splitter, 1988; Schock *et al*, 1998). However, LGLs could also be maintained in the absence of exogenous IL-2 for days, weeks and sometimes months. Interestingly, OvHV-2-infected bovine LGLs did not express IL-2 mRNA even after stimulation with concanavalin A (conA) (Schock *et al*, 1998) and LGL culture supernatants did not display detectable levels of IL-2 activity (Schock, 1996). This is in contrast with normal uninfected T cells that produce IL-2 when activated (Meuer *et al*, 1984, Schock *et al*, 1998). Schock's study (1998) did however show that bovine OvHV-2-infected LGL cell lines express mRNA for the cytokines IL-4, IL-10, IFN-γ and TNF-α but not IL-1β or IL-2. These observations raise the possibility that endogenous growth factors other than IL-2 may maintain LGL cell survival for a limited period of time.

Since Schock's study (1998), the cytokine IL-15 has been described as a T cell growth factor. This cytokine was of interest to the present study as it has similar actions to IL-2 and can induce the expression of IL-2Rα (Kumaki *et al*, 1996). In the mouse and man, IL-15 promotes the proliferation of T cells and NK cells and of relevance to the pathogenesis of MCF, induces CTL and LAK activity (Armitage *et al*, 1995; Carson *et al*, 1994; Gamero *et al*, 1995; Giri *et al*, 1994, 1995b; Grabstein

et al, 1994). Unlike IL-2, which is produced by T cells, IL-15 is predominantly produced by accessory cells, including monocytes (Carson *et al*, 1995; Doherty *et al*, 1996), dendritic cells (Jonuleit *et al*, 1997), fibroblasts and a variety of other non-lymphoid tissues (Grabstein *et al*, 1994). While T cells are not normally a source of IL-15, transcripts of the cytokine have been detected in HTLV-1-transformed T cell lines (Azimi *et al*, 1998). Induction of IL-15 expression by peripheral blood mononuclear cells (PBMC) in human herpesviruses 6 and 7 (HHV-6 and HHV-7) - infected individuals (Atedzoe *et al*, 1997; Flamand *et al*, 1996) has also been reported. These observations prompted the question of whether MCF-virus infection can also upregulate IL-15 expression in LGL cells and thus explain the IL-2 independent phenotype observed in LGL cell lines.

In this study, MCF virus-infected LGL cell lines established from naturally infected cattle and experimentally infected rabbits were characterised in a series of phenotypic assays and compared to control uninfected lymphoblasts. In particular, the hypothesis that MCF virus-induced IL-15 mediates the lymphoproliferation and cytotoxic activity of the LGLs was tested. The objectives of this study were as follows:

1. To test the whether IL-15 acts as a growth factor for the LGLs.

2. To test whether IL-15 maintains or enhances the cytotoxic phenotype of the LGL cell lines.

3. To test the production of IL-15 in LGL cell lines.

A)

B)

25μm
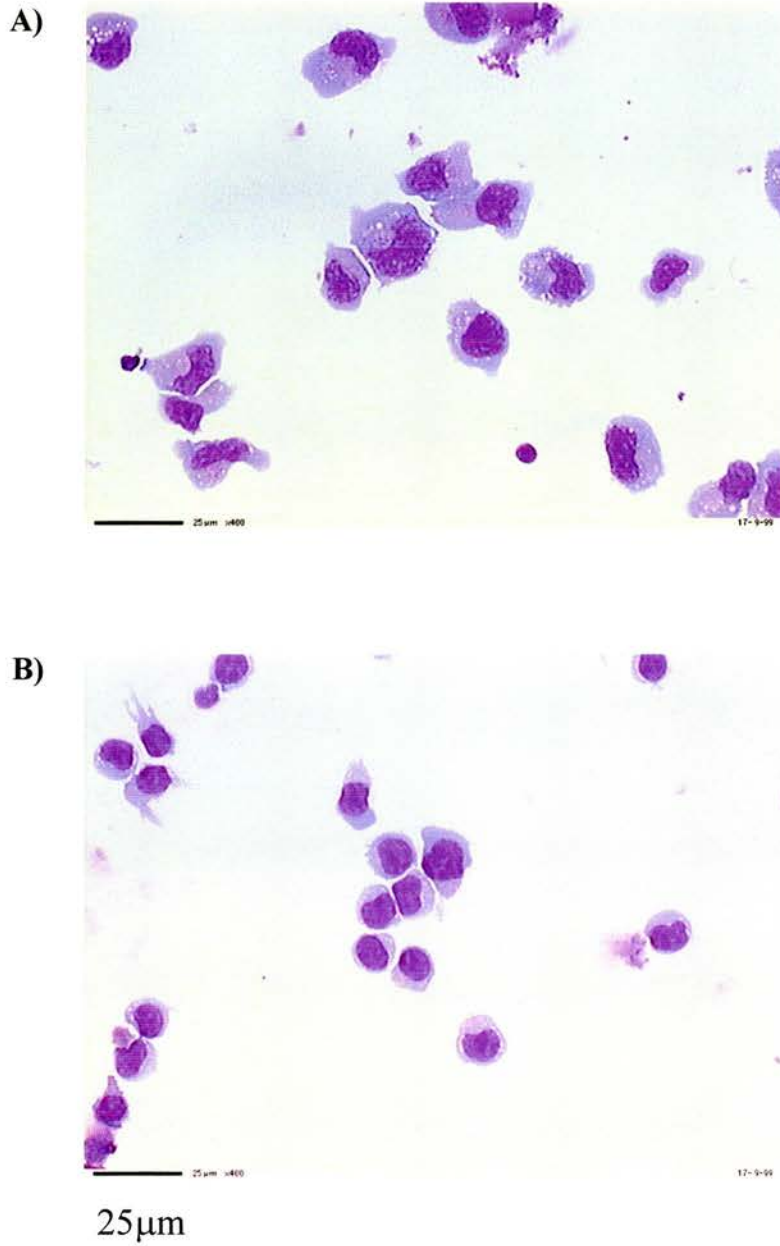
Fig3.1: An OvHV-2 infected cattle cell line (BJ1035) showing the morphology of large granular lymphocytes (LGLs).
Morphology of **A)** BJ1035 and **B)** bovine lymphoblasts (controls) revealed by Leishman's staining - see chapter 3.2.2. Note the large kidney-shaped nuclei, large cytoplasm and vacuoles/granules in **A)** compared to morphology of **B)**.

3.2 Materials and methods

3.2.1 Cells

LGL cell lines:

BJ1035, BJ1044, BJ1104 and BJ1196 were bovine LGL cell lines derived from natural cases of OvHV-2-induced MCF. These bovine cell lines did not survive the course of this study due to difficulty in resurrecting the cells from liquid nitrogen following a move to new laboratories. Therefore, new cell lines had to be established to allow more data to be generated. These cell lines (BJ1857, BJ1859 and BJ1860) were generated from rabbits experimentally infected with OvHV-2 (BJ1857) or AlHV-1 (BJ1859, BJ1860). The rabbit cell lines BJ1001 and BJ1263 (both infected with AlHV-1) stored in liquid nitrogen were successfully resurrected and included in these studies. Infection protocols for the LGL cell lines were as described in Chapter 2.

Control cells:

Fresh PBMCs or conA-stimulated lymphoblasts were used as control cells. These cells were collected from uninfected rabbit or bovine blood or lymph nodes and the lymphoblasts were generated as described in Chapter 2.

3.2.2 Analysis of cell phenotype

Morphological analysis:

Cytocentrifuge preparations at a density of 5×10^5 cells / ml were prepared from cell suspensions and the morphology of the LGL cell lines and the control lymphocytes was revealed by staining of cells with Leishman's stain (Chapter 2.3.3).

FACS analysis:

All LGL cell lines and control PBMCs (bovine and rabbit) were phenotyped by FACScan analysis (see Chapter 2.3.1) using murine monoclonal antibodies against CD2, CD3, CD4, CD5, CD8, WC1 (expressed on $\gamma\delta$ T cells), CD21 and CD45 (Table 3.1 and 3.2). For the characterisation of bovine cells, where bovine antigen-specific antibodies were not available, ovine / bovine cross-reactive antibodies were used. For the characterisation of rabbit lymphocytes, mouse monoclonal antibodies

specific for rabbit cell surface antigens were used. It should be noted that these were only available for a limited range of surface markers. Murine monoclonal antibodies (of different IgG isotypes) to border disease virus (a gift from G. Entrican, Moredun Research Institute) were used as antibody isotype controls.

BLT esterase cytochemistry and quantitation:

Cytotoxic enzyme activity over a range of IL-15 concentrations was measured by BLT (benzyloxycarbonyl-L-lysine thiobenzyl) esterase activity. BLT esterase is a serine protease (tryptase) induced in cytotoxic cells (Griffiths and Mueller, 1991; Haig *et al*, 1996). The activity of BLT esterase is measured by an assay that relies on an enzyme-substrate reaction between BLT esterase and BLT. The reaction produces a yellow product that can be visualised in cells by light microscopy or quantified by absorbance at 412nm. The BLT esterase assay was performed on cytocentrifuge preparations, cell lysate samples (to measure intracellular enzyme) and culture supernatant samples (to measure secreted enzyme) as described in Chapter 2.3.4.

3.2.3 Detection of virus-specific proteins in LGL cell lines

The immunofluorescence antibody test (IFAT) was used to demonstrate the presence of virus proteins in OvHV-2-infected cell lines and their absence in control uninfected lymphoblasts. Cytocentrifuge preparations of LGL and control cells were prepared as previously described (Chapter 3.2.2). Serum antibodies from a cow ('Jezebel') that survived OvHV-2 infection recognise undefined OvHV-2 proteins and are useful as a probe for OvHV-2 infection of cells (Milne and Reid, 1990). 'Jezebel' serum was added to slides at 1:10 or 1:20 dilution in PBS for 30mins at 37°C following a blocking stage with 1:20 normal horse serum. Bovine serum from a MCF-negative cow was used as a negative antibody control. After each incubation stage the slides were washed 2 x 10mins in PBS. The anti-sheep FITC conjugate was added at 1:50 dilution to all the slides and incubated as before.

The presence of virus proteins in AlHV-1-infected LGLs was demonstrated by RT-PCR detection of a protein first defined by Handley (1993) as a potential virulence factor for AlHV-1 and more recently defined as the protein encoded by ORF50 (Ensser *et al*, 1997; H. Wright, personal communication). The amino acid

sequence encoded by ORF50 has homology to the EBV R transactivator protein produced as an immediate early gene product (Ensser *et al*, 1997). The details regarding these experiments and the role of this protein in the virus life cycle are not discussed in this Chapter but will be presented in Chapters 5 and 6.

3.2.4 Cell survival assays in the absence of exogenous cytokines

The bovine OvHV-2-infected MCF cell lines BJ1035, BJ1044 and control bovine conA activated lymphoblasts were washed twice in IMDM to remove any residual IL-2. Cells were then seeded at 2, 1, 0.5 or 0.25 $x10^6$/ml IMDM, plated out in 48-well plates at 1ml per well and incubated in a humidified atmosphere of 5% CO_2 at 37°C. Viable cell numbers were determined by nigrosine exclusion (Chapter 2.2.6) and performed every 24hrs for 4 days or longer. Cytocentrifuge samples were also taken for the detection of BLT esterase in these cells (Chapter 3.2.2).

In addition, viable cell numbers in LGL and control cells cultured in rhIL-2-free medium for 3 days were recorded on at least three separate occasions prior to use in signal transduction experiments (Chapter 4).

3.2.5 Cytokine response assays

Human recombinant (hr) IL-2 expressed in *E.coli* (>98% purity, specific activity 18 x 10^6 units/mg) was purchased from Eurocetus UK Ltd (Harefield, UK). Human recombinant IL-15 expressed in *E.coli* (>98% pure, specific activity > 2 x 10^6 units/mg) was purchased from Peprotech EC Ltd (London, UK). After a thorough wash in IMDM cells were set up in 48-well plates at a density of 0.5 x 10^6/ml in duplicate or triplicate wells (using 500μl per well). IL-2 or IL-15 was added to each well in a range of concentrations and cells incubated under standard conditions (see Chapter 2.2.2). Cell growth was measured as changes in viable cell counts (measured by nigrosine exclusion - Chapter 2.2.6) after time in culture. Based on preliminary experiments, cell growth was recorded for IL-2 and IL-15 after 3 days and 6 days in culture. 50μl volumes of lysates containing 10% of total cell population per well (lysed in water) were taken for determination of BLT esterase activity in cell lysates. 50μl volumes of neat culture supernatant were taken for the

determination of BLT esterase activity secreted by cells into the culture supernatant (see Chapter 3.2.2).

Table 3.1: Mouse monoclonal antibodies used in FACS analysis of bovine cells.

Specificity	Clone	Ig Isotype	Source	Reference
boCD2	CC42	IgG1	IAH	Davis and Splitter, 1991
boCD4	CC8	IgG2a	IAH	Bensaid and Hadam, 1991
bo/ovCD5	CC17	IgG1	IAH	Howard and Leibold, 1991
bo/ovCD8	CC63	IgG2a	IAH	MacHugh and Sopp, 1991
boWC1	CC15	IgG2a	IAH	Morrison and Davis, 1991
boCD21	CC21	IgG1	IAH	Naessens and Howard, 1991
boCD45	CC1	IgG1	IAH	Bembridge et al, 1993

IAH = Institute of Animal Health (Compton, UK); bo = bovine-specific; ov = ovine-specific; WC1 = workshop cluster 1. WC1 is expressed on $\gamma\delta$ T cell receptor[+] (TCR) cells. CD21 is expressed on B cells. CD45 (also known as leukocyte common antigen - LCA) was included as a positive control as it is present on all lymphocytes.

Table 3.2: Mouse monoclonal antibodies used in FACS analysis of rabbit cells.

Specificity	Clone	Ig Isotype	Source	Reference
rbt.CD4	KEN-4	IgG2a	Research Diagnostics	Kotani et al, 1993
rbt.CD5	KEN-5	IgG1	Serotec	Kotani et al, 1993
rbt.CD8	12C7	IgG1	Serotec	Civin et al, 1984
rbt.IgM	NRBM	IgG1	Serotec	Idogawa et al, 1997

Antibodies were obtained from Research Diagnostics Inc., Flanders, USA or Serotec Ltd, Oxford, UK. rbt = rabbit-specific. KEN5 detects antigen on rabbit T cells. IgM is expressed on B cells.

3.2.6 Immunocytochemical detection of IL-15

Normal rabbit monocytes or adherent cells from peritoneal lavage and spleen cell suspensions were grown on coverslips as a putative source of IL-15-expressing positive controls. As fibroblasts are known to express IL-15, the mouse fibroblast cell line LTK⁻ (Spandidos and Wilkie 1983) was also grown on coverslips and included as a positive control. The cells were stimulated for 24h with 10µg/ml lipopolysaccharide (LPS from *S. minnesota* - Sigma Aldrich, Gillingham, UK) to induce IL-15 production. MCF virus-infected LGLs were stimulated overnight (or not) with 10µg/mlConA+10ng/mlTPA and cytospins prepared. Cells were fixed in cold acetone.

After an initial wash in PBS/0.05%Tween 80 the slides were blocked in 1:50 normal horse serum and normal rabbit serum in PBS/high salt (0.5M NaCl in PBS) for 30mins at 25°C. Normal goat IgG (at 1, 5 and 10µg/ml) was used as negative control. Goat anti-human IL-15 (L-20) (Santa Cruz Biotechnology, Santa Cruz, USA), specific for a carboxy terminal peptide of the human IL-15 precursor, was diluted in PBS/high salt to 1, 5 and 10µg/ml and incubation proceeded for 30 minutess at 25°C. Following 2 x 10 minute washes in PBS/Tween80 the biotinylated horse anti-goat IgG antibody (2µg/ml) was added and incubated as before. A Vectastain ABC-alkaline phosphatase kit and Vector Red substrate (Vector Laboratories, Peterborough, UK) was used to detect bound antibodies.

3.2.7 Detection of IL-15 by western blot

Cell lysates were prepared using a 1% IGEPAL-CA630 lysis buffer (1% IGEPAL-CA630, 10mM Tris pH7.2, 150mM NaCl, 1mM activated Na_3VO_4, 1mM NaF) from rabbit MCF cell lines BJ1857 (infected with OvHV-2), BJ1859 and BJ1860 (infected with AlHV-1). Uninfected, conA-stimulated lymphoblasts derived from rabbit lymph nodes were used as controls. Cells were washed in IMDM to remove rhIL-2 then given further stimulation (or not) with 10µg/ml conA and 10ng/ml TPA for 18 hours prior to lysis. Cell lysate from approximately 10^6 cells was loaded into a well of a 15% acrylamide gel. Recombinant human IL-15 (Peprotec EC Ltd, London, UK) was loaded into two wells at 100ng or 200ng per

well. Electrophoresis and western blots were performed as before (Chapter 2.4.2 and 2.4.4). Anti-IL-15 (Santa Cruz Biotechnology, Santa Cruz, USA) was used at 1µg/ml to probe the nitrocellulose membrane. The cell lysates were also concentrated four-fold in Vivaspin concentrators (with a 10kDa membrane) (Vivascience, Lincoln, UK) and analysed in the same manner.

3.2.8 Detection of IL-15mRNA in rabbit MCF cell lines

RT-PCR and Southern blots were used to detect whether or not LGL cell lines transcribe IL-15 mRNA. The IL-15 primers and probes used in this study were based on the bovine IL-15 sequence, as the rabbit IL-15 has not been sequenced (Table 3.3). RT-PCR and Southern blots were performed by the method described in Chapters 2.5.1 and 2.5.2. This was performed on RNA samples from the rabbit AlHV-1-infected LGL lines, BJ1001, BJ1263, BJ1857, BJ1860 and rabbit OvHV-2-infected cell line BJ1265. Control cells were uninfected lymphoblasts isolated from rabbit spleen or lymph nodes. Cells were either taken from culture with exogenous IL-2, without exogenous IL-2 or stimulated with 10µg.ml conA and 5ng/ml TPA.

In a preliminary study, an oligonucleotide sequence specific for the 231-270bp region of bovine IL-15 was used as an internal probe to detect and identify the products of the RT-PCR reaction in the rabbit AlHV-1-infected LGL cell line, BJ1001.

Table 3.3: Primers used for the detection of IL-15 by RT-PCR and Southern blot.

Specificity	Function	Sequence	T_m	Product Size
IL-15	5' primer	CATATTTGAGAAGTACTTCCATCCAG	72°C	469bp
	3' primer	TGTGCAAATGTTCATCAACACTCC	64°C	
	internal probe	GCACTGCATCGCTGTTACTTTGCAATTGGGAT GAGCATCA	114°C	
GAPDH	5' primer	CCTTCATTGACCTCAACTACAT	56°C	380bp
	3' primer	CCAAAGTTGTCATGGATGACC	58°C	

The primers used for IL-15 detection cover the 17-485bp region of the bovine IL-15 sequence. The internal probe covers the 231-270bp region of the same sequence. GAPDH = glyceraldehyde-3-phosphate dehydrogenase - used as a control to measure equal loading of mRNA in RT-PCRs. T_m = melting temperature

3.3 Results

3.3.1 Phenotypic analysis of MCF LGL cell lines

The OvHV-2-infected cattle LGL cell lines BJ1035, BJ1044, BJ1104, and BJ1196 were phenotyped by FACS analysis. Rabbit LGL cell lines from three infected rabbits were also phenotyped. These rabbits were infected with AlHV-1 (two rabbits) or OvHV-2 (two rabbits). One of the rabbits infected with OvHV-2 died without developing a febrile reaction. The reason for this was not established. From the remaining rabbits, three cell lines (one per rabbit) were generated - BJ1857, BJ1859 and BJ1860 (see Chapter 2.1.2). BJ1857 was infected with OvHV-2 while BJ1859 and BJ1860 were infected with AlHV-1. The results presented here are representative of at least three separate analyses.

The phenotypic analysis of the cattle cell lines showed that the majority (>96%) of the LGLs in each cell line was $CD2^+$ (summarised in Table 3.4). None of the LGL cell lines contained $CD4^+$ cells but all cell lines contained variable percentages of $CD8^+$ cells, except BJ1035, which was $CD4^-CD8^-$. A proportion ranging from 5% to 16% of the cells in BJ1104 and 1044 respectively (both OvHV-2-infected) expressed the WC1 antigen specific to $\gamma\delta TCR^+$ cells. The majority (>96%) of cells in lines BJ1035 and BJ1044 were $CD5^+$ and $CD2^+$.

The phenotype of the rabbit cell lines was determined using the limited number of anti-rabbit CD antibodies available. The results (see Table 3.5) show that none of the cell lines expressed surface IgM. Of the two established rabbit cell lines (i.e. > 3 years old) the majority (>99%) of BJ1263 cells expressed CD5. At least 50% of cells in BJ1263 expressed the CD8 marker. Cells in BJ1001 expressed neither CD5 nor CD8. Only small proportions of cells in the cell lines BJ1263 and BJ1001 expressed the CD4 marker. The phenotype of the LGL cultures BJ1857, BJ1859 and BJ1860 stabilised after 5 months in culture. The presence of $CD5^+$ cells was observed in 50% of cells in BJ1859. Neither BJ1857 nor BJ1860 cells expressed CD5. The proportion of cells expressing CD8 varied between each cell line. None of these cell lines expressed CD4.

All antibodies used in this study reacted against antigens expressed on control PBMCs. Some sample FACS profiles are illustrated in Fig 3.2.

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Table 3.4: Detection of cell surface markers on bovine OvHV-2-infected LGL cell lines.

Cell Line	Period in Culture	CD2 (%)	CD4 (%)	CD5 (%)	CD8 (%)	T19 (%)	CD21 (%)	CD45 (%)
BJ 1035	20months	98	<1	98	<1	<1	<1	96
BJ 1104*	27months	<1	<1	nd	93	5	4	99
BJ 1044	19months	96	<1	96	56	16	<1	87
BJ 1196	4months	98	<1	nd	52	<1	nd	nd

Values represent % of total live cell population staining positively with antibody. *nd* = not determined. *performed by J. McCallum.

Table 3.5: Detection of cell surface markers on rabbit MCF cell lines.

Cell Line	Period in Culture	CD4 (%)	CD5 (%)	CD8 (%)	sIgM (%)
BJ1001	59months	8	<1	<1	<1
BJ1263	40months	2	99	50	<1
BJ1857	5months	<1	<1	12	<1
BJ1859	12months	<1	50	50	<1
BJ1860	12months	<1	<1	45	<1

BJ1001 and BJ1263 are established cell lines from AlHV-1-infected rabbits. BJ1857 is a cell line derived from an OvHV-2-infected rabbit. BJ1859 and BJ1860 are both derived from AlHV-1-infected rabbits.
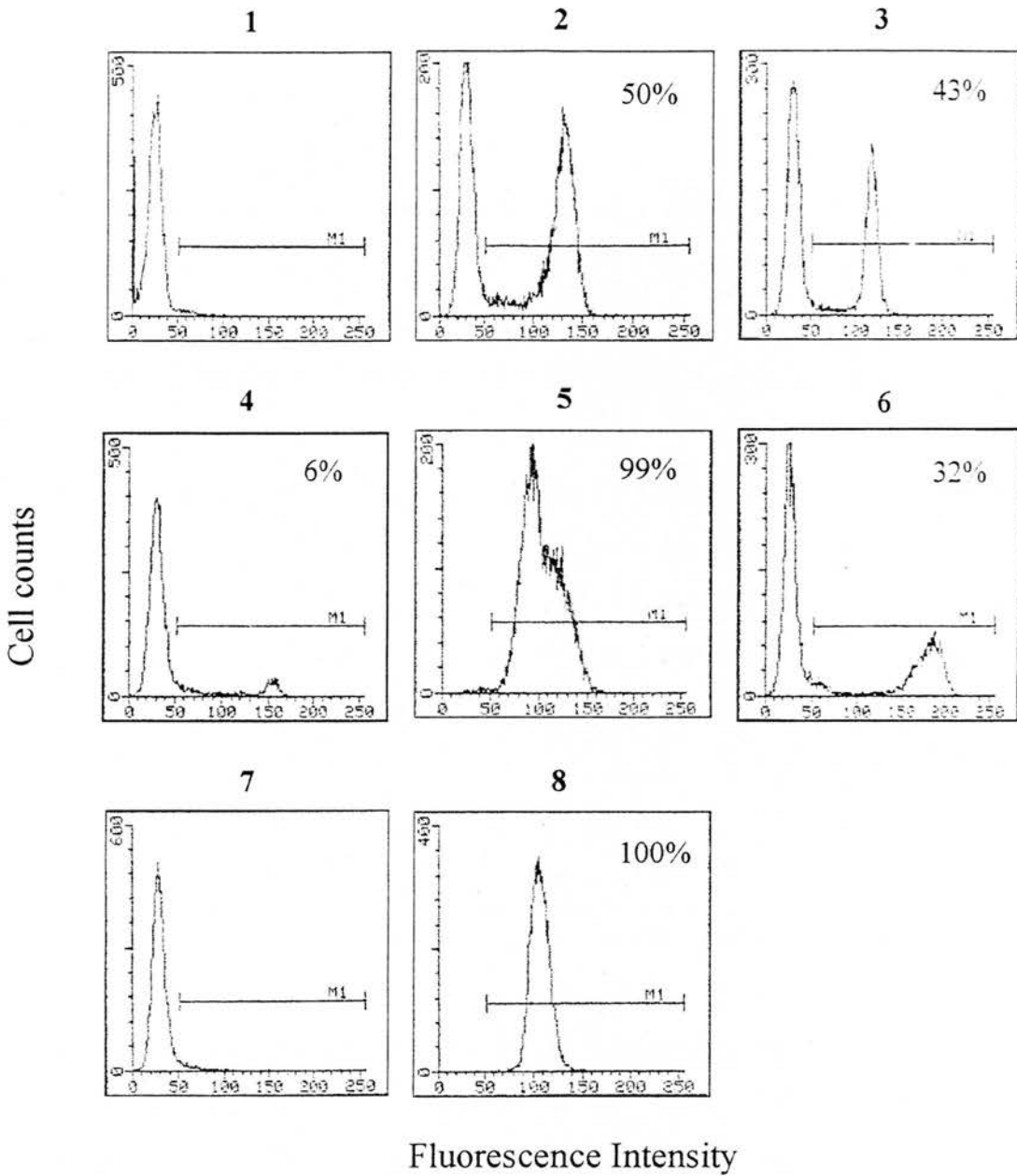
Fig 3.2: Sample FACS profile for bovine conA-stimulated lymphoblasts (continues on next page).
1) FITC conjugate alone. 2) anti-CD2. 3) anti-CD4. 4) anti-CD8.
5) anti-CD5. 6) anti-WC1. 7) anti-CD21. 8) anti-CD45.
M1 = marker gate for positive cells
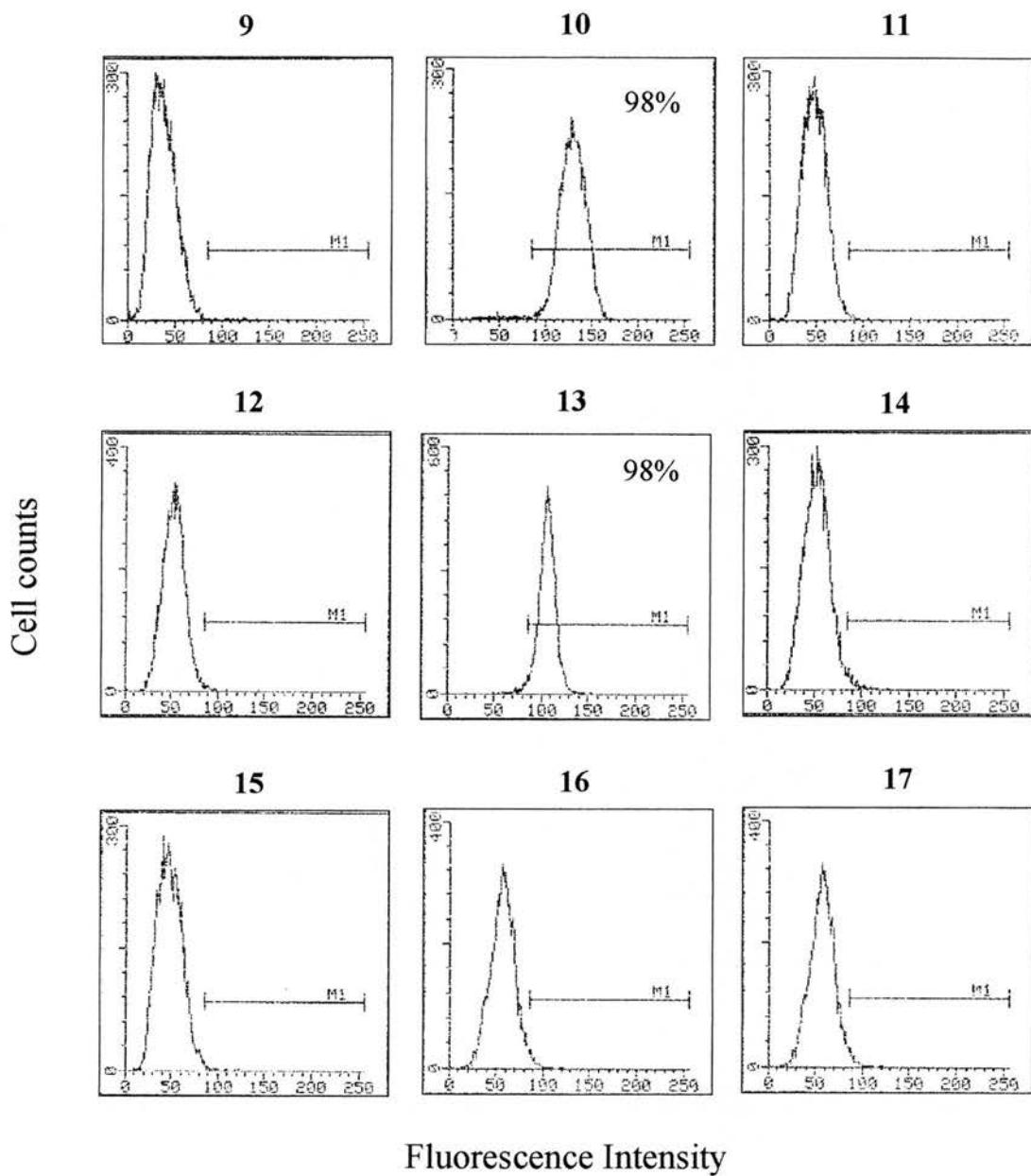
Fig 3.2 (continued): Sample FACS profile for bovine LGL cell line BJ1035.
9) FITC conjugate alone. **10)** anti-CD2. **11)** anti-CD4. **12)** anti-CD8.
13) anti-CD5. **14)** anti-WC1. **15)** ant-CD21. **16)** anti-CD45.
17) anti-border disease virus (control). **M1** = marker gate for positive cells
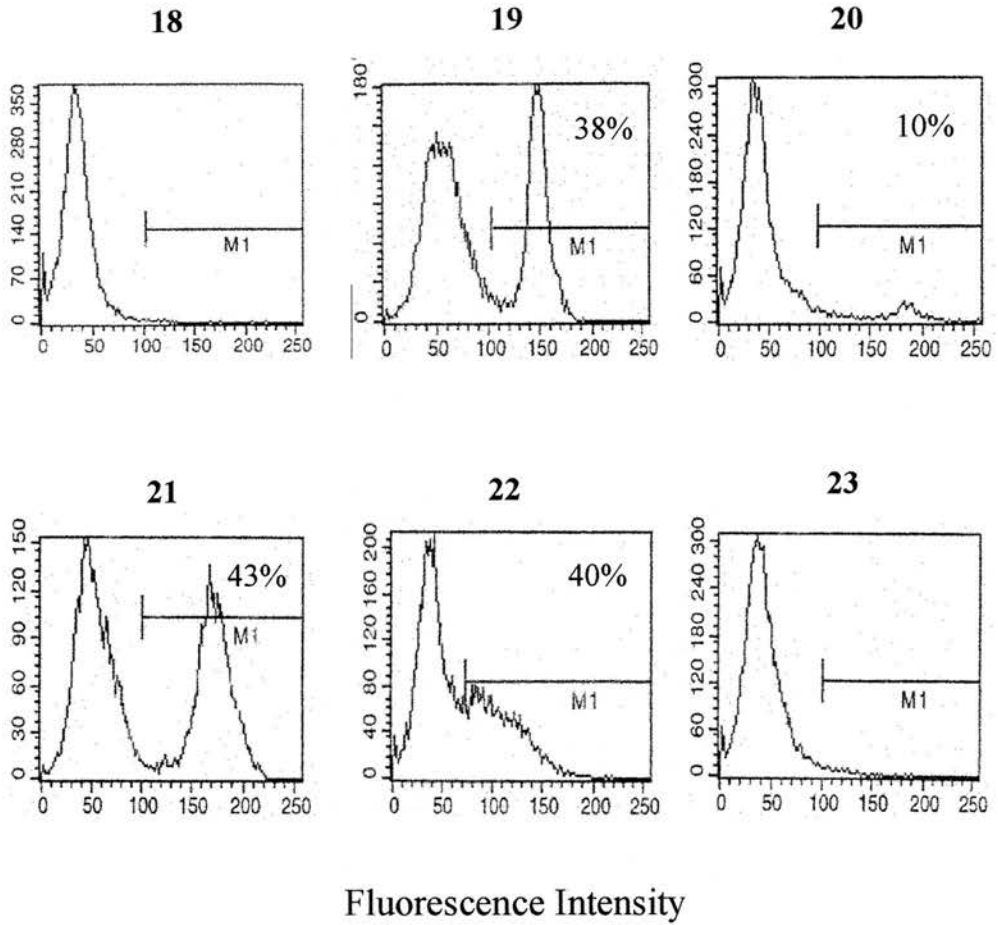
Fig 3.2 (continued): Sample FACS profile for rabbit mononuclear cells from lymph nodes.
18) FITC conjugate alone. **19)** anti-CD4. **20)** anti-CD8. **21)** anti-CD5.
22) anti-IgM. **23)** anti-border disease virus (control). **M1** = marker gate for positive cells.
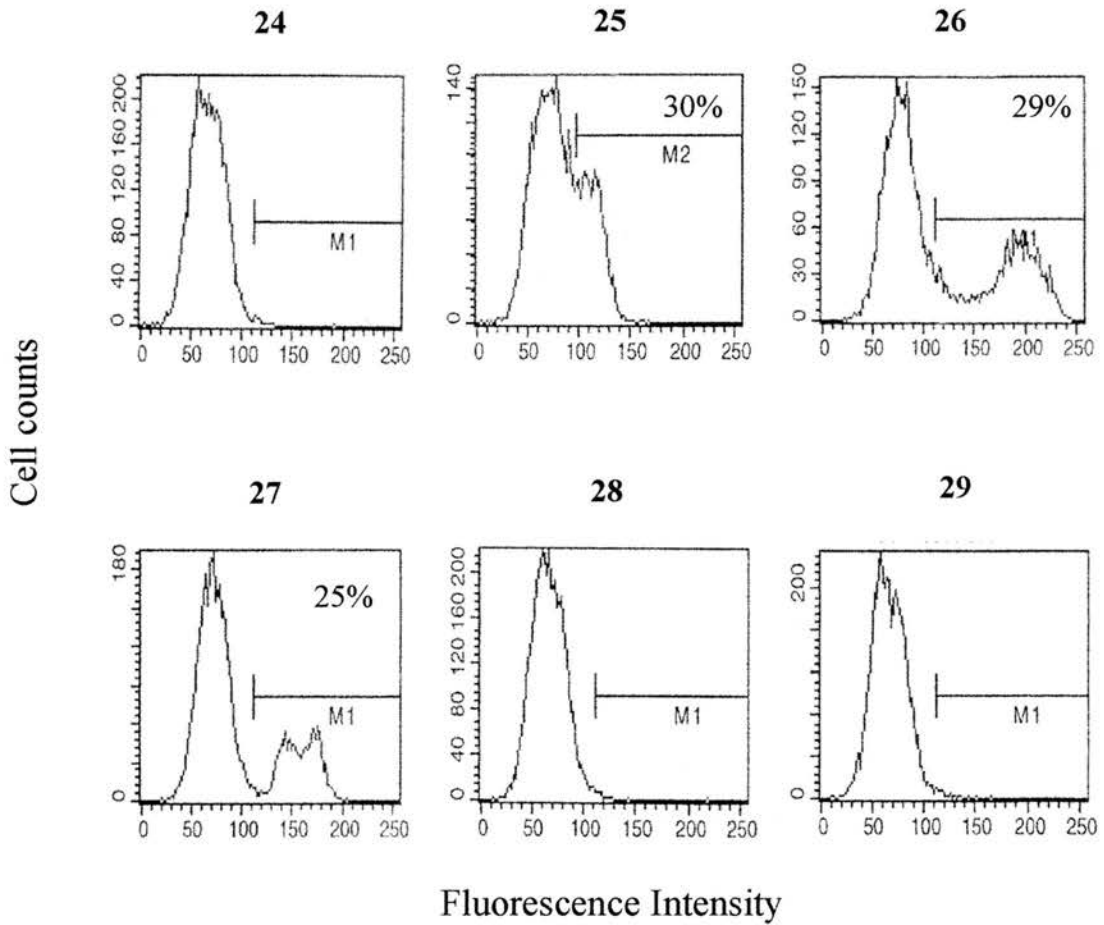
Fig 3.2 (continued): Sample FACS profile for rabbit LGL cell line BJ1859. (Analysis made after 5 months in culture).
24) FITC conjugate alone. **25)** anti-CD4. **26)** anti-CD8. **27)** anti-CD5.
28) anti-IgM. **29)** anti-border disease virus (control). **M1** = marker gate for positive cells.

3.3.2 OvHV-2 convalescent bovine serum detects viral antigens in the nucleus of LGL cell lines

In order to confirm the virus infection of the OvHV-2-infected cell lines the OvHV-2 convalescent bovine serum ('Jezebel' serum) was used in an indirect fluorescent antibody test to detect viral antigen. Over 90% of cells in BJ1035 and BJ1265 (derived from a rabbit infected with OvHV-2) were positive for viral antigen(s) in nuclei of LGL cells (Fig. 3.3b). A qualitative difference in staining was observed when an uninfected bovine control serum was used on BJ1035. Some non-specific cytoplasmic staining was seen in BJ1035 using this control serum but no nuclear staining was present (Fig 3.3a). In control uninfected conA-stimulated lymphoblast cells nuclear staining was not detected using either the 'Jezebel' or the control serum.

Virus infection of the AlHV-1-infected cell lines (BJ1859 and BJ1860) was confirmed by the presence of mRNA for the viral protein encoded in ORF50. These results are presented and discussed in Chapter 6.
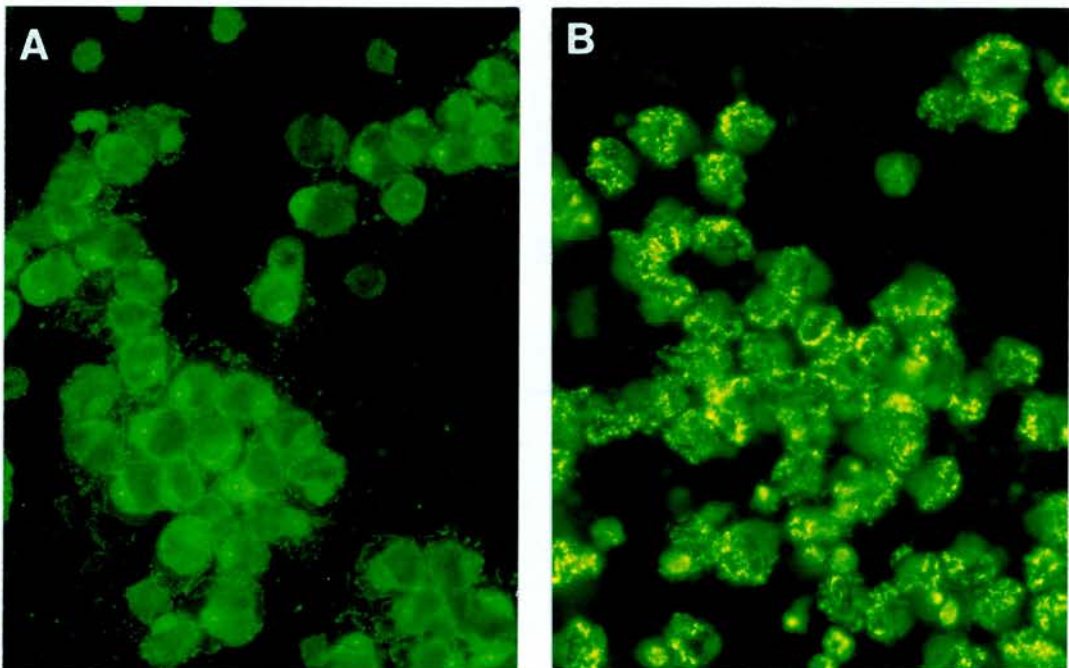
Fig. 3.3: Punctate nuclear staining detected by IFAT using a convalescent bovine serum (photographs were taken using equal exposures).
A) Bovine LGL cell line BJ1035 incubated with bovine **control serum**.
B) BJ1035 incubated with 1: 20 **'Jezebel' serum**.

3.3.3 Growth of LGL cell lines in the absence of exogenous IL-2

The growth of LGL cell lines was tested and compared to growth of uninfected conA-stimulated lymphoblasts in the absence of rhIL-2 over 4 days. The cells were set up in these cultures at different initial cell densities to determine if this might also have an effect on survival. The data shown in Fig 3.4 represent those from duplicate wells as cells were in limited supply at the time of this experiment. Therefore, the following trends were recorded although no statistical analysis was performed.

Both OvHV-2 infected bovine cell lines (BJ1035 and BJ1044) cultured in medium alone could survive without exogenous IL-2 for at least a week (and up to 46 days in one instance with BJ1035 if medium was refreshed and cultures were split when cell densities reached over 2×10^6/ml). This survival was characterised by a small initial decline in viable cell numbers followed by a recovery of viable cell numbers three to four days after culture. In contrast, the control uninfected con-A-stimulated lymphoblasts tended to decline from day 0 of culture without exogenous IL-2. Any survival of control cells was short-lived and could not be maintained at over 50% viability for as long as the LGL cell lines. The greatest difference in the number of viable cells between the LGL cell lines (BJ1035 and BJ1044) controls occurred after 4 days in culture (Fig 3.4a-c, e-g), except when the initial density was 2×10^6 cells/ml (Fig 3.4d, h). The viable cell numbers in the LGL lines after 4 days in culture also exceeded the initial population at day 0 and the cells maintained at least 50% viability during this time. The recovery of cell numbers over four days appears to be dependent on initial cell density where an initial density of 1×10^6/ml gave the greatest difference in viable cell numbers between LGL cell lines and controls after four days in culture (Fig 3.4c, g). For this reason, 10^6 cells/ml was the chosen cell density for the inhibition and the signal transduction studies (Chapter 4).

The survival of rabbit LGL cell lines in the absence of hrIL-2 has been observed for 5 to 10 days. This period of survival exceeds that of control uninfected rabbit lymphoblasts, which decline in numbers and viability (to less than 70% viable) after 30 hours without hrIL-2.

In a series of subsequent analyses, the viable cell count following 3 days in culture without exogenous IL-2 was recorded for LGL and control cells set up at an

initial density of 10^6 cells/ml prior to use in signal transduction assays (Chapter 4). These data, generated from at least three different experiments, are shown in Fig 3.4i and confirms the trend observed for the time-course assay described above. Hence, at an initial density of 10^6 cells/ml LGLs survive, while control cells die in the absence of exogenous IL-2 for 3 days (in this instance).

3.3.4 LGL cell lines show cytotoxic granular morphology in the presence and absence of IL-2

The BLT esterase assay determines the presence of the enzyme in cytoplasmic granules. Its expression is associated with functionally cytotoxic cells (Griffiths and Mueller 1991; Haig *et al*, 1996). In the presence of recombinant human IL-2, OvHV-2-infected bovine LGL cell lines BJ1035, BJ1044 and rabbit OvHV-2-infected LGL cell line BJ1265 grown in culture have cytoplasmic granules containing BLT esterase (observed as a yellow stain within the granules - Fig.3.5b). While the majority of BJ1265 cells expressed BLT esterase, the intensity of staining was lower than that observed in BJ1035. This staining was detected in LGL cell lines in the absence of IL-2. In one case - BJ1035 cultured in the absence of exogenous IL-2 for 1 month - cells still maintained good LGL morphology with cytoplasmic granules that were positive for BLT esterase.

Less than 1% of uninfected, conA-stimulated lymphoblast cells were positive for BLT esterase. The levels of BLT esterase were very low by comparison to BJ1035 cells although cytoplasmic granules could be seen (Fig. 3.5a). After a short period in culture without exogenous IL-2, the few remaining live cells showed lower intensity of BLT esterase staining than those cultured with IL-2.
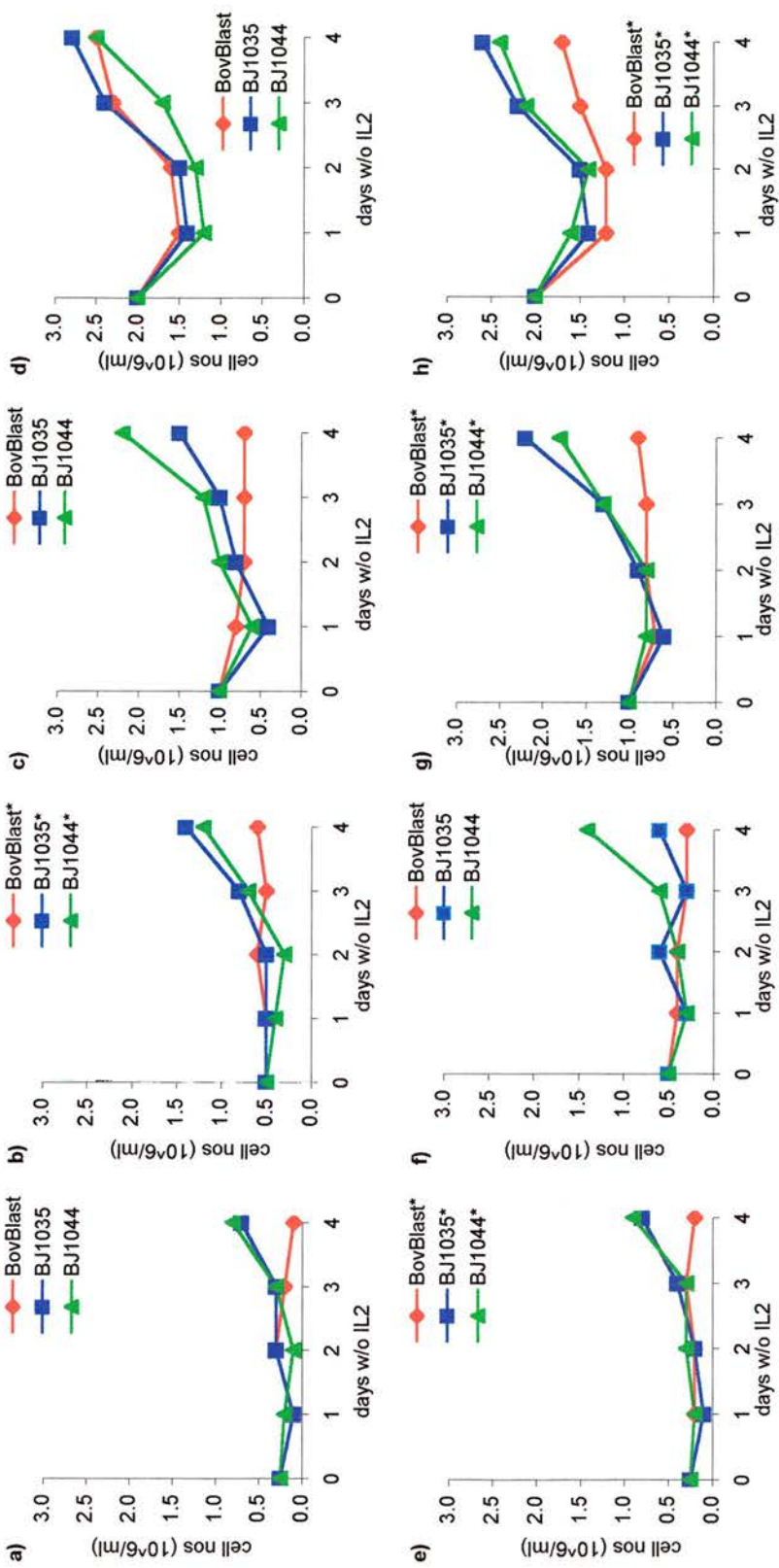
Fig 3.4: Dose-dependent and time course of IL-2-independent growth of LGL cell lines in culture.
Single well data (and duplicates, indicated by '*') for cell survival and growth in the absence of exogenous hrIL-2 over four days in culture. Cells were set up at an initial density of 0.25×10^6 cells/ml (a, e), 0.5×10^6 cells/ml (b, f), 1×10^6 cells/ml (c, g) and 2×10^6 cells/ml (d, h). BJ1035, BJ1044 = bovine OvHV-2-infected LGL cell lines. BovBlast = bovine conA-stimulated control cells. (Data from three or more separate cultures are described on the next page (Fig3.4i)).
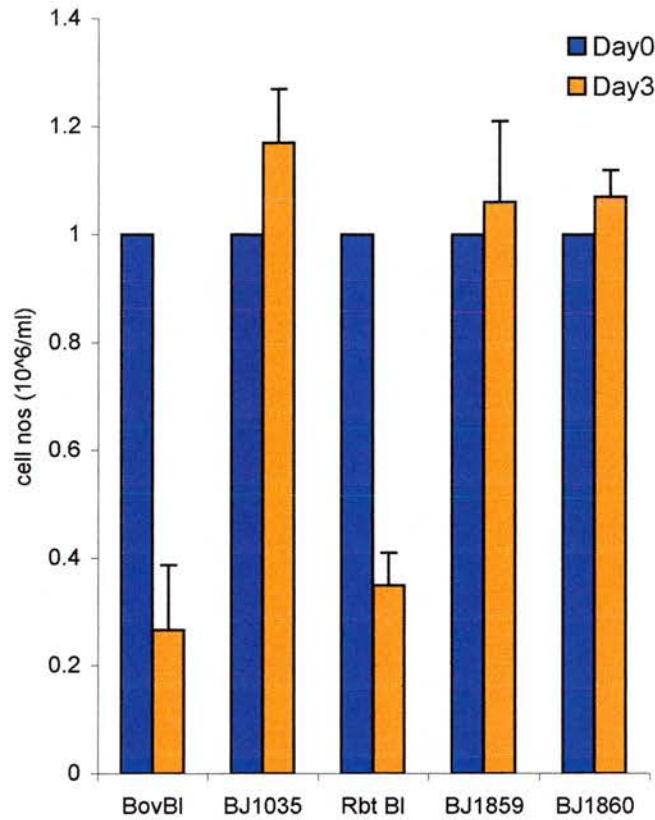
Fig 3.4i: IL-2 independent growth and survival of LGL cell lines in culture.
Cells (set up at an initial density of 10^6 cells/ml) were cultured in the absence of exogenous IL-2 for 3 days. Mean viable cell counts and standard error of the mean (s.e.m.) for repeated experiments are shown above.
BJ1035 (n=5); bovine OvHV-2$^+$ LGL cell line
BJ1859 (n=4), BJ1860 (n=3); rabbit AlHV-1$^+$ LGL cell lines
BovBl (n=3); bovine conA-stimulated control cells
RbtBl (n=4); rabbit conA-stimulated control cells
n = number of experiments

A)

B)

25μm
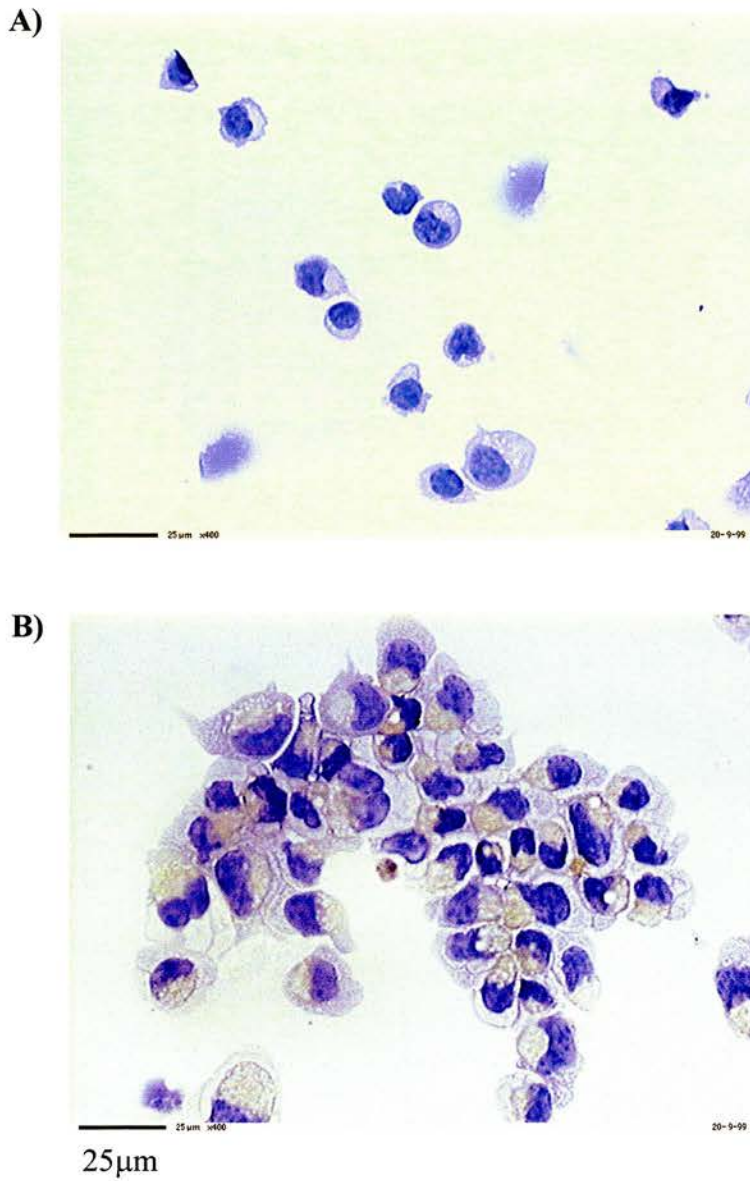
Fig. 3.5: Expression of BLT esterase in cytoplasmic granules /vacuoles of LGL cell lines compared to control uninfected cells.
A) Control, uninfected, conA-stimulated bovine lymphoblasts.
B) Bovine OHvV-2-infected cell line, BJ1035. The yellow stain indicates the presence of BLT esterase.

3.3.5 LGL cell lines respond to human recombinant interleukin 15

LGL cell lines and control cells were tested for their response to human recombinant IL-15. Cell growth and expression of secreted or intracellular (stored) BLT esterase per culture were recorded. Response to IL-2 was also measured for comparison. Uninfected, conA-activated lymphoblasts were used as control cells. The following data from bovine LGL cell lines are representative of two separate experiments.

The established OvHV-2-infected bovine cell line BJ1035 and control cells showed a dose-dependent proliferative response to IL-15 (0.01 to 100ng/ml) and IL-2 (1 to 50U/ml) (Fig 3.6a, b). While the addition of both IL-2 (at 20U/ml) and IL-15 (0.1, 1.0 and 10ng/ml) increased the growth response of both control and BJ1035 cells when compared to the response with IL-15 alone, this increase was not significant (data not shown). A similar response was observed in the AlHV-1-infected rabbit cell lines BJ1859 and BJ1860 and in rabbit control cells on addition of IL-15 (0.1 to 100ng/ml) and IL-2 (1 to 400U/ml) (Fig 3.7a, b). The addition of IL-2 at 50U/ml along with different concentrations of IL-15 (0.1, 1.0, 10 and 50ng/ml) to the culture medium significantly increased the growth response of BJ1859 and BJ1860 when compared to IL-15 alone ($p \leq 0.02$).

In the OvHV-2-infected LGL cell line BJ1035 and control cells the levels of BLT esterase in culture supernatants was unaffected by the dose of cytokine added. However, there was a dose-dependent rise in the intracellular BLT esterase activity in the total population of BJ1035 cells per culture with increasing concentrations of IL-2 and IL-15 (Fig 3.6c, d). The uninfected controls expressed lower levels of intracellular BLT esterase compared to BJ1035 ($p \leq 0.0007$) and were less sensitive to IL2 or IL-15 mediated enhancement of BLT esterase activity. However, at IL-15 concentrations of 100ng/ml, the BLT esterase stored in the total population of BJ1035 cells per culture had declined from peak levels and intracellular BLT levels in control and BJ1035 cell cultures were comparable (Fig 3.7c). Rabbit LGLs and control cells also showed similar patterns in BLT esterase expression in response to IL-2 and IL-15. The intracellular levels of BLT esterase in rabbit control cell cultures were comparable to rabbit LGL cultures only when using 100ng/ml IL-15 in

the culture medium (Fig 3.7d, f). Otherwise, levels of intracellular BLT esterase in AlHV-1-infected rabbit LGL cell cultures were higher than in control cells ($p < 0.01$) and showed an IL-2 and IL-15 dose-dependent increase (Fig 3.7c-f). A combination of IL-2 at 50U/ml and IL-15 at different concentrations (0.1, 1.0, 10, 50 ng/ml) did not produce a significant increase in intracellular BLT esterase levels when compared with IL-15 alone (data not shown). BLT esterase levels in all rabbit cell culture supernatants were not affected by the addition of IL-2 or IL-15 into the culture medium.

Therefore, these experiments demonstrate the ability of IL-2 and IL-15 to stimulate the proliferative response of the LGL cell lines tested here. In addition, both IL-2 and IL-15 are capable of stimulating and /or maintaining the production of the BLT esterase cytotoxic enzyme in LGL cell cultures. In contrast, only high concentrations of IL-15 induced the production of cytotoxic enzymes in control cells. This could indicate that the dose response observed in LGL cell lines represents the maintenance of BLT esterase production while the dose-response of control cells represents the induction of enzyme in a small proportion of the cells.
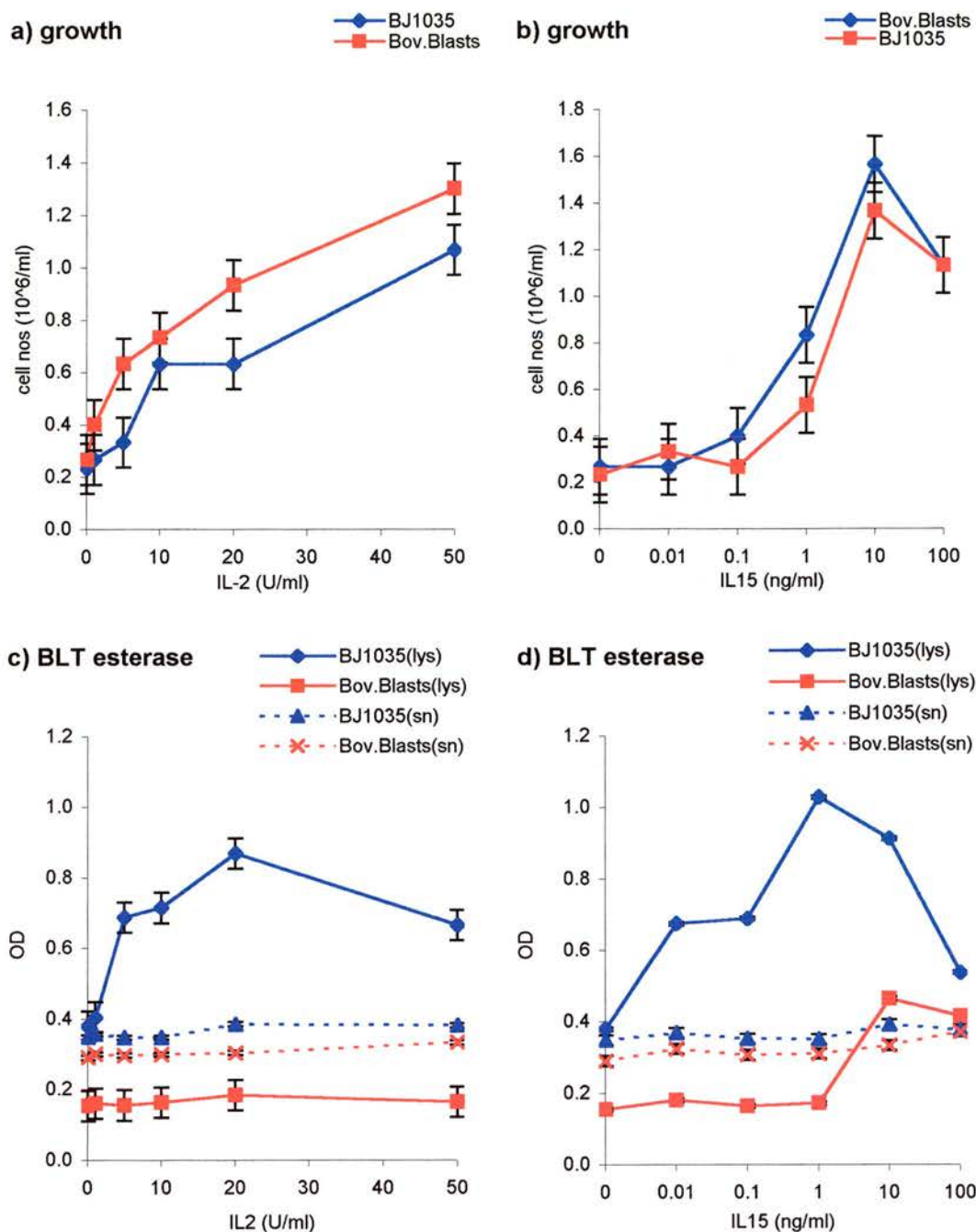
Fig 3.6: Dose-dependent growth and BLT esterase activity in the bovine OvHV-2-infected LGL cell line, BJ1035.

Cells and supernatants were harvested after 6 days in culture with the respective cytokines. BLT esterase activity of cell supernates (sn) and lysates (lys) represent secreted and stored enzyme respectively. Stored enzyme data represent activity present in 10% of the total population of cells per culture. Analysis of enzyme activity was as described in Chapter 2 (materials and methods). Error bars indicate standard error of the mean (s.e.m.). (Statistical analysis performed by ANOVA).

Fig 3.7: Dose-dependent growth and BLT esterase activity in the rabbit AlHV-1-infected LGL cell lines, BJ1859 and BJ1860.
Cells and supernatants were harvested after 6 days in culture with the respective cytokines.
a-b) Growth of LGL cells and control cells in hrIL-2 or hrIL-15. BJ1859+ and BJ1960+ represent data where 50U/ml hrIL-2 with different concentrations of hrIL-15 were added to the cultures.
c-f) (see following page for **d-f)**. BLT esterase activity of cell supernates (sn) and lysates (lys) represent secreted and stored enzyme respectively. Stored enzyme data represent activity present in 10% of the total population of cells per culture. Analysis of enzyme activity was as described in Chapter 2 (materials and methods). Error bars indicate standard error of the mean (s.e.m.). (Statistical analysis performed by ANOVA).
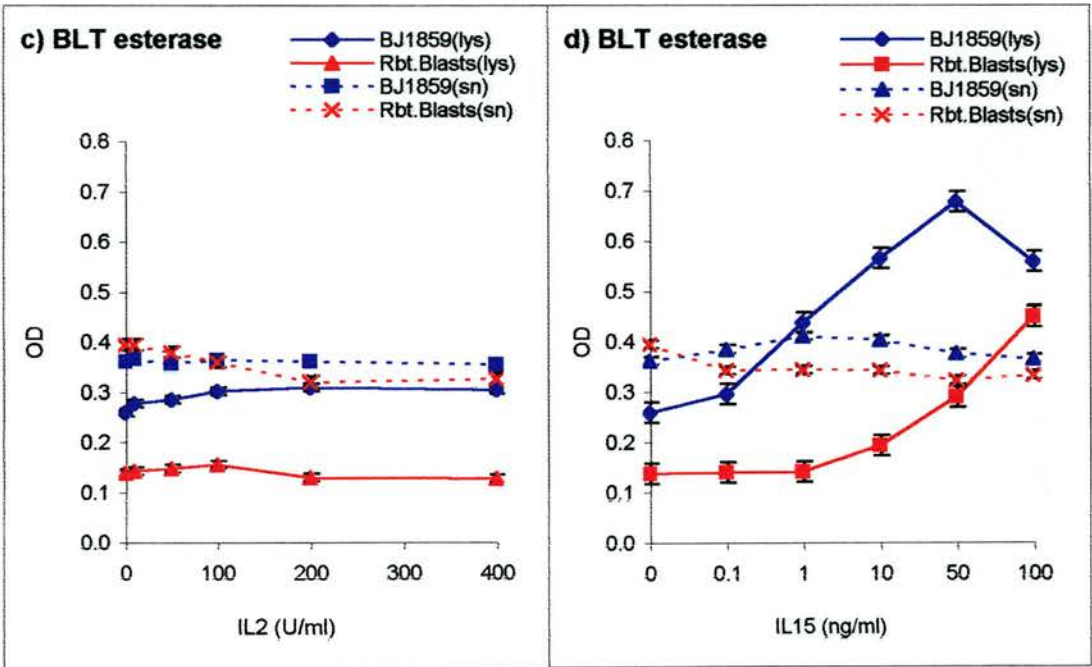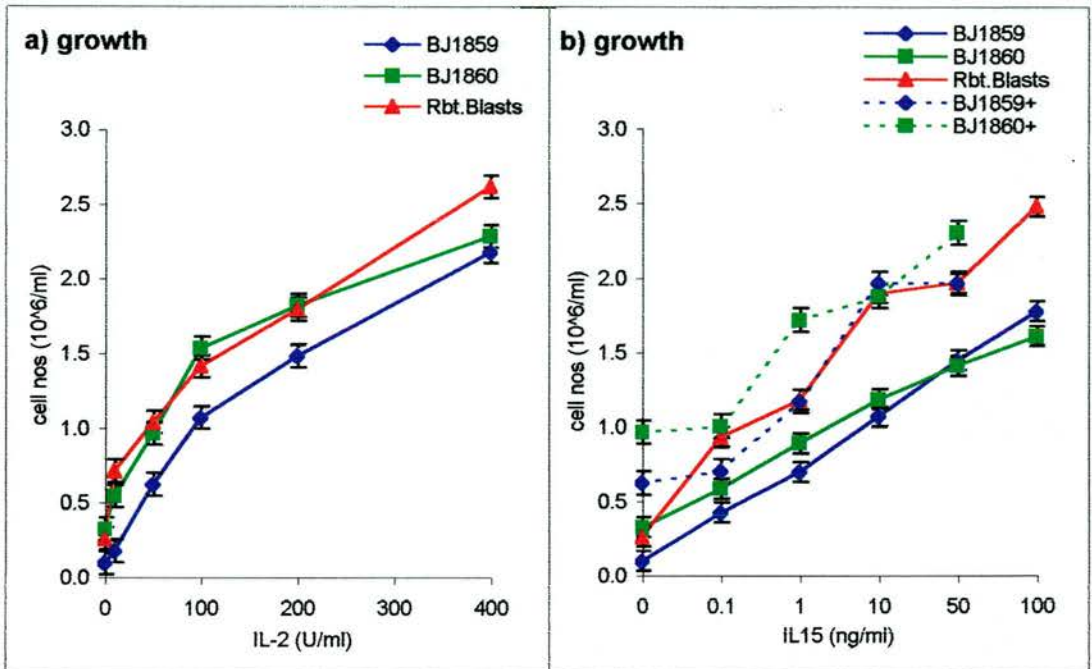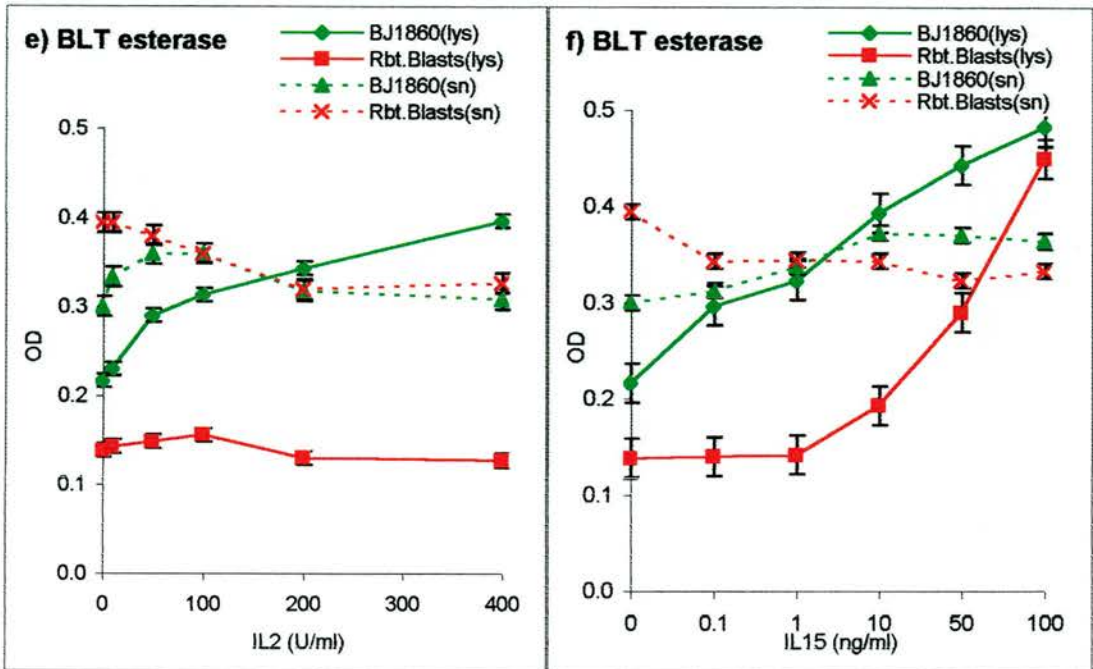
Fig 3.7: (continued).

3.3.6 IL-15 expression by MCF virus-infected LGLs

An immunocytochemical assay performed with the anti-IL-15 antibody (described in Chapter 3.2.6) did not detect any IL-15 in the positive control cells, rabbit monocytes and mouse LTK⁻ cells. Western blotting analysis confirmed that this antibody recognised purified recombinant human IL-15 but did not detect any IL-15 in lysates prepared from the rabbit AlHV-1-infected LGL cell lines BJ1859, BJ1860 or the rabbit OvHV-2 infected cell line BJ1857 (Fig 3.8).

3.3.7 Expression of IL-15 messenger RNA in LGL cell lines

A previous study (C. Rensche, personal communication) by RT-PCR and Southern blotting using an internal primer as a probe (Table 3.1) showed expression of IL-15 mRNA in the rabbit AlHV-1-infected LGL cell line, BJ1001 (Fig 3.9). This present study repeated and extended the previous investigation of IL-15 expression by RT-PCR in a variety of rabbit LGL cell lines (Table 3.5). IL-15 mRNA was not detectable in any of the cell lines, including BJ1001 (Fig 3.10).
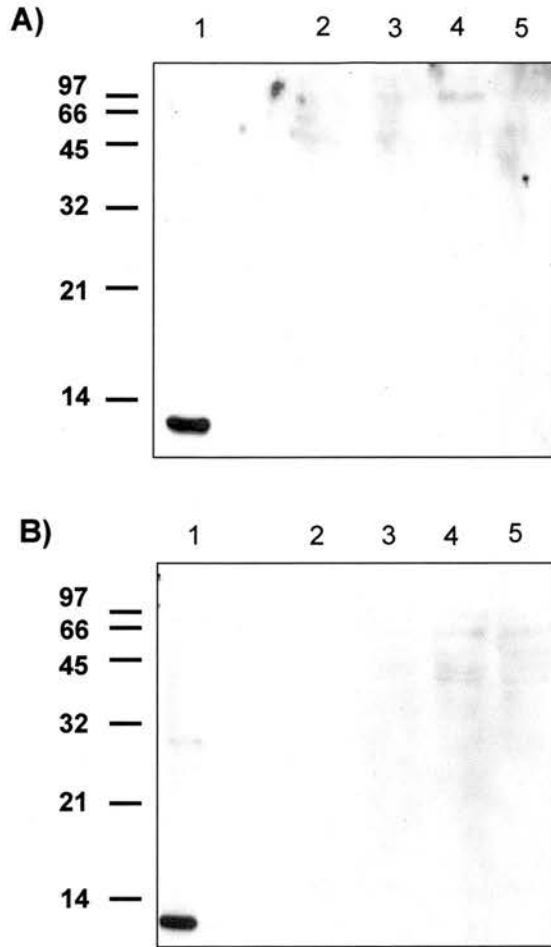
Fig3.8: Western blot analysis of the reactivity of an IL-15-specific antibody (clone L-20) to rhIL-15 and LGL cell lysates.
A) Lane 1: rhIL-15 (100ng). Lane 2: BJ1857 (rabbit OvHV-2^{+}) (not stimulated). Lane 3: BJ1857 (stimulated with conA/TPA, 24hrs). Lane 4: BJ1859 (rabbit AlHV-1^{+})(not stimulated). Lane 5: BJ1859 (stimulated).
B) Lane 1: rhIL-15 (200ng). Lane 2 - BJ1860 (rabbit AlHV-1^{+}) (not stimulated). Lane 3: BJ1860 (stimulated). Lane 4: uninfected rabbit control cells (not stimulated). Lane 5: uninfected rabbit control cells (stimulated).

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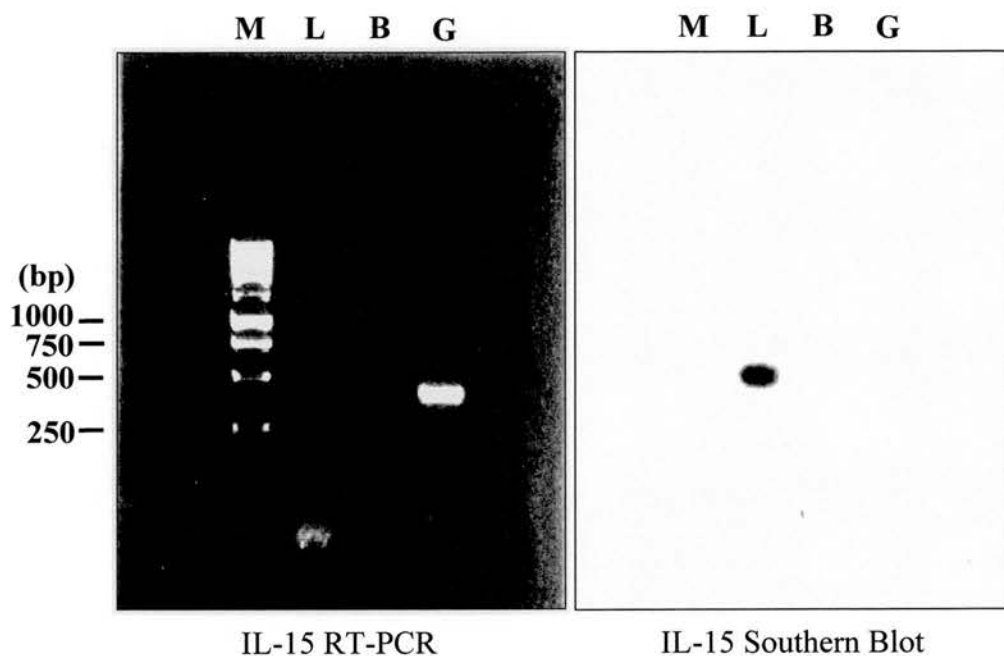
Fig 3.9: IL-15 PCR and Southern Blot.
M = markers; L = rabbit AIHV-1+ cell line, BJ1001; B = uninfected rabbit lymphoblasts; G = GAPDH. Primer used in Southern blot was specific for the 231-270bp region of the bovine IL-15 sequence.

Table 3.6: Cells used in detection of IL-15 by RT-PCR.

Sample Number	Cell type	Virus
1	BJ1857+	OvHV-2
2	BJ1857	"
3	BJ1860	AlHV-1
4	BJ1860-	"
5	BJ1860+	"
6	BJ1860-	"
7	Rabbit lymphoblast (from **lymph nodes**)	Uninfected
8	Rabbit lymphoblast (from **spleen**)	Uninfected
9	BJ1001	AlHV-1
10	BJ1265	OvHV-2
11	BJ1263+	AlHV-1
12	Rabbit lymphoblast (from **lymph nodes**)	Uninfected
13	Rabbit lymphoblast + (from **lymph nodes**)	Uninfected
14	Rabbit lymphoblast (from **lymph nodes**)	Uninfected
15	Rabbit lymphoblast (from **spleen**)	Uninfected
16	BJ1860	AlHV-1
17	BJ1001	"
18	BLANK	---

Sample numbers refer to those indicated in **Fig 3.10**. - = cells cultured without IL-2 (for a minimum of 24 hours). + = cells stimulated with $10\mu g/ml$ conA + 5ng/ml TPA. All other cells were cultured in the presence of hrIL-2. All LGL cell lines were derived from rabbits.
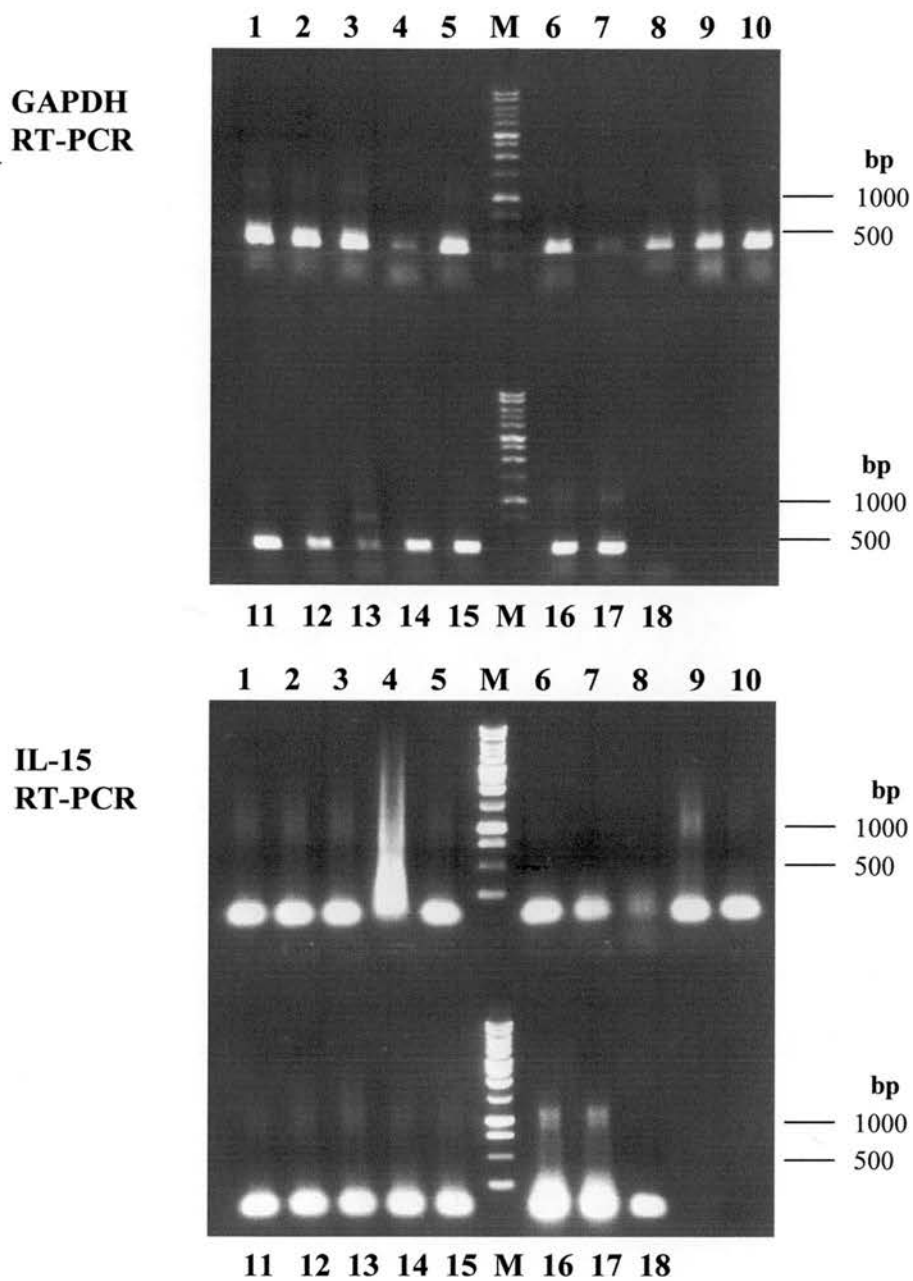
Fig3.10: IL-15 RT-PCR of rabbit LGL cell lines and control uninfected lymphoblasts from rabbit spleen and lymph nodes.
Samples are as indicated in **Table 3.5**. The expected PCR product size for GAPDH = 380bp and IL-15 = 469bp.

3.4 Discussion

3.4.1 Phenotypic and functional analysis

The results confirmed findings from other studies (Cook and Splitter, 1988; Burrells and Reid, 1991; Reid *et al*, 1989; Schock *et al*, 1998) and showed that cell lines established from MCF-affected rabbits infected with AlHV-1 and cattle infected with OvHV-2, have the morphology of large granular lymphocytes (LGLs) and proliferate in the absence of exogenous cytokines for longer periods than control cells. The expression of serine proteases such as BLT esterase (a tryptase) has been associated with cytotoxic activity in T and NK cells following target cell-mediated stimulation (Griffiths and Mueller, 1991). However, the presence of IL-2 in *in vitro* cultures can also induce the expression of serine proteases (Liu *et al*, 1989). Therefore, the expression of BLT esterase in LGL cells cultured without exogenous IL-2 indicates that LGL cells have the phenotype of constitutively activated cytotoxic cells. The cytotoxic activity of the LGL cells on target cells was not demonstrated in this study. The chromium release assay where LGLs are exposed to ^{51}Cr-labelled target cells would need to be performed to demonstrate the cytotoxic activity of these LGL cells. However previous studies using ^{51}Cr-release assays have demonstrated the cytotoxic activity of LGL cells derived from tissues of MCF affected animals (Reid *et al*, 1985). In addition, the occasional use of feeder cell monolayers (which are rapidly destroyed by LGL cells) in the routine culture of the LGL cell lines used in the current study support the idea that these LGLs are functionally cytotoxic. In culture supernatants, the level of BLT esterase detected was not influenced by the dose of cytokine. This could suggest that a steady state between secretion and breakdown of BLT esterase is occurring.

On the basis of the presence of the cell surface antigens CD2, CD4, CD8, CD5 and CD6, previous studies identified the cells in cell lines derived from MCF-affected animals as T cells (Burrells and Reid, 1991; Schock *et al*, 1998). Experimentally infected rabbit LGL cell lines have also been phenotyped in a similar fashion (Reid *et al*, 1983; Schock and Reid 1996). In the present phenotypic analyses, the surface antigen phenotype (with respect to lymphocyte-specific markers) of all of the cells in rabbit and cattle LGL cell lines could not be established

using the available antibodies. However, in some cattle LGL cell lines (BJ1035 and BJ1044) the expression of both CD2 and CD5 on almost 100% of cells and the absence of CD21 expression suggested a T cell phenotype. Thus, in the cell lines BJ1035 and BJ1044 the possibility that CD5 expression is due to the presence of B cells is eliminated. In other LGL cell lines tested where 100% of cells do not express CD2 and CD5 (or where expression of these markers could not be established), the absence of CD21 or surface IgM suggests that these cell lines also do not contain B cells. In some cases, the expression of CD5 at low levels along with CD2 and CD8 expression could describe the cell surface phenotype of NK cells, $\alpha\beta$ T cells or $\gamma\delta$ T cells (Meeusen et al, 1988; Howard and Naessens 1993). Clearly, more information is required to phenotype these LGL cell lines further. For example, the co-expression of these antigens on LGL cells has not been determined but would be helpful for the identification of LGL cell phenotype. An analysis of CD2 expression in rabbit LGL cell lines should be studied once a rabbit CD2-specific antibody becomes available. The detection of CD3 (which forms part of the T cell receptor complex not found on NK cells) was attempted using two antibodies reactive to cattle and rabbit CD3 respectively (Davis et al, 1993; Jones et al, 1993). Attempts to phenotype cattle and rabbit LGL cells using the CD3-specific antibodies gave inconclusive results despite having positive reactivity against PBMCs. Therefore, the expression of the definitive T-cell specific marker CD3 should be re-evaluated (by using different antibodies if possible or by RT-PCR) to confirm (or not) a T cell phenotype. This would enable the identification of these CD2$^+$ cells as T cells rather than NK cells that can also express CD2 (Perussia et al, 1983).

The phenotypic analysis of LGL cells is complicated by the occurrence of phenotypic drift over time in culture. For example, in the present study the OvHV-2-infected cell line BJ1104 did not express CD2. In a previous analysis of this cell line, this marker was expressed (Schock et al, 1998). A disparity between the past and present analysis of the cell lines BJ1035 and BJ1044 was also observed (Schock et al, 1998). Phenotypic drift was also observed in the rabbit LGL cell lines. This was demonstrated by the enrichment of CD8 expressing cells and loss of CD4 expressing cells in rabbit LGL cell lines during the first few months of culture. This phenomenon has been described previously where short-term cultures from AlHV-1-

infected rabbits were shown to contain significantly fewer CD4$^+$ cells and more CD8$^+$ cells than cultures from an uninfected animal (Schock and Reid, 1996). The phenotypic drift of the LGL cell lines after short periods in culture may reflect the adaptation of the cultures to conditions *in vitro* despite the presence of virus. In addition, it is possible that these short-term cultures represent a mixture of uninfected lymphocytes activated in response to infection of other cells as well as the virus infected LGLs that eventually become established cell lines. Thus, established LGLs may be a better reflection of the cells involved in pathogenesis than early cell cultures. At the other extreme, LGL cell lines established *in vitro* for years show some disparities between different phenotypic analyses. This may suggest that long-term culture influences the expression of cell surface antigens or that there is a genuine shift in phenotype due to expression of viral genes. Alternatively, the emergence of different populations of cells over time may reflect the fact that the LGL cell lines are not cloned cell lines. However, it is not clear in this study whether the differences in the phenotypic analyses is due to the emergence of a different population of cells or due to changes in expression of specific markers on the same cells. Difficulties in defining a constant cell surface phenotype has been encountered in the past (Schock, 1996). This may simply indicate that like other herpesviruses, the MCF viruses have the ability to infect more than one cell type.

In conclusion, there are important features common to all LGL cell lines irrespective of cell surface phenotype:

1) The majority of MCF virus-infected cells exhibit the morphology of large granular lymphocytes after approximately one month in culture

2) LGL cell lines are not B cells

3) LGL cell lines have a cytotoxic phenotype (most cells are BLT esterase$^+$), which supports the results showing that a proportion of these cells express surface antigens of T cells or NK cells

4) LGL cell lines express virus genes or transcripts

5) Irrespective of phenotype and whatever the reasons for phenotypic drift LGL cell lines maintain their ability to cause MCF in susceptible rabbits when used as an inoculum.

3.4.2 Growth characteristics of LGL cell lines in culture: the role of IL-15

The lack of requirement for exogenous IL-2 by LGL cell lines over a limited period of time is comparable to the growth characteristics of some HVS-transformed T cells (Meinl *et al*, 1995). HVS-transformed cell lines require high cell densities for optimum growth. This was also a characteristic of LGL cell lines in this study. Moreover, survival in IL-2 deficient medium was cell density-dependent. These characteristics suggest firstly that the LGL cell lines, due to MCF virus infection, may secrete a growth factor(s) into the medium that acts in an autocrine fashion. This is also supported by the fact that the LGL cultures cannot be maintained if all of the culture medium is changed during passage. Secondly, these characteristics also suggest that cell to cell contact may be an important factor for cell survival. In the case of HVS-transformed T cells, stimulation through the interaction of CD2 with its ligand CD58 is thought to be important for continuous growth of these cells in culture (Mittrücker *et al*, 1992). The stimulation of LGL cells through direct cell to cell contact may induce the activated phenotype observed in these cells. The virus-mediated production of cytokines by LGLs could be facilitated through the interaction of surface molecules such as CD2 on LGL cells. Indeed, even in the absence of direct cell to cell contact the close proximity of cells to each other may be sufficient to maintain the cycle of virus-induced cytokine stimulation of LGL cells. The finding that survival of LGL cell lines without exogenous IL-2 exceeds that of control uninfected cells has been exploited in these experiments to reveal characteristics of LGL phenotype and function associated with virus infection of these cells.

Despite the ability to grow in culture without exogenous IL-2, previous studies have shown that bovine OvHV-2-infected LGL cell lines do not transcribe IL-2. However, IL-2Rα is present on the cell surface and the cells respond to IL-2 (Shock *et al*, 1998). The complete removal of LGL culture supernatant during routine passage of these cells is detrimental to optimum LGL growth. Partial characterisation of cytokines expressed by bovine OvHV-2-infected LGL cells demonstrated the presence of IL-4, IL-10, IFN-γ and TNF-α but not IL-2 or IL-1β mRNA (Schock *et al*, 1998). Therefore, these or other undefined cytokines in culture supernatant may support the growth and survival of LGL cells in the absence

of exogenous IL-2. The presence of growth factors in LGL supernatants have been tested by their ability to stimulate uninfected PBMCs (Schock, 1996). The results of these studies showed the existence of low levels of stimulatory activity in LGL culture supernatants. However, it is difficult to determine whether this was due to low levels of growth factor secretion by LGLs or due to growth factor sequestration on receptors in LGL cultures. The evidence in the present study suggesting the recovery of LGL cell numbers following a few days in culture in the absence of exogenous hrIL-2 would indicate that LGLs do produce their own growth factors. Of the cytokines known to be transcribed by LGLs, none were likely candidates for allowing IL-2-independent growth. Therefore, the virus-induced production of IL-15 by LGL cells was suggested as a mechanism for survival in culture in the absence of exogenous and endogenous IL-2.

The shared effects of IL-2 and IL-15 are mediated by the use of a common cytokine receptor - the IL-2Rβγ (Grabstein *et al*, 1994; Carson *et al*, 1994; Giri *et al*, 1994). The IL-2Rβγ along with IL-2Rα form the three components of the IL-2 receptor (Smith, 1988). The IL-2Rβ and γ chains mediate signal transduction through non-receptor tyrosine kinases (NRPTKs) (Miyazaki and Taniguchi, 1996; Siegel et al, 1987). Cell activation induces the expression of the IL-2Rα (Cantrell and Smith, 1983; Koyasu *et al*, 1991; Uchiyama *et al*, 1981). The association of the IL-2Rα with the βγ chains mediates high affinity binding ($K_d = 10^{-11}$ M) of IL-2 to this receptor complex (Kondo *et al*, 1986; Robb, 1986; Takeshita *et al*, 1992). IL-15 uses the IL-2Rβγ for low affinity binding and intracellular signalling (Carson *et al*, 1994; Giri *et al*, 1994). Alternatively, the response of cells to IL-15 can be mediated through the binding of IL-15 to its high affinity ($K_d = 10^{-11}$ M) receptor, IL-15Rα in association with IL-2Rβγ on lymphoid cells (Giri *et al*, 1995a.b; Anderson *et al*, 1995). The expression of the IL-15Rα has been detected in T cell, B cell and macrophage cell lines and NK cells as well as in non-lymphoid tissue (Giri *et al*, 1995b).

The results of the present study suggests that hrIL-15 stimulates LGL cell proliferation and maintains or enhances BLT esterase expression. IL-15 also stimulated the proliferation and at high concentrations enhanced the expression of BLT esterase in control uninfected lymphoblasts. The elevation of BLT esterase

activity in control cells at high IL-15 concentrations could suggest the presence of a small proportion of cytotoxic cells within the control lymphoblast population in which BLT esterase is induced. The proliferative response of LGL cells and control cells to IL-15 suggests that these cells express a functional receptor for IL-15. The results also indicate that IL-15 could replace IL-2 as has been demonstrated in adult T cell leukaemia cell lines (Yamada *et al*, 1998). In this case, the response to IL-15 was mediated by the expression of IL-15Rα. Whether the binding of IL-15 on LGL cells takes place through the low affinity IL-2Rβγ or the high affinity IL-15Rα/IL-2Rβγ complex is not known. The receptor usage by either control or LGL cells was not determined as the important finding in this study was that virus-infected LGL cells express higher levels of BLT esterase than uninfected cells and that IL-15 enhances BLT expression in LGL cells but not in uninfected cells. Therefore, the implication is that the MCF viruses infect cells capable of cytotoxicity or that virus infection itself may enhance the cytotoxic ability of infected and uninfected bystander lymphocytes *in vivo*.

The induction of cellular IL-15 by the T cell transforming virus HTLV-1 through Tax-1 (a viral transactivating protein) (Azimi *et al*, 1998) has been observed. As viral homologues of IL-15 have not been observed in the genomic sequence of AlHV-1 (Ensser *et al*, 1997) any IL-15 produced in LGL cells must be of cellular origin. RT-PCR detection of IL-15 in rabbit LGL cell lines and controls was unsuccessful. This was probably due to use of primers based on the bovine IL-15 sequence, as the sequence for rabbit IL-15 is not available. This may have consequences for the specificity of the primers for rabbit IL-15 and thus explain the problems in detecting the IL-15 transcript in rabbit cells. An initial analysis (Christine Rensche, personal communication) showed a positive result on one occasion. The inability to repeat this may be due to transient expression of IL-15 (giving a negative result) or may be due to the presence of contaminating cells (in Rensche's study) that can produce IL-15 (giving a positive result). However, comparison of the bovine IL-15 sequence chosen for the generation of primers with that of human, mouse, rat and pig IL-15 shows a high degree of homology to each other (Fig 3.11). The failure of the anti- human IL-15 antibody to detect IL-15 in rabbit control and LGL cell lysates may be due to the lack of sensitivity of the

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western blot or the lack of antibody reactivity with rabbit IL-15. Analysis of bovine LGLs for IL-15 mRNA or protein was not done as bovine LGLs were not available at the time as attempts to resurrect these cells from liquid nitrogen had failed. This could be explained by the reactivation of virus replication in LGLs or simply due to cell death on return to culture. The detection of IL-15 protein in tissues from MCF virus-infected animals using anti-IL-15 antibodies was attempted but the results were inconclusive due to problems with non-specific antibody reactivity. Due to time restrictions, the detection of IL-15 in bovine control tissues was not attempted. This has since been achieved. In conclusion, given the inability to find a suitable IL-15-producing uninfected rabbit positive control the question of IL-15 expression in rabbit LGL cells remains unanswered.

Further work with other anti-IL-15 antibodies may be useful and RT-PCR or *in situ* mRNA hybridisation analysis on bovine LGL cell lines may be informative. The detection of IL-15 mRNA by RT-PCR in bovine LGLs is currently being investigated. Any future attempts to detect IL-15 mRNA in rabbit LGLs could benefit from the introduction of alternative nucleotides in the primer sequence at the non-homologous sites.

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                                    20          30          40
(5'primer)BOVIL15 -CATATTTGAGAAGTACTTCCATCCAG-
          HUMIL15 -CACATTTGAGAAGTATTTCCATCCAG-
          MUSIL15 -CATATATGAGGAATACATCCATCTCG-
          RATIL15 -CATATATGAGGAATACATCCATCTTG-
          PIGIL15 -CATGTTTGAGAAGTACTTGCATCCAG-
                   **   *  ****  *  **   *  ****   *


                                   470         480
(3'primer)BOVIL15 -TGTCCAAATGTTCATCAACACTTC-
          HUMIL15 -TGTCCAAATGTTCATCAACACTTC-
          MUSIL15 -TGTCCAAATGTTCATCAACACGTC-
          RATIL15 -TGTCCAAATGTTCATCAACACGTC-
          PIGIL15 -CGTGCAAATGTTCATCAACCCTTC-
                   **  ***************  *  **
```

Fig 3.11: Sequence alignment of bovine IL-15 primers to IL-15 sequences from other species.

'*' Indicate sites of sequence identity between species. Scale indicates nucleotide position with respect to the start codon (ATG). BOV = bovine; HUM = human; MUS = mouse; RAT = rat; PIG = pig.

Chapter 4

Signal transduction in MCF virus-infected cell lines

4.1 Introduction

In the previous chapter, MCF virus-infected cells were demonstrated to have the morphology of large granular lymphocytes; have the ability to survive and grow in the absence of exogenous cytokines for longer periods of time than uninfected cells; constitutively express the cytotoxicity-associated enzyme BLT esterase; display a phenotype most closely resembling T cells or NK cells and respond to IL-15. Some of these characteristics are similar to those observed in HVS-transformed cell lines (Meinl *et al*, 1995). The mechanism for T cell dysfunction following HVS infection involves viral interference with T cell signalling molecules. For instance, the transforming protein Tip interacts with the T cell-associated signal transduction molecule, *lck* - a *src* kinase (Biesinger *et al*, 1995; Jung *et al*, 1995a; Lund *et al*, 1996). This kinase is involved in T cell signalling via the T cell receptor (TCR), the IL-2 receptor and other surface molecules such as CD2 and CD4 (Crabtree and Clipstone, 1994). The HVS transforming proteins known as STPs also bind to *ras* (which is involved in signalling downstream of *lck*) (Jung and Desrosiers, 1995) and *fyn* (another *src* kinase involved in the early stages of the signalling pathway) via interaction with *c-src* (Lee *et al*, 1997). Similarly, the EBV proteins LMP-1 and 2A block the transduction of signals from the B cell receptor (BCR) by binding the kinases *lyn* and *syk* (Miller *et al*, 1995) or by binding signalling molecules normally associated with the TNF receptor (Izumi and Keiff, 1997; Mosialos *et al*, 1995). Therefore, these two gammaherpesviruses have evolved mechanisms that target lymphocyte *src* kinases and other downstream signalling molecules in order to control virus life cycle and pathogenicity. In this chapter, the possibility that the MCF viruses express proteins that interfere with lymphocyte signalling is explored. This form of virus-host interaction could explain the proliferative and constitutively cytotoxic phenotype of the LGL cell lines. Given this background an analysis of T cell-associated kinases (eg *lck*, *fyn* and ZAP-70) in MCF virus-infected cell lines is therefore appropriate.

The strategy adopted for this study was to compare the phenotype of MCF virus-infected cattle and rabbit LGLs with uninfected 'resting' or activated lymphocytes from the respective species. The objective was to determine whether

infected LGLs had altered signalling pathways compared to uninfected control lymphocytes. This objective was approached from two directions. First, the indirect approach used biochemical inhibitors of signal transduction pathways to define general pathways utilised by the LGLs. The specific inhibitors used and their mode of action will be discussed in more detail over the course of this chapter. The second approach was to target specific signalling molecules based on the outcome of the inhibitor studies. To do this, signalling molecules were isolated from cell lysates by immunoprecipitation. The state of activation of the precipitated kinases can then be assessed (either by the presence of phosphorylated tyrosine residues or by testing the ability of the kinase to transfer radioactive phosphate onto a known substrate). The presence of tyrosine kinases (such as *lck* and *fyn* and ZAP-70) were also assessed by examining the presence of tyrosine phosphorylated proteins in whole cell lysates. In these studies, the activating effect on the LGLs of exogenous IL-2 could mask any virus-induced effect within the cells. For this reason, LGL cell lines were deprived of exogenous IL-2 for at least 30 hours before analysis of signalling molecules and pathways.

4.2 Materials and Methods

4.2.1 Inhibitors

Rapamycin (Calbiochem-Novabiochem, San Diego, CA) - an inhibitor of S6 kinase - and genistein (TCS Biologicals, Botolph Claydon, UK) - a general tyrosine kinase inhibitor - were diluted in DMSO before use. Cyclosporin A (Sandoz, Sandimmun, Switzerland) – an inhibitor of the Ca^{2+}/calcineurin pathway - and sodium orthovanadate (Sigma Aldrich, Gillingham, UK) – a phosphatase inhibitor - were diluted in water.

Experimental protocol:

OvHV-2-infected LGL cell lines (BJ1035, BJ1104) were grown in hrIL-2-deficient medium for 3-4 days then transferred to 96-well plates at a density of 10^6 cells/ml (100µl per well). The rabbit AlHV-1-infected cell line BJ1859 was also set up at 10^6 cells/ml in 96-well plates following culture for 2-3days in hrIL-2-deficient medium. The inhibitors were added to each well at a range of concentrations (see results). As a carrier control, DMSO alone was added to a well at the maximum concentration used in each inhibitor study. The growth response of the IL-2-independent cell lines to the addition of inhibitors was measured as numbers of viable cells (determined by nigrosine exclusion) per ml of culture. Data were collected over three days in culture.

4.2.2 Cell proliferation assay using ^{3}H-thymidine

Antibodies that cross-link the T cell receptor and activate rabbit and bovine T cells are not available. Therefore, the T cell mitogen conA was used in this study. To test the proliferative response of LGL and control cells to different concentrations of mitogen the ^{3}H-thymidine incorporation assay was used. 100µl aliquots of cells at a density of 2 x 10^6 cells/ml IMDM were cultured in 96-well plates for two days. The cells were then incubated with 50ml of ^{3}H-thymidine (0.5µCi/well) for a further 18 hours. Cells were harvested onto membranes and ^{3}H-thymidine incorporation measured in a β-scintillation counter (Tri-carb 2500 TR, Packard, Meriden, USA).

4.2.3 Stimulation of lymphocyte signalling molecules and preparation of cell lysates

The bovine OvHV-2-infected LGL cell line BJ1035 and the rabbit AlHV-1-infected cell lines BJ1859 and BJ1860 were used in this study. The bovine LGL cell line BJ1035 was cultured for 48 hours to 22 days in the absence of exogenous IL-2. Samples were taken at various points during this period of IL-2-deficient culture. The rabbit LGL cell lines were also taken from culture between 48 and 96 hours after deprivation of exogenous IL-2. Cell viability at this stage was >70%. Control rabbit and bovine PBMC or conA-activated lymphoblasts were also deprived of exogenous IL-2 for up to 30 hours. After this point, cell viability in control cell cultures dropped below 70%. The maintenance of IL-2-independent cultures is described in Chapter 2.2.4.

IL-2-deprived control and LGL cells were stimulated using 10µg/ml conA for 0, 3, 5, 10 and/or 30 minutes at 37°C in serum-free medium. The dose of conA was determined by dose-response experiments using the phosphotransferase assay (Chapter 2.4.5). The preparation of cell lysates following stimulation of cells proceeded as described in Chapter 2.4.1. Cell lysates were used in western blot analysis or in subsequent immunoprecipitation protocols (see below).

4.2.4 Immunoprecipitation (IP) of signal transduction molecules

Signal transduction molecules were immunoprecipitated from cell lysates using specific antibodies at the concentrations indicated (Table 4.1) and as described in Chapter 2.4.1. Immunoprecipitated samples were prepared for western blotting or phosphotransferase assays as described in Chapter 2.

4.2.5 SDS-PAGE and western blot analysis

Whole cell lysates or immunoprecipitates from cell lysates were prepared for SDS-PAGE as described in Chapter 2.4.2 and analysed by western blot (Chapter 2.4.4). For the detection of phosphotyrosine in proteins, nitrocellulose membranes

were incubated in 4G10 antibody (diluted 1:1000 in wash buffer (0.5M NaCl, 0.5% (v/v) Tween 80 in PBS). *Lck* and *fyn* were detected using the same antibodies as described for immunoprecipitation and used at a concentration of 1:1000 in wash buffer.

4.2.6 Determination of *lck* and *fyn* activity by *in vitro* phosphotransferase assay

Immunoprecipitation of *lck* and *fyn* were performed as described in Chapter 2.4.1 using the antibodies in Table 4.1. The activity of these kinases was determined in the phosphotransferase assay as described in Chapter 2.4.5.

Table 4.1: Antibodies used for immunoprecipitation (IP) and western blot.

Antibody	Specificity	Species reactivity	Mol. Wt. (of IP antigen)	Antibody Isotype
3A5	p56*lck* peptide (aa54-222)	human/ mouse/rat	56/59kDa (doublet)	mouse IgG2b
15	p59*fyn* peptide (aa85-206)	human/ mouse/rat	59kDa	mouse IgG1
4G10	phospho-tyrosine	all species	protein-dependent	mouse IgG2bκ

All antibodies (except 4G10) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. 4G10 was purchased from Upstate Biotechnology, Lake Placid, NY. All antibodies were used at a final concentration of 1-2µg/ml.

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4.3 Results

4.3.1 Signal transduction inhibitor studies: effect on cell growth

To determine the ability of biochemical inhibitors of signal transduction pathways to inhibit the growth response of IL-2-independent cells these chemicals were added to culture medium and cell growth observed over 3-4 days. These experiments were designed around inhibitor concentrations demonstrated to affect T cell lines as reported in previous studies (Baldari and Telford, 1994; Gajewski *et al*, 1995; Luo *et al*, 1993). It should be noted at this point that the interpretation of results from these experiments were made with the knowledge that appropriate control cells could not be included in this study. As this experiment measures the ability of inhibitors to reverse the IL-2-independent phenotype of LGL cells, the appropriate control cells would need to be constitutively activated lymphoblasts with T/NK cell phenotype and the ability to survive IL-2 deprivation. As these control cells were not available, the results of these experiments were used merely to indicate possible trends in LGL signalling. The data from these experiments represent single observations. Since then some of these experiments have been repeated using triplicate wells and are indicated by error bars - which represent standard error of the mean (see Fig 4.1).

The cell lines BJ1035, BJ1104 (bovine OvHV-2 infected cells) and BJ1859 (rabbit AlHV-1 infected cells) were cultured without IL-2 for 2-3 days prior to addition of inhibitors. In the absence of inhibitors, IL-2 independent survival was maintained for a further 3 days. The growth response of these cells on addition of an inhibitor differed according to the inhibitor used. Cyclosporin A (CsA) showed a dose-dependent inhibitory effect on IL-2 independent cell growth in BJ1035 after 3 days in culture with CsA (Fig 4.1a). Sodium orthovanadate had a small inhibitory effect on cell growth in BJ1035 following 3 days of culture with $100\mu M$ Na_3VO_4 (Fig 4.1b). The inhibition of S6 kinase using rapamycin (which blocks the entry of resting T cells into the cell cycle) did not inhibit the growth of BJ1035 or BJ1104 in IL-2-deficient medium (Fig 4.1c). Addition of genistein at $100\mu M$ to BJ1035 cultures (Fig 4.1d) inhibited cell survival and growth over 3 days (p<0.01, compared

with medium control values). A similar response was noted in BJ1859 (rabbit, AlHV-1-infected) cultures using genistein at 10µM and 100µM (p<0.01) (Fig 4.1d). The response of BJ1104 to genistein followed the same trend as that observed in BJ1035 and BJ1859 although statistical analysis was not performed as the BJ1104 data represents single observations.

4.3.2 Mitogen stimulation of LGL and control cells

The proliferative response of LGLs and control cells (deprived of exogenous IL-2 for approximately 30 hours) to conA ± TPA stimulation was assessed by ^{3}H-thymidine incorporation over three days and the results shown in Table 4.2. The results show that mitogen induces proliferation of bovine and rabbit control lymphoblasts. However, both rabbit and bovine LGL cells (deprived of exogenous IL-2 for 2 and 4 days respectively) showed poor (if any) proliferative responses to mitogen. The use of TPA in addition to conA did not alter the response observed with conA alone.

4.3.3 Analysis of tyrosine phosphorylated proteins in total cell lysates

The role of protein-tyrosine kinase signaling molecules in MCF virus infection was suggested by the response of LGL cell lines to genistein. To determine whether MCF-virus infection was associated with differences between control and LGL cell lines the presence of tyrosine phosphorylated proteins in cell lysates of IL-2-deprived cultures (with and without 5 and 10 minutes of stimulation with conA) was investigated. During this period of stimulation, the non-receptor tyrosine kinases (NRPTKs) such as *lck* and *fyn* are likely to be activated and phosphorylated. The presence of tyrosine phosphorylated proteins was assessed by western blot using the phosphotyrosine-specific antibody, 4G10. Fresh peripheral blood mononuclear cells (PBMCs) or conA-stimulated lymphoblasts were used as controls. The data shown represents the best two of three repeated experiments.

In unstimulated rabbit AlHV-1-infected LGL cell lines BJ1859 and BJ1860 tyrosine-phosphorylated proteins of approximately 90, 70, 55-59, 48, 38 and 21 kDa were detected (Fig 4.2a) and was observed in repeated 4G10 western blots (Fig

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4.2b). After 5 minutes stimulation with conA changes in the intensity of some bands were observed, though this was not consistent in repeat analyses. ConA-stimulated control rabbit lymphoblasts expressed a similar set of phosphorylated tyrosine bands as those observed in the unstimulated rabbit LGL cell lines. These same bands appeared at low levels in the unstimulated rabbit lymphoblast cell lysates.

Stimulated bovine control PBMCs or lymphoblasts and unstimulated bovine OvHV-2-infected cell line BJ1035 expressed tyrosine-phosphorylated proteins of approximately 90, 70, 55-59, 48, 38, and 21 kDa (Fig4.2c, d). Unstimulated control lymphoblasts expressed low levels of tyrosine-phosphorylated proteins compared to stimulated control cells. Tyrosine-phosphorylated proteins were not detectable in unstimulated PBMCs. After stimulation, BJ1035 exhibited changes in the intensity of some bands while others appeared unchanged. The changes in band intensity following stimulation were also observed in a repeated analysis (Fig4.2d). However, these changes in band intensity were not consistent between repeated analyses (Fig 4.2c, d).

Fig 4.1: Growth response of LGL cell lines to signal transduction inhibitors.

Cell lines BJ1035, BJ1104 (bovine, OvHV-2-infected) and BJ1859 (rabbit, AlHV-1-infected) were seeded at a density of 10^6 cells/ml (100μl per well) at day 0. Viable cell counts were performed after 3 days of culture in the following inhibitors: **a)** CsA = cyclosporin A, **b)** Na_3VO_4 = sodium orthovanadate, **c)** rapamycin and **d)** genistein. med. control = medium only. Error bars indicate mean ± sem for triplicate wells. Where error bars are absent, data represent single observations.
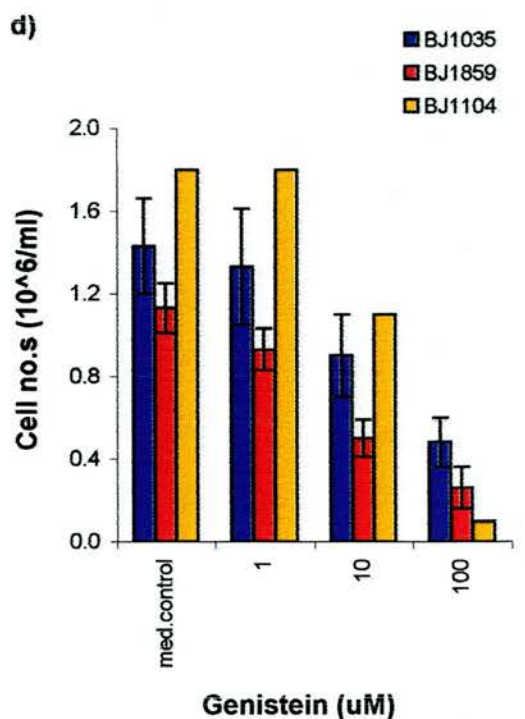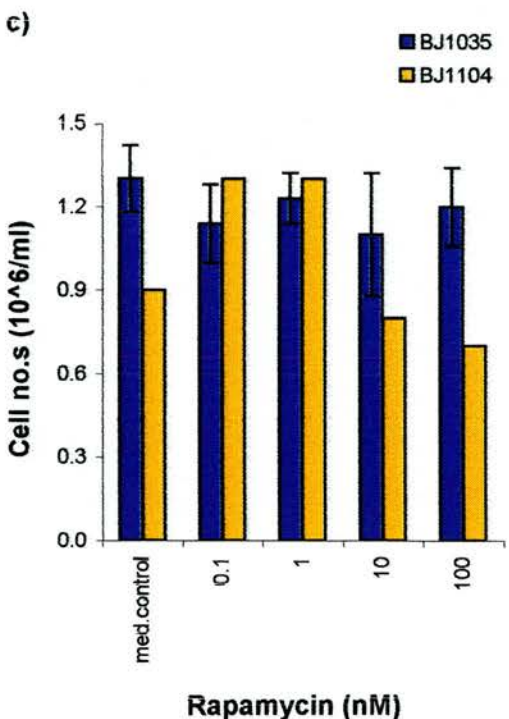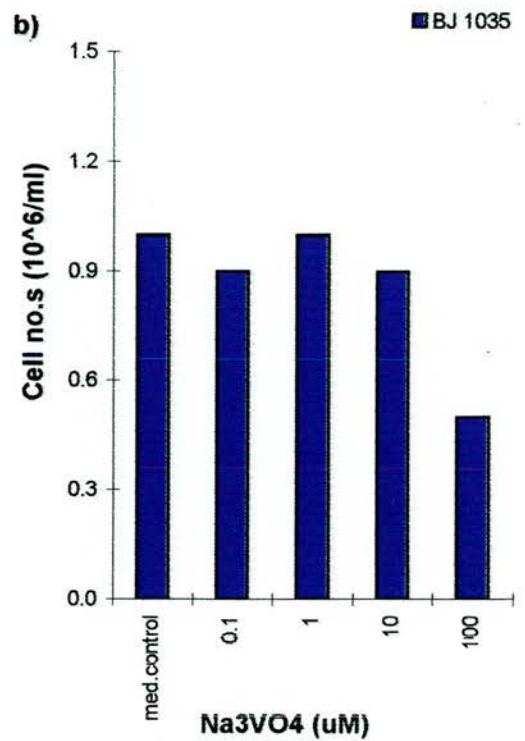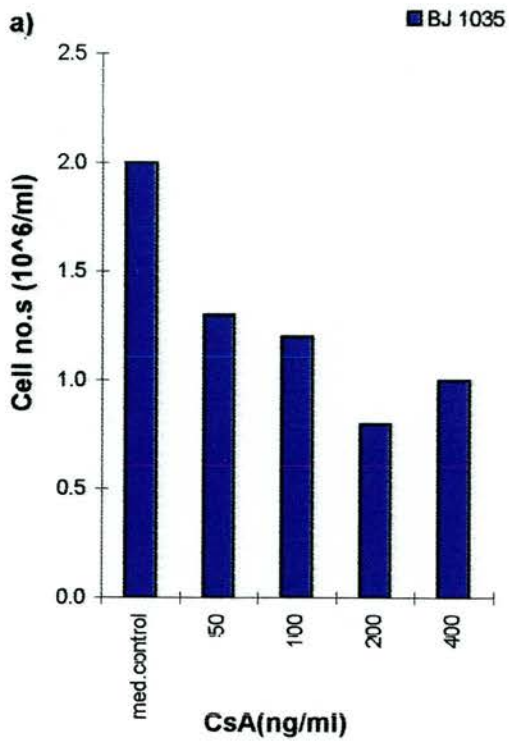
Table 4.2: Mitogen stimulated proliferation of LGLs and control cells.

	BJ1859	BJ1860	BJ1035	BovBlast	RbtBlast
medium only	2498±131	1292±22	1060±21	132±12	22±1.2
TPA only	1245±68	1376±116	1448±71	896±72	130±9
conA (2.5)	2044±110	1342±98	856±41	3160±148	2312±168
conA (5)	2467±106	1825±146	634±5	4820±246	4307±249
conA (10)	1349±126	1648±108	1368±102	2734±98	1030±68
conA (1.25) + TPA	2233±102	1650±78	1240±39	4668±236	3220±49
conA (2.5) + TPA	2402±114	1727±232	893±171	5796±342	4704±137
conA (5) + TPA	2264±242	1924±110	1403±125	5204±124	1489±35

BJ1859 and BJ1860 are rabbit, AlHV-1-infected LGL cell lines while BJ1035 is a bovine, OvHV-2-infected LGL cell line. Control cells are rabbit (RbtBlasts) or bovine (BovBlasts) conA-stimulated lymphoblasts. Data are mean counts per minute (cpm) and error bars represent standard error of the mean (sem) from triplicate samples in the ^{3}H-thymidine incorporation assay. TPA used at 5ng/ml alone or in combination with conA (mg/ml) used at doses shown in parenthesis.

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Fig 4.2: Phosphotyrosine proteins in LGL and control lysates.

4G10 anti-phosphotyrosine western blot of lysates from unstimulated (-) cells or cells stimulated for 5 (5) or 10 (10) minutes with 10μg/ml conA and separated by SDS-PAGE. The best two of three repeated experiments are shown.

A and **B**) Control rabbit conA-stimulated lymphoblasts (L/blasts) and the AlHV-1-infected LGLs, BJ1859 and BJ1860.

C and **D**) Control bovine peripheral blood mononuclear cells (PBMC), control bovine conA-stimulated lymphoblasts (L/blasts) and the OvHV-2-infected LGL, BJ1035.
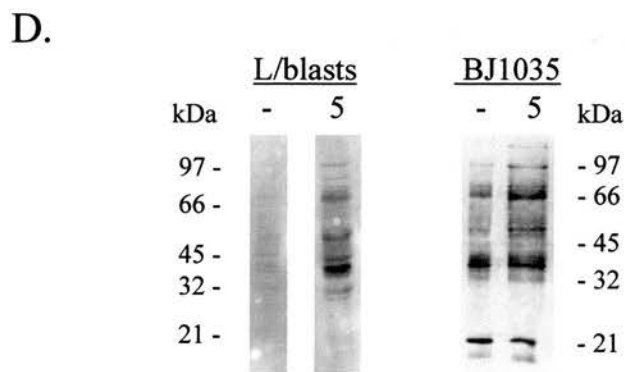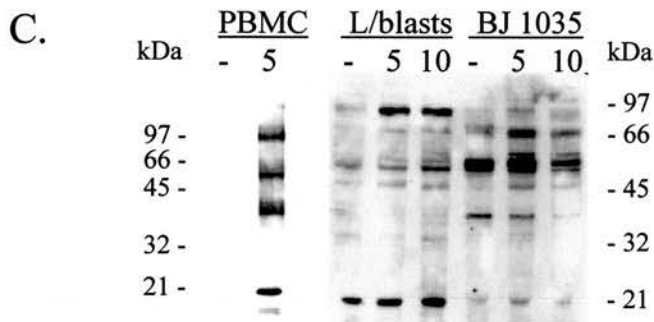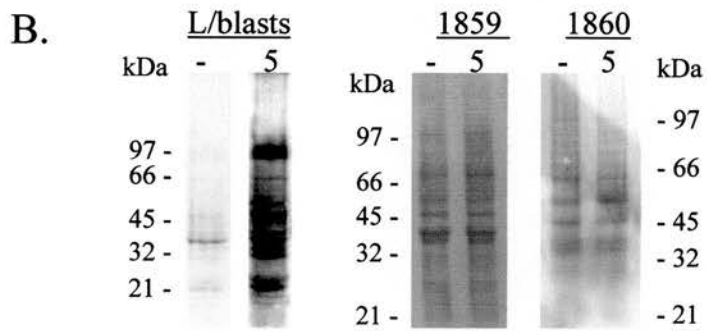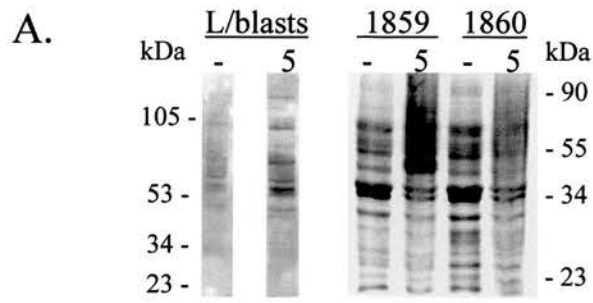
4.3.4 Detection of *src* kinases in LGLs

Western blot analysis of the whole cell lysates of rabbit or bovine LGL cells and control cells with antibodies to *src* kinases and the *syk* kinase ZAP-70 identified only two antibodies - anti-*fyn* and anti-*lck* that reacted with the p55 and p56/59kDa proteins respectively. ZAP-70 could not be detected. The cells were stimulated (or not) with 10μg/ml of conA for 5 and 30 minutes. The concentration of conA was optimised by dose-repsonse experiments using the phosphotransferase assay.

Activity of *lck* and *fyn* kinases was assessed by phosphotransferase assay. This assay measures the amount of radioactive $[\gamma^{-32}P]$-ATP incorporated into a *src* kinase substrate (enolase) by a precipitated kinase. The level of $[\gamma^{-32}P]$-ATP incorporated into enolase was evaluated by autoradiography and by β-scintillation counting. In earlier experiments, only β-scintillation counts were collected. The activity of *lck* and *fyn* were assessed in the rabbit AlHV-1-infected cell lines BJ1859 and BJ1860 and the bovine OvHV-2-infected cell line BJ1035. The data shown represents of the best two of three repeated experiments.

In control rabbit lymphoblasts and the AlHV-1-infected cell lines BJ1859 and BJ1860, immunoprecipitated *fyn* kinase was activated and phosphorylated the substrate, enolase (Fig 4.3). In control cells, the degree of *fyn* activation in 'rested' unstimulated cells was low but increased to high levels following a 5 minute stimulation with conA (Fig 4.3a, d, e). The level of activity observed after 5 minutes stimulation declined after 30minutes stimulation. In contrast, the *fyn* kinases in BJ1859 and BJ1860 were equally activated with and without conA stimulation (Fig 4.3a, d, e).

In 'rested' unstimulated bovine control lymphoblasts, *fyn* exhibited very low levels of activation. *Fyn* was maximally activated following stimulation of control cells with conA for 5 minutes. This activity declined after 30 minutes of stimulation (Fig 4.4a). However, OvHV-2 infected BJ1035 cells exhibited similar levels of fyn activation with or without conA stimulation (Fig 4.4a, d, e). Fig 4.3b, c and Fig 4.4b, c show that for each of the cell types there was equal loading of enolase and immunoprecipitated *fyn* was used in the analysis of enolase phosphorylation by autoradiography.

In control rabbit PBMCs after stimulation with conA; rabbit control lymphoblasts after stimulation with conA and AlHV-1 infected rabbit LGL cell lines BJ1859, BJ1860 either before or after conA stimulation *lck* kinase was activated and phosphorylated enolase (Fig 4.5). In contrast to the control cells, the LGL cell lines BJ1859 and BJ1860 exhibited a high level of *lck* activation in the absence of conA that did not increase after conA stimulation of the cells. In unstimulated bovine control lymphoblasts, *lck* phosphorylation of enolase substrate was low and maximally active after 5 minutes stimulation compared to 30 minutes stimulation with conA. In contrast, *lck* was highly active in unstimulated bovine OvHV-2 cell line BJ1035 and this activity was not increased by conA stimulation of the cells (Fig 4.6). In all experiments, control samples containing enolase and $[\gamma\text{-}^{32}P]$-ATP but without immunoprecipitated kinases did not show any detectable phosphorylation of enolase (Fig 4.5a and 4.6a). For each of the cell types there was an approximately equal loading of enolase and immunoprecipitated *lck* (Fig 4.5b, c and 4.6b, c). In Fig 4.5b the loading of enolase in the gel with samples of BJ1859 and BJ1860 is not shown as the quality of the stained gel was poor on two occasions.

Fig 4.3: *Fyn* **kinase activation in AIHV-1-infected rabbit LGLs (BJ1859 and BJ1860) and control rabbit cells.**

A. Autoradiograph of SDS-PAGE gel showing enolase phosphorylation by *fyn* kinase immunoprecipitated (IP) from LGLs and control lymphoblasts either unstimulated (-) or stimulated with conA ($10\mu g/ml$) for 5 minutes (5) or 30 minutes (30).
B. Coomassie blue stained gel. Control for protein loading of enolase in each lane.
C. Western blot (anti-fyn) control for equal loading of IP *fyn*.
D. Quantitation of enolase phosphorylation by β-scintillation counts per minute (cpm) of enolase bands excised from the SDS-PAGE gels shown above. C = control lymphoblasts; 59 = BJ1859; 60 = BJ1860. Cells were unstimulated (0) or stimulated with conA for 5 or 30 minutes.
E. Quantitation of enolase phosphorylation by β-scintillation cpm from a separate experiment where enolase bands were excised from gels without autoradiography (thus no images of gels are available). PBMC = peripheral blood mononuclear cells. Cells were unstimulated (0) or stimulated with conA for 5 or 30 minutes.
These data represent the best two of three repeated experiments.
Note: cpm shown in **D** and **E** were not taken at the same time in relation to the start of the assay therefore the radioactivity in these samples will have been at different stages of decay. For this reason, the results above must be compared within a group rather than between groups. In these gels, lysates from 8×10^6 cell equivalents were loaded per lane.

Fig 4.4: *Fyn* kinase activation in the OvHV-2-infected bovine LGL, BJ1035 and control bovine cells.

A. Autoradiograph of SDS-PAGE gel showing enolase phosphorylation by *fyn* kinase immunoprecipitated (IP) from LGLs and control lymphoblasts either unstimulated   (-) or stimulated with conA (10μg/ml) for 5 minutes (5) or 30 minutes (30).
B. Coomassie blue stained gel.   Control for protein loading of enolase in each lane.
C. Western blot (anti-*fyn*) control for equal loading of IP *fyn*.
D. Quantitation of enolase phosphorylation by β-scintillation counts per minute (cpm) of enolase bands excised from the SDS-PAGE gels shown above.   C = control lymphoblasts; 35 = BJ1035.   Cells were unstimulated (0) or stimulated with conA for 5 or 30 minutes.
E. Quantitation of enolase phosphorylation by β-scintillation cpm from a separate experiment where enolase bands were excised from gels without autoradiography (thus no images of gels are available).   PBMC = peripheral blood mononuclear cells.   35 = BJ1035.   Cells were unstimulated (0) or stimulated with conA for 5 or 30 minutes.

These data represent the best two of three repeated experiments.

Note: cpm shown in **D** and **E** were not taken at the same time in relation to the start of the assay therefore the radioactivity in these samples will have been at different stages of decay. For this reason, the results above must be compared within a group rather than between groups. In these gels, lysates from 8×10^6 cell equivalents were loaded per lane.
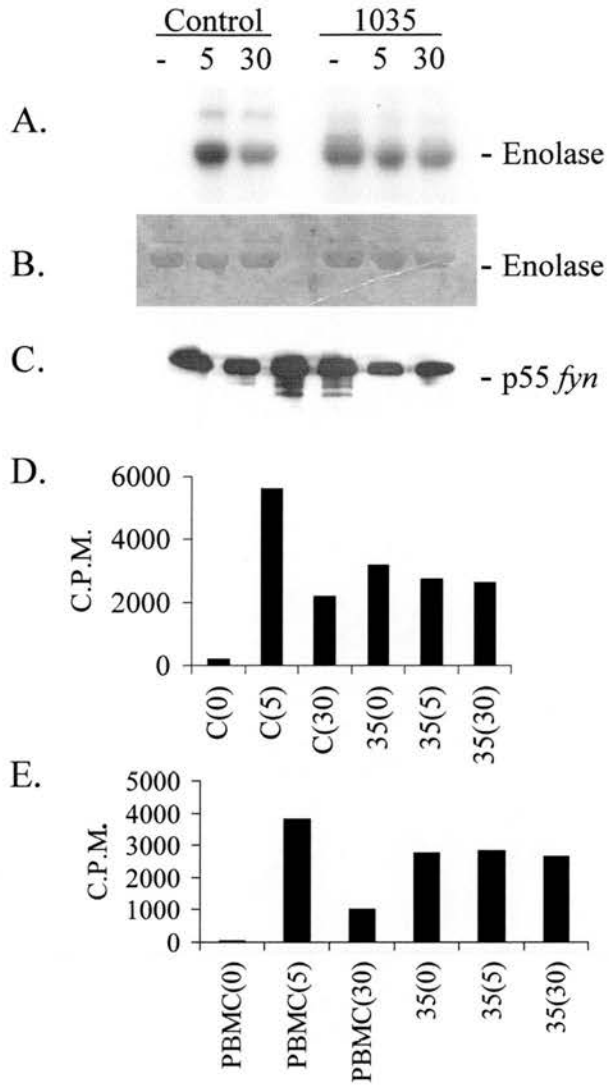
Fig 4.5: *Lck* **kinase activation in AlHV-1-infected rabbit LGLs (BJ1859 and BJ1860) and control rabbit cells.**

A. Autoradiograph of SDS-PAGE gel showing enolase phosphorylation by *Ick* kinase immunoprecipitated (IP) from LGLs and control lymphoblasts/ peripheral blood mononuclear cells (PBMC) either unstimulated　(-) or stimulated with conA (10μg/ml) for 5 minutes (5).
B. Coomassie blue stained gel.　Control for protein loading of enolase in each lane.
C. Western blot (anti-*Ick*) control for equal loading of IP *Ick*.
D. Quantitation of enolase phosphorylation by β-scintillation counts per minute (cpm) of enolase bands excised from the SDS-PAGE gels shown above.　L/blast = control lymphoblasts; PBMC = peripheral blood mononuclear cells; 59 = BJ1859; 60 = BJ1860.　Cells were unstimulated (0) or stimulated with conA for 5 minutes.
E. Quantitation of enolase phosphorylation by β-scintillation cpm from a separate experiment where enolase bands were excised from gels without autoradiography (thus no images of gels are available).　PBMC = peripheral blood mononuclear cells.　Cells were unstimulated (0) or stimulated with conA for 5 minutes.
These data represent the best two of three repeated experiments.
Note: cpm shown in **D** and **E** were not taken at the same time in relation to the start of the assay therefore the radioactivity in these samples will have been at different stages of decay.　For this reason, the results above must be compared within a group rather than between groups.
In these gels, lysates from 8×10^6 cell equivalents were loaded per lane. Coomassie blue stain for enolase loading for the BJ1859 and BJ1860 samples (**B**) was of poor quality and is therefore not shown.

A. L/blasts PBMC Enol con 1859 1860
    - 5   - 5   - 5   - 5

- Enolase

B.

- Enolase

C.

- p56/59 *lck*

D.

E.

135
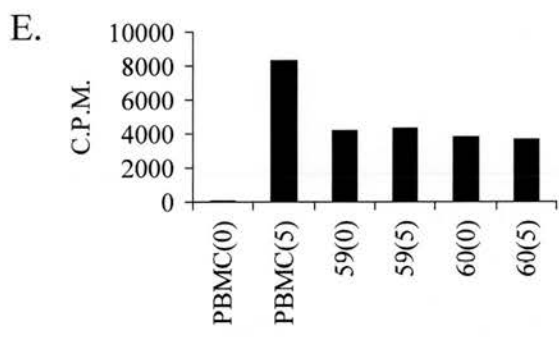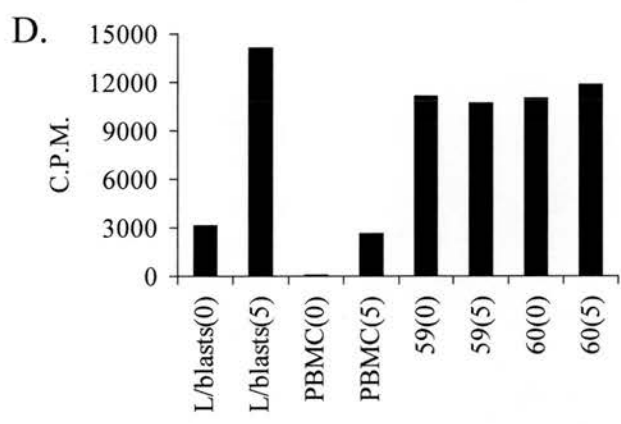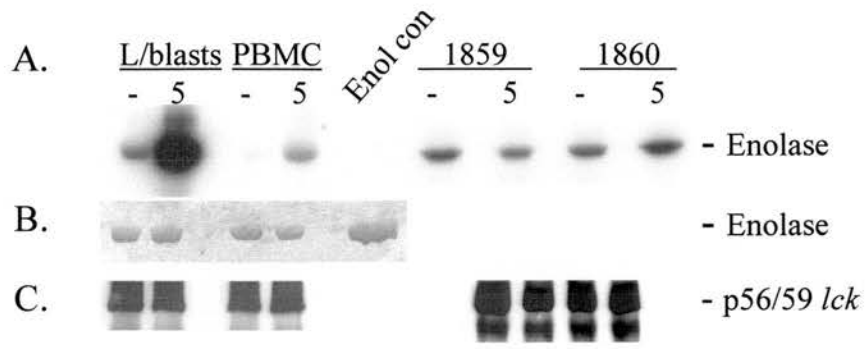
Fig 4.6: *Lck* **kinase activation in the OvHV-2-infected bovine LGL, BJ1035 and control bovine lymphoblasts.**

A. Autoradiograph of SDS-PAGE gel showing enolase phosphorylation by *lck* kinase immunoprecipitated (IP) from LGLs and control lymphoblasts either unstimulated (-) or stimulated with conA (10μg/ml) for 5 minutes (5) or 30 minutes (30).

B. Coomassie blue stained gel. Control for protein loading of enolase in each lane.

C. Western blot (anti-*lck*) control for equal loading of IP *lck*.

D. Quantitation of enolase phosphorylation by β-scintillation counts per minute (cpm) of enolase bands excised from the SDS-PAGE gels shown above. C = control lymphoblasts; 35 = BJ1035. Cells were unstimulated (0) or stimulated with conA for 5 or 30 minutes.

E. Quantitation of enolase phosphorylation by β-scintillation cpm from a separate experiment where enolase bands were excised from gels without autoradiography (thus no images of gels are available). PBMC = peripheral blood mononuclear cells.

These data represent the best two of three repeated experiments.

Note: cpm shown in **D** and **E** were not taken at the same time in relation to the start of the assay therefore the radioactivity in these samples will have been at different stages of decay. For this reason, the results above must be compared within a group rather than between groups. In these gels, lysates from 8 x 10^6 cell equivalents were loaded per lane.
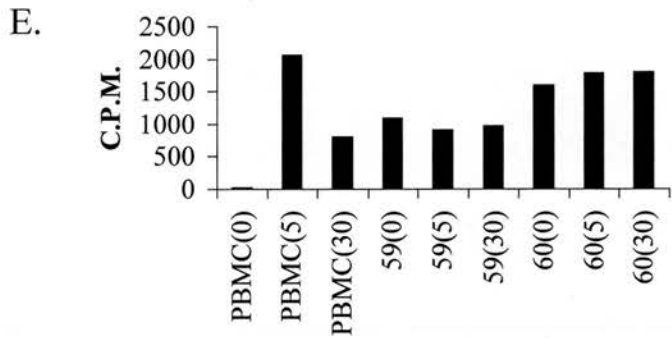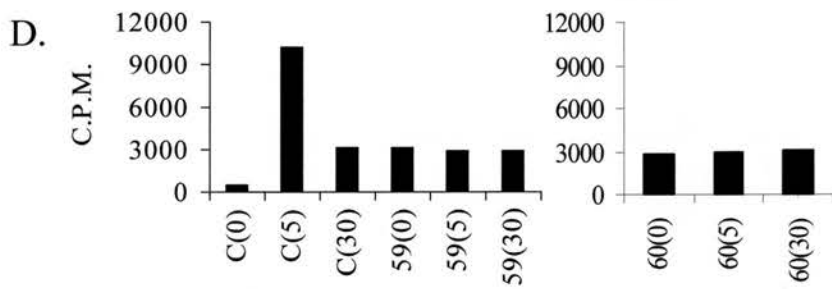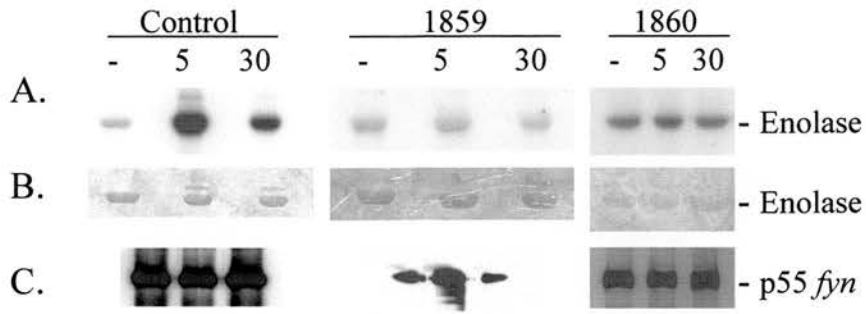
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A.   Control   1035 LGL
      -   5   30   -   5   30   enol.

- Enolase

B.

- Enolase

C.

- p56/59 *lck*

D.

E.
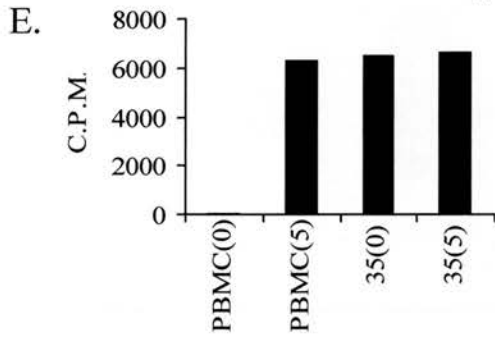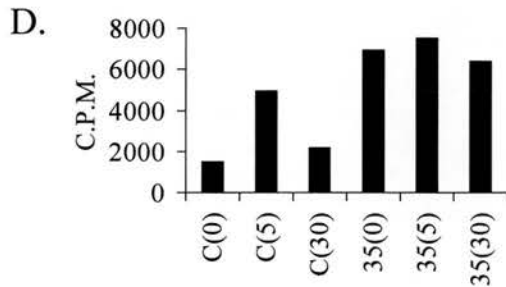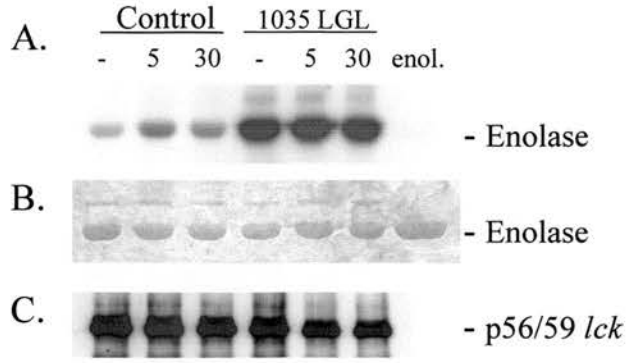
4.4 Discussion

4.4.1 Inhibitor studies suggest involvement of early signalling events of T cell activation in LGL cells

Several characteristics of LGL cell lines from MCF-affected animals have been used to differentiate them from uninfected control cells. The ability of LGLs to survive or grow in the absence of exogenous cytokines for longer periods than control cells and the LGL's constitutive cytotoxic activity are two such characteristics and may be dependent on viral gene products interfering with signalling molecules. As viral genes or proteins were not available at the time of this study, the characteristics of these cell lines could not be attributed to a specific virus-host protein-protein interaction. Thus, the inhibitor study allowed for a more general investigation of signalling mechanisms that might be responsible for the phenotype of LGL cells cultured in the absence of exogenous IL-2.

Cyclosporin A binds an intracellular protein called cyclophillin A. This complex then binds and inactivates calcineurin (a phosphatase). This protein is involved in the Ca^{2+}/calcineurin pathway that mediates cytokine production and proliferation of T cells (McKeon, 1991). The effect of CsA in inhibiting the lymphoproliferative response in OvHV-2 infected rabbits has been demonstrated (Buxton *et al*, 1984). This immunosuppressant has also been shown to inhibit the growth of bovine OvHV-2-infected cell lines in a time and dose-dependent manner (Schock, 1996). The results presented here follows the trend observed in previous studies which implicates the involvement of T cell or NK cell signalling molecules in exogenous IL-2-independent LGL growth. Phosphatases such as calcineurin and CD45 are involved in T cell signal transduction pathways following TCR or cytokine receptor engagement (Amrein and Sefton, 1988; Crabtree and Clipstone 1994; Koretzky *et al*, 1990). The involvement of phosphatases in LGL factor-independent growth is suggested by the effect of high concentrations of sodium orthovanadate on bovine OvHV-2-infected cell line BJ1035. Rapamycin inhibits signal transduction pathways through the IL-2 and other cytokine receptors (Chung *et al*, 1992). However, inhibition of IL-2-independent growth by rapamycin was not observed in the two LGL cell lines tested. This would appear to suggest that IL-2 receptor

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mediated signalling is not critical for IL-2 independent growth. The effect of genistein on growth of cell lines tested here was the most significant and implicates tyrosine kinases as mediators of IL-2-independent LGL growth.

It should be noted that the results of studies using CsA, sodium orthovanadate or rapamycin were not conclusive due to lack of replicate samples. This was due to the limited supply of cells as a consequence of the death of cell lines during the course of this study combined with the failure to resuscitate fresh cell lines from frozen stocks. Subsequent to this study, the effect of rapamycin and genistein on the cell lines BJ1035 and BJ1859 were repeated using triplicate samples and included in Fig 4.1. Statistical analysis was performed on these new data (and indicated by the presence of error bars). At present, any conclusions made from these data remain tentative and are further complicated by the difficulty in distinguishing between inhibition of signalling pathways and toxicity due to these chemicals as both phenomena are measured by cell death. However, this study has proved useful in suggesting potential areas of LGL growth control that should be investigated in more detail.

4.4.2 Targeting specific signalling molecules

Non-receptor tyrosine kinases found in T cells (*lck*, *fyn* and ZAP-70) are located near the cell membrane and are the first signalling molecules activated on engagement of the T cell receptor (TCR) complex and other cell surface molecules such as CD2 and CD4 (Crabtree and Clipstone, 1994). On activation, these signalling molecules become phosphorylated on tyrosine residues. ConA (a T cell mitogen) and TPA (a phorbol ester) stimulate polyclonal T cell proliferation through receptors rich in mannose residues including the TCR complex and by the activation of protein kinase C respectively.

While conA-activated lymphoblasts serve well as uninfected controls with a T cell phenotype, it was often difficult to obtain a fully rested population even after an overnight deprivation of exogenous cytokines. Using fresh PBMCs as 'resting' controls was useful for assessing the state of activation of MCF-derived cell lines. The results suggested that MCF virus infection of LGLs is associated with the constitutive activation of *lck* and *fyn* tyrosine kinases. There were more similarities

than differences in the pattern of tyrosine phosphorylation between control lymphoblasts and LGL cell lines after stimulation as measured by the 4G10 antibody western blot. This suggested that tyrosine kinases were activated following stimulation of both control and LGL cells. The important observation was that in the absence of stimulation tyrosine phosphorylated proteins were detected in LGL cells but not PBMCs. This may suggest that LGL cells are constitutively active in the absence of exogenous IL-2. However, the identity of the phosphorylated proteins has not been determined. Antibodies specific for human, mouse and rat ZAP-70 did not react convincingly with any proteins in the rabbit or bovine cell lysates. However *lck* and *fyn* were revealed using antibodies specific for human, mouse and rat *lck* and *fyn* in western blots of cell lysates at 56/59kDa and 55kDa respectively.

An indication of constitutively active kinase activity in the LGLs emerged from the phosphotransferase assays of immunoprecipitated *lck* and *fyn*. Using enolase as a substrate for the precipitated kinases, the phosphotransferase assays indicated that *lck* and *fyn* were constitutively activated in both rabbit and bovine LGL cells compared to control 'resting' cells. The fact that conA stimulation did not substantially alter the level of *lck* and *fyn* activation in the LGL cells suggest that these kinases are maximally activated in these cells. Previous studies showed that OvHV-2-infected LGL cell lines exhibit low levels of proliferation in response to conA stimulation (Schock, 1996). This result was confirmed in the cell lines examined in this study and extended the results to AlHV-1-infected rabbit LGL cells. Therefore, the lack of enhanced proliferation of LGL cells to conA (or conA + TPA) stimulation may be explained by the inability of *lck* and *fyn* kinase activity to be stimulated further by these mitogens. An alternative explanation for the relative lack of responsiveness of LGLs to conA compared to control cells could be due to lack of expression of conA receptors in the LGL cell lines. However, the aggregation of LGL cells and control cells in the presence of conA indicates the binding of this mitogen to the cells (data not shown). The data presented here confirms the preliminary observations from inhibitor studies suggesting the involvement of tyrosine kinases in LGL cell lines. Moreover, this study is the first description of the responsiveness of rabbit AlHV-1-infected LGLs to conA.

The constitutive activation of *lck* and *fyn* in LGL cell lines suggested by this study warranted a more thorough investigation of the downstream consequences of MCF virus infection. Further investigation of the MAPKs (mitogen-activated protein kinases) *erk1* and *erk2*, which are activated following stimulation through various membrane receptors (Pelech and Sanghera, 1992), was performed following the completion of the present study. The results showed that MAPKs were constitutively activated in MCF virus-infected LGL cell lines (Swa *et al*, in press).

In conclusion, an association between MCF virus infection of LGL cells and the constitutive activation of *lck, fyn* and MAPK has been suggested. This may point to the possibility that virus could interact directly or indirectly with *lck, fyn* and MAPK. However, it should be noted that this has not been shown. However given more recent understanding of the molecular biology of AlHV-1 (Ensser *et al*, 1997) subsequent to this study, the search for viral proteins that interact with signalling molecules is currently being undertaken.

Chapter 5

Expression of recombinant AlHV-1 proteins (ORF50, A6 and A7) in *E. coli* and production of specific antisera

5.1 Introduction

In Chapter 3, the results demonstrated that MCF virus infection of LGL cells was associated with an altered phenotype. In addition, interference with *lck* and *fyn* kinase signalling pathways was also associated with MCF virus infection (Chapter 4). However, the involvement of specific virus proteins in the generation of the LGL phenotype and function has not been established. The following chapters describe specific virus proteins associated with virulence, which may be important in MCF pathogenesis and describe experiments to test whether these proteins could account for the ability of LGLs to cause disease in rabbits.

Until recently, only limited sequence data was available on the AlHV-1 genome and to an even lesser extent on OvHV-2. However, the AlHV-1 C500 genome had been characterised by restriction enzyme analysis (Handley *et al*, 1995). AlHV-1 C500 exists in two forms - the cell-associated virulent form and the cell-free attenuated form. The second form of C500 is generated upon serial passage in culture (Plowright, 1986). Handley *et al* demonstrated differences in the restriction enzyme profiles between virulent and attenuated forms of C500. These differences were encompassed in three fragments. These fragments were cloned and sequenced. When sequenced, these three clones (of approximately 3.5kbp each) were found to share 2kbp of DNA. The differences in nucleotide sequence between these clones were thought to be due to rearrangements in the viral genome during the transition from virulence to attenuation. However, more recent results indicate that the rearrangements that occur at the right terminal end of the attenuated C500 genome are different from that published by Handley *et al* (1995) (H.Wright, personal communication). The new data is consistent with the translocation of a fragment of DNA from the middle of the virulent C500 genome to the right terminal end of the attenuated genome. Any proteins deleted or altered during the rearrangements were potential virulence factors with a role in viral pathogenesis. Protein translation of the DNA clone encompassing the middle of the virulent C500 genome revealed three ORFs. These were denoted ORFs 50, A6 and A7 respectively in Ensser's analysis of the complete genomic sequence for AlHV-1 C500 (Ensser *et al*, 1997).

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One of the distinctive characteristics of LGLs is that they have the ability to cause MCF when inoculated into rabbits. This would suggest the presence of a productive and virulent virus in the LGLs. The study of virulence-associated proteins is therefore important due to their potential role in pathogenesis and their expression in LGLs could explain the ability of such cell lines to induce MCF when used as an inoculum in rabbits.

In this chapter, the expression of ORF50, A6 and A7 as bacterial recombinant proteins and the production of rabbit polyclonal antisera specific for them is described. These reagents were then used to detect the presence of ORF50, A6 and A7 at the protein level in virus-infected cells (Chapter 6). The expression of recombinant proteins in *E. coli* is a common method of expressing foreign proteins and is used for production of high yields. The cloned genes were inserted into the vector, pTrXFus, that contained an ampicillin resistance gene to aid selection of transformed *E. coli* and the bacteriophage λ promoter (P_L) to drive expression, which is induced by tryptophan (Mieschendahl *et al*, 1986). This expression system uses thioredoxin (TrX) as the peptide fusion partner and was chosen for its ability to express high levels of soluble fusion proteins (Holmgren, 1985; La Vallie *et al*, 1992). This fusion partner was also useful as a means of identification of the recombinant proteins in the absence of virus protein-specific antisera as monoclonal antibodies to TrX were available. These recombinant fusion proteins were then used as antigens to raise specific polyclonal antisera in rabbits. The generation of a glutathione S-transferase (GST)-recombinant ORF50 product and specific antisera following purification of the antigen by electroelution was previously achieved by R. Ireri at Moredun (Ireri, 1999). In this study, similar methods were used to produce, purify and test the reagents specific for the products of ORFA6 and A7. The further purification by affinity chromatography of the antisera to the products of ORF50, A6 and A7 is also described.

5.2 Materials and Methods

5.2.1 Expression of recombinant AIHV-1 proteins in *E. coli*

The DNA encoding ORF50, A6 and A7 were amplified by PCR, inserted into the vector pTrXFus and expression induced in *E coli* as described in Chapter 2.5.3 using the primers shown in Table 5.1. Recombinant thioredoxin fusion proteins were harvested from *E. coli* by lysis as described in Chapter 2.5.3. Recombinant proteins in the *E. coli* supernatant and pellet material were identified using an anti-thioredoxin antibody in a Western blotting analysis (Chapter 2.4.4). For the purposes of clarity, these recombinant proteins will be described in the text as ORF50-TrX, ORFA6-TrX and ORFA7-TrX.

5.2.2 Purification of recombinant proteins by electroelution

200µl of crude *E. coli* supernatant was prepared in the same volume of 2x Laemmli sample buffer (62.5mM Tris-HCl pH7.8, 10% sucrose, 2% SDS, 0.001% bromophenol blue, 5% β–mercaptoethanol). Meanwhile, insoluble proteins in the pellet fraction (resulting from 20mls of induced broth - see Chapter 2.5.3) were resuspended in 200µl of 1x Laemmli sample buffer. Proteins were visualised during separation by Coomassie Blue SDS-PAGE (see Chapter 2.4.3). Recombinant ORFA6 and A7 bands were excised from the gel according to their molecular weight.

Gels containing the excised protein band were roughly chopped and proteins removed by electroelution as described in Chapter 2.4.6. The electroeluted material was collected and concentrated in Vivaspin concentrators (with a 10kDa cut-off membrane) (Vivascience, Lincoln, UK) with PBS to a final volume of 500µl. This material was stored at 4°C while aliquots were taken to assess the purity and concentration of the material.

Table 5.1: Primers used for the production of DNA sequences for ORF50, A6 and A7 of AlHV-1 for recombinant protein synthesis.

Specificity	Function	Sequence	T_m	Product Size
ORF50	5' primer	Bam HI GAACCAGGATCCAATGTTTAGCGGAGGGA	88°C	1349bp
	3' primer	Sal I ACACTGTCGACTATGGCTATGGTCTGGTCA	90°C	
ORFA6	5' primer	Bam HI TCTTTGGGATCCCATGCATAAGCACTCTGC	90°C	648bp
	3' primer	Pst I TTTTAACTGCAGTGTAAAGGTACCTTTTTG	80°C	
ORFA7	5' primer	Bam HI TGGCTGGATCCTGAGATGTTGGCAGAAATG	90°C	759bp
	3' primer	Sal I GCTAGTCGACAGTTATCCATGGACGGGTGC	94°C	

Primers for ORF50 DNA were generated prior to the publication of the full AlHV-1 sequence by Ensser (1997) and covers the ORF50 DNA sequence from 73571bp – 74919bp (thus 746bp smaller than the sequence for ORF50 published by Ensser). The primers for ORFA6 DNA cover the sequence from 75213bp - 75860bp. Similarly, ORFA7 primers cover 76554bp - 77312bp. [Tm = melting temperature].

5.2.3 Testing the purity and concentration of electroeluted material

A 20μl aliquot of the electroeluted material was separated by SDS-PAGE and analysed by western blot with an anti-thioredoxin antibody (diluted 1: 5000). Aliquots of purified ORFA6-TrX were placed alongside samples of bovine serum albumin (BSA) at different concentrations (between 0.05 - 25mg/ml) on an SDS-PAGE gel. Proteins were visualised by silver staining (see Chapter 2.4.3) and the concentration of the electroeluted sample was calculated on a BIORAD densitometer using BSA as standards. The concentration of purified insoluble ORFA7-TrX was estimated by the size of the band in a Coomassie stained SDS-PAGE gel.

5.2.4 Production of polyclonal rabbit antibodies against recombinant AlHV-1 proteins

For the preparation of polyclonal antibodies, two rabbits were immunised with the electroeluted ORFA6-TrX or ORFA7-TrX as described in Chapter 2.1.2. The antigens used were prepared as follows. ORFA6-TrX from 2mls of crude *E. coli* supernatant was electroeluted and the material was concentrated to a final volume of 1.5ml. The protein concentration was determined as described above. This material was divided between the two rabbits for each set of inoculations. Similarly, the equivalent of 3 pellets of ORFA7-TrX/*E. coli* insoluble protein were purified by electroelution and divided between two rabbits for each set of inoculations.

5.2.5 Affinity purification of polyclonal sera on a protein A-sepharose column

Once reactivity of sera from inoculated rabbits to the respective recombinant proteins had been confirmed by western blot (see below) the rabbits were bled. Blood was allowed to clot and serum was collected by centrifugation at 900 x g for 10 minutes. The serum was further purified on a protein A-sepharose column by FPLC to produce an IgG fraction, which was detected by a shift in absorbance at 280nm (see Chapter 2.4.7).

Samples were either tested for reactivity against the original antigen or aliquoted and stored (in PBS/0.05% azide) at -20°C for future use.

5.2.6 Testing for reactivity of antisera by SDS-PAGE and western blot

To test the reactivity of sera, recombinant proteins (in reducing Laemmli sample buffer) were loaded in one large well (using a 0.5mm comb) of a BIORAD Mini Protean II system and transferred to nitrocellulose (see Chapter 2.4.4). Strips of this nitrocellulose were then blotted with each fraction of purified sera and the 'flow through' (unbound protein) fraction at two dilutions (1:100 and 1:500). Anti-thioredoxin was included as a positive control (and used at 1:5000). Western blots were developed with TMB stabilised substrate for HRP (Promega, Southampton, UK) or with ECL reagents (Amersham International, Little Chalfont, UK).

5.3 Results

5.3.1 Thioredoxin recombinant proteins encoded by ORF50, A6 and A7 of AlHV-1 have variable solubility

All the recombinant proteins appeared as the dominant protein in the *E. coli* lysate. This was determined by Coomassie staining following separation of the soluble and insoluble fractions in a reducing gel. However, only the ORFA6-TrX appeared in the soluble fraction of the *E.coli* lysate. The proteins ORF50-TrX and ORFA7-TrX appeared in the insoluble fraction of the *E coli* lysate (Fig 5.1).

Western blot with anti-thioredoxin antibody confirmed these dominant bands to be thioredoxin recombinant proteins. ORF50-TrX, ORFA6-TrX and ORFA7-TrX have a molecular weight of 70-75kDa, 40kDa and 40-45kDa respectively (Fig 5.2).

5.3.2 Recombinant proteins ORFA6-TrX and ORFA7-TrX can be successfully purified by electroelution for inoculating rabbits

In order to purify recombinant proteins for use as an inoculum for raising antibodies in rabbits, electroelution of these proteins from Coomassie Blue-SDS-PAGE gels was performed. Recombinant proteins in these gels were identified by their respective molecular weights established in the previous section (5.3.1).

The electroeluted material from the ORFA6-TrX Coomassie Blue gel contained a thioredoxin protein of 40kDa (Fig 5.3a). This protein was the dominant protein in the purified material. The concentration of the pooled sample was approximately 100μg/ml (using known concentrations of BSA to give a standard curve - Fig 5.3c). This equates to approximately 7.5μg recombinant protein /ml bacterial culture. The thioredoxin recombinant ORFA7-TrX was successfully purified in a similar way (Fig 5.3b) and its identity confirmed by the presence of a protein of the predicted molecular weight that was detectable by western blot with anti-thioredoxin. The concentration of electroeluted ORFA7-TrX was estimated to be approximately 100μg/ml by the intensity of the protein band following staining with Coomassie blue.
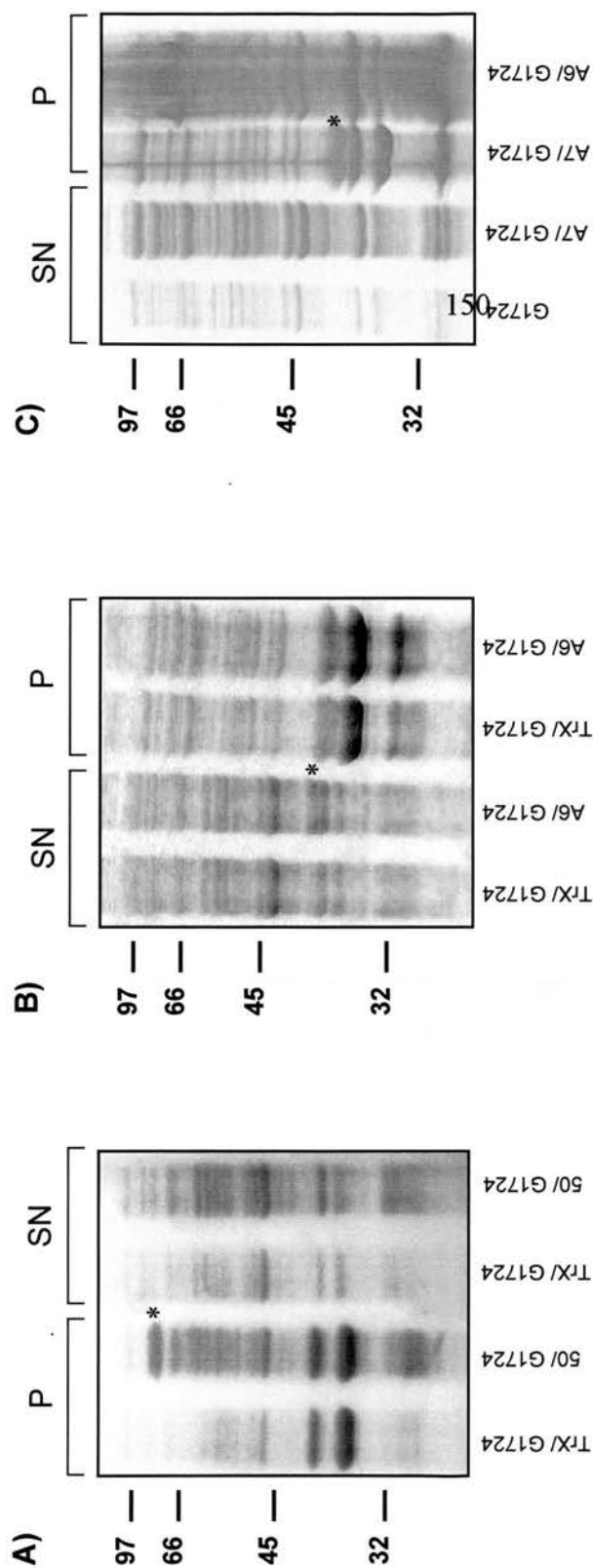
Fig 5.1: Recombinant proteins in *E. coli* lysates detected by Coomassie blue staining.
A) TrX/ G1724 = *E.coli* + thioredoxin (control); 50/ G1724 = *E.coli* + ORF50-TrX protein.
B) Trx/ G1724 = (control); A6/ G1724 = *E.coli* + ORFA6-TrX protein.
C) G1724 (SN) = *E.coli*, uninduced (control); A6/ G1724 (P) = (control); A7/ G1724 = *E.coli* + ORFA7-TrX protein.
ORF50-TrX and ORFA7-TrX were present in the insoluble pellet (P) fraction of the lysate while ORFA6-TrX was present in the soluble supernatant (SN) fraction of the lysate. '*' to right of lane indicates recombinant protein band. Molecular weight markers (kDa) are indicated to the left of each gel.

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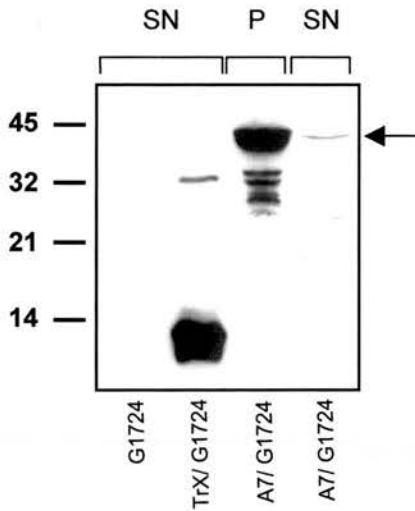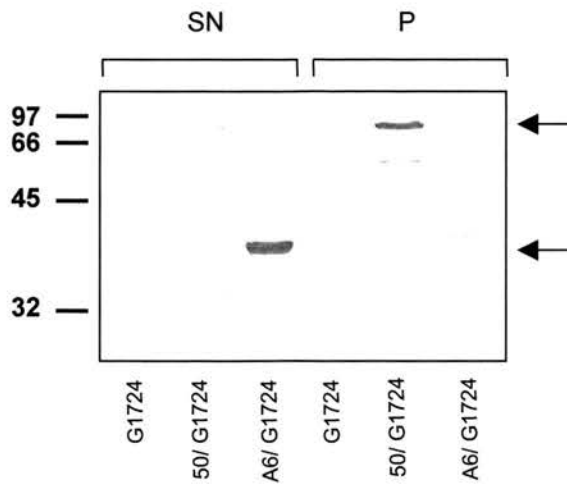
Fig 5.2: Western blot analysis of recombinant proteins in *E. coli* lysates using an anti-thioredoxin antibody (diluted 1: 5000).
G1724 = *E.coli*, uninduced (control); 50/ G1724 = *E.coli* + ORF50-TrX protein; A6/ G1724 = *E.coli* + ORFA6-TrX protein; A7/ G1724 = *E.coli* + ORFA7-TrX protein; TrX/ G1724 = *E.coli* + thioredoxin (control). SN = soluble fraction of lysate; P = insoluble pellet fraction of the lysate. Molecular weight markers (kDa) are indicated to the left of each blot. Recombinant proteins are indicated by arrows.
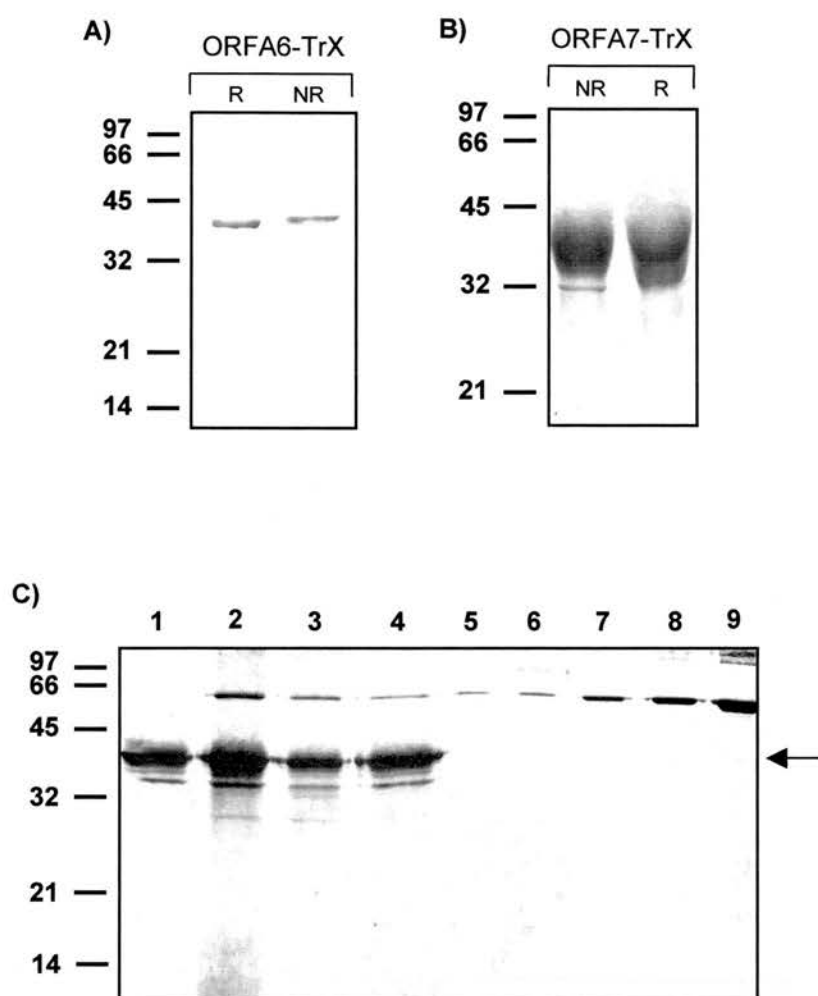
Fig 5.3: Electroeluted samples of ORFA6 and ORFA7 - thioredoxin fusion proteins.
A) Western blot analysis of electroeluted ORFA6-TrX using an anti-thioredoxin antibody detected by TMB substrate reaction. R = reduced NR = non-reduced.
B) Coomassie Blue stain of electroeluted ORFA7-TrX.
C) Silver stain of electroeluted ORFA6-TrX indicated by arrow (lanes 1-3: batch 1-3, lane 4: pooled sample) and different concentrations of BSA (lanes 5-9: 0.05, 0.5, 2.5, 5.0, 25mg/ml).
Molecular weight markers (kDa) are indicated to the left of each blot or gel.

5.3.3 Polyclonal antisera react with the respective recombinant protein

5.3.3.1 Antisera specific for ORF50-GST fusion protein reacts with ORF50-TrX

Further purification on a protein-A sepharose column of whole serum from a rabbit (#28) inoculated with ORF50-GST recombinant protein was performed. The shift in absorbance at 280nm indicated the presence purified IgG in the eluted fractions. A total of five 1ml fractions were eluted from the column using 0.5ml of crude serum - hence the serum was diluted by a factor of ten. These eluted fractions were able to detect ORF50-TrX along with a number of other *E. coli* proteins by western blot (after a further dilution of 1:100 and 1:500). The IgG fractions were reactive against reduced and non-reduced ORF 50-TrX. No proteins were detected using the 'flow through' fraction which contains antibodies and proteins not bound to the protein-A sepharose column (Fig 5.4).

5.3.3.2 Rabbits generate a lower antibody response to ORFA6-TrX recombinant protein

Rabbits (#88 and #89) inoculated with ORFA6-TrX protein did not appear to produce as much IgG as rabbit #28 as the shift in absorbance at 280nm was not as large as that seen with eluted IgG fractions from rabbit #28. Using 0.5mls of crude serum from rabbit #88 and #89, four 2ml IgG fractions and three 2ml IgG fractions respectively were eluted from protein-A sepharose (resulting in a total serum dilution of 1:16 and 1:12). A further dilution of 1:100 and 1:500 was made for western blot analysis against the ORFA6-TrX fusion protein. All eluted fractions tested from rabbit #88 (Fig 5.5a) but not rabbit #89 (data not shown) were reactive to reduced and non-reduced ORFA6-TrX. The 'flow through' fraction in both cases was negative for reactive antibodies. However, whole sera from both rabbits were able to detect ORFA6-TrX (Fig 5.5b).
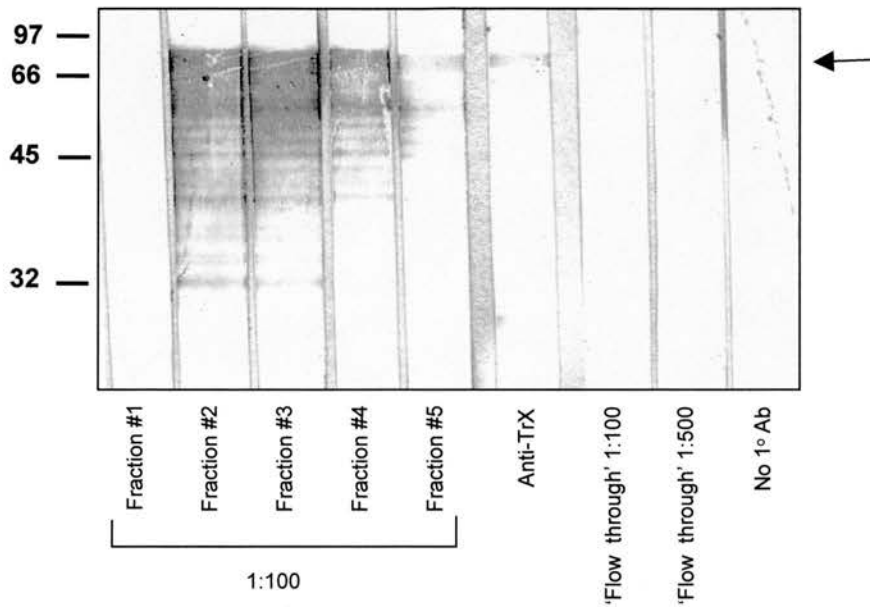
Fig 5.4: Western blot analysis of purified (IgG) anti-ORF50 antiserum against ORF50-TrX recombinant protein.
Fraction #1-#5 = purified IgG eluted from a protein-A sepharose column. 'Flow through' = antibodies/proteins from whole sera that did not bind to affinity column. Recombinant protein is indicated by the arrow. Molecular weight markers (kDa) are indicated to the left of the blot.
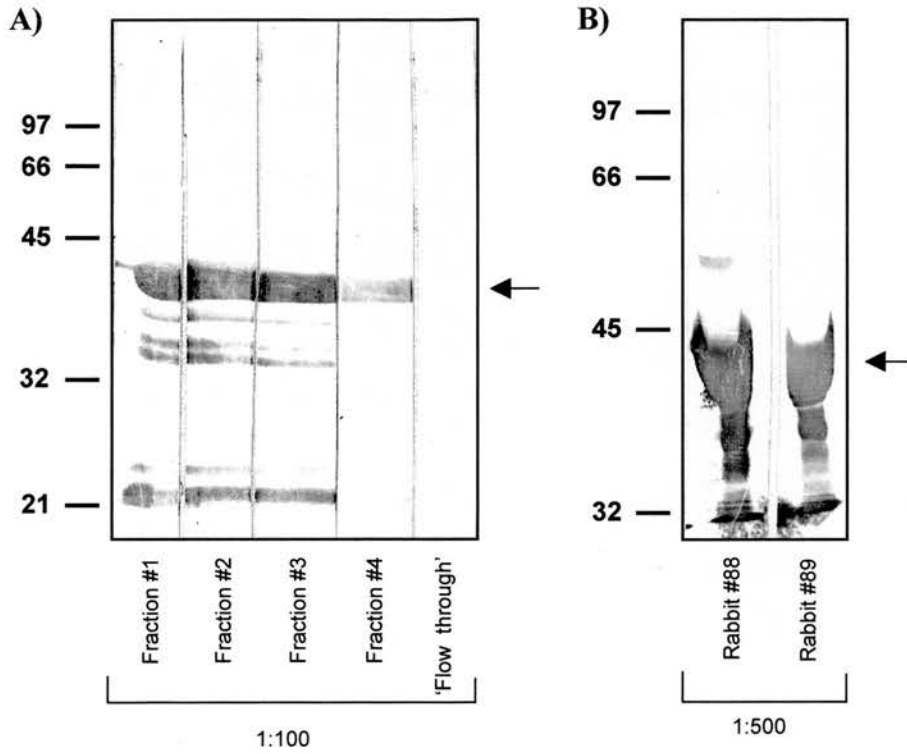
Fig 5.5: Western blot analysis of anti-ORFA6 antiserum reactivity against recombinant ORFA6-TrX.
A) IgG fractions (#1 - #4) and unbound antibodies/proteins ('Flow through') produced during purification of ORFA6-TrX antisera using a protein-A sepharose column.
B) Whole ORFA6-TrX antisera from rabbit #88 and #89.
Recombinant proteins are indicated by arrows. Molecular weight markers (kDa) are indicated to the left of the blots.
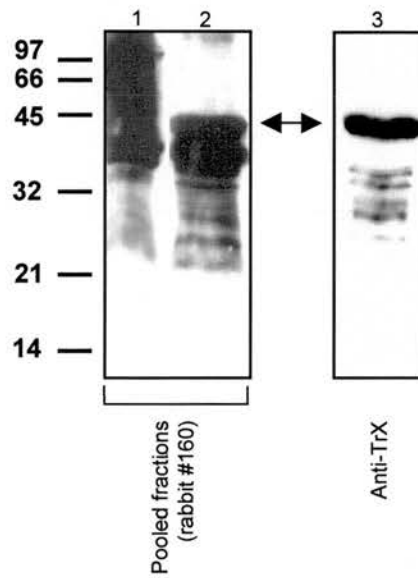
Fig 5.6: Western blot analysis of purified (IgG) anti-ORFA7 antiserum against recombinant ORFA7-TrX.
Lane 1: G1724 pellet (control) = insoluble fraction of an *E. coli* lysate.
Lanes 2-3: A7/ G1724 pellet = insoluble fraction of an *E. coli* + ORFA7-TrX lysate. Recombinant proteins are indicated by arrows. Molecular weight markers (kDa) are indicated to the left of the blots.

5.3.3.3 Pooled fractions of IgG to ORFA7-TrX are more reactive to the recombinant protein than single fractions alone

Using whole sera from rabbit #159 and #160 (inoculated with ORFA7-TrX), a shift in absorbance at 280nm was detected in the eluted IgG fractions from a protein-A sepharose column. A larger shift in absorbance was observed in the eluted IgG fractions from rabbit #160 than from rabbit #159. This suggested that the IgG response of rabbit #160 to full length ORFA7-TrX was greater than that produced by rabbit #159. From an original volume of 0.5ml whole serum four 2ml fractions were eluted from the protein-A sepharose column for each rabbit (a total serum dilution of 1:16). Western blot reactivity of these sera at 1:100 and 1:500 against ORFA7-TrX was patchy with fraction 2 and 3 producing the strongest response (data not shown). Western blots using pooled fractions of IgG to ORFA7-TrX (from rabbit #160) followed by ECL development showed reactivity to ORFA7-TrX and a smaller but equally abundant *E. coli* protein (Fig 5.6). The IgG fractions were reactive against reduced and non-reduced ORF A7-TrX. Normal rabbit serum in all cases was not reactive against any of the recombinant proteins.

5.3.3.4 Purification of terminal sera from rabbits #28, #88 and #160

While the protein A-sepharose column was successful in purifying IgG fractions from whole sera, the yield (as assessed by shift in O.D.$_{280}$) was not always high. In order to ensure the optimal binding of IgG a new protein-A sepharose column was packed before proceeding with the final purification of terminal sera. This resulted in high levels of IgG binding for all three sera (of which purification of sera to ORFA6-TrX is shown in Fig 5.7). The fractions collected were pooled (giving a total volume of 5ml for each serum) then concentrated in PBS to the original volume of 0.5ml. Using the Pierce assay the IgG levels in rabbit #28, #88 and #160 were approximately 2, 1 and 3mg/ml respectively.
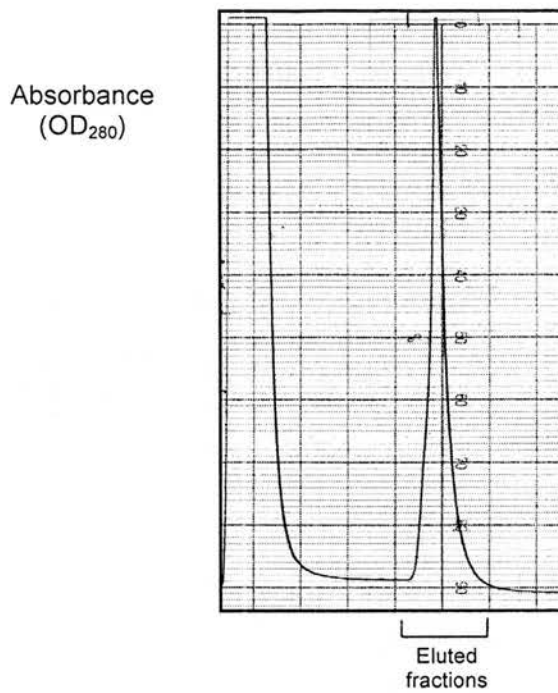
Fig 5.7: Antiserum to ORFA6-TrX purified on a protein A–sepharose column.
IgG fractions were eluted from the column using a glycine elution buffer and detected by a shift in absorbance at 280nm.

5.4 Discussion

The production of proteins encoded by ORF50, A6 and A7 produced as TrX fusion proteins was successful although some degradation of the fusion proteins was observed despite the presence of leupeptin and EDTA (both protease inhibitors) in the lysis buffer. This could be improved by addition of other protease inhibitors or by reducing the induction period. The induction period of 4 hours was chosen as a compromise between the recommended 2-7 hours (ThioFusion^{TM} Expression System Manual, Invitrogen) to limit degradation while allowing time for higher yields. For the purpose of use as an immunogen, the level of expression and degradation were acceptable following this period of induction. Had there been problems with expression a more thorough investigation of optimum induction periods would have been carried out.

The use of electroelution as opposed to affinity purification allowed for the selection of only the full-length fusion protein, eliminating any degradation products. In addition, the use of affinity purification requires the use of soluble proteins. As ORFA7-TrX was insoluble further solubilisation and refolding procedures would have to be performed prior to affinity purification. The uncertainty of regaining the native tertiary structure by these procedures made electroelution the method of choice for purification of proteins for use as an inoculum for immunising rabbits to raise antisera. Other strategies to increase the expression of soluble proteins could be investigated further (e.g. lowering the temperature during induction, use of different strains of *E. coli* or different vectors). Following the publication of the full sequence of AlHV-1, ORFA7 was predicted encode a putative glycoprotein (Ensser *et al*, 1997). The absence of post-translational protein glycosylation in *E. coli* would have produced an ORFA7-TrX recombinant protein without the normal glycosyation found in native ORFA7 product. The lack of glycosylation may also explain the insolubility of ORFA7-TrX. The purification of ORFA6-TrX and ORF A7-TrX by electroelution gave a high yield. The protein concentration was determined by densitometry of silver stained gels using (bovine serum albumin) BSA to create a standard graph. In this instance, protein concentration was not determined by the

colorimetric Pierce assay as the electroeluted proteins had been stained with Serva blue and would have given false absorbance readings in the Pierce assay.

The antisera produced were reactive to the recombinant protein of interest although cross-reactivity to other *E. coli* proteins was also present. Each eluted IgG fraction of sera from rabbits inoculated with recombinant protein was tested individually with the initial intention to keep only the fractions with the lowest non-specific reactivity. However, after the final purification all fractions were pooled to maximise the titre of specific IgG and thus increase the chances of reactivity to native proteins in infected cells. If necessary, it would have been possible to purify these IgG fractions even further on affinity columns coupled to each recombinant protein.

In the next chapter, these polyclonal antibodies will be used to study the expression of ORF50, A6 and A7 at the protein level in LGL cell lines and in AlHV-1-infected bovine turbinate monolayers.

Chapter 6

Detection of ORF50, A6 and A7 products in MCF virus-infected bovine turbinate cells and LGL cell lines using specific antisera

6.1 Introduction

In the previous chapter three recombinant proteins translated from three open reading frames encoded in a region of AlHV-1 C500 that undergoes rearrangement after passage in culture (Handley *et al*, 1995) were expressed and specific antibodies raised to these proteins. The low-passage cell-associated and virulent form of C500 contains the ORFs 50, A6 and A7. These genes were located in the middle of the genome at approximately 72-76kbp. After extensive passage, C500 becomes cell-free and attenuated. In the attenuated virus a block of the AlHV-1 genome containing ORF50 appears to be translocated to the right hand end of the genome next to the terminal repeats and inverted with respect to transcriptional direction. During this translocation, the promoter region of ORF50 is lost (H. Wright, personal communications). This block of translocated genome also contained a truncated version of ORFA6, which was found adjacent to a truncated version of ORFA10 (H. Wright, personal communication) (Fig 6.1). The proteins encoded in ORF50, A6 and A7 were of interest as the rearrangements described at the right hand end of the AlHV-1 genome were associated with loss of virulence. It is proposed that these three proteins may be important in the switch from virulence to attenuation.

The antisera to the recombinant proteins ORF50-TrX, ORFA6-TrX and ORFA7-TrX recognised the respective recombinant proteins described in Chapter 5. In this chapter, the expression of the natural proteins encoded by ORF50, A6 and A7 was investigated in virus-infected bovine turbinate cells to test the ability of the sera to recognise native viral protein. To determine whether these potential virulence factors are associated with the ability of LGL cell lines to cause disease in rabbits the expression of ORF50, A6 and A7 was also investigated in these cells. Protein was detected by indirect fluorescent antibody tests (IFAT) using the anti-sera produced in Chapter 5. RT- PCR using primers for the genes encoding these proteins were also performed to assess expression at the mRNA level.
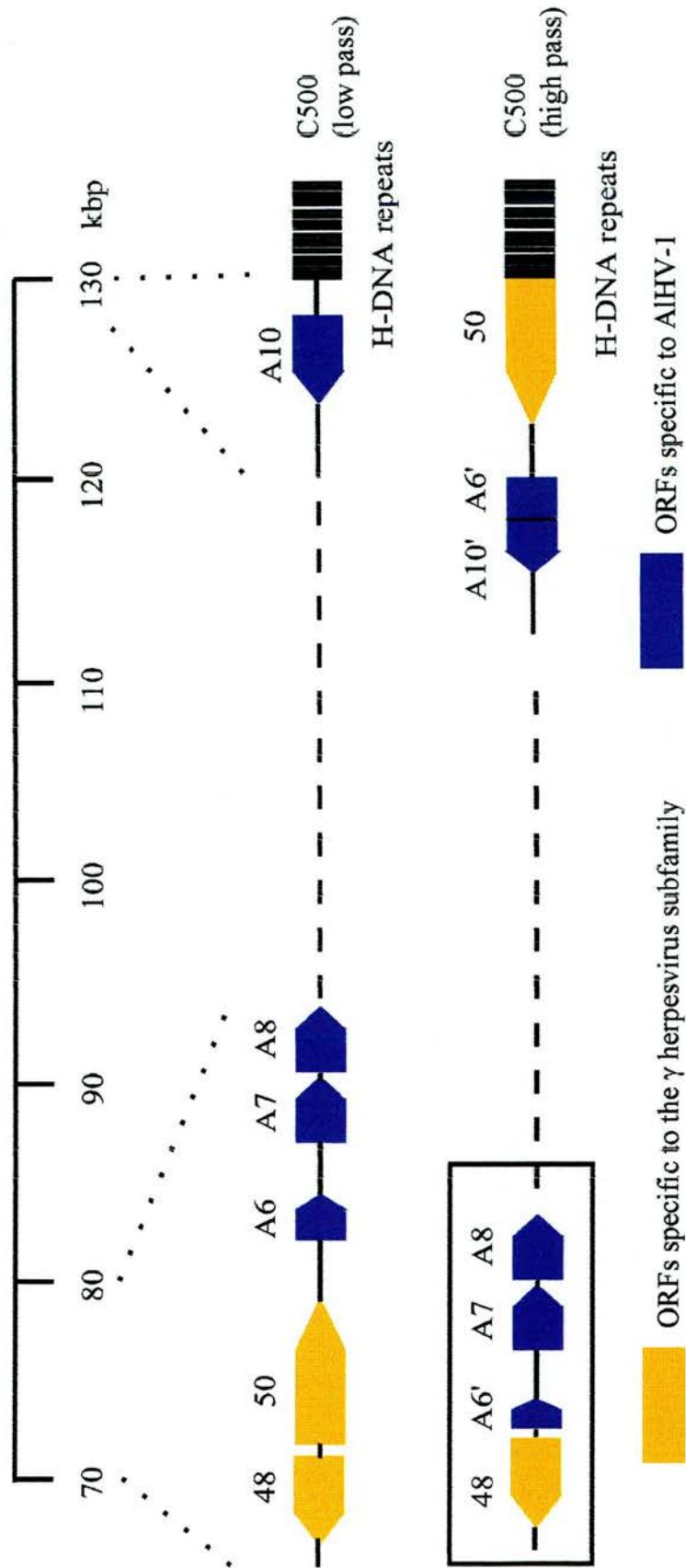
Fig 6.1: Diagrammatic representation of the genomic rearrangements at the right-hand end of the AlHV-1 genome during the virulence to attenuation switch.

ORF48 = EBV BRRF2 homologue; ORF50 = EBV BRLF1 homologue; ORFA6 = EBV BZLF1 positional homologue; ORFA7 = EBV BZLF2 positional homologue; ORFA8 = EBV BZLF3 positional homologue; ORFA10 = putative glycoprotein. ORFX' = truncated gene. H-DNA = non-coding terminal repeat sequences. The genomic organisation for C500 (low pass) represents that published by Ensser et al (1997) while the rearranged genome of C500 (high pass) is based on analysis of restrictions fragments (H. Wright, personal communication).

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6.2 Materials and Methods

6.2.1 Sera used for the detection of AlHV-1 proteins

Terminal sera from rabbits inoculated with recombinant proteins described in Chapter 5 were used in these studies. Both whole serum and purified IgG fractions were used.

6.2.2 MCF virus-infected cells used for the detection of AlHV-1 proteins by IFAT

Bovine turbinate (BT) cells grown on coverslips were infected with fully virulent cell-associated AlHV-1 C500 (pass no. 4-5 i.e. 'low pass'), attenuated cell-free AlHV-1 C500 (pass no.>350 i.e. 'high pass') or the attenuated cell-free AlHV-1 WC11. The coverslips were mounted on microscope slides and fixed in ice cold acetone. See Chapter 2.2.5 for a detailed protocol.

Rabbit LGL cell lines infected with OvHV-2 (BJ1857) or AlHV-1 (BJ926, BJ1859 and BJ1860) were resuspended in PBS to a density of 5×10^5 cells/ml. From these suspensions, cytocentrifuge preparations were fixed in acetone as above.

6.2.3 Detection of ORF50, A6 and A7 protein in MCF virus-infected cells by IFAT

Detection of AlHV-1 proteins was performed by indirect immunocytochemistry as described in Chapter 2.3.2. Normal horse serum (Gibco BRL, Paisley, UK) was used as a blocking serum for the secondary antibody (a donkey anti-rabbit polyclonal - SAPU, Carluke, UK). Normal rabbit serum was used as a negative control. For the positive control, terminal serum from a rabbit infected with AlHV-1 C500 (BJ652) was purified on a protein A-sepharose column (see Chapter 2.4.7) to produce a pooled IgG fraction.

Fixed cells were incubated in normal horse serum (NHS) diluted 1: 50 in PBS for 30 minutes at 37°C. Primary sera to the recombinant proteins were diluted 1:20 in PBS and the slides incubated as before. The control sera were applied at the same

dilution. The secondary FITC (fluorescein isothiocyanate) anti-rabbit conjugate was applied at a 1:50 dilution and incubated under the same conditions. Washing with PBS was performed after each incubation stage as described in Chapter 2.3.2. Slides were mounted and viewed under a UV microscope.

6.2.4 Detection of ORF50, A6 and A7 mRNA in MCF virus-infected cells

The detection of mRNA was performed by RT-PCR using the primers for ORF50, A6 and A7 indicated in Table 6.1 and by the method described in Chapters 2.5.1 and 2.5.2. BT cells used for this study were infected with AlHV-1 WC11 or C500 (low or high passage). Uninfected BT cells were included as negative controls. LGL cell lines used for this study were BJ1859, BJ1860 or BJ1263 (all rabbit AlHV-1-infected cells).

Table 6.1: Primers used for the detection of ORF50, A6 and A7 mRNA of AlHV-1.

Specificity	Function	Sequence	T_m	Product Size
ORF50	5' primer	TTCTATTGTCCACTGCCTCATCTC	72°C	503bp
	3' primer	TGACTGTGGTTCACTTGCTTCCAG	72°C	
ORFA6	5' primer	CCCATTTCAGCATTTAGCCCTCTG	72°C	541bp
	3' primer	TTGGGTCCTCTCACTACTAGCTTG	72°C	
ORFA7	5' primer	CAATTGGCAACCTAACCTTTCCCC	72°C	535bp
	3' primer	AAATCCTCTTGGCCACACAGACAG	72°C	
GAPDH	5' primer	CCTTCATTGACCTCAACTACAT	62°C	380bp
	3' primer	CCAAAGTTGTCATGGATGACC	62°C	

The primers for ORF50, A6 and A7 covered nucleotide positions 73405 - 73907, 75259 - 75800 and 76722 - 77256 respectively. The numbering is based on the published AlHV-1 sequence (Ensser *et al*, 1997). Tm = melting temperature.

6.3 Results

6.3.1 Expression of ORF50, A6 and A7 protein in AlHV-1-infected bovine turbinate (BT) cultures

Using whole rabbit polyclonal serum, ORF50 and A6 protein could be detected in BT cultures. Serum to ORF50 protein detected protein that was localised to the nucleus of virus-infected cells showing CPE. This staining was brightest in BT cells infected with low pass C500 and distributed mainly in the large multinucleate syncitia (Fig 6.2a). In high pass C500-infected BT cell cultures, round refractile cells showed positive staining (Fig 6.2b). Positive staining in WC11-infected BT cells was also found in areas of round refractile cells (Fig 6.2c). The use of purified IgG to ORF50 protein gave similar results (Fig 6.2e-g). Staining with whole sera or purified IgG to ORFA6 protein was also localised to the nucleus of cells showing CPE and similar differences between strains of AlHV-1 were observed (Fig 6.2i-k, m-o). Specific staining was not observed using the serum to ORFA7 protein in whole or purified form.

Using BJ652 serum (from a rabbit infected with AlHV-1 C500), the majority of staining in infected BTs was localised to the nucleus and reminiscent of the staining seen with sera to ORF50 and A6 proteins. The distribution of positive cells was limited to areas of CPE and was again reminiscent of staining observed using sera to ORF50 and A6 proteins (Fig 6.2q-s). In addition, some cytoplasmic staining was observed using the BJ652 serum.

Neither the BJ652 serum nor the sera to ORF50 and A6 proteins could detect any staining in uninfected BTs other than low levels of non-specific cytoplasmic staining (Fig 6.2d, h, l, p, t). The normal rabbit serum controls were negative in all cases (Fig6.2u-x).
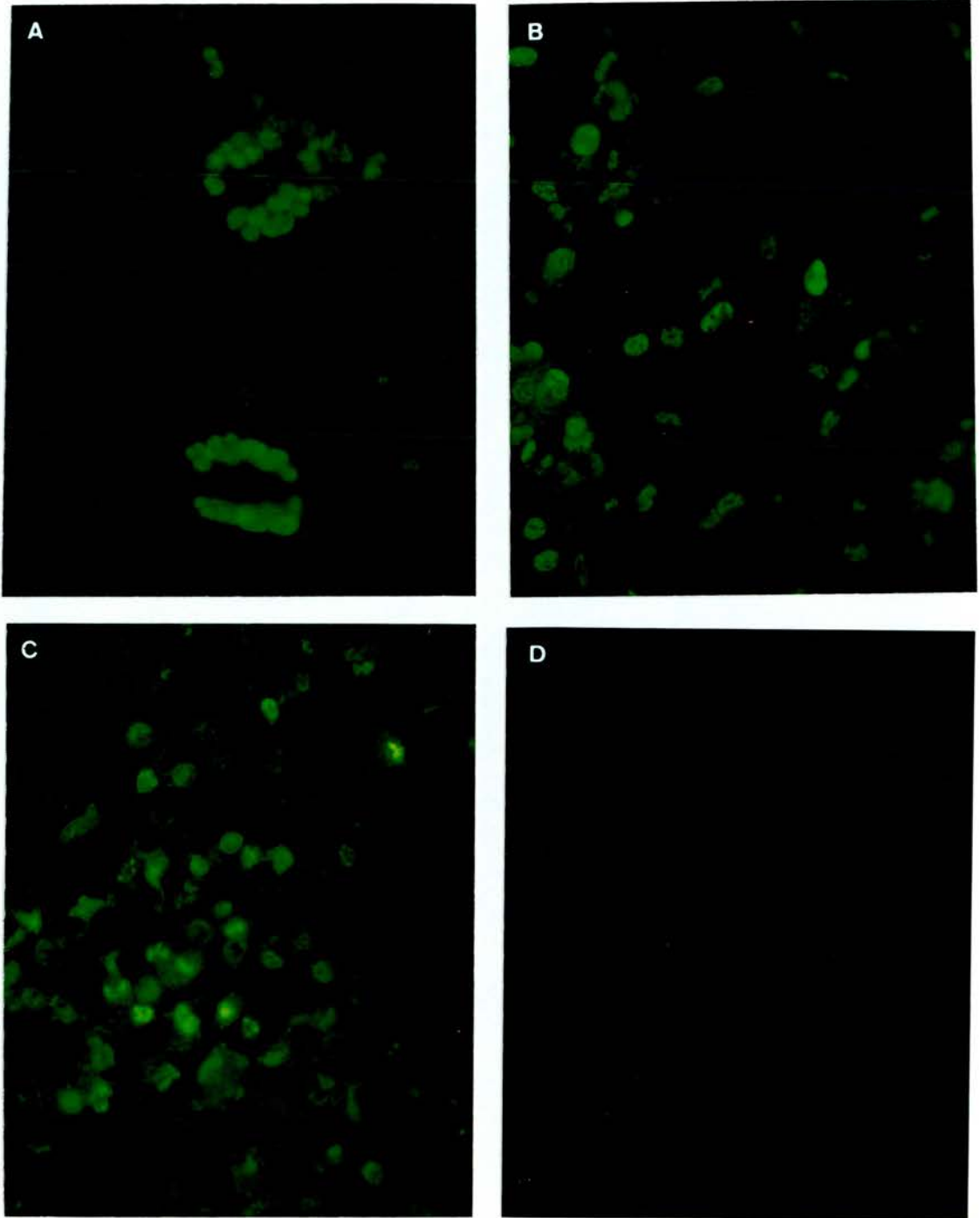
Fig 6.2: Determination of ORF50 and ORFA6 protein expression in AlHV-1 infected BT cells (continues on next page). Magnification, x200. Photographs were taken using equal exposures.

A) Whole ORF50 serum against BT cells infected with AlHV-1 C500 (low pass)
B) Whole ORF50 serum against BT cells infected with AlHV-1 C500 (high pass)
C) Whole ORF50 serum against BT cells infected with AlHV-1 WC11
D) Whole ORF50 serum against uninfected BT cells
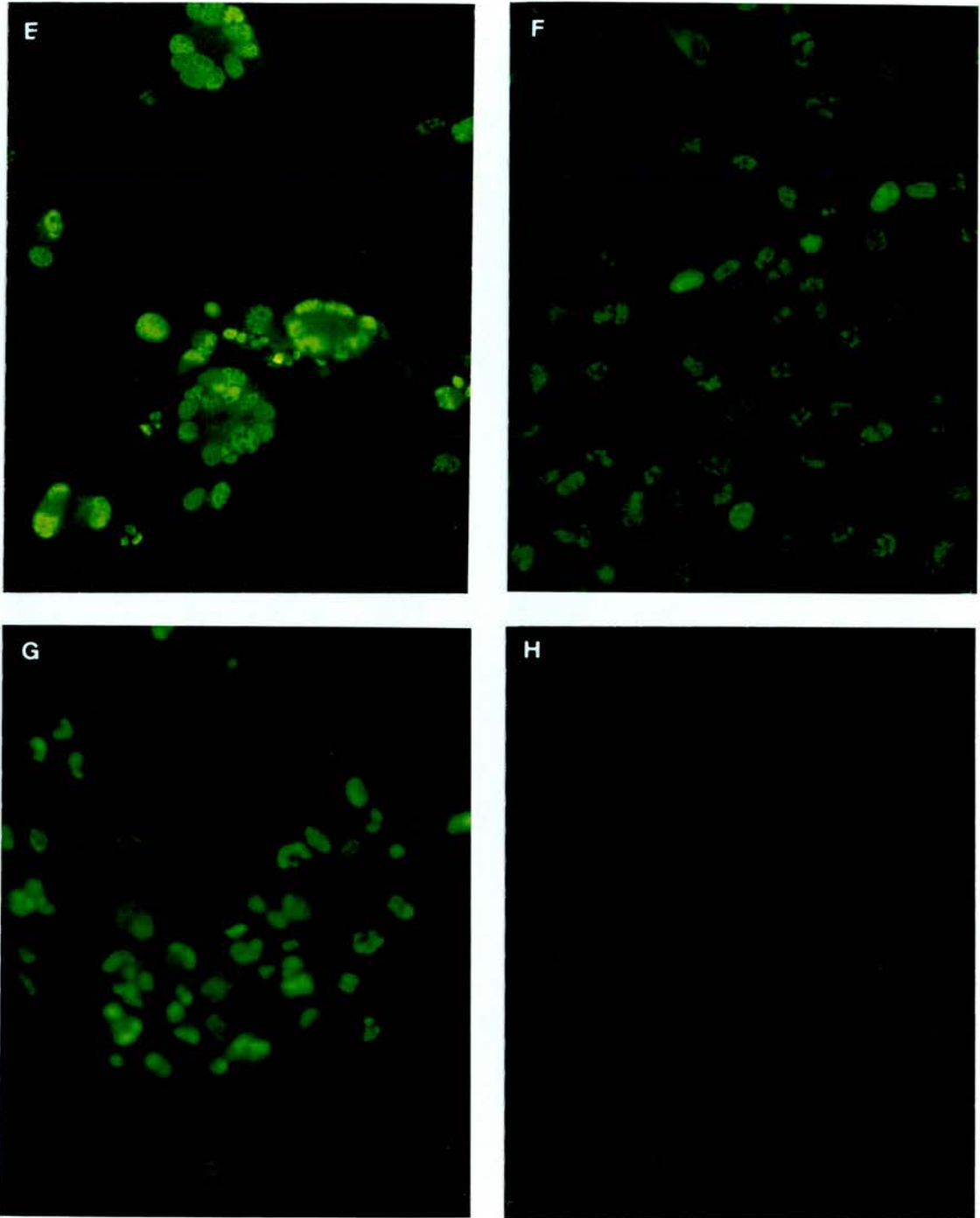
Fig 6.2 (continued): Determination of ORF50 and ORFA6 protein expression in AlHV-1 infected BT cells. Magnification, x200. Photographs were taken using equal exposures.

E) Purified ORF50 serum against BT cells infected with C500 (low pass)

F) Purified ORF50 serum against BT cells infected with C500 (high pass)

G) Purified ORF50 serum against BT cells infected with WC11

H) Purified ORF50 serum against uninfected BT cells
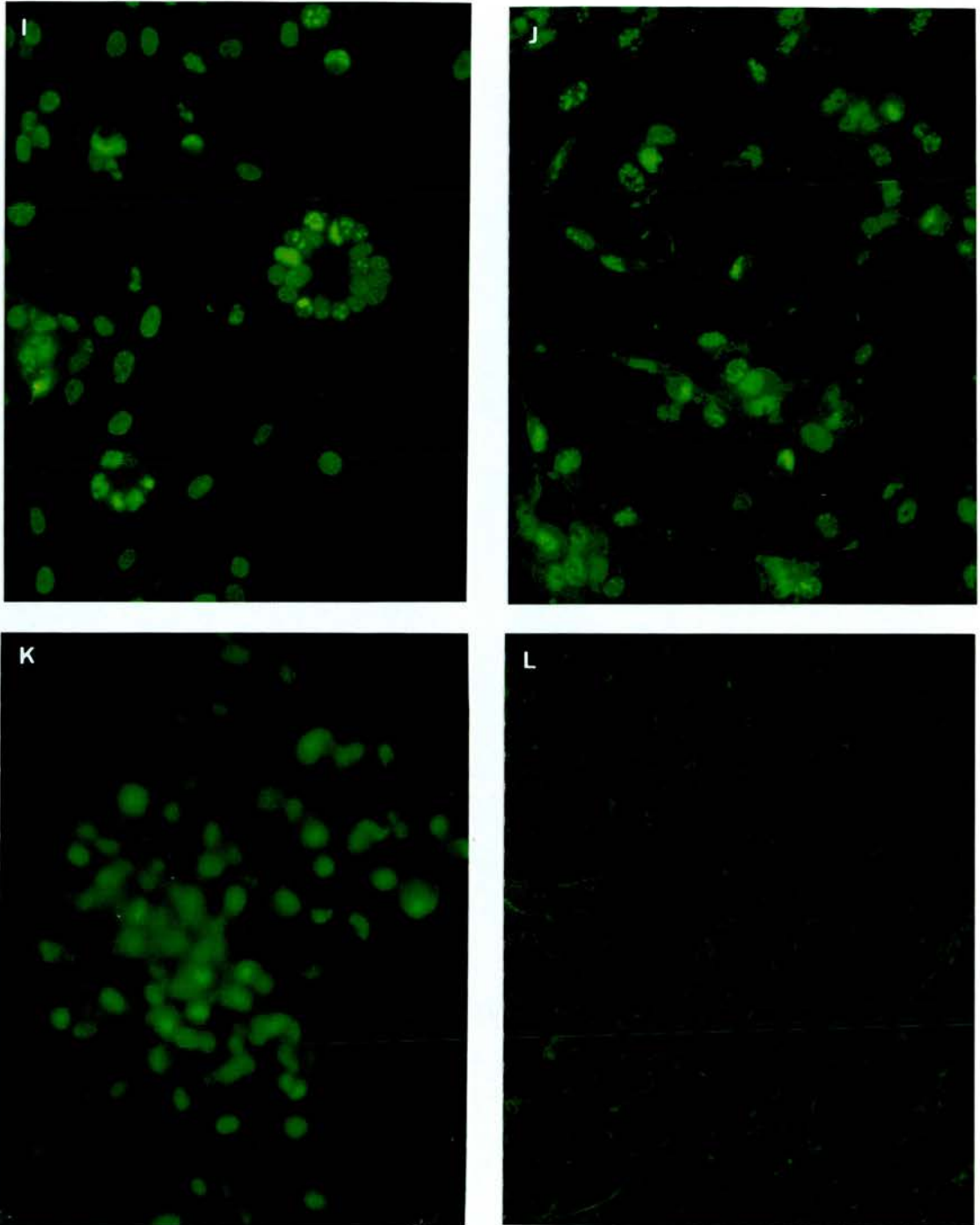
Fig 6.2 (continued): Determination of ORF50 and ORFA6 protein expression in AlHV-1 infected BT cells Magnification, x200. Photographs were taken using equal exposures.

 I) Whole ORFA6 serum against BT cells infected with AlHV-1 C500 (low pass)
J) Whole ORFA6 serum against BT cells infected with AlHV-1 C500 (high pass)
K) Whole ORFA6 serum against BT cells infected with AlHV-1 WC11
L) Whole ORFA6 serum against uninfected BT cells
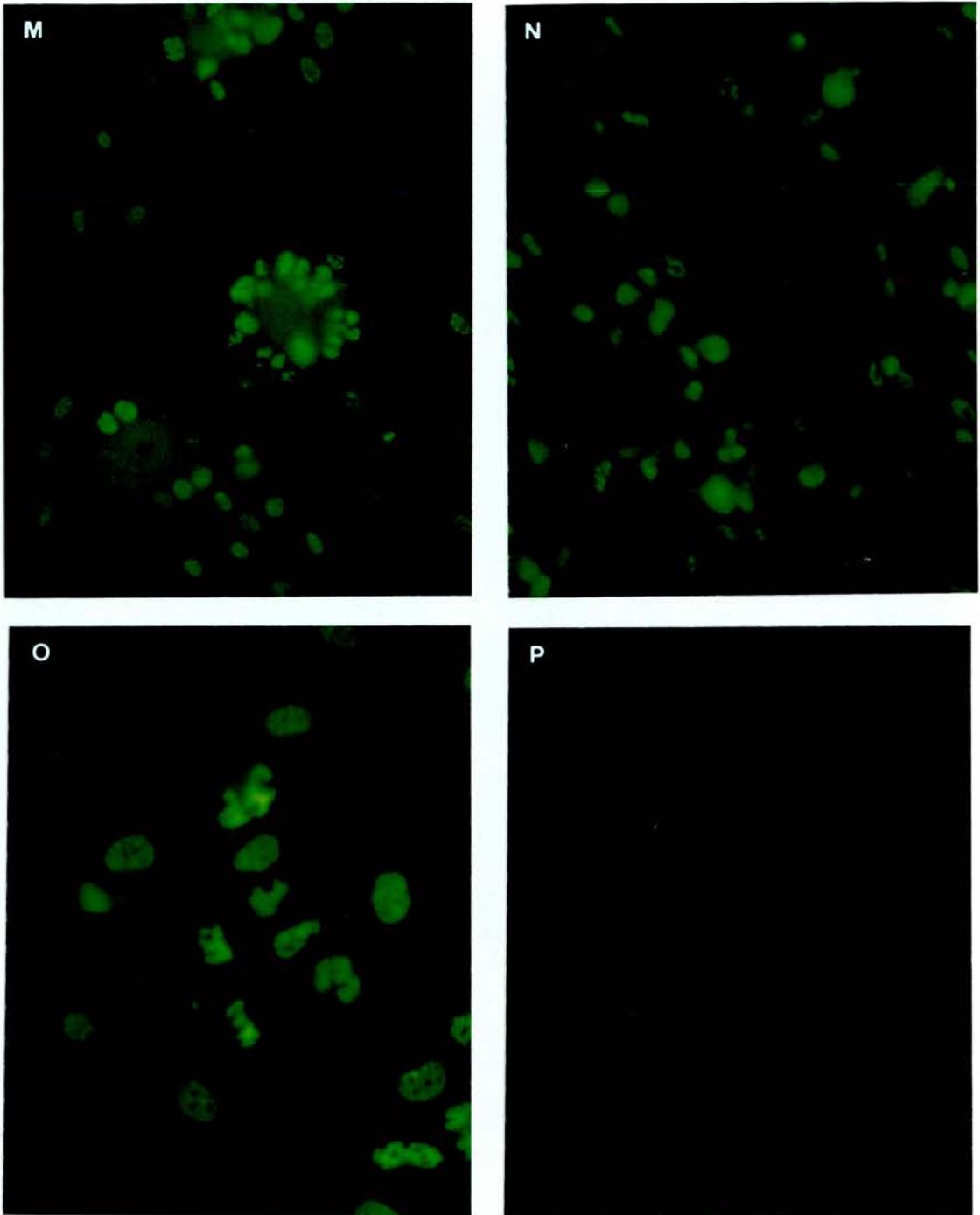
Fig 6.2 (continued): Determination of ORF50 and ORFA6 protein expression in AlHV-1 infected BT cells. Magnification, x200 (except O). Photographs were taken using equal exposures.

M) Purified ORFA6 serum against BT cells infected with C500 (low pass)
N) Purified ORFA6 serum against BT cells infected with C500 (high pass)
O) Purified ORFA6 serum against BT cells infected with WC11 (x400)
P) Purified ORFA6 serum against uninfected BT cells
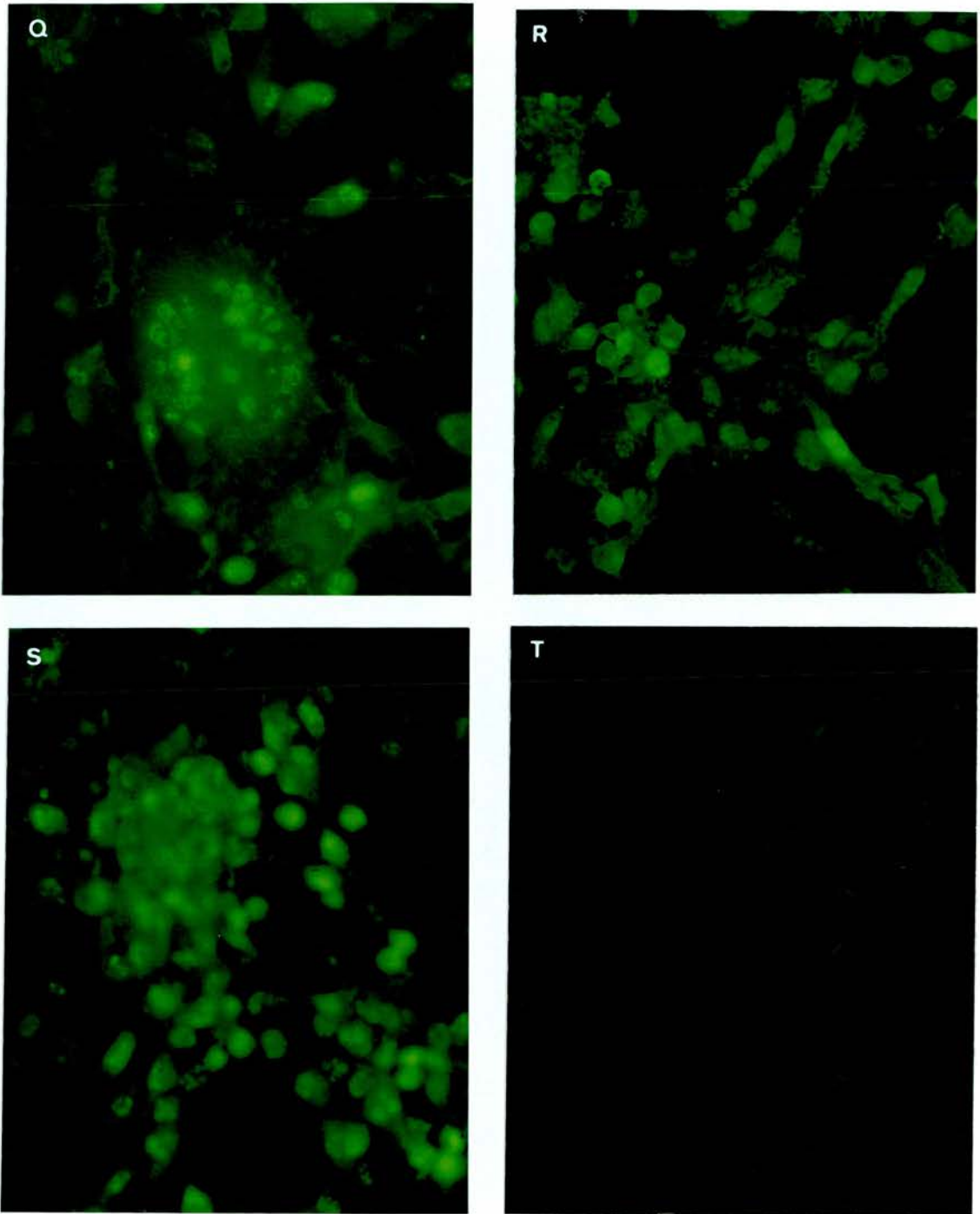
Fig 6.2 (continued): Determination of ORF50 and ORFA6 protein expression in AlHV-1 infected BT cells (positive control serum). Magnification, x200. Photographs were taken using equal exposures.
Q) Purified BJ625 serum against BT cells infected with AlHV-1 C500 (low pass)
R) Purified BJ625 serum against BT cells infected with AlHV-1 C500 (high pass)
S) Purified BJ625 serum against BT cells infected with AlHV-1 WC11
T) Purified BJ625 serum against uninfected BT cells
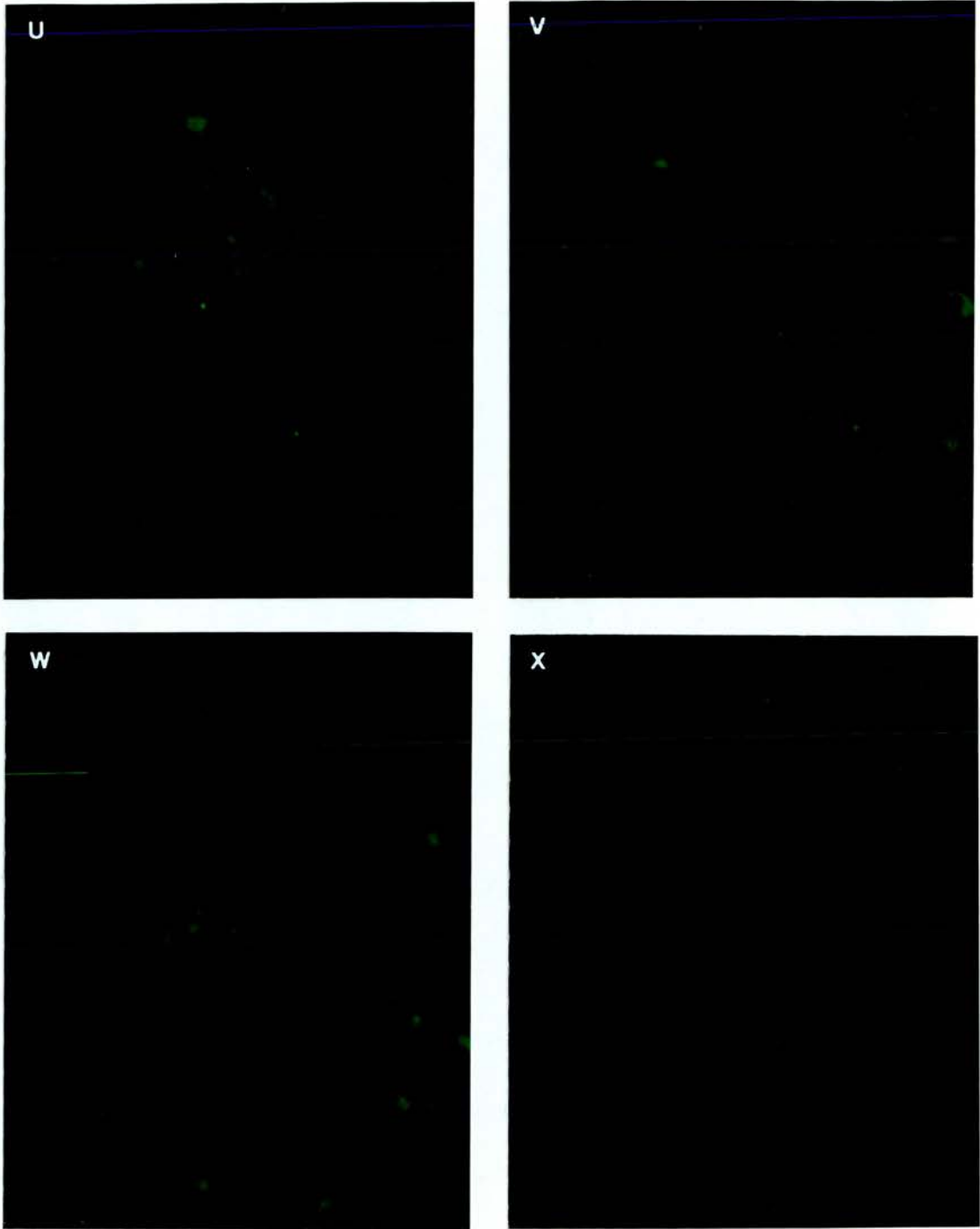
Fig 6.2 (continued): Determination of ORF50 and ORFA6 protein expression in AlHV-1 infected BT cells (negative control serum). Magnification, x200. Photographs were taken using equal exposures.

  U) Normal rabbit serum against BT cells infected with AlHV-1 C500 (low pass)
  V) Normal rabbit serum against BT cells infected with AlHV-1 C500 (high pass)
 W) Normal rabbit serum against BT cells infected with AlHV-1 WC11
  X) Normal rabbit serum against uninfected BT cells

6.3.2 Expression of ORF50, A6 and A7 protein in rabbit LGL cell lines

After repeated experiments on cell lines BJ1857, 1859 and 1860 the results were inconclusive with only BJ1859 showing some positive staining on one occasion using the sera against ORF50-GST.

6.3.3 Expression of ORF50, A6 and A7 mRNA in MCF virus-infected cells

Parallel experiments were undertaken using AlHV-1 infected bovine turbinate cells where expression of ORF50, A6 and A7 transcripts was detected by RT-PCR using the primers indicated in Table 6.1. As in the IFAT experiments, bovine turbinate cells were harvested once 50-70% of cells showed signs of CPE. However, despite the use of DNase the detection of mRNA was hampered by the presence of excess viral DNA making interpretation of results difficult.

However, a PCR using the same primers on total DNA showed the presence of the genes ORF50, A6 and A7 in both low and high pass C500 (Fig 6.3). In the WC11 isolate the genes ORF50 and A6 but not A7 were detected (Fig 6.3). Uninfected BTs were negative in all cases.

The experiment was also repeated on AlHV-1-infected rabbit LGL cell lines (BJ1859, BJ1860 and BJ1263). Messenger RNA from ORF50, A6 and A7 was detected in BJ1859 (Fig 6.4a). Lower levels of ORF50, A6 and A7 mRNA was also detected in the other cell lines tested (BJ1860 and BJ1263). As a negative control, PCR was performed on the same RNA samples as above without reverse transcriptase and showed that viral DNA was not present. GAPDH mRNA was detected in equal quantities in all cells used (Fig 6.4b).

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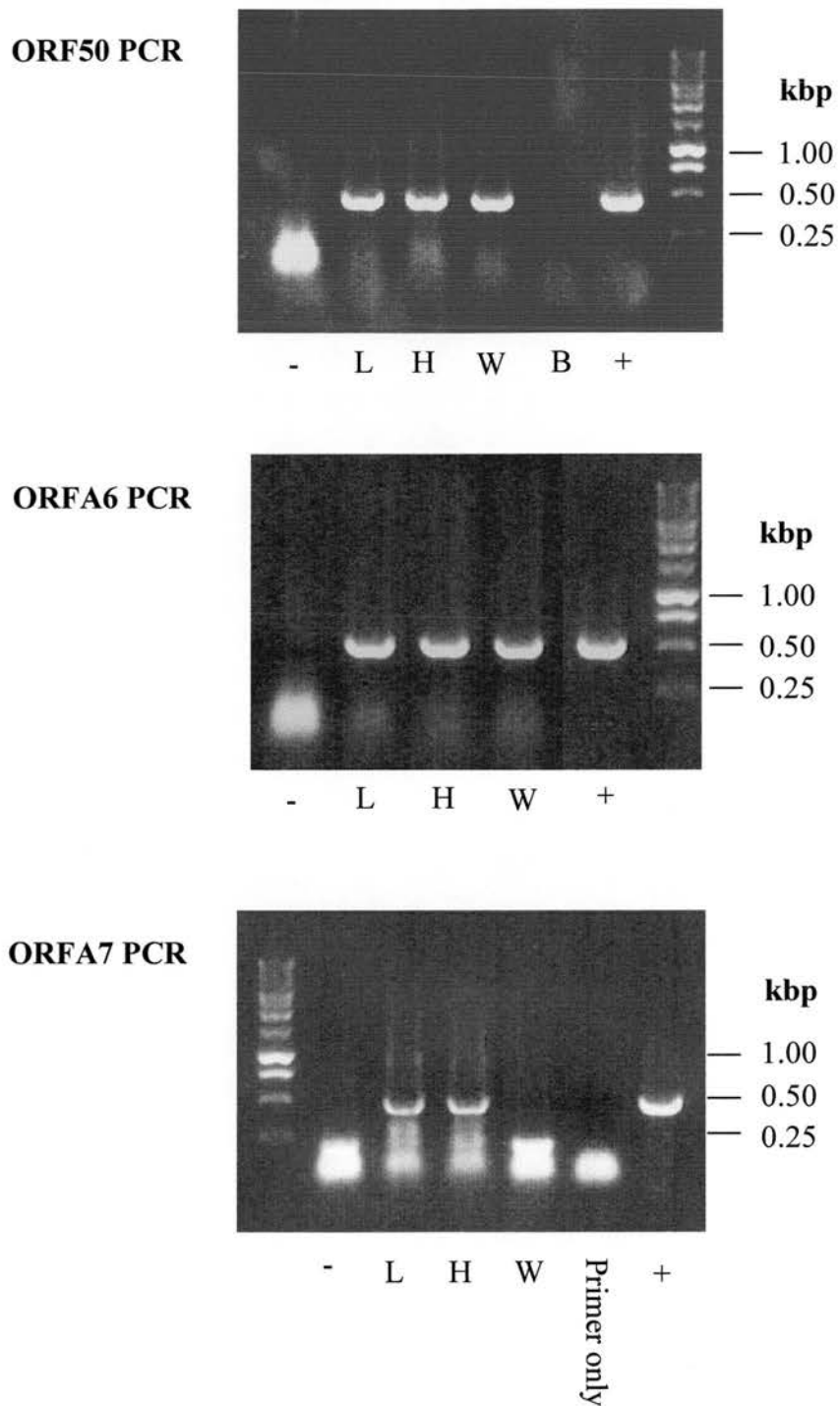
Fig 6.3: Detection of DNA encoding ORF50, A6 and A7 by PCR in AIHV-1-infected BT cells.

Primers used for these PCR reactions are described in **Table 6.1**.
- = Uninfected BT cells. + = Purified AIHV-1 DNA. **L** = BTs infected with low pass C500. **H** = BTs infected with high pass C500. **W** = BTs infected with WC11. **B** = Blank (no primers). Size of DNA (kbp) indicated to the right of gels.

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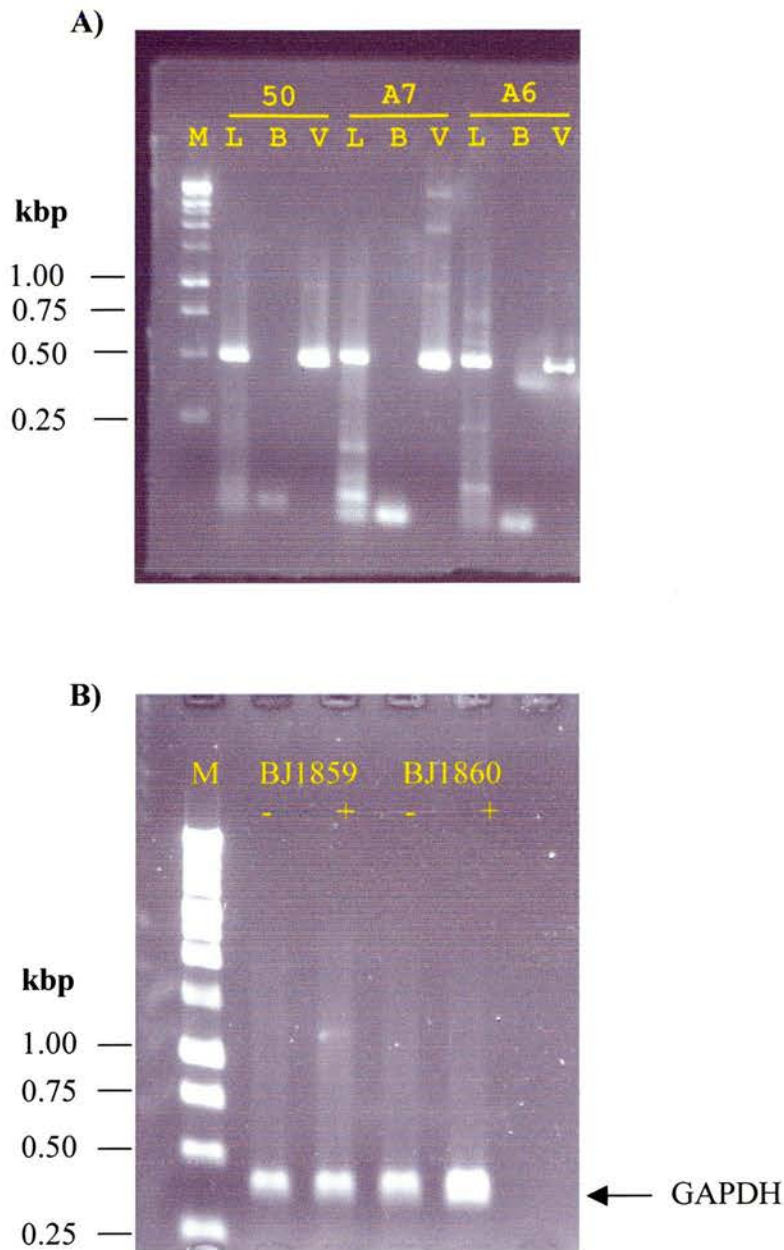
Fig 6.4: Expression of mRNA from ORF50, A6 and A7 in rabbit cells.
A) M = molecular weight markers. L = LGL (BJ1859, AlHV-1⁺). B = uninfected
lymphoblasts. V = purified AlHV-1.
B) BJ1859 and BJ1860 (LGLs infected with AlHV-1). M = markers.
 - = unstimulated. + = stimulated with 10µg/ml con A + 5ng/ml TPA.
GAPDH = glyceraldehyde-3-phosphate dehydrogenase.   Size of DNA (kbp) are
indicated to the left of gels.

6.4 Discussion

6.4.1 Expression of ORF50, A6 and A7 in infected BT cells

The results presented here show that in virus-infected BT (fibroblast) cells the different strains of C500 produce different forms of CPE. In low pass (virulent) C500-infected BT cells, CPE is characterised by presence of syncitia. In contrast, attenuated high pass C500 and WC11-infected BT cells showed CPE in the form of round refractile cells. This pattern of CPE is consistent with previous observations of CPE in AlHV-1-infected fibroblast cells (Ferris *et al*, 1976; Harkness and Jessett, 1981). However, proof of AlHV-1-infection in the previous studies required supporting evidence showing AlHV-1 protein expression in infected fibroblast cells by the use of specific sera. This was demonstrated in the present study by the use of antisera to the proteins encoded in ORF50, A6 and by the use of serum from a rabbit infected with AlHV-1 (strain C500, low pass).

The results shown here demonstrated the ability of antisera to ORF50-TrX and ORFA6-TrX to detect native viral antigens in infected BT cells. Whether the failure of the antisera to the ORFA7 product to detect viral antigen reflects a genuine lack of expression in BT cells or merely low-affinity antibody binding is not known. Alternatively, the difficulty in detecting ORFA7 at protein level may reflect the limited sensitivity of immunocytochemistry compared to RT-PCR. This would have been addressed by RT-PCR analysis of ORF50, A6 and A7 mRNA expression in infected BT cells. However, the analysis in virus-infected BT cells was hampered by the presence of excess amounts of viral DNA despite treating samples with DNase. The results obtained by the less sensitive method of mRNA detection, the northern blot, were inconclusive. However, the detection of PCR products for ORF50, A6 and A7 from DNA samples of BT cells infected with low and high pass C500 demonstrated that none of these genes as defined by the primers were deleted following extensive passage. The absence of a PCR fragment for ORFA7 in WC11 may indicate that this region of the AlHV-1 genome has a high degree of susceptibility to genetic alterations. This is supported by evidence that the neighbouring ORF (A8) is also truncated in WC11 (Ireri, 1999).

From the rearrangements thought to occur at the right hand end of AlHV-1 genome following attenuation (H. Wright, personal communication, Fig 6.1) the expected results of the present study might have been the loss of ORF50 and A6 expression upon attenuation. In addition, ORFA7 does not appear to be affected by the predicted rearrangements and could theoretically be expressed in the attenuated virus. The results of this study are in contrast with this prediction where expression of proteins encoded by ORF50 and A6 but not ORFA7 was observed in the attenuated virus. In a study by Handley, the translocation of a region of the attenuated C500 from the middle to the right-hand end of the unique DNA (see Fig 6.1) is also repeated at the left-hand end of the genome (Handley, 1993; Handley *et al*, 1995). Sequence analysis of the left end of the attenuated genome has not been performed to confirm this observation. It is therefore possible that more rearrangements are present than has been described so far. If this is true, the presence of a transcriptionally active ORF50 at the left end of the genome could explain the expression of ORF50 protein in the attenuated virus. Likewise, the translocation of the full ORFA6 sequence could explain the detection of ORFA6 protein in both virulent and attenuated C500. If so, the translocation of the full ORFA6 sequence could also have consequences for the expression of the adjacent gene, ORF A7 if its promoter sequences are within the sequence coding for ORFA6. Alternatively, the presence of a mixed culture of virulent and attenuated viruses in high pass C500 is a possibility. This hypothesis would not conflict with the generally attenuated nature of the high pass C500 cultures as presence of a small percentage of virulent virus in this culture could be detectable by causing infection of BT cells whilst not being sufficient to induce disease.

The function of proteins encoded in ORF50, A6 and A7 in the virus life cycle has not been demonstrated. However, protein database analysis indicated that the protein encoded by ORF50 showed similarity to a protein sequence in bovine herpesvirus 4 (BHV-4). This BHV-4 sequence is a homologue of the EBV BRLF1 gene product (R transactivator) an immediate early protein involved in the regulation of EBV BZLF1 transcription (van Santen, 1993). The equivalent protein in HVS is encoded by ORF50 and is situated in the middle of the unique DNA (Albrecht *et al*, 1992). As the ORFs of gammaherpesviruses are collinear (Roizman *et al*, 1992) the

positional homologue of AlHV-1 ORFA6 is BZLF1 of EBV (Ensser *et al*, 1997). BZLF1 encodes a Z transactivator, another immediate early protein. EBV BRLF1 and BZLF1 are expressed during viral replication (Countryman and Miller, 1985; Farrell *et al*, 1989; Hardwick *et al*, 1988; Rooney *et al*, 1989). The homology of AlHV-1 ORF50 and the positional homology of ORF A6 to the EBV BRLF1 and BZLF1 respectively would suggest that the proteins encoded by ORF50 and A6 could be immediate early proteins. In EBV lytic replication requires both BRLF1 and BZLF1 gene products (Holley-Guthrie *et al*, 1990; Miller, 1990). If ORF50 and A6 are functional homologues of BRLF1 and BZLF1, their expression in BT cells showing viral CPE (a sign of lytic replication) could indicate their role in initiation of lytic replication. However, structural nor functional homology between the proteins encoded by ORF50, A6 and A7 with other proteins has been identified. In addition, the expression of ORF50 and A6 in high pass C500 where virulence (or disease induction) is lost suggests that ORF50 and A6 are not virulence factors.

The presence of ORF50 expression in high pass C500 could be explained in another way. ORF50 of AlHV-1 also has homology to HVS ORF50 encoding HVS-R transactivator (Albrecht *et al*, 1992; Ensser *et al*, 1997). Both ORF50 sequences contain an intron and in HVS encode splice variants designated ORF50a and ORF50b (Whitehouse *et al*, 1997). While ORF50a was transcribed from a promoter present upstream of exon1 (in the non-coding DNA strand of the neighbouring ORF48), the shorter ORF50b was transcribed in the delayed early phase from a promoter region within the second exon. If this is also true for ORF50 of AlHV-1, it might explain the ability of sera to detect ORF50 protein in both low and high pass C500. In other words, in high pass C500 the translocation of ORF50 to the right terminal end only affects transcription from the first promoter. Therefore, the presence of a promoter region in exon 2 of ORF50 could allow the expression of a splice variant. The existence of a second promoter in ORF50 has yet to be determined.

6.4.2 Expression of ORF50, A6 and A7 in rabbit LGL cell lines

The detection of ORF50, A6 and A7 by IFAT was difficult to interpret owing to non-specific reactivity using normal serum and in uninfected rabbit control cells. This cross-reactivity is unlikely to be due to reactivity with other known herpesvirus antigens as these sera were obtained from specific-pathogen free rabbits. The results for the IFAT experiments were therefore inconclusive. The RT-PCR analysis demonstrated low and variable levels of mRNA in the three cell lines tested and suggest that the corresponding proteins are expressed at low levels and that levels of expression differ between cell lines. This has also been shown for other viral genes currently being analysed (H. Wright, personal communication). Alternatively, mRNA with a short half-life may explain the variable levels of mRNA. The relative insensitivity of immunofluorescence for detecting low levels of protein may contribute to the difficulty in detecting ORF50, A6 and A7 at protein level.

MCF LGL cell lines have consistently been shown to contain viral DNA and protein. The replicative state of virus in LGLs is unknown. While virions have yet to be detected in LGLs, these cells can induce MCF when inoculated into rabbits. All these features of LGLs suggest the presence of productive virus in these cells. This hypothesis is supported by the findings of the present study. The detection of mRNA for ORF50, A6 and A7 in BJ1859 and other LGL cell lines indicates that a replicating virus is present at low levels and could explain the ability of LGLs to transmit disease to rabbits. Recent work has shown that ORF50 of AlHV-1 transactivates ORF6, 57 and A9 (Frame and Dalziel, 1999). This is homologous to the function of ORF50 of HVS (in turn homologous to EBV ORF50), which encodes the R transactivator - an immediate early/ early protein - that transactivates ORF57 expression (Albrecht *et al*, 1992; Whitehouse *et al*, 1998). Therefore, if ORF A6 of AlHV-1 is also a functional homologue of EBV BZLF1, ORF50 and A6 like EBV BRLF1 and BZLF1 could mediate the initiation of productive replication required for disease transmission.

Chapter 7

General Discussion

The pathology associated with malignant catarrhal fever is characterised by lymphocyte hyperplasia in lymphoid tissues, lymphoblast infiltration of non-lymphoid tissues and widespread tissue necrosis followed by death. The experimental transmission of MCF using lymphocyte cell suspensions from blood, lymphoid and non-lymphoid tissue and the generation of lymphoblastoid cell lines from such cell suspensions suggested that these cells may be important in MCF pathogenesis.

Whether derived from AlHV-1 or OvHV-2-infected animals, lymphoblastoid cell lines have the morphology of large granular lymphocytes (LGLs). More importantly, the detection in LGL cell lines of MCF virus DNA or protein and the ability of LGL cell lines to transmit MCF to experimentally inoculated rabbits support their role in pathogenesis. However, the mechanism of virus-infected LGL-mediated pathogenesis is not known.

The results of this study demonstrated several important phenotypic differences between MCF virus-infected LGL cell lines and uninfected control cells. Some of these findings confirmed previous observations such as the ability of LGLs to survive and grow without the need for exogenous IL-2 for longer periods than control cells (Reid *et al,* 1989). The expression of BLT esterase (a tryptase and activation marker for cytotoxic cells (Griffiths and Mueller, 1991; Haig *et al*, 1996)) in IL-2-independent LGLs showed that the LGLs displayed the phenotype of constitutively active and cytotoxic cells.

The most important and novel findings of this study were firstly that the active and cytotoxic phenotype of LGL cell lines could be maintained by the cytokine IL-15 (Chapter 3). At high doses, IL-15 could also develop increased numbers of BLT esterase expressing cells in control lymphoblast cultures. Thus, IL-15 is a potential stimulus for the generation and maintenance of LGL and cytotoxic lymphocytes. Secondly, this study demonstrated the constitutive activation of the T cell tyrosine kinases, *lck* and *fyn* in LGL cells in the absence of exogenous IL-2 (Chapter 4). This suggested that virus infection of the LGLs was associated with the activated phenotype of the LGLs, which was similar to that of mitogen-activated control lymphocytes. The observed characteristics of the LGL cells reported here

were associated with MCF virus infection of these cells. However, specific interaction(s) between virus and host proteins have not been demonstrated.

Finally, the viral proteins encoded by ORF50, A6 and A7 of AlHV-1 C500 were of interest as the area of the genome encoding these proteins becomes rearranged on attenuation of the virus (where attenuation is described as the loss of disease induction). These proteins were thought to be putative virulence proteins and their expression in LGLs may explain the ability of such cells to transmit disease. The work presented in this study showed that the LGL cells expressed mRNA for ORF50, A6 and A7 at variable levels (Chapter 6). Despite the difficulty in detecting the protein products of ORF50, A6 and A7 in LGLs, the RT-PCR results suggested that a low level of replicative AlHV-1 C500 may be present in LGL cell lines. This conclusion is supported by the observation that ORF50 and A6 share positional homology with EBV R and Z transactivators, which are required for the initiation of EBV replication.

During lytic replication of attenuated C500 in BT cells, ORF50 and ORFA6 at DNA and protein level and ORFA7 at DNA level were detected (Chapter 6). Thus, the detection of the proteins encoded by ORF50 and A6 in the attenuated C500 suggested two possibilities. Firstly, that these proteins may not be virulence factors as was initially proposed. Alternatively, the proposed rearrangement of the attenuated genome may not represent all the genomic changes that occur on attenuation. Furthermore, the detection of DNA encoding ORF50, A6 and A7 in the attenuated C500 virus indicates that the loss of disease induction using attenuated virus cultures may not be due to the physical loss of these genes but rather a loss of transcriptional activity. An alternative explanation for the detection of ORF50 and A6 protein in apparently attenuated C500 cultures could be the presence of virulent virus amongst the attenuated virus cultures at a level that is too low to induce disease.

Mechanisms for LGL dysfunction

The ability of LGL cells to survive in culture without exogenous IL-2 for longer periods than control cells and their dependence on cell density for optimal growth suggested that their survival could be mediated though the production of

endogenous cytokines. It had been proposed previously that immune dysregulation was responsible for the pathogenesis of MCF. One hypothesis was that MCF virus-induced production of IL-2 caused the initial proliferation of T cells (Schock 1996). However, it was subsequently discovered that IL-2 was not transcribed by OvHV-2-infected cell lines and no IL-2 activity could be detected in culture supernates (Schock, 1996; Schock *et al.* 1998) despite the fact that some of these lines could survive and grow in the absence of exogenous IL-2. Lack of IL-2 mRNA has not been measured in AlHV-1-infected LGL cell lines. However, the absence of IL-2mRNA in OvHV-2-infected LGL cell lines would suggest that endogenous IL-2 production does not substitute for exogenous IL-2. It also suggests that IL-2 induction by LGLs following virus infection is not important in MCF pathogenesis. This led to the hypothesis that other endogenous cytokines are responsible for the proliferative and cytotoxic phenotype of the LGL cells. An investigation into the cytokines expressed by OvHV-2-infected LGLs showed the presence of mRNA for IL-4, IL-10, TNFα and IFNγ while IL-2 and IL-1β could not be detected (Schock, 1996; Schock *et al*, 1998).

The temporal expression of these cytokines and therefore their role in MCF virus-infected LGLs *in vivo* has not been determined. However, it would be possible to speculate that TNFα and IFNγ, with their antiviral activities, could limit spread of virus to neighbouring cells (if the viruses do not have anti-TNF/IFN mechanisms of immune evasion). In contrast, the expression of IL-4 and IL-10 could benefit virus survival by inhibiting the response of Th1 (helper T) cells that regulate antiviral activities such as cytotoxicity and macrophage activation. In this respect, IL-10 is encoded in the genomes of several herpesviruses including EBV where it is thought to be expressed during lytic replication following primary infection thereby inhibiting local T cell and NK cell-mediated immunity (Moore *et al*, 1993). In OvHV-2-infected LGLs, the origin of the IL-10 mRNA (i.e. whether viral or cellular) could not be determined (Schock *et al*, 1998). However, these cytokines do not display activities that could induce LGL proliferation and auto-stimulation leading to IL-2-independence in culture.

The cytokine IL-15, which is known to stimulate the proliferation of T cells and NK cells and develop or maintain their cytotoxic phenotype (Grabstein *et al*,

1994) was proposed as an alternative candidate. In addition, induction of IL-15 in transformed T cells by HTLV-1 (the virus associated with an adult T cell leukaemia) has been demonstrated. This virus induces the transcription of the IL-15 gene through the binding of Tax-1 (a viral transactivating protein) to the promoter region of the IL-15 gene. It was shown that HTLV-1 infection or Tax-1 transfection led to a 3-fold increase in levels of IL-15 mRNA in T cells (Azimi *et al*, 1998). In this study, LGL cells responded to human recombinant IL-15 in a dose-dependent manner. At low concentrations IL-15 maintained or enhanced the cytotoxic phenotype of LGLs but not control cells. These results suggests that LGL cells express receptors for IL-15 and that IL-15 can substitute for or co-stimulate with IL-2 in maintaining the active cytotoxic phenotype of LGL cells *in vitro*.

Whether IL-15 is produced by LGL cells or by another cellular source *in vivo* is not known. An attempt was made to test the hypothesis that AlHV-1 or OvHV-2 might induce IL-15 production in infected cells that stimulate proliferation and cytotoxicity of the LGL cells. Analysis of IL-15 mRNA expression in LGL cell lines by RT-PCR did not demonstrate the induction of IL-15 in these cells. However the primers used for the detection of IL-15 in rabbit LGL cell lines were based on the bovine IL-15 sequence (which has a high degree of sequence identity to the IL-15 sequence of other species) as the sequence for rabbit IL-15 has not been reported (Chapter 3). In addition, the antibody to IL-15 did not detect rabbit or bovine IL-15, therefore the production of IL-15 by LGLs or cells in tissues of MCF-affected rabbits could not be determined. The absence of a reliable positive control for detection of rabbit IL-15 was also a problem. Time constraints meant that positive controls for rabbit or bovine IL-15 could not be performed. All the data available on the expression of IL-15 in various cell types come from human or murine studies. From these, IL-15 was produced most readily in cell lines (frequently of epithelial or fibroblast origin) and in activated monocyte enriched PBMCs (Grabstein *et al*, 1994). Given more time, a more comprehensive study of IL-15 expression in cell lines or normal macrophages from cattle may reveal a source of IL-15 that is detectable using the primers used for the RT-PCR analysis. Alternatively, new primers could be constructed that have a greater chance of hybridising with IL-15 of rabbit origin. This work is currently underway. As for the detection of rabbit or bovine IL-15 by

western blot, this can only be achieved if known IL-15 mRNA+ cells express protein that can be detected by putative cross-species reactive anti-IL-15 antibodies. Antibodies specific for rabbit or bovine IL-15 have not been developed. Consequently, whether IL-15 is expressed in LGL cells *in vitro* or *in vivo* is not known.

The fact that LGLs respond to hrIL-2 and that IL-2Rα has been detected on some cell lines would indicate that this response is mediated through the high affinity IL-2R (IL-2Rαβγ). With an affinity or K_d of 10^{-11} M the high affinity IL-2R can be stimulated by as little as 10pM of IL-2 (Minami *et al*, 1993). The affinity of the IL-15 receptors has been most thoroughly examined in the murine system. Activated peripheral blood T cells express only one type of IL-15 receptor (IL-15R) - the high affinity receptor comprising the subunits IL-15Rα and IL-2Rβγ - with a K_d of 10^{-11} M (Giri *et al*, 1995a). In contrast with T cells, murine NK cells express a small number of high affinity IL-15 receptors (IL-15Rα/IL2Rβγ) along with intermediate affinity IL-15 receptors comprising only the IL-2Rβγ subunits ($K_d = 10^{-10}$M). Therefore, irrespective of whether LGLs are T cells or NK cells the results, by extrapolation from murine studies, would suggest that LGLs would be capable of responding to IL-15. This was proven *in vitro* by the IL-15 response assays described in Chapter 3. Thus, although production of IL-15 by LGLs was not demonstrated in this study it is possible to speculate that if LGLs or other cells *in vivo* did produce even small quantities of IL-15, it is possible that LGLs could respond.

Given the chemoattractant properties of IL-15 (Oppenheimer-Marks *et al*, 1998; Sancho *et al*, 1999) it is tempting to speculate that recruitment of LGL cells in response to IL-15 may explain the lymphoblast infiltration of non-lymphoid tissue and lymphoproliferation observed in MCF. The source of IL-15 *in vivo* may be virus-infected LGLs or more likely inflammatory cells in affected tissue. Accumulation of virus-infected lymphoblast cells to non-lymphoid tissue could be mediated by production of IL-15 in the tissues following virus infection. Subsequent proliferation of recruited lymphoblasts may provide new target cells for virus infection.

The production of IL-15 may also promote the clearance of MCF virus-infected cells by developing and activating cytotoxic T lymphocytes (CTLs) or NK

cells. The production of IL-15 as a host defence mechanism against viral infection has been reported. For example, IL-15 enhances stimulation and proliferation of virus-specific cytotoxic T cell responses in HIV infection (Chehimi *et al*, 1997; Kanai *et al*, 1996). In HHV-6 and HHV-7 infection activation of NK cell activity via IL-15 has also been reported (Atedzoe *et al*, 1997; Flamand *et al*, 1996). If IL-15 is involved in a host defence mechanism during MCF infection, it does not seem to be protective in that the disease is largely fatal. The persistence of virus and the high mortality rates due to MCF may be due to the infection of antiviral effector cells (i.e. CTLs and NK cells) by the MCF viruses.

IL-15 has been implicated in disease pathogenesis of inflammatory diseases such as rheumatoid arthritis and ulcerative colitis (Kirman and Nielsen, 1996; McInnes *et al*, 1996, 1997). In addition, the progression of hepatitis C virus (HCV)-associated chronic liver disease has been related to high levels of serum IL-15 (Kakumu *et al*, 1997). Furthermore, in LDGL (lymphoproliferative disease of granular lymphocytes) proliferation and cytotoxicity of granular lymphocytes was achieved via IL-15 produced by PBMC isolated from LDGL patients (Zambello *et al*, 1997). This disease shares several characteristics with MCF including excessive proliferation of primed granular lymphocytes (of T cell phenotype) against unknown antigens. These cells did not express mRNA for IL-15 or IL-2. Phenotypic similarity between the cells involved in LDGL and MCF-derived LGL cell lines now extends to their response to IL-15. Hence, even if LGL cell lines do not express IL-15 their ability to respond to IL-15 from another source may be an important part of MCF pathogenesis. In this respect, the occasional observation of macrophage cell lines derived from MCF-affected animals (Mushi and Rurangirwa, 1981d) would suggest that monocytes (and possibly other antigen presenting cells) are also targets for MCF virus infection and potentially another source of virus-induced IL-15.

The induction of IL-15 (if present) is not likely to be the only mechanism responsible for the constitutive activation of LGL cells. In other herpesviruses, continuous growth and maintenance of virus in infected cells may be mediated through the inhibition of apoptosis by the expression of the anti-apoptotic protein, bcl-2 (Chapter 1). The fact that human herpesvirus 8 (HHV-8) (Boshoff and Weiss, 1998), EBV and HVS encode a bcl-2 homologue indicates the importance of bcl-2 in

the virus life cycle. Viral bcl-2 may function by inhibiting apoptosis induced at the early stages of virus infection allowing subsequent infection and transformation of the infected cell. In AlHV-1 a bcl-2 homologue has been found and is encoded in ORF A9 (Ensser *et al*, 1997). The expression of this ORF has been detected in LGL cell lines (H Wright, personal communication) may, in part, explain their continuous survival *in vitro* and the lack of requirement for exogenous IL-2. The expression of viral bcl-2 may also play a role in the lytic virus cycle *in vivo* by delaying the induction of apoptosis following viral infection of a cell and thus allowing viral replication to proceed. Expression of bcl-2 could also mediate continuous survival of the infected cell leading to immortalisation of the cell and allowing persistence of the viral genome in a latent state. This function may be important in sheep where OvHV-2 is thought to be latent in B cells (Baxter *et al*, 1997) or in the case of MCF-recovered cows where the virus is thought to be latent (Plowright *et al*, 1972). The putative anti-apoptotic-function of ORF A9 is currently being investigated.

There is another possible mechanism for the observed exogenous IL-2-independent proliferation and cytotoxicity of the LGL cell lines. MCF virus-infection of LGL cell lines was associated with the constitutive activation of the *src* tyrosine kinases, *lck* and *fyn*. These are non-receptor protein tyrosine kinases (NRPTKs) expressed in T lymphocytes (Crabtree and Clipstone 1994) and are involved in the early stages of cell surface receptor-mediated signalling. In MCF virus-infected LGLs, the activation of *lck* and *fyn* may indicate that MCF viruses exert their lymphoproliferative effect at least in part by by-passing the signals initiated from antigen or cytokine cell surface receptors. The consequence of constitutive *lck* and *fyn* activation in LGL cells is not known. Recently, the altered activity of the mitogen-activated protein kinases (MAPKs) - *erk1* and *erk2* - in LGL cell lines has been demonstrated (Swa *et al*, in press). This analysis was performed following the completion of the present study. The results showed that MAPK is constitutively activated in LGL cells infected with the MCF viruses. In normal cells, MAPKs can be activated by a variety of stimuli including those generated by the T cell receptor and cytokine receptors (Schwartz and Baron, 1999). *Lck* and *fyn* are known to activate the MAPKs via the activation of the GTP-binding protein *ras* and the kinase *raf* (Minami *et al*, 1993; Pelech and Sanghera, 1992; Schwartz and Baron,

1999). Therefore, constitutive activation of *lck, fyn* and MAPK could be the mechanism by which the virus-associated proliferative and possibly cytotoxic phenotype of LGL cells is achieved.

The importance of kinase binding motifs is demonstrated by the HVS proteins Tip and STP (encoded in ORF 1 by a bicistronic mRNA) and by the EBV protein LMP-2A. These proteins in EBV and HVS have little sequence homology to each other but the presence of kinase binding motifs is common to both. For instance, the binding of the HVS protein Tip to the tyrosine kinase *lck* is mediated by two motifs on Tip that makes up the *lck*-binding domain. The physical association of Tip and *lck* modulates *lck* activity (Wiese *et al*, 1996) and leads to the activation of other kinases downstream of *lck* including the JAKs (Janus kinases) and the STATs (signal transducers and activators of transcription). JAKs and STATs are signalling molecules associated with cytokine receptor-mediated signalling. As Tip is required for transformation of T cells (Duboise *et al*, 1998a) the activation of the JAK/STAT pathway might indicate that transformation is achieved by by-passing the normal cytokine receptor mediated proliferation signals. The activity of p21*ras* was also altered by HVS via the interaction of viral STP with *ras*. STP is another HVS protein required for T cell transformation (Jung and Desrosiers, 1995) showing that different downstream signalling pathways can be used by the virus to achieve the same goal. Therefore, the deregulation of NRPTK activity by HVS may be an important mechanism for virus persistence or dissemination. The EBV protein LMP-2A also associates with NRPTKs. EBV is associated with lymphoproliferative diseases and tumours in man infecting B cells, persisting in a latent state and transforming B cells to continuous growth (Fields *et al* 1996; Sinclair and Farrell, 1995). During latency a limited number of genes are expressed including latent membrane protein 2A (LMP-2A) (Farrell, 1995). This protein is one of the transforming factors of EBV and is constitutively phosphorylated (Miller *et al*, 1995). LMP-2A has been shown to bind the B cell-specific kinases, *lyn* and *syk* that mediate signals through the B cell receptor (Burkhardt *et al*, 1992; Longnecker and Miller 1996). This binding is mediated through the presence of kinase binding domains on LMP-2A (Burkhardt *et al*, 1992; Miller *et al*, 1995). The interaction of LMP2A with *lyn* and *syk* downregulates the activity of *lyn* and *syk* following B cell

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receptor cross-linking. This interaction blocks the signals from the B cell receptor and inhibits virus reactivation from latency (Miller *et al*, 1994). Therefore, LMP2A is thought to play a role in allowing persistence of the virus in B cells by establishing and maintaining latency.

The importance of NRPTKs to virus life cycles or pathogenesis is demonstrated by the number of other viruses known to bind cellular kinases. For example viral interaction with NRPTKs is demonstrated by the HIV protein nef - a protein required for HIV replication and pathogenesis *in vivo* (Jamieson *et al*, 1994; Kestler *et al*, 1991). The interaction of nef with NRPKs including *lck* leads to altered T cell activation pathways and enhanced infectivity (Collette *et al*, 1996; Du *et al*, 1995). The polyoma middle T antigen (mT) and HTLV-1 Tax (p40) also induce activation of NRPTKs which is involved in the transformation of T cells (Courtneidge, 1985; Courteidge *et al*, 1991). This demonstrates that modulation of NRPTKs has been used by several viruses to maintain virus survival in the host cell.

The functional consequence of *lck, fyn* and MAPK activation in LGL cell lines is not known. The viral proteins of HVS and EBV illustrated above appear to be involved primarily in maintenance of latency and cellular transformation. However, MCF LGL cells exhibit infectivity in rabbits (thus are not wholly latent) and are not associated with neoplasia *in vivo* (thus not transformed). Therefore, direct homologies between virus/ host interactions in HVS/EBV-infected cells and MCF virus-infected cells may not be present. However, viruses need not share homologous proteins to share similar mechanisms of pathogenesis. A direct comparison between the epidemiology and the lymphoproliferative disease caused by herpesvirus saimiri and the MCF viruses has shown many similarities (Chapter 1). Therefore, it is possible that HVS and the MCF viruses share similar pathogenic mechanisms. In the case of EBV there seems little if any similarity between EBV and MCF virus infection, at first glance. It appears that while all the viruses involved are gammaherpesviruses, the disease caused by MCF is acute and usually lethal while EBV infection of humans is chronic and largely asymptomatic with occasional disease. However, a closer inspection suggests that the comparison made above is of viruses at different stages of infection. A closer comparison can be made between MCF and primary EBV infection that results in infectious mononucleosis (IM)

(Rickinson and Kieff, 1996). Primary lytic infection of epithelial cells in the throat is followed by viraemia and latent infection of B cells. The immunopathological features of IM include increased numbers of activated T cells and virus-specific antibodies in the blood, infiltration of internal organs by these T cells and transformation of infected B cells. EBV-transformed B cells have been shown to produce cytokines that activate T cells *in vitro* (Wallace *et al*, 1982). It is thought that T cell proliferation associated with IM is the direct consequence increased cytokine production by EBV-infected B cells (Foss *et al*, 1994). The mechanisms underlying these processes may also explain the proliferation and infiltration of non-lymphoid organs by T cells observed in MCF. The constitutive activation of *lck* and *fyn* and possibly other virus interactions with NRPTKs could facilitate the dysregulation of the cytokine network and lead to activation and recruitment of bystander cells.

Given the results of the present study and given that the positional homologue of AlHV-1 ORF A1 in HVS is STP/Tip, functional analysis of ORFA1 is currently being performed. While kinase-binding motifs have not been identified in AlHV-1 genes, interaction of virus proteins with cellular (possibly signalling) proteins is being investigated.

The role of proteins encoded by ORF50, A6 and A7; putative virulence proteins in AlHV-1 life-cycle and pathogenesis

Viral proteins that might be involved in the generation of the LGL phenotype came from restriction enzyme analyses of the virulent and attenuated forms of AlHV-1 C500 (Handley *et al*, 1995; H. Wright, personal communication) where virulence was defined as the ability to cause disease. This work showed the presence of a region of the AlHV-1 C500 genome that was rearranged after attenuation of the virus (following extensive passage in culture). This rearranged region contained three genes encoded by ORF50, A6 and A7. Therefore, the affect of the rearrangements on these genes was associated with attenuation of AlHV-1 C500 or loss of disease induction.

More recent investigation of the rearrangements in the AlHV-1 C500 genome following attenuation (H. Wright personal communication) showed that ORF50, A6

and A7 are found in the middle (~72-75kbp) of the virulent C500 genome. On attenuation a part of this region of the genome was translocated to the right hand terminus, inverted with respect to direction of transcription and inserted between the terminal repeats and the end of the unique DNA (the coding region). The translocated region at the right-hand end of the attenuated C500 virus contains all but the promoter sequences of ORF 50 (which was located next to the terminal repeats) and a truncated 5' portion of ORF A6 (which was found next to a truncated ORF A10). ORF A7 remained in its original position in the middle of the genome (Fig 6.1). These rearrangements are therefore associated with attenuation of the virus. The effect of the translocation on the expression of the proteins encoded by these ORFs was not known. Therefore, whether or not the proteins encoded by ORF50, A6 and A7 were virulence proteins were investigated (Chapter 6). From the rearrangements described and assuming no point mutations (or other changes) affect these genes, the expected transcription pattern would show the loss of ORF50 protein, the loss of ORFA6 protein and presence of ORFA7 protein in the attenuated AlHV-1 C500 virus.

The results of this study showed that both ORF50 and ORFA6 protein expression is present in BT cells infected with virulent and attenuated AlHV-1 while ORFA7 protein expression was not detected in BT cells infected with either virus. PCR detection of the DNA encoding ORF50, A6 and A7 showed that none of these genes appeared to be truncated after attenuation of the virus as is suggested by the rearrangements that occur at the right-hand end of the genome. These results may be interpreted in different ways. Firstly, the rearrangements described at the right-hand end of the genome may not be the only ones to occur on attenuation of the virus. In the original assessment of the attenuated C500 genome, the translocated segment found on the right-hand terminal position was repeated at the opposite end of the unique DNA (Handley *et al*, 1995). Thus, it is possible that the complete ORFA6 sequence and a transcriptionally active ORF50 are present at the left-hand terminal region. This would explain the presence of the proteins encoded by ORF50 and A6 in both virulent and attenuated C500. Sequence analysis of the left-terminal region or the middle of the attenuated virus genome has not been determined. The significance of the rearrangements of the AlHV-1 genome is still unclear and a more

extensive evaluation of the translocations that occur on attenuation must be performed.

The presence of ORF50 and A6 proteins in both virulent and attenuated virus-infected BT cells would suggest that these proteins are not directly or solely involved in the induction of disease. The absence of ORFA7 protein in the presence of its DNA might suggest that this gene is not transcribed in either the virulent or the attenuated virus. However, the presence of mRNA for ORF50, A6 and A7 in C500-infected BT cells could not be determined due to the presence of excess viral DNA in RT-PCR experiments, despite the use of DNase (Chapter 6). If ORFA7 transcription is assumed in the virulent virus, the hypothetical translocation in the attenuated virus of ORF50 and ORFA6 to the left end of the genome may also disrupt the upstream regulatory sequences controlling ORFA7 transcription. The loss of ORFA7 following attenuation of AlHV-1 C500 could explain the loss of disease induction if information regarding its positional homologue in EBV extends to functional homology. ORFA7 has a positional homologue in EBV (i.e. BZLF2, which has 17.1% sequence identity to ORFA7). In EBV, BZLF2 encodes a glycoprotein (gp42) (Li et al, 1995) that associates with the EBV surface glycoproteins gH and gI and interacts with MHC class II (Li et al, 1997; Spriggs et al, 1996). Interference with gp42 binding using antibodies or by deletion mutation blocks B cell transformation and virus entry into the cell (Li et al, 1997; Miller and Hutt-Fletcher 1988; Wang and Hutt-Fletcher 1998). Furthermore, release of virions (and therefore lytic replication) was not disrupted in the gp42 deletion mutant. Therefore, the loss of transcription of ORFA7 could explain the loss of disease induction and the presence of virus replication in BT cells infected with attenuated virus.

Alternatively, the presence of ORF50 and A6 at protein level could suggest the presence of a mixture of virulent and attenuated virus in the high pass C500 cultures used in this study. Indeed, in Handley's work the *Hind*III clone vir1 thought to originate from the virulent C500 (Handley et al, 1995) was subsequently found to be a sequence from the attenuated virus (H. Wright, personal communication). Therefore, it is likely that the presence of even a small number of virulent genomes in high pass attenuated C500 virus cultures could account for the detection of ORF50 and A6 protein in high pass C500-infected BT cells. The attenuation of high pass

C500 may not be compromised by this hypothesis. As attenuation refers to loss of induction of disease, it is possible that the titre of virulent virus in high pass C500 is too low for disease induction.

In LGL cell lines, the replicative state of the MCF viruses is unknown. The results of this study showed the expression of ORF50, A6 and A7 mRNA in LGLs and support the hypothesis that in LGLs there is at least a low level of virus replication. The low and variable levels of mRNA encoding ORF50, A6 and A7 in different LGL cell lines may indicate that not all cells in a cell line express these genes. However, the presence of ORF50, A6 and A7 mRNA in LGLs does not conclusively demonstrate the presence of a replicative virus as the whole set of lytic genes need to be expressed for viral replication to occur. In the MCF viruses, lytic genes have not been determined. In order to prove the presence of a replicative virus in LGL cell lines a more extensive search for virions (e.g. by electron microscopy) would need to be performed. Electron microscopy of OvHV-2-infected LGL cell lines performed subsequent to this study has revealed the presence of herpesvirus virions (H. Reid, unpublished observations) and supports the hypothesis that LGL cell lines contain replicative virus.

The sequence homology of ORF50 and positional homology of ORFA6 to immediate early proteins in EBV and HVS support the hypothesis that LGL cell lines could contain replicating virus. ORF50 is the positional equivalent of EBV BRLF1 (an R transactivator) and HVS ORF50 (Ensser et al, 1997). EBV BRLF1 is expressed in the immediate-early (I.E.) phase of the viral lytic replication cycle that is followed by the delayed-early (D.E.) and late (L.) phase culminating in the formation and release of mature virions (Honess and Roizman, 1974). The EBV R transactivator is required for the reactivation of the lytic cycle in latently infected B cells (Miller, 1990). ORF50 of HVS has also been demonstrated to act as a transactivator expressed during the immediate early and delayed early phases of lytic replication (Whitehouse et al, 1998). Recent investigations have revealed that ORF50 of AlHV-1 has similar functions to ORF50 of HVS, as a transactivator of ORF57, 6 and A9 (Frame and Dalziel, 1999). This work provides further evidence for the presence of replicative virus in LGL cell lines. The positional homologue of ORFA6 in EBV is BZLF1 (which has 19.5% sequence identity to the ORFA6

sequence). This is another gene transactivator and co-operates with the BRLF1 gene product in the switch from the latent to lytic cycle in B cells (Miller, 1990). However, the function of ORFA6 protein has not been determined. The positional homology of ORFA7 to EBV BZLF2, which encodes a glycoprotein with an role in virus entry (Li et al, 1997; Miller and Hutt-Fletcher, 1988) also supports the hypothesis that AlHV-1 in LGLs is capable of replication. The role of these AlHV-1 proteins in virus replication or disease induction needs to be assessed (e.g. by the generation of virus gene deletion mutants). Work is currently in progress to generate a ORF50 deletion mutant.

The translocation of the region of AlHV-1 C500 in the middle of the genome to the right-hand terminal position may affect the transcription on attenuation of the gene adjacent to the translocated region (i.e. the gene encoded in ORFA10). Similarly, the loss of genes from the middle of the genome may affect transcription of genes adjacent to ORF50 and A7 (i.e. the genes encoded in ORF48 and ORFA8). Therefore, the effect of attenuation on the transcription of ORF48, A8 and A10 should be investigated. ORF A8 is particularly interesting, as it is a positional homologue of EBV BZLF3 (gp350/220), MHV-68 M7 (gp150) and HVS and EHV-2 ORF51 (Ensser et al, 1997; Stewart et al, 1996; Telford et al, 1995). The glycoproteins gp350/220 mediate attachment of the virus to B cells via CD21 (CR2) (Fingeroth et al, 1984) and plays a role in entry of virus into cells as antiserum directed to gp350/220 inhibits EBV infection (Tanner et al, 1987). The protein is therefore considered a virulence factor for EBV. The ORFs at the left terminal location of AlHV-1 (i.e. A1 and A2) may be affected if rearrangements also occur at this end of the attenuated genome. AlHV-1 ORF A1 and A2 have no sequence homology to other herpesvirus genes. However, they share positional homology with the HVS gene encoding Tip and STP - the two transforming factors (Duboise et al, 1998a; Lee et al, 1997; Wiese et al, 1996).

The number and identification of genes expressed in infected LGL cells are not known. In sheep, where OvHV-2 latently infects B cells, there is no apparent disease. In disease-susceptible animals, rare cases of recovery from MCF have been reported. In such cases, the virus also appears to become latent. Therefore, it is possible that a protective immune response is present in such animals during the

initial acute phase of the disease. The appearance of disease in susceptible animals would indicate that viral gene expression in LGLs does not raise a protective immune response. In addition, the infection by the MCF viruses of antiviral effector cells may lead to the immune dysfunction and pathology associated with MCF. Unfortunately, studies of latent gene expression in carrier animals cannot be performed due to lack of information on OvHV-2 genes and lack of latently infected cell lines from AlHV-1-infected wildebeest. However, virus gene expression in LGLs is currently being investigated that will be helpful in determining the pathogenesis of MCF.

The work presented here has improved our understanding of LGL dysfunction in MCF and identified possible means by which this dysfunction may occur. Insights into MCF pathology have consequently been gained. The results presented in this study form the basis for further work. For instance, AlHV-1 proteins will be expressed from a number of putative virulence genes to look for binding to cellular signalling or other molecules. The putative anti-apoptotic function of ORF A9, which encodes an AlHV-1 homologue of bcl-2, is currently being investigated. An ORF50 deletion mutant virus is being generated to determine the function of the protein encoded by this gene. The number of viral genes expressed in LGL cells will also be investigated by mRNA analysis. The work on IL-15 is currently being completed. Thus, the work presented in this thesis has prompted continuing and new avenues of investigation for future research in MCF.

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Appendix

Constitutive activation of Lck and Fyn tyrosine kinases in large granular lymphocytes infected with the γ-herpesvirus agents of malignant catarrhal fever

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SUMMARY

Large granular lymphocytes (LGL) with a T or natural killer (NK) lymphoblast morphology and indiscriminate (non-major histocompatibility complex-linked) cytotoxicity for a variety of target cells can be derived in culture from the tissues of animals infected with either alcelaphine herpesvirus-1 (AlHV-1) or ovine herpesvirus-2 (OvHV-2). In this study, LGL survival in the absence of exogenous interleukin-2 was inhibited by the protein kinase inhibitor genestein, but not the p70 s6 kinase inhibitor rapamycin. Constitutive activation of the src kinases Lck and Fyn was demonstrated in a bovine LGL line infected with OvHV-2 and in two rabbit LGL lines infected with AlHV-1. The p44 erk1 and p42 erk2 mitogen-activated protein kinases (MAPK) were also constitutively activated in the LGLs but not control T cells. Lck and Fyn kinase activity in the LGLs did not increase after mitogen (concanavalin A or concanavalin A plus phorbol ester) stimulation of the cells, in contrast to control T cells. Control T cells, but not the LGLs, proliferated after mitogen stimulation. An analysis of tyrosine phosphorylated proteins in the cells indicated that the LGLs exhibited some similarities and differences to activated control T cells. The results demonstrate that the activated phenotype of the LGLs, associated with malignant catarrhal fever virus infection and in the absence of exogenous interleukin-2, involves constitutively activated Lck and Fyn kinases. These are normally crucial for the initial activation of T cells via several cell-surface receptors (e.g. the T-cell receptor and CD2). The inability of the LGLs to proliferate in response to mitogen may be due to an inability of Lck and Fyn to become further activated after mitogen stimulation.

INTRODUCTION

Malignant catarrhal fever (MCF) is a fatal lymphoproliferative disease of farmed and wild ungulates.[1–3] The known causative agents are two γ-herpesviruses, alcelaphine herpesvirus-1 (AlHV-1) and ovine herpesvirus-2 (OvHV-2). AlHV-1 infects and replicates in blue wildebeest (*Connochaetes taurinus*), causing no apparent disease. In contrast, when MCF-susceptible species become infected they develop MCF, which is usually fatal. OvHV-2 infects and replicates in sheep, which do not become diseased. However, a proportion of cattle, deer, or other susceptible species that are in the vicinity of infected sheep can become infected and develop MCF which again is normally fatal. Rabbits develop MCF after experimental infection with AlHV-1 or OvHV-2 that is characteristic of the disease in ungulates.[1,3,4]

MCF is characterized by degenerative changes in multiple tissues, associated with the infiltration of large numbers of lymphocytes and hyperplasia of lymphoid organs. In OvHV-2-infected cattle or rabbits, submandibular and mesenteric (MLN) lymph nodes, appendix and spleen become enlarged. This is followed by invasion of non-lymphoid tissue by lymphocytes and tissue necrosis in both lymphoid and non-lymphoid tissues.[1,3,4] In AlHV-1-infected animals, the pathology is generally similar except that popliteal lymph nodes are larger and MLN are smaller than in OvHV-2-induced disease. The current hypothesis is that MCF is caused by the auto-destruction of tissues by indiscriminately cytotoxic lymphocytes, produced as a consequence of MCF virus infection.

Large granular lymphocytes (LGL) containing viral transcripts can be derived from MCF virus-infected animal lymph node, spleen, corneal and other affected tissues and grown in culture.[1–4] The LGL can transmit MCF when injected into rabbits or other susceptible species. The LGL are normally grown in medium containing interleukin-2 (IL-2), but can also be grown in the absence of IL-2 and other exogenously added cytokines. The phenotype of the cultured cells from OvHV-2-infected cattle is generally characteristic of T cells or natural

killer (NK) cells. CD4$^+$ and CD8$^+$ T cells as well as CD4$^-$ CD8$^-$ T cells have been grown in culture.[3,5,6]

Characteristics of OvHV-2 virus-infected bovine T cells in culture are: large granular lymphocyte morphology; the ability to survive or grow in culture in the absence of exogenous IL-2 (or other cytokines) for longer periods than non-infected cells; constitutive non-major histocompatibility complex (MHC) -associated cytotoxicity; T-cell or NK-cell surface alloantigen phenotype; lack of IL-2 mRNA and protein expression; and lack of mitogen-stimulated proliferation in culture.[1,3,6,7] Rabbit AlHV-1-infected LGLs have some of the above properties but are not as well characterized.[1,3] The viruses of MCF have several features in common with other T-cell-tropic γ-herpesviruses, particularly herpesvirus saimiri (HVS). HVS naturally infects squirrel monkeys without disease but causes a lymphoproliferative disease associated with lymphomas and leukaemias of T-cell origin following infection of disease-susceptible primates.[8] HVS will infect human T cells in culture that can be cytotoxic and grow in the absence of exogenous IL-2.[9]

A common feature of HVS and Epstein–Barr virus (EBV) γ-herpesviruses is the presence of viral proteins that interfere with lymphocyte signal transduction molecules, particularly the non-receptor protein tyrosine kinases (NRPTK)[10] . In EBV, latent membrane protein-2A (LMP-2A) binds to and inhibits the activity of the B cell NRPTKs Lyn (a *src* kinase) and Syk[11] HVS-transformed monkey and human T cells contain stp and tip virus proteins.[9,12,13] The tip protein is a membrane protein that binds to and alters the function of the NRPTK Lck (a *src* kinase).

By targeting NRPTKs, many viruses have evolved an effective mechanism for controlling the virus life cycle and pathogenicity by altering the signalling pathways of infected cells.[10] The T-cell-associated *src* kinases Lck and Fyn are NRPTKs that are recruited to the T-cell receptor complex and other receptor structures (e.g. CD4, CD8 and CD2) within minutes of T-cell stimulation via these receptors. They link with a range of downstream signalling pathways including the ras/ raf-1/erk MAPK pathway to control T-cell activation, proliferation and differentiation (reviewed in refs 14 and 15). Lck and Fyn are therefore candidate molecules for interference by the MCF viruses AlHV-1 and OvHV-2.

The genome sequence of AlHV-1 has recently been elucidated.[16] However, an amino acid homology search of the virus open reading frames (ORFs) for putative binding factors for host signalling molecules has been unsuccessful.

The objective of this study was to determine whether the IL-2-independent growth of LGLs from MCF-affected animals was associated with protein kinase modulation in the LGLs compared to lymphocytes from uninfected control animals. In particular, we wished to compare the activation status of the *src* kinases Lck and Fyn in AlHV-1- and OvHV-2-infected LGLs, compared to uninfected T cells, grown in culture in the absence of exogenous IL-2.

MATERIALS AND METHODS

Viruses and cells

The fully virulent C500 strain of AlHV-1 was maintained by passage in rabbits.[1,3,5] The AlHV-1-infected LGL cell lines 1859 and 1860 were generated by injecting C500-infected rabbit

Table 1. Phenotype of the MCF virus-infected cell lines

Cells	Virus	Surface phenotype (%)*	BLT-esterase (%)†
1859	AlHV-1	CD5$^+$(50%), CD4$^-$, CD8$^+$(50%), IgM$^-$	>80
1860	AlHV-1	CD5$^-$, CD4$^-$, CD8$^+$ (45%), IgM$^-$	>75
BJ1035	OvHV-2	CD2$^+$, CD4$^-$, CD8$^-$, CD21$^-$	>90

*CD$^+$ cells were >98% positive unless shown otherwise in parentheses.
†BLT-esterase$^+$ cells detected by cytochemistry (result shown based on two separate analyses). Control T cells (rabbit and bovine) were <5% BLT-esterase$^+$, except for one rabbit CD8$^+$ (95% positive) T-cell line that was 22% BLT-esterase$^+$.

lymphoid cells intravenously into two rabbits. Two days after a rise in rectal temperature above 40°, the rabbits were killed. MLN and spleen cells were harvested, single cell suspensions were prepared and cultured at $5 \times 10^6 - 10 \times 10^6$ cells/ml in Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal calf serum (FCS) and 200 U/ml IL-2 (Eurocetus, Amsterdam, the Netherlands) in 25-cm^2 flasks. Large granular lymphocytes developed after 1–3 weeks of splitting and refeeding the cells with IL-2 on an approximately weekly basis. After 8 weeks the phenotype of the cells had stabilized (Table 1). Virus genes and proteins were detected in the LGL using polymerase chain reaction (PCR) for the AlHV-1 P1 gene (ORF 50, an early viral R-transactivator gene[16]) and indirect immunofluorescence using serum antibody to AlHV-1 proteins from an infected rabbit, using techniques described previously.[17,18]

For the P1 DNA PCR, a guanidinium isothiocyanate extract of cells was analysed using the following primers: 5′ TTCTATTGTCCACTGCCTCATCTC; and 3′ TGACTG-TGGTTCACTTGCTTCCAG. PCR amplification was performed using the Expand^R long template PCR (Boehringer Mannheim, Mannheim, Germany). Amplified product (503 bp) was analysed on agarose gels stained with ethidium bromide and visualized under UV light.

For IL-2-independent growth, 1859 and 1860 LGL were washed in medium and cultured in 24-well tissue culture plates at 10^6/ml. For this study, the rabbit LGL were taken from culture between 48 and 96 hr after deprivation of exogenous IL-2, when cell viability was >70%.

Control rabbit or bovine cells were peripheral blood mononuclear cells (PBMC) derived from blood after centrifugation over lymphoprep (Nyegard, Oslo, Norway). T-cell blasts were also used. These were derived from 2×10^6/ml PBMC stimulated with 5 µg/ml concanavalin A (Con A) for 3 days. Then, 2×10^5 lymphoblasts/ml medium were expanded for 1–4 weeks in 200 U/ml IL-2. The cells were split and refed with the IL-2 and fresh medium every 3–5 days. The control cells were negative for AlHV-1 or OvHV-2 viruses, using the tests described above and below. For IL-2-independent growth, 10^6 rabbit or bovine T-cell blasts were cultured in IMDM. The cells did not survive at >70% viability at 10^6/ml (or less) for more than 30 hr and during this time the majority of viable lymphoblasts reverted to a 'resting' medium/large lymphocyte morphology.

The BJ1035 LGL cell line was derived from a cow naturally infected with OvHV-2 in 1994.[6] The T cells were routinely

passaged in IMDM containing 200 U/ml IL-2. BJ1035 was also maintained for 12 weeks in IMDM without exogenous IL-2. The cells were replenished with medium and readjusted to 10^6/ml approximately every week. The cells exhibited a doubling time of 7–8 days under these conditions. The presence of OvHV-2 DNA in the cells was detected by PCR as described previously.[19] OvHV-2 virus proteins were detected within the cells using antibodies from the serum of a convalescent cow and an indirect immunofluorescence technique.

Antibodies

Murine monoclonal antibodies (mAb): anti-rabbit CD4 (clone KEN-4); anti-rabbit CD8α (clone 12C6); anti-rabbit CD5 (clone KEN-5); anti-rabbit immunoglobulin M (IgM; clone NRBM) were purchased from Serotec (Slough, UK). The mAbs to bovine antigens: anti-bovine CD4 (clone CC8); anti-bovine CD8 (clone CC63); anti-bovine CD2 (clone CC42); anti-bovine CD21 (clone CC21) were gifts from the Institute for Animal Health, Compton, Berkshire, UK. The murine anti-phosphotyrosine ·mAb (clone 4G10) was obtained from Upstate Biotechnology (Lake Placid, NY). The murine anti-Lck (clone 3A5), anti-Fyn (clone 15), and anti-phosphorylated (activated) erk1 (clone sc-7383) mAbs and the rabbit anti-erk1 antibody (sc-94) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Isotype-matched control murine mAbs to Border Disease virus antigens were a gift from G. Entrican (Moredun Research Institute). The AlHV-1 and OvHV-2 antibodies were obtained from the serum of an AlHV-1-infected rabbit and a cow that survived OvHV-2 infection, respectively. The antibodies recognize uncharacterized virus antigen(s) located in the nuclei and perinuclear areas of infected cells. They do not react with uninfected cells.

Cell phenotype analysis

Cell-surface antigens were detected by indirect immunofluorescence and fluorescence-activated cell scan (FACscan) analysis (Becton Dickinson, Mountain View, CA) using standard procedures.

Cytotoxic cell BLT-esterase activity was detected in the LGL and control cells using the tryptase-specific substrate *N*-α-benzyloxy-carbonyl-L-lysine thiobenzyl ester (BLT, Sigma, Poole, UK) as described previously.[20] BLT-esterase is expressed by activated cytotoxic cells.[20,21]

Treatment of LGL cells by genestein and rapamycin

Virus-infected LGLs in IL-2-deficient IMDM were added to wells of a 96-well tissue culture plate at 10^5 cells/well (100 μl/well). The protein kinase inhibitor genestein (TCS, Botolph Claydon, Buckingham, UK) was added to the wells to give final concentrations of 1 μM, 10 μM and 100 μM. The 1035 LGL line was also treated with the p70 s6 kinase inhibitor rapamycin (TCS, UK) at 0·1 nM, 1 nM, 10 nM and 100 nM. Cell growth was measured as the numbers of viable cells (determined by nigrosine exclusion) per ml on day 3 of culture.

Cell activation and lysate production

Antibodies that cross-link the T-cell receptor and activate rabbit and bovine T cells are not available. Instead, the T-cell-stimulating mitogen Con A (ICN, Thame, UK) with and without the phorbol ester 12-O-Tetradecanoylphorbol 13-acetate (TPA; Sigma) was used in this study. For T-cell

proliferation, different concentrations of Con A (±TPA) were used to stimulate 100 μl aliquots of 2×10^6 cells/ml IMDM (2·5% FCS) in the wells of 96-well plates. After 2 days of culture, the cells were incubated with 50 μl [³H]thymidine (0·5 μCi/well) for a further 18 hr. Cells were harvested onto membranes and [³H]thymidine incorporation measured in a β-scintillation counter (Tri-carb 2500 TR, Packard, Meriden, CT).

For cell stimulation, 10 μg/ml Con A was added (±5 ng/ml TPA) to 5×10^6 or 10^7 PBMC or IL-2-deprived T cells or LGL in 1 ml serum-free medium at 37° in microfuge tubes for various periods of time. The doses of Con A and TPA used were determined by dose–response kinase-activation (phosphotransferase) experiments. Activation reactions were stopped by centrifugation of the cells at 10 000 *g* for 10 seconds in a microfuge at 4°. Supernatants were discarded and cells were lysed with ice-cold lysis buffer (1% Triton-X, 150 mM NaCl, 1 mM sodium vanadate, 1 mM sodium fluoride, 10 mM Tris–HCl pH 7·5) containing protease inhibitors (Complete®, Boehringer Mannheim). Then, 50 μl of lysis buffer was added to cells for subsequent analysis of tyrosine-phosphorylated proteins using the 4G10 antibody and Western blot. Prior to immuno-precipitation, 1 ml of lysis buffer was added to cells. Clarified lysates were obtained by centrifugation in a microfuge at 10 000 *g* for 20 min at 4°. Protein concentrations in the 50 μl lysates were within the 10–14 mg/ml range and in the 1 ml lysates were within the 1–2 mg/ml range as determined by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). For sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), an equal volume of cell lysate from each cell preparation was added to the wells of the gels, the amount being expressed as cell-equivalents (i.e. the number of cells lysed to make the lysate).

Immunoprecipitation and Western blot analyses

Lysates were precleared with murine or rabbit IgG-agarose for 1 hr at 4° followed by centrifugation and removal of the agarose beads. Primary Lck, Fyn, or control mAb (final concentration 1 μg/ml) was added to lysates for 1–2 hr at 4°; 20 μl of protein-A/G-agarose (Santa Cruz) was added and lysates were incubated for 1 hr at 4°. Agarose immunoprecipitates were centrifuged and washed three times in lysis buffer. A 5-μl sample (of 25 μl total volume) was taken at this stage for Western blot analysis of immunoprecipitated Lck or Fyn kinases. For the phosphotransferase assay, the agarose complexes containing Lck and Fyn were washed twice more with kinase buffer (20 mM Tris–HCl pH 7·5, 10 mM MgCl₂, 5 mM MnCl₂).

For Western blot analysis, samples of lysate were boiled in reducing sample buffer (containing 2-mercaptoethenol) and proteins were separated by SDS–PAGE using 10% or 12% Proseive-50 gels (FMC, Rockland, WA) and a Mini-Protean II apparatus (Biorad, Hemel Hampstead, UK). Proteins were transferred to nitrocellulose membranes using a semi-dry transfer apparatus (Sigma). Membranes were blocked in 5% non-fat milk (Marvel) for 1 hr, and dilutions of primary antibodies were added in wash buffer (0·5% Tween-80 in phosphate-buffered saline) for 1 hr (or overnight at 4°). Membranes were washed (2 × 10 min) in wash buffer and the horseradish peroxidase-conjugated goat anti-mouse (or anti-rabbit) IgG second-stage antibody was added for 1–2 hr at

room temperature. After a final wash, proteins were detected using the enhanced chemiluminescence (ECL) method (Amersham, Little Chalfont, UK). Control antibodies did not immunoprecipitate antigen from cell lysates.

In vitro phosphotransferase assay

The activation of immunoprecipitated Fyn and Lck kinases was assayed by kinase-mediated *in vitro* phosphotransferase of [^{32}γ]-ATP (Amersham) to the *src* kinase substrate acid-treated rabbit muscle enolase (Sigma). Twenty-five microlitres of kinase buffer containing 25 μM ATP (Sigma), 400 μg/ml acid-treated enolase, and 320 μCi/ml [^{32}γ]-ATP was added to the agarose immunocomplexes in microfuge tubes. The samples were incubated at 30° for 10 min in a water bath. Then, 6 μl of fourfold concentrated electrophoresis sample buffer (500 mM Tris–HCl pH 6·8, 8% SDS, 20% glycerol, 0·012% bromophenol blue, 8% β-mercaptoethanol) was added to the samples which were incubated for a further 20 min at room temperature. The samples were boiled for 5 min and fractions were separated by SDS–PAGE using 10% or 12% Proseive gels. The gels were fixed and stained with Coomassie blue (0·25% Coomassie blue in 45% methanol, 10% acetic acid), destained (40% methanol, 10% acetic acid), dried and exposed to X-ray film. Kinase activity was detected as a band of [^{32}γ]-ATP-phosphorylated enolase (molecular weight 48 000). In order to quantify the phosphorylation of enolase, the 48 000 MW enolase bands were excised from the gels and radioactivity was measured in a β-scintillation counter. Incorporation of [^{32}γ]-ATP into enolase was calculated for each sample as follows: [counts per minute (c.p.m.) enolase in the presence of kinase]−(c.p.m. enolase in control samples without immunoprecipitated kinase).

Mitogen-activated protein kinases (MAPK) activation analysis

Activated p42 and p44 MAPK is phosphorylated on tyrosine and threonine residues at positions 185 and 183, respectively, and was detected in cell lysates by Western blot using the p42/44 MAPK-phosphotyrosine-specific mAb sc-7383. Non-phosphorylated MAPK was detected using the erk1-specific antibody sc-94 that cross-reacts with erk2.

RESULTS

Phenotype of MCF virus-infected cell lines

The LGL cell lines consisted of >96% LGL, of which >80% stained positive for either AlHV-1 (1859 and 1860) or OvHV-2

(1035) proteins by immunofluorescence. Pl mRNA was detected by reverse transcription (RT) -PCR in the rabbit AlHV-1$^+$ LGL but not in control rabbit cells. OvHV-2 DNA was detected by PCR in the bovine 1035 LGL line, but not in control uninfected bovine T cells.

In the absence of exogenous IL-2 for at least 4 days in the cultures, the LGL survived or grew and maintained a lymphoblastoid appearance and constitutive expression of BLT-esterase, an activated cytotoxic cell tryptase (Table 1).

Table 1 shows the cell-surface phenotype of the MCF virus-infected LGL cell lines. The phenotype of approximately half of the cells of the AHV-1$^+$ rabbit LGL could not be typed with the available antibodies. The LGL did not contain B cells (IgM$^-$ or CD21$^-$).

Control rabbit and bovine T cells ($n=12$) derived from Con-A-stimulated blood mononuclear cells (MNC) were: CD5$^+$ (>90%) or CD2$^+$ (>90%) IgM$^-$ (<5%) or CD21$^-$ (<5%), CD4$^+$ (range 8–90%) and/or CD8$^+$ (range 4–95%).

Mitogen-stimulated MCF virus-infected LGL do not proliferate in culture

Table 2 shows that Con A or Con A plus TPA stimulated a proliferation signal ([^{3}H]thymidine incorporation into dividing cells) in control rabbit or bovine T-cell blasts that had been deprived of exogenous IL-2 for approximately 30 hr. However, the AlHV-1-infected rabbit LGL (1859 and 1860) and the OvHV-2-infected bovine LGL line (1035) that had been deprived of IL-2 for approximately 48 hr and 4 days, respectively, did not respond to Con A or Con A/TPA stimulation with any significant increase in [^{3}H]thymidine incorporation into the cells above that of unstimulated cells. [^{3}H]Thymidine incorporation into unstimulated LGL cells but not into control uninfected T cells showed that at least a proportion of the LGL (but not the control T cells) were dividing in the absence of exogenous IL-2. Con A and Con A plus TPA stimulated cell aggregate formation in both control T cells and the LGL. This, along with a minor Con A dose-dependent effect on [^{3}H]thymidine incorporation into LGL indicated that Con A was capable of stimulating a response in the LGL.

Tyrosine phosphorylated proteins in total cell lysates

Tyrosine phosphorylated proteins were detected in the cell lysates of control T cells deprived of IL-2 for 30 hr and MCF

Table 2. Mitogen-stimulated proliferation of LGL and control T cells

Stimulus	1859 LGL	1860 LGL	1035 LGL	Bovine T cells	Rabbit T cells
Medium control	2498±131	1292±22	1060±21	132±12	22±1·2
Con A, 10 μg/ml	1349±126	1648±108	1368±102	2734±98	1030±68
Con-A, 5 μg/ml	2467±106	1825±146	634±5	4820±246	4307±249
Con A, 2·5 μg/ml	2044±110	1342±98	856±41	3160±148	2312±168
Con A (5)+TPA*	2264±242	1924±110	1403±125	5204±124	1489±35
Con A (2·5)+TPA	2402±114	1727±232	893±171	5796±342	4704±137
Con A (1·25)+TPA	2233±102	1650±78	1240±39	4668±236	3220±49
TPA	1245±68	1376±116	1448±71	896±72	130±9

Data are counts per minute ± SEM from replicate samples in the [^{3}H]thymidine incorporation assay. * TPA used at 5 ng/ml alone or in combination with Con A used at the doses shown in parentheses (in μg/ml).
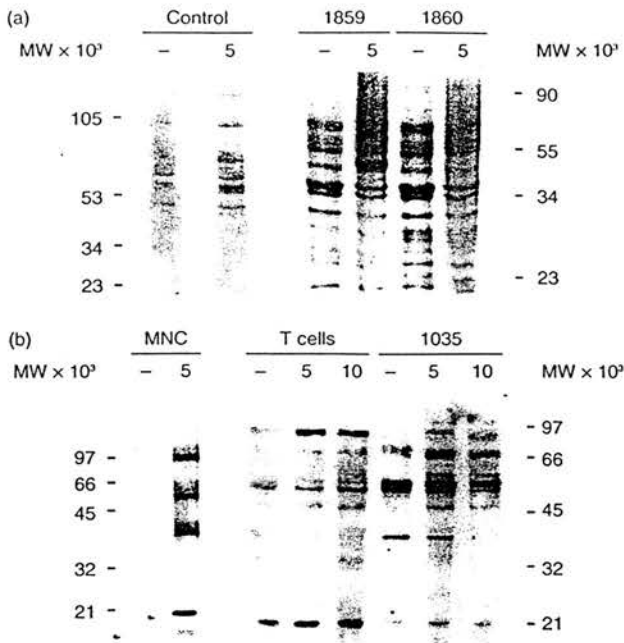
Figure 1. Phosphotyrosine proteins in LGL and control cell lysates. 4G10 anti-phosphotyrosine Western blot of lysates from unstimulated (−) cells or cells stimulated for 5 (5) or 10 (10) min with 10 μg/ml Con A and subjected to SDS–PAGE. (a) Control rabbit T cells and the AHIV-1$^+$ LGL 1859 and 1860. (b) Control bovine blood mononuclear cells, control bovine T cells and the OvHV-2$^+$ LGL 1035.

Table 3. Inhibition of LGL growth by genestein but not rapamycin

Inhibitor	1035 LGL (cells/ml) × 10^6	1859 LGL (cells/ml) × 10^6
Genestein		
Medium control	1·43 ± 0·23	1·13 ± 0·12
1 μM	1·33 ± 0·28	0·93 ± 0·10
10 μM	0·90 ± 0·20	0·50 ± 0·09*
100 μM	0·48 ± 0·12*	0·26 ± 0·10*
Rapamycin		
Medium control	1·30 ± 0·12	ND
0·1 nM	1·14 ± 0·14	ND
1 nM	1·23 ± 0·09	ND
10 nM	1·10 ± 0·22	ND
100 nM	1·20 ± 0·14	ND

Viable cell counts (mean ± SEM for triplicate wells) performed after 3 days of culture. All cells set up at 10^6/ml on day 0. ND, not determined. * $P < 0.01$ compared with medium control values.

Genestein inhibits the IL-2-independent growth of LGLs

Table 3 shows that exogenous IL-2-independent survival/ growth of the OvHV2$^+$ bovine LGL line 1035 and the AlHV-1$^+$ rabbit LGL line 1859 was inhibited in a dose-dependent way by the protein kinase inhibitor genestein. The 1035 LGL cell growth was not inhibited by rapamycin. The rapamycin experiment was not performed on the rabbit LGLs as they died during the course of these studies and could not be resurrected from frozen stock.

virus-infected LGL deprived of IL-2 for at least 48 hr, with and without Con A stimulation. This was to determine whether infection with virus was associated with any differences in tyrosine-phosphorylated protein expression between the LGL cell lines and control T cells. In particular proteins phosphorylated within 10 min of Con A stimulation were compared, as these are likely to reveal the NRPTKs.

The unstimulated rabbit AlHV-1-infected LGL cell lines 1859 and 1860 expressed phosphotyrosine proteins at MW of approximately 21 000, 30 000, 38 000, 42 000, 48 000, 56 000 and 59 000 (Fig. 1a) that were also seen in repeated 4G10 blots. Five minutes after Con A stimulation the intensity of some bands (e.g. p30, p38, p42) decreased while there was an increase in the intensity of a p50 band in the example shown (Fig. 1a), but not in repeated analyses of the same LGL. Control rabbit T cells expressed phosphoproteins with MW of approximately 38 000, 42 000, 55 000, 59 000, 70 000, 90 000 and 130 000 5 min after Con A stimulation (Fig. 1a).

Control stimulated bovine PBMNC or T-cell blasts and the unstimulated and stimulated bovine OvHV-2-infected cell line BJ1035 expressed phosphotyrosine proteins at approximately 21 000, 38 000, 48 000, 56 000, 59 000, 70 000 and 90 000 (Fig. 1b). Control, unstimulated T-cell blasts exhibited low level expression of phosphotyrosine proteins compared to stimulated cells. Unstimulated PBMC did not express any detectable phosphotyrosine proteins. BJ1035 cells, when compared to unstimulated cells after stimulation with Con A showed an increase in 48 000, 61 000, 70 000 and 90 000 protein staining with 4G10, whereas 56 000 and 59 000 protein expression was not visibly altered.

Fyn and Lck kinases are constitutively active in the MCF virus-infected cell lines

Figure 2 shows that Con A stimulated an increase in Fyn activity in control cells whereas in the LGLs, Fyn was activated in the absence of Con A and did not increase in activity after Con A stimulation. In control cells, the degree of activation was low or not detectable in 'rested' unstimulated cells, but was high in cells stimulated for 5 min with Con A. Fyn kinase activity had declined by 30 min compared to 5 min after stimulation (Fig. 2a,d). In contrast, the Fyn kinases in the 1859, 1860 and 1035 cells were approximately equally activated in both the unstimulated and Con-A-stimulated cells (Fig. 2a,d).

Figure 2(b,c) shows that for each of the cell types, there was an equal loading of enolase and an approximately equal loading of immunoprecipitated Fyn in the gels.

Figure 3 shows that Lck kinase activated and phosphorylated enolase when Lck was immunoprecipitated from: control rabbit blood mononuclear cells after Con A stimulation; control rabbit or bovine T cells particularly after Con A stimulation; AlHV-1-infected 1859 and 1860 LGLs and the OvHV-2-infected 1035 LGL cells either before or after Con A stimulation (Fig. 3a,d). In contrast to the control cells, the LGL exhibited Lck activation in the absence of Con A that did not increase after Con A stimulation of the cells (Fig. 3a,d). Figure 3(b,c) show that for each of the cell types, there was an equal loading of enolase (except for 1859 and 1860 cells in Fig. 3(b) where the quality of the stained gel was poor on two
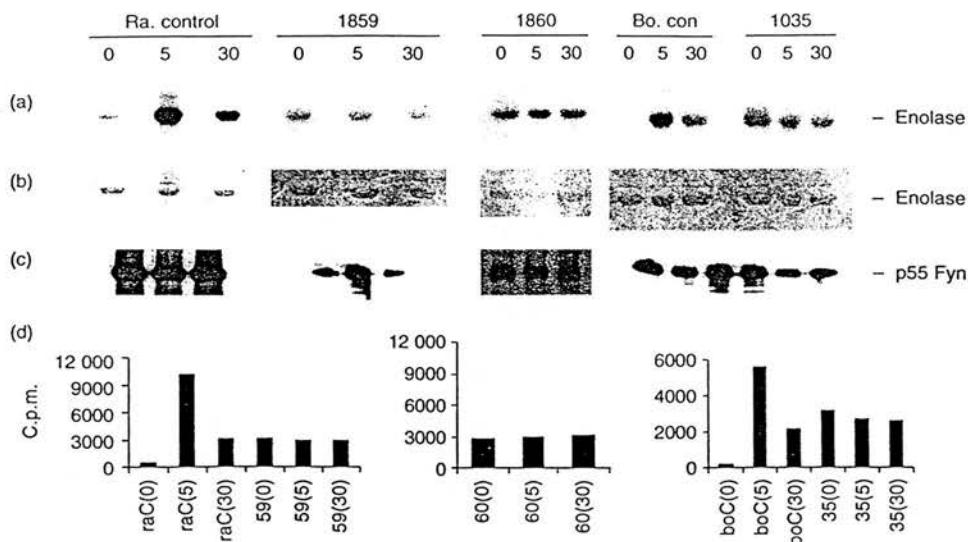
Figure 2. Fyn kinase activation in the LGL lines and control T cells. (a) Autoradiograph of SDS–PAGE gel showing enolase phosphorylation by Fyn kinase immuno-precipitated from LGL and control rabbit (ra) and bovine (bo) T cells either unstimulated (0) or stimulated with Con A (10 μg/ml) for 5 min (5) or 30 min (30). (b) Coomassie blue stain, control for protein loading in each lane (showing enolase band). (c) Western blot (anti-Fyn) control for immunoprecipitated Fyn loading. (d) Quantification of enolase phosphorylation by β-scintillation counts per minute (c.p.m.) of enolase bands cut out of the SDS–PAGE gels. C, control T cells; 59, 1859 LGL; 60, 1860 LGL; 35, 1035 LGL; unstimulated (0) or stimulated for 5 or 30 min with Con A. Lysates from 8×10^6 cell equivalents were loaded in each of the lanes of the gels. Note that a comparison of the intensity of Fyn-mediated enolase phosphorylation between control and LGL cells, and between LGL cell lines may not be possible as different exposure times were often used in the development of the autoradiographs. Furthermore, β-scintillation analysis of the enolase bands cut from the gels of the different cell types did not always take place at the same relative time following the gel run.

occasions and is not shown) and an approximately equal loading of immunoprecipitated Fyn in the gels.

In all experiments, enolase was not phosphorylated in control samples containing enolase and [^{32}γ]-ATP without the immunoprecipitated kinases, as detected after autoradiography of the SDS–PAGE gels [e.g. Fig 3(a) compared to Fig. 3(b)], or by β-scintillation assay of the excised enolase bands. The inclusion of TPA with ConA as a stimulus for the cells generated similar results (data not shown) to those shown using ConA only.

P42/44 MAPK are constitutively activated in the LGLs

To determine whether activated Lck or Fyn was associated with the activation of downstream signalling pathways, LGL and control cell lysates were analysed for p42/44 MAPK activation (phosphorylation). Figure 4(a,b) shows that the LGL cell lines constitutively expressed phosphorylated MAPK that did not increase in signal intensity after Con A stimulation of the cells for 10 min. The control Con-A-stimulated, IL-2-deprived rabbit and bovine T cells expressed phosphorylated MAPK, whereas the unstimulated cells expressed little or no detectable phosphorylated MAPK.

DISCUSSION

The infiltration of lymphoid and non-lymphoid tissues by large lymphoblastoid cells and tissue necrosis are characteristic features of MCF. The LGL isolated from the tissues of animals with MCF exhibit an activated phenotype in culture. They are

large lymphoblasts that can grow in the absence of exogenous cytokines and are indiscriminately cytotoxic, killing various target cells in a non-MHC-dependent manner.[1,6,7,22]

A mechanism for the constitutive activation of the LGL is provided by this study in which the *src* kinases Lck and Fyn were constitutively activated in the LGL in cultures lacking exogenous IL-2, to the extent that further activation by the mitogen Con A was not possible. The OvHV-2$^+$ bovine LGL line was the same in this respect as the two AlHV-1$^+$ rabbit LGL lines. The survival or growth of the LGL with a lymphoblast morphology and activated cytotoxic phenotype (BLT-esterase$^+$ – demonstrated in this study for the first time) in the absence of exogenous IL-2 suggests that, at least in the OvHV-2$^+$ LGL where IL-2 is not produced, IL-2 is not essential for the maintenance of an activated LGL phenotype. The activation characteristics of the LGL are associated with infection of the cells with MCF viruses, although no viral proteins have so far been identified that interact with T-cell (or any other cell) proteins. Control (uninfected) T lymphoblasts in culture do not survive in the absence of exogenous IL-2 and do not develop a LGL cytotoxic cell phenotype characteristic of MCF virus-infected cells. PBMC and T-cell blasts were appropriate controls in these experiments as they contain the principal cell types that express Lck and Fyn kinases in uninfected animals. Furthermore, the LGLs are most similar to these normal cell types in spite of a phenotype altered by infection. An issue that needs to be resolved is whether the LGL are T cells or NK cells. Lck and Fyn are active in the early stage of NK-cell activation, for example via Fcγ receptors.[23] However, Lck-deficient, Fyn-deficient and ZAP-70-deficient
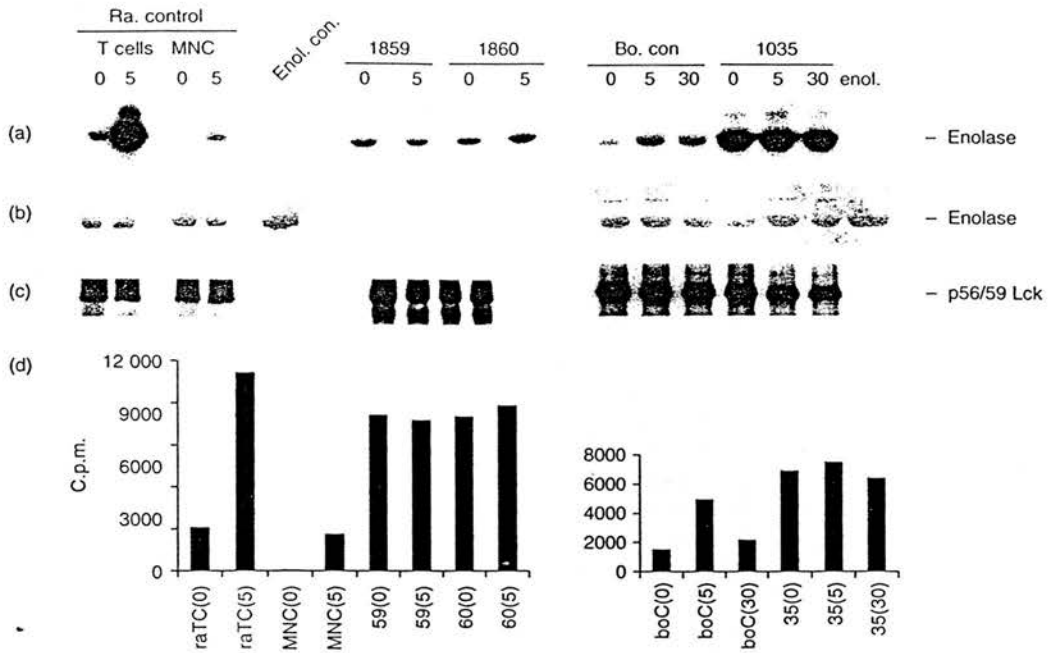
Figure 3. Lck kinase activation in the LGL lines and control T cells. (a) Autoradiograph of SDS–PAGE gel showing enolase phosphorylation by Lck kinase immunoprecipitated from the LGL and control bovine (bo) and rabbit (ra) T cells either unstimulated (0) or stimulated with Con A (10 µg/ml) for 5 min (5) or 30 min (30). (b) Coomassie blue stain, control for protein loading in each lane (showing enolase band). (c) Western blot (anti-Lck antibody) control for immunoprecipitated Lck loading. (d) Quantification of enolase phosphorylation by β-scintillation c.p.m. of enolase bands cut out of the SDS–PAGE gels. TC, control T cells, 59, 1859; 60, 1860; and 35, 1035 LGL, unstimulated (0) or stimulated for 5 min and 30 min with Con A. Lysates from 8×10^6 rabbit control and all of the LGL cell equivalents were loaded in each of the lanes of the gels. Lysates from 4×10^6 bovine control cell equivalents were loaded in each of the lanes of the appropriate gel. Note that a comparison of the intensity of Lck-mediated enolase phosphorylation between control and LGL cells, and between LGL cell lines may not be possible as different exposure times were often used in the development of the autoradiographs. Furthermore, β-scintillation analysis of the enolase bands cut from the gels of the different cell types did not always take place at the same relative time following the gel run.

Figure 4. Activation of p42/44 MAPK in the LGL lines and control T cells. (a) Anti-phosphorylated p42/44 MAPK (erk1 and erk2) Western blot of whole cell lysates (1.5×10^6 cell equivalents/lane) of LGL cell lines and control rabbit and bovine T cells either unstimulated (−) or stimulated with Con A for 10 min (+). It is not clear whether p42 or p44 or both forms of MAPK have been phosphorylated. (b) Anti-p42/44 MAPK Western blot of the cell lysates described in (a). Note that both p42 and p44 forms of MAPK have been detected.

mice do not exhibit defects in natural killing despite defects in T-cell function.[24] The conclusions of the present study are not affected, but there will be a need in future studies to define whether the LGL are T cells or NK cells.

The inhibition of 1035 LGL growth/survival in the absence of IL-2 by genestein but not rapamycin (albeit in only one LGL line studied) suggests that IL-2R-dependent signalling may not be involved in IL-2-independent LGL growth. Rapamycin inhibits biochemical events required for the progression of

IL-2-stimulated T cells from G_1 to S phase of the cell cycle.[25] Furthermore, in a recent study, IL-2-independent growth of HVS-transformed T cells was associated with constitutive activation of the protein kinases Lck and ZAP-70 but not of JAK/STAT kinases associated with interleukin signalling.[26]

Lck and Fyn are *src* kinases involved in the initiation of T-cell activation via the T-cell receptor (TCR) or other structures such as CD2, CD4, CD8 and the IL-2 receptor.[14,15] They are the first known signalling molecules to be recruited to the TCR after antigen or other external stimulation of the TCR. The kinases become activated by phosphorylation of activation motifs and phosphorylate immunoreceptor tyrosine-base activation motifs (ITAMs) on TCR CD3 and ζ chains. This in turn initiates a series of signalling events that leads to T-cell gene transcription and expression of an activated T-cell phenotype. Lck and Fyn are enzymatically inactive in resting cells.[15,27] The constitutive action of inhibitory kinases such as Csk and phosphatases such as CD45 ensure that the *src* kinases are only active for a short period of time while recruited to a relevant surface receptor, such as the TCR.[28]

Given this background, it was interesting that in the LGL, Lck and Fyn were always activated regardless of Con A or Con A and TPA stimulation. In control cells this was not the case and in addition there was a predicted rapid up-regulation followed by a decline of kinase activity after Con A stimulation. In these experiments, it was not always possible

to compare basal levels of the kinases in control cells versus the LGLs. However, basal levels in the control cells probably represent residual activated cells in an otherwise 'rested' population. This was supported by the lack of kinase activity in unstimulated PBMNCs in all cases. If Lck and Fyn were at or near maximum activation in the LGL, then this would explain why any further stimulation of the cells by mitogen did not occur. An alternative explanation of the refractile response of LGLs to the mitogen is that they did not express receptors for Con-A (mannose-rich structures such as the TCR), as they had an unusual phenotype compared to control T cells (Table 1). This was apparently not the case as the LGL and the control T cells aggregated in the presence of Con A (indicating Con-A-binding to the cells). Furthermore, there was a tendency for the LGL to exhibit a small dose-dependent response to Con A or Con A plus TPA (Table 2). A lack of responsiveness of OvHV-2$^+$ bovine LGL to Con A had been reported previously.[6] In this study the comparative lack of responsiveness of AlHV-1$^+$ rabbit LGL compared to control rabbit T cells to Con A (or Con A plus TPA) is demonstrated for the first time.

Interference with *src* kinase function (activation or inhibition) in lymphocytes is a feature of other γ-herpesvirus infections.[10–12] EBV LMP2A protein binds to and inhibits the *src* kinase Lyn in B cells, preventing B-cell activation and presumably preventing the re-activation of EBV from the latent state in the B cells.[11] HVS Tip binds to Lck and interferes with Lck function in infected T cells.[12] Interestingly, deletion of either Tip or Stp in HVS-infected T cells inhibits the exogenous IL-2-independent growth of the cells.[9] With the full genome sequence of AlHV-1 C500 strain available,[16] the next stage of this project is to determine which viral proteins (if any) are responsible for the unusual LGL phenotype. In LGLs treated with the tyrosine kinase inhibitor genestein, IL-2-independent growth of the cells was inhibited. The identity of the tyrosine kinases that have been inhibited by genestein in the LGL remains to be determined, but Lck and Fyn must be candidates.

A direct association of transient Lck and Fyn kinase activation and aspects of T-cell activation has been demonstrated in human and murine T cells.[14,15] However, an association of these kinases with the activation of cytotoxic cell effector function is less clear. Different downstream signalling pathways are engaged after Lck and Fyn activation in T cells that control different aspects of T-cell activation. Of particular interest is the ZAP-70 kinase, which is intimately linked with *src* kinase regulation of T-cell activation. In patients with ZAP-70 mutations, peripheral T cells do not synthesize IL-2 and are refractile to TCR stimulation.[29,30] Unfortunately there was no ZAP-70 antibody available to study this kinase in the LGL. However, the ras/raf/erk (p42/44 MAPK) signalling pathway is downstream of Lck and Fyn and known to be activated by these *src* kinases.[31] In this study, p42/44 MAPK was constitutively activated along with Lck and Fyn in the LGL cell lines. Although p42/44 MAPK can be activated by a variety of mitogenic stimuli, including those generated by the TCR and cytokine receptors,[31] it is likely that its activation in the LGLs was by Lck and/or Fyn.

The examination of tyrosine-phosphorylated proteins in LGL versus control T cells in general indicated both similarities and differences. Between 5 and 7 phosphotyrosine proteins were detected in the 20 000–100 000 MW range in the LGL

and control cells within 3–5 min after mitogen stimulation. Those of ~ 55 000–59 000 MW probably include Lck and Fyn. The 70 000 band might be ZAP-70. In mouse and man Lck is a doublet of 56 and 59 kDa in western blots and Fyn is a 59 000 MW protein. In this study, the dominant form of Lck (where discernible) was the 59 000 MW moiety and Fyn in all the bovine and rabbit cells was a 55 000 MW protein.

The present results are important as they demonstrate that the earliest signalling molecules in T-cell activation are constitutively active in MCF virus-infected LGL, and that they cannot be further activated by mitogen stimulation. This is important because it was also a possibility that signalling molecules downstream of Lck and Fyn were activated, bypassing the NRPTKs such that Lck and Fyn were not required for the observed activation state of the cells.

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