Co-evolution of Host and Virus:

Mechanisms of Host Resistance and Virus Attenuation

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DECLARATION

I herby certify that the work embodies in this thesis is my original work. Assistance with rabbit handling was provided by employees of the CSIRO Gungahlin animal house facility. Statistical analysis was undertaken by Mr J Wood and Ms J Searle, Statistical Consulting Unit, The Australian National University.

(Susie Collins)

To Matt,

for your unending patience, support and love

and to all my family and friends

who have supported my seemingly never-ending journey.

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ABSTRACT

The interaction between myxoma virus and the European rabbit is one of the best documented natural models of host/ virus co-evolution. Although there were many studies which documented the development of this co-evolution, the cellular and molecular mechanisms behind the co-evolution are not clear. This thesis examined the role of lymphocytes in resistance of rabbits to myxomatosis and in attenuation of myxoma virus strains. Primary lymphoid cells were isolated from susceptible (laboratory) and resistant (wild) rabbits and infected *in vitro* with the virulent SLS or attenuated Ur strain of myxoma virus. The effect of lymphocyte activation on the interaction between the lymphoid cells and myxoma virus was examined by the addition of the T-cell mitogen, concanavalin A (Con A) to the primary cultures.

The permissivity of lymphoid cells to myxoma virus replication significantly increased with the addition of Con A. When this was observation was examined at a cellular level it was found that myxoma virus preferentially replicated in lymphocytes that were activated or in the G2/M stage of the cell cycle. However, once lymphocytes were infected myxoma virus completely abrogated T-cell activation processes, such as the increase in cell size and CD25 expression. Myxoma virus also down-regulated MHC-I and CD4 expression on the surface of activated primary lymphocytes and RL-5 cells. These results suggested that activated cell signaling pathways may play a critical role during myxoma virus pathogenesis.

The increase in SLS titres with T-cell activation was due to an increase in virus production in every infected cell. For Ur infection, however, the increase in titres with T-cell activation, which was only detected in lymphoid cell cultures from laboratory

rabbits, was due to an increase in the number of infected cells. Ur did not replicate in activated lymphoid cells from wild rabbits.

It was also observed that in Ur infections there was a reduction in lymphocyte viability late in infection. This was presumably due to the failure of Ur to inhibit cell death pathways that are initiated on cell cycle arrest. In contrast, SLS was able to maintain lymphocyte viability late in infection. Genetic analysis identified a lesion in the M005 ORF in Ur where there was an insertion of a cytosine relative to the M005 ORF in SLS. The insertion of this cytosine induces a frameshift, potentially resulting in production of a truncated M005 protein. M005 has been previously implicated in lymphocyte survival, so this lesion may interfere with the biological function of this protein leading to the inability of Ur to maintain lymphocyte viability at late time points. This may then lead to a reduction in viral titres and viral dissemination *in vivo* compared to SLS and is the first identified potential attenuation mechanism for myxoma virus.

This thesis is also the first to show a potential mechanism for resistance of rabbits to myxoma virus. There was a small but statistically significant reduction in the permissivity of lymphocytes from resistant rabbits for myxoma virus replication compared to lymphocytes from susceptible rabbits. Unstimulated mock-infected lymphocytes from wild rabbits also had greater cell surface expression of CD4 and CD43 and stimulated lymphocytes had greater expression of MHC-I than lymphocytes from laboratory rabbits which may enhance the early immune response against the virus. Taken together, a lower ability of myxoma virus to replicate in lymphoid cells from wild rabbits in addition to a potentially more efficient and effective early innate immune response in wild rabbits compared to laboratory rabbits, may be responsible for resistance of rabbits to myxomatosis.

A secondary part of this thesis was the production and characterisation of antibodies specific for rabbit IL-2 and IFNγ, as representative T_H1 cytokines, and rabbit IL-4, IL-6 and IL-10, as representative T_H2 cytokines. Synthetic peptides corresponding to potential epitopes on these cytokines were designed conjugated to the immunogenic carrier protein and injected into rats (and chickens for the IL-2 synthetic peptides).

Antibodies to IL-4, IL-6, IL-10 and IFNγ were generated after immunization of rats with synthetic peptide/conjugate. These antibodies worked to differing extents in immunofluorescence, Western blot assays, flow cytometry and tissue sections. Production of anti-peptide sera to rabbit IL-2 was attempted but was unsuccessful. The ability of the antibodies produced to recognize native forms of rabbit cytokines will enable future investigation of the role of cytokine expression in the attenuation of myxoma virus and genetic resistance to myxoma virus. However, their most useful role is likely to be in immunostaining in tissue sections rather than in immunostaining of infected cultures as this will enable clarification of the immune response elicited by the host rabbit on infection with myxoma virus.

In conclusion, this thesis showed that the interaction between myxoma virus and T-cells may be critical for determining the outcome of infection and postulated that it is this interaction that ultimately determines resistance of rabbits to myxomatosis and attenuation of the virus. This thesis also presented a model for resistance of rabbits to myxomatosis and a model for attenuation of myxoma virus.

ABBREVIATIONS

^{3}H-T	tritiated thymidine
APC	Antigen presenting cells
APS	ammonium persulphate
ANU	Australian National University
BFA	buffered formaldehyde acetone
Bref A	Brefeldin A
BSA	bovine serum albumin
CMV	cytomegalovirus promotor
Con A	Concanavalin A
CPE	cytopathic effects
cpm	counts per minute
CTL	cytotoxic T-lymphocyte
DTT	dithiothreitol
EEV	extracellular enveloped virion
ER	endoplasmic reticulum
FADD	Fas associated death domain
FCS	fetal calf serum
FSC	forward scatter
HRP	horseradish peroxidase
IFN	interferon
IL	interleukin
IMV	intracellular mature virion
kb	kilobases
LPS	lipopolysaccharide
Lu	Lausanne

mcKLH	mariculture keyhole limpet hemocyanin
MEM	minimal essential media
MHC	major histocompatibility class
MIP	macrophage inhibitory protein
MRV	malignant rabbit virus
NK	natural killer cell
ORF	open reading frame
pfu	particle forming units
PI	propidium iodide
RT	room temperature
SFV	Shope fibroma virus
SLS	standard laboratory strain
SSC	side scatter
TGF	transforming growth factor
TIR	terminal inverted repeat
TNF	tumor necrosis factor
Ur	Uriarra strain

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Perhaps the most studied and documented natural model of host/virus co-evolution in mammals is that of myxoma virus and the European rabbit, *Oryctolagus cuniculus.* Myxoma virus is the causative agent of myxomatosis and was released in Australia in the summer of 1950-51 in order to control the wild European rabbit population, which was immunologically and evolutionarily naïve with respect to myxoma virus. The initial epidemic of myxoma virus was estimated to have killed greater than 99% of infected wild rabbits. However in the years following, there was rapid selection for attenuated strains of myxoma virus and for rabbits resistant to myxomatosis (reviewed in Fenner and Fantini 1999).

Leukocytes are one of the key players during myxoma virus pathogenesis. Fenner and Woodroofe (1953) first identified leukocytes as important for *in vivo* amplification, dissemination and pathology of myxoma virus. More recently it has been shown that lymphocytes, in particular T-cells, are critical for myxoma virus pathogenesis as myxoma virus localizes to the T-cell zones in lymph nodes from infected rabbits (Best and Kerr 2000) and replicates in the rabbit $CD4^+$ T-cell line, RL-5 (Barry et al., 1995; Macen et al., 1996; Barry et al., 1997). It has also been postulated that T-cells are one of the key cell types that disseminate myxoma virus systemically (Kerr and Best 1998). This is supported by studies using recombinant myxoma viruses with genes essential for replication in T-cells deleted, which show virus strains that are unable to replicate in T-cells are attenuated *in vivo* (Opgenorth et al., 1992a; Macen et al., 1996).

This thesis investigates the role of T-cells in co-evolution of myxoma virus with the European rabbit by examining infection of lymphoid cells from susceptible and resistant rabbits with virulent and attenuated strains of myxoma virus. The original virulent strain

of myxoma virus, the 'standard laboratory strain' (SLS), released in the field in 1950 is available, as well as an attenuated strain, Uriarra (Ur), isolated from the field in 1953. In addition, laboratory rabbits that are susceptible to myxomatosis and wild rabbits selected in the field for resistance to myxomatosis are also available. The accessibility of these resources puts our laboratory in a unique position to study host/virus co-evolution in this natural model.

The introduction to this thesis will firstly detail the characteristics of myxoma virus and the historical development of myxoma virus attenuation and wild rabbit resistance. Following from this, the cellular responses to virus infection and the importance of T-cells and T-cell activation and replication in these responses will be described. Then, the immunomodulatory mechanisms used by myxoma virus to overcome the rabbit immune response will be discussed and finally, the aims of this thesis set out.

1.2 Myxoma Virus

1.2.1 Myxoma Virus

Myxoma virus is the causative agent of myxomatosis, a systemic lethal disease of European rabbits, *Oryctolagus cuniculus*. Myxoma virus is one of six members of the *Leporipoxvirus* genus (Fenner 1979). All six of the *Leporipoxvirus* species cause a benign, cutaneous fibroma in their respective natural host, but they cause differing extents of disease in *O. cuniculus* (Fenner and Ratcliffe, 1965; Fenner and Ross, 1994). The natural host of each of these virus species and the general disease each causes in *O. cuniculus* are listed in Table 1.1. The use of 'myxoma virus' in this thesis refers to the South American myxoma virus species.

TABLE 1.1. NATURAL HOST AND DISEASE PHENOTYPE IN *ORYCTOLAGUS CUNICULUS* INDUCED BY *LEPORIPOXIVRUS* SPECIES

Leporipoxvirus Species	Natural Host*	Disease in *Oryctolagus cuniculus*
South American Myxoma Virus	*Sylvilagus brasiliensis*	generalized lethal disease, gross external pathology
Californian Myxoma Virus	*Sylvilagus bachmani*	generalized lethal disease, few external signs
Shope Fibroma Virus	*Sylvilagus floridanus*	benign, cutaneous fibroma
Squirrel Fibroma Virus	*Sciurus carolinensis*	benign, cutaneous fibroma
Western Squirrel Fibroma Virus	*Sciurus griseus griseus*	not determined
Hare Fibroma Virus	*Lepus europaeus*	benign, cutaneous fibroma

* All virus species cause a benign cutaneous fibroma in their natural host

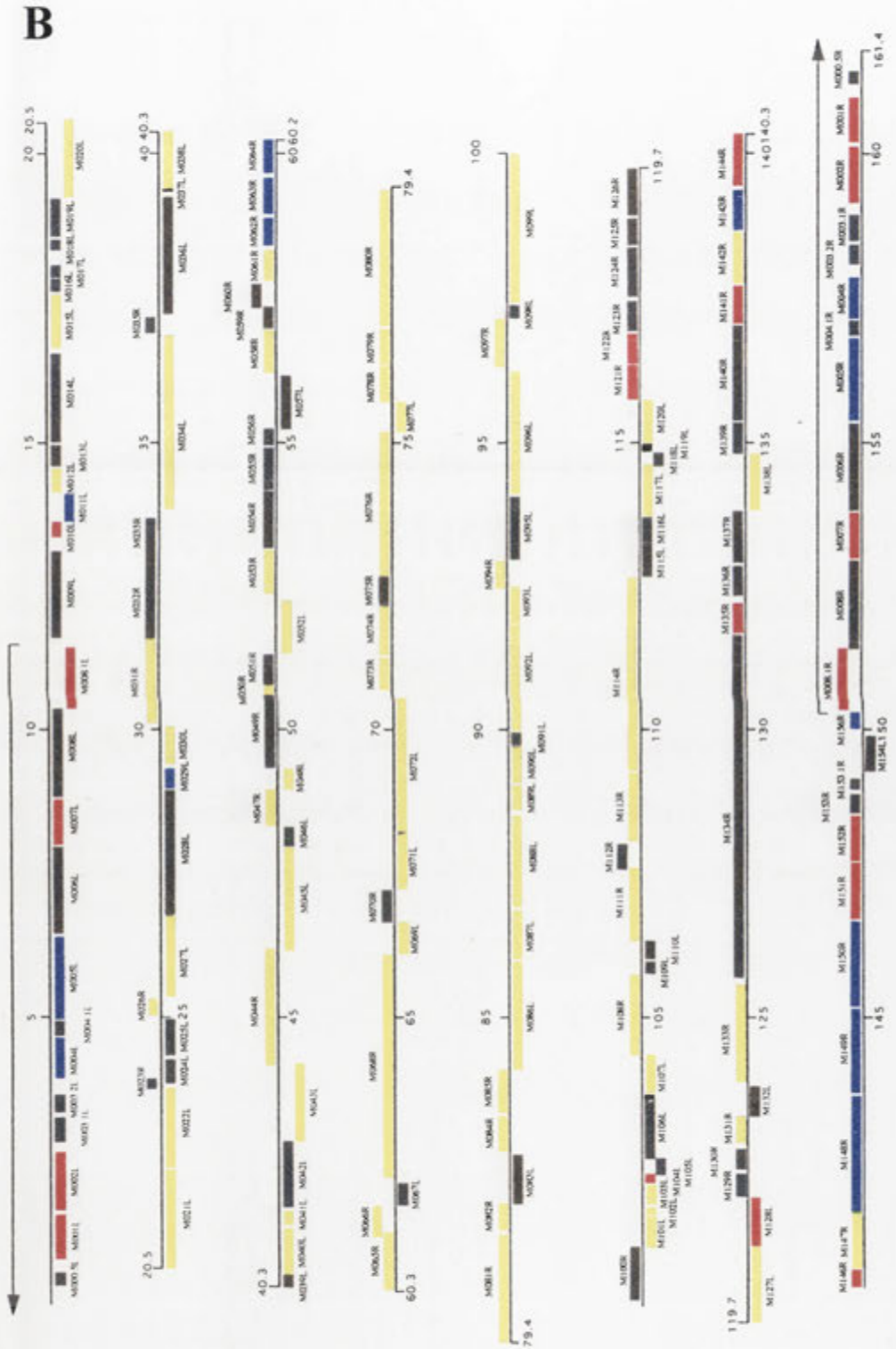
The *Leporipoxvirus* genus is part of the *Poxviridae* family. Poxviruses have linear, double-stranded DNA genomes. The genome of myxoma virus is 161.8 kilobases (kb) (Cameron et al., 1999). Each end of the genome is composed of a covalently closed single stranded hairpin loop and 11.5 kb terminal inverted repeat (TIRs), which is typical of poxviruses (Cameron et al., 1999). The TIRs and adjacent regions of the genome typically encode 'virulence' genes (Cameron et al., 1999; Moss 2001). Virulence genes encode proteins that disrupt and circumvent the host anti-viral immune response and that block apoptosis of infected cells. The genes in this region are variable between members of the *Poxviridae* family and tend to be specific to particular virus species or virus genera. The central region of the genome encodes structural and house-keeping genes (e.g. genes encoding mRNA transcription and translation factors, virion morphogenesis factors). Many of these genes are essential for the production of

infectious virus progeny and are highly conserved among the *Poxviridae* family. Figure 1.1A shows a schematic of the myxoma virus genome.

Sequencing of the genome from the Lausanne (Lu) strain of myxoma virus identified 425 potential open reading frames (ORFs) with a minimum length of 50 amino acids (Cameron et al., 1999). Of these, 159 are predicted to be co-linear expressed genes based on homology to known poxvirus genes and by comparison to predicted ORFs in Shope fibroma virus (SFV). One of these ORFs, M037L, encodes a 32 amino acid protein, but has been identified based on comparisons with genomes from other poxviruses. The 159 ORFs were numbered sequentially starting from the left end of the genome, based on the nomenclature of *Melanoplus sanguinipes* and *Molluscum contagiosum* (as shown in Cameron et al., 1999). A diagrammatic representation of the 159 assigned ORFs of myxoma virus is shown in Figure 1.1B. The ORF names used in this thesis are defined as in Cameron et al. (1999) without the direction of transcription

FIGURE 1.1. THE MYXOMA VIRUS GENOME

(A) Schematic of the myxoma virus genome. The genome is linear double-stranded DNA and contains a covalently closed single-stranded hairpin loop and terminal inverted repeats (TIR) at each end. The central region of the genome encodes structural and house-keeping genes, which tend to be conserved among the *Poxviridae* family. The genes encoding proteins with putative immunomodulatory functions are generally situated at the ends of the genome and vary between members of the *Poxviridae* family. (B) Linear map of myxoma virus genome (adapted from Cameron et al, 1999). The genome is 161.8 kb (161.4 kb excluding the TIR) and contains 159 assigned ORF. ORFs are numbered sequentially, starting at the left end of the genome. The 'R' and 'L' designate the direction of gene transcription. Genes with predicted immunomodulatory functions are shown in red, those predicted to encode proteins related host range or anti-apoptotic activity are coloured blue. The conserved genes (genes encoding house-keeping and structural proteins) are shown in yellow. The TIR (purple) encodes genes M001 to M008.1.


B

stated, and will be described as genes (e.g. M001 gene). For this thesis, the putative proteins translated from these genes will share the gene name (e.g. M001).

1.2.2 Myxoma Virus Morphology

The myxoma virus virion appears brick-shaped when viewed by electron microscopy and morphologically is indistinguishable from the prototype poxvirus, vaccinia virus (*Orthopoxvirus* genus) (Fenner 1996). Studies of vaccinia virus have identified two infectious forms, the intracellular mature virion (IMV) and extracellular enveloped virion (EEV). The IMV consists of a biconcave nuclear core, containing the DNA genome and core membrane, and two lateral bodies surrounded by a lipoprotein membrane. An additional membrane derived from the Golgi encloses the IMV to form the EEV. Myxoma virus infection also generates IMV and EEV (Padgett et al., 1964). Figure 1.2 shows an electron micrograph of a myxoma virus IMV and a schematic of a poxvirus virion.

In vaccinia virus, IMV forms 80-95% of the total virus produced by the cell with the remainder being EEV (Moss 2001). However, EEV is considered the primary infectious form of virus for *in vivo* and *in vitro* dissemination. This is because the EEV does not rely on the loss of cell membrane integrity (e.g. cell lysis), which is required for release of IMV from the cell (Vanderplasschen et al., 1998).

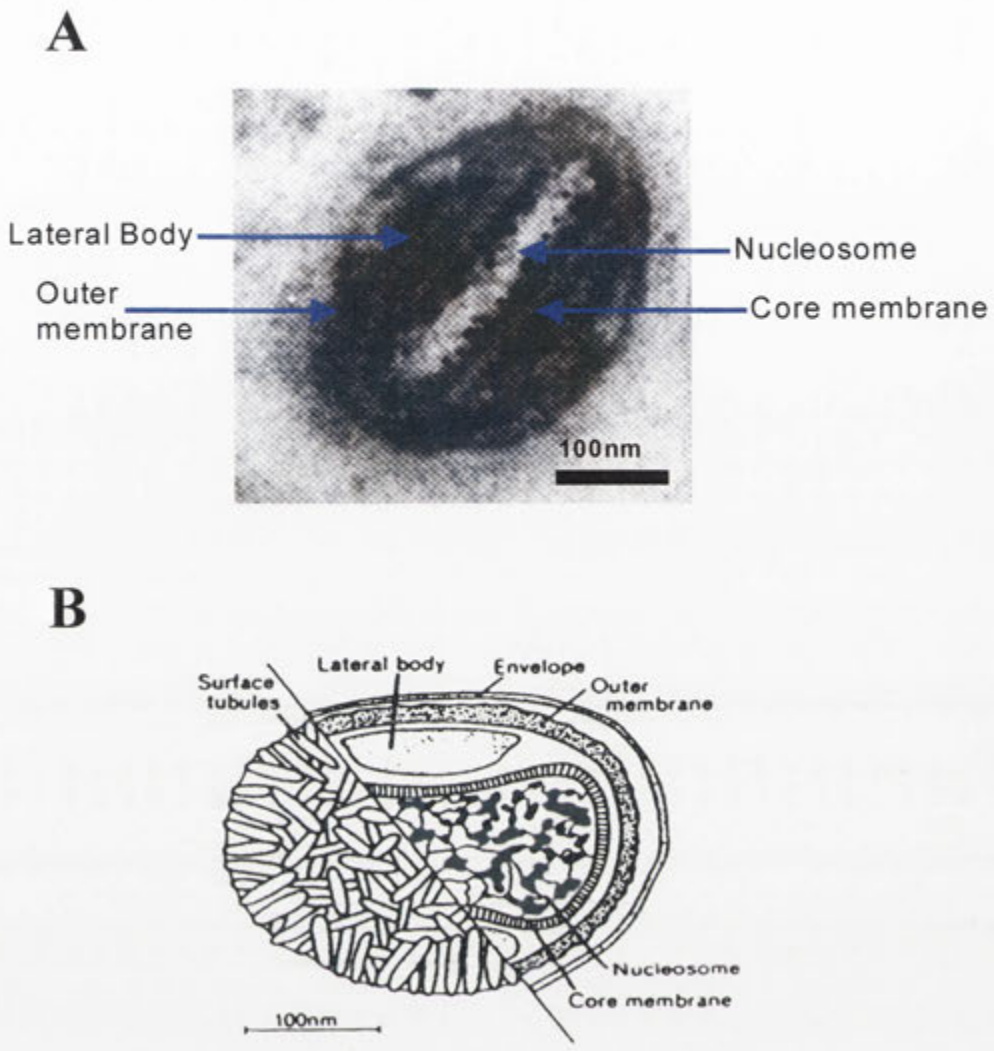


FIGURE 1.2. MYXOMA VIRUS VIRION

(A) An electron micrograph of the intracellular mature myxoma virus virion (IMV) (Fenner, personal communication) and (B) schematic of a typical poxvirus virion (taken from Fenner, 1996). A poxvirus virion is made up of the nucleosome, containing the DNA genome, and is surrounded by the core membrane. An additional lipoprotein membrane (outer membrane) surrounds this core and the two lateral bodies. This is the IMV form of the virus. A Golgi-derived membrane envelopes the virions in the EEV form.

1.2.3 Poxvirus Replication Cycle

Poxvirus replication has been elucidated by studying that of vaccinia virus and is outlined in this section and in Figure 1.3 (reviewed in Moss 2001).

The first steps in the poxvirus replication cycle are attachment of a virion to the cell membrane, fusion with the cell membrane and entry of the viral core into the cell cytoplasm (Step 1, Figure 1.3). Attachment to and fusion of a virion with the cell membrane will be discussed in more detail in Section 1.2.3.1. Once the virion has entered the cell, replication of the virus begins. Replication of poxviruses occurs exclusively in the cytoplasm in defined areas called virosomes. The machinery required to transcribe early genes is packaged within the viral core and transcription of these genes is initiated from early promoters after partial uncoating of the core (Step 2, Figure 1.3). These early transcripts encode immunomodulatory proteins, host-range proteins, intermediate transcription factors and proteins involved in replication of the viral genome, in addition to proteins that complete uncoating of the viral core. Once the core is uncoated (Step 3, Figure 1.3), replication of the viral genome can begin (Step 4, Figure 1.3). At this time, gene transcription from intermediate and late promoters is initiated.

Replication of the poxvirus genome results in the formation of concatemeric DNA, which is cleaved into unit genomes and packaged, along with early transcription factors required during the next replication cycle (Step 5, Figure 1.3), and assembled at the late stage of infection into an immature virion. Maturation of the virion occurs leading to formation of IMV (Step 6, Figure 1.3) (Beaud 1995; (Moss 2001). Virions destined for the extracellular environment acquire an additional membrane from the Golgi and are transported to the cell surface via actin tail polymerization (Step 7, Figure 1.3) (Frischknecht et al., 1999). At the cell surface, the virion fuses with the cellular membrane and either EEV is released or the virion stays adhered to the cell surface forming a cell-associated enveloped virion (Step 8, Figure 1.3) (reviewed in Moss 2001).

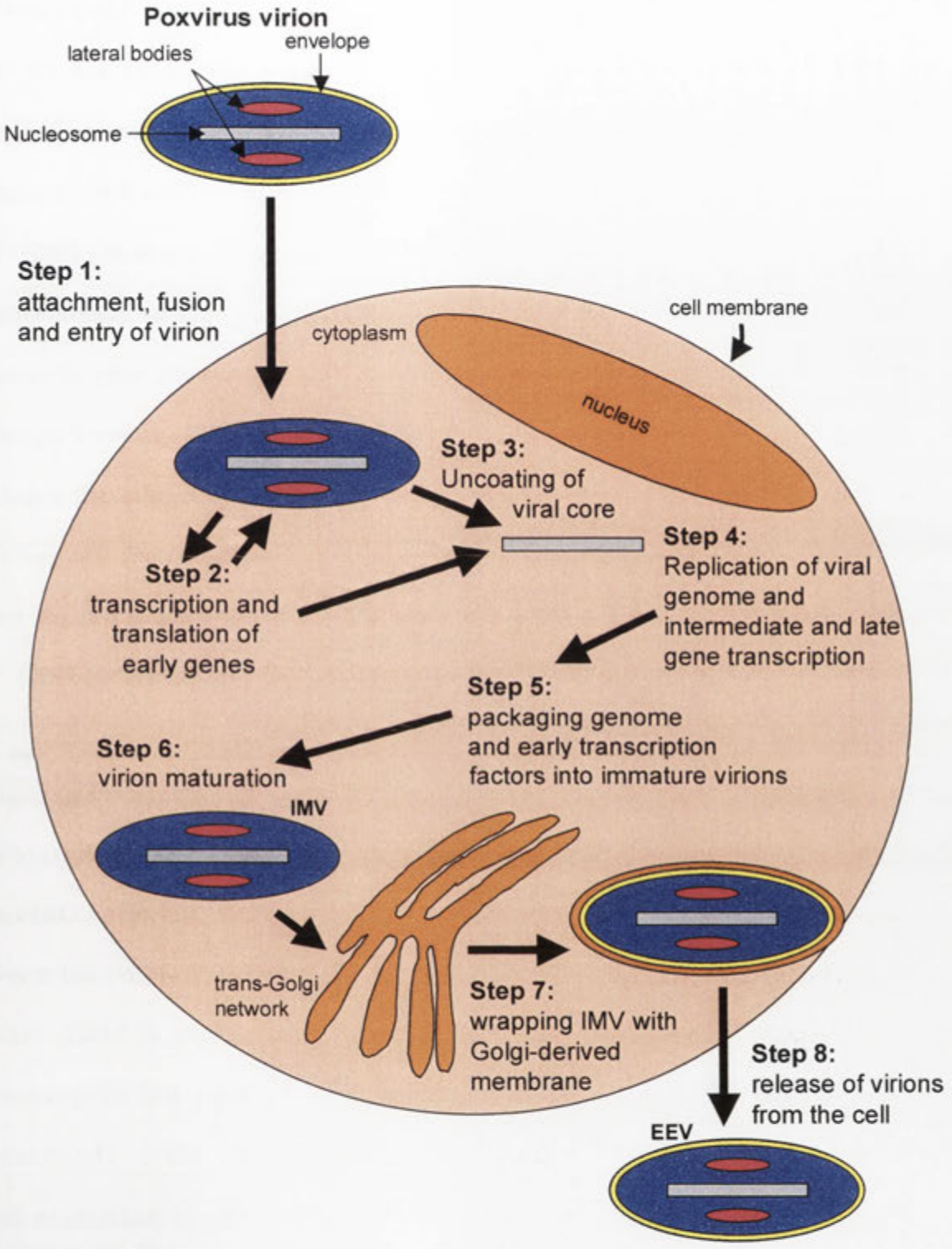


FIGURE 1.3. THE REPLICATION CYCLE OF POXVIRUSES

Step 1: Poxvirus virions attach and fuse to the cell membrane, releasing viral cores into the cytoplasm. Step 2: Early genes are transcribed and translated. Step 3: The viral core is uncoated and replication of the viral genome begins (Step 4). Step 5: The DNA genome and early transcription factors required for the next poxvirus cycle are packaged into immature virions. Late gene expression follows, and assembly of the virus particle occurs. Step 6: The virion matures to an IMV. The IMV may be wrapped in a second, Golgi-derived membrane (Step 7), transported to the cell membrane and released from the cell as EEV (Step 8). Figure adapted from Moss (2001).

1.2.3.1 Virion Attachment, Fusion and Entry

Attachment to, and fusion with the cell membrane differs between the two infectious forms of virion, as there are additional viral-encoded proteins associated with EEV that are not present in IMV (Payne 1979; Payne 1980). The differences between vaccinia virus IMV and EEV in cell membrane attachment and fusion have been illustrated experimentally (Vanderplasschen and Smith 1997; Vanderplasschen et al., 1998). The cell surface receptors responsible for vaccinia virus attachment have not been elucidated (Vanderplasschen and Smith, 1997). However, it has been shown that entry of vaccinia virus IMV is dependent on temperature, pH (Doms et al., 1990) and cell signaling cascades (Locker et al., 2000). In contrast, EEV entry does not require cell signaling cascades (Locker et al., 2000) even though it is also dependent on pH. The fact that signaling cascades are not required for EEV entry into a cell may explain why cell entry of EEV is more rapid than that of IMV (Payne and Norrby 1978; Doms et al., 1990).

A number of proteins putatively involved in myxoma virus virion attachment and fusion have been identified based on homology to other poxvirus proteins. The products of the M035, M041, M055, M071, M088, M098, M102, M103, M107 and M115 ORFs are predicted to be associated with the IMV membrane (Cameron et al., 1999) and may be implicated in IMV attachment and fusion. For EEV, the products of M022, M121, M122 and M126 ORFs are associated with the EEV envelope and are putatively implicated in EEV attachment and fusion (Cameron et al., 1999). The putative properties of each of these IMV and EEV-associated proteins, in addition to their poxvirus homolog and relative conservation among the *Poxviridae* family, are shown in Table 1.2.

TABLE 1.2. PUTATIVE PROPERTIES OF IMV- AND EEV-ASSOCIATED PROTEINS ENCODED BY MYXOMA VIRUS

	Myxoma Virus ORF*	Vaccinia Virus Homolog	Putative Properties	Conservation among Poxviruses+
IMV-associated proteins	M035	E10R	•hydrophobic N-terminus •transmembrane domain •redox function	conserved
	M041	I5L	•hydrophobic •basic •structural protein •transmembrane	semi-conserved
	M055	L1R	•myristylated •hydrophobic N-terminus	conserved
	M071	H3L	•hydrophobic •heparin binding •immunodominant	semi-conserved
	M088	D13L	•rifampicin resistance	conserved
	M098	A9L	•putative signal peptide •transmembrane domain	conserved
	M102	A13L	•predicted secreted •hydrophobic N-terminus	semi-conserved
	M103	A14L	•phosphorylated •myristoylated •associated with A17L/M107	conserved
	M107	A17L	•associated with A27L and A14L/M103 •morphogenesis factor •neutralizing antibody	semi-conserved
EEV-associated proteins	M115	A27L	•fusion protein •neutralizing antibody •heparin binding •EEV formation	semi-conserved
	M022	F13L	•non-glycosylated •palmitylated •envelope protein •EEV antigen	conserved
	M121	A33R	•lectin-like glycoprotein •transmembrane	semi-conserved
	M122	A34R	•hydrophobic N-terminus •C-type lectin domain •transmembrane •NK cell receptor homolog •N-glycosylated	semi-conserved
	M126	A37R	•unknown	semi-conserved

* Information in this table was taken directly from Cameron et al. (1999) and Moss (2001)

+ Proteins with conservation classified as 'semi-conserved' share 20% to 50% amino acid identity and those classified as 'conserved' proteins share greater than 50% amino acid identity, with at least one gene outside the *Leporipoxvirus* genus but within the poxvirus family.

The discrete entry mechanisms of myxoma virus IMV and EEV particles have not been investigated. In fact, there are very few studies on myxoma virus in the early stages of infection. One study that has examined myxoma virus infection, showed that infection is dependent on tyrosine phosphorylation signaling pathways, as infection was blocked by herbimycin A (tyrosine kinase inhibitor) and tyrophostin AG490 (Jak 2 inhibitor) (Lalani et al., 1999b; Masters et al., 2001). Although not stated, it is probable that the virus used in these studies consisted predominantly of the IMV form of myxoma virus because of the method of preparing virus stocks. This suggests that myxoma virus IMV also requires signaling cascades to enter a cell, and suggests that myxoma virus IMV enters the cell by a similar mechanism to vaccinia virus IMV.

Lalani et al. (1999b) also found pre-treatment of cells permissive for infection with either the growth factor RANTES (Regulated-upon Activation, Normal T-cell Expressed and Secreted) or an anti-CC chemokine receptor-5 antibody that blocks HIV infection, also blocked infection of myxoma virus. Pretreatment of cells with other chemokines, such as macrophage inhibitory protein (MIP)-1α or MIP-1β or with other signal cascade inhibitors, such as pertussis toxin (G-protein inhibitor) or PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*] pyrimidine, generalized phosphatase inhibitor) did not block infection (Lalani et al., 1999). These results suggest that some aspects of HIV entry may be similar to that of myxoma virus and that signaling pathways involving tyrosine phosphorylation and that are linked to RANTES or CC-chemokine receptors may be critical for myxoma virus infection.

1.3 MYXOMATOSIS

Infection of naïve European rabbits with virulent strains of myxoma virus causes the lethal, systemic disease, myxomatosis. Myxomatosis is characterized by multiple

internal and external lesions, both at the site of virus inoculation and in tissues throughout the infected rabbit. Clinical signs of myxomatosis include swelling of the eyelids, conjunctival inflammation, anogenital edema and respiratory difficulties (Best and Kerr 2000). In contrast, in its natural host, *S. brasiliensis*, myxoma virus infection is generally not fatal and is characterized by mucoid cutaneous tumors localized to the site of infection (reviewed in Kerr and McFadden 2002; Zuniga 2002).

Myxoma virus is transmitted by arthropod vectors, such as mosquitoes and fleas. Transmission is passive, meaning myxoma virus does not replicate in the arthropod vector; rather myxoma virus virions adhere to the mouthparts of the arthropod, which transfers the virions to the epidermis or dermis of another rabbit during the arthropod's next feeding (Aragao, 1943). In Australia, epidemics of myxomatosis usually occur annually in the spring and summer, but can occur at any time depending on the availability of vectors and susceptible rabbits (Fenner and Ratcliffe 1965; Fenner and Ross 1994).

A model for myxoma virus pathogenesis has been postulated by Best et al. (Best 1998; Best et al., 2000; Best and Kerr 2000). This model is outlined in Figure 1.4 and is described below. Myxoma virus enters the dermis of the host from the mouthpart of a biting arthropod. In the dermis, the virus infects MHC-II$^+$ dendritic-like cells that transport the virus to the lymph node draining the site of infection. Once at the lymph node the virus can infect other cells, but most importantly lymphocytes and monocytes, which disseminate it systemically to other tissues. Lymphocytes and monocytes also transport myxoma virus back to the dermis/epidermis where the virus can be picked up by the next biting arthropod.

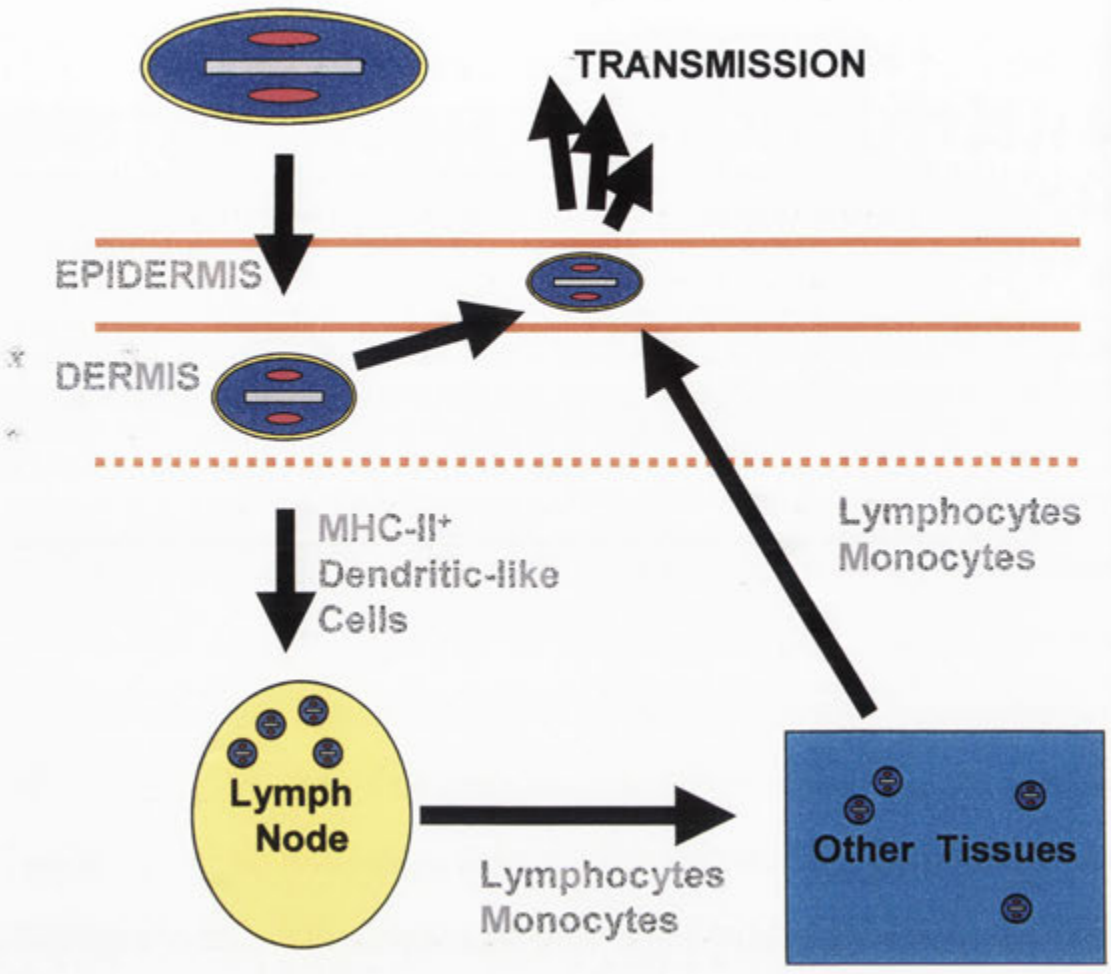


FIGURE 1.4. MODEL FOR THE PATHOGENESIS OF MYXOMA VIRUS

Myxoma virus enters the dermis of the host from the mouthpart of a biting arthropod. The virus infects MHC-II$^+$ dendritic-like cells which transport it to the lymph node. At the lymph node the virus can infect lymphocytes and monocytes which disseminate the virus systemically to other tissues and also transport myxoma virus back to the dermis/epidermis where it can be picked up by the next biting arthropod and transmitted to another rabbit. Figure adapted from Kerr and Best (1998).

An inflammatory and immune response against myxoma virus is initiated at the skin and lymph node. The interaction between the rabbit immune response and myxoma virus determines subsequent pathogenesis and ultimately determines the outcome of myxoma virus infection. This interaction will be influenced by the virulence of the virus and the degree of resistance of the rabbit. These factors will be discussed in detail in Section 1.6.

Infection of European rabbits, that are immunologically naïve with respect to myxoma virus, with virulent strains of this virus typically ends with the death of the infected rabbit. The mechanism of death is obscure, but can be is associated with opportunistic infection of the upper respiratory tract with Gram-negative bacteria. Death from virulent South American strains of myxoma virus has been previously attributed to respiratory obstructions (Hobbs 1928; Strayer and Sell 1983). The lethality of myxoma virus in naïve *O. cuniculus* made it an attractive candidate as a biological control agent for the wild European rabbit population of Australia.

1.4 Myxoma Virus as a Biological Control Agent for the Wild European Rabbit

Wild European rabbits (wild rabbits) were deliberately introduced into Australia in 1859 and spread across the continent at an estimated rate of 100-300 km per year. The clearing of land, introduction of domestic livestock, and control of predators by settlers and pioneers all assisted the establishment and spread of the wild rabbit population (reviewed in Myers et al., 1994).

Initial trials of the effectiveness of myxoma virus as a biological control agent were undertaken in the 1930s using Moses' strain of South American myxoma virus (referred to in subsequent years as the 'standard laboratory strain' or 'SLS') (Moses 1911; Martin 1936; Bull and Mules 1944). These trials were unsuccessful, with the virus failing to become established in the wild rabbit population and not spreading beyond the sites of its introduction (Myers et al., 1954). Further field trials with myxoma virus were performed after World War II and, in the summer of 1950-1951, there was an explosive epidemic of myxomatosis, initiated from experimental release sites in southeastern Australia. During this time, *Culex annulirostris* mosquitoes were in plague proportions

and were probably the chief vectors for the myxomatosis epidemic as the incidence of disease closely followed waterways infested with these mosquitoes (Ratcliffe et al., 1952; Myers et al., 1994). The spread of myxoma virus at this time was augmented by extensive wild rabbit inoculation campaigns throughout southern Australia (Ratcliffe et al., 1952; Fenner et al., 1953). By 1954, myxomatosis had extended throughout the entire geographic range of the wild rabbit (Fenner and Ratcliffe 1965).

The myxomatosis epidemic in the summer of 1950-51 was estimated to have killed greater than 99% of infected wild rabbits. However, this level of effectiveness of myxoma virus as a biological control agent was short-lived, with the rapid emergence of both attenuated myxoma virus strains and rabbits resistant to myxomatosis (Myers et al., 1954; Marshall and Fenner 1958). The attenuated strains were selected for in the field as they allowed the infected rabbit to survive for a longer period, thereby giving the virus a longer time for transmission. As these strains were less virulent, they also enabled the survival of occasional rabbits with some degree of inherent resistance to myxomatosis. Thus, the emergence of attenuated virus strains and resistant rabbits was the result of interactions between the virus and host. This co-evolution of myxoma virus and wild *O. cuniculus* provided an exciting opportunity to study a natural model of host/virus co-evolution, which had followed the introduction of a novel pathogen into a completely naïve species. As a result, long-term studies monitoring virus virulence and resistance of wild rabbits were initiated. These studies are described in the following section.

1.5 Co-evolution of Host and Virus

1.5.1 Development of Attenuated Strains of Myxoma Virus

To study the changing virulence of myxoma virus, field isolates collected during sequential annual epizootics were assigned a virulence grade, from grade I through to grade V, depending on the mortality rate and mean survival time of inoculated naïve laboratory rabbits (Table 1.3) (Fenner and Marshall 1957). Grade I strains were the most virulent (least attenuated), killing 100% of inoculated laboratory rabbits with an average survival time of less than 13 days. Grade V strains were the least virulent (most attenuated) with less than 50% mortality and prolonged survival time for inoculated laboratory rabbits. Grade II-IV strains were intermediate between the two. For example grade III strains had a mortality rate of 70-95% and a mean survival time for inoculated laboratory rabbits of 17-28 days (Fenner and Marshall 1957).

Table 1.3. Mean Survival Time and Mortality Rate of Naïve Laboratory Rabbits Inoculated with Myxoma Virus Strains of Different Virulence Grades

Virulence Grade* of Myxoma Virus	Mean Rabbit Survival Time	Rabbit Mortality Rate (%)
I	< 13 days	100
II	13-16 days	95-99
III	17-28 days	70-95
IV	29-50 days	50-70
V	not relevant	< 50

* Table taken from Kerr and Best (1998) from data in Fenner and Marshall (1957).

SLS, the original strain of myxoma virus introduced into the wild rabbit population in 1950, is a grade I strain. Although SLS was not the only strain of myxoma virus released in Australia in an attempt to control the wild rabbit population, experimental evidence indicates that all myxoma virus strains isolated from the field are derived genetically from SLS (Saint et al., 2001). By 1952-53, the proportion of virus field isolates of grade I virulence had fallen from 100% to approximately 13%, and only comprised 1-2% of the field isolates between 1975 and 1981 (Figure 1.5) (Marshall and Fenner 1960). Grade III and IV strains became dominant in the field and comprised greater than 60% of the virus isolated between 1952 and 1954, and greater than 90% between 1975 and 1981 (Figure 1.5) (Marshall and Fenner 1960). Virus strains of grade II and V virulence only constituted a small proportion of strains isolated from the field between 1952 and 1981. In recent years, virus strains of grade I virulence have been regularly isolated from the field (Saint et al., 2001; Kerr et al., 2003). This will be discussed further in Section 1.5.3.

The reason strains of grade III and IV virulence initially became dominant in the field between 1952 and 1981 was due to the enhanced transmission of strains with this virulence at this time. As previously described, myxoma virus is transmitted passively by blood-feeding arthropod vectors. Thus, the probability that myxoma virus would be transmitted to an uninfected rabbit depends on virus attached to the mouthparts of the arthropod vector. This in turn would depend on the titre of virus in the skin of the infected rabbit that initially probed for a blood meal (Fenner et al., 1952; Day et al., 1956; Fenner et al., 1956).

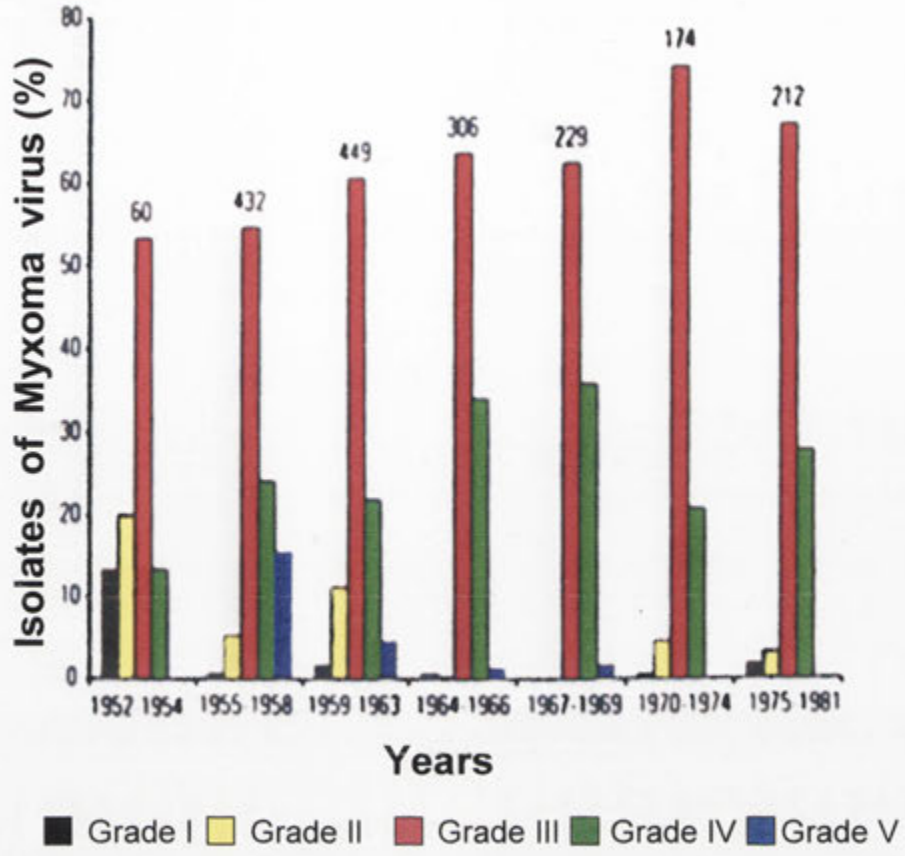


FIGURE 1.5. ATTENUATION OF MYXOMA VIRUS
The proportion of myxoma virus strains of each grade isolated from Australian wild rabbits between 1952 and 1981 are shown. Grades are defined in Table 1.3. The numbers of isolates tested during each period is indicated above each group of columns. Figure from Kerr and Best (1998) from data in Marshall and Fenner (1960).

Experimentally it was shown that a virus titre in the skin of greater than 10^7 pock-forming units per gram of skin (pfu/g) was required for effective transmission by arthropod vectors (Day et al., 1956; Fenner et al., 1956). The skin titre in inoculated rabbits, of myxoma virus strains of different virulence grades over time is shown in Figure 1.6. If the virus was highly virulent (grade I) then it would kill its rabbit host within a few days of reaching skin titres required for effective transmission. However, if it was too attenuated (grade V), then the host would control replication of the virus, thereby rapidly reducing skin titres and the period of time those titres are over the transmission threshold. It must be noted that strains of grade V were able to reach titres sufficient for transmission for a limited time. Infection with virus strains of grade III or

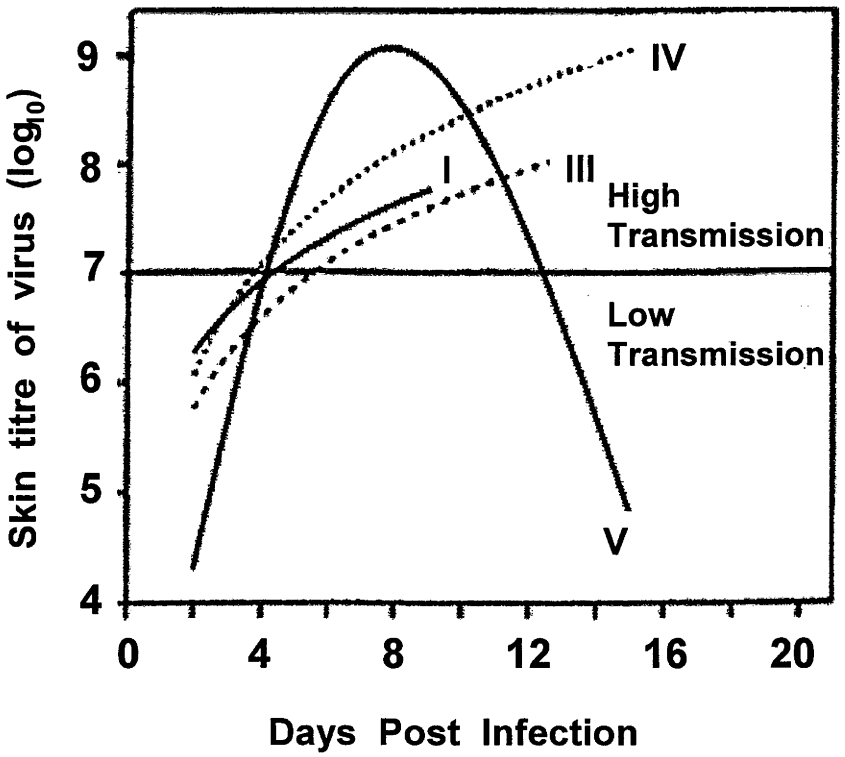


FIGURE 1.6. TRANSMISSIBILITY OF STRAINS OF MYXOMA VIRUS OF DIFFERENT VIRULENCE GRADES

The titre of myxoma virus strains of grade I, III, IV and V virulence in the skin of inoculated rabbits over time is shown. A skin titre of 10^7 has been shown experimentally to be the threshold for effective transmission by arthropod vectors. Figure from Kerr and Best (1998) from data in Fenner et al. (1956) for grade I, III and IV data, and Best and Kerr (2000) for grade V data.

grade IV virulence enabled the virus to reach transmissible titres in the skin and allowed the infected rabbit to survive for longer periods. Together these increased the chances of successful transmission. Thus, virus strains of grades III and IV virulence would have had the highest probability for transmission by arthropod vectors and, over time, were more likely to out-compete strains of other grades.

1.5.2 Development of Rabbit Resistance to Myxomatosis

As previously stated, the emergence of attenuated strains of myxoma virus allowed the survival of rabbits with some inherent resistance to myxomatosis. Environmental

factors, such as ambient temperature, may have augmented the development of resistance in wild rabbit populations as, experimentally, naïve laboratory rabbits had a greater survival rate after challenge with myxoma virus if housed at high temperatures (Marshall 1959). Thus the hot Australian summer, especially in inland regions, may well have aided the survival of naturally infected wild rabbits with some degree of inherent resistance to myxomatosis (Marshall 1959; Marshall and Douglas 1961).

Studies during the 1950s illustrated the rapid development of genetic resistance of wild rabbits to myxomatosis. In one of these studies, rabbit kittens were taken from the Lake Urana (NSW) area each year prior to the seasonal epidemic of myxomatosis (Marshall and Fenner 1958). Kittens were not challenged until at least 4 months of age. Only kittens that were seronegative with respect to myxoma virus were used in resistance experiments as these animals had presumably not previously been exposed to myxoma virus. The kittens were challenged with myxoma virus and the clinical signs of disease recorded. The strain of myxoma virus used, KM13, was of grade III virulence and killed 90% of inoculated wild rabbit kittens at the beginning of the study (Figure 1.7). After the fourth epidemic in the Lake Urana area, the mortality rate of challenged kittens had fallen to approximately 50%, and further decreased to approximately 26% by the seventh epidemic (Figure 1.7).

The development of resistance was also illustrated by the changes in the severity of clinical disease, with the proportion of infected rabbits exhibiting mild clinical symptoms increasing from 0-2% to 30% over the seven epidemics (Marshall and Fenner 1958; Marshall and Douglas 1961; Fenner and Ratcliffe 1965). The decrease in severity of clinical signs as rabbits became increasingly resistant to myxomatosis was an important factor in the field, as rabbits with mild clinical signs were more likely to

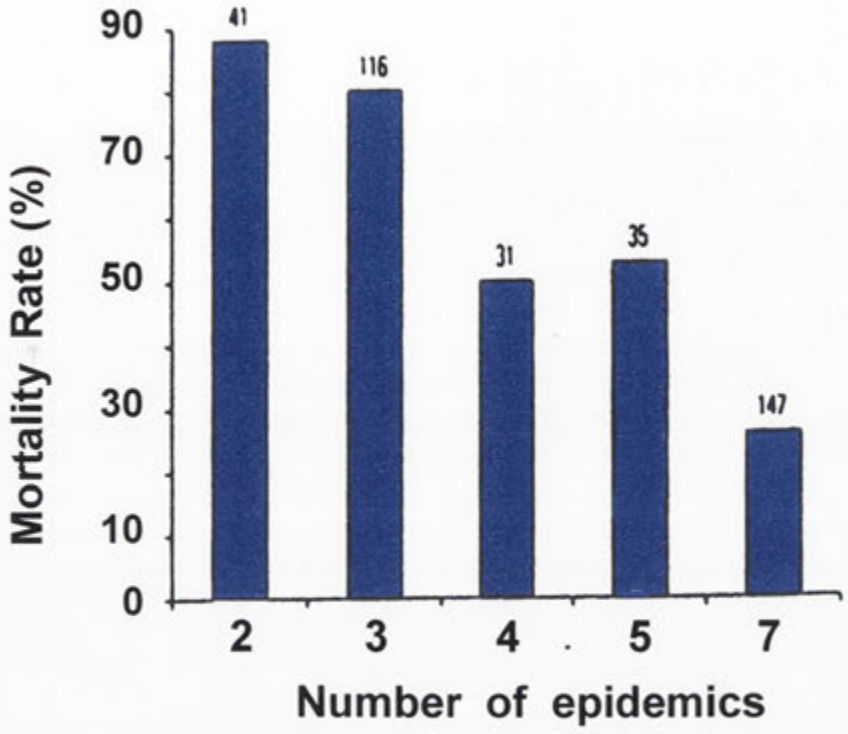


FIGURE 1.7. DEVELOPMENT OF RESISTANCE OF WILD RABBITS TO MYXOMATOSIS

The mortality rate of wild rabbit kittens from the Lake Urana area challenged with the KM13 strain of myxoma virus (grade III virulence) was determined. The mortality rate is expressed as a percentage of total number of rabbits tested (numbers above each column). Rabbits were caught and challenged after 2, 3, 4, 5 or 7 epidemics. Graph from Kerr and Best (1998) from data in Marshall and Fenner (1958) and Marshall and Douglas (1961).

avoid predator attack and less likely to succumb to dietary pressures during the progression of the disease, compared to rabbits with severe clinical signs, which may have survived in cage trials (reviewed in Kerr and Best 1998).

1.5.3 Co-evolution in Recent Years

Co-evolution between myxoma virus and the Australian wild rabbit is still occurring. Rabbit resistance has developed to the point where strains of myxoma virus with intermediate virulence, as judged in laboratory rabbit assays, are quickly controlled by the wild rabbit host. The mortality rate of the present day wild rabbits infected with grade I strains of myxoma virus, such as SLS, is often less than 50% (Kerr et al., 2003).

For titres of myxoma virus to be maintained over the threshold for effective transmission in the skin of these rabbits, the virus has had to become increasingly virulent. This has been observed in recent years with the re-emergence in the field of myxoma virus strains of grade I virulence, as measured in naïve laboratory rabbits (Saint et al., 2001).

1.6 Interactions Between the European Rabbit and Myxoma Virus

The inherent resistance of an infected rabbit to myxomatosis combined with the virulence of the infecting myxoma virus strain, intercurrent disease and the environment will determine whether the rabbit restricts virus replication, clears the virus from its system and recovers from disease, or whether virus replication is unrestrained, the rabbit develops severe myxomatosis, fails to clear the virus and eventually dies. The outcome of this dynamic interaction is ultimately determined by the interaction of the host and pathogen at the cellular and molecular level.

1.6.1 Cell Permissivity for Virus Replication

All viruses require cellular resources for replication. If a virus cannot replicate in a cell then it will not survive in that host and subsequently will not be propagated to a new host. The ability of a virus to replicate in a cell, or the ability of the cell to allow virus replication, defines the permissivity of that cell to virus replication. The permissivity of a cell to virus replication is critical for virus propagation and for host-virus interactions. Differences in cell permissivity for virus replication are hypothesized as the mechanism for attenuation of some viruses and for host resistance to some viruses (Brinton et al., 1998). Mechanisms of virus attenuation and of host resistance can affect one or more

stages in the virus replication cycle. These stages include attachment of the virion to the cell membrane, entry of the virion into the cell, transcription of early and late viral genes, replication of the viral genome, virion assembly and maturation, and virion egress. If the virus cannot interact appropriately with cellular proteins and maintain the viability of the cell, then virus replication will be severely limited.

1.6.1.1 Cell Permissivity for Virus Replication: Considerations for Myxoma Virus Attenuation

A reduced ability of the virus to infect a host cell, due to changes in the envelope proteins of the virion, has been shown as the mechanism of attenuation for some viruses, including Japanese encephalitis virus (Ni and Barrett 1998), yellow fever virus (Dunster et al., 1999), vaccinia virus (Dallo and Esteban 1987; Engelstad and Smith 1993; McIntosh and Smith 1996; Mathew et al., 1998) and tick-bourne encephalitis virus (Labuda et al., 1994). Other attenuated viruses, such as attenuated avian-human influenza A virus, are able to gain entry into the cell but cannot effectively interact with the host cellular machinery (Snyder et al., 1987) or, like Sendai virus, cannot prevent induction of cell death (apoptosis) on infection (Itoh et al., 1998).

The ability of some *Leporipoxviruses* to replicate in infected rabbit lymphocytes *in vitro* has been shown to correlate with virus virulence in rabbits. Shope fibroma virus (SFV), which is highly attenuated in rabbits, does not replicate in rabbit lymphocytes *in vitro* as it cannot effectively inhibit virus-induced cellular apoptosis (Strayer et al., 1985; Macen et al., 1996). Additionally, recombinant myxoma viruses with one of M011 (M11L, Opgenorth et al., 1992a; Macen et al., 1996), M002 (MT2, Macen et al., 1996), M004 (MT4, Barry et al., 1997) or M005 (MT5, Mossman et al., 1996) ORFs deleted are all unable to replicate in rabbit lymphocytes; of these modified viruses all result in

apoptosis in infected lymphocytes *in vitro* and are all highly attenuated in rabbits. Interestingly, all of these deletion mutants are able to replicate to similar levels to wild type virus in fibroblast cell lines and, for the M005 deletion virus, in monocytic cell lineages (Mossman et al., 1996). This suggests that it is the ability of *Leporipoxviruses* to replicate in lymphocytes that is critical for pathogenesis.

Investigation of replication of the virulent and attenuated strains of myxoma virus *in vitro* has been limited. Preliminary work has shown that both the virulent SLS and attenuated Ur strains of myxoma virus are able to infect and replicate in the rabbit $CD4^+$ T-cell line, RL-5 (Dr P. Kerr, personal communication) and in primary rabbit lymphocytes (Best and Kerr 2000). However, significant differences between the two virus strains in their ability to replicate in these cells were not observed. These preliminary studies suggest that attenuation of the Ur strain of myxoma virus is not due to differences in replication. The details of these studies will be considered in more detail in Section 1.7 and in chapter 3. Thus, it is not known whether natural attenuation of myxoma virus is related to changes in the ability of the virus to infect and replicate in primary rabbit cells.

1.6.1.2 Cell Permissivity for Virus Replication: Considerations for Resistance to Myxomatosis

A change in cell permissivity to virus replication has also been postulated as a mechanism of host resistance to a virus (e.g. mice resistant to flavivirus infection, reviewed in Brinton and Nathanson 1981). Investigation of the permissivity of primary rabbit cells to myxoma virus replication *in vitro* is also limited. One study showed no difference in replication of the SLS strain of myxoma virus in primary fibroblast or lymphocytes from laboratory (susceptible) or wild (resistant) rabbits (Best and Kerr

2000). However, differences in cell permissivity to virus replication may not have been detected because the cells were not stimulated with mitogen, which may increase permissivity. This will be further discussed in chapter 3 of this thesis. Any difference in the permissivity of a cell for virus replication may be sufficient for reduced virus replication and reduced expression of immunomodulatory proteins, which may help the host mount an effective anti-viral immune response and quickly control and clear the virus.

The current hypothesis for resistance of rabbits to myxomatosis is that the rabbit controls systemic spread of myxoma virus and reduces virus replication in distal tissues. This is hypothesized to be due to an enhanced effective, early innate immune response, controlling virus replication enough to allow development of an effective adaptive cellular immune response (Kerr and McFadden 2002). Any changes in the virus or in the permissivity of rabbit lymphocytes that decreases the ability of the virus to replicate may aid control of the virus by the rabbit and thereby contribute to resistance.

1.6.2 Host Response to Virus Infection

The mammalian immune response is made up of two interconnected systems; the innate and the adaptive immune responses. Both of these systems are activated on virus infection. The innate immune response can alter the permissivity of a cell to virus replication by production of interferon, whereas the adaptive immune response is, responsible for generating immunological memory. Both these responses aid the control and clearance of the virus by the host. The activities of the innate and adaptive immune responses require interaction and co-operation.

1.6.2.1 The Innate Immune Response

The innate immune response is the first line of defense against pathogens and includes the physical barrier of the skin and mucous membranes as well as having a cellular component. Although the innate immune response is non-specific, its activity ultimately determines the specific, adaptive immune response (reviewed in Medzhitov and Janeway 1997). The innate immune response involves effector cells, such as natural killer (NK) cells, macrophages, neutrophils and the complement proteins (reviewed in Parkin and Cohen 2001).

The innate immune response has a variety of ways to combat virus infections. In the extracellular environment, complement proteins can opsonise virions thereby inducing their phagocytosis by macrophages and neutrophils (reviewed in Greenberg and Grinstein 2002; Uthaisangsook et al., 2002). If a virus is inside a cell, interferon (IFN) -γ and tumor necrosis factor (TNF) can inhibit viral replication and TNF can directly kill some virus-infected cells (reviewed in Benedict et al., 2003). In addition, the type II interferons (IFNα and IFNβ) are also key components of the anti-viral innate immune response and are secreted by fibroblasts and leukocytes.

Natural killer cells and macrophages are also able to directly kill virus-infected cells. The activity of these cells is regulated by a balance between stimulatory and inhibitory signals. The inhibitory signals are given by major histocompatibility complex (MHC) -I expression on the surface of cells (reviewed in Raulet et al., 2001; French and Yokoyama 2003). If a cell expresses abnormally low MHC-I levels, which is common in virus infection, then NK cells will induce lysis of that cell. Natural killer cells induce apoptosis in their target cells by either formation of a pore in the target cell membrane and release of granzyme B into the cell or by signaling through the Fas receptor

(reviewed in Parkin and Cohen 2001). Natural killer cells are activated by IFNα and IFNβ, which are secreted by virus-infected cells (reviewed in Decker et al., 2002). Interferon-α and IFNβ can also induce a non-permissive state in cells for virus replication (reviewed in Decker et al., 2002). Other cytokines also play roles in this response. On activation, NK cells secrete IFNγ and interleukin (IL) -2. Interferon-γ secretion by NK cells is increased by TNF and IL-12 (reviewed in Trinchieri 2003). Tumor necrosis factor also induces expression of the IL-2 receptor α-chain, which then enables IL-2 to stimulate NK cell proliferation and activation (reviewed in Parkin and Cohen 2001). The actions of the innate immune response result in a non-specific defense against the virus and, because of the general nature of the response, are often not sufficient to resolve virus infection. To provide a specific immune response against the virus, an adaptive immune response is activated. The activation of the adaptive immune response is aided by expression of cytokines during the innate immune response.

1.6.2.2 The Adaptive Immune Response

The adaptive immune response is a specific response against the invading pathogen and involves T- and B-cells. T-cells are activated by antigen presenting cells (APCs). Cells able to present antigen to effector cells include activated B-cells, dendritic cells and macrophages. APCs present antigen in association with MHC-I (expressed on all nucleated cells) or MHC-II (expressed on APCs cells). MHC-I presents peptides from processed antigen derived from the intracellular environment such as viral antigen, whereas MHC-II presents peptides from processed antigen derived from the extracellular environment, such as B-cell processed antigen or soluble toxins.

Two major types of T-cells, cytotoxic T-lymphocytes (CTLs, $CD8^+$ T_C-cells) and helper T-cells ($CD4^+$ T_H cells) have been identified in mice and similar subsets have been identified in other species, such as human. Cytotoxic T-lymphocytes recognize intracellular-derived antigen associated with MHC-I and respond by killing virus-infected cells, whereas T_H cells recognize and respond to antigen in association with MHC-II and are involved in mediating interactions between cells of the immune system during an immune response (Santana and Rosenstein 2003). Activation of murine $CD4^+$ T-cells leads to generation of T_H1 or T_H2 T-cell subsets (reviewed in Asnagli and Murphy 2001). The induction of a T_H1 or T_H2 immune response, by T_H1 and T_H2 $CD4^+$ T-cell subsets respectively, can determine the outcome of the virus/host interaction. Classification of T_H cells into these subsets originated in mouse models and is a useful tool for categorizing immune responses. $CD8^+$ T-cells have also been shown to differentiate into T_C1 and T_C2 sub-types based on cytokine secretion (reviewed in Parkin and Cohen 2001). The role of these subsets is still the subject of research.

T_H cell subsets identified in mice are defined in Table 1.4. The immune response mediated by T_H1 $CD4^+$ T-cells is classified as the anti-viral cell-mediated immune response and is characterized by production of IFNγ and IL-2 by activated T_H1-cells (Santana and Rosenstein 2003). Interleukin-2 stimulates proliferation of T-cells and proliferation and functioning of NK cells (reviewed in Karupiah et al., 1990; Kuby 1997). Interleukin-2 also promotes the differentiation of $CD8^+$ T-cells into CTLs (reviewed in Parkin and Cohen 2001). As previously described, IFNγ can inhibit virus replication in infected cells. IFNγ can also enhance macrophage activity, increase MHC-I and MHC-II expression, thereby enhancing antigen presentation. IFNγ inhibits the development of T_H2 cells and the T_H2 immune response.

TABLE 1.4. T_H-CELL SUBSETS

T_H-cell Subset	Function of Subset	Cytokines Secreted	Source	Target Cells	Cytokine Functions
T_H1	• Cell-mediated response • Activation of CTLs	IL-2	• T_H1 cells	• $CD4^+$ T-cells • $CD8^+$ T-cells • Some NK cells	• T-cell proliferation • NK cell proliferation and function • Differentiation of $CD8^+$ T-cells
		IFNγ	• T_H1 cells • CTL • NK cells	• Unifected cells • Macrophages	• Inhibits viral replication • Enhances macrophage activity • Increases expression of MHC-I and MHC-II • Inhibits T_H2 response
T_H2	• Antibody-mediated response • B-cell activation	IL-4	• T_H2 cells • Mast cells • NK cells	• B-cells • T-cells • Macrophages • Mast cells	• Stimulates proliferation and differentiation of B- and T-cells • Upregulates MHC-II expression on B-cells • Increases phagocytic activity • Inhibits T_H1 response
		IL-5	• T_H2 cells • Mast cells	• Activated B-cells • Eosinphils	• Stimulates proliferation and differentiation of eosinophils (and B-cells in mice)
		IL-6	• T_H2 cells • Monocytes/ macrophages	• Proliferating B-cells • Plasma cells	• Promotes terminal differentiation of B-cells • Stimulates antibody secretion
		IL-10	• T_H2 cells	• Macrophages • Antigen presenting cells	• Suppresses cytokine production • Down-regulates MHC-II expression • Antagonizes effects of IL-2 and IFNγ
T_H3	• Regulation of T_H1 and T_H2 responses	TGFβ	• T_H3 cells	• T_H1 and T_H2 cells	• Anti-proliferative function • Inhibits T_H1 and T_H2 responses

Information obtained from Kelso, A (1998) Cytokines: Principles and prospects. *Immunol Cell Biol* **76**(4);300-317.

The T_H2 immune response primarily involves events leading to the production of antigen-specific antibodies (reviewed in Parkin and Cohen 2001). Activation of $CD4^+$ T_H2 cells leads to the production of IL-4, IL-5, IL-6, and IL-10 (Table 1.4) (reviewed in (Asnagli and Murphy 2001). IL-4, IL-5 and IL-6 stimulate the differentiation and proliferation of B-cells and, for IL-5, eosinophils (Parkin and Cohen 2001). Eosinophils are involved in protection against parasites. IL-4 also increases MHC-II expression on B-cells, enhances phagocytic activity and inhibits the T_H1 immune response. IL-10 antagonizes the effects of T_H1 cytokines such as IL-2 and IFNγ and decreases MHC-II expression and cytokine production.

There is also evidence of a T_H3 cytokine pattern produced by murine $CD4^+$ T-cells (reviewed in Coffman and Romagnani 1999; Mosmann and Sad 1996). The T_H3 response has not been well documented, but is characterized by the secretion of transforming growth factor (TGF) -β. TGFβ has an anti-proliferative effect and inhibits leukocyte activation. $CD4^+$ T_H3 cells do not secrete IL-2, IFNγ, IL-4 or IL-10 and inhibit both the T_H1 and T_H2 immune responses (reviewed in Coffman and Romagnani 1999; Mosmann and Sad 1996).

For viral infections, the overall aim of the adaptive immune response is to activate a response that is specific for the invading virus and that ultimately controls and clears the virus. The adaptive immune response also generates immunological memory so that subsequent infections will be more quickly controlled.

1.6.2.3 The Rabbit Immune System

Although the rabbit has been extensively used in immunology for production of anti-sera, it has not been the animal of choice for delineation of immunological concepts

with the majority of the data regarding the host immune response delineated using mice or *in vitro* assays. The ability to extensively examine the rabbit immune system and thereby determine the applicability of the murine and *in vitro* work to the rabbit, is severely hindered by the paucity of rabbit-specific reagents and the fact that inbred rabbits are not routinely available. However, it has been shown that the rabbit immune system is functionally similar to that of other mammals (reviewed in Mage 1998). The rabbit has a complement system and array of MHC antigens and leukocyte markers that are similar to those identified in mice and humans (reviewed in Mage 1998). The rabbit T-cell population has been shown to contain $CD8^+$ and $CD4^+$ T-cells. However, different to the mouse, rabbit peripheral blood leukocytes contain a population of cells that express both T- and B-cell markers. For example, both activated T-cells and B-cells express MHC-II (reviewed in Mage 1998). The importance of this for the rabbit immune system is not clear. However, generally it can be concluded that the rabbit immune response will involved similar activities to those described in Sections 1.6.2.1 and 1.6.2.2.

1.6.2.4 T-cell Activation and Proliferation

Presentation of viral antigen by APCs to T-cells initiates interactions between a variety of immune cells and accessory molecules, leading to activation of multiple T-cell signaling pathways and transcription of genes involved in T-cell differentiation, proliferation and clonal expansion (Hollsberg 1999). The interaction of IL-2 with the IL-2 receptor is a key event in T-cell proliferation. The IL-2 receptor has three subunits that are not all continuously expressed. Expression of the α-subunit (CD25) is regulated by T-cell activation signals. Removal of these signals stops expression of CD25, thereby curtailing subsequent IL-2 signaling and cell proliferation (Cantrell and Smith 1984).

Cell proliferation involves the progression of cells through the cell cycle. Figure 1.8 shows the stages of the eukaryotic cell cycle, which have been elucidated by studying proliferation of yeast (reviewed in Bartek and Lukas 2001; Stewart and Pietenpol 2001). Quiescent cells are in G0 phase of the cell cycle. If these cells are activated, they move into G1 phase where the cell prepares for DNA replication. The G1 checkpoint ensures there is no damaged DNA and that the environment is favorable for the cell to divide before the cell enters S phase (Stewart and Pietenpol, 2001). After DNA replication, which occurs in S phase, the cells enter G2 phase. G2 is the gap between DNA replication and mitosis (M). The G2 checkpoint ensures that all DNA has replicated successfully prior to cell division.

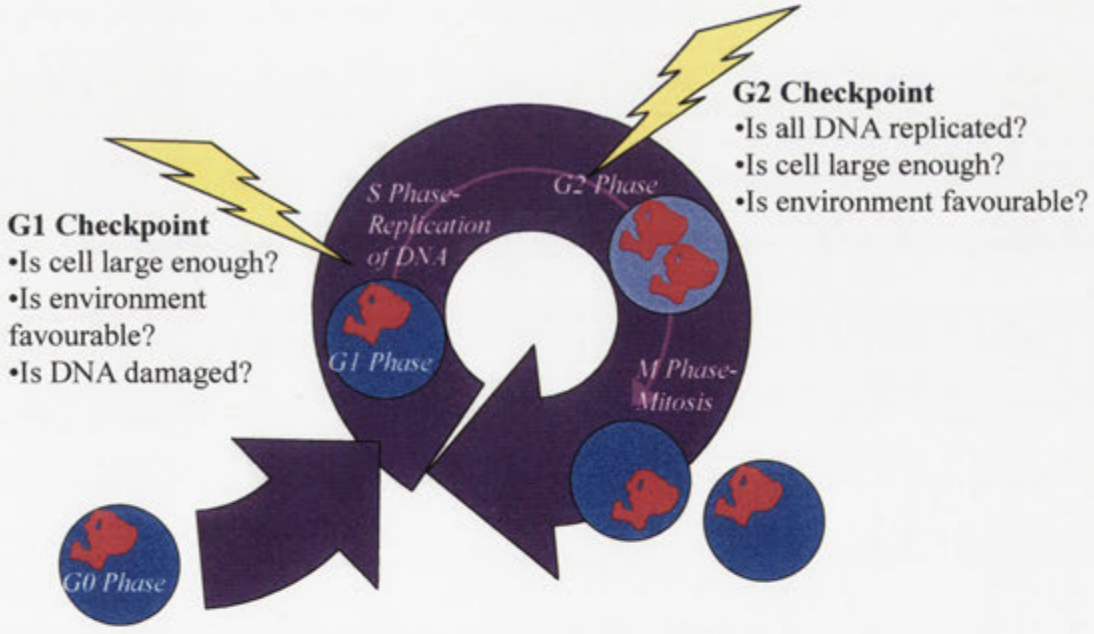


FIGURE 1.8. THE EUKARYOTIC CELL CYCLE

The stages of the eukaryote cell cycle. Cell activation stimulates cells in G0 to enter G1 phase. After the cells pass through the G1 checkpoint, S phase is entered. DNA replication occurs and then the cell enters G2. Passage through the G2 checkpoint leads to M phase (mitosis) and cell division. Figure adapted from Alberts et al. (1994).

Both the G1 and G2 checkpoints ensure that the cell is large enough to continue through the cell cycle. The role of cell size checkpoints in cell cycle progression of mammalian cells is unclear.

A recent study showed that Schwann cells (adherent neural cells) did not co-ordinate their growth with cell cycle progression (Conlon and Raff 2003). However, this finding may not apply to lymphocytes and other cells that can proliferate in suspension, coordinating cell growth with cell cycle progression as just described for yeast.

1.6.2.5 Apoptosis

Cell cycle progression is inextricably linked to programmed cell death (apoptosis) as failure of a cell to pass a checkpoint induces apoptosis (Kerr et al., 1972; Wyllie et al., 1980). Apoptosis is also an essential part of the immune response through death of infected or unhealthy cells and by regulation of the immune system. There are three main phases of apoptosis: induction, effector and degradation. These are presented in Figure 1.9. Apoptosis can be induced through a 'death receptor', such as Fas or TNFR1, through CTL or NK cell activity or external stimuli such as cellular stress. Apoptosis is effected through proteolytic cascades involving caspases (cysteine proteases), cytochrome c and other death substrates (e.g. poly(ADP-ribose) polymerase, lamins, and cell cycle regulators). The effector stage commits the cell to apoptosis. Once the cell is committed to apoptosis, the degradation phase begins. This last phase leads to the morphological features of apoptosis, which are characterized by nucleolysis, chromatinolysis, proteolysis and cytolysis.

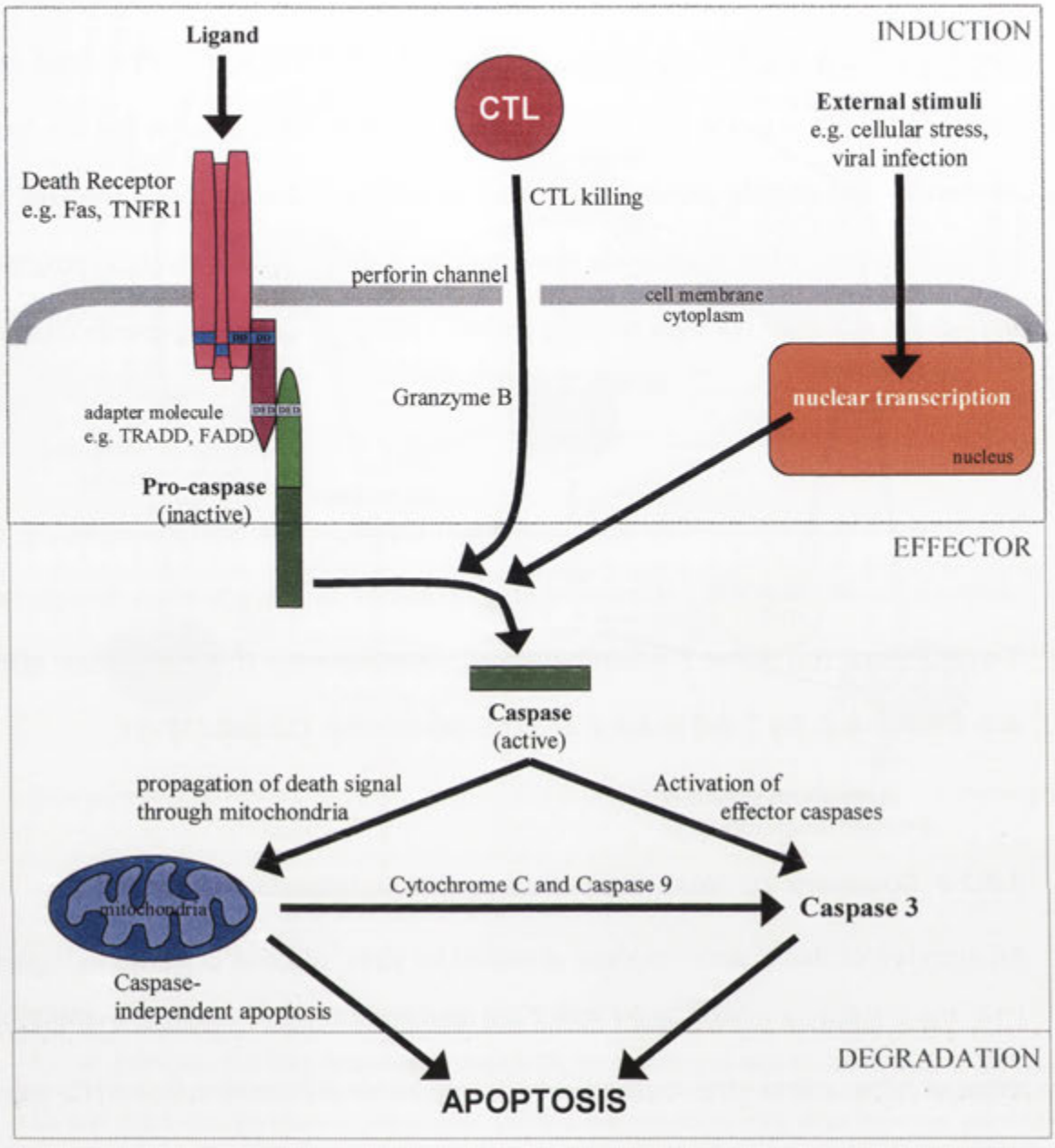


FIGURE 1.9. APOPTOSIS

The three main phases of apoptosis- induction, effector and degradation, and the major factors involved in each is depicted. Induction of apoptosis can be through either interaction with a death receptor, cytotoxic (CTL) killing or external stimuli, such as virus infection. Apoptotic signals are transduced via caspases during the effector phase, leading to the degradation phase where the morphological changes of the cell are observed. Figure is adapted from Turner and Moyer (1998) and Everett and McFadden (2001).

The stage of the cell cycle that a cell is in determines its susceptibility to apoptosis (Buttyan et al., 1988; Colotta et al., 1992; Marti et al., 1994; Nishioka and Welsh 1994). Activation of various cyclins and cyclin-dependent kinases, which are proteins involved

in cell cycle regulation and progression, have been shown to correlate with the onset of apoptosis (Shi et al., 1994; Meikrantz and Schlegel 1996; Yao et al., 1996; Evan and Littlewood 1998; Guo and Hay 1999). There has recently been a report that the Fas-associated death domain protein (FADD) has an additional domain that is involved in cell proliferation. When this domain is altered it abolishes the cell's ability to progress through the cell cycle but does not alter the cell's ability to undergo apoptosis (Hua et al., 2003).

The previous experiences of the lymphocyte also appear to determine susceptibility to apoptosis. In one study, IL-2 stimulation of lymphocytes prior to activation through the T-cell receptor (i.e. naïve T-cells) led to cell death, whereas IL-2 stimulation after activation through the T-cell receptor led to cell proliferation (Lenardo 1991).

1.6.2.6 Summary of Host Response to Virus Infection

An overview of the immune response generated by virus infection is shown in Figure 1.10. Virus infection initiates both innate and adaptive immune responses. The innate response helps control virus replication by killing of virus-infected cells by NK cells and by production of factors, such as IFNα and IFNβ, which induce an anti-viral state in cells. Infected cells can also undergo programmed cell death (apoptosis). The innate response ultimately determines the adaptive immune response as it is the first response the host initiates against the invading pathogen. The adaptive immune response against a virus involves activation of $CD8^+$ T-cells, which directly kill infected cells and $CD4^+$ T-cells, which differentiate into T_H1 or T_H2 cell subsets. The T_H1 response activates NK cells and CD8+ T-cells and stimulates IFNγ production. The T_H2 response helps mediate the activity of B-cells and other APCs. Activation of these cells in turn helps

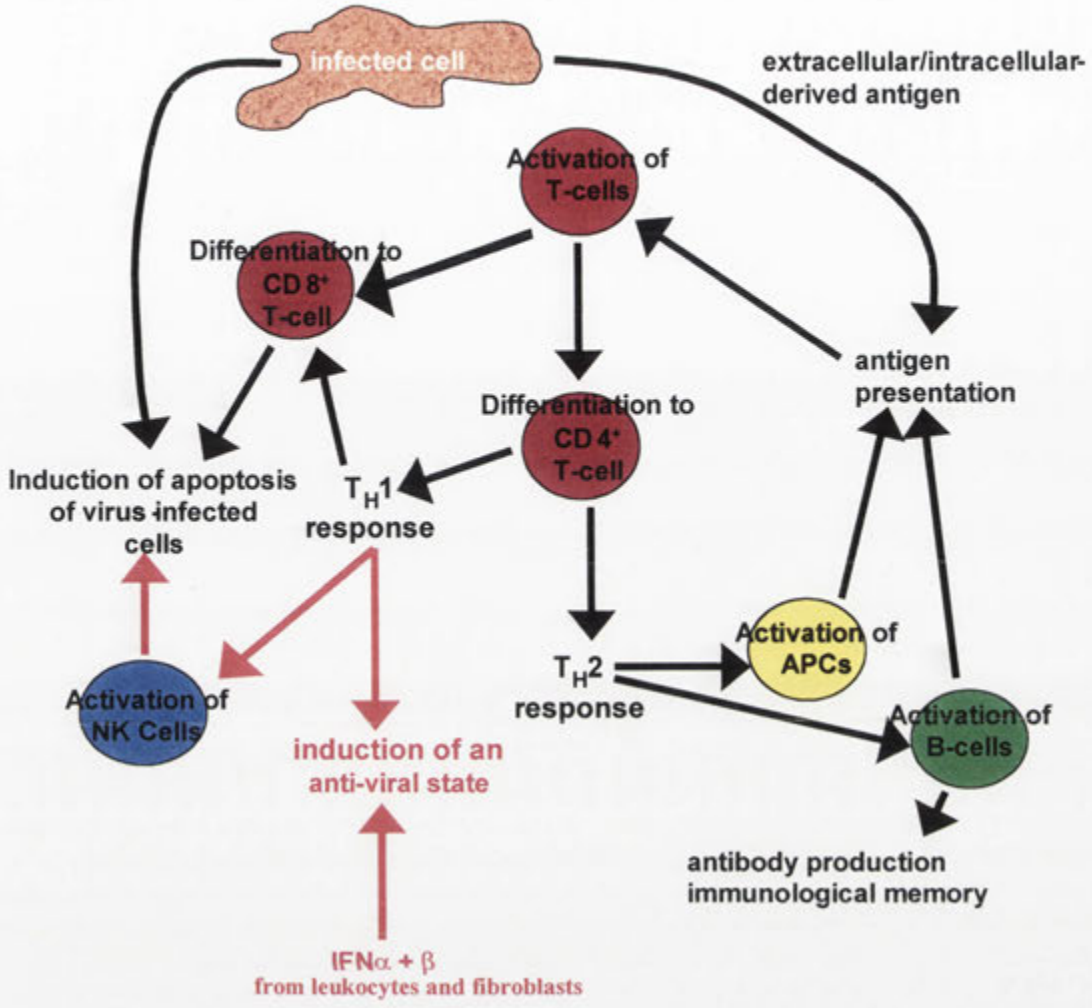


FIGURE 1.10. HOST IMMUNE RESPONSE TO VIRUS INFECTION

On virus infection, the host responds through both the innate (red arrows) and adaptive (black arrows) immune responses. The innate response is non-specific and includes natural killer (NK) cells and cytokines, particularly interferons (IFNs), and acts to control virus infection prior to activation of the specific adaptive immune response. Induction of antigen-specific $CD4^+$ T-cells during the adaptive immune response leads to generation of a T_H1 or T_H2 response. T_H1 response activates $CD8^+$ T-cells. $CD8^+$ T-cells and NK cells non-specifically kill virus-infected cells. Virus-infected cells may also undergo apoptosis without $CD8^+$ T-cell or NK cell help. The T_H2 response activates antigen presentation by antigen presenting cells (APC). APCs present extracellular and intracellular-derived viral antigen to T-cells. Activation of B-cells leads to production of virus-specific antibody, which can neutralise virus particles and generate immunological memory.

activate more T-cells through antigen presentation, and leads to antibody production and immunological memory.

1.6.3 Specific Action of a Virus Against the Host Immune Response

The outcome of a virus infection is determined by the interaction between the virus and the cells of the host immune system. For poxvirus infections, $CD8^+$ T-cells, $CD4^+$ T-cells, IFNγ, nitric oxide and TNF are all involved in control of poxvirus replication (Ruby and Ramshaw 1991; Sambhi et al., 1991; Karupiah et al., 1998) and in poxvirus clearance from the host (Spriggs et al., 1992; Karupiah et al., 1996; Karupiah 1998). The production of antigen-specific antibodies is not crucial for control of poxvirus infection but may be essential for complete clearance of the virus (Karupiah 1998).

An effective host anti-viral immune response has been postulated to be the mechanism of host resistance to some poxviruses. For example, various studies have postulated that resistance of inbred mouse strains to ectromelia virus infection is associated with an increased CTL–mediated immune response (O'Neill and Brenan 1987), early enhanced non-specific host defenses, such as NK cells and IFNα/β (Jacoby et al., 1989) and induction of a T_H1 cytokine response as opposed to a T_H2 cytokine response (Karupiah 1998). Genetic analyses of mice resistant to ectromelia virus infection have determined that the main genetic component is in gene(s) within the natural killer gene complex (Delano and Brownstein 1995). In studies of other viruses early IFN production has been implicated in resistance of mice to encephalomyocarditis virus infection (Pozzetto and Gresser 1985) and herpes simplex virus type 2 infection (Pedersen et al., 1983).

Many viruses have evolved mechanisms to mediate, modify or survive the host anti-viral immune response by directly interfering with the activity of the cells of the host immune system. For example, inhibition or inappropriate activation of T-cell proliferation or cellular apoptosis may have a drastic effect on the outcome of virus

infection as these functions are central to the host anti-viral immune response. Some of the mechanisms developed by viruses to interfere with T-cell proliferation and cellular apoptosis will be described in the following sections.

1.6.3.1 T-cell Proliferation and Virus Infection

Inhibition of T-cell proliferation leads to improper T-cell functioning and immunosuppression. Viruses can abrogate cell proliferation by arresting the cell cycle. For example, herpes simplex virus type I arrests cells at the G1/S boundary in growing cells and entry to G1 in quiescent cells, by degrading a component of human kinetochores (Lomonte and Everett 1999) and by inhibiting the increase in cyclin D1 and D3, which are involved in progression through G1 (Ehmann et al., 2000; Song et al., 2000). Measles virus (Schnorr et al., 1997; Engelking et al., 1999), bovine herpesvirus type 1 (Hanon et al., 1997) and human papilloma virus (Wiman 1993; Lee and Cho 2002) arrest cells in the G1 or G0 stage of the cell cycle.

Other viruses have been shown to alter T-cell function by stimulating cell cycle progression. For example, productive HIV-I replication requires T-cell proliferation (Forsdyke 1991), as non-dividing (G0) T-cells restrict virus replication at a stage prior to import of viral DNA into the nucleus (Stevenson et al., 1995). Adenovirus, herpesviruses and human papillomavirus have also been shown to stimulate cell cycle progression by either inhibiting the activity of negative cell cycle regulators or by enhancing the activity of positive cell cycle regulators (reviewed in Swanton and Jones 2001).

Poxviruses have been shown to alter T-cell function by either stimulating or inhibiting T-cell proliferation. For example, inactivated parapox virus stimulates proliferation of

$CD4^+$ T-cells leading to increased production of the T_H1 anti-viral cytokines IL-2, TNF and IFNγ (Fachinger et al., 2000). Malignant rabbit fibroma virus (MRV), a recombinant between SFV and myxoma virus, suppresses T-cell proliferation (Strayer et al., 1983; Strayer et al., 1986a; Strayer et al., 1986b; Strayer and Leibowitz 1986; Heard et al., 1990). This suppression has been postulated to be due to a T-cell derived factor that stimulates T-cell suppression mechanisms. The MRV-induced suppression of lymphocyte proliferation is postulated to be directly related to the ability of MRV to replicate in lymphocytes (Strayer et al., 1985; Stern et al., 1997; Heard et al., 1990).

Other poxviruses can inhibit or stimulate lymphoid cell proliferation depending on the system studied. Vaccinia virus has been shown to transiently inhibit proliferation of peripheral blood mononuclear cells (Mathew et al., 2000) and stimulate proliferation of human leukocytes (McFarland et al., 1980) and rabbit B- and T-cells (Elfenbein and Rosenberg 1973). Fowlpoxvirus can also stimulate proliferation of epithelial cell lines (Tanizaki et al., 1989), whereas variola virus inhibits proliferation of epithelial cell lines (Ono and Kato 1968).

1.6.3.2 Apoptosis and Virus Infection

Apoptosis can be initiated by an infected cell (i.e. cell suicide), or can be induced in virus-infected cells by CTLs or NK cells. Many viruses have evolved mechanisms to either inhibit or stimulate apoptosis. By inhibiting apoptosis, and therefore keeping the host cell alive, a virus will prolong the period it has to replicate and produce infectious progeny. On the other hand, induction of apoptosis by a virus may assist dissemination of the virus, by freeing intracellular virions (reviewed in McFadden and Barry 1998; Everett and McFadden 1999). Apoptosis may also aid suppression of the anti-viral immune response, as phagocytic cells are induced to secrete immunosuppressive

factors, such as IL-10, on engulfment of apoptotic cells (Voll et al., 1997) and apoptotic cells themselves secrete TGF-β which inhibits T_H1 and T_H2 responses (Chen et al., 2001).

There are a number of different viral mechanisms to mediate apoptosis. Homologs of the apoptosis inhibitor, Bcl-2, are encoded by Epstein Barr virus (BHRF-1) (Henderson et al., 1993), adenovirus (E1B-19K) (Rao et al., 1992) and African swine fever virus (A179L/5-HL) (Brun et al., 1996). Inhibitors of caspase activity are encoded by African swine fever virus (A224L/4CL) (Nogal et al., 2001) and baculoviruses (p35, IAP) (Bertin et al., 1996; Zoog et al., 1999). Poxviruses also encode a variety of anti-apoptotic factors, such as serpins and caspase inhibitors. Those encoded by myxoma virus will be described in Section 1.6.4.1. Extensive reviews of other virus-encoded anti-apoptotic factors can be found in Collins (1995), Razvi and Welsh (1995), Everett and McFadden (1999) and Roulston et al. (1999).

1.6.4 Immunomodulation by Myxoma Virus

Myxoma virus encodes proteins that interfere with the innate and adaptive immune responses, cell proliferation and apoptosis. Table 1.5 summarizes the putative immunomodulatory, anti-apoptotic or host-range proteins identified by Cameron et al. (1999), their promotor type (early, intermediate or late), conservation between different poxviruses and predicted cellular localization. These characteristics determine when and where virus-encoded proteins act and suggest potential roles during myxoma virus pathogenesis. Myxoma virus immunomodulatory proteins include growth factors as well as proteins that interfere with antigen presentation, recognition and co-stimulation. These have been recently reviewed by Kerr and McFadden (2002), Zuniga (2002) and Everett and McFadden (2002).

TABLE 1.5. IMMUNOMODULATORY, ANTI-APOPTOTIC AND HOST-RANGE PROTEINS ENCODED BY MYXOMA VIRUS

ORF*	Promotor Type	Conservation within poxviruses	Predicted Localization	Putative Function/ Structure of Protein
M001	Early	semi-conserved	extracellular	chemokine binding protein
M002	Early	semi-conserved	extracellular / intracellular	TNF receptor homolog apoptosis regulator
M004	Early	semi-conserved	endoplasmic reticulum	apoptosis regulator RDEL motif
M005	Early	semi-conserved	cytoplasmic	apoptosis regulator ankyrin-like, host-range RNase3 domain
M007	Early	semi-conserved	extracellular	IFNγ receptor homolog, α-chain
M008.1	Late	unique	extracellular	anti-inflammatory serpin
M010	Early	semi-conserved	extracellular	EGF-like growth factor
M011	Early	semi-conserved	integral membrane protein	apoptosis regulator
M029	Early	semi-conserved	cytoplasmic	IFN-resistance PKR inhibitor host-range
M062	Early	semi-conserved	cytoplasmic	virulence factor, host-range
M063	Early	semi-conserved	unknown	apoptosis regulator, host-range DAXX-like motif
M064	Early	semi-conserved	cytoplasmic	host-range, virulence factor
M104	Late	semi-conserved	transmembrane	receptor-like fragment
M121	Early	semi-conserved	transmembrane (EEV?)	NK cell receptor homolog EEV glycoprotein C-type lectin
M122	Late	semi-conserved	transmembrane (EEV?)	NK cell receptor homolog EEV glycoprotein C-type lectin domain
M128	Early?	unique	transmembrane	CD47 homolog (integrin-associated protein)
M135	Early	semi-conserved	transmembrane/ extracellular	IL-1/IL-6 receptor-like
M141	Early	unique	transmembrane	OX-2 homolog immunoglobulin domain
M143	Late	semi-conserved	cytoplasmic	apoptosis regulator zinc ring finger protein
M144	Early?	semi-conserved	transmembrane	complement control protein homolog CD46 homolog
M146	Early?	semi-conserved	cytoplasmic?	nonessential virulence factor
M148	Early	semi-conserved	cytoplasmic?	ankyrin-like, host-range
M149	Early	unique	cytoplasmic	ankyrin-like, host-range
M150	Early	unique	cytoplasmic	ankyrin-like, host-range
M151	Early	semi-conserved	cytoplasmic	serpin apoptosis regulator
M152	Early?	unique	cytoplasmic	Zinc finger RNA binding serpin-like
M156	Late	semi-conserved	cytoplasmic	interferon resistance S1 RNA binding e-IF2α homolog

* Open reading frames (ORFs) encoding proteins with putative immunomodulatory function are shown in red. ORFs encoding proteins with putative functions designated as anti-apoptotic or host-range are shown in blue. Host-range proteins mediate the interaction between viral and cellular proteins. Information in table taken from Cameron et al. (1999).

Figure 1.11 shows the steps in the host immune response with which some of these virus-encoded proteins interfere. Examples of these factors include NK cell receptor

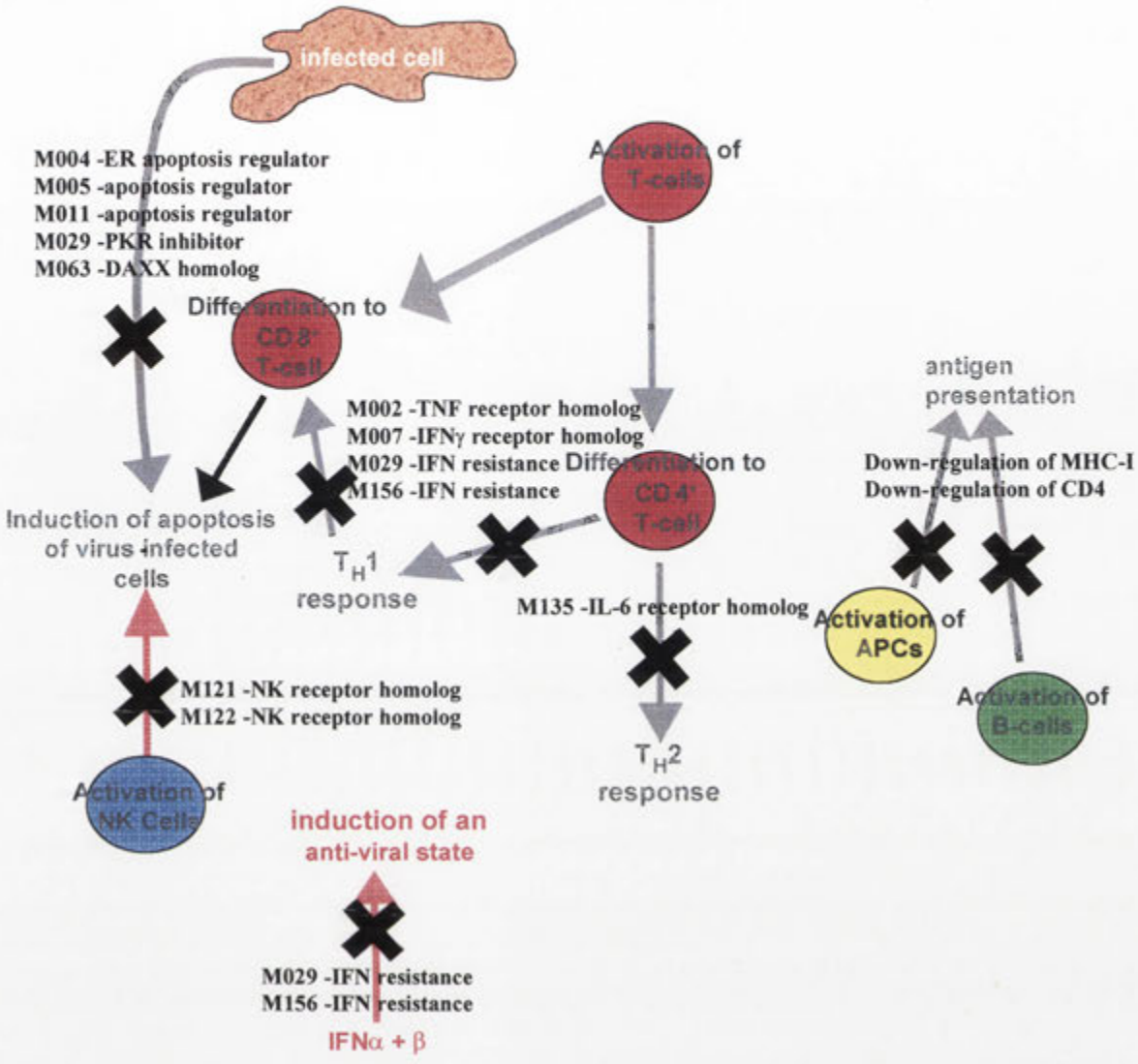


FIGURE 1.11. INTERACTIONS BETWEEN THE HOST IMMUNE RESPONSE AND MYXOMA VIRUS

The cells involved in the host immune response, as described in Figure 1.10, are shown. Myxoma virus encodes factors that interfere with many of these pathways. In particular myxoma virus putatively encodes NK cell receptor homologs and proteins involved in IFN resistance. This disrupts the innate immune response. Myxoma virus also encodes homologs to receptors for cytokines, which help inhibit both T_H1 and T_H2 responses. In addition, myxoma virus interferes with antigen presentation by down-regulating MHC-I and CD4 on the surface of infected cells. Myxoma virus interferes with apoptosis by encoding a variety of anti-apoptotic factors.

homologs, cytokine and chemokine receptor homologs, anti-inflammatory and anti-apoptotic proteins, such as serine proteinase inhibitors (serpins) and caspase inhibitors. Relevant to this thesis are the anti-apoptotic and host-range proteins and the down-regulation of host proteins, which are described in the next section.

1.6.4.1 Anti-Apoptotic and Host-Range Factors

The list of anti-apoptotic factors identified in the myxoma virus genome by Cameron et al. (1999) includes so called 'host-range' proteins. Host-range proteins are postulated to mediate the interaction between viral and cellular proteins and, therefore, determine cell permissivity for viral replication or cell-type able to be infected, rather than species specificity. If the interactions between virus and host proteins fail, due to a defective gene for example, apoptosis may be induced. It is for this reason that they are included with anti-apoptotic factors. Some putative myxoma virus host-range proteins have been identified based on homology to host-range proteins from other species, although none of these has been extensively studied. The following sections describe the current knowledge of myxoma virus-encoded proteins with anti-apoptotic or host-range function.

1.6.4.1.1 M002

The M002 (MT-2) gene encodes a TNF receptor homolog in addition to having an anti-apoptotic domain. Truncated versions of M002 that are unable to bind TNFα are still able to prevent apoptosis, suggesting that these functions are distinct and spatially separated in the native protein (Schreiber et al., 1997). M002 acts intracellularly to block apoptosis. *In vitro,* the M002 deletion virus induced apoptosis of infected RL-5 cells and non-adherent leukocytes, but not adherent leukocytes, such as monocytes and macrophages (Macen et al., 1996). This suggests that M002 plays a role in maintaining the viability of infected monocytes and macrophages.

1.6.4.1.2 M004

The myxoma virus M004 (M-T4) gene is encoded under the control of an early promotor and its protein product localizes to the endoplasmic reticulum (ER) (Cameron et al., 1999). The ER retention-like sequence –RDEL (Arg, Asp, Glut, Leu) in M004 is not essential for ER retention, but its presence increases the biological half-life of M004 (Hnatiuk et al., 1999). Infection of the RL-5 cell line or primary rabbit peripheral blood lymphocytes with the M004 deletion virus results in the rapid induction of apoptosis when compared to wild type virus (Barry et al., 1997). Apoptosis of rabbit peripheral blood mononuclear cells will limit spread of myxoma virus as these cells are involved in virus dissemination. This may account for failure of the M004 deletion virus to produce secondary lesions *in vivo* (Barry et al., 1997).

1.6.4.1.3 M005

M005 (M-T5) acts early in infection to prevent apoptosis. This protein contains seven ankyrin repeat domains. Ankyrin domains are involved in protein-protein interactions and protein folding (Lambert et al., 1990). Infection of RL-5 cells with recombinant virus in which both copies of the M005 gene have been disrupted, abrogates host and viral protein synthesis, leading to apoptosis of these cells (Mossman et al., 1996). Apoptosis did not occur in adherent monocyte or primary rabbit fibroblast cell cultures infected with M005 deletion virus suggesting that M005 is critical for infection of lymphocytes. *In vivo* studies with the M005 deletion virus showed a greatly reduced formation of secondary lesions, suggesting the M005 deletion virus was not capable of systemic dissemination (Mossman et al., 1996). This was postulated to be due to the rapid induction of apoptosis of infected lymphocytes and an effective immune response at the inoculation site (Mossman et al., 1996).

1.6.4.1.4 M011

The M011 gene product, M11L, is a type II transmembrane protein that is targeted to the mitochondria (Everett et al., 2000). It is expressed early in infection and has important anti-apoptotic and anti-inflammatory activity (Opgenorth et al., 1992a; Cameron et al., 1999). *In vitro,* infection of RL-5 cells and cells of the monocyte/macrophage lineage with the M011 deletion virus resulted in apoptosis with extensive DNA fragmentation (Macen et al., 1996; Everett et al., 2000). Further studies revealed the M011 protein blocks staurosporine-induced apoptosis by preventing mitochondrial permeability transition (Everett et al., 2000). *In vivo*, the M011 deletion virus decreased cellular proliferation and hemorrhage in primary lesions, but increased edema and leukocyte infiltration compared to wild type virus (Opgenorth et al., 1992). The M011 deletion virus is highly attenuated *in vivo.* These results suggest that M011 helps to inhibit the pro-inflammatory response by inhibiting apoptosis of infected leukocytes.

1.6.4.1.5 M151

M151 (SERP-2), the second of three putative serpins encoded by myxoma virus, is expressed intracellularly, early in infection (Petit et al., 1996). The M151 deletion virus is attenuated *in vivo* compared to wild type virus (Messud-Petit et al., 1998). Elevated levels of apoptosis in lymph nodes draining the inoculation site and a greater inflammatory response were also observed in rabbits infected with the M151 deletion virus. This protein binds human interleukin-1β converting enzyme (ICE), thereby preventing the processing of pro-IL-1β to IL-1β *in vitro*. M151 also inhibits granzyme B (Turner and Moyer 1998). However, biochemical studies have shown that

M151 is only a weak inhibitor of ICE and granzyme B *in vitro* (Turner et al., 1999). A recombinant cowpox virus with the myxoma virus M151 gene does not block apoptosis in cowpox virus-infected cells (Turner et al., 1999), indicating M151 activity is specific to myxoma virus infection.

1.6.4.2 Additional Immunomodulatory Functions

Myxoma virus also circumvents the rabbit immune response by down-regulating key proteins involved in antigen presentation and co-stimulation. For example, CD4 expression was down-regulated from the surface of RL-5 cells infected with myxoma virus (Barry et al., 1995). During infection, CD4 is targeted to lysosomes by the myxoma virus-encoded ubiquitin ligase, MV-LAP, where it is subsequently degraded (Mansouri et al., 2003). The MV-LAP ORF was identified in the T1 strain of myxoma virus by Guerin et al. (2002), 621 bp downstream of M152. It is a combination of the M153 and M153.1 ORF identified in Lu by Cameron et al. (1999). This latter group subsequently re-sequenced this area of the Lu genome and found that the complete MV-LAP ORF was also present in Lu (Mansouri et al., 2003). Down-regulation of CD4 on infected T_H cells potentially inhibits both the cell-mediated and antibody-mediated immune responses as CD4 is not available for appropriate $CD4^+$ T_H cell functioning. This may potentially cause T-cells and B-cells to become anergic, due to the lack of co-stimulation, and would drastically disrupt the anti-viral immune response (Mueller and Jenkins 1995; Schwartz 1997).

Myxoma virus also induces the endosomal/lysosomal degradation of MHC-I (Zuniga et al., 1999). This viral-induced degradation is not only of cell surface MHC-I, but also of intracellular post-Golgi MHC-I associated with β_2-microglobulin. Even though MHC-I down-regulation is not observed until late in infection (Zuniga et al., 1999), it is

mediated by MV-LAP (Guerin et al., 2002). The degradation of MHC-I has been postulated to provide myxoma virus with a means of reducing MHC-I molecules that are associated with processed viral proteins (Zuniga et al., 1999), and thus inhibiting recognition of virus-infected cells by $CD8^+$ T-cells.

The action of MV-LAP has been proposed to help protect the infected cells from immune surveillance (Guerin et al., 2002). The MV-LAP deletion virus induces fewer secondary lesions, indicating decreased virus dissemination, and milder respiratory complications compared to infection with wild type virus. In addition, the inoculation site of infected rabbits had a predominantly monocytic immune response compared to a predominately polymorph response in wild type virus infections. MV-LAP also down-regulated CD95 (Fas) molecules *in vitro* (Guerin et al., 2002) but a change in lymphocyte apoptosis, as measured by the TUNEL assay, was not observed during *in vivo* infections of the MV-LAP deletion virus (Guerin et al., 2002).

1.6.4.3 Summary

Myxoma virus encodes 27 predicted immunomodulatory, anti-apoptotic or host-range proteins, examples of those with anti-apoptotic or host-range functions are described above. These experiments show that deletion of only a single gene can dramatically alter myxoma virus virulence, indicating the complexity of the interactions between host and virus. However, entire genes do not have to be deleted to alter the outcome of infection. Small changes in immunomodulatory proteins, or the regulation of their products, rendering them non-functional or semi-functional, may also have drastic consequences on the outcome of infection.

Ultimately the interaction between myxoma virus and the host rabbit leads to either myxoma virus producing enough infectious progeny and immunomodulatory factors to overcome the host immune response, or the host immune response controlling the extent of virus replication leading to virus control and clearance. These factors are determined by both the characteristics of the virus and the ability of the host rabbit's cells to sustain virus replication.

1.7 Lymphoid Cells in Myxoma Virus Pathogenesis

Lymphoid cells are the key effectors of the immune system and are central to effective functioning of the anti-viral immune response, as they are involved in cytotoxic killing of virus-infected cells and are involved in regulating the immune response through production of cytokines and other molecules (Section 1.6.2). Leukocytes and lymphoid tissues have been identified as important for *in vivo* amplification and dissemination (Fenner and Woodroofe 1953; Best and Kerr 2000) and are one of the major sites of pathology during myxoma virus infection (Hurst 1937; Best et al., 2000). Additionally, it has also been shown that myxoma virus is able to infect and replicate in the rabbit $CD4^+$ T-cell line, RL-5 (Barry et al., 1995; Macen et al., 1996; Barry et al., 1997), and that myxoma virus localizes to the T-cell zones of rabbit lymph nodes during *in vivo* infection (Best et al., 2000). These results suggest an important role for T-cells in myxoma virus pathogenesis.

Myxoma virus inhibits T-cell action by encoding immunomodulatory factors that compromise cytokine and chemokine networks and other lymphocyte effector functions (Section 1.6.4). Best (1998) has also shown that lymphoid cells isolated from susceptible rabbits infected with virulent myxoma virus did not proliferate in response to the T-cell specific mitogens Concanavalin A (Con A) and phytohaemagglutinin

(PHA). However, response to the B-cell mitogen, lipopolysaccharide (LPS), was unchanged. Infection of laboratory rabbits with the attenuated Ur strain of myxoma virus did not affect the response of lymphoid cells to stimulation *in vitro* with T-cell mitogens. It is unknown whether a similar effect on T-cell responsiveness is observed in rabbits resistant to myxomatosis. The mechanism of suppression of T-cell responsiveness is also unknown but may involve one of the many immunomodulatory proteins encoded by this virus, or, similarly to MRV, may involve a T-cell-derived suppressive factor (Section 1.6.3.1).

Activity of the lymphoid cells may also contribute to myxoma virus attenuation. *In vivo* dissemination of the attenuated Ur strain of myxoma virus from the lymph node draining the inoculation site to distal tissues is delayed compared to virulent SLS (Best and Kerr 2000). Systemic dissemination has been postulated to involve T-cells as well as monocytes and macrophages (Best et al., 2000; Best and Kerr 2000). In addition, Best et al. (2000) showed differences in the pathology of the draining lymph node between SLS and Ur infection of laboratory rabbits. In SLS-infections, apoptosis of uninfected lymphocytes was observed 4-6 days post infection and there was a failure to repopulate the node, whereas in Ur infections of laboratory rabbits, the node was repopulated and apoptosis was only prominent from 6 days post infection. The reduced dissemination of Ur may be hypothesized to be due to Ur being unable to replicate as efficiently in lymphocytes and therefore unable to control the host anti-viral immune response.

Lymphoid cells may also be involved in resistance to myxomatosis as control of myxoma virus replication by wild rabbits is first evident at the lymph node draining the inoculation site. Infection of resistant rabbits with the virulent SLS strain of myxoma

virus has been shown to result in a significantly reduced viral titre in the lymph node draining the inoculation site when compared to susceptible laboratory rabbits, even though virus reached the draining lymph node at similar times (Best and Kerr 2000). A decrease in dissemination of virus to distal tissues, as measured by virus titre, was also observed in wild rabbits compared to laboratory rabbits. In this case lymphocytes from wild rabbits may effectively control virus replication leading to the host being able to mount an effective immune response against the virus.

1.8 Summary and Aims of this Study

The mechanisms for attenuation of myxoma virus and resistance to myxoma virus are still unclear but recent studies have implicated a major role for lymphoid cells. Extensive study of the effects of myxoma virus infection on primary lymphoid cells from resistant rabbits has not previously been undertaken. The goal of this thesis is to investigate the role of lymphoid cells in attenuation of myxoma virus and resistance to myxomatosis. This was carried out by isolating lymphoid cells from susceptible (laboratory) and resistant (wild) rabbits and infecting the cell cultures *in vitro* with the virulent SLS or attenuated Ur strains of myxoma virus and examining the subsequent interactions.

Firstly, lymphoid cell permissivity for myxoma virus replication was examined to determine whether this is a critical factor in resistance of wild rabbits to myxoma virus or in attenuation of the Ur strain of myxoma virus. The requirement for cell activation for cellular permissivity for virus replication was analyzed by addition of a T-cell mitogen to the cell cultures. These results are described in chapter 3.

As apoptosis was a major feature of all infections from the Best et al. (2000) study and has been postulated to play a role in attenuation and resistance, the cell cycle was examined in infected cell cultures (chapter 4). The genetic basic for the cell death that was observed in Ur infections from examination of the cell cycle was investigated by sequencing viral genes with putative anti-apoptotic functions and looking for mutations that may be responsible for changes in apoptosis.

The role of activation of lymphoid cells in myxoma virus pathogenesis was revisited in chapter 5, by analysis of expression of cell surface proteins critical for the immune response.

Chapter 6 details production and characterization of anti-rabbit cytokine antibodies. The early innate immune response has been postulated as important for resistance to myxoma virus. The ability to undertake immunological studies in rabbits is limited due to the paucity of rabbit specific reagents and the fact that inbred rabbits are not available. The antibodies produced in chapter 6 were used to characterize the cytokine production during myxoma virus infection.

The results obtained in this thesis will help clarify the cellular and molecular mechanisms of host/virus co-evolution.

Chapter 2

Materials and Methods

2.1 Cells and Their Culture

2.1.1 Cells and Cell Lines

The rabbit kidney cell line, RK13 (ATCC- CCL-37) was used to produce working stocks of the Standard Laboratory Strain (SLS) and Uriarra (Ur) strain of myxoma virus. The African green monkey kidney cell line, Vero (ATCC- CCL-81) was used to titre virus stocks and during analysis of myxoma virus replication.

Primary rabbit lymphoid cells, prepared from rabbit lymph node, and the rabbit T-cell line, RL-5 (obtained from Dr I. Ramshaw, JCSMR, Canberra, Australia) (Kaschka-Dierich et al., 1982; Wilkinson et al., 1992), were used to examine the effects of myxoma virus infection on rabbit lymphoid cells.

COS-7 cells (green monkey kidney cell line) (ATCC- CRL-1651) were used for expression of proteins from plasmid DNA.

2.1.2 Cell Culture Media

RK13 and Vero cells were cultured in minimal essential medium (MEM, Life Technologies) supplemented with 10% fetal calf serum (FSC) (CSL, Melbourne Australia), 2 mM L-glutamine (Invitrogen), 0.225% $NaHCO_3$ (Merck), 60 µg/ml penicillin G (CSL), 100 µg/ml streptomycin (ICN) and 250 ng/ml amphotericin (GibcoBRL). In this thesis, MEM containing all supplements is referred to as 'complete MEM'.

RL-5 cells and primary rabbit lymphoid cells were cultured in RPMI 1640 medium (JCSMR Media Facilities, Canberra, Australia) supplemented with 10% FCS, 2 mM $_L$-glutamine, 0.17% β-mercaptoethanol (Sigma), 60 μg/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin. Supplemented RPMI 1640 is referred to in this thesis as complete RPMI 1640.

COS-7 cells were grown in DMEM media (JCSMR Media Facilities, ANU) supplemented with 10% FCS, 2 mM $_L$-glutamine, 10 mM HEPES (Sigma), 0.17% β-mercaptoethanol, 60 μg/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin. In this thesis, DMEM with all these supplements is referred to as complete DMEM.

2.1.3 Reconstitution of Cell Lines

Aliquots of RK13, Vero, RL-5 and COS-7 cells, stored in 20% dimethyl sulphoxide (DMSO) (Sigma)/ 80% FCS in liquid nitrogen, were thawed at 37°C for 1-2 min, after which 1 ml of complete MEM (RK13, Vero), complete RPMI 1640 (RL-5), or complete DMEM (COS-7), warmed to 37°C, was added to the cell suspension. The cells were then transferred to a 15 ml tube and an additional 10 ml of warm media was gradually added over 5 min to each tube. The cells were centrifuged at 270×g for 5 min, and washed once with 10 ml of warm media. After a final resuspension in warm media, the cells were transferred to a tissue culture flask which was incubated overnight in a humidified incubator at 37°C and 5% CO_2 in air. Cell cultures were checked every day and sub-cultured when confluent as described in Section 2.1.4 (RK13, Vero and COS-7 cells) or Section 2.1.5 (RL-5 cells).

2.1.4 Sub-culturing of RK13, Vero and COS-7 Cell Lines

RK13, Vero and COS-7 cells are adherent cell lines. For sub-culturing, the cell monolayers were washed twice with trypsin diluent (140mM NaCl (BDH), 5 mM KCl (Ajax Chemicals), 0.4 mM Na_2HPO_4 (BDH), 0.4 mM KH_2PO_4 (Ajax Chemicals), 0.3 mM K_2EDTA (Ajax Chemicals), 6 mM $NaHCO_3$, pH 7.3). After washing, the cell monolayers were covered with a thin layer of 0.04% trypsin (Sigma) in trypsin diluent and incubated for 5-10 min at 37°C to detach the cells from the flask. The resulting cell suspension was transferred to a 50 ml tube containing 10 ml of complete MEM (RK13 and Vero) or complete DMEM (COS-7) and centrifuged at 270×g for 5 min. The cell pellet was resuspended in complete MEM (RK13 and Vero) or complete DMEM (COS-7). The concentration of the cells was determined using a haemocytometer. An appropriate number of cells, depending on the size of the flask required, were seeded into a new flask and the cells incubated in a humidified incubator at 37°C and 5% CO_2 in air.

2.1.5 Sub-culturing RL-5 Cells

RL-5 cells are suspension cells, but cell cultures have a significant proportion of cells adhering to the bottom of tissue culture flasks. For this reason, flasks containing RL-5 cells were first incubated at 4°C for approximately 30 min prior to harvesting in order to detach the adherent cells. Cell cultures were then decanted and centrifuged at 270×g for 5 min and the cell pellet resuspended in complete RPMI 1640. An appropriate volume of cells, depending on the size of the tissue culture flask required, was then transferred to a new flask and the cells incubated in a humidified incubator at 37°C and 5% CO_2 in air. RL-5 cells were sub-cultured the day before they were used for an experiment to

ensure that actively replicating cells were used in experiments. This also helped to prevent inconsistencies in results due to cells in different stages of growth.

2.1.6 Preparation of Primary Lymphoid Cell Culture from Rabbit Lymph Nodes

2.1.6.1 Rabbit Breeds and Housing

Laboratory European rabbits (*Oryctolagus cuniculus*) and Australian wild rabbits (*Oryctolagus cuniculus*) were bred and housed at the CSIRO Gungahlin animal house facility. The wild rabbits used as breeding stock were captured from the field and confirmed as naïve to myxoma virus. All wild rabbits used in this thesis were three generations removed from the field and, as such, neither the experimental rabbits nor their direct parents had been exposed to myxoma virus. Details of housing and care are given elsewhere (Best and Kerr 2000). All animal work followed ANU and CSIRO/NHMRC guidelines for animal usage and was approved by the CSIRO Sustainable Ecosystems Animal Ethics Committee. In this thesis, 'susceptible rabbits' are laboratory rabbits and 'resistant rabbits' are wild rabbits.

2.1.6.2 Preparation of Rabbit Lymphoid Cell Cultures

Lymphoid cell preparations were made from the popliteal lymph nodes of laboratory and wild rabbits (Kruisbeck 1993). Lymph nodes were dissected from the rabbit and any adherent, non-lymphoid tissue was removed. Each lymph node was cut into small pieces and passed through a 50 μm steel mesh using a glass pestle, in order to break up clumps of cells. The steel mesh had been pre-wet with RPMI 1640. To optimize the number of cells obtained from the lymph nodes, 50 ml of RPMI 1640 was poured through the mesh. The

cell suspension obtained after this process was centrifuged at 270×g for 5 min, resuspended in 10 ml of RPMI 1640 and centrifuged again at 270×g for 5 min. The cells were washed twice with RPMI 1640 after which they were resuspended in 5 ml of complete RPMI 1640 and transferred to a T25 tissue culture flask (Life Technologies). The rabbit lymphoid cell cultures were incubated overnight in a humidified incubator at 37°C and 5% CO_2 in air. The following day the cells in suspension were removed and the viability of the lymphoid cells was determined by trypan blue exclusion (Section 2.1.6.3). Only preparations with viability greater than 95% were used for further experiments. The cells adherent to the tissue culture flask were discarded.

2.1.6 3 Trypan Blue Exclusion

Trypan blue exclusion gives a measure of cell viability; cells take up trypan blue when their membranes are leaky, which occurs during cell death. An equal volume of 0.4% trypan blue (GibcoBRL) and rabbit lymphoid cells were mixed and the percentage of viable (clear) and non-viable (blue) cells was calculated after counting the cells using a haemocytometer. Approximately 200 cells were counted each time.

2.2 Viruses and their Culture

2.2.1 Derivation of Viruses

SLS was the original strain of myxoma virus released in Australia in the summer of 1950-51. SLS is of grade I virulence and is 100% lethal for laboratory rabbits. The SLS stock used in this study was derived from a rabbit tissue stock freeze-dried by Professor Frank Fenner in 1953. It has since been passaged twice through RK13 cells and once

through laboratory rabbits. The virulence of SLS derived from this seed stock has been recently confirmed (Robinson et al., 1999).

Ur is of grade V virulence and infected laboratory rabbits have a survival rate greater than 95%. All infected laboratory rabbits develop clinical myxomatosis (Fountain et al., 1997; Kerr, unpublished data). The Ur stock used in this thesis was derived from plaque-purified stocks produced in 1989 (Russell and Robbins 1989), which in turn were derived from Uriarra/2/53-1, a strain isolated from the field in 1953 (Mykytowycz 1953). The Ur stock was subsequently passaged through tissue culture (CV-1 cells, RK13 cells) and laboratory rabbits. The virulence of the Ur stocks has been recently confirmed by inoculation of laboratory rabbits (Fountain et al., 1997; Kerr et al., 1999).

The Lausanne (Lu) strain of myxoma virus was released in France in 1952, it has since spread across the entire European continent (Fenner and Ross 1994). Similarly, to the co-evolution in Australia, attenuated strains of virus and resistant rabbits emerged in Europe (Fenner and Chapple 1965; Ross and Sanders 1977; Ross and Sanders 1984; Ross and Sanders 1987; Fenner and Ross 1994). Lu is highly lethal in laboratory rabbits and is more virulent in Australian wild rabbits than SLS (reviewed in Kerr and Best 1998).

2.2.2 Preparation of Seed and Working Stocks of Myxoma Virus

SLS, Ur and Lu virus stocks were obtained from Ms B. Inglis (School of Biochemistry and Molecular Biology, ANU). To prepare seed and working stocks of virus, RK13 cells were grown to confluency in a T75 tissue culture flask (Life Technologies). Media was removed from the flask leaving approximately 2 ml behind. Virus stock (0.5 ml),

was added to the flask. The RK13 cells were then incubated at 37°C for 1 h with frequent agitation, after which an additional 15 ml of complete MEM was added to the flask. The cells were incubated in a humidified incubator at 37°C and 5% CO_2 in air for 48h. After this time, the monolayers were checked for cytopathic effects (CPE). If CPE was observed, the flasks were frozen at -70°C. If CPE was not observed, flasks were left overnight and checked for CPE the following day. This was continued until CPE was observed, at which point the flasks were frozen at -70°C. The frozen flasks were freeze/thawed twice and 5ml aliquots were frozen at -70°C. This virus preparation became the seed stock.

To obtain a working stock of virus, RK13 cells were grown to confluency in a T180 tissue culture flask (Nalge Nunc International) and infected as described above using 5 ml of seed stock. Once CPE was observed, the cells and media were freeze/thawed twice and transferred to Oak Ridge tubes. The cell/virus suspension was then centrifuged for 2 h in a Sorvall SS-34 rotor at 12,000 rpm and 4°C. The supernatant was decanted and the cell/virus pellet resuspended gently and slowly in 1 ml of complete MEM. All suspensions were pooled and the Oak Ridge tubes washed with an additional 1 ml of complete MEM, which was added to the pooled suspensions. The pooled suspensions were sonicated using a MSE 100 Watt Ultrasonic Disintegrator, (3 cycles of 15 sec sonication then 45 sec rest) at 4°C, to release virus from the cells. Aliquots of 1 ml of the cell/virus suspension were stored at -70°C.

2.2.3 Plaque Assay for Determining Virus Concentration

Vero cells were seeded at 0.8×10^6 cells per well in 6-well plates (Life Technologies) and incubated overnight in a humidified incubator at 37°C and 5% CO_2 in air. Each

virus sample was then titrated with a minimum of three dilutions, plated in duplicate. Thus, one 6-well plate was the minimum requirement for each virus sample. Media was aspirated from each well, leaving approximately 100 µl behind. Serial dilutions of 100 µl of the virus samples were set up (10^{-3}, 10^{-4} and 10^{-5} for titration of working virus stocks) and added to the corresponding well in the 6-well plate. The plates were incubated at 37°C for 1 h with frequent agitation. After this time, an additional 3 ml of complete MEM was added and the plates incubated in a humidified incubator at 37°C and 5% CO_2 in air, for 5 to 7 days, depending on the time taken for virus plaques to appear.

When plaques were detected, the media was removed and 1 ml of 10% formalin (Merck) was added to each well to fix the cell monolayers. The plates were incubated for 1 h at room temperature (RT) after which the formalin was removed and 1 ml of 0.1% crystal violet (Sigma) was added to each well to enhance plaque visualization. After a further 1 h, the monolayers were washed with tap water, left to dry and the number of plaques counted. Titres were expressed as plaque forming units (pfu)/ml and were the mean of duplicate titrations.

2.3 Infection of Rabbit Lymphoid Cell Cultures and RL-5 Cells with SLS and Ur

2.3.1 Infection of Rabbit Lymphoid Cell Cultures Prior to Mitogen-Stimulation

For analysis of myxoma virus infection prior to mitogen stimulation, primary lymphoid cells isolated from laboratory or wild rabbits were either mock infected (media without

virus) or infected with SLS or Ur at a multiplicity of infection (moi) of 3 or 0.1. Cells were infected for 1 h at 37°C with frequent agitation in a total volume less than 500 µl. The appropriate number of cells, depending on the experiment undertaken, was added to wells in a 24-well plate (Life Technologies). Complete RPMI 1640 was added to give a total volume of 1 ml per well and Concanavalin A (Con A) (Sigma) was added at this point to the required samples to a final concentration of 5 µg/ml. This concentration has been previously shown to be optimal for stimulation of primary lymphoid cells (Cartledge 1995).

2.3.2 Infection of Mitogen-Stimulated Rabbit Lymphoid Cell Cultures

To analyze the effects of myxoma virus infection on rabbit lymphoid cells that were infected after mitogen stimulation, cells were isolated as described in Section 2.1.6.2, and seeded at 1×10^6 cells per well in 24-well plates. Complete RPMI 1640 was added to give a final volume of 1 ml per well and Con A was added to give a final concentration of 5 µg/ml. Cell cultures were incubated with Con A for 4, 24, 48 or 72 h, after which the cells were harvested, counted and either mock-infected with media or infected with SLS or Ur at a moi of 3 for 1 h at 37°C with frequent agitation, in a total volume less than 500 µl. The cells were then re-plated in 24-well plates with 1 ml of complete RPMI 1640 containing 5 µg/ml of Con A.

2.3.3 Infection of RL-5 Cells

RL-5 cells were mock-infected with media or infected with SLS or Ur at a moi of 3 or 0.1 as described for primary lymphoid cells (Section 2.3.1). Con A was not used for the experiments involving myxoma virus infection as RL-5 cells are already actively

proliferating. Con A stimulation of RL-5 cell cultures was tested during analysis of IFNγ expression (Section 2.13) and was added to cell cultures to a final concentation of 5 μg/ml.

2.4 Flow Cytometric Analysis of Myxoma Virus Infection and Cellular DNA Content

2.4.1 Myxoma Virus Infection and DNA Content of Primary Rabbit Lymphocytes

Myxoma virus infection and DNA content were measured concurrently using the same samples. In cell cultures stimulated with Con A after infection with myxoma virus (Section 2.3.1), cells were seeded at 2×10^6 cells per well in a 24-well plate and one well from each infection was harvested at 4, 12, 24, 36, 48 and 72 h. In cell culturesinfected after stimulation (Section 2.3.2), cells were stimulated for 4, 24, 48 or 72 h and harvested after infection for an additional 12 or 24 h.

The harvested cells were centrifuged at 3,000 rpm in a microfuge and resuspnded in 100 μl PBS (137 mM NaCl, 2.7 mM KCl, 4 mM Na_2HPO_4, 0.18 mM KH_2PO_4). To fix and permeabilise the cells, 400 μl of methanol (Merck):acetone (Merck) (1:1 v/v) was added. After a 10 min incubation at RT, the cells were washed twice with PBS and resuspended in 100 μl of anti-myxoma virus monoclonal antibody (3b6e4; Fontain et al., 1997) diluted 1:800 in 0.1% bovine serum albumin (BSA) (Amersham Pharmacia Biotech) in PBS. To examine non-specific binding, 100 μl of mouse ascites fluid diluted 1:800 in 0.1% BSA/PBS, was used in place of the 3b6e4 antibody. The cells were incubated at 37°C for 1 h, then washed twice with 0.1% BSA/PBS befce being

resuspended in 100 µl of anti-mouse IgG-FITC conjugate (1:40 dilution, in 0.1% w/v BSA/PBS) (Silenus). After incubation for 30 min at RT in the dark, 500 µl of 10 µg/ml of propidium iodide (PI) (Sigma) in PBS was added and the cells were incubated on ice in the dark for a further 30 min. PI binds DNA and was used to analyse cell viability and stage of cell cycle. The proportion of cells infected with myxoma virus and PI staining of DNA were analyzed on a Becton Dickinson FACSort; forward scatter (FSC)- Voltage E-1, AmpGain 9.17, Mode Lin; side scatter (SSC)- Voltage 203, AmpGain 1.00, Mode Log; FL1-H- Voltage 381, AmpGain 1.00, Mode Log; FL3-H- Voltage 319, AmpGain 1.00, Mode Log. The experiment was repeated using lymphoid cell cultures from three laboratory and three wild rabbits.

2.4.2 DNA Content of Infected RL-5 Cells

Myxoma virus infection was not examined in RL-5 cells as this has previously been done (Barry et al., 1995; Macen et al., 1996; Barry et al., 1997). To examine DNA content, RL-5 cells were mock-infected with media or infected with SLS or Ur at a moi of 3 or 0.1 for 1 h as previously described (Section 2.3.3), and seeded at 1×10^6 cells/well in a 24-well plate. Cells were harvested at 4, 24, 48 and 72 h post infection and resuspended in 100 µl PBS. To fix and permeabilise the cells, 400 µl of methanol:acetone (1:1 v/v) was added. After 10 min incubation at RT, the cells were washed twice with PBS and resuspended in 500 µl of 10 µg/ml PI diluted in PBS. The cells were incubated on ice in the dark for 30 min. PI staining of DNA was analyzed on a Becton Dickinson FACSort using the settings previously described (Section 2.4.1). This experiment was repeated on three separate occasions.

2.4.3 Data Analysis of Myxoma Virus Infection

The proportion of cells infected with myxoma virus was determined by analysis of density plots of side scatter (SSC) (cell granularity) versus FL1-H using CellQuest software (Becton Dickinson Immunocytometry Systems). FL1-H detects FITC fluorescence and therefore correlates to binding of the 3b6e4 antibody. Graphs were generated of the proportion of infected cells versus time. These results are described in chapter 3. Samples were also gated based on cell size and those gated sub-populations were analyzed separately for myxoma virus infection as just described. These results are also described in chapter 3.

2.4.4 Data Analysis of Cellular DNA Content

2.4.4.1 Cell Viability

The proportions of viable primary lymphoid cells, as measured by trypan blue exclusion (Section 2.1.6.3), was used to confirm the gating used in flow cytometry to separate non-viable from viable cells. Viability was examined on the flow cytometer usingFL-3, which detects PI fluorescence. The gate used was set up such that the major population of viable cells (G1/G0 stage of the cell cycle) had a fluorescence intensity of PI staining greater than 10^2 units. Therefore, non-viable cells were defined as those events with fluorescence intensity between 10^1 and 10^2 units and viable cells were defined asthose events with fluorescence intensity greater than 10^2 units. It must be noted that the gating of 'non-viable cells' includes cell fragments in addition to apoptotic and necrotic cells. Cell debris had a fluorescence intensity $<10^1$ units and as such was not includedin the gatings. These results are described in chapter 4.

Viability of RL-5 cells is presented as histogram plots of FL3-H and tables of the proportion of non-viable cells as calculated using CellQuest software.

2.4.4.2 Stage of Cell Cycle

Staining with PI identified cells in G1/G0 (single copy of DNA), G2/M (two copies of DNA) and Sub G1 (non-viable cells) sub-populations. Histograms of DNA content were gated with respect to events classified as G1/G0, G2/M or Sub G1, and the proportion of events in each sub-population was calculated using CellQuest software. Additional two-color analysis was performed to determine stages of the cell cycle where viral antigen was detected to examine the permissivity of cells in different stages of the cell cycle to myxoma virus replication. To investigate this, FL1-H (myxoma virus infection) and FL3-H (DNA content) were examined concurrently. The results are described in chapter 4.

2.5 Virus Replication

Rabbit lymphoid cell cultures were infected with SLS or Ur at a moi of 3 or 0.1, and stimulated with Con A (5 μg/ml final concentration) as described in Section 2.3.1. Cells were harvested at 4, 12, 24, 36, 48 and 72 h post infection, freeze/thawed twice and sonicated using a MSE 100Watt Ultrasonic Disintegrator (3 cycles of 15 sec sonication and 45 sec rest) at 4°C. These cell/virus suspensions were stored at -70°C. Virus titres were measured by duplicate plaque assay on Vero cell monolayers as previously described (Section 2.2.3).

Titres were expressed as pfu/input cell and graphed as pfu/input cell versus time. Data was also converted to pfu/infected cell using the mean proportion of infected cells as

determined in Section 2.4.3. Virus replication was examined in infected lymphoid cell cultures from three laboratory and three wild rabbits. Results of virus replication are described in chapter 3.

2.6 Proliferation of Rabbit Lymphocytes and RL-5 Cells

Rabbit lymphoid cells and RL-5 cells were mock-infected with media or infected with SLS or Ur at a moi of 3 or 0.1 and stimulated with Con A as described in Section 2.3.1 for primary cell cultures and Section 2.3.3 for RL-5 cell cultures. Cells were plated in triplicate in a 96-well plate at a concentration of 3×10^5 cells/well for primary lymphoid cells, or 3×10^4 cells/well for RL-5 cells, in a final volume of 100 µl. The plates were incubated for 0, 24, 48 or 72 h. At each time point, cell proliferation was analyzed by tritiated thymidine (^{3}H-T) incorporation (Section 2.6.1) or CellTitre 96® AQ_{ueous} One Solution Assay (Section 2.6.2). Proliferation assays were performed in lymphoid cell cultures from three laboratory and three wild rabbits, and in RL-5 cell cultures on three separate occasions.

2.6.1 ^{3}H-T Incorporation

In this assay, ^{3}H-T is incorporated into cellular DNA during its replication. Therefore, this assay measures actively replicating cells. At each time point (0, 24, 48 and 72 h) 50 µl of media containing 10 µCi of ^{3}H-T was added to each well. Primary cell cultures were then incubated for 18 h in a humidified incubator at 37°C and 5% CO_2 in air. RL-5 cells were incubated for 4 h. At the end of the incubation, the plates of cells were frozen. The plates were freeze/thawed twice prior to harvesting of the cells using a Packard Filtermate 196 Cell Harvester and analysis on a Packard Top-Count Microplate Scintillation Counter (Packard Instrument Co.) as per the manufacturer's instructions.

2.6.2 CellTitre 96® AQ_{ueous} One Solution Assay

The CellTitre 96® AQ_{ueous} One Solution assay (Promega) is a colorimetric assay in which absorbance at 490 nm is directly proportional to the number of live cells in a culture. Metabolically active cells reduce MTS tetrazolium compound to a colored formazan product, the absorbance of which can be read at 490 nm. Cells that are not metabolically active will not cause a color change.

Rabbit lymphoid cells and RL-5 cells were set up as described above. At each time point (0, 24, 48 and 72 h) 20 µl of CellTitre 96® AQ_{ueous} One Solution Reagent was added to each well and the plates incubated in the dark at 37°C. After 4 h, the absorbance at 490 nm was read on a microplate reader. This incubation period was previously shown to produce an optimal signal: noise ratio for primary lymphocytes (Dr P. Kerr, unpublished data).

2.6.3 Presentation of Cell Proliferation Data

Results from the ^{3}H-T incorporation assay and CellTitre assay were scaled such that ^{3}H-T incorporation (as measured as counts per minute), or absorbance at 490 nm, of mock-infected cell cultures at the 4 h time point was equal to 1 unit. Proliferation data was plotted as relative ^{3}H-T incorporation, or relative absorbance at 490 nm versus time.

2.7 Analysis of Cell Surface Protein Expression

2.7.1 Cell Surface Protein Expression in Rabbit Lymphoid Cell Culture Infected with Myxoma Virus Prior to Mitogen Stimulation

Rabbit lymphoid cell cultures and RL-5 cells were mock-infected with media or infected with SLS or Ur at a moi of 3 or 0.1 as described previously (Section 2.3.1 and Section 2.3.3), seeded at a concentration of 2×10^6 cells/well (primary cell cultures) or 0.5×10^6 cells/well (RL-5 cells) and incubated in a humidified incubator at 37°C and 5% CO_2 in air. Cell cultures were incubated with or without 5 µg/ml Con A, which was added after an initial 1 h infection. Preliminary experiments using 24 and 48 h time points demonstrated that the greatest level of cell surface marker expression was after 48 h incubation. Therefore, cell cultures were harvested and analyzed for expression of proteins on the cell surface after 48 h.

After harvesting, the cells were washed twice with PBS and resuspended in 100 µl of a specific monoclonal antibody (1:20 diluted in 0.1% BSA/PBS). The antibodies tested were specific for rabbit CD25, CD4, CD43, CD45, KEN-5, MHC-I or MHC-II (Serotec). Mouse ascites fluid, at a dilution of 1:20 in 0.1% BSA/PBS, was used instead of the primary antibody to measure non-specific binding of the monoclonal antibodies to the cells. Cells were incubated for 1 h at 37°C after which they were washed twice with 0.1% BSA/PBS. Next, the cells were resuspended in 100 µl of anti-mouse IgG-FITC (1:40 dilution in 0.1% BSA/PBS) and incubated at RT in the dark for 30 min. Prior to analysis, 500 µl of 0.1% BSA/PBS was added to each sample. Samples were analyzed for expression of the specific marker on a Becton Dickinson FACSort flow cytometer. Flow cytometer instrument settings for primary cell cultures were FSC-

Voltage E-1, AmpGain 9.17, Mode Lin; SSC- Voltage 203, AmpGain 1.00, Mode Log, with the following FL1-H settings;- for analysis using CD25, CD4, CD43, MHC-I and MHC-II monoclonal antibodies, FL1-H voltage was set at 450; for the monoclonal antibody specific for CD45 FL1-H voltage was 350; and for the KEN-5 monoclonal antibody FL1-H voltage was 390. For RL-5 cells, instrument settings were identical to those used for primary cell cultures for the respective cell surface marker, with the exception of CD25, where the FL1-H voltage was 400, and MHC-I, where the FL1-H voltage was 380. The expression of cell surface markers was examined on three separate occasions using lymphoid cells prepared from three laboratory, three wild rabbits and three different cell cultures of RL-5 cells.

2.7.2 Cell Surface Protein Expression in Rabbit Lymphoid Cell Cultures Stimulated Prior to Infection with Myxoma Virus

Rabbit lymphoid cells were stimulated with Con A and infected with myxoma virus as described in Section 2.3.2. After the cells were infected, they were incubated for 24 or 48 h, at which time they were harvested and analyzed for expression of CD25 or MHC-I as described in Section 2.7.1. Experiments were repeated in lymphoid cell cultures from three laboratory and three wild rabbits.

2.7.3 Data Analysis

Cell surface marker expression was presented as histogram overlays of protein expression and density plots of cell size versus protein expression generated using CellQuest software. Plots were divided into 'low' (fluorescence intensity less than 10^2 units) and 'high' (fluorescence intensity greater than 10^2 units) expression levels. The median fluorescence intensity of cell surface marker expression in cell cultures infected

with myxoma virus was also determined and the mean of three replicate samples was calculated.

Cell cultures were also gated based on cell size into large and small cells (Section 2.8). The mean median fluorescence intensity corresponding to cell surface marker expression in each of these sub-populations was determined using CellQuest software.

2.8 Analysis of Cell Size and Granularity in Primary Lymphocytes and RL-5 Cells

Mitogen stimulation of cells is not only associated with proliferation, but also with changes in cell morphology, for example cell size and granularity (Webster et al., 1995). Cell size and granularity were analyzed as FSC (forward scatter- cell size) and SSC (side scatter- cell granularity) and are collected by the flow cytometer automatically with every sample. Cell size data was presented as histogram overlays of cell size and density plots of cell granularity versus cell size. The proportion of large and small cells was calculated. The data used in this analysis was that from Section 2.7. Instrument settings for FSC and SSC for primary cells and RL-5 cells were as listed in Section 2.7.1.

The size of cells in cell cultures infected with myxoma virus after stimulation with Con A were examined as density plots of myxoma virus infection versus cell size. These plots were divided into quadrants. The proportion of cells in each quadrant was calculated using CellQuest software. Large and small cell sub-populations were also examined for myxoma virus infection (Section 2.4.3) and cell surface protein expression (Section 2.7.3).

Cell granularity was examined as density plots of cell size versus cell granularity and histogram overlays of cell granularity. These results are described in chapter 3.

2.9 Statistical Analysis

Statistical analysis of virus replication, the proportion of cells infected and cell proliferation were undertaken. All data expressed as a percentage were logit transformed, the remainder being log transformed. Statistical analysis was by analysis of variance (ANOVA) with components of variance between and within rabbits. All main effects and interactions were tested for significance. A p value <0.05 was deemed significant. Analyses were done using S-Plus (Insightful Corporation, 2001). These statistical analyses were performed by Mr J. Wood and Ms J. Searle at the Statistical Consulting Unit, ANU.

ANOVA analysis was performed on the proportions of cells in Sub G1, G1/G0 and G2/M. Infection regimes were compared at each time point and data from the one infection regime was compared over the 72 h time course. ANOVA analysis was also performed on the expression of cell surface proteins. Mock-infected and SLS- and Ur-infected cultures were compared to each other. A p value of <0.05 was deemed significant. Discussion of the expression of cell surface markers was not restricted to only significant differences. The levels of expression of some proteins, although not significant, were discussed as it was considered that the changes may have some biological significance.

2.10 Analysis of Anti-Apoptotic, Host-Range and Immunomodulatory Viral Genes

2.10.1 Preparation of Viral DNA

Total DNA (cellular and viral) was purified from working stocks of Lu, SLS and Ur strains of myxoma virus prepared as described in Section 2.2.2. For each virus strain, a 100 µl volume of working stock was added to 100 µl of 2× lysis buffer (100 mM Tris (Progen) pH 7.5, 5 mM K_2EDTA pH 8.0, 200 mM NaCl, 0.2% SDS (BDH), 0.1 mg/ml proteinase K (Sigma)) and incubated at 58°C for 2 h. The samples were centrifuged at 14,000 rpm in a microfuge for 15 min. The supernatant was removed (approximately 200 µl) from each sample and added to an equal volume of phenol (Amresco):chloroform (Ajax Chemicals) (1:1 v:v), then incubated at RT for 20 min with frequent inverting. The samples were centrifuged again at 14,000 rpm for 5 min and the aqueous supernatant (top layer) transferred to fresh tubes. An equal volume of isopropanol (Merck) and a 1/10 volume of 3 M Na acetate (Merck) were added to each tube and mixed with the contents of the tube. The samples were incubated at RT for a minimum of 1 h and centrifuged at 14,000 rpm for 15 min to pellet the DNA. The pellet was rinsed with 1 ml 70% ethanol (Merck) and dried in a Speed Vac SC100 (Savant) (~5min). The DNA was resuspended in 50 µl of MilliQ water.

2.10.2 Primers for Amplification of Myxoma Virus Open Reading Frames

Primers for M002, M004, M005, M062, M063, M064, M128, M151, M152 and M156 open reading frames (ORFs) were designed from the published Lu gene sequences (Cameron et al., 1999). The primers used to sequence the above ORFs are shown in

Table 2.1, and were synthesized by Proligos (formerly Genset Oligos, Lismore, Australia). Primers were diluted to 5 μM stocks and stored at -20°C.

2.10.3 PCR Amplification of Myxoma Virus ORFs

A 1 μl volume of each primer (5 μM stock) from a pair was added to 1 μl of 10× buffer (Fisher Biotech), 1 μl of Mg^{2+} (Fisher Biotech), 1 μl of dNTPs (Fisher Biotech), 0.2 μl of Taq polymerase (Fisher Biotech), 3.3 μl of ddH_2O and 1 μl of viral DNA (prepared in Section 2.10.1). The ORFs were amplified using a capillary thermal cycler (Corbett Research).

The following PCR cycle was used for amplification;

1× { 95°C for 3 min

35× { 95°C for 15 sec
53°C, 55°C or 57°C (depending on the Tm of the primer pairs) for 15 sec
72°C for 90 sec repeated

1× { 72°C for 3 min and 25°C for 1 min.

TABLE 2.1. PRIMERS USED FOR SEQUENCING VIRUS ORFS

ORF	Primers	Expected PCR Product Size (bp)	ORF Size (bp)	Melting Temperature (Tm)
M002*	5′ external primer CTAATTGGCACCTTCTACGTC	5' fragment-730bp	1064bp	57°C
	3′ external primer CGCGAATAATACACACAGGC			55°C
	5′ internal primer CTGCACAAAATGTCCTCGG	3' fragment-653bp		53°C
	3′ internal primer GCTCGTTCCGTAGTATTCCG			57°C
M004	5' primer GAAGAGTTCCTGGTTTGGG	809bp	714bp	53°C
	3' primer GCGAACCAACAGTCCGAGTG			59°C
M005*	5′ external primer GTTGATATAACCCCGGACGAG	5' fragment-727bp	1428bp	59°C
	3′ external primer CAAATCGATAGGATAGGACC			57°C
	5′ internal primer GTTGGAACGTAAGATCAGCC	3' fragment-850bp		53°C
	3′ internal primer CAACACCTTTCGATTAAAGACG			57°C
M062	5' primer CAGCGACCTCTAGGACGTC			57°C
	3' primer CCCACGCTCGTAATGCTCAG	543bp	477bp	59°C
M063	5' primer TGTGAGCATTACGAGCGTGG			59°C
	3' primer CCTCCATGTTTACGGCAACG	732bp	648bp	57°C
M064	5' primer AGCGACGATTATACCAGCAG			55°C
	3' primer TGTCCATAGATACCGGTTCC	785bp	612bp	55°C
M128+	5′ external primer CGTTCTACTACGTACGACGT.	External fragment-900bp	846bp	58°C
	3′ external primer GCATTCTTGAAGAACTTGCC			58°C
	3′ internal primer GCTTTGCCCAGCAGATAGTGC	Internal fragment-679bp		59°C
M151*	5′ external primer TAAGTGCGATTATGGAG	5' fragment-999bp	1001bp	43°C
	3′ external primer CTAACGGATACAGGAGAG			49°C
	5′ internal primer CGACAGCGTCGGACGTC	3' fragment-1044bp		53°C
	3′ internal primer GAAGTGACTCTACCCAG			47°C
M152	5' primer GGTAGAGTCACTTCTCCC			51°C
	3' primer GGCTACTGTTGTAAACATGG	889bp	801bp	53°C
M156	5' primer CGTAGCGTATACATAATGCCG			57°C
	3' primer CGACGTTAAACATAAGACGAGG	376bp	309bp	59°C

* M002, M005 and M151 were amplified in two overlapping sections, 5' and 3' ends

\+ M128 was amplified over the entire gene sequence and from an internal primer

2.10.4 Cloning of PCR Product

Each PCR product was purified (described in Section 2.10.4.1), cloned into a vector (described in Section 2.10.4.2) and amplified in competent DH5α *E. coli* cells (described in Section 2.10.4.4). The vector containing the amplicon was purified from the bacterial cells (described in Section 2.10.4.5) and the amplicon sequenced (described in Section 2.10.5).

2.10.4.1 Purification of the PCR Product

The PCR product was separated from the primers on a 1% agarose gel in TAE buffer (40 mM Tris, 1% acetic acid (Merck), 1 mM K_2EDTA) at 90 V for approximately 1 h. To visualize the DNA, the gel was stained in 1 μg/ml of ethidium bromide (Sigma) for 30 min before destaining in ddH_20 for an additional 30 min. If an amplicon of appropriate size was visible when the gel was viewed on a UV transluminator, the DNA band was excised and the DNA extracted from the gel matrix using the UltraClean DNA Purification Kit (Mo Bio Laboratories Inc) as per the manufacturer's instructions. Components in the solutions were not defined by the manufacturer. Briefly, the gel slice was weighed and 3 equivalent volumes of ULTRA SALT were added. The gel slice was melted at 55°C and 5 μl of BRESA-BIND glass beads were added. The solution was mixed on a turntable for 10 min at RT and centrifuged at 14,000 rpm in a microfuge for 1 min. The pellet was then washed three times with BRESA-WASH solution. The resulting pellet was resuspended in 10 μl of ddH_20 and incubated at 55°C for 5 min. The solution was centrifuged again as above and the 10 μl supernatant containing the eluted PCR product was removed and stored at -20°C.

2.10.4.2 Ligation of PCR Product into the Plasmid Vector

To ligate the PCR product into a plasmid vector, 7 µl of the PCR product was added to 1 µl of pGEM-T Easy vector (Promega), 1 µl of T4 DNA ligase (Promega) and 1 µl of 10× ligase buffer (Promega) and incubated overnight at 16°C.

2.10.4.3 Preparation of Competent DH5α E. coli Cells

Competent DH5α *E. coli* cells were used to amplify the cloned PCR product. To prepare the competent cells, one loop of DH5α *E. coli* cells from a glycerol stock (obtained from Ms B. Inglis) was streaked onto a LB plate and incubated overnight at 37°C. A single bacterial colony was picked and used to inoculate 20 ml of LB broth, which was incubated overnight at 37°C in a shaking incubator. The following morning, two flasks containing 1 L of LB broth were inoculated with 10 ml of culture and incubated at 37°C and 200 rpm until the OD_{600} was between 0.5 and 0.6 units (~2-3h). Once this OD was reached, the cultures were equilibrated on ice water and all following procedures were carried out gently at 2-4°C.

The cultures were divided equally between six pre-chilled 500 ml bottles and centrifuged in a G5-3 rotor (Sorvall) at 4,500 rpm, 4°C for 15 min. The supernatant was drained quickly and the pellet gently resuspended in 5 ml of ice-cold sterile MilliQ water. After addition of extra ice-cold MilliQ water to a final volume of 500 ml, the suspensions were centrifuged at 4,500 rpm and 4°C for 15 min. At the end of the 15 min, the pellets were resuspended in 5 ml ice-cold MilliQ water. Suspensions were combined into two bottles and MilliQ water was added again to a final volume of 500 ml. The suspensions were centrifuged at 4,500 rpm and 4°C for 15 min. The supernatants were discarded and the pellets resuspended in 5 ml of ice-cold 10%

glycerol in ddH_2O. At this point, the suspensions were transferred to two pre-chilled 30 ml Oak Ridge tubes and ice-cold 10% glycerol in ddH_2O was added to give a final volume of 25 ml. The samples were centrifuged in a Sorvall SS-34 rotor at 5,000 rpm, 4°C for 15 min. The supernatants were drained and the pellets resuspended in 1 ml of ice-cold 10% glycerol in ddH_2O. Cells were aliquoted (40 µl) into pre-chilled eppendorf tubes on ice, which were then stored at -70°C.

2.10.4.4 Transformation of DH5α E. coli Cells

For transformation, 1 µl of ligation mix was added to 40 µl of competent DH5α *E. coli* cells that had been thawed on ice. The cells were electroporated at 2.5 kV after which 400 µl of LB broth was added and the cells incubated for 1 h at 37°C. A 100 µl aliquot of the cell suspension was plated on a LB plate supplemented with 10 µg/ml of ampicillin (Sigma), 10 µg/ml of X-gal (Progen) and 10 µg/ml of IPTG (Progen). Ampicillin selected for colonies that had taken up the plasmid during transformation. X-gal and IPTG were used in color selection of vector containing the amplicon. In this assay, white colonies contain vector with the inserted PCR product and blue colonies contain vector without the PCR product inserted. The plates were incubated overnight at 37°C. Three white colonies were picked from each plate and grown overnight at 37°C in a shaking incubator in 3 ml of LB broth containing 10 µg/ml of ampicillin.

2.10.4.5 Isolation of Plasmid DNA

Plasmid DNA was isolated using a DNA plasmid miniprep kit (Qiagen) as per the manufacturer's instructions. Components in the solutions were not defined by the manufacturer. Briefly, 3 ml of culture was spun down and the cells resuspended in 250 µl of resuspension solution (P1). P2 lysing solution (250 µl) was added and the tubes

inverted 6 times. After this, 350 µl of neutralizing solution (P3) was added and again the tubes were inverted 6 times. The suspensions were centrifuged at 14,000 rpm for 10 min in a microfuge, after which the supernatants were transferred to a miniprep filter. The filters were centrifuged at 14,000 rpm for 1 min and washed with 500 µl of PB solution. The filters were centrifuged again in a microfuge at 14,000 rpm for 1 min and washed with 750 µl of PE buffer. After this wash, the filters were centrifuged at 14,000 rpm for 30 sec to remove all traces of PE buffer. To elute the plasmid DNA from the filter, 50 µl of ddH_2O was added to the filter and incubated at RT for 1 min. The filters were centrifuged at 14,000 rpm for 1 min and the 50 µl containing the plasmid DNA was removed. The absorbance at 260 nm and 280 nm of a diluted aliquot of the plasmid DNA solution was determined. The DNA concentration was calculated by multiplying the 260 nm absorbance reading by 50 µg DNA/ml and by the dilution factor. The 260 nm/280 nm ratio was also calculated to estimate the purity of the DNA samples. The samples were stored at -20°C.

2.10.5 Nucleotide Sequencing Reactions

The plasmid DNA containing the gene of interest, produced in Section 2.10.4, was used as the template for sequencing reactions. Plasmid DNA (200 µg) was added to 4 µl of Big Dye reaction mix (Applied Biosystems) and 2 µl of M13 forward or M13 reverse primer (Biomolecular Resource Facility, JCSMR, Canberra, Australia). The sequencing reaction mix was made up to a final volume of 10 µl with ddH_2O.

Sequencing reactions were done using a capillary thermal cycler (Corbett Research) with the following steps;

1× { 96°C for 30 sec

25× { 50°C for 15 sec
60°C for 4 min

1× { 25°C for 1 min

After sequencing, 25 µl of 100% ethanol and 1 µl of 3 M Na acetate was added to each sample, which was then incubated on ice for 10 min. The samples were centrifuged in a microfuge at 14,000 rpm for 20 min before being washed twice with 70% ethanol and left to air dry. The samples were processed by the Biomolecular Resource Facility (JCSMR, Canberra, Australia) using an Applied Biosystems gel system.

2.10.6 Analysis of Nucleotide Sequences

Initially, only ORFs from the Ur strain of myxoma virus were sequenced and compared with the published Lu strain of myxoma virus. If these sequences differed, then the ORF from SLS and Lu were sequenced independently and compared. To check for differences between the nucleotide sequences obtained, an alignment of the nucleotide sequences was done using ClustalW (alignment- full, matrix- iub, gap open- 10, gap extension- 0.05, gap distance- 8) (http://ebi.ac.uk). For genes that were different between sequence obtained in this thesis and the published Lu sequence, all six ORFs of the virus gene were translated using MacVector (Oxford Molecular Inc). The resulting protein sequences were aligned using the above programs. Analysis of molecular weight, pI, amino acid composition and hydrophilicity (Kyte and Doolittle scale) was undertaken using MacVector. Analysis of putative protein transmembrane domains was done using the Dense Alignment Surface (DAS) Transmembrane Prediction Server (http://www.sbc.su.se/~miklos/DAS/) (Cserzo et al., 1997). Putative protein domains

were identified using PROSITE (all patterns, profiles and rules scanned, patterns with a high probability of occurring were included) (http://au.expasy.org/cgi-bin/scanprosite). These results are described in chapter 4.

2.11 Production of Antibodies Specific for Rabbit Cytokines

2.11.1 Synthetic Peptides Derived from Rabbit Cytokine

Two synthetic peptides for each of rabbit IL-2, IL-4, IL-6, IL-10 and IFNγ were designed with assistance from Dr H. Perkins (School of Biochemistry and Molecular Biology, ANU) and Dr P. Kerr (CSIRO Sustainable Ecosystems). Peptides were synthesized by the Biomolecular Resource Facility (JCSMR, Canberra, Australia). The names of each peptide are as follows; IL-2 helix B, IL-2 C/D loop, IL-4 helix A, IL-4 helix C, IL-6 helix E, IL-6 tail, IL-10 C/D-hD, IL-10 Nterm, IFNγ helix D, IFNγ hb-B/C. Each peptide was modeled on the cytokine from which it was derived, using the 3D structure viewer, Cn3D 4.0, available from the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov:80/Structure/CN3D/cn3d.shtml). Alignments of rabbit, rat, human and chicken sequences, where available, for each cytokine were aligned using ClustalW (http://ebi.ac.uk) using the settings listed in Section 2.10.6.

2.11.2 Immunization of Rats with Synthetic Peptides

2.11.2.1 Conjugation of Synthetic Peptides to mcKLH

Each synthetic peptide was conjugated to mariculture keyhole limpet hemocyanin (mcKLH) using the Imject® Maleimide Activated mcKLH kit (Pierce) as per the manufacturer's instructions. Components in the solutions were not defined by the

manufacturer. Briefly, 2 mg of each peptide was dissolved in a 300 µl volume of conjugation buffer. The IL-2 helix B, IL-2 C/D loop, IL-4 helix A, IL-4 helix C, IL-6 helix E and IFNγ hb-B/C synthetic peptides were not soluble in conjugation buffer so were dissolved in guanidine (Sigma) or DMSO, as listed in Table 2.2. For each synthetic peptide, 2 mg of carrier protein was dissolved in 200 µl ddH_20 and added to the dissolved synthetic peptide. The carrier protein and synthetic peptide were mixed for 2 h at RT on a rocker.

TABLE 2.2. SOLUBILITY OF SYNTHETIC PEPTIDES

Synthetic Peptide	Solubility
IL-2 helix B	Soluble in 10% 7 M guanidine
IL-2 C/D loop	Partially soluble in 7 M guanidine
IL-4 helix A	5 M guanidine
IL-4 helix C	20% DMSO
IL-6 helix E	Partially soluble in 7 M guanidine
IL-6 tail	Imject® Conjugation buffer (Pierce)
IL-10 C/D-hD	Imject® Conjugation buffer (Pierce)
IL-10 Nterm	Imject® Conjugation buffer (Pierce)
IFNγ helix D	Imject® Conjugation buffer (Pierce)
IFNγ hb-B/C	Soluble in 10% 7 M guanidine

Conjugated synthetic peptides were separated from unconjugated synthetic peptides and carrier proteins by column chromatography. Each D-Salt Desalting column was washed with 5 column volumes of Imject® purification buffer (Pierce) prior to addition of 500 µl of the synthetic peptide/carrier protein mixture. A separate column was used for each synthetic peptide. Aliquots of 500 µl of Imject® purification buffer were added to the column to elute the conjugated peptides. Fractions of 500 µl were collected and the absorbance at 280 nm of each fraction was determined. The fractions with the conjugate

(as shown by increased absorbance) were pooled. The pooled fractions were passed through a 0.22 μm filter and stored at -20°C. The columns were reconstituted by washing with five column volumes of ddH_20 and were stored at 4°C.

2.11.2.2 Rat Breeds and Housing

Female Wistar rats older than 6 weeks were obtained from the JCSMR Specific Pathogen Free Animal Facility (Canberra, Australia) and housed and cared for at the School of Biochemistry and Molecular Biology, ANU, using standard housing and care practices. All animal work followed ANU guidelines for animal usage and was approved by the ANU Animal Experimentation Ethics Committee.

2.11.2.3 Inoculation Protocol

Each peptide-conjugate was thawed at 37°C. An equal volume of peptide-conjugate and Freunds incomplete adjuvant (GibcoBRL) was mixed by pipetting and vortexing until an emulsion was formed. The peptide-conjugate emulsion (100 μl) was injected subcutaneously into each rat behind each shoulder; thus, each rat was injected with a total of 200 μl. Two rats were used for each peptide-conjugate. Rats were boosed twice with the same volume of peptide-conjugate emulsion at fortnightly intervals. Each boost was accompanied by a tail bleed (~50 μl of blood was collected) and the collected sera were analyzed for the presence of antibodies specific for the particular cytokine from which the peptide was derived. Two weeks after the last boost, the rats were anaesthetized using chloroform and blood collected by cardiac puncture. Dr P. Kerr assisted with blood collection. The blood was left to clot at 4°C overnight after which the samples were centrifuged at 14,000 rpm in a microfuge and the sera removed. The sera were stored at -20°C.

2.11.3 Analysis of Rat Sera for Recognition of Rabbit Cytokines

2.11.3.1 Preparation of Plasmid DNA Expressing Rabbit Cytokines

Glycerol stocks of DH5α *E. coli* cells transformed with plasmids expressing rabbit IL-2, IL-4, IL-6, IL-10 or IFNγ were obtained from Dr H. Perkins and Ms B. Inglis. Each cytokine gene had been cloned into pcDNA3.1 vector under the control of the immediate early cytomegalovirus promotor and with signal sequences for expression in mammalian tissue culture systems. Each gene was cloned such that it was in frame with a six-Histidine tag. Glycerol stocks of the transformed DH5α *E. coli* cells were thawed slowly on ice. One loop of cells was transferred to 20 ml of LB broth and grown overnight at 37°C in a shaking incubator. A larger culture volume was used than that in Section 2.10.4.5 to enable greater replication of *E. coli*, and thus the plasmid. Plasmid DNA was isolated from the culture using the DNA plasmid miniprep kit (Qiagen) as per the manufacturer's instructions. Details of this protocol are outlined in Section 2.10.4.5. The concentration of plasmid DNA was determined by measuring absorbance at 260 nm as described in Section 2.10.4.5 and the DNA was stored at -20°C.

2.11.3.2 Transfection of Vero Cells

Vero cells were seeded at 0.8×10^6 cells per well in a 24-well plate. Plates were incubated overnight in a humidified incubator at 37°C and 5% CO_2 in air. Vero cells were transfected with plasmid DNA produced in Section 2.11.3.1. For each well transfected, the volume of DNA stock containing 2 μg of plasmid DNA was made up to 50 μl with MEM. In a separate tube, 3 μl of Lipofectamine 2000 (Invitrogen) per well was added to MEM to a final volume of 50 μl per well. The plasmid DNA/MEM and

Lipofectamine/MEM solutions were incubated separately at RT for 5 min. The plasmid DNA/MEM solution was then added to the Lipofectamine/MEM solution and these solutions were incubated together for 20 min at RT. The Vero cell monolayers were washed twice with MEM, after which 100 µl of plasmid DNA/Lipofectamine solution was added to each well. An additional 200 µl of MEM supplemented with 10% FCS was added per well and the cells incubated in a humidified incubator at 37°C and 5% CO_2 in air, for 24 h.

2.11.3.3 Detection of Rabbit Cytokines by Immunofluorescence

Vero cell monolayers transfected with plasmid-expressing rabbit cytokines (Section 2.11.3.2) were washed twice with PBS and incubated at RT for 10min in 200 µl of methanol:acetone (1:1). The cells were then washed twice with PBS and incubated with 200 µl of the primary antibody. Primary antibody was either the rat serum specific for the cytokine the cells had been transfected with (test samples) or sera from rats' not immunized (negative control). The primary antibodies were diluted 1:20 in 3% BSA/PBS. The anti-His (C term) antibody (Invitrogen) diluted 1:500 in 3% BSA/PBS was used as the positive control. The plates were incubated at RT for 2 h on a platform rocker after which the monolayers were washed three times with 3% BSA/PBS. Secondary antibody, diluted 1:40 in 3% BSA/PBS, in a volume of 200 µl was added to each well. For the rat sera the secondary antibody was anti-rat Ig-FITC (Silenus) and for the anti-His C-term antibody, the secondary antibody was anti-mouse Ig-FITC (Silenus). The plates were incubated in the dark for a further 1 h before being washed three times with 3% BSA/PBS. Fluorescence of the monolayers was examined using a Fluorvert FS (Leica) fluorescence microscope. Photographs were taken of the cell monolayers using a Wild MPS 46/52 Leica camera.

To determine if antibodies specific for murine IL-2 and IL-10 cross-reacted with rabbit cytokines, a 1:20 dilution of anti-mouse IL-2, anti-mouse IgG IL-10 or anti-mouse IgM IL-10 (obtained from Dr A. Ramsay, JCSMR, Canberra, Australia) in 3% BSA/PBS was used as the primary antibody in place of the rat sera. The negative control was mouse ascites fluid (1:20 dilution in 3% BSA/PBS) and the secondary antibody was anti-mouse Ig-FITC. The anti-His C-term antibody was used, as described in the previous paragraph, as the positive control.

2.11.4 Immunization of Chickens with Synthetic IL-2 Peptides

As no positive staining of rat sera was detected for synthetic peptides derived from IL-2, production of anti-rabbit IL-2 antibodies was attempted in chickens.

2.11.4.1 Chicken Breeds and Housing

Chickens were obtained from Bellchamber Produce, Fyshwick (ACT). Chickens were housed at the Research School of Biological Sciences Animal Facility (ANU) using standard housing and care practices. All animal work followed ANU guidelines for animal usage and was approved by the ANU Animal Experimentation Ethics Committee.

2.11.4.2 Inoculation Protocol

Approximately 3 ml of blood was collected from the superficial vein under the wing of each chicken prior to inoculation. Chickens were inoculated at two sites in the thigh muscle with 100 µl of either IL-2 helix B or IL-2 C/D loop conjugated to mcKLH, emulsified with Freund's incomplete adjuvant, prepared as for the rat inoculations (Section 2.11.2.3). Therefore, each chicken was inoculated with a total of 200 µl of

emulsion. The chickens were boosted with the peptide-conjugate emulsion after four weeks and again two weeks later. To test the progress of the immunization protocol, the chickens were bled (~1 ml) from the superficial vein on the underside of the wing, prior to the second boost. Two chickens were inoculated with each synthetic peptide.

2.11.4.3 Infection of Vero Cells with SLS-IL2 and Examination for Antibodies to IL-2

Infection of cells with a recombinant myxoma virus (SLS) that expressed rabbit IL-2 (Junankar 1999) was used as a source of rabbit IL-2 to test for the presence of specific antibodies in the chicken sera. Vero cells were seeded in 24-well plates, at a concentration of 2×10^5 cells/well and incubated overnight in a humidified incubator at 37°C and 5% CO_2 in air. Media was aspirated from each well. A moi of 1 of recombinant SLS-expressing rabbit IL-2 was added to each well in a total volume of 100 µl of complete MEM. The plates were incubated at 37°C for 1 h with frequent agitation. After this time, an additional 1ml of complete MEM was added and the plates incubated for 24 h in a humidified incubator at 37°C and 5% CO_2 in air.

Monolayers were examined for IL-2-specific antibodies as described in Section 2.11.3.3. The primary antibodies used for immunofluorescence were either the anti-myxoma virus monoclonal antibody (3b6e4) diluted 1:800 in 3% BSA/PBS as the positive control, or the chicken sera diluted 1:20 in 3% BSA/PBS as the test samples. The negative control was the sera taken from each chicken prior to immunization. Primary antibody binding was detected using anti-mouse-FITC or anti-chicken-FITC (Sigma) at a dilution of 1:40 in 3% BSA/PBS. Fluorescence was analyzed using a Fluorvert FS (Leica) fluorescence microscope and photographs were taken of the cells using a Wild MPS 46/52 Leica camera.

2.11.5 DNA Immunization of Rats

Due to the lack of success in producing anti-rabbit IL-2 antibodies using synthetic peptide-conjugates, DNA immunization of rats using a plasmid containing the rabbit IL-2 gene was performed.

2.11.5.1 Preparation of Plasmid DNA for Immunisation

The plasmid expressing rabbit IL-2 under the CMV promoter, pMagicIL-2, was obtained from Mr M. Adams (School of Biochemistry and Molecular Biology, ANU). The concentration of the plasmid stock was determined by measuring the OD_{260}. A 400 µl aliquot of the plasmid stock was taken and added to 1 ml of 100% ethanol and 40 µl of 3 M Na acetate. The mixed sample was incubated on ice for 10 min and centrifuged at 14,000 rpm in a microfuge for 15 min. The DNA pellet was washed twice with 70% ethanol and resuspended in sterile saline such that 100 µl of solution contained 50 µg of DNA.

2.11.5.2 Inoculation Protocol

Female Wistar rats were obtained and housed as previously described (Section 2.11.2.2). A 100 µl solution of saline containing 50 µg of plasmid DNA was injected intramuscularly into two sites on each of two rats. Rats were bled after two weeks and boosted twice at two-week intervals using the peptide-conjugate emulsion as described in Section 2.11.2.3. The boost in this case was similar to previous inoculations of peptide-conjugates except that both IL-2 conjugates were added in the same emulsion. Therefore, each rat had a total of 100 µl of Freund's incomplete adjuvant and 50 µl of each IL-2 peptide-conjugate. Rats were then tail bled (Section 2.11.2.3) and the sera tested for antibodies specific for rabbit IL-2 as described in Section 2.11.3.

2.12 Characterization of Rat Sera

Only rat sera positive for rabbit cytokines were characterized. Sera were titrated on transfected Vero cell monolayers to determine optimal working dilutions for immunofluorescence. Immunoblot analysis and flow cytometric analysis of the sera were also performed. The results for antibody characterization are presented in chapter 6.

2.12.1 Titration of Rat Sera

Vero cell monolayers were transfected and analyzed as described in Section 2.11.3.2 and Section 2.11.3.3. Rat sera were used as the primary antibody and were diluted 1:20, 1:40, 1:100 or 1:500 in 3% BSA/PBS. The secondary antibody (anti-rat Ig FITC) was used at a 1:40 dilution. Photographs were taken of the monolayers to analyse fluorescence using a Fluorvert FS fluorescence microscope and Wild MPS 46/52 Leica camera.

2.12.2 Immunoblot Analysis of Rat Sera

Western analysis of the rat sera was performed. Samples for analysis were prepared from either cells infected with recombinant myxoma virus-expressing rabbit cytokines (described in Section 2.12.2.1) or from cells transfected with plasmid DNA-expressing rabbit cytokines (described in Section 2.12.2.2).

2.12.2.1 Preparation of Samples from Virus-Infected Cells

RK13 cells were seeded at 0.8×10^6 cells/well in 6-well plates and grown overnight in a humidified incubator at 37°C and 5% CO_2 in air. Monolayers were washed three times

with MEM. Cells were infected with a moi of 1 of recombinant myxoma viruses expressing one of IL-4, IL-6, IL-10 or IFNγ (obtained from Dr H. Perkins and Ms B. Inglis) for 1 h at 37°C with frequent agitation. After this initial infection, the unbound virus was removed from the cells, by washing three times with MEM. MEM (1 ml) was added to each well and the cells incubated for 48 h in a humidified incubator at 37°C and 5% CO_2 in air. After this time the supernatants were collected and the monolayers were frozen. Processing of supernatants and monolayers are described in the following sections.

2.12.2.1.1 Processing of Supernatants from Virus-Infected Cells

Five volumes of cold acetone were added to each supernatant sample to precipitate the protein. The samples were incubated at -20°C for 10 min, then centrifuged at 10,000 rpm at 4°C for 5 min in a Sorvall SS-34 rotor. The supernatants were discarded and the precipitate washed again with cold acetone and centrifuged as above. The supernatants were again removed and the precipitate left to air dry, then resuspended in 100 µl of 2× sample loading buffer (62.5 mM Tris, 10% glycerol, 2% SDS, 0.04% bromophenol blue (Sigma)) and stored at -20°C.

2.12.2.1.2 Processing Cell Monolayers from Virus-Infected Cells

The cell monolayers were thawed and 200 µl of 2× sample loading buffer was added to each well. The bottom of each well was scraped and the cell extracts removed and boiled for 5 min to denature the cellular DNA. The cell extracts were stored at -20°C.

2.12.2.2 Preparation of Samples from Transfected Cells

Rat anti-rabbit antibodies specific for IL-6 and IFNγ could not be detected when virus-infected cells were used as the source of antigen. As an alternative source of antigen, COS-7 cells were transfected with pcDNA3.1 containing IL-6 or IFNγ as detailed in Section 2.11.3.2 for Vero cells, except complete DMEM was used as the media. Transfected COS-7 cells were incubated for 48 h, after which the supernatants were removed and processed as described in Section 2.12.2.1.1. The monolayers were processed as described in Section 2.12.2.1.2. One well from each transfection was analyzed for transfection efficiency as described in Section 2.11.3.3 using rat sera previously shown, by immunofluorescence to contain antibodies specific for rabbit IL-6 or IFNγ.

2.12.2.3 Fractionation of Supernatants and Cell Extracts by SDS-PAGE

Protein extracted from supernatants and cell extracts were fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in non-reducing and reducing conditions. A 9% resolving gel was made from 2.5 ml of resolving gel buffer (0.4% SDS, 1.5 M Tris), 3 ml of 30% bis/acrylamide (Amresco), 4.5 ml of ddH_2O, 50 µl of 10% ammonium persulphate (APS) (Bio-Rad) and 5 µl of Temed (Bio-Rad). The resolving gel was poured into a mini-gel former (Bio-Rad) and ddH_2O was layered on top. Once the gel was set, the water was removed and a 3.6% stacking gel poured on top. The stacking gel consisted of 1.25 ml of stacking gel buffer (0.4% SDS, 0.5 M Tris), 600 µl of 30% bis/acrylamide, 3.25 ml of ddH_2O, 50 µl of 10% APS and 5 µl of Temed. A 10 well comb was placed in the stacking gel, which was left to set.

For non-reduced samples, 20 µl of protein samples at RT, were loaded onto the gel. For reduced samples, 2 µl of 1M dithiothreitol (DTT) (Sigma) was added to 20 µl of sample, which was then boiled for 5 min prior to loading on the gel. Pre-stained Benchmark protein ladder (Invitrogen), 5 µl, was also loaded onto a lane of each gel. The SDS-PAGE gels were run with Tris-glycine running buffer (12 mM Tris, 96 mM glycine (Merck), 1.7 mM SDS) at 100 V for approximately 60 min using the Mini-Protean II system (Bio-Rad). The gels were either stained as described in Section 2.12.2.4 or transferred to a PDVF membrane as described in Section 2.12.2.5.

2.12.2.4 Protein Detection by Staining SDS-PAGE Gels

The protein gels were initially stained with Coomassie brilliant blue stain (1 g of brilliant blue dye (Progen) in 550 ml of methanol:acetic acid: ddH_2O 5:1:5 v:v:v) overnight on a rocker and subsequently destained with destaining solution (25% ethanol (Merck), 10% acetic acid in ddH_2O). The gels were then silver stained using the Bio-Rad silver stain kit according to the manufacturer's instructions. Components of each solution in the kit were not specified. Briefly, the gels were fixed in 200 ml of 40% methanol/10% acetic acid for 60 min, then twice in 200 ml of 10% ethanol/5% acetic acid for 30min. The oxidizer solution was prepared according to manufacturer's instructions. The gels were incubated for 10 min in 100 ml of oxidizer before being washed three times, each in 200 ml of ddH_2O for 10 min. The silver reagent was also prepared as per the manufacturer's instructions and the gels incubated in 100 ml of silver reagent for 30 min. After this time the gels were washed once in 200 ml of ddH_2O for 2 min. To visualize the protein bands, 100 ml of developer solution was added and the gels incubated for approximately 2 min at RT, at which point the developer solution was discarded and 100 ml of fresh developer solution was added. The gels were

incubated for a further 5 min at RT and the developer solution again changed. To stop development, 200 ml of 5% acetic acid solution was added.

2.12.2.5 Electroblotting of Protein to a PDVF Membrane

Proteins were transferred to a PDVF membrane (Millipore) using a Hoefer TE 70 semi-dry transfer unit according to the manufacturer's instructions. The transfer stack was assembled containing 9 sheets of 3MM paper soaked in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3), PDVF membrane (prewet with methanol and then with transfer buffer), the protein gel and an additional 9 sheets of 3 MM paper soaked in transfer buffer. The transfer was run for 1 h at 40 mA with the voltage capped at 50 V. After 1 h, the membrane was stained with 0.5% Ponceau S solution (Gelman Sciences) for 5 min and subsequently destained with ddH_2O to check for transfer of protein.

2.12.2.6 Immunodetection of Cytokines

Membranes were incubated in 10% (w/v) skim milk in TBS buffer (137 mM NaCl, 2.7 mM KCl, 25 mM Tris) overnight at 4°C. The membranes were washed at RT three times with TTBS buffer (100 µl of Tween 20 in 100 ml TBS) for 5 min and once with TBS for 5 min. Membranes were then incubated with primary antibody in antibody-binding buffer (1% skim milk and 50 µl Tween 20 in 100 ml TBS). Rat sera that had tested positive in Section 2.11.3.3 were used as the primary antibody and were diluted 1:200 in 10 ml of antibody binding buffer. As a positive control for samples from virus-infected cells, polyclonal anti-myxoma virus rabbit serum was used, diluted 1:5,000 in 10 ml of antibody binding buffer. For samples prepared from transfected cells, anti-His C-term antibody was used diluted 1:5,000 in 10 ml of antibody binding buffer. After the

addition of the primary antibody, membranes were incubated at RT on a platform rocker for 2 h. Next, the membranes were washed once for 10 min with TTBS then three times for 5 min with TTBS and finally, once for 5 min with TBS. All washes were done at RT with agitation. Secondary antibody was prepared (1:1,000 dilution in 10 ml of antibody binding buffer). For the rat sera this was anti-rat-HRP (Silenus), for the polyclonal anti-myxoma virus rabbit serum this was anti-rabbit-HRP and for anti-His C-term antibody this was anti-mouse-HRP (Silenus). Membranes were incubated in 10 ml of secondary antibody solution for 1 h at RT on a platform rocker, before being washed once with TTBS for 10 min then three times for 5 min with TTBS. The final wash was for 5 min in TBS. Detection of the bound HRP antibody was done using either enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) (described in Section 2.12.2.8.1) or SigmaFast (3,3'-diaminobenzidine (DAB) peroxidase substrate) (Sigma) (described in Section 2.12.2.8.2).

2.12.2.7 Stripping and Re-probing Membranes

Membranes were stripped in 100 mM β-mercaptoethanol, 2% SDS and 62.5 mM Tris at 50°C for 30 min with agitation. The membranes were then washed twice for 10 min each with TTBS and blocked in 10% skim milk in TBS for 1 h at RT on a platform rocker. The membranes were washed once more with TTBS and once with TBS before detection using ECL (as described in Section 2.12.2.7.1) to confirm stripping success.

If no binding was evident on the membranes, they were re-probed with a different primary antibody and processed as detailed in Section 2.12.2.6.

2.12.2.8 Visualization of Rabbit Cytokines

2.12.2.8.1 ECL Detection

ECL is a non-radioactive method for detection of proteins conjugated directly or indirectly with horseradish peroxidase-labeled (HRP-labeled) antibodies. Briefly, an equal volume of detection solution 1 (Amersham Pharmacia Biotech) and detection solution 2 (Amersham Pharmacia Biotech) were mixed, poured onto the protein-bound side of the membrane and incubated for precisely 1 min at RT. The excess detection reagent was then drained and the membrane wrapped in cling wrap and placed in a film cassette. Under a safelight in a dark room, a piece of autoradiography film was placed on top of the membrane and the cassette closed. Initial exposure was for 15 sec and the film was developed immediately in an automatic film processor (Kodak). Once the film had been developed other exposure times were used depending on the signal strength.

2.12.2.8.2 SigmaFast Detection

Bound secondary antibody conjugated to HRP was also detected using the SigmaFast kit. After washing the unbound secondary antibody from the membrane as described in Section 2.12.2.6, membranes were incubated in 10 ml of SigmaFast solution for 10-30 min until protein bands were visualized. SigmaFast solution was made according to the manufacturer's instructions; one of each 3,3'diaminobenzidine tablet set was dissolved in 5 ml of ddH_20. The reaction was stopped by the addition of 10 ml of ddH_20.

2.13 Flow Analysis of Rabbit Cytokines

Flow cytometric analysis was undertaken to investigate whether the rabbit cytokine anti-sera could be used as a tool for examining the response of lymphoid cells to myxoma virus infection. The results from this section are described in chapter 6.

2.13.1 Optimization of IFNγ Detection in RL-5 Cells Using Flow Cytometry

RL-5 cells constitutively express IFNγ (Seto et al., 1997) and so were used to optimize experimental conditions for detection of cytokines in rabbit lymphocytes by flow cytometry. RL-5 cells, 2×10^6 per well, were seeded in a 24-well plate and incubated overnight with and without 5 μg/ml Brefeldin A (Bref A) (Sigma). Bref A reduces secretion of proteins from the cell by inhibiting their movement from the Golgi apparatus. As cytokines are secreted proteins, Bref A was used to sequester cytokines for intracellular detection. RL-5 cells were also incubated with and without 5 μg/ml Con A to determine whether stimulation with Con A activated signaling pathways involved in IFNγ expression that were potentially not constitutively activated.

RL-5 cells were harvested and centrifuged at 270×g and resuspended in 100 μl of PBS. To permeabilise the cells, 400 μl of methanol:acetone (1:1) was added and the cells incubated for 10 min at RT. The cells were then washed twice with PBS and resuspended in 100 μl of primary antibody. The primary antibody, either normal rat serum or serum from rats inoculated with IFNγ hb-B/C was diluted 1:20 in 0.1% BSA/PBS. As controls, two additional RL-5 cell samples were resuspended in 100 μl of 0.1% BSA/PBS without either serum. Cells were incubated at 37°C for 1 h, after which

they were washed twice with 0.1% BSA/PBS. Samples with either normal rat serum or IFNγ hb-B/C rat serum and one of the control samples were resuspended in 1:40 dilution of anti-rat-FITC (Silenus) in 0.1% BSA/PBS. The second control sample was resuspended in 100 µl of 0.1% BSA/PBS without secondary antibody. Cells were incubated at RT in the dark for 30 min. Prior to analysis on a Becton Dickinson FACSort flow cytometer, 500 µl of PBS was added to each sample. Instrument settings for the flow cytometer were FSC- voltage E-1, AmpGain 9.17, Mode Lin; SSC- voltage 203, AmpGain 1, Mode Log; FL1-H- voltage 443, AmpGain 1, Mode Log. IFNγ expression was analyzed as histogram overlays of FL1-H (IFNγ expression), which were generated using the CellQuest software.

2.13.2 Analysis of IFNγ in RL-5 Cells Infected with SLS or Ur

To determine whether IFNγ could be detected in cells infected with myxoma virus, 2×10^6 RL-5 cells were infected with SLS or Ur at a moi of 3 as described in Section 2.3.3, and incubated for 24 h with or without 5 µg/ml Bref A in a humidified incubator at 37°C and 5% CO_2 in air. IFNγ expression was examined as described in Section 2.13.1.

The results showed that normal rat serum non-specifically bound to cells when cell cultures had been infected with SLS or Ur. To try to reduce this non-specific binding, different permeabilisation and blocking techniques were tried.

2.13.2.1 Examination of Different Permeabilising Solutions

In all earlier experiments methanol:acetone had been used to permeabilise cells. To examine the effects of other permeabilisation techniques, RL-5 cells were infected with

SLS at a moi of 3 (Section 2.3.3) and either methanol:acetone, 70% ethanol or buffered formaldehyde acetone (BFA) (0.05 mM Na_2HPO_4, 0.7 mM KH_2PO_4, 45% acetone, 25% formalin, pH6.8) was used for permeabilisation. Samples were analyzed for IFNγ expression as described in Section 2.13.1.

2.13.2.2 Blocking Non-Specific Binding With Fetal Calf Serum or High Concentrations of BSA

RL-5 cells were infected and permeabilised with methanol:acetone, 70% ethanol or BFA. After permeabilisation, samples were incubated with 1:10 dilution of fetal calf serum (FCS) in PBS for 1 h at 37°C. The samples were washed twice with 0.1% BSA/PBS before being resuspended in primary antibody and analyzed for IFNγ expression by flow cytometry as described in Section 2.13.1. This experiment was also repeated two more times using 1% BSA/PBS and 3% BSA/PBS instead of 0.1% BSA/PBS for antibody dilutions and for washing cells.

2.14 Immunofluorescence of Tissue Sections From Rabbits Infected with Myxoma Virus

To analyze the immune response to myxoma virus *in vivo*, the expression of IFNγ and IL-4 in lymph node sections from laboratory and wild rabbits infected with SLS or Ur four days post infection were examined.

2.14.1 Sectioning Tissues for Analysis

Lymph nodes from laboratory and wild rabbits infected with SLS or Ur were used for analysis. Rabbit lymph nodes draining the virus inoculation site and contralateral lymph

nodes held at -70°C in Tissue-Tek (Sakura) blocks were obtained from Dr P. Kerr. Sections of 5-7 μm were cut using a cryostat and placed on microscope slides that had been coated with 0.01% poly-L-lysine (Merck) (v/v in ddH_2O) and air dried. Slides were labeled and fixed by immersing in formyl-calcium (10% formaldehyde (40%) (Merck), 10% 1 M $CaCl_2$ (Merck) in ddH_2O) for 5 min, dipping in cold acetone, immersing in chloroform: acetone (1:1) for 5 min at -20°C, dipping in cold acetone and washing twice in PBS. The slides were air dried and stored at -70°C.

2.14.2 Immunodetection of Rabbit Cytokines

Prior to immunodetection of rabbit cytokines, tissue sections were blocked overnight in 3% BSA/PBS, washed once with 1% BSA/PBS then three times with 0.01% Tween 20/PBS. Slides were placed in a humidified chamber and 100 μl of primary antibody diluted in 1% BSA/PBS was added. The primary antibodies used in these experiments were normal rat serum (negative control), anti-rabbit IL-4 helix A rat serum and anti-rabbit IFNγ hb-B/C rat serum (test samples) and the monoclonal antibody specific for anti-rabbit CD43 (positive control). The rat sera were diluted 1:40 in 1% BSA/PBS whereas the CD43 monoclonal antibody was diluted 1:200 in 1% BSA/PBS. Slides were incubated in a humidified chamber at 37°C for 2h. Sections were washed with 1% BSA/PBS and three times with 0.01% Tween 20/PBS. The secondary antibody (anti-rat-FITC or anti-mouse-FITC) was diluted 1:100 in 1% BSA/PBS, and 100 μl was added to each tissue section. The sections were then incubated in a humidified chamber at 37°C in the dark for 1 h. The slides were again washed once with 1% BSA/PBS and three times with 0.01% Tween 20/PBS before being mounted with FluoroGuard anti-fade (Bio-Rad) under a coverslip and examined using a confocal microscope. The results are presented in chapter 6.

Chapter 3

Lymphocyte Permissivity to Myxoma Virus Replication

p values for chapter 3

Proportion of Cells Infected

Con A significantly increased the proportion of cells infected. p values for this are shown in the table below.

Time (h)	p value
4	<0.02
12	<0.00001
24	<0.0003
36	<0.00001
48	<0.00001
72	<0.002

The proportion of Ur-infected cells dropped significantly at 72 h, with a p value <0.02

The proportion of cells infected with SLS or Ur when lymphoid cell cultures from laboratory and wild rabbits were stimulated with Con A for 4 h prior to infection was significantly lower compared to cell cultures stimulated for longer periods prior to infection, with a p value <0.001

Viral Replication

Con A significantly increased the titre of SLS. The p values for this are shown in the table below. The increase in Ur titres as a result of Con A activation was only significant in domestic rabbits ($p<0.03$).

Time (h)	p value
4	not significant
12	not significant
24	<0.003
36	<0.00001
48	<0.00001
72	<0.00001

Viral titres in wild rabbit lymphoid cell cultures were significantly lower than those in domestic rabbit lymphoid cell cultures. The p values for this are shown in the table below.

Time (h)	p value
4	<0.0004
12	<0.0006
24	<0.03
36	<0.00001
48	<0.0003
72	<0.00001

3.1 INTRODUCTION

Following the introduction of myxoma virus into the Australian wild rabbit population in the 1950s there was a rapid selection for rabbits that were genetically resistant to myxomatosis. The development of this resistance in rabbits has been well described (Fenner et al., 1953; Myers et al., 1954; Marshall and Fenner 1958; Marshall and Douglas 1961). More recently, the pathogenesis of myxoma virus in resistant rabbits has been experimentally examined and compared to that in susceptible rabbits (Best et al., 2000; Best and Kerr 2000). Although these studies put forward a model for resistance of rabbits to myxomatosis, no mechanism for resistance has been experimentally identified. Because of the major role of lymphocytes play in the pathogenesis of myxomatosis, it may be postulated that any decrease in the permissivity of lymphocytes for myxoma virus replication diminishes the severity of clinical disease (Fenner and Woodroofe 1953; Best and Kerr 2000). This is supported by other studies which have shown that myxoma viruses that are unable to replicate in lymphocytes, due to the insertional inactivation of genes that are essential for virus replication, have an attenuated phenotype in susceptible European rabbits (reviewed in Kerr and McFadden 2002; Zuniga 2002). The permissivity of lymphocytes from resistant wild rabbits to myxoma virus replication has been examined in unstimulated cells (Best and Kerr 2000). The aim of this chapter is to further investigate the relationship between lymphocyte permissivity and resistance of rabbits to myxomatosis.

Outside the skin, T-cells have been proposed to be the primary cell for myxoma virus replication and dissemination *in vivo* (Best and Kerr 2000). This is because myxoma virus antigen is detected in T-cell zones of the lymph node of infected rabbits (Best et al., 2000) and myxoma virus replicates in the rabbit $CD4^+$ T-cell line, RL-5 (Barry et al., 1995; Macen et al., 1996; Barry and McFadden 1997).

Additionally, MRV, a laboratory recombinant between myxoma virus and a small component of Shope fibroma virus (SFV), replicates in T-cells *in vitro* and is able to disseminate in *O. cuniculus,* whereas SFV does not replicate in T-cells and does not disseminate in *O. cuniculus* (Strayer et al., 1985). These studies indicate an association between replication in lymphocytes and dissemination *in vivo.*

T-cell permissivity for myxoma virus replication may be controlled at several stages of infection: attachment of the virion to the cell surface, entry of the virion into the cell, early and late viral gene transcription and translation, virion assembly and maturation and virion egress. Differences in permissivity between lymphocytes from susceptible and resistant rabbits to virus replication could be due to obstruction at any one of these stages. Best and Kerr (2000) have previously examined the permissivity of lymphocytes from resistant rabbits to myxoma virus replication. This study did not detect any *in vitro* difference in either virus replication (measured by plaque assay) or in the proportion of cells expressing a late viral protein (measured by immunofluorescence) in primary fibroblast and lymphocyte cultures from laboratory and wild rabbits (Best and Kerr 2000).

However, in hindsight and in the light of other evidence discussed below, a more extensive investigation is warranted to examine differences between lymphocytes from laboratory and wild rabbits in their permissivity for myxoma virus replication. Firstly, lymphoid cells in the Best and Kerr (2000) study were not stimulated with mitogen. Mitogen stimulation activates multiple cell signaling pathways, and has been shown to increase cell permissivity to myxoma virus infection (Lalani et al., 1999). Activation of cells in this way may enhance *in vitro* differences between lymphoid cells from susceptible and resistant rabbits that are too small to be detected in the absence of

activation. Activation of T-cells is an important part of the anti-viral immune response (reviewed in Sloan-Lancaster and Allen 1996; Hollsberg 1999; Bi and Altman 2001; Sims and Dustin 2002). Hence, any differences between activated lymphocytes from susceptible and resistant rabbits in permissivity for virus replication may considerably affect the outcome of myxoma virus infection.

Secondly, Best and Kerr (2000) only tested the virulent SLS strain of myxoma virus. The use of an attenuated strain of myxoma virus may increase the sensitivity of the assay for detecting small differences in permissivity between lymphocytes from susceptible and resistant rabbits. Finally, the proportion of lymphoid cells infected with myxoma virus was estimated by microscopic examination of cells for immunofluorescence staining for a late viral protein. A more sensitive quantitative analysis, for example, by using flow cytometry, may enhance the detection of differences in virus replication in infected lymphocytes from susceptible and resistant rabbits. Therefore, the hypothesis that lymphocytes from genetically resistant rabbits are less permissive for myxoma virus replication than lymphocytes from susceptible rabbits, would be more comprehensively tested in experiments incorporating these parameters.

To test this hypothesis, the following questions were addressed;

1) Can resistance of rabbits to myxomatosis be attributed to differences between lymphoid cells from susceptible and resistant rabbits in their permissivity to myxoma virus replication?
2) Does mitogen stimulation affect the permissivity of lymphoid cells to myxoma virus replication?

In these experiments, primary lymphoid cells isolated from lymph nodes of susceptible (laboratory) and resistant (wild) rabbits were infected *in vitro* with either the virulent SLS or attenuated Ur strain of myxoma virus. Cell cultures were prepared from rabbit lymph nodes as described in chapter 2 (Section 2.1.6), and left overnight to remove adherent cells, such as monocytes and macrophages, which are known to be permissive for myxoma virus infection (Macen et al., 1996; Mossman et al., 1996; Everett et al., 2000). It has been estimated that ~57% of cells in rabbit lymph nodes are T-cells and ~37% are B-cells (Mage 1998). Thus, the lymphoid cell cultures prepared for this thesis would have consisted predominantly of T- and B-cells, but would also include small populations of other non-adherent cells, such as NK cells. Because T-cells are considered the main target of myxoma virus outside of the skin (Best and Kerr 2000), the effect of activation of lymphocyte signaling pathways on SLS or Ur infection was analysed by addition of the T-cell specific mitogen, Con A, to the lymphoid cell cultures from resistant and susceptible rabbits.

3.2 RESULTS

3.2.1 Permissivity of Rabbit Lymphoid Cells for Myxoma Virus Replication

The ability of the virulent SLS and attenuated Ur strains of myxoma virus to replicate in lymphoid cell cultures from laboratory (susceptible) and wild (resistant) rabbits was examined by measuring the titre of virus produced by the cultures and the proportion of lymphoid cells infected. Virus titre was determined by plaque assay on Vero cell monolayers. The proportion of cells infected was determined by flow cytometric analysis of cells immunofluorescently stained for a myxoma virus late protein using the monoclonal antibody, 3b6e4. To examine the effects of low and high moi, rabbit lymphoid cell cultures were infected with SLS or Ur at a multiplicity of infection (moi) of 0.1 or 3 plaque-forming units (pfu)/input cell. If all cells are equally permissive for infection, then a moi of 3 theoretically leads to approximately 95% of cells being infected with at least one pfu of virus, and a moi of 0.1 results in a low proportion of cells initially infected, with the virus able to spread from initially-infected cells to uninfected cells.

3.2.1.1 Replication of Myxoma Virus in Rabbit Lymphoid Cell Cultures

Lymphoid cells were infected with either SLS or Ur at a moi of 0.1 or 3 and analyzed for virus replication by plaque assay, 4, 12, 24, 36, 48 and 72 h post infection. Cultures were incubated with or without the T-cell mitogen, Con A. All experiments were replicated using lymphoid cell cultures prepared from three laboratory and three wild rabbits. Virus replication was expressed as mean pfu/input cell for each infection and

data are presented as pfu/input cell over time. Statistical analysis of virus replication was by ANOVA with a p value <0.05 deemed significant.

3.2.1.1.1 SLS Titres in Unstimulated and Con A-Stimulated Lymphoid Cell Cultures from Laboratory and Wild Rabbits

Unstimulated lymphoid cell cultures from laboratory rabbits produced low titres (mean <5 pfu/input cell) of SLS regardless of moi (Figure 3.1A and 3.1B, open squares, dotted line). In these infections, SLS titres remained negligible (<2 pfu/input cell) until 36 h post infection after which they increased to a mean of 5 pfu/input cell by 72 h (Figure 3.1A and 3.1B). Unstimulated lymphoid cell cultures from wild rabbits appeared to be less permissive for SLS replication than those from laboratory rabbits, with the mean titre remaining less than 2 pfu/input cell for all time points for both moi (Figure 3.1E and 3.1F).

Con A stimulation significantly increased the titres of SLS produced by rabbit lymphoid cell cultures ($p<0.05$) (Figure 3.1A, 3.1B, 3.1E and 3.1F filled square/circles, solid line). When lymphoid cell cultures from either laboratory or wild rabbits were infected with SLS at a moi of 0.1, titres increased after 24 h to reach a mean of 11 pfu/input cell by 72 h (Figure 3.1A and 3.1E). When a moi of 3 was used for the initial infection, SLS titres increased after the 12 h time point and plateaued at 36-48 h. By 72 h, there were 19 pfu/input cell and 9 pfu/input cell for lymphoid cell cultures from laboratory and wild rabbits respectively (Figure 3.1B and 3.1F).

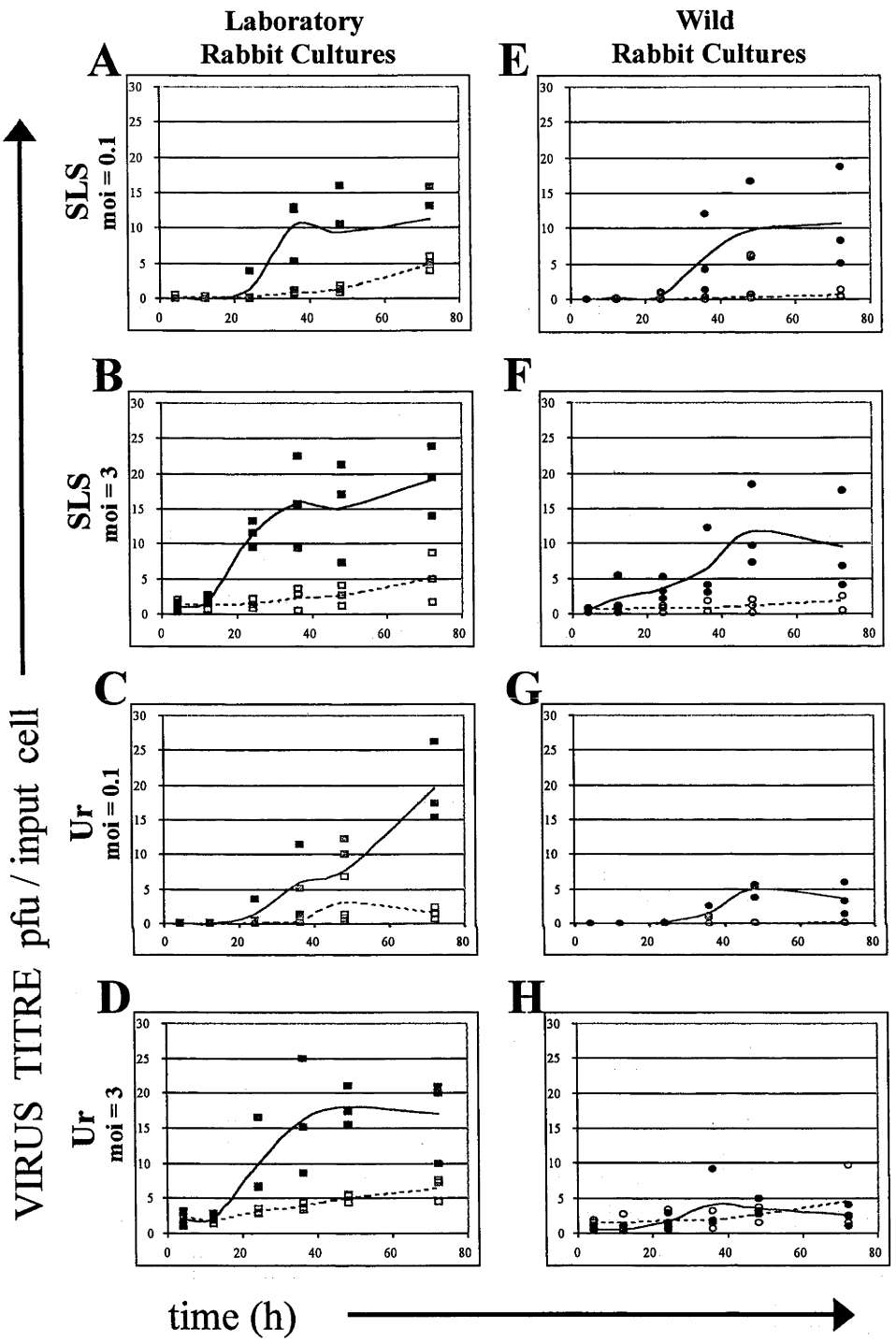


FIGURE 3.1. TITRES OF SLS AND UR IN LYMPHOID CELL CULTURES FROM LABORATORY AND WILD RABBITS

Primary lymphoid cells isolated from (A-D) laboratory or (E-H) wild rabbits were infected with either SLS, at a moi of 0.1 (A, E) or 3 (B, F), or Ur at a moi of 0.1 (C, G) or 3 (D, H). Samples were harvested 4, 12, 24, 36, 48 or 72 h post infection. The time course was undertaken in the presence (solid) or absence (open) of 5μg/ml Con A. Virus was released from the cells by freeze/thawing and sonication and titres determined by plaque assay on Vero cell monolayers. Graphs show results from lymphoid cell cultures prepared from three laboratory and three wild rabbits, with each point representing a different rabbit. Solid lines indicate the mean titre in Con A-stimulated lymphoid cell cultures, whereas dotted lines indicate the mean titre in unstimulated lymphoid cell cultures.

3.2.1.1.2 Ur Titres in Unstimulated and Con A-Stimulated Lymphoid Cell Cultures from Laboratory and Wild Rabbits

In unstimulated lymphoid cell cultures from laboratory rabbits infected with Ur at a moi of 0.1 or a moi of 3, a mean titre of 2 and 6 pfu/input cell respectively, was reached 72 h post infection (Figure 3.1C and 3.1D). Infection of unstimulated lymphoid cell cultures from wild rabbits with Ur at a moi of 0.1 did not produce infectious virus particles to any extent over the 72 h time course (mean <1 pfu/input cell) (Figure 3.1G). Infection of unstimulated cell cultures from wild rabbits, with a moi of 3, produced a mean of 5 pfu/input cell by 72 h (Figure 3.1H).

Con A significantly increased Ur titres only in lymphoid cell cultures from laboratory rabbits ($p<0.05$). In these cultures, Ur titres increased after 24 h to 20 pfu/input cell by 72 h, when a moi of 0.1 was used (Figure 3.1C). In cultures infected with Ur at a moi of 3, titres increased after 12 h and plateaued at 36 h with 18 pfu/input cell produced by 72 h (Figure 3.1D). A similar increase in Ur titres was not observed in lymphoid cell cultures from wild rabbits as Ur titres remained less than 5 pfu/input cell for all infections at all time points ($p<0.05$) (Figure 3.1G and 3.1H). However, in the low moi, Con A stimulation tended to increase Ur titres when compared to unstimulated lymphoid cell cultures from wild rabbits, but this was not significant at the 95% confidence interval (CI) (Figure 3.1G).

3.2.1.1.3 Summary of Myxoma Virus Replication

Using plaque assays to measure viral titre in Con A-activated lymphoid cell cultures from different rabbits gave varied results, as illustrated by the scattered data points. In spite of this variation, statistical analysis of the plaque assay results showed that Con A activation significantly increased SLS titres in cultures from both rabbit types. However

for Ur infections, Con A activation only significantly increased viral titres in lymphoid cell cultures from laboratory rabbits. The lack of Ur replication in Con A-stimulated lymphoid cell cultures from wild rabbits clearly differentiated between the two rabbit types. In addition, the replication of myxoma virus was significantly less efficient overall in stimulated lymphoid cell cultures from wild rabbits than in lymphoid cell cultures from laboratory rabbits ($p<0.05$). These results suggest that the use of the virulent SLS strain in unstimulated cultures in previous studies (Best and Kerr, 2000) has masked potential differences in lymphocyte permissivity for myxoma virus replication between the two rabbit types. This will be discussed in more detail in Section 3.3.1.

Infection of cultures with the low moi resulted in a low proportion of cells initially infected compared to a moi of 3, where approximately 95% of cells would be infected with at least one pfu. Therefore, in cultures infected at the low moi, virus would have to spread from cell to cell. This would explain why viral titres were increased with Con A stimulation after 12 h in cultures infected with a moi of 3 and after 24 h in cultures infected with a moi of 0.1.

Lymphoid cell cultures from resistant rabbits were statistically significantly less permissive for virus replication than cell cultures from susceptible rabbits. This may have important implications for resistance to myxomatosis and will be considered further in the discussion to this chapter.

3.2.1.2 Proportion of Rabbit Lymphoid Cells Infected with Myxoma Virus

The proportion of cells infected with myxoma virus was determined, using flow cytometry, by immunostaining infected lymphoid cells with a monoclonal antibody (3b6e4) specific for an intracellular myxoma virus protein expressed late in infection. Lymphoid cell cultures from laboratory and wild rabbits were mock-infected with medium or infected with SLS or Ur at a moi of 0.1 or 3. Cells were examined 4, 12, 24, 36, 48 and 72 h post infection. Lymphoid cell cultures were incubated in the presence or absence of Con A.

Lymphoid cell cultures were mock-infected or infected with myxoma virus and the binding of either mouse ascites fluid or the 3b6e4 monoclonal antibody. Figure 3.2A shows that the binding of the 3b6e4 antibody is specific for myxoma virus infection, as a shift of the peak to the right was observed in samples incubated with the 3b6e4 antibody, compared to the mouse ascites fluid control (Figure 3.2A). Figure 3.2B and 3.2C show density plots of cell granularity (SSC) versus 3b6e4 antibody binding (FL1-H) and histogram plots of 3b6e4 antibody binding respectively in mock-infected and myxoma virus-infected lymphoid cell cultures. It was assumed that all cells in the gated sub-population staining for the myxoma virus protein (Figure 3.2- blue ovals and brackets) were infected because mock-infected lymphoid cell cultures did not fluoresce at these intensities.

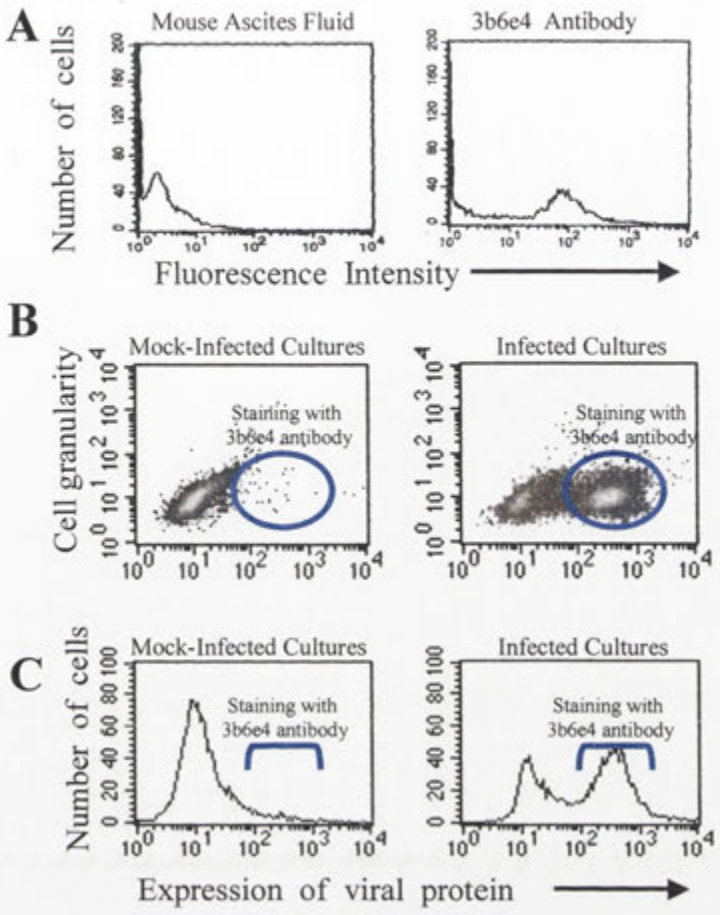


FIGURE 3.2. FLOW CYTOMETRIC ANALYSIS OF MYXOMA VIRUS INFECTION OF RABBIT LYMPHOID CELLS

Myxoma virus infection of rabbit lymphoid cells was analysed by flow cytometry. (A) To determine non-specific binding, rabbit lymphoid cells were infected with myxoma virus and stained using mouse ascites fluid. This was compared to staining of infected cells using the anti-myxoma monoclonal antibody, 3b6e4, which is specific for an intracellular myxoma virus protein. (B) Density plots of cell granularity versus myxoma virus protein expression and (C) histogram plots of protein expression versus number of cells (counts) were generated using CellQuest software. Blue circles and brackets are those sub-populations of cells expressing the viral protein.

The sub-population of infected cells was gated as shown in Figure 3.2 and the proportion of events in this gate was calculated using CellQuest software. All experiments were replicated by using lymphoid cell cultures prepared from three individual laboratory and three individual wild rabbits. Samples of these cultures were used in the analysis of virus replication. Statistical analysis of the proportion of infected cells was by ANOVA with a p value less than 0.05, significant.

3.2.1.2.1 Proportion of Rabbit Lymphoid Cells Infected with SLS

In lymphoid cell cultures from both laboratory and wild rabbits, incubated in the absence of Con A, the proportion of SLS-infected lymphocytes increased steadily after 24 h to reach 13% by 72 h when a moi of 0.1 was used (open squares and circles Figure 3.3A and 3.3E). However, when a moi of 3 was used, differences in the proportion of infected cells were observed between unstimulated lymphoid cell cultures from laboratory and wild rabbits. In cultures from laboratory rabbits, 21% of cells were infected by 12 h and 40% by 72 h post infection (Figure 3.3B). In contrast, in cell cultures from wild rabbits infected with SLS at a moi of 3, the proportion of infected cells rose steadily from 24 h to reach 30% at 72 h (Figure 3.3F). These cultures also had similar proportions of infected cells at 4 and 12 h post infection to lymphoid cell cultures from laboratory rabbits but lower proportions of infected cells at 24 h post infection (4%) and all subsequent time points. This may indicate events occurring within the first 24 h are important for resistance in unstimulated lymphoid cells from wild rabbits.

Con A significantly increased the proportion of cells infected with SLS in lymphoid cell cultures from both laboratory and wild rabbits ($p<0.05$). The increase in proportion of rabbit lymphoid cells infected for both moi was similar, reaching approximately 70%, but occurred around 24 h with the higher moi, compared to 36 h with the low moi (solid squares and circles Figure 3.3A, 3.3B, 3.3E and 3.3F). In all SLS infections, the proportion of cells infected was slightly less towards the end of the time course with approximately 60% of cells infected at 72 h in all cultures. The exception to this was SLS in lymphoid cell cultures from wild rabbits.

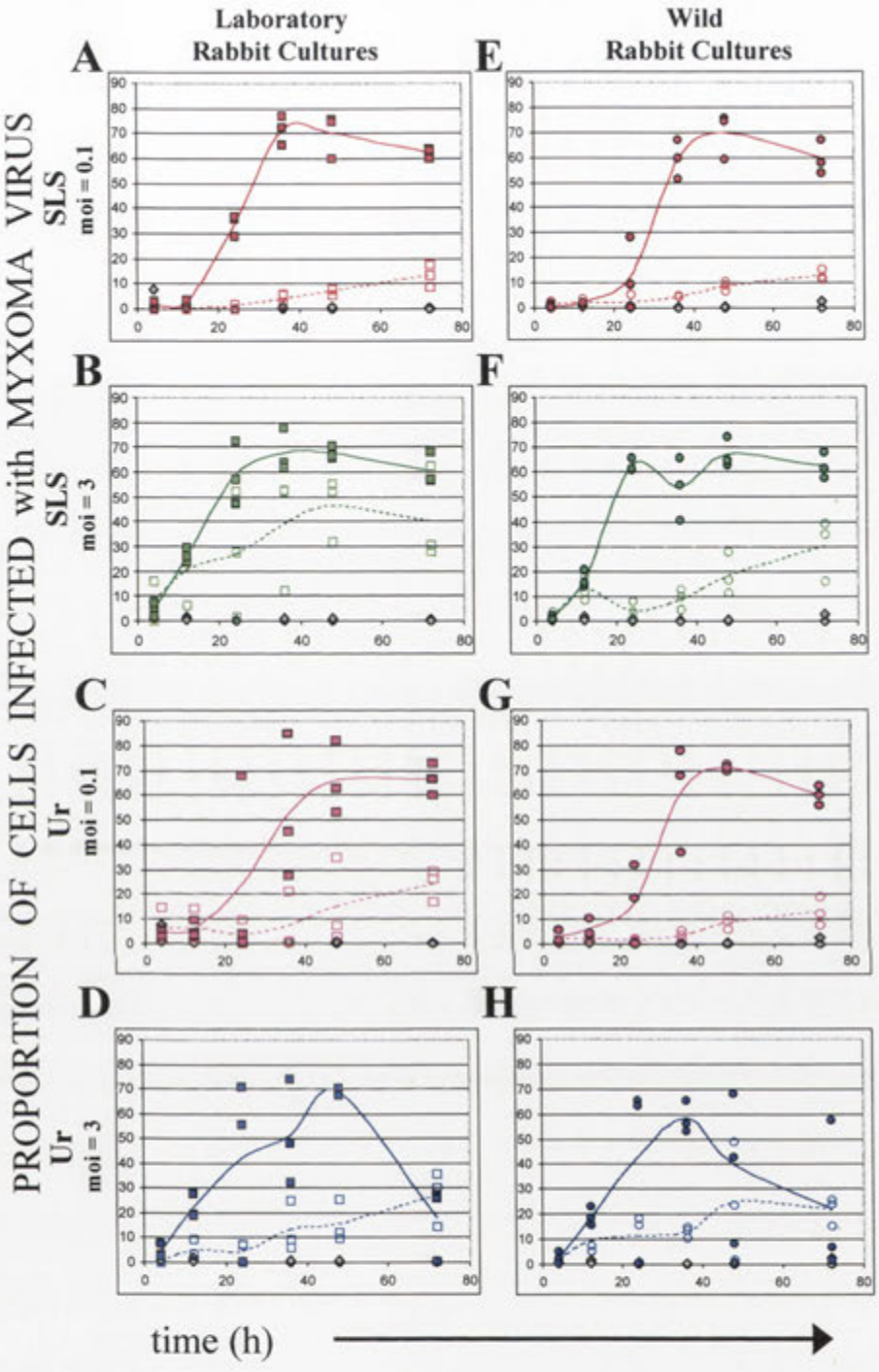


FIGURE 3.3. PROPORTION OF CELLS IN RABBIT LYMPHOID CELL CULTURES INFECTED WITH SLS OR UR

Rabbit lymphoid cells isolated from (A-D) laboratory or (E-H) wild rabbits were infected with either SLS, at a moi of 0.1 (A, E) or 3 (B, F), or Ur, at a moi of 0.1 (C, G) or 3 (D, H). Cells were harvested 4, 12, 24, 36, 48 or 72 h post infection. The time course was undertaken in the presence (solid squares) or absence (open squares) of 5μg/ml Con A. The proportion of cells infected was determined by immunostaining for an intracellular late viral protein and analysis on a Becton Dickinson FACSort using the associated CellQuest software, as shown in Figure 3.2. Graphs show data points for lymphoid cell cultures prepared from three laboratory and three wild rabbits. Grey data points indicate mock-infected controls (solid and open diamonds indicate with and without Con A respectively). Solid lines indicate the mean proportion of infected cells in Con A-stimulated cultures, whereas dotted lines indicate the mean proportion of infected cells in unstimulated cultures.

3.2.1.2.2 Proportion of Rabbit Lymphoid Cells Infected with Ur

The proportion of Ur-infected cells was similar over the 72 h time course between lymphoid cell cultures from both rabbit types regardless of moi. In unstimulated cultures from laboratory rabbits, the proportion of Ur-infected cells reached 24% and 27% by 72 h, when infected with a moi of 0.1 or 3 respectively (Figure 3.3C and 3.3D). In unstimulated cultures from wild rabbits the proportion of cells infected increased to 13% and 22% by 72 h post infection when a moi of 0.1 and 3 were used respectively (Figure 3.3G and 3.3H).

Con A stimulation significantly increased the proportion of cells infected with Ur in lymphoid cell cultures from both laboratory and wild rabbits ($p<0.05$). In infections with a moi of 0.1, the proportion of infected cells reached 66% and 71% by 48 h in lymphoid cell cultures from laboratory and wild rabbits respectively (Figure 3.3C and 3.3G). In lymphoid cell cultures from laboratory rabbits, the proportion of infected cells did not change during the remainder of the experiment. The proportion of infected cells fell to 60% by 72 h in lymphoid cell cultures from wild rabbits.

When a moi of 3 was used in lymphoid cell cultures from laboratory rabbits, the proportion of cells infected with Ur increased significantly with Con A stimulation to 69% at 48 h ($p<0.05$). After this time point, the proportion of cells infected dropped significantly to 18% at 72h ($p<0.05$) (Figure 3.3D). A similar result was observed in wild rabbits lymphoid cell cultures infected with Ur. In these cultures, the proportion of cells infected rose to 58% by 36 h (Figure 3.3H), after which it dropped to 22% at 72 h. This significant decrease was not observed in cultures infected with Ur at a low moi or in cultures infected with SLS.

3.2.1.2.3 Summary of the Proportion of Infected Lymphoid Cells

Con A stimulation had a significant effect on the proportion of cells infected with myxoma virus in rabbit lymphoid cell cultures ($p<0.05$). The maximum proportion of lymphocytes infected with SLS was similar to that infected with Ur when cultures were stimulated. This was regardless of type of rabbit from which the lymphocytes were derived, and directly contrasts to virus replication, where Ur did not replicate in stimulated lymphoid cell cultures from wild rabbits, but did replicate in stimulated cultures from laboratory rabbits.

Although approximately 70-80% of cells were infected when either a high or low moi was used, there was a lag in reaching the 70-80% in cell cultures infected with a moi of 0.1 compared to infections with a moi of 3. For example, 70-80% of cells were infected with SLS 36-48 h after Con A stimulation, when a moi of 0.1 was used compared to 24-36 h for infections when a moi of 3 was used. Supporting this, the proportion of cells infected was significantly different between the two moi only until 36 h ($p<0.05$).

The significant drop in the proportion of cells infected with Ur in Con A-stimulated lymphoid cell cultures at late time points contrasts to SLS infections, where no large decrease was apparent. This decrease was observed in cell cultures from both laboratory and wild rabbits after infection with Ur at a moi of 3. This indicates that there was a difference between the virulent and attenuated myxoma virus strains in their replication in primary rabbit lymphocytes. The difference was not due to the properties of the lymphocytes since the same effect was observed in infected lymphoid cell cultures from both laboratory and wild rabbits. Therefore, there must be a difference between SLS and Ur that becomes apparent during *in vitro* infection of lymphocytes.

3.2.1.3 Proportion of Cells Infected in Rabbit Lymphoid Cell Cultures Stimulated with Con A Prior to Infection

Stimulation of lymphoid cells significantly increased both viral titre (Section 3.2.1.1) and proportion of infected cells (Section 3.2.1.2). However, the cells were stimulated after an initial 1 h infection. Therefore, stimulation would not be affecting attachment to the cell surface, but rather, would be acting on processes after this point, such as entry of the virion into the cell, early and late gene transcription and translation, virion assembly and maturation or virion egress. Stimulation of specific cell signal pathways has already been shown to be critical for myxoma virus infection (Lalani et al., 1999; Masters et al., 2001). However, those experiments were undertaken on cell lines that were permanently activated. To examine myxoma virus infection of primary lymphoid cell cultures that were activated prior to infection, cell cultures were stimulated with Con A for 4, 24, 48 and 72 h before infection with SLS or Ur at a moi of 3 for an additional 12 or 24 h. Myxoma virus infection was examined by flow cytometric analysis of 3b6e4 binding as described in Section 3.2.1.2.

The proportion of cells infected when cultures were stimulated with Con A prior to infection are tabulated in Table 3.1. For comparison, the mean and range of proportion of cells infected when cell cultures were stimulated with Con A *after* infection, as described in Section 3.2.1.2, is also included.

TABLE 3.1. MEAN PROPORTION OF CELLS INFECTED* WHEN RABBIT LYMPHOID CELL CULTURES WERE STIMULATED WITH CON A PRIOR TO SLS OR UR INFECTION

Period of Con A Stimulation *Prior* to Infection	SLS				Ur			
	Laboratory Rabbit Lymphoid Cell Cultures		Wild Rabbit Lymphoid Cell Cultures		Laboratory Rabbit Lymphoid Cell Cultures		Wild Rabbit Lymphoid Cell Cultures	
	12 h infection	24 h infection	12 h infection	24 h infection	12 h infection	24 h infection	12 h infection	24 h infection
4 h[θ]	11%	21%	8%	21%	8%	15%	8%	19%
24 h	13%	32%	12%	46%	12%	44%	16%	38%
48 h	16%	52%	23%	32%	16%	48%	18%	30%
72 h	12%	42%	28%	63%	13%	41%	26%	58%
Mean and Range in Proportion of Cells Infected when Cell Cultures were Stimulated *after* Infection[+]	Mean 26%	Mean 59%	Mean 17%	Mean 63%	Mean 22%	Mean 42%	Mean 19%	Mean 43%
	Range 24% - 30%	Range 48% - 73%	Range 14% - 21%	Range 61% - 66%	Range 19% - 28%	Range 0% - 71%	Range 16% - 23%	Range 0% - 65%

* The proportion of cells infected was determined through flow cytometry by immunostaining for an intracellular viral protein. The mean from experiments using lymphoid cell cultures from three laboratory and three wild rabbits is tabulated.

[+] For comparison, the mean proportion and range of proportion of cells infected after 12 and 24 h when cultures were stimulated with Con A after infection with SLS or Ur at a moi of 3, is also shown.

[θ] 4 h Con A stimulation prior to infection caused significantly less cells to be infected then all other infections shown above. No significant difference was observed between the other infections.

3.2.1.3.1 Infection of Con A-Stimulated Lymphoid Cell Cultures from Laboratory and Wild Rabbits

The proportion of cells infected with SLS or Ur when lymphoid cell cultures from laboratory and wild rabbits were stimulated with Con A for 4 h prior to infection was significantly lower compared to cell cultures stimulated for longer periods prior to infection ($p<0.05$) (Table 3.1). This was also significantly less than those in cell cultures that were stimulated after infection. For example, lymphoid cells from laboratory rabbits, infected with SLS for 24 h after stimulation with Con A for 4 h had 21% of cell infected compared to 59% when Con A stimulation followed infection. This was also lower than in cultures stimulated for longer periods prior to infection. Comparing this with previous results, where cultures were stimulated with Con A after infection, shows that the proportion of cells infected in cultures stimulated for 4 h with Con A prior to infection, was less than half that in cultures that were infected prior to stimulation. These results raise some interesting questions, as they suggest that a short period of lymphocyte activation prior to infection makes the cells refractory to productive infection. Alternatively, a longer period of Con A stimulation resulted in a greater proportion of lymphocytes infected, suggesting that events occurring late in lymphocyte activation have either less effect on or increase the permissivity of lymphocytes for myxoma virus replication.

Lymphoid cell cultures from wild rabbits that were stimulated with Con A for 48 h prior to infection for 24 h with either SLS or Ur at a moi of 3, had a lower proportion (not statistically significant at the 95% CI) of cells infected than if cultures were stimulated for 24 or 72 h prior to infection. For example, 32% of lymphocytes were infected with SLS in cultures that were stimulated for 48 h prior to infection for 24 h, compared to

46% and 63% if cultures were first stimulated for 24 or 72 h (Table 3.1). This effect was not observed in cultures from laboratory rabbits. The reason for this is not clear.

Experiments in Section 3.2.1.2 and 3.2.1.3 indicate that there is an upper limit of 60-80% of cells in rabbit lymphoid cell cultures susceptible to myxoma virus infection. This limit was reached in cell cultures stimulated with Con A after infection for 24-48 h and in cultures stimulated with Con A for 72 h prior to infection for 24 h. This, taken together with the increase in virus replication in Con A-stimulated cell cultures, implied that either mitogen stimulation produced more target cells for the virus, or Con A-stimulated cells were more permissive for infection and that only a small proportion of cells were appropriately activated in unstimulated cell cultures. To address these issues, proliferation assays (Section 3.2.2) and a more comprehensive analysis of viral titre per infected cell (Section 3.2.3) and cell activation, as measured by cell size (Section 3.2.4), were undertaken.

3.2.2 Con A-Induced Proliferation of Rabbit Lymphoid Cells

Proliferation of rabbit lymphoid cells was measured by two methods. The first of these was tritiated thymidine (^{3}H-T) incorporation, which measures DNA replication. The second, the CellTitre 96® AQ_{ueous} One Solution Cell Proliferation Assay, is a colorimetric assay where the mitochondrial activity in metabolically active cells causes the reduction of a MTS tetrazolium compound to a colored formazan product, the absorbance of which can be read at 490 nm. Cells that are not viable are not metabolically active and, therefore, will not cause a color change. Hence, the absorbance at 490nm is directly proportional to the number of live cells.

The use of these two proliferation assays enabled examination of somewhat different time points because the primary lymphoid cell cultures were incubated for 18 h with ^{3}H-T prior to harvest, whereas in the CellTitre Proliferation Assay, samples were measured after a 4 h incubation with the MTS reagent. Thus, the ^{3}H-T assay measured whether cells were actively replicating their DNA for a period after each time point whereas the CellTitre assay provided a snapshot of the number of metabolically active cells at each time point.

All infections were analysed in triplicate and repeated in primary lymphoid cell cultures prepared from three different laboratory and three different wild rabbits. Because similar results were obtained for all rabbit lymphoid cell cultures, only results from one rabbit of each type is shown here, with the replicates for ^{3}H-T and CellTitre Proliferation Assay in Appendix 1 and 2 respectively. The ability of infected RL-5 cells to proliferate was also examined. Results are presented as column graphs with values adjusted such that ^{3}H-T incorporation or absorbance at 490 nm of unstimulated mock-infected cell cultures at the 0 h time point was equal to 1 unit.

3.2.2.1 ^{3}H-T Incorporation as a Measure of Proliferation of Rabbit Lymphoid Cells Infected with SLS or Ur

^{3}H-T incorporation into mock-infected cell cultures was first detectable at the 24 h time point (24 h mock-infection and 18 h incubation with ^{3}H-T) and peaked at the 48 h time point (48 h mock-infection and 18 h incubation with ^{3}H-T) (Figure 3.4), with the exception of one wild rabbit sample where the peak in ^{3}H-T incorporation was at the 72 h time point (72 h mock-infection and 18 h incubation with ^{3}H-T) (see Appendix 1).

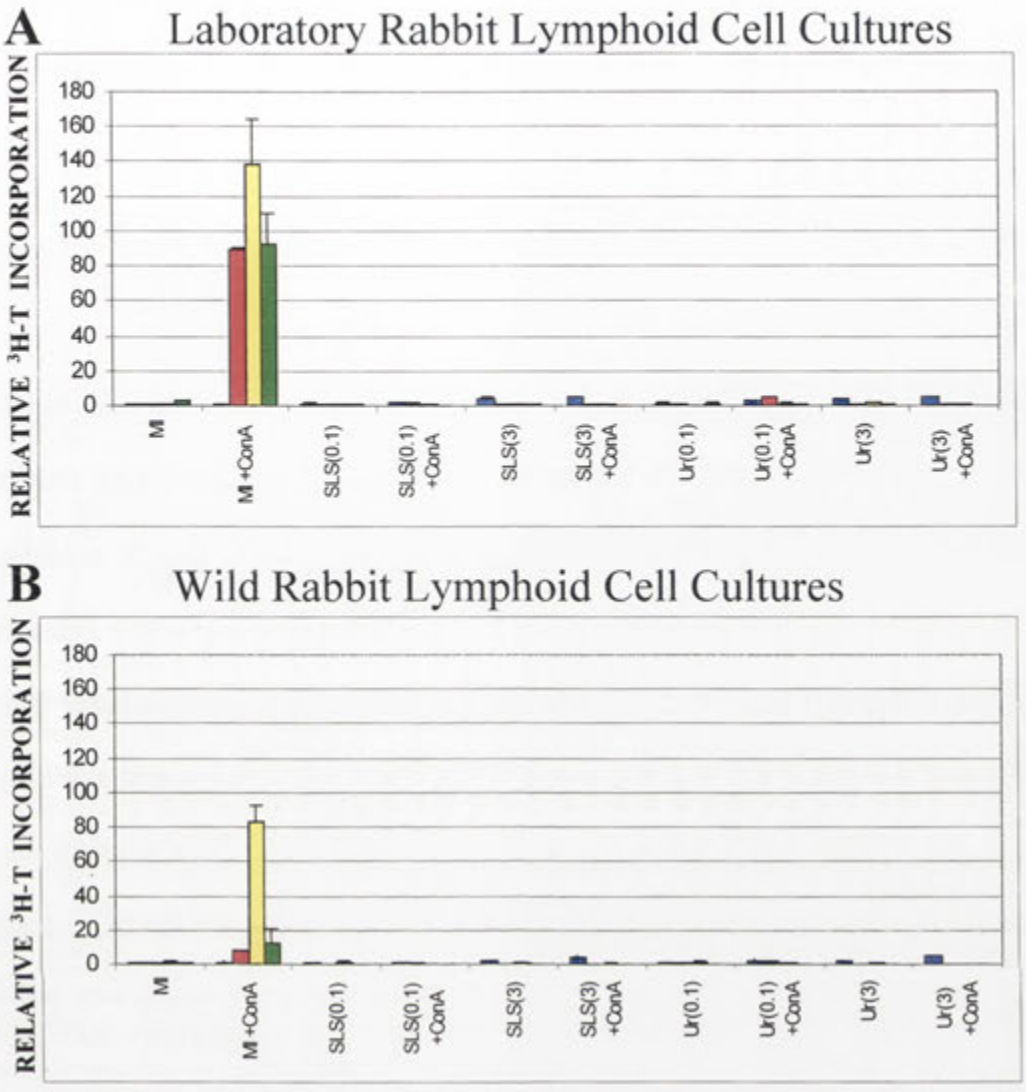


FIGURE 3.4. ^{3}H-T INCORPORATION IN PRIMARY LYMPHOID CELLS ISOLATED FROM LABORATORY OR WILD RABBITS AND INFECTED WITH SLS OR UR

The proliferation of lymphoid cells from (A) laboratory and (B) wild rabbits was measured using tritiated thymidine (^{3}H-T) incorporation. Lymphoid cells were mock-infected (MI) or infected with SLS or Ur at a moi of 0.1 or 3 pfu/input cell, for one hour prior to the 0 h time point. Con A was added at 0 h. ^{3}H-T was added at 0■, 24■, 48■ and 72 h■ post infection. Pulsing was for 18h prior to analysis on a Top Count Microplate Scintillation Counter. Values were adjusted such that ^{3}H-T incorporation of MI cultures at 0 h (0 h incubation and 18 h incubation with ^{3}H-T) was equal to 1 unit. Reactions were done in triplicate and repeated on cultures prepared from three laboratory and three wild rabbits (a representative result is shown here, see Appendix 1 for replicates). Error bars represent standard errors.

Infection with SLS or Ur completely abrogated Con A-induced proliferation of lymphoid cells from both laboratory and wild rabbits, as measured by ^{3}H-T incorporation (Figure 3.4). This occurred even at low moi. Unstimulated rabbit

lymphoid cells did not proliferate in the absence of mitogen, nor did they proliferate in response to myxoma virus infection in unstimulated cell cultures (Figure 3.4).

3.2.2.2 MTS Assay as a Measure of Proliferation of Rabbit Lymphoid Cells Infected with SLS or Ur

Total abrogation of Con A-induced proliferation of primary lymphoid cells by SLS and Ur was also observed using the CellTitre 96® AQ_{ueous} One Solution Cell Proliferation Assay (Figure 3.5). In most cases, the peak absorbance due to Con A stimulation of mock-infected cell cultures was observed 72 h post infection (Figure 3.5, Appendix 2). The reason this differs from the peak in ^{3}H-T incorporation is most probably due to the different times required for analysis. ^{3}H-T incorporation is measured over an 18 h period whereas the CellTitre assay is a colorimetric assay that measures live cell numbers at each time point. The optimal time for incubation with the MTS reagent to maximize the signal to noise ratio for primary rabbit lymphocytes was previously determined to be 4 h (Dr P. Kerr, personal communication).

The absorbance at 490 nm was lower in virus-infected cell cultures than in mock-infected cell cultures. As the CellTitre assay measures the number of live cells, this suggests that both SLS and Ur may induce cell death in infected lymphocytes. Mitochondrial activity, as measured in the CellTitre assay, in unstimulated primary rabbit lymphoid cells did not change substantially over the time course and was not altered by myxoma virus infection (Figure 3.5).

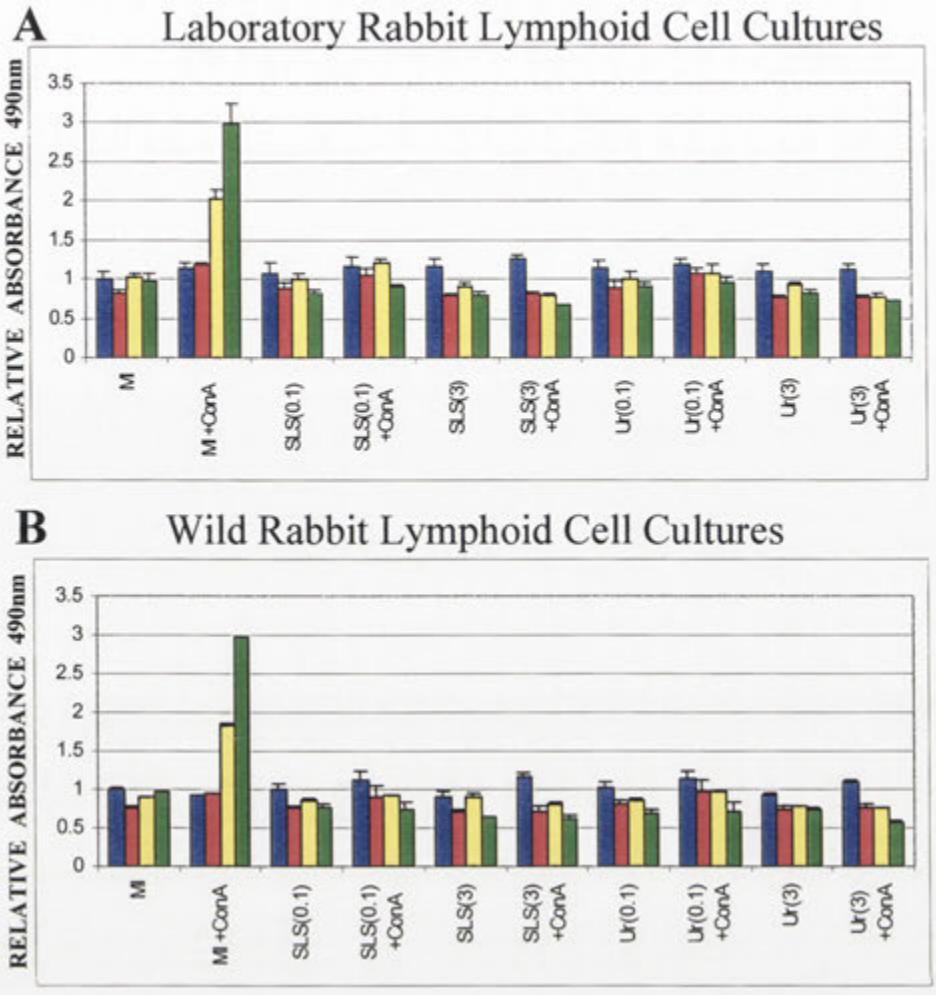


FIGURE 3.5. PROLIFERATION OF PRIMARY LYMPHOID CELLS ISOLATED FROM LABORATORY OR WILD RABBITS AND INFECTED WITH SLS OR UR

The proliferation of lymphoid cells from (A) laboratory and (B) wild rabbits was measured using the CellTitre 96® AQ_{ueous} One Solution Cell Proliferation Assay. Lymphoid cells were mock-infected (MI) or infected with SLS or Ur at a moi of 0.1 or 3 pfu/input cell, for one hour prior to the 0 h time point. Con A was added at 0 h. The MTS reagent was added at 0■, 24■, 48■ and 72 h■ post infection. Absorbance was read at 490 nm after a 4 h incubation with the MTS reagent. Values were adjusted such that the absorbance of MI samples at 0 h was equal to 1 unit. Reactions were done in triplicate and repeated using cultures from three laboratory and three wild rabbits. A representative result is shown here, with replicates in Appendix 2. Error bars represent standard errors.

3.2.2.3 Proliferation of RL-5 Cells Infected with SLS or Ur

Since most studies analyzing myxoma virus infection have utilized the RL-5 rabbit T-cell line, ^{3}H-T and CellTitre proliferation assays were undertaken on infected RL-5 cells to determine whether infection with myxoma virus could also inhibit proliferation of these cells. As RL-5 cells are continuously proliferating, they incorporated ^{3}H-T at 0 h

and did not require Con A activation. This contrasted with primary lymphoid cell cultures, which only proliferated once Con A was added.

As with primary lymphoid cells, infection of RL-5 cells with SLS or Ur at either moi totally abrogated proliferation measured by both the ^{3}H-T incorporation and CellTitre assay (Figure 3.6). This experiment was repeated three times with different RL-5 cells. Results shown in Figure 3.6 are from one set of experiments, with results from the other two sets of experiments in Appendix 3. The absorbance in cultures infected with myxoma virus was lower than that in mock-infected cultures (Figure 3.6B), suggesting that myxoma virus induced cell death in some infected cells.

3.2.2.4 Summary of Cell Proliferation

Myxoma virus infection totally abrogated proliferation of primary rabbit lymphoid cells and RL-5 cells. Thus, it can be concluded that the increase in proportion of cells infected and increase in viral titres in primary rabbit lymphoid cell cultures observed when cultures were stimulated with Con A was not due to an increase in the number of target cells. In fact, infection with myxoma virus even at a low moi prevented cell proliferation, suggesting that inhibition of lymphocyte proliferation may be a critical immunosuppressive mechanism during myxoma virus infection.

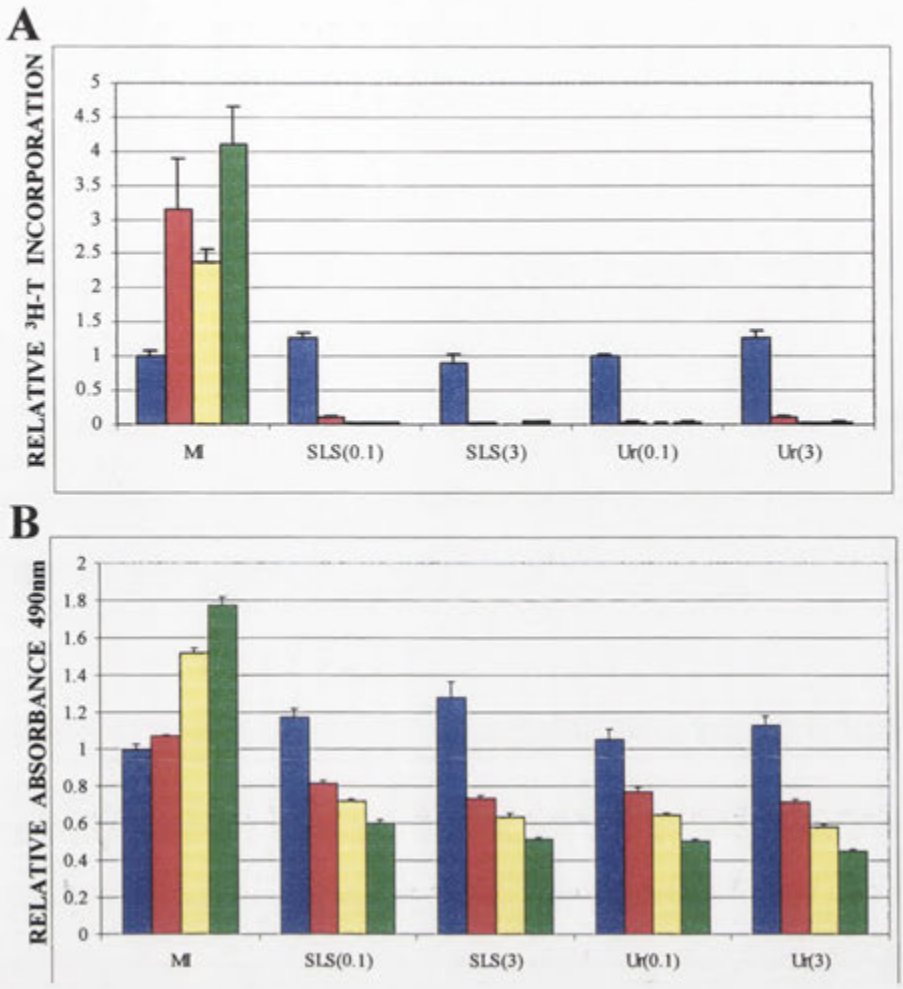


FIGURE 3.6. PROLIFERATION OF THE RABBIT T-CELL LINE, RL-5, INFECTED WITH SLS OR UR

Proliferation of RL-5 cells was measured using (A) tritiated thymidine (^{3}H-T) incorporation or (B) CellTitre 96® AQ_{ueous} One Solution Cell Proliferation Assay. RL-5 cells were mock-infected (MI) or infected with SLS or Ur at a moi of 0.1 or 3 pfu/input cell for one hour prior to beginning of time course. ^{3}H-T or MTS reagent was added at 0■, 24■, 48■ and 72 h■ post infection. Samples were pulsed for 4 h with ^{3}H-T or MTS reagent. Values were adjusted such that the ^{3}H-T incorporation or absorbance at 490 nm of MI samples at 0 h was equal to 1. Reactions were done in triplicate and the experiments repeated three times. Result from one of the sets of experiments is shown. Error bars represent standard errors.

3.2.3 Titre of Myxoma Virus per Infected Cell

The previous section showed that the increase in viral titre and proportion of cells infected observed after Con A stimulation was not due to increased cell numbers. To examine whether the increase in viral titre and proportion of cells infected could be attributed to individual infected cells producing higher titres of virus, analysis was undertaken by adjusting virus titre per *input* cell to virus titre per *infected* cell. This conversion was performed by taking the mean proportion of cells infected from Figure 3.3, and, using the mean virus titre from Figure 3.1, calculating pfu/infected cell.

3.2.3.1 SLS Titre per Infected Cell

Infected lymphoid cells from laboratory rabbits stimulated with Con A produced up to 4 fold more infectious SLS virus particles than unstimulated lymphoid cells (Table 3.2). The difference between unstimulated and Con A-stimulated cultures was greatest 24 and 48 h post infection. For example, 48 h post infection there were 5.7 and 22.5 pfu/infected cell in unstimulated and Con A-stimulated lymphoid cell cultures from laboratory rabbits, infected with SLS respectively.

TABLE 3.2. MEAN TITRE* PER INFECTED CELL

Time Post Infection	SLS				Ur			
	Laboratory Rabbit Cell Cultures		Wild Rabbit Cell Cultures		Laboratory Rabbit Cell Cultures		Wild Rabbit Cell Cultures	
	- Con A	+ Con A	- Con A	+ Con A	- Con A	+ Con A	- Con A	+ Con A
12 h	6.9	7.4	5.6	13.4	36.0	10.0	15.5	2.8
24 h	5.5	19.5	19.2	5.7	66.7	23.4	16.5	3.8
48 h	5.7	22.5	6.2	17.5	32.3	25.9	11.2	9.1
72 h	12.8	31.6	6.2	15.2	24.1	94.1	21.2	11.3

* Mean virus titres were analyzed with respect to mean number of infected cells to obtain mean titre (pfu)/infected cell. pfu/infected cell was calculated at 12, 24, 48 and 72 h after infection with SLS or Ur at a moi of 3.

Infected lymphoid cells from wild rabbits stimulated with Con A produced 2 to 3 fold more infectious SLS particles than their unstimulated counterparts. For example, 48 h post infection there was 6.2 pfu/infected cell in unstimulated lymphoid cell cultures from wild rabbits compared to 17.5 pfu/infected cell in stimulated lymphoid cell cultures from wild rabbits. The exception to this was the 24 h time point. At this time point, SLS-infected unstimulated lymphoid cells from wild rabbits produced 19.2 pfu/infected cell, whereas SLS-infected stimulated cells produced 5.7 pfu/infected cell (Table 3.2). In addition, there were a lower proportion of lymphocytes infected in unstimulated cell cultures compared to stimulated cell cultures from wild rabbits, but similar viral titres. This resulted in the 3 to 4 fold decrease in pfu/infected cell observed when cultures were stimulated.

Overall, these results show that in SLS infections, Con A stimulation increased virus replication in individual infected cells.

3.2.3.2 Ur Titre per Infected Cell

Con A did not increase the pfu/infected cell in rabbit lymphoid cell cultures infected with Ur (Table 3.2). For example, in unstimulated lymphoid cells, from laboratory rabbits, infected with Ur there were 32.3 pfu/infected cell 48 h post infection, compared to 25.9 pfu/infected cell in cultures stimulated with Con A. Similar results were observed in lymphoid cells from wild rabbits. The one exception to this result was 72 h after Ur infection of stimulated lymphoid cells from laboratory rabbits. In this infection there was 94.1 pfu/infected cell in Con A-stimulated cells compared to 24.1 pfu/infected cell in unstimulated cells. This result is most likely skewed by the large decrease in proportion of cells infected (Figure 3.3), but similar viral titres at this time point (Figure 3.1). The reason for this decrease in proportion of infected cells is postulated to be due

to an increase in cell death and will be discussed in Section 3.3.5 and examined in chapter 4.

Thus, for Ur, the increase in viral titres observed with Con A stimulation appeared to be due to an increase in the number of cells infected and not due to an increase in replication in individual infected cells as shown for SLS.

3.2.4 Analysis of Myxoma Virus Infection and Con A-Induced Changes in Cell Morphology

Con A stimulation of lymphoid cells is not only associated with cell division but also with an increase in cell size and granularity. It was postulated in Section 3.2.1.3. that activation of cultures with Con A either produced more target cells for the virus, or made the cells more permissive for infection. Section 3.2.2 examined the first possibility and demonstrated that more target cells for myxoma virus infection were not produced when cultures were stimulated with Con A, as myxoma virus completely abrogated lymphocyte proliferation. This section investigates the second possibility; that activated lymphoid cells are more permissive for myxoma virus replication. One method of identifying activated cells is by an increase in their size, which can be measured by flow cytometry (Tsao et al., 1993; Webster et al., 1995). Experiments in this section will firstly examine the effects of myxoma virus infection on cell size (Section 3.2.4.1), and then investigate whether stimulated cells are more permissive for myxoma virus replication by gating large and small cells and examining 3b6e4 binding in these sub-populations (Section 3.2.4.2). The cultures used in these experiments were those used to examine the expression of cell surface proteins (chapter 5). Cultures previously used in this chapter to examine myxoma virus infection were not used for

examining cell size as permeabilisation, which was essential for the 3b6e4 monoclonal antibody binding may have affected cell size results.

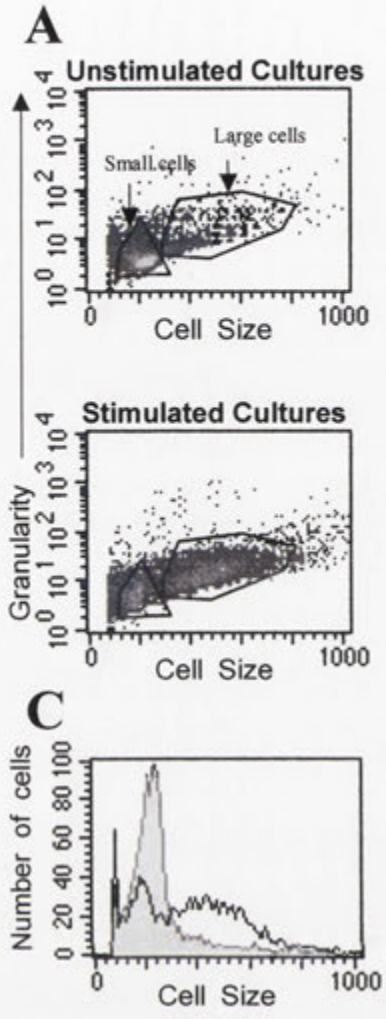
3.2.4.1 Con A-Induced Changes in Lymphocyte Size

3.2.4.1.1 Cell Size in Rabbit Lymphoid Cell Cultures Infected with Myxoma Virus Prior to Stimulation with Con A

Lymphoid cell cultures from laboratory and wild rabbits were infected with SLS or Ur at a moi of 0.1 or 3 and incubated for 48 h in the presence or absence of Con A. Cell size was analysed as forward scatter (FSC) using flow cytometry. Data was presented as density plots of cell granularity versus cell size and histogram overlays of cell size. The proportion of large and small cells was calculated using CellQuest software.

Two sub-populations based on cell size (forward scatter- FSC) were gated in mock-infected rabbit lymphoid cell cultures as shown in Figure 3.7A. The proportion of cells in each sub-population, 'large' and 'small', was calculated using CellQuest software. The gates used were identical for all infections and tried to encompass discrete large and small sub-populations of every infection. As a result they do not include the entire cell culture and proportions will not add up to 100%.

Con A increased the proportion of large cells from 7% to 50% and from 4% to 60% over the 48 h in mock-infected lymphoid cell cultures from laboratory and wild rabbits respectively (Figure 3.7B). There was a corresponding decrease in the proportion of small cells in these cultures.



B

Mean proportion of unstimulated and Con A-stimulated MI cells in each sub-population, as defined by cell size (FSC), 48 h post-infection.

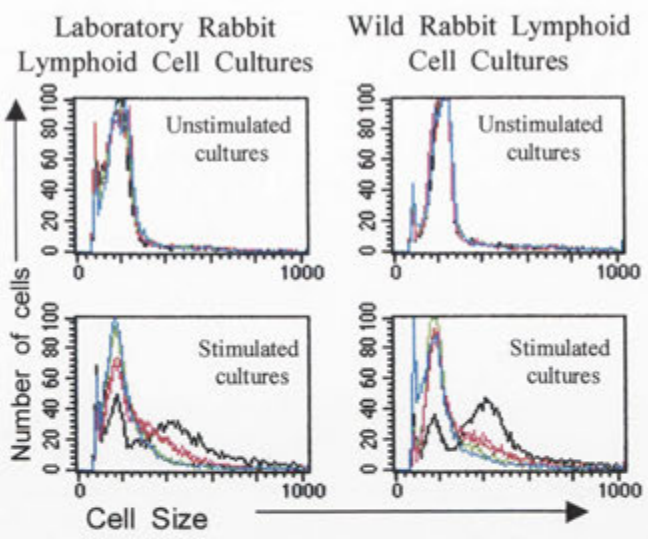
		MI	MI + ConA
Laboratory Rabbit Lymphoid Cell Cultures	Small cells	71%	27%
	Large cells	7%	50%
Wild Rabbit Lymphoid Cell Cultures	Small cells	83%	24%
	Large cells	4%	60%

FIGURE 3.7. THE EFFECTS OF CON A STIMULATION ON THE SIZE OF MOCK-INFECTED LYMPHOCYTES FROM LABORATORY AND WILD RABBITS

The size of mock-infected (MI) lymphocytes from laboratory and wild rabbits was analysed with or without Con A stimulation, 48 h post infection. Lymphocyte size was examined as forward scatter (FSC) by flow cytometry. (A) A representative density plot of MI cultures showing the large and small cell sub-populations. (B) The proportion of cells in the large and small sub-population was calculated using CellQuest software. Numbers are mean proportions from infections of cultures from three different rabbits. (C) A histogram overlay showing the increase in cell size in stimulated mock-infected cell cultures compared to unstimulated mock-infected cultures. The shaded region represents cell size in unstimulated cell cultures and the black line represents cell size in Con A-stimulated cultures. Plots were generated using the CellQuest software.

Infection of lymphoid cell cultures with either SLS or Ur abrogated the Con A-induced increase in proportion of large cells (Figure 3.8). This appeared to be somewhat proportional to moi as both SLS and Ur at a moi of 0.1 did not inhibit the increase in

A



B

		Laboratory Rabbit Lymphoid Cell Cultures		Wild Rabbit Lymphoid Cell Cultures	
		unstimulated	Con A-stimulated	unstimulated	Con A-stimulated
Small cells	Mock-infected	71%	27%	83%	24%
	SLS (0.1)	70%	55%	84%	50%
	SLS (3)	70%	60%	83%	69%
	Ur (0.1)	72%	56%	83%	44%
	Ur (3)	76%	53%	81%	63%
Large cells	Mock-infected	**7%**	**50%**	**4%**	**60%**
	SLS (0.1)	**7%**	**19%**	**5%**	**25%**
	SLS (3)	**7%**	**13%**	**4%**	**9%**
	Ur (0.1)	**10%**	**23%**	**4%**	**32%**
	Ur (3)	**6%**	**17%**	7%	**13%**

FIGURE 3.8. THE EFFECTS OF CON A STIMULATION ON THE SIZE OF LYMPHOCYTES INFECTED WITH SLS OR UR

(A) Data is presented as histogram overlays of cell size. Lymphocytes from laboratory and wild rabbits were mock-infected (black) or infected with either SLS at a moi of 0.1 (red) or 3 (green), or Ur at a moi of 0.1 (pink) or 3 (blue). Cell size was analysed 48 h post-infection in lymphocytes isolated from three laboratory and three wild rabbits (a representative sample is shown). Cultures were infected with and without Con A stimulation. (B) Mean proportion of rabbit lymphoid cell cultures in each size sub-population. The large and small cell sub-populations were gated on density plots, as shown in Figure 3.7, and the mean percentage of cells comprising each population, was calculated using CellQuest software.

proportion of large cells to as great an extent as the same virus at a moi of 3, although proportions were not greatly different from each other. For example, as seen in Figure 3.8B, lymphoid cell cultures from laboratory rabbits, infected with SLS at a moi of 0.1, had 19% of cells classified as large but only 13% were classified as large under the same conditions when a moi of 3 was used. In Ur-infected lymphoid cell cultures from laboratory rabbits, 23% of cells were classified as large in infections with a moi of 0.1 and 17% of cells were classified as large in infections with a moi of 3. Similar results were observed in lymphoid cell cultures from wild rabbits (Figure 3.8). Concurrent with the abrogation of cell size in SLS- and Ur-infected cell cultures stimulated with Con A, there was an increase, relative to mock-infected cell cultures, in the proportion of cells classified as small.

SLS and Ur infection at either moi did not affect cell size in unstimulated lymphoid cell cultures from laboratory and wild rabbits, as similar proportions of cells in the large and small sub-populations to those in the corresponding unstimulated mock-infected cell cultures were observed (Figure 3.8B).

3.2.4.1.2 Cell Size in Rabbit Lymphoid Cell Cultures Stimulated with Con A Prior to Infection

Investigation of whether myxoma virus could alter cell size if cells were activated prior to infection was undertaken. Cultures were stimulated for 4, 24 or 48 h before mock-infection or infection with SLS or Ur at a moi of 3, for 24 or 48 h. Results are presented as histogram overlays of cell size and proportions of large and small cells, calculated as shown in Figure 3.7. Cell cultures stimulated with Con A for 4 h prior to infection will be described in Section 3.2.4.1.2.1, followed by cell size in cell cultures stimulated for 24 and 48 h prior to infection (Section 3.2.4.1.2.2).

3.2.4.1.2.1 Cell Size in Rabbit Lymphoid Cell Cultures Stimulated with Con A for 4 h Prior to Infection with SLS or Ur

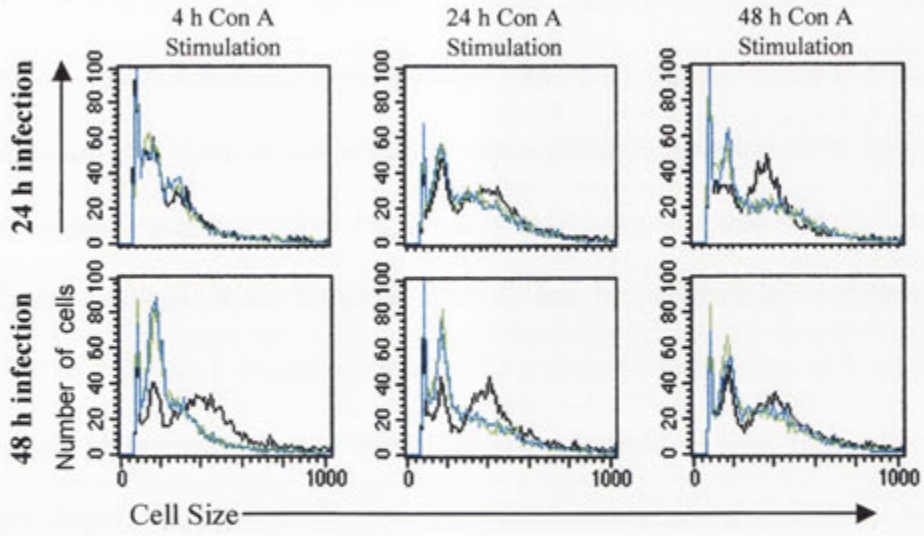
In lymphoid cell cultures from laboratory rabbits, the proportion of cells in the large sub-population when cultures were stimulated for 4 h prior to mock-infection was 42% at 24 h post infection and rose to 61% at 48 h (Figure 3.9). Concurrent with this, there was a decrease in cells classified as small in mock-infected lymphoid cell cultures.

Infection with either SLS or Ur prevented the increase in proportion of large cells in cultures that had been stimulated with Con A for 4 h prior to infection (Figure 3.9). For instance, in lymphoid cell cultures from laboratory rabbits infected with SLS or Ur, the mean proportion of large cells remained between 32-38% during the 48 h period of infection. The reason for the sizeable population of large cells in infected cell cultures is presumably due to an increase in cell size during the 4 h of Con A stimulation prior to infection. The mean proportion of small cells in myxoma virus-infected cell cultures remained between 62-68% in infected lymphoid cell cultures from laboratory rabbits (Figure 3.9B).

Infection of lymphoid cell cultures from wild rabbits with either SLS or Ur also inhibited the increase in proportion of large cells induced by Con A. For example, 48 h after infection, 60% of mock-infected cells were in the large cell sub-population, whereas 27% and 34% of cells were large in SLS- and Ur-infected cell cultures respectively (Figure 3.9B).

These results show that myxoma virus either abrogates the increase in cell size associated with activation if infection occurs 4 h after stimulation or may induce death in large cells. This will be examined in chapter 4.

A



B

		Laboratory Rabbit Lymphoid Cell Cultures						Wild Rabbit Lymphoid Cell Cultures					
		MI		SLS		Ur		MI		SLS		Ur	
		Small cells	**Large cells**	Small cells	**Large cells**	Small cells	**Large cells**	Small cells	**Large cells**	Small cells	**Large cells**	Small cells	**Large cells**
4 h Con A Stimulation	**24 h Infection**	58%	**42%**	63%	**37%**	62%	**38%**	44%	**56%**	56%	**44%**	51%	**49%**
	48 h Infection	39%	**61%**	68%	**32%**	68%	**32%**	40%	**60%**	73%	**27%**	66%	**34%**
24 h Con A Stimulation	**24 h Infection**	43%	**57%**	52%	**48%**	48%	**52%**	29%	**71%**	41%	**59%**	27%	**63%**
	48 h Infection	40%	**60%**	63%	**37%**	63%	**37%**	21%	**79%**	44%	**56%**	42%	**58%**
48 h Con A Stimulation	**24 h Infection**	41%	**59%**	56%	**44%**	52%	**48%**	21%	**79%**	31%	**69%**	27%	**73%**
	48 h Infection	36%	**64%**	48%	**52%**	56%	**44%**	22%	**78%**	39%	**61%**	36%	**64%**

FIGURE 3.9. MEAN PROPORTION OF LARGE AND SMALL RABBIT LYMPHOID CELLS IN CULTURES STIMULATED WITH CON A PRIOR TO INFECTION WITH SLS OR UR

Lymphoid cells from (A) laboratory and wild rabbits that had been stimulated with Con A for 4, 24 or 48 h prior to mock-infection (MI) (black) or infection with SLS (green) or Ur (blue) at a moi of 3, were analysed for cell size, 24 and 48 h post-infection. Data is presented as histogram overlays of cell size of a representative result. (B) The 'large' and 'small' populations were gated on density plots, as shown in Figure 3.7, and the mean proportion of cells comprising each sub-population was calculated. The experiment was undertaken using lymphoid cell cultures prepared from three laboratory and three wild rabbits and the mean calculated.

3.2.4.1.2.2 Cell Size in Rabbit Lymphoid Cell Cultures Stimulated with Con A for 24 or 48 h Prior to Infection with SLS or Ur

Cells were stimulated with Con A for 24 and 48 h prior to mock-infection or infection with SLS or Ur for an additional 24 or 48 h. The proportion of cells classified as large was calculated and mean value for each group of three rabbits presented as shown in Figure 3.7.

In mock-infected lymphoid cell cultures prepared from laboratory rabbits and stimulated with Con A for 24 or 48 h, the proportion of large cells was 57% and 59% respectively after 24 h mock-infection and 60% and 64% respectively after 48 h mock-infection (Figure 3.9B). If lymphoid cell cultures from laboratory rabbits, stimulated for 24 were infected with SLS then 48% and 37% of cells were large after 24 h and 48 h infection respectively. For comparison, 33% of lymphoid cells from laboratory rabbits were large after 24 h of Con A stimulation prior to infection (data not shown). If the period of stimulation prior to SLS infection was 48 h then 44% of cells were large after infection for 24 h and 52% of cells were large after infection for 48 h (Figure 3.9). Similar results were observed in Ur-infected cell cultures.

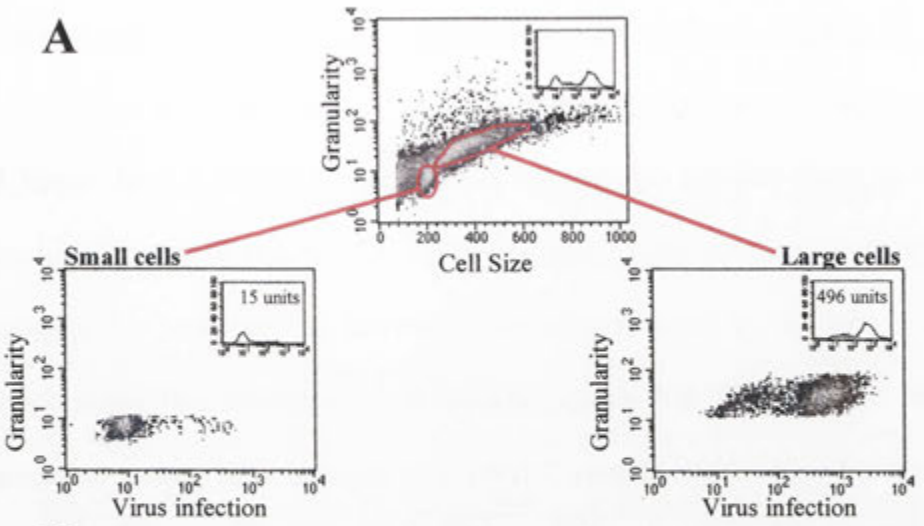
These results suggest that the size of stimulated cells is maintained after infection with SLS or Ur and that the longer stimulation occurs prior to infection, the lower the ability of myxoma virus to alter cell activation. Similar results were observed in lymphoid cell cultures from wild rabbits, although the proportions of large cells in the majority of infections were greater than the corresponding infection in cell cultures from laboratory rabbits. This indicates that any difference between rabbit types in the ability of lymphoid cells to be activated after infection with myxoma virus is small.

3.2.4.2 Permissivity of Large and Small Sub-Populations of Laboratory and Wild Rabbit Lymphoid Cells to Infection with SLS or Ur

3.2.4.2.1 Lymphoid Cell Cultures Infected Prior to Con A Stimulation

To determine whether large cells are more permissive for myxoma virus replication, further analysis of large and small cell sub-populations in data collected from Section 3.2.1 was undertaken. The method used for this analysis is shown in Figure 3.10A. The flow cytometric data from unstimulated and Con A-stimulated lymphoid cells from laboratory and wild rabbits that had been mock-infected or infected with SLS or Ur at a moi of 3 were analysed for virus infection, 48 h post infection. The mean median fluorescence intensity of virus infection was determined for each rabbit, from the histogram plot of granularity versus fluorescence intensity of anti-myxoma virus antibody (3b6e4) binding. The mean median fluorescence intensity of anti-myxoma antibody binding was then calculated for large and small cell sub-populations in cultures prepared from each group of three laboratory or three wild rabbits. The mean median fluorescence intensity is a quantitative measure of protein expression and is less likely to be skewed by very high or low antibody binding in a minor cell sub-population. Analysis in this way would be important for examining virus infection in large and small cell sub-populations as Section 3.2.4.1 showed that myxoma virus infection inhibited the increase in cell size associated with Con A activation, resulting in a low proportion of large cells. The difference between the mean median fluorescence intensity in SLS- or Ur-infected and mock-infected cell cultures was calculated. These numbers are tabulated in Figure 3.10B.

A



B

		Laboratory Rabbit Lymphoid Cell Cultures		Wild Rabbit Lymphoid Cell Cultures	
		unstimulated	Con A-stimulated	unstimulated	Con A-stimulated
SLS	Small cells	4	3	0	3
	Large cells	**162**	**535**	**62**	**592**
Ur	Small cells	3	22	12	1
	Large cells	**55**	**195**	**191**	**438**

FIGURE 3.10. MEAN MEDIAN FLUORESCENCE INTENSITY OF ANTI-MYXOMA VIRUS ANTIBODY STAINING IN LARGE AND SMALL CELL SUB-POPULATIONS

Unstimulated and Con A-stimulated lymphoid cells from laboratory and wild rabbits that had been mock-infected or infected with SLS or Ur at a moi of 3 were analysed for virus infection 48 h post-infection. (A) The method used for the analysis. The sub-populations were gated on density plots of cell size versus granularity and plotted on density plots of granularity versus virus infection (3b6e4 antibody binding) and histogram plots of virus infection (inset). The median fluorescence intensity corresponding to 3b6e4 antibody binding was determined (indicated by numbers). The example shown here is Con A-stimulated lymphoid cell cultures from a laboratory rabbit infected with Ur. (B) Mean median fluorescence intensity of anti-myxoma virus antibody staining in large and small cell sub-populations. The mean net difference in fluorescence intensity of large and small populations between mock-infected and virus-infected cell cultures was calculated by subtracting values in mock-infected cell cultures from those in virus-infected cell cultures. Means are for each group of three rabbits.

The mean median fluorescence intensity of anti-myxoma virus staining in the small cell sub-population of either Con A-stimulated or unstimulated cell cultures infected with

either SLS or Ur, was 0 to 22 fluorescence units above mock-infected controls (Figure 3.10B). In contrast, the fluorescence intensity of anti-myxoma virus staining in large cells from cell cultures infected with SLS or Ur ranged from 55 to 191 fluorescence units above mock-infected cell cultures in the absence of Con A, and ranged from 195 to 592 fluorescence units above mock-infected cell cultures in the presence of Con A. Greater expression of viral protein was observed in the large cell sub-population compared to the small cell sub-population from lymphoid cell cultures from both laboratory and wild rabbits (Figure 3.10B). The expression of greater amounts of viral protein in the large cell sub-population compared to the small cell sub-population is hypothesized to be associated with increased virus replication in large cells. This is supported by the Con A-induced increase in mean median fluorescence, which, as previously shown, is associated with an increase in viral titre. The fact that stimulation of Ur-infected lymphoid cell cultures from wild rabbits had a mean median fluorescence intensity of 438 units (compared with 592 units for cultures infected with SLS), but infectious progeny were not produced will be discussed in Section 3.3.5.

3.2.4.2.2 Lymphoid Cell Cultures Stimulated with Con A Prior to Infection

The previous sections showed expression of a late myxoma virus protein was higher in large cells. Although this suggests myxoma virus preferentially replicated in large cells, the experiments are not able to show whether myxoma virus preferentially infects large cells as cultures were infected prior to stimulation. To investigate whether myxoma virus preferentially infected large cells, cultures were Con A-stimulated for 48 h prior to infection with SLS or Ur at a moi of 3, for an additional 48 h. Con A stimulation of mock-infected cell cultures for 48 h would lead to 50-60% of lymphoid cells being activated. Hence, it can be assumed that half of the cells in the culture will be activated prior to infection.

Results were similar between lymphoid cell cultures from three laboratory and three wild rabbits, hence only one sample is shown in Figure 3.11 (laboratory rabbit). Density plots of myxoma virus infection versus cell size were divided into quadrants. The proportion of cells in each quadrant was calculated using CellQuest software. Fluorescence intensity above 10^2 was taken to correspond to infection of cells with myxoma virus.

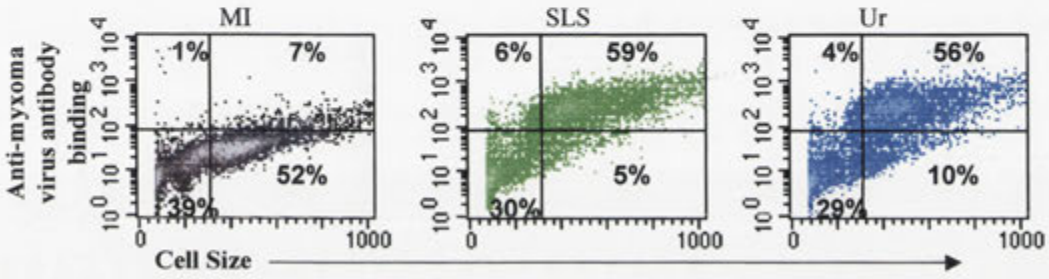


FIGURE 3.11. POPULATION OF LYMPHOID CELLS INFECTED WITH MYXOMA VIRUS
Lymphoid cells from laboratory and wild rabbits were stimulated with Con A for 48 h prior to mock-infection (MI) (black) or infection with SLS (green) or Ur (blue) at a moi of 3, for an additional 48 h. Density plots of myxoma virus infection versus cell size were generated using CellQuest software. Results were similar between lymphoid cell cultures from different rabbits, hence only one sample is shown here (laboratory rabbit). The percentages are the proportion of cells in each quadrant- large/high fluorescence of anti-myxoma virus antibody (3b6e4) binding (upper right quadrant), large/low fluorescence of 3b6e4 antibody binding (lower right quadrant), small/high fluorescence of 3b6e4 antibody binding (upper left quadrant), small/low fluorescence of 3b6e4 antibody binding (lower left quadrant).

Figure 3.11 shows that myxoma virus preferentially infected large cells. For example, of the 65% of lymphoid cells that had a fluorescence intensity of 3b6e4 binding greater than 10^2, 59% were classified as large and the other 6% were small. Similar results were obtained with Ur infections where, of the 60% of lymphoid cells with a fluorescence intensity of 3b6e4 binding greater than 10^2, 56% of them were classified as large and the other 4% as small. This is compared to mock-infected cell cultures, which show a total background staining of 8% of cells with a fluorescence intensity greater than 10^2 (Figure 3.11). As Con A is a T-cell specific mitogen and approximately 57% of cells in

rabbit lymph nodes are T-cells (Mage 1998), these results suggest that activated T-cells are the sub-population permissive for myxoma virus replication.

3.2.4.3 Con A-Induced Changes in Lymphocyte Granularity

Granularity was examined 48 h post infection in lymphoid cell cultures that were either mock-infected or infected with SLS or Ur at a moi of 0.1 or 3. Granularity of a cell can change in response to activation and may be altered by myxoma virus infection. Granularity (side scatter- SSC) is collected automatically by the flow cytometer at the same time as FSC. Side scatter detects light scattered by a particle 90° from the incident laser beam and is related to the internal structure and granularity of that cell.

Figure 3.12 shows Con A-stimulation increased granularity of mock-infected rabbit lymphoid cell cultures. Myxoma virus infection did not alter cell granularity in unstimulated cell cultures or in cell cultures stimulated with Con A after infection (Figure 3.13).

The presence of viral replication factories might be expected to deflect the incidence laser beam. However, myxoma virus did not substantially alter granularity in cell cultures that were stimulated with Con A prior to infection (Figure 3.14). Those cell cultures that did have a slight change in granularity following myxoma virus infection were lymphoid cells from wild rabbits infected for 48 h after stimulation for 48 h. As this increase was not seen in all panels, the increase in granularity was not due to viral replication factories.

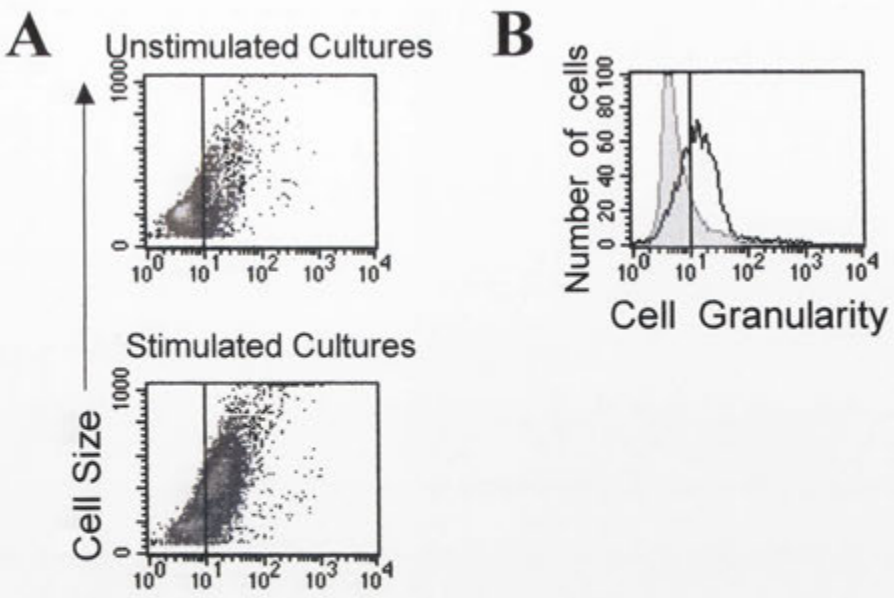


FIGURE 3.12. THE EFFECTS OF CON A STIMULATION ON THE GRANULARITY OF LYMPHOCYTES FROM LABORATORY AND WILD RABBITS

The granularity of mock-infected lymphocytes from laboratory and wild rabbits was analysed with and without Con A stimulation, 48 h post infection. Granularity was detected as side scatter by flow cytometry (horizontal axes). Plots shown are representative (A) density and (B) histogram plots of all samples. The histogram overlay shows the increase in cell granularity when Con A was added to the cultures. The shaded region represents mock-infected cell cultures and black line represents Con A-stimulated cultures. A line has been drawn at a fluorescence intensity of 10^1 to aid visual analysis of the histogram plots. Plots were generated using the CellQuest software.

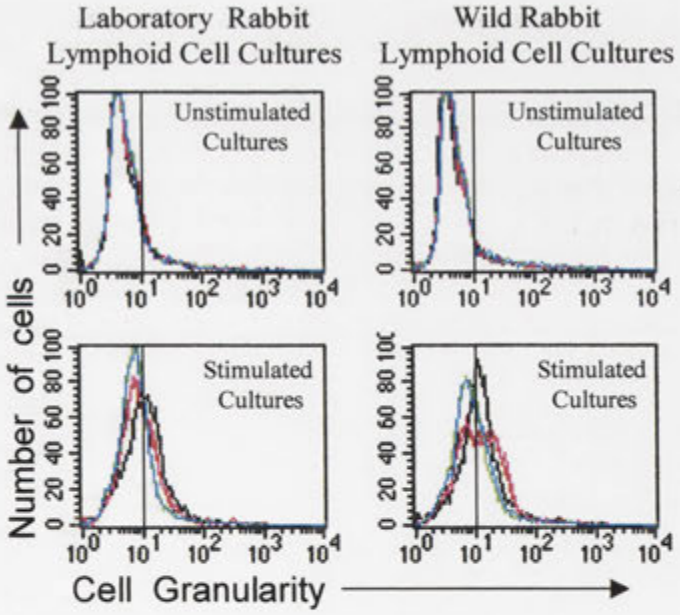


FIGURE 3.13. CHANGES IN CELL GRANULARITY IN INFECTED LYMPHOCYTES FROM LABORATORY AND WILD RABBITS

Lymphocytes from laboratory and wild rabbits were mock-infected (black) or infected with either SLS, at a moi of 0.1 (red) or 3 (green), or Ur, at a moi of 0.1 (pink) or 3 (blue). Granularity was analysed in lymphoid cell cultures isolated from three laboratory and three wild rabbits (a representative sample is shown) with and without Con A stimulation. Data is presented as histogram overlays of cell granularity.

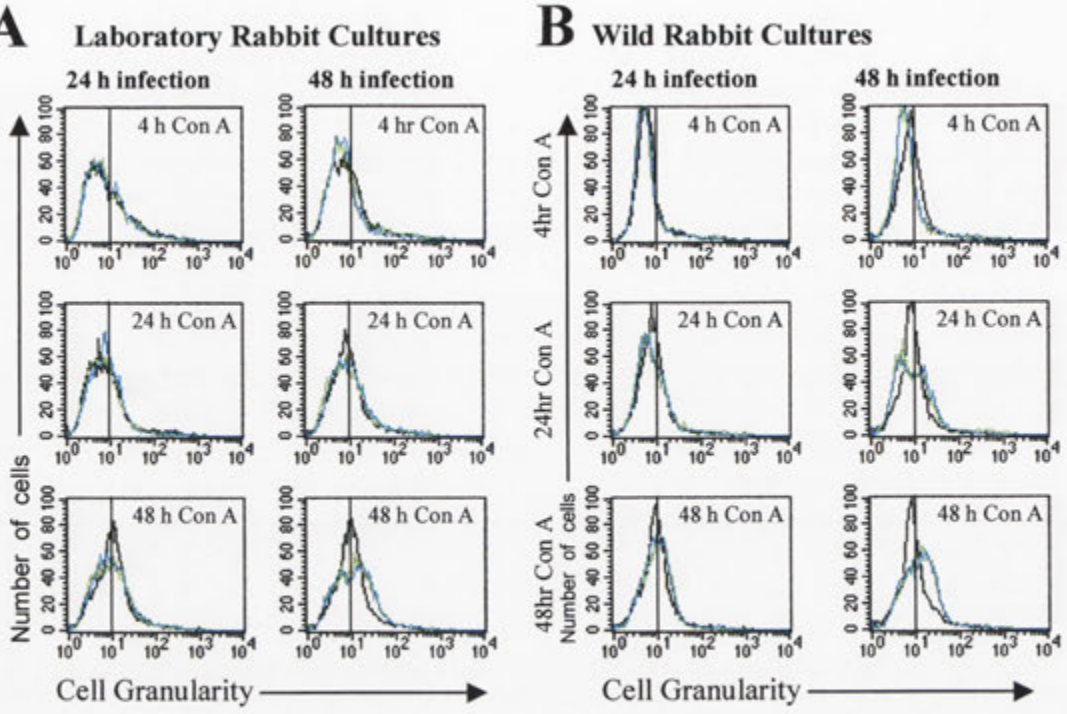


FIGURE 3.14. CHANGES IN CELL GRANULARITY IN RABBIT LYMPHOCYTES STIMULATED WITH CON A PRIOR TO INFECTION WITH MYXOMA VIRUS

Lymphoid cells from (A) laboratory and (B) wild rabbits that had been stimulated with Con A for 4, 24 or 48 h prior to mock-infection (black) or infection with SLS (green) or Ur (blue) at a moi of 3, were analysed for cell granularity 24 and 48 h post infection. The experiment was undertaken using lymphocytes isolated from three laboratory and three wild rabbits. A representative sample is shown here. Data is presented as histogram overlays of cell granularity.

3.2.4.4 RL-5 Cell Size and Granularity

The size and granularity of RL-5 cells mock-infected or infected with SLS or Ur at a moi of 3 was also examined 48 h post infection. Figure 3.15 shows that infection with myxoma virus did not alter the size of RL-5 cells. However, granularity was increased after infection with either strain of myxoma virus. These results are different to those obtained for primary cultures, as granularity in primary lymphoid cell cultures was not altered by myxoma virus infection, and may be related to RL-5 cells being immortalized.

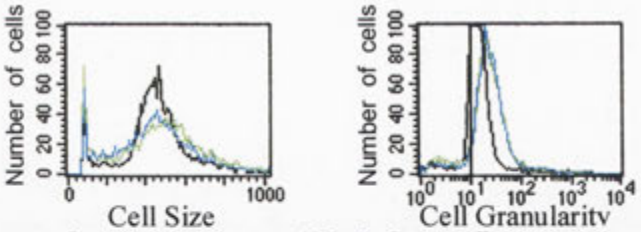


FIGURE 3.15. SIZE AND GRANULARITY OF RL-5 CELLS INFECTED WITH SLS OR UR

The size and granularity of RL-5 cells mock-infected (black) or infected with SLS (green) or Ur (blue) at a moi of 3 were analysed 48 h post infection. Cell size was detected as forward scatter and granularity as side scatter by flow cytometry. The above histogram overlays were generated using the associated CellQuest software. The experiment was repeated three times, a representative sample is shown here.

3.2.5 Summary

Table 3.3 shows a comparison of the results from this chapter between infection with the virulent SLS and attenuated Ur strains of myxoma virus and between lymphoid cell cultures from susceptible laboratory and resistant wild rabbits. Differences between SLS and Ur may reflect a cellular basis for attenuation of the virus. Difference between laboratory and wild rabbits may reflect a cellular basis for resistance.

TABLE 3.3. SUMMARY OF SLS AND UR INFECTION OF LYMPHOID CELL CULTURES FROM LABORATORY AND WILD RABBITS

	SLS versus Ur	Laboratory Rabbits versus Wild Rabbits
Virus Replication*	• Ur did not replicate in Con A-stimulated lymphoid cell cultures from wild rabbits	• Con A did not increase Ur replication in lymphoid cell cultures from wild rabbits, but did in infected cultures from laboratory rabbits • Myxoma virus has a significantly reduced ability to replicate in lymphoid cell cultures from wild rabbits compared to cultures from laboratory rabbits
Proportion of Cells Infected+	• Proportion of cells infected with Ur dropped significantly 72 h post infection compared to the proportion of cells infected with SLS	• SLS infected a greater proportion of cells at early time points (<48 h) in unstimulated lymphoid cell cultures from laboratory rabbits compared to wild rabbits
Titre per infected cell$^{\theta}$	• Con A increased SLS replication in infected cells • The Con A-induced increase in Ur titre was predominantly due to increase in proportion of cells infected	• No difference in titre per infected cell was observed between rabbit types

* Analysis of virus replication was by plaque assay and is shown in Figure 3.1

+ Analysis of proportion of cells infected was by detection of a late viral protein by flow cytometry and is shown in Figure 3.3.

$^{\theta}$ Titre per infected cell was calculated from the mean virus titres and mean proportion of cells infected and is listed in Table 3.2.

3.3 Discussion

This chapter investigated if the genetic resistance of wild rabbits to myxoma virus could be attributed to differences in the permissivity of lymphocytes from susceptible and resistant rabbits for virus replication. The results showed:

1. Lymphoid cell cultures from resistant rabbits were statistically significantly less permissive for virus replication (as measured by plaque assay) than cell cultures from susceptible rabbits.
2. Con A activation significantly increased SLS titres and the proportion of cells infected with SLS in cultures from both rabbit types. Con A activation also increased the proportion of Ur-infected cells in lymphoid cell cultures from both rabbit types. However, Con A stimulation did not increase Ur titres in lymphoid cell cultures from wild rabbits significantly above that of unstimulated cultures.
3. The increase in SLS titres observed with Con A stimulation of rabbit lymphoid cell cultures was due to increased virus production in every infected cell. For Ur infection, however, the increase in titres observed with Con A stimulation was due to the increased number of infected cells.
4. Both strains of myxoma virus, although preferentially replicating in stimulated lymphocytes, completely abrogated T-cell activation (measured by lymphocyte proliferation and cell size).
5. A key difference between SLS and Ur identified in this chapter was the dramatic drop in proportion of cells infected with Ur 48-72 h post infection compared to cultures infected with SLS.

3.3.1 Differences in Permissivity of Lymphoid Cells from Susceptible and Resistant Rabbits to Virus Replication

Lymphoid cell cultures from resistant rabbits were significantly less permissive for mxyoma virus replication than lymphoid cell cultures from susceptible rabbits. This was highlighted in Con A-stimulated wild rabbit lymphoid cell cultures infected with Ur, where the mean titre of three replicates did not rise above 5 pfu/ input cell over the 72 h. In contrast, the mean Ur titre reached 17 pfu/input cell in lymphoid cell cultures from laboratory rabbits. The small but statistically significant reduction in titre of myxoma virus in lymphoid cell cultures from resistant rabbits indicated that there is a measurable difference *in vitro* between lymphocytes from laboratory and wild rabbits. Viral titres were measured by plaque assay on Vero cell monolayers and, as such, actually measured production of infectious viral progeny, which is the end stage of the virus life cycle.

There are many stages when the permissivity of the lymphocyte to myxoma virus replication have been impeded, including virus entry, viral DNA and protein synthesis, virion assembly and maturation and virion egress. The mechanism for the reduced permissivity of lymphocytes from wild rabbits for myxoma virus replication is most probably related to virion assembly and maturation. This is because experiments in this chapter examined myxoma virus infection by staining for a 42 kDa late virus protein. As similar proportions of cells expressing this protein in lymphoid cell cultures from laboratory and wild rabbits were detected, this suggests that resistance is operating after expression of this protein. This could include events late in the virus life cycle, such as virion assembly and maturation or egress. However, any problems in virion egress would not have been detected in these experiments as the cells were sonicated to release virions prior to determination of titres by plaque assay. Further experiments examining

viral morphogenesis will be needed to determine which step(s) in the virion assembly and maturation pathway is hindered in lymphocytes from wild rabbits. Alternatively, resistance may involve increased cell death in cell cultures from wild rabbits which is initiated after late protein expression. This would lead to reduced titres because cells are killed before infectious virus progeny are produced. Cell death during myxoma virus infection is examined in chapter 4.

A reduced ability of myxoma virus to replicate *in vivo* in lymphocytes during infection of resistant rabbits compared to infection of laboratory rabbits, may aid control of virus replication and dissemination by the wild rabbit immune response. If virus replication is reduced, it may be expected that the overall transcription and translation of viral genes and proteins would also be reduced (some early gene products would be present as these are packaged in the virion). Lower expression levels of any immunomodulatory viral proteins may diminish the extent to which myxoma virus can suppress the immune response leading to the development of an effective adaptive immune response by the host. Lower expression of proteins involved in virus replication may reduce the numbers of infectious progeny, which will also diminish the extent to which myxoma virus can suppress the immune response.

In the model presented by Best and Kerr (2000) resistance is postulated as due to an effective innate immune response, involving effectors such as natural killer (NK) cells and IFNα/β. These are postulated to effectively control viral replication at the lymph node draining the inoculation site in the first 2-4 days of infection. The early control of virus infection is postulated to enable an effective anti-viral adaptive immune response to develop, for which cytotoxic T-cells (CTLs) are critical in virus control and clearance (Best 1998; Best and Kerr 2000). Reduction in permissivity of lymphoid cells to

myxoma virus replication, no matter how small, will reduce viral replication and will aid the host in controlling and clearing the virus.

Although Best and Kerr (2000) did examine permissivity of lymphoid cells from susceptible and resistant rabbits to myxoma virus replication and found no difference, these experiments were not done with the attenuated Ur strain of myxoma virus and were not done with mitogen stimulation. The results in this chapter show that the use of an attenuated virus and mitogen stimulation did increase the sensitivity of the assay as a significant difference between lymphocytes from laboratory and wild rabbits were observed. This will be discussed further in the next section.

3.3.2 The Effect of Stimulation on the Permissivity of Lymphoid Cells to Myxoma Virus Replication

The T-cell mitogen, Con A, was used to stimulate lymphoid cell cultures. Con A induces extensive crosslinking of cell membrane proteins by binding α-D-mannose and α-D-glucose, which are part of the glycosaminoglycans (GAGs) that are commonly linked to membrane glycoproteins (Smith and Goldstein 1967). Crosslinking of cell membrane proteins mimics receptor activation and initiates multiple cell signaling pathways resulting in T-cell activation and proliferation (Smith and Goldstein 1967). It has previously been reported that, generally, isolated rabbit T-cells respond to Con A whereas rabbit B-cells do not (Sell 1979). A T-cell specific mitogen was used because T-cells are considered critical for myxoma virus pathogenesis. Myxoma virus antigen is detected in T-cell zones of the lymph node of infected rabbits (Best et al., 2000) and myxoma virus replicates in the rabbit $CD4^+$ T-cell line, RL-5 (see Section 1.7). However, myxoma virus has also been shown to infect monocytes and macrophages

(Macen et al., 1996; Mossman et al., 1996; Everett et al., 2000). T-cells have been shown to be important in the pathogenesis of other *Leporipoxviruses*. For example, malignant rabbit virus (MRV), which is a recombinant between myxoma virus and Shope fibroma virus, grows best in T-cells, moderately in B-cells, and least efficiently in adherent cells, such as monocytes and macrophages (Strayer et al., 1987).

Results in this chapter show stimulation of lymphoid cell cultures with Con A significantly increased lymphocyte permissivity to myxoma virus replication. The increase in permissivity of lymphoid cells to myxoma virus replication due to Con A may be due to interactions at one or more levels. These include virion entry, viral DNA and protein synthesis, virion assembly and maturation and virion egress. As discussed in the previous section, infected cells were detected by binding of a monoclonal antibody specific for a myxoma virus late protein. More cells expressed this late viral protein in stimulated cell cultures compared to unstimulated cell cultures, indicating the actions of Con A on virus replication must occur prior to late protein expression. It is postulated that Con A increases virion entry into the cell. The reasons for this are discussed below.

Poxviruses exist in two infectious forms, intracellular mature virion (IMV) and extracellular enveloped virion (EEV), which have different cell entry mechanisms (Moss 2001). The virus stocks used in this thesis consisted primarily of IMV, as intracellular free virus was released from infected cells by freeze/thawing and sonication. Studies of vaccinia virus have shown that vaccinia IMV entry into a cell requires activated signaling pathways (Locker et al., 2000). Locker et al. (2000) also showed that tyrosine kinase activity was involved in these signaling pathways. Tyrosine kinases play a key signaling role during the T-cell activation process and are more than likely activated by Con A (Fraser et al., 1993; Isakov 1993; Isakov et al., 1994).

Tyrosine kinases have also been shown to be critical for permissive and productive myxoma virus infection (Lalani et al., 1999; Masters et al., 2001). Thus in a simple model, Con A may increase the activity of specific tyrosine kinase signaling pathways that are critical for the infection of lymphocytes with myxoma virus.

However, lymphocytes were also successfully infected with myxoma virus in the absence of Con A stimulation. This result may be accounted for by either a proportion of cells in unstimulated cell cultures appropriately activated without the need for an external stimulus, or infection of cells with the EEV form of myxoma virus. In vaccinia virus, the EEV form of virion does not require activated cell signaling pathways for infection (Locker et al., 2000). However, it is not known whether the EEV form of myxoma virus uses a similar process for infection. It is unlikely that there were large amounts of EEV in the infecting virus stocks (~1% reviewed in Moss 2001). Therefore, it can be concluded that in the unstimulated cell cultures there were probably cells appropriately activated that could be preferentially infected by myxoma virus.

3.3.3 The Sub-population of Lymphoid Cells Permissive for Myxoma Virus Replication

The observation that only 60-80% of cells were permissive for myxoma virus replication suggests that there are particular sub-population(s) in primary lymph node cultures susceptible to myxoma virus infection. Previous studies have identified lymphocytes (Fenner et al., 1953), and in particular T-cells (Best and Kerr 2000), as critical for myxoma virus infection. Results from this thesis suggest that activated T-cells are the main cell sub-population in rabbit lymphoid cell cultures that are permissive for myxoma virus infection. This is because a T-cell mitogen significantly

increased virus replication and the proportion of cells infected and higher levels of late protein were detected in the large cell sub-population, which are activated T-cells. T-cells account for ~57% of the lymph node population (reviewed in Mage 1998). Therefore, there must be other cells in lymph node cell cultures permissive for myxoma virus infection.

These other cells could be B-cells or cells of the monocytic lineage. Preliminary studies *in vitro* of myxoma virus infection of spleen cells, which have a higher proportion of B-cells than lymph nodes, showed a smaller population of infected cells when examined by flow cytometry compared to lymph node preparations (data not shown). This suggests that B-cells are not a major target for myxoma virus infection although they are able to be infected. Supporting this, MRV preferentially replicates in T-cells, although it can also replicate in B-cells (Strayer et al., 1987). However, these results do not rule B-cells out as playing a role in myxoma virus infection. Monocytes and macrophages are also permissive for myxoma virus infection and also play a role in myxoma virus dissemination *in vivo* (Macen et al., 1996; Mossman et al., 1996; Everett et al., 2000), but were not examined in this thesis. Strayer et al. (1987) have shown that MRV replicates less efficiently in adherent cells such as monocytes and macrophages then it does in B-cells. If this is also the case for myxoma virus then it can be concluded that monocytes and macrophages do not play as important a role as T-cells in myxoma virus pathogenesis.

3.3.4 Does Myxoma Virus Affect the Response of Rabbit Lymphoid Cells to T-cell Mitogens?

Although mitogen stimulation significantly enhanced myxoma virus replication in lymphocytes, paradoxically myxoma virus inhibited some features associated with lymphocyte activation, such as an increase in cell size and cell proliferation. Abrogation of proliferation is associated with arrest of the cell cycle at one of two cell cycle checkpoints, G1 or G2. The fact that the increase in cell size (FSC) and replication of cellular DNA (^{3}H-T incorporation) are both inhibited suggests that myxoma virus is arresting the lymphocytes in the G1 stage of the cell cycle. The effect of myxoma virus infection on the cell cycle is investigated in chapter 4.

The results from this chapter indicate that the viral mechanism for inhibition of cell proliferation is independent of the signaling pathways that increase myxoma virus replication. This is because myxoma virus infection at a low moi completely abrogated proliferation but virus could still spread from cell to cell, as seen by the increase in proportion of cells expressing late protein and increase in virus replication. This is highlighted in the low moi, where peak infections were observed after 36-48 h but proliferation was inhibited after 24 h (see Figure 3.3). The fact that infection with the low moi also potently suppressed lymphocyte proliferation at a similar time to infection with the high moi indicates that virus replication is not required for the anti-proliferative effect. Inhibition of T-cell proliferation may be due to either binding of non-infectious virions, which are present in the virus preparations, to the cell surface, or due to the action of a potent anti-proliferative factor either derived from an early viral gene product or secreted by lymphoid cells immediately or shortly after virus binding and entry into the cell.

The presence of secreted factors, either of viral or cellular origin, which halt lymphocyte proliferation has been suggested by previous studies. Strayer et al. have shown that infection with MRV suppressed T-cell proliferation (Strayer et al., 1988; Strayer et al., 1983; Strayer et al., 1986; Strayer et al., 1988), possibly due to a T-cell-derived factor that activated T-cell suppression mechanisms. As MRV virus is similar to myxoma virus, it could be assumed that similar mechanisms are operating in the suppression of lymphocyte proliferation. At this stage, it is difficult to make a convincing argument for either a T-cell-derived factor inducing suppression or suppression being induced by viral particles binding to cell surfaces, as the hypothesized anti-proliferative MRV protein has not been characterised. In addition, attempts to replicate the anti-proliferative effect of T-cell supernatants described by Strayer et al (1988) have not been consistent (Cartledge 1995). However, any anti-proliferative factor would have to have potent inhibitory activity or would have to be secreted in sufficient concentrations to inhibit lymphocyte proliferation regardless of the moi, as myxoma virus infection with both low and high moi completely abrogated lymphocyte proliferation by 24 h.

The most likely explanation for the inhibition of lymphocyte proliferation is the presence of high amounts of non-infectious particles in the virus stocks that bind to T-cells and impede proliferation signals or stimulate inhibitory signals. High levels of non-infectious virus particles are typically present in stocks of virus with large DNA genomes, such as myxoma virus.

From these results, it can be hypothesized that the ability of myxoma virus to increase production of infectious progeny *in vivo* during cell activation but inhibit cell proliferation will cause severe disease in European rabbits. This is because during an

immune response, cells proliferate and differentiate in response to activation signals. Myxoma virus replication and infection will be enhanced by these activated cell signaling pathways, but the virus will abrogate proper T-cell functioning by inhibiting proliferation and activation. This will disrupt the immune response and potentially lead to immunosuppression while still allowing myxoma virus amplification and dissemination in lymphocytes. Differences between virulent and attenuated virus strains may be related to other interactions between virus and host, such as dissemination. Results from this chapter that may shed light on this are discussed in the next section.

3.3.5 Is Attenuation of Ur Associated with a Reduced Ability to Productively Infect Lymphocytes?

In this chapter, three major differences were observed between SLS and Ur infection of rabbit lymphoid cell cultures. The first was the difference in viral titre/infected cell in lymphoid cell cultures. Analysis of titre/infected cell showed that the increase in replication of the attenuated Ur strain of myxoma virus in rabbit lymphoid cell cultures stimulated with Con A was a result of an increase in the number of cells infected. This was because the number of infectious virus particles produced for every infected cell was greater in unstimulated cell cultures compared to stimulated cell cultures. In contrast, the increase in virus titres observed in virulent SLS infections was due to an increase in the number of infectious particles produced per SLS-infected cell.

The difference in titre/infected cell between the virulent and attenuated myxoma virus strains when cells were activated may have consequences *in vivo*. Activation of T-cells is an important part of the immune response (reviewed in Sloan-Lancaster and Allen 1996; Hollsberg 1999; Bi and Altman 2001; Sims and Dustin 2002) and requires

specific stimulatory and co-stimulatory signals. In the lymph node this is usually provided by dendritic cells (Jenkins et al., 2001). In this thesis the T-cell mitogen, Con A, was used to mimic T-cell activation. Con A cross-links cell surface proteins and as such initiates signaling through many pathways. Although Con A stimulation may not be an accurate representation of events *in vivo*, as a variety of cell signaling pathways are activated that may not necessarily be activated during *in vivo* activation, it is probable that at least one of the pathways that Con A activates is that which is involved in *in vivo* activation of T-cells. The non-specific nature of Con A, with regards to the cell signaling pathways activated, does not enable detailed examination of which signaling pathways are required for myxoma virus infection, but rather enables examination of the broad effect of T-cell activation on virus infection.

During the following discussion it must be remembered that lymphocytes will only play a role in myxoma virus pathogenesis once the virus reaches the lymph node draining the inoculation site. So the differences observed in this chapter in the ability of SLS and Ur to replicate in activated T-cells will not affect the early events in myxoma virus pathogenesis which occur in the skin, but rather will impact on events after 24 h when myxoma virus has reached the lymph node draining the inoculation site as detected by immunofluorescence (Best and Kerr 2000).

In the case of SLS, T-cell activation *in vitro* leads to more cells becoming infected and an increase in virus replication in every infected cell. This potentially increases the chances of SLS "surviving" *in vivo* as there is more virus able to infect new cells. T-cell activation also leads to more cells infected with Ur *in vitro* but, unlike SLS infections, Ur replication within individual infected cells is not enhanced. Thus, potentially not as many infectious progeny will be produced *in vivo* once the virus reaches the lymph node

draining the inoculation site, leading to a lower probability of infection of new cells. This may lead to better virus control and clearance by the host at the lymph node draining the inoculation site or at other lymphoid tissues, based simply on a reduced number of infectious Ur virions produced per infected cell. Studies of myxoma virus infection have shown that titres of Ur in lymph nodes draining the inoculation site and in distal tissues tend to be lower when compared to SLS (Best and Kerr 2000). Although one reason for this may be that the ability of Ur to replicate is lower *in vivo* as supported by the results just described, it could also be due to an enhanced anti-viral immune response by the host that inhibit virus replication.

The second difference between SLS and Ur was in their ability to replicate in stimulated lymphocytes from wild rabbits, which was discussed in Section 3.3.1. However, it must be remembered that lymphoid cells from wild rabbits exhibit resistance to myxoma virus replication and it may be the resistance of lymphocytes rather than attenuation of Ur (or a combination of both) that is responsible for this finding. As previously noted, permissivity to myxoma virus replication can be impeded at a number of levels. In lymphoid cell cultures from wild rabbit both SLS and Ur had a similar proportion of cells infected as measured by staining for a late virus protein, but the amount of infectious virus particles produced in lymphoid cell cultures from wild rabbits infected with Ur was significantly less than in cultures infected with SLS. These results indicate that the difference between SLS and Ur is operating after late protein expression in events late in the virus life cycle, such as virion assembly and maturation or egress. As discussed previously, any problems in virion egress would not be seen as the cells were sonicated to release virions prior to determination of titres by plaque assay. This suggests that Ur virions have a reduced ability to assemble or mature in lymphoid cells from wild rabbits compared to SLS.

Alternatively, attenuation of Ur may involve increased cell death in cell cultures from wild rabbits initiated after late protein expression. Increased cell death late in infection may also be responsible for the significant drop at 72 h in the proportion of cells infected with Ur compared to SLS. The dramatic decrease in proportion of cells infected with Ur was the third major difference observed between SLS and Ur. Cell death pathways are induced on cell cycle arrest, which occurs when cell proliferation is abrogated. It is expected that these pathways are activated after infection with both SLS and Ur, but as only Ur showed a drop in proportion of cells infected at 72 h, it could be postulated that SLS is more effective at inhibiting the induction of cell death at cell cycle checkpoints than Ur. The possibility that a difference in cell death is involved in myxoma virus attenuation is examined experimentally in the next chapter.

Chapter 4

Analysis of Cell Viability, Cell Cycle and Genetic Mechanism of Attenuation

p values for chapter 4

Proportion of Cells in Sub G1

Con A significantly increased the proportion of events classified as Sub G1 in mock-infected cultures over the 72 h time period. The p values for lymphoid cell cultures from susceptible and resistant rabbits are <0.02 and <0.001 respectively.

The proportion of Sub G1 events was less in stimulated SLS-infected cell cultures compared with stimulated mock-infected cell cultures in cell cultures from both rabbit types, but this difference was only significant in lymphoid cell cultures from wild rabbits (p value <0.03).

Compared to SLS-infected cell cultures, Ur-infected cell cultures had significantly higher proportion of events classified as Sub G1 72 h posit infection in lymphoid cell cultures from both rabbit types. For cell cultures from susceptible rabbits the p value is <0.05 and for cell cultures from resistant rabbits, the p value is <0.04.

Proportion of Cells in G1/G0

When mock-infected cultures were stimulated with Con A, the proportion of cells in G1/G0 significantly decreased over the 72 h time course. For cell cultures from susceptible rabbits the p value is <0.01 and for cell cultures from resistant rabbits, the p value is <0.0005.

When cell cultures infected with SLS were stimulated with Con A, there was a significantly greater proportion of events in G1/G0 at 48 h (cell cultures from laboratory rabbits only) and 72 h (cell cultures from wild rabbits only) post infection compared to mock-infected cell cultures, and a significantly greater proportion of events in G1/G0 at 72 h compared to Ur-infected cell cultures (both rabbit types). The p values for this are shown in the table below.

Significance between Con A stimulated MI and SLS-infected cultures, 48 h pi	**p value**
Cell cultures from susceptible rabbits	<0.02
Cell cultures from resistant rabbits	not significant
Significance between Con A stimulated MI and SLS-infected cultures, 72 h pi	**p value**
Cell cultures from susceptible rabbits	not significant
Cell cultures from resistant rabbits	<0.03
Significance between Con A stimulated SLS and Ur-infected cultures, 72 h pi	**p value**
Cell cultures from susceptible rabbits	<0.05
Cell cultures from resistant rabbits	<0.05

4.1 INTRODUCTION

There was a dramatic drop in the proportion of cells infected with Ur after 48 h in stimulated lymphoid cell cultures (chapter 3). The decrease in proportion of cells infected with Ur was observed in cell cultures from both rabbit types, but was not observed when cell cultures were infected with SLS. This result clearly differentiated virulent SLS myxoma virus from attenuated Ur myxoma virus. Chapter 3 also showed that both SLS and Ur abrogated lymphocyte proliferation, as measured by ^{3}H-T incorporation and the CellTitre assay. Inhibition of proliferation is associated with arrest of the cell cycle at one of the cell cycle checkpoints (described in Section 1.6.1.3). Failure of a cell to pass a cell cycle checkpoint can induce programmed cell death (apoptosis) (Shi et al., 1994; Meikrantz and Schlegel 1996; Yao et al., 1996; Evan and Littlewood 1998; Guo and Hay 1999). Differences between SLS and Ur in their ability to inhibit apoptosis induced by cell cycle arrest may account for the differences in proportion of cells infected at late time points.

Therefore, it could be hypothesized that the drop in proportion of Ur-infected lymphocytes at late time points *in vitro* is due to removal of these cells by apoptosis or other forms of cell death. Further, Ur fails to inhibit cell death pathways that are induced on cell cycle arrest. The inability of Ur to maintain lymphocyte viability *in vivo* would lead to a lower proportion of infected cells, which would limit Ur dissemination and amplification and lead to a milder clinical disease. In contrast, it is postulated that SLS is able to maintain the viability of infected lymphocytes after cell cycle arrest at late time points *in vitro*. If SLS was also able to maintain lymphocyte viability *in vivo,* it would lead to more efficient virus replication and dissemination and more severe clinical disease compared to Ur infection. These hypotheses can be investigated by

examining and comparing lymphocyte viability in cell cultures infected with SLS and Ur.

Previous studies have shown that recombinant myxoma viruses with genes deleted that are essential for replication in lymphocytes are attenuated *in vivo* and induce apoptosis in lymphocytes *in vitro* (Opgenorth et al., 1992a; Macen et al., 1996; Barry et al., 1997). Shope fibroma virus also induces apoptosis in lymphoid cells *in vitro,* fails to replicate in lymphocytes and is attenuated *in vivo.* These studies suggest attenuation, at least in some cases, is associated with apoptosis of lymphocytes. Thus, it could be postulated that Ur has a lesion that alters production or function of proteins involved in apoptosis or related processes. These changes result in the failure of Ur to maintain lymphocyte viability after cell cycle arrest and contribute to Ur attenuation.

This chapter addresses the following questions;

1) Is there a difference between SLS and Ur in their ability to maintain lymphocyte viability after cell cycle arrest?
2) Can any difference in lymphocyte viability that is observed, be attributed to genetic differences in anti-apoptotic or host-range genes between SLS and Ur?

These questions were addressed using flow cytometric analysis of lymphocytes prepared from susceptible laboratory and resistant wild rabbits, infected *in vitro* with virulent SLS or attenuated Ur strain of myxoma virus. Propidium iodide staining of cellular DNA enabled analysis of cell viability and identification of cell cycle stages. To determine whether genetic differences exist between the SLS and Ur strains of myxoma virus, 10 viral genes implicated in inhibition of apoptosis, host-range or immunomodulation by Cameron et al. (1999) were sequenced.

4.2 RESULTS

4.2.1 Cell Viability and Cell Cycle Analysis of Infected Rabbit Lymphocytes

4.2.1.1 Experimental Approach

Cells that are viable (in G1/G0 and G2/M stages of the cell cycle) can be distinguished from non-viable cells, cell fragments and debris using flow cytometry, based on intensity of propidium iodide (PI) staining of cellular DNA (Figure 4.1A). This is because the amount of genomic DNA in a cell in G2/M will be twice that of a cell in G1/G0 as, by G2/M the genomic DNA has replicated but cytokinesis has not been completed. The flow cytometer counts and analyses 'events', which can be either a viable cell, apoptotic body or necrotic cell, cell fragment or cell debris (Becton Dickinson Immunocytometry Systems Reference Manual). Because there is twice as much DNA in cells in G2/M than G1/G0, more PI will bind to these cells, increasing the fluorescence intensity that is measured for that event. Apoptotic or necrotic cells, which have fragmented DNA, and cell fragments containing DNA will have less DNA per event measured than cells in G1/G0 and will therefore have lower fluorescence levels (Becton Dickinson Immunocytometry Systems). In this thesis, the term 'Sub G1 phase of the cell cycle' is used to describe these non-viable apoptotic and necrotic cells and cell fragments. The term 'viable' is used to describe cells in G1/G0 and G2/M.

Cells were assayed for viability by trypan blue exclusion immediately prior to setting up all experiments and only preparations with greater than 95% of cells excluding trypan blue were used. These cell cultures, with greater than 95% viability, were mock-infected or infected with SLS or Ur at a moi of 3 and analysed 4, 24, 48 and 72 h post infection

for stage of cell cycle by PI staining of cellular DNA. Cell cultures prepared from three individual laboratory and three individual wild rabbits were used.

To confirm the flow cytometric gating of cells according to viability, the proportion of events detected as viable cells after cell cultures were incubated for 48 h was compared to that calculated using trypan blue exclusion. Results showed 79% of events (of 10,000 counted) were classified as viable after 48 h by flow cytometry and 84% of cells (of approximately 200 counted) were classified as viable by trypan blue exclusion. The higher proportion of non-viable events when samples were examined by flow cytometry (21% compared to 16%) is possibly explained by the presence of cell fragments in the non-viable cell gate, which would not be counted in the trypan blue exclusion assay. This indicates that analysis of cell viability by flow cytometry is more powerful than that of trypan blue exclusion. Furthermore, flow cytometry also enables more than one cellular feature to be examined at a time. For example, myxoma virus infection can be examined concurrently with cellular DNA content to analyse whether cells infected with virus are in a particular stage of the cell cycle. This gives flow cytometry an advantage over trypan blue exclusion when examining the effects of myxoma virus infection on rabbit lymphoid cells.

The flow cytometer was calibrated such that events corresponding to the G1/G0 sub-population had a fluorescence intensity greater than 10^2 units and events classified as Sub G1 had a fluorescence intensity between 10^1 and 10^2 units (Figure 4.1B). Using this method, distinct viable (G1/G0 and G2/M) and non-viable (Sub G1) cell sub-populations were identified. Cell culture debris had a fluorescence intensity less than 10^1 units and so was not included. CellQuest software was used to gate sub-populations from density plots of cell size versus PI staining, into Sub G1 (non-viable), G1/G0 and

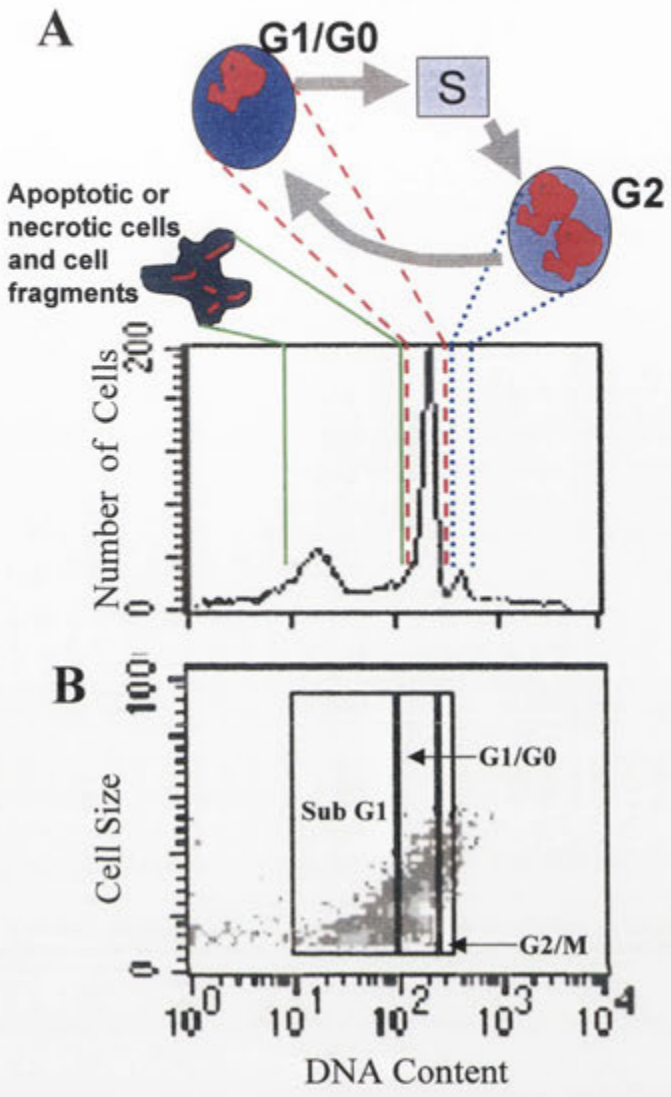


FIGURE 4.1. METHOD USED TO ANALYZE CELL VIABILITY AND CELL CYCLE STAGES
(A) The cell cycles through G1/G0 where the cell has one copy of DNA, and S phase (DNA replication) to G2/M phase where the cell has two copies of DNA. The flow cytometer identifies and analyses 'events'. As there is twice as much DNA in G2/M than G1/G0, more propidium iodide (PI) will bind per event, shifting the fluorescence intensity peak to the right in the histogram plots. Cells undergoing apoptosis or necrosis and cell fragments will be detected as events, these sub-populations will have fragmented DNA and so will bind less DNA per event. Hence a shift to the left in fluorescence intensity will occur. (B) Cells were gated into Sub G1, G1/G0 and G2/M based on PI staining of DNA. The proportion of cells in each of these sub-populations was calculated using CellQuest software. These proportions are graphed in subsequent figures.

G2/M phases and to calculate the proportion of events in each. The density plots of cell size versus DNA content (Figure 4.1B) were graphed as a histogram plot.

4.2.1.2 Proportion of Events Classified as Sub G1 (Non-viable cells)

There was no significant difference at the 95% confidence interval (CI) between unstimulated and Con A-stimulated mock-infected cultures in the proportion of events classified as Sub G1 (fluorescence intensity 10^1-10^2 units) over the 72 h time period. However, there was a significant difference in the proportion of events classified as Sub G1 in Con A-stimulated mock-infected cell cultures from both laboratory and wild rabbits when the duration of stimulation was examined (ANOVA, $p<0.05$) (Figure 4.2). For example, the proportion of events in Sub G1 in stimulated lymphoid cell cultures from laboratory rabbits was 23% 4 h post mock-infection, and 43% 72 h post mock-infection. In wild rabbit cell cultures, 14% of events were in Sub G1 at 4 h post mock-infection compared to 58% at 72 h post mock-infection. This illustrates that stimulation of cell cycling by a T-cell mitogen is sufficient to induce cell death in uninfected rabbit lymphocytes. In unstimulated mock-infected cell cultures the proportion of Sub G1 events did not significantly change over the time course.

The high proportion (15-35%) of non-viable events in mock-infected cultures (stimulated and unstimulated) after 4 h indicates that the process of mock-infection is responsible for some of the Sub G1 sub-population, as all cultures had greater than 95% of cells viable by trypan blue exclusion prior to setting up the experiment.

The proportion of events in Sub G1 in SLS-infected cell cultures did not differ significantly at the 95% CI with different periods of Con A stimulation. This sub-population was also not significantly different in SLS-infected cell cultures compared to mock-infected cell cultures at 4, 24 or 48 h post infection (95% CI). At 72 h post infection, however, the proportion of Sub G1 events was less in stimulated SLS-infected cell cultures compared with stimulated mock-infected cell cultures in cell cultures from

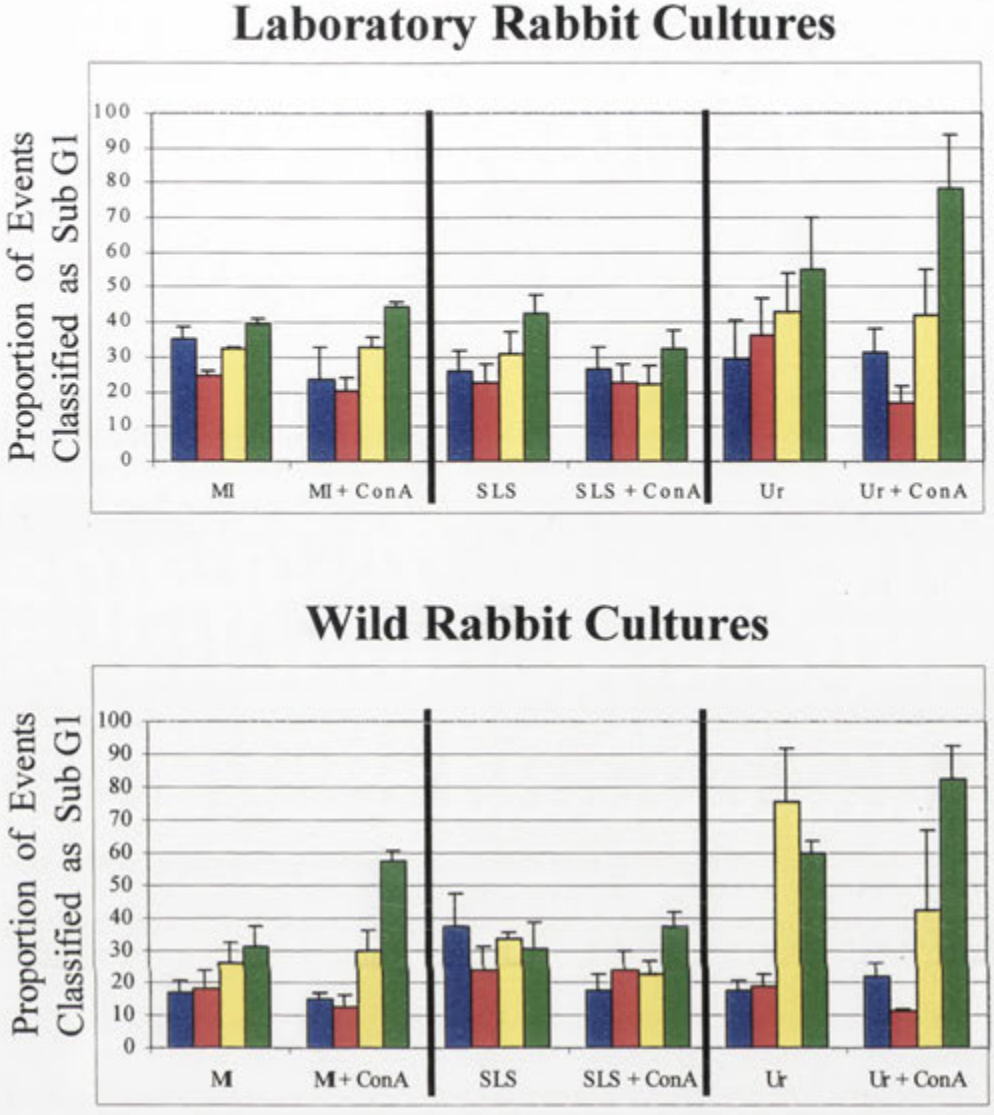


FIGURE 4.2. PROPORTION OF EVENTS CLASSIFIED AS SUB G1 IN RABBIT LYMPHOID CELL CULTURES MOCK-INFECTED OR INFECTED WITH SLS OR UR

Laboratory and wild rabbit lymphoid cell cultures were mock-infected or infected with SLS or Ur at a moi of 3, in the presence or absence of Con A. Samples were analysed at ■4, ■24, ■48 and ■72 h post infection for PI staining of DNA. The proportion of events classified as Sub G1 was calculated as shown in Figure 4.1. Infections were repeated for lymphoid cell cultures prepared from three laboratory and three wild rabbits, the mean of which is shown here. Error bars represent one standard error.

both rabbit types, but this difference was only significant in lymphoid cell cultures from wild rabbits ($p<0.05$) (Figure 4.2).

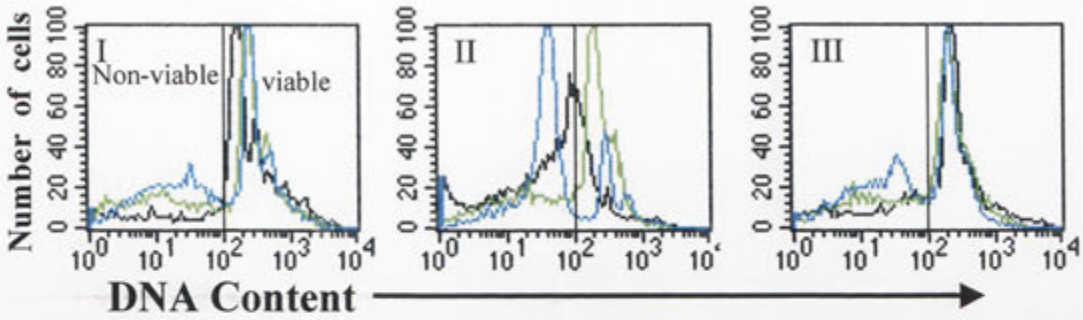
Compared to SLS-infected cell cultures, Ur-infected cell cultures had significantly higher proportions of events classified as Sub G1 72 h post infection in lymphoid cell cultures from both rabbit types ($p<0.05$). There was no significant difference between SLS- and Ur-infected cell cultures at earlier time points. Comparison of unstimulated and stimulated Ur-infected cell cultures at each time point showed Con A stimulation did not significantly change the proportion of Sub G1 events in these cultures ($p<0.05$). The Sub G1 sub-population in Ur-infected cell cultures was not significantly different from that in mock-infected cell cultures at 72 h, however it did tend to be greater in Ur-infected cell cultures (Figure 4.2).

In conclusion, SLS maintained lymphocyte viability significantly above that of mock-infected and Ur-infected cell cultures at late time points ($p<0.05$).

4.2.1.2.1 Viability of Infected RL-5 Cells

To determine whether Ur also induced cell death compared to SLS in the rabbit T-cell line, RL-5, RL-5 cells were mock-infected or infected with SLS or Ur at a moi of 3 and analysed 72 h post infection as detailed in Figure 4.1. Histogram overlays of DNA content in mock-infected and infected RL-5 cells are shown in Figure 4.3 for three independent experiments. In cell cultures infected with Ur, the mean proportion of viable cells was 49% at 72 h. This was significantly less than in mock-infected or SLS-infected cell cultures where 78% of events in SLS-infected RL-5 cell cultures and 82% of events in mock-infected cell cultures were classified as viable ($p<0.05$) (Figure 4.3B). These results show that Ur is also unable to maintain lymphocyte viability in infected RL-5 cells. However, in these RL-5 cell cultures there was no significant difference in proportion of events classified as viable between mock-infection and SLS-infection.

A



B Mean Proportion of Viable Cells at 72 h

Mock-infected	SLS	Ur
82%	78%	49%

FIGURE 4.3. VIABILITY OF RL-5 CELLS INFECTED WITH SLS OR UR
(A) RL-5 cells were mock-infected (black line) or infected with either SLS (green line) or Ur (blue line) at a moi of 3. Cell viability was determined by propidium iodide (PI) staining of DNA and was analyzed by flow cytometry, where events with a fluorescence intensity between 10^1 and 10^2 units were classified as the non-viable sub-population and events with a fluorescence intensity above 10^2 units were classified as viable cells. A fluorescence intensity of 10^2 units is shown as a vertical black line. Cell viability was examined in three cultures, represented by I, II, III. The histogram overlays were generated using the CellQuest software. (B) The mean proportion of viable cells in mock-infected and SLS- and Ur-infected RL-5 cell cultures was calculated.

4.2.1.3 Proportion of Lymphocytes in G1/G0 Stage of the Cell Cycle

There was no significant difference ($p<0.05$) in the proportion of unstimulated mock-infected lymphocytes in G1/G0 phase of the cell cycle over the 72 h time course with 60% and 77% at 4 h in cell cultures from laboratory and wild rabbits respectively to 56% and 64% respectively at 72 h (Figure 4.4). However, when mock-infected cell cultures were stimulated with Con A, the difference in proportion of lymphocytes in G1/G0 was significant ($p<0.05$) over the 72 h time course, with 65% and 73% of events in G1/G0 at 4 h post infection in cell cultures from laboratory and wild rabbits respectively, and 49% and 39% of events respectively at 72 h post infection.

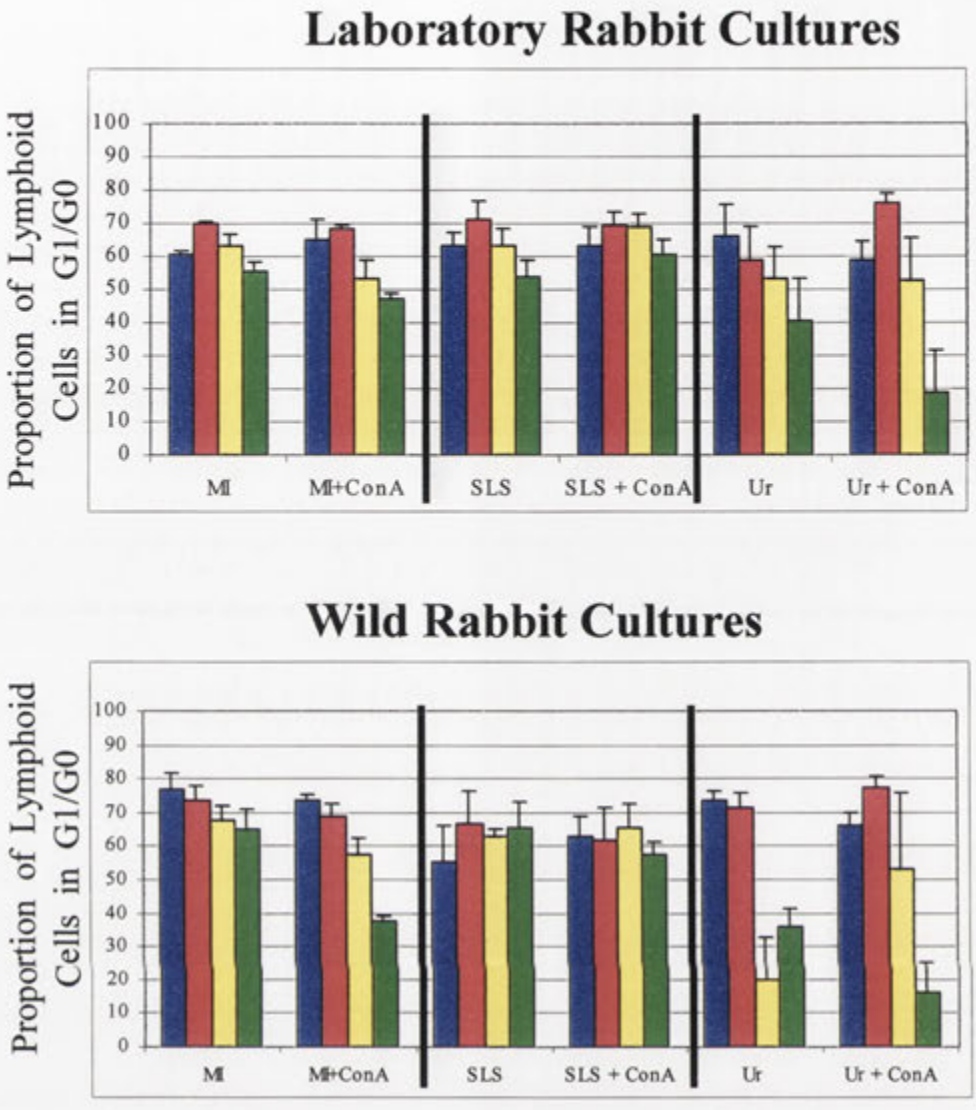


FIGURE 4.4. PROPORTION OF CELLS IN G1/G0 STAGE OF THE CELL CYCLE IN RABBIT LYMPHOID CELL CULTURES MOCK-INFECTED OR INFECTED WITH SLS OR UR

Laboratory and wild rabbit lymphoid cell cultures were mock-infected or infected with SLS or Ur at a moi of 3, in the presence of absence of Con A. Samples were analysed at ■4, ■24, ■48 and ■72 h post infection for propidium iodide (PI) staining of DNA. The proportion of cells in G1/G0 was calculated as shown in Figure 4.1. Infections were repeated for lymphoid cell cultures prepared from three laboratory and three wild rabbits, the mean of which is shown here. Error bars represent one standard error.

When cell cultures were infected with either SLS or Ur in the absence of mitogen, the proportion of cells in G1/G0 was not significantly different to those in mock-infected cell cultures at the same time points ($p<0.05$). However, cell cultures infected with Ur tended to have lower proportions of events in G1/G0 compared to SLS-infected and mock-infected cell cultures (Figure 4.4).

When cell cultures infected with SLS were stimulated with Con A, there was a significantly greater proportion of events in G1/G0 at 48 h (cell cultures from laboratory rabbits only) and 72 h (cell cultures from wild rabbits only) post infection compared to mock-infected cell cultures, and a significantly greater proportion of events in G1/G0 at 72 h compared to Ur-infected cell cultures (both rabbit types) ($p<0.05$) (Figure 4.4). There was no difference between Ur- and mock-infected cell cultures (stimulated and unstimulated) in the proportion of events in G1/G0 at any time point ($p<0.05$), however the proportion of cells in G1/G0 in Ur-infected cultures was less than that in mock-infected cultures at 48 and 72 h.

In conclusion, there were a significantly higher proportion of cells in G1/G0 in SLS-infected cell cultures compared to mock-infected and Ur-infected cell cultures. This is reflected by the lower proportion of Sub G1 events in SLS-infected cell cultures compared to mock-infected and Ur-infected cell cultures as described in Section 4.2.1.2.

4.2.1.4 Proportion of Lymphocytes in G2/M Stage of the Cell Cycle

Con A stimulation more than doubled the proportion of cells in G2/M stage of the cell cycle in mock-infected rabbit lymphoid cell cultures (Figure 4.5). However, the proportion of cells in G2/M (mostly about 5%) was much lower generally than those in G1/G0 (mostly about 60%). The peak in proportion of cells in G2/M in cell cultures infected with myxoma virus was 4-24 h post infection with generally lower proportions compared with mock-infected cultures after this time. No significant difference in proportion of events in G2/M was observed between infections using SLS or Ur or infections of cultures from laboratory or wild rabbits for any time point ($p<0.05$).

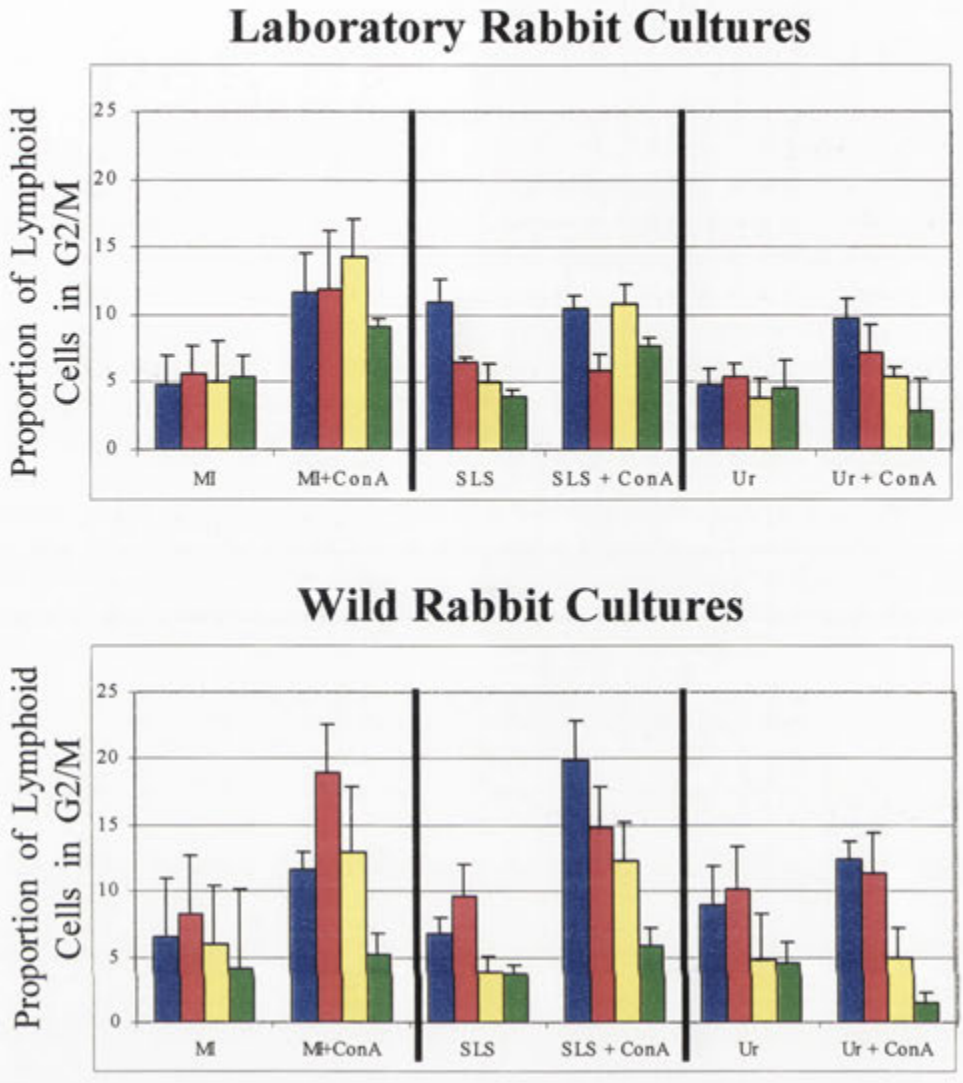


FIGURE 4.5. PROPORTION OF CELLS IN G2/M STAGE OF THE CELL CYCLE IN RABBIT LYMPHOID CELL CULTURES MOCK-INFECTED OR INFECTED WITH SLS OR UR
Laboratory and wild rabbit lymphoid cell cultures were mock-infected or infected with SLS or Ur at a moi of 3, in the presence of absence of Con A. Samples were analysed at ■4, ■24, ■48 and ■72 h post infection for propidium iodide (PI) staining of DNA. The proportion of cells in G2/M was calculated as shown in Figure 4.1. Infections were repeated for lymphoid cell cultures prepared from three laboratory and three wild rabbits, the mean of which is shown here. Error bars represent one standard error.

The fact that some cells in myxoma virus-infected cultures are in G2/M early in the time period suggests that the mechanism used by myxoma virus to inhibit cell proliferation is initiated in the middle to late stages of the virus life cycle. These results appear to conflict with those in chapter 3 which showed no increase in either ^{3}H-T or absorbance (CellTitre assay). However, the ^{3}H-T assay detects replicating DNA as ^{3}H-T is

incorporated into the replicating DNA strands, whereas propidium iodide is an intercalating agent and as such is incorporated into existing DNA strands. Therefore, the two results can both exist.

4.2.1.5 Summary of Cell Viability and Cell Cycle

The Sub G1 sub-population was present 72 h post infection in infected lymphoid cell cultures from both laboratory and wild rabbits and infected RL-5 cells. In primary lymphocyte cell cultures the proportion of events in Sub G1 was increased by Con A stimulation. However, the Sub G1 sub-population was statistically significantly greater in Ur-infected cultures. This indicates that Ur is unable to maintain lymphocyte viability at late time points compared to SLS. In contrast, SLS maintained cell viability at late time points even when cell cultures were stimulated with Con A. The maintenance of viability in stimulated SLS-infected cell cultures appeared to be strongly related to cells in G1/G0, as stimulation of SLS-infected cell cultures led to significantly more cells in G1/G0 than mock-infected controls ($p<0.05$) but similar proportions of cells in G2/M at 72 h. Therefore, the ability of SLS to maintain the viability of cells appears to be linked to G1/G0 arrest. This supports the results from chapter 3, which show SLS (and Ur) inhibit ^{3}H-T incorporation indicating cells are not progressing into S phase.

4.2.2 Analysis of Myxoma Virus Antigen in Events Classified as Sub G1, G1/G0 and G2/M

In chapter 3 it was shown that cells stimulated with Con A were more permissive for infection than unstimulated cells. Con A stimulation of lymphocytes will generate sub-populations of activated cells and of dividing cells which could not be differentiated in chapter 3. This section examines which stage of the cell cycle infected cells are in and

also examines the permissivity of dividing cells (those lymphocytes in the G2/M stage of the cell cycle) for myxoma virus replication.

4.2.2.1 Experimental Approach

Cell cultures were stimulated with Con A for 48 h prior to infection with SLS or Ur for 24 h. Cells were gated as Sub G1, G1/G0 or G2/M on a density plot of cell size versus DNA content as shown in Figure 4.1. Each of these sub-populations was analyzed for binding of the anti-myxoma virus monoclonal antibody, 3b6e4, by plotting side scatter (SSC- light scattered 90° to direction of incident laser) versus virus infection (Figure 4.6). From the density plots of cell granularity versus virus infection, histogram plots of virus infection were generated. The proportion of cells expressing viral antigen and therefore infected, were calculated from the histogram plots.

To compensate for the fact that G1/G0 events constitute the major sub-population present in rabbit lymphoid cell cultures, the probability that an event in a particular stage of the cell cycle would also stain positive for viral antigen was calculated. This was done by dividing the proportion of events in each sub-population that were expressing viral antigen by the total number of events in that sub-population. Analysis of data in this way showed there was a higher probability that an event, if classified as G2/M, will express viral antigen after 24 h of infection compared to events in G1/G0 or Sub G1 (Table 4.1). Interestingly, lymphoid cell cultures from wild rabbits had a lower probability of staining positive for viral antigen after 24 h than those in laboratory rabbit cell cultures (Table 4.1). This may indicate a lower permissivity of lymphocytes from wild rabbits to myxoma virus replication and supports the finding in chapter 3, where significantly lower virus titres were produced in lymphocytes from wild rabbits compared to lymphocytes from laboratory rabbits (chapter 3, Figure 3.1).

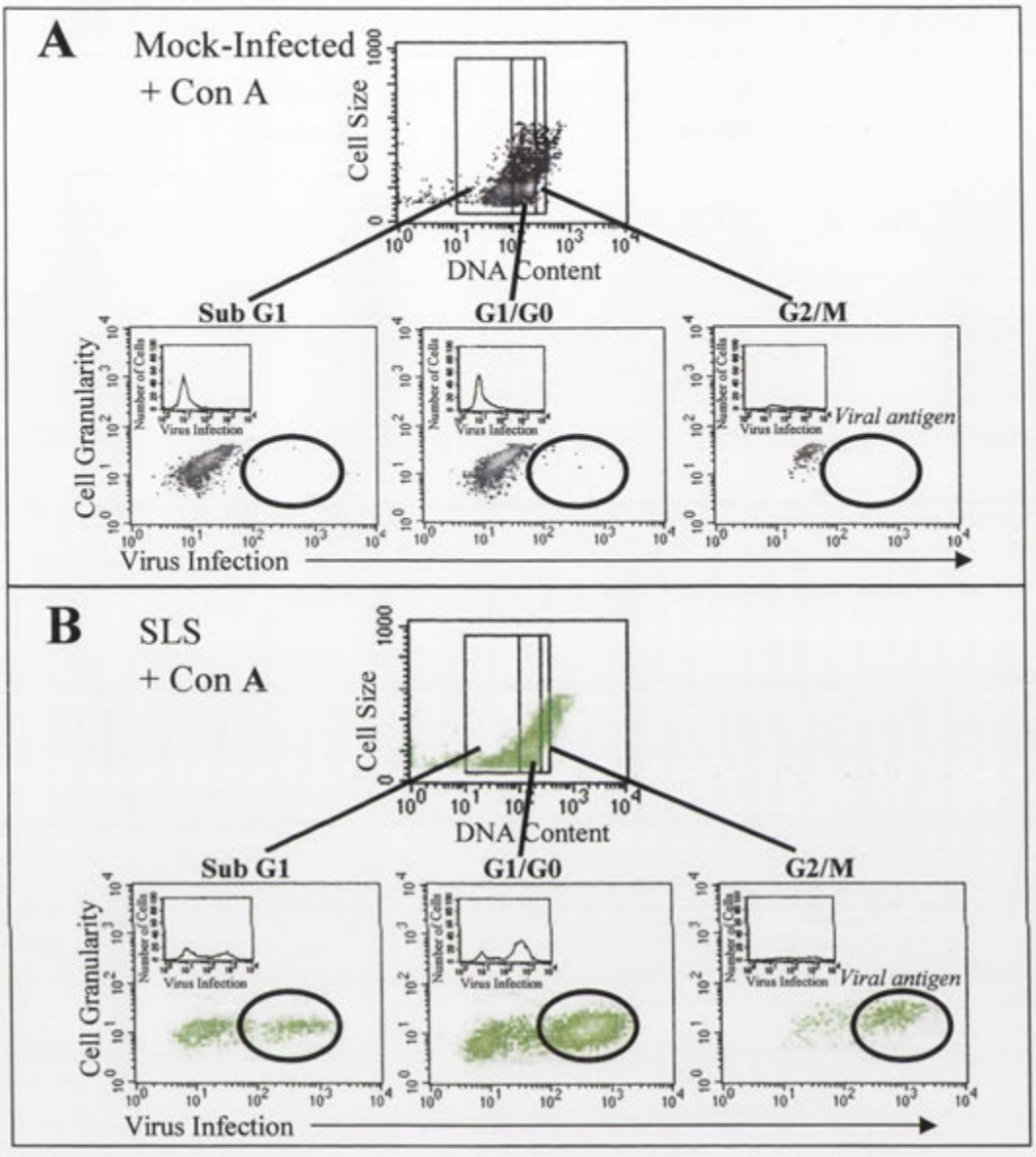


FIGURE 4.6. METHOD USED TO ANALYZE VIRAL ANTIGEN IN EVENTS CLASSIFIED AS SUB G1 G1/G0 OR G2/M

Rabbit lymphoid cells were analyzed for stage of the cell cycle in conjunction with virus infection. Two examples are shown in this figure to illustrate the methodology used. Con A-stimulated laboratory rabbit lymphoid cell cultures were (A) mock-infected or (B) infected with SLS. Density plots of cell size versus DNA content were generated for all infections. Events were gated, based on DNA content, as either in Sub G1, G1/G0 or G2/M as shown in Figure 4.1. Each of these sub-populations was then analyzed for expression of a myxoma virus antigen (circled regions) by plotting cell granularity versus virus infection. A histogram plot (inset) of virus antigen expression is also shown.

TABLE 4.1. PROBABILITY THAT AN EVENT IN SUB G1, G1/G0 OR G2/M WILL STAIN POSITIVE FOR VIRAL ANTIGEN

	Laboratory Rabbit Lymphoid Cultures			Wild Rabbit Lymphoid Cultures		
	Sub G1	G1/G0	G2/M	Sub G1	G1/G0	G2/M
SLS	40%* replicates = 46, 37, & 37%	70% replicates = 71, 73, & 66%	86% replicates = 75, 93, & 91%	16% replicates = 23, 12, & 14%	46% replicates = 45, 58, & 34%	68% replicates = 75, 68, &61%
Ur	32% replicates = 15, 36, & 43%	71% replicates = 72, 71, & 69%	84% replicates = 88, 92, & 72%	18% replicates = 30, 17, & 6%	37% replicates = 46, 28, & 36%	62% replicates = 66, 53, & 67%

* Probabilities were calculated by dividing the proportion of infected cells 24 h post infection in each sub-population by the total number of cells in that sub-population. Figures are the means of cultures from three laboratory or three wild rabbits. Probabilities for individual replicate cultures are also shown.

4.2.2.2 Detection of Viral Antigen Stages of Cell Cycle

To analyse in more detail the stage of the cell cycle where viral antigen was detected, the median fluorescence intensity corresponding to viral antigen was calculated and averaged for lymphoid cell cultures stimulated with Con A prior to infection for 24 h for each group of three rabbits. Cultures stimulated for 4, 24, 48 or 72 h prior to infection were examined. Analysis of mean median fluorescence intensity gives a more accurate idea of what is happening in terms of myxoma virus infection in each of the sub-populations without data being skewed by outliers. However, the absolute values of mean medium fluorescence intensity cannot be compared between groups as they reflect concentration of viral antigen rather than the total amount in each sub-population.

Table 4.2 shows the difference in late protein expression, as measured by mean median fluorescence intensity, between mock-infected and virus-infected rabbit lymphoid cells. The results were obtained by calculating the difference in mean median fluorescence

intensity of events in Sub G1, G1/G0 or G2/M, between mock-infected and SLS- or Ur-infected cell cultures.

The mean median fluorescence intensity in cell sub-populations in lymphoid cell cultures from laboratory rabbits generally increased the longer the cells were stimulated prior to infection, supporting previous evidence that stimulation of cells increases their permissivity for virus replication (Table 4.2). This was a function of stimulation and not infection, as all cell cultures were analyzed after 24 h of infection. The greatest differences between mock-infected and virus-infected lymphocyte cell cultures from laboratory rabbits were observed when cell cultures were stimulated for either 48 or 72 h prior to infection. This contrasts to infections of lymphoid cell cultures from wild rabbits, where there is a decrease in fluorescence intensity in all sub-populations when cell cultures were stimulated with Con A for 48 h prior to infection compared to those stimulated for 24 or 72 h (Table 4.2). This decrease in mean median fluorescence intensity at this time point in cell cultures from wild rabbits was also noted in chapter 3 (Table 3.1).

In all infections of lymphoid cell cultures (laboratory and wild rabbit) stimulated for 4, 24 or 48 h, events in G2/M had a mean median fluorescence intensity difference greater than that of events in G1/G0 or Sub G1 (Table 4.2). This suggests that either more virus protein is being produced in cells in this sub-population or that virus protein accumulates in cells in G2/M by 24 h post infection. The increased production or accumulation of viral antigen may be related to increased virus replication in cells with activated signaling pathways, as shown in chapter 3, and as would be found in lymphocytes in G2/M stage of the cell cycle.

TABLE 4.2. DIFFERENCE IN MEAN MEDIAN FLUORESCENCE INTENSITY* CORRESPONDING TO MYXOMA VIRUS ANTIGEN EXPRESSION BETWEEN MOCK-INFECTED AND VIRUS-INFECTED RABBIT LYMPHOID CELLS CLASSIFIED AS SUB G1, G1/G0 OR G2/M

		Laboratory Rabbit Lymphoid Cultures				Wild Rabbit Lymphoid Cultures			
Stage of Cell Cycle	Virus Strain	4 h Con A stimulation	24 h Con A stimulation	48 h Con A stimulation	72 h Con A stimulation	4 h Con A stimulation	24 h Con A stimulation	48 h Con A stimulation	72 h Con A stimulation
Sub G1	SLS	13	24	103	76	10	104	<0	8
	Ur	<0	61	77	137	15	117	<0	67
G1/G0	SLS	45	118	193	254	11	429	104	401
	Ur	11	190	292	283	17	378	19	337
G2/M	SLS	117	357	239	484	288	772	272	813
	Ur	61	454	476	456	126	598	288	725

* The mean median fluorescence intensity of anti-myxoma antibody binding was determined for events in Sub G1, G1/G0 and G2/M. The difference between the mean median fluorescence intensity in mock-infected and virus-infected cultures was calculated. Cultures were stimulated for 4, 24, 48 or 72 h prior to infection for 24 h.

4.2.3 Summary of Cell Viability and Cell Cycle Examined by Flow Cytometry

The previous sections examined cell viability and cell cycle in rabbit lymphoid cell cultures infected with myxoma virus. In Ur-infected cultures there was a significant decrease in the number of viable cells 72 h post infection compared to cultures infected with SLS. In contrast, SLS maintained cell viability in infected cultures significantly above that of mock-infected cultures. These results support the hypothesis that the decrease at 72 h in the proportion of Ur-infected stimulated rabbit lymphoid cells observed in chapter 3, is due to the inability of Ur to completely inhibit cell death pathways, whereas SLS is able to inhibit cell death.

Cells in G2/M were more permissive for infection. Cells in G2/M would have activated signaling pathways because they are dividing. This is consistent with the results in chapter 3 that show activated cells were more permissive for infection. However, infection of cells in G1/G0 would probably play a larger role in mxyoma virus pathogenesis, as G1/G0 constitutes the major cell sub-population in lymphoid cell cultures.

4.2.4 Sequence Analysis of Anti-Apoptotic and Host-Range Genes

The results in the previous Section clearly differentiated the attenuated Ur and virulent SLS strains of myxoma virus. One cause of these differences may be mutations in the viruses' anti-apoptotic or host-range genes as potential mechanisms for Ur attenuation. Myxoma virus encodes a wide variety of anti-apoptotic and host-range proteins that facilitate virus-host interactions and help maintain lymphocyte viability. The increase in

Sub G1 events in Ur-infected cell cultures 72 h post infection may be attributable to changes in one of these genes.

4.2.4.1 Selection of Genes to be Sequenced

Nucleotide and amino acid sequence homology of anti-apoptotic, host-range or immunomodulatory ORFs between the two *Leporipoxvirus* species with published genome sequences, the virulent Lu strain of myxoma virus and attenuated SFV, were examined to determine which genes were least conserved and therefore may be associated with *Leporipoxvirus* attenuation. Homology was determined for all putative anti-apoptotic, host-range or immunomodulatory ORFs identified by Cameron et al (1999).

The majority of genes were between 70-80% homologous between Lu and SFV in nucleotide identity regardless of putative function (data not shown). The ORF with the least homology between Lu and SFV, M077, putatively encodes a structural protein. The wide variation in homology between the Lu and corresponding SFV ORF, particularly the structural and housekeeping genes which tend to be more conserved across poxvirus species, indicated that nucleotide and amino acid homologies were not useful as a basis for identifying genes potentially involved in *Leporipoxvirus* attenuation. Selection of the ten immunomodulatory, anti-apoptotic and host-range genes for sequencing was thus based primarily on putative gene product function and only partly on homology between ORF sequence in SFV and Lu.

Ten genes that were identified by Cameron et al (1999) as having potential immunomodulatory, anti-apoptotic or host-range function were selected for sequencing based primarily on gene product function. The ten anti-apoptotic, host-range and

immunomodulatory genes selected for sequencing were M002, M004, M005, M062, M063, M064, M128, M151, M152 and M156. Table 4.3 lists these genes and the putative properties of their protein products.

TABLE 4.3. THE ORF SEQUENCED AND THE FUNCTIONS OF THEIR PROTEIN PRODUCTS

ORF	Gene Product Function(s)	Reference
M002	-TNF receptor homolog -blocks apoptosis -deletion virus induces apoptosis in non-adherent leukocytes	Macen et al. (1996)
M004	-deletion virus has reduced *in vivo* virulence and dissemination -deletion virus has reduced primary lesions	Barry et al. (1997)
M005	-deletion virus inhibits cell and viral protein synthesis -deletion virus has reduced *in vivo* virulence and dissemination -deletion virus fails to initiate cellular immune response in lymphoid organs	Mossman et al. (1996)
M062	-unknown -putative host range/ virulence factor	Cameron et al. (1999)
M063	-apoptosis regulator -domain homology to Fas-binding death associated protein	Cameron et al. (1999)
M064	-unknown -putative host range/ virulence factor	Cameron et al. (1999)
M128	-CD47 homolog (integrin-associated protein)	Cameron et al. (1999)
M151	-SERP-2 -binds human ICE -prevents processing of pro-IL-1β to IL-1β -inhibits granzyme B -deletion virus produces milder disease symptoms -deletion virus causes reduced secondary lesions -deletion virus lowers rabbit mortality -deletion virus elevates levels of apoptosis in lymph nodes draining inoculation site -deletion virus increases inflammatory response at the vascular level	Messud-Petit et al. (1998) Petit et al. (1996)
M152	-SERP-3 -deletion virus has reduced virulence and produces milder disease symptoms -deletion virus causes rapid inflammatory response -deletion virus does not cause significant apoptosis -deletion virus does not produce secondary lesions in lymph node draining inoculation site	Guerin et al. (2001)
M156	-eIF2α homolog -putative role in interferon resistance and RNA binding	Cameron et al. (1999)

4.2.4.2 Sequence of Selected Immunomodulatory, Anti-Apoptotic and Host-Range Genes in SLS and Ur

The strategy followed was that each of these ORFs was sequenced in the Ur strain of myxoma virus. If these Ur sequences differed from that of the published Lu strain of myxoma virus then the same ORFs in the Lu and SLS strains of myxoma virus were sequenced. Each ORF was sequenced separately from three independent PCR amplifications.

Results of sequencing the ten genes are shown in Table 4.4. The M002, M004, M062, M063, M064, M128, M151 and M156 ORFs were identical between Ur and the published Lu sequences. Differences were observed between the Ur sequence of M005 and M152 and the published Lu sequences, hence sequencing of these genes in SLS and Lu was undertaken. These results are discussed in detail in the following sections.

TABLE 4.4. SEQUENCING RESULTS

ORF in Lu Strain of Myxoma Virus	Difference between Lu and Ur	Difference between Lu and SLS	Difference between Ur and SLS
M002	NO DIFFERENCE	-	-
M004	NO DIFFERENCE	-	-
M005	$T_{33}\rightarrow C$ Insertion of C_{34} Insertion of C_{35} $G_{293}\rightarrow A$	$T_{33}\rightarrow C$ Insertion of C_{34} $G_{292}\rightarrow A$	Insertion of C_{35}
M062	NO DIFFERENCE	-	-
M063	NO DIFFERENCE	-	-
M064	NO DIFFERENCE	-	-
M128	NO DIFFERENCE	-	-
M151	NO DIFFERENCE	-	-
M152	$G_{196}\rightarrow A$ $A_{625}\rightarrow G$ $A_{684}\rightarrow G$ Deletion of T_{778}	$G_{196}\rightarrow A$ $A_{625}\rightarrow G$ $A_{684}\rightarrow G$ Deletion of T_{778}	-
M156	NO DIFFERENCE	-	-

4.2.5 Sequence Analysis of M005

4.2.5.1 Mutations in the M005 ORF

In the M005 ORF, there were three nucleotide differences in SLS compared to Lu and four nucleotide differences in Ur compared to Lu (three of which are the changes found in the SLS strain). The first difference from Lu was a change of T_{33} in Lu to C_{33} in SLS and Ur (Figure 4.7). The second difference was an insertion in both SLS and Ur with respect to Lu, of a C at position 34. Ur had an insertion of a second C at this point compared to SLS (Figure 4.7). The final mutation in the M005 ORF was downstream of these insertion(s) and was from G_{291} in Lu to A_{292} and A_{293} in SLS and Ur respectively (Figure 4.8).

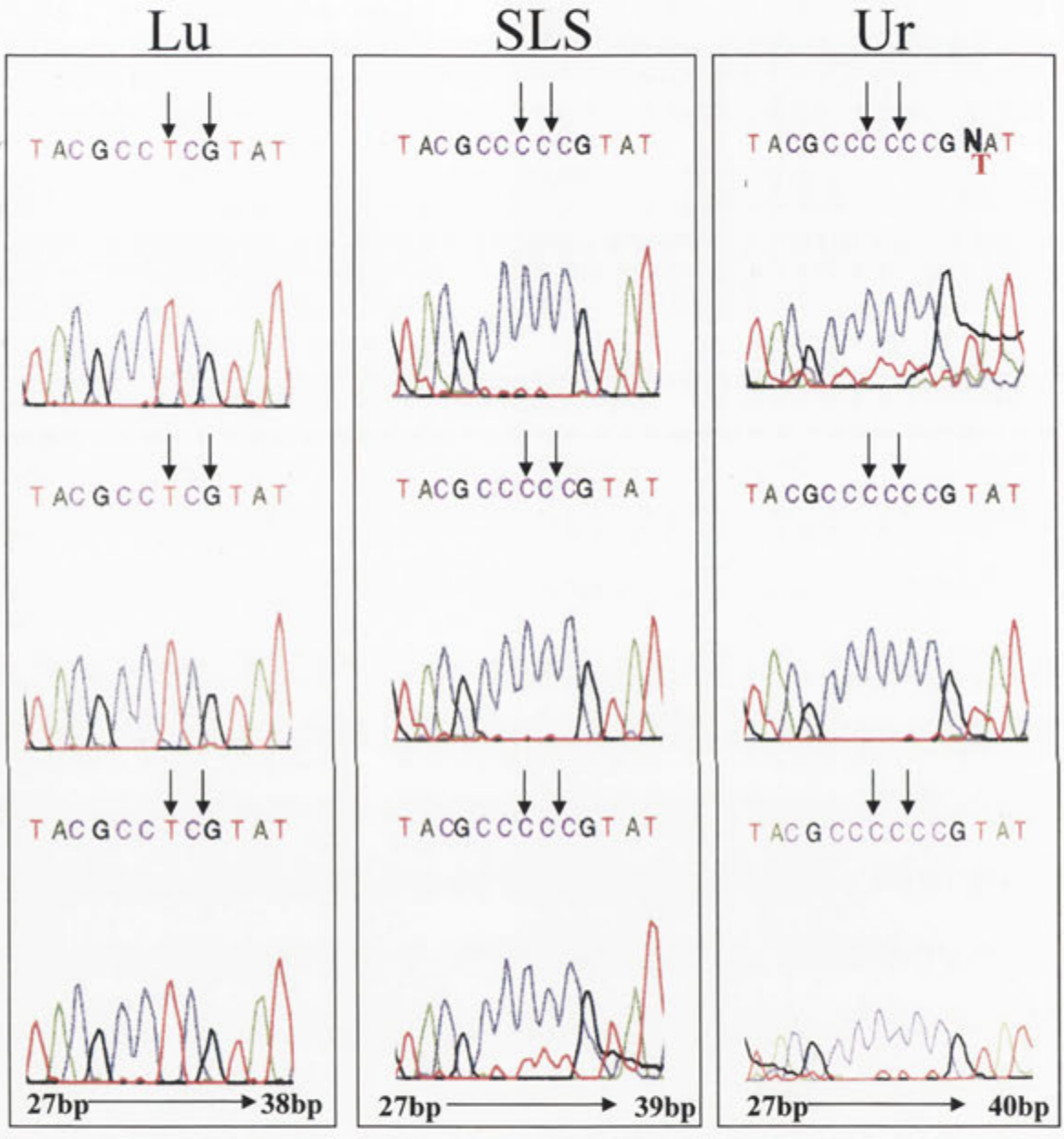


FIGURE 4.7. NUCLEOTIDE DIFFERENCES 33-35 NUCLEOTIDES FROM THE FIRST ATG IN THE M005 ORF

Three sequence traces of the 27-38bp region of M005 ORF in Lu, SLS and Ur. The first difference in sequence was 33 nucleotides from the start codon, with a T in Lu and a C in SLS and Ur. There was also an insertion of a C after nucleotide 34 in the SLS strain and an insertion of two Cs in the Ur strain, with respect to Lu. The positions of these differences are indicated by the arrows. The M005 ORF was sequenced in all three virus strains from three independent PCR amplifications as shown.

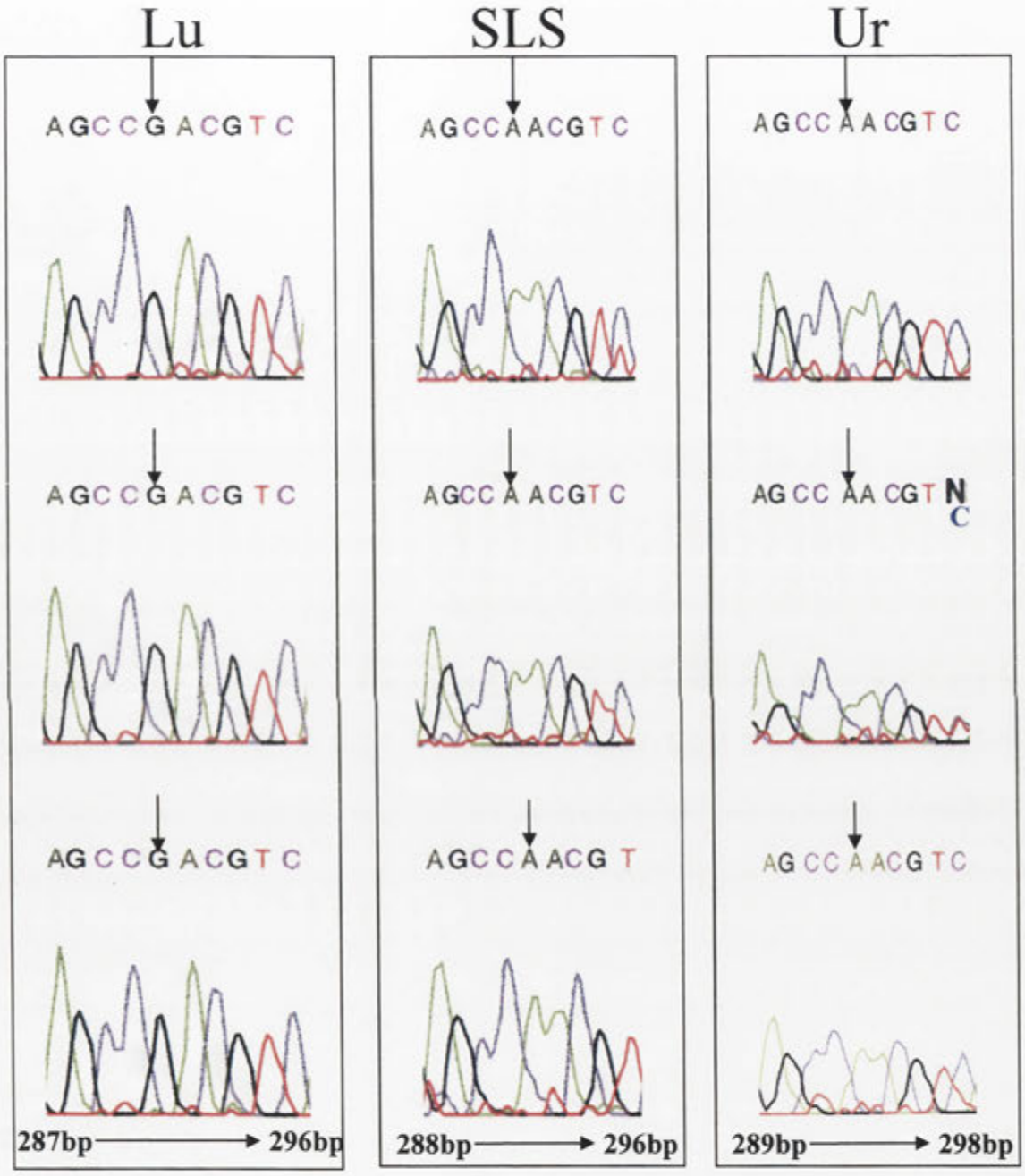


FIGURE 4.8. NUCLEOTIDE DIFFERENCES 287-298 NUCLEOTIDES FROM THE FIRST ATG IN THE M005 ORF

Three sequence traces of the 287-298bp region of M005 ORF in Lu, SLS and Ur. The difference was G_{291} in Lu and A_{292} and A_{293} in SLS and Ur respectively (indicated by an arrow). The M005 ORF was sequenced in all three virus strains from three independent PCR amplifications as shown.

4.2.5.2 Implications of Nucleotide Mutations for M005 Protein Expression

The insertion of C at position 34 in SLS compared with Lu results in a frameshift from the 11th codon, such that the protein encoded, when starting at the putative start codon in the Lu sequence, is only 78 amino acids long (red sequence in Figure 4.9A). In Ur, the protein encoded when this start site is used is 22 amino acids long before a stop codon is reached (red sequence in Figure 4.9B). Therefore, if M005 translation in SLS and Ur starts at the same start codon as in Lu, only truncated proteins will be produced from the ORF. To identify any other potential translation start codons all possible ORFs were translated for the SLS and Ur M005 sequences using MacVector. In the case of SLS, an ATG 17 nucleotides downstream from the Lu translation start site was found (green sequence Figure 4.9). Translation from this ATG in SLS would encode a protein 478 amino acids in length that is identical to the Lu M005 protein except for the first ten amino acids of Lu M005, which, in SLS have been changed to five, non-homologous amino acids. After these five amino acids, the frameshift is rectified and the amino acid sequences between Lu and SLS are identical. If this ATG were to be used as the start of translation in Ur a 73 amino acid protein would be produced with no homology to either M005 from SLS or Lu (green sequence in Figure 4.9B). A 16 amino acid protein is produced from this ATG in Lu (data not shown).

In Ur, a protein identical to the Lu M005 C-terminal 382 amino acids would be produced if the ATG 303 nucleotides from the Lu M005 ATG were to be used. In this scenario, the first 101 amino acids of the M005 protein in Ur will have been lost.

An alignment of the homologous M005 amino acid sequences in Lu, SLS and Ur is shown in Figure 4.10. For SLS and Ur, translation needs to occur at the ATG 17 and

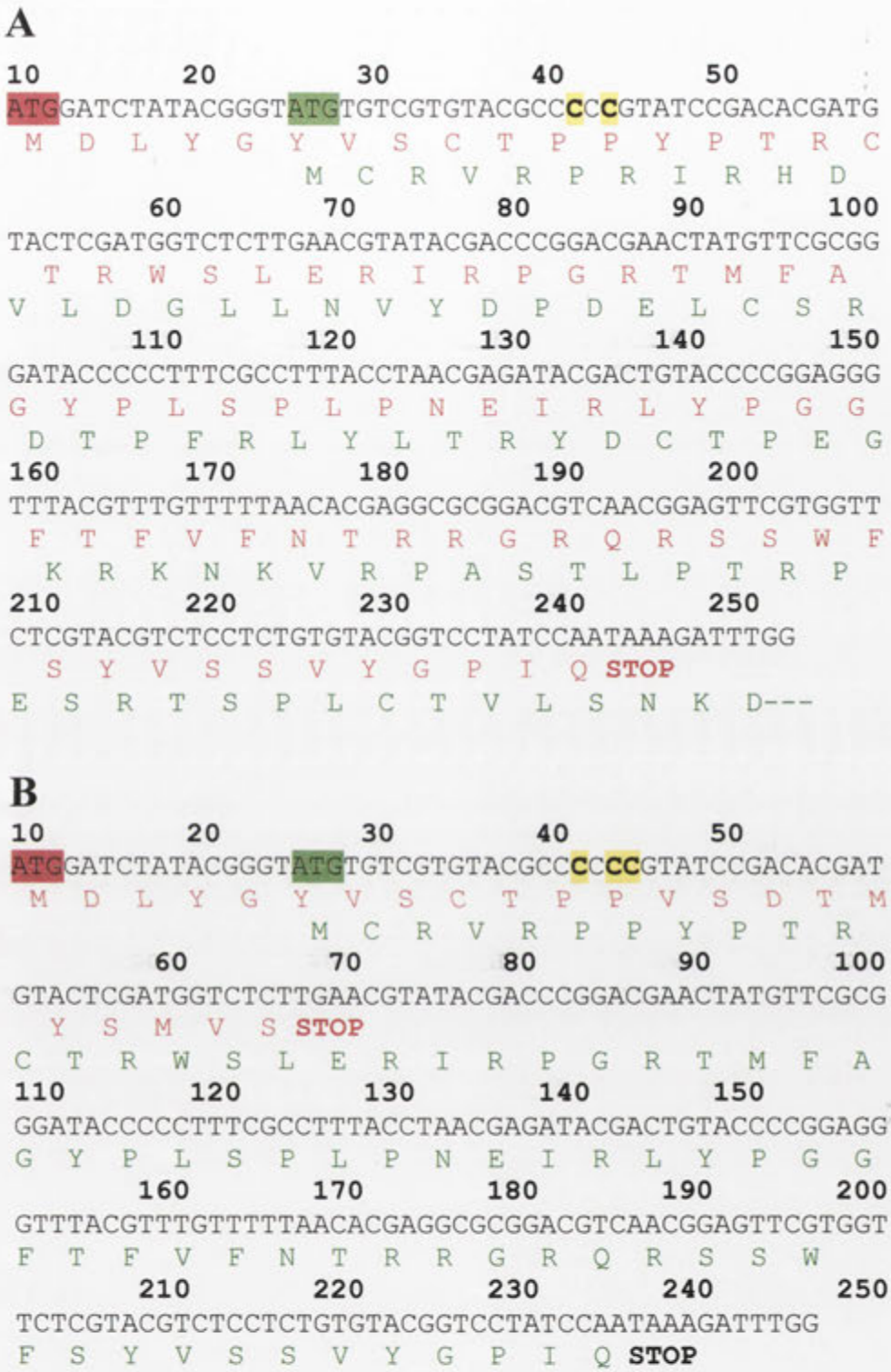


FIGURE 4.9. TRANSLATION OF THE M005 ORF FROM SLS AND UR WHEN READING FROM THE FIRST AND SECOND ATG

(A) SLS and (B) Ur nucleotide sequence and translation when the first (red) and second ATG (green) in the M005 ORF are used. Yellow shading indicates differences in nucleotide sequence between the virus strains.

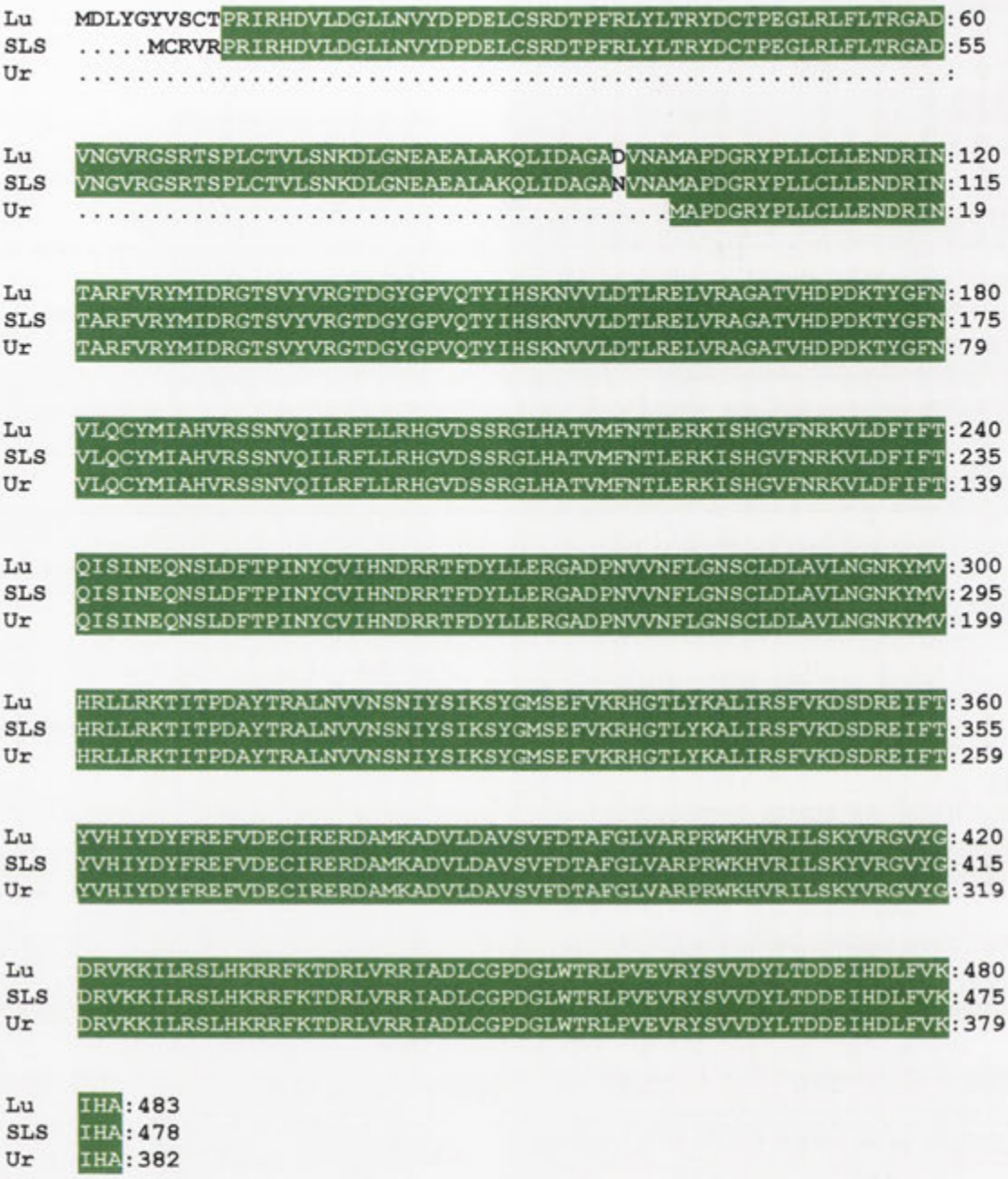


FIGURE 4.10. ALIGNMENT OF M005 ORF TRANSLATIONS FROM LU, SLS AND UR STRAINS OF MYXOMA VIRUS

The M005 ORF was sequenced from the Lu, SLS and Ur strains of myxoma virus. The nucleotide sequences were translated, aligned and shaded based on amino acid identity. The ORFs chosen were based on homology to Lu M005.

303 nucleotides respectively downstream of the Lu M005 translation start site. Initiation of translation at these sites will generate a 478 and 382 amino acid protein in SLS and Ur respectively. It is unknown whether translation occurs from either of the initiation sites other than that proposed for Lu. However, it is presumed that SLS and Ur encode at least partial M005 homologs as infection with either of these strains does not lead to the high levels of apoptosis observed early in lymphocyte infection with the M005 deletion virus (Mossman et al., 1996). The rest of this section will assume that the 478 amino acid SLS M005 protein and the 382 amino acid Ur M005 protein are produced. Other possibilities are examined in the discussion of this chapter.

The final mutation in the M005 ORF was from G_{291} in Lu to A_{292} and A_{293} in SLS and Ur respectively. Assuming that translation of the M005 protein in SLS starts at the ATG 17 nucleotides downstream from the Lu start site, the mutation at position 292 changes the 97th amino acid from aspartic acid in Lu, to asparagine in SLS. The change from aspartic acid to asparagine does not alter the predicted ankyrin repeat region. This amino acid is not present in the truncated M005 protein that is postulated to be encoded by Ur.

4.2.5.3 Analysis of the Putative Protein Products from the M005 ORF

4.2.5.3.1 Amino Acid Composition

The differences in the M005 ORF in SLS and Ur would change the expected molecular weight and isoelectric points (pI) of the putative M005 proteins, but would not affect the percent of non-polar, polar, acidic and basic amino acids. The predicted molecular weight of M005 in Lu is 55.5 kDa, and, if translation from the $A_{17}TG$ for SLS and $A_{303}TG$ for Ur occurs, is 55 kDa and 44.4 kDa respectively. The differences in amino

acid sequence would increase the estimated pI of M005 from 9.34 for Lu to 9.57 and 9.65 for SLS and Ur respectively. However, all proteins would be composed of approximately 40% non-polar amino acids, 30% polar amino acids, 12% acidic amino acids and 18% basic amino acids. The conservation of amino acid composition may indicate conservation of the function of the putative translated proteins even though the complete N-terminal amino acid sequences are not retained.

4.2.5.3.2 Protein Domains in M005

Table 4.5 lists the domains found in M005, their consensus sequence and function and their position in Lu M005. The domains were identified using the PROSITE database. PROSITE searches and identifies amino acid patterns within protein sequences. M005 is predicted to have 7 ankyrin repeats and a RNase3 domain (Cameron et al., 1999). Figure 4.11 shows the position of these domains in the M005 amino acid sequence.

Using the PROSITE database, M005 in Lu contains, in addition to the two domains listed above, one tyrosine sulfonation site, one cAMP- and cGMP-dependent protein kinase phosphorylation site, eight protein kinase C phosphorylation sites, eight casein kinase II phosphorylation sites, one tyrosine kinase phosphorylation site and six N-myristoylation sites. The domain pattern of these latter sites is commonly found in proteins, and their predicted presence may thus be spurious. For example, the protein kinase C phosphorylation site, [ST]-x-[RK], only consists of three amino acids with a S or T separated from a R or K by any other amino acid. This pattern is commonly found in proteins, so the presence of a functional site must be experimentally determined.

TABLE 4.5. PUTATIVE PROTEIN DOMAINS IN M005

Domain*	Consensus Sequence	Function	Position in Lu protein sequence
Ankyrin Repeat	~33 amino acids and form β-hairpin followed by two α-helices	· Protein-protein interactions	· 105-140 · 250-282 · 283-315 repeat region 32-315
RNase3 domain	[DEG]-[KRQT]-[LM]-E-[FYW]-[LV]-G-D-[SARH]	· Digestion of dsRNA and processing ribosomal RNA precursors	· 218-292
Tyrosine Sulfonation site	-	· Regulation of protein function	· 461-475
cAMP- and cGMP-dependent protein kinase phosphorylation site	[RK](2)-x-[ST] S or T = phosphorylation site	· Regulation of protein function	· 223-226
Protein kinase C phosphorylation site	[ST]-x-[RK] S or T = phosphorylation site	· Regulation of protein function	· 10-12 · 77-79 · 121-123 · 159-161 · 208-210 · 326-328 · 354-356 · 354-356 · 437-439
Casein kinase II phosphorylation site	[ST]-x(2)-[DE] ST = phosphorylation site	· Regulation of protein function	· 42-45 · 77-80 · 159-162 · 169-172 · 243-246 · 286-289 · 354-357 · 391-394 · 464-467 · 470-473
Tyrosine kinase phosphorylation site	[RK]-x(2)-[DE]-x(3)-**Y** Y = phosphorylation site	· Regulation of protein function	· 456-463
N-myristoylation site	G-{EDRKHPFYW}-x(2)-[STAGCN]-{P} G = N-myristoylation site	· Site for addition of C14 fatty acid to protein	· 58-63 · 63-68 · 96-101 · 205-210 · 211-216 · 274-279

* Information on protein domains, their consensus sequence and function were obtained from PROSITE database

yellow shading indicates that this site is not present in SLS or Ur

red shading indicates that this site is not present in Ur

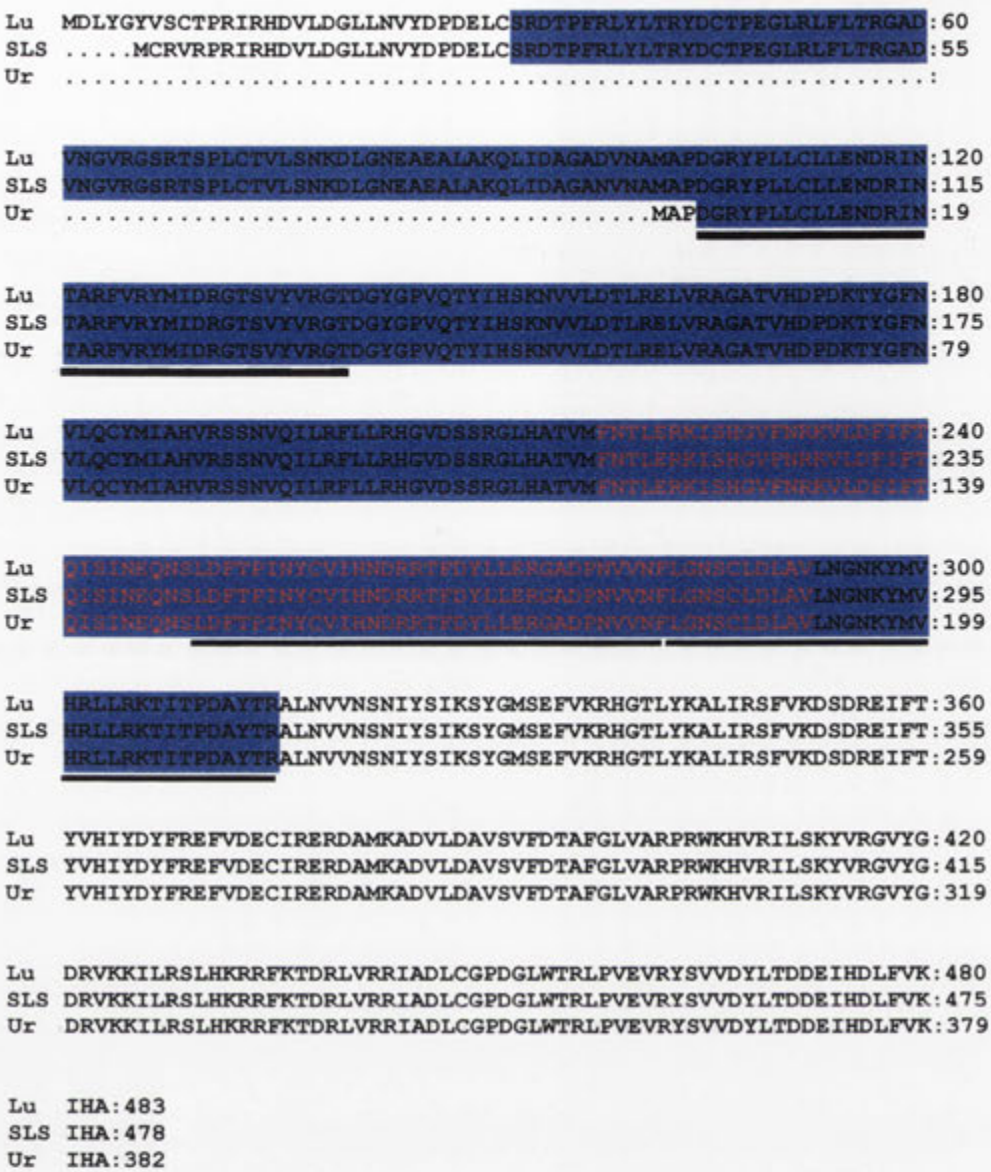


FIGURE 4.11. PUTATIVE PROTEIN DOMAINS IN M005
Putative domains of M005 in Lu, SLS and Ur were determined using the PROSITE database. PROSITE identified an ankyrin repeat region (blue shading), ankyrin repeats (black line) and RNase3 domain (red text). In Ur the ankyrin repeat domain is truncated.

Changes in the predicted SLS M005 protein deletes one of the protein kinase C phosphorylation sites, whereas the predicted Ur M005 protein has deletion of two protein kinase C phosphorylation sites, two casein kinase II phosphorylation sites and three N-myristoylation sites. In addition, the ankyrin repeat region is truncated in Ur M005 compared to Lu M005. The differences in Ur M005 are due to the deletion of the N-terminal amino acids of the putative M005 protein and not to differences in amino acid sequence.

4.2.5.3.3 Secondary Structure

Secondary structure was predicted by combining the predictions using the Chou-Fasman (CF) method and Robson-Garnier (RG) method (MacVector). The CF method assigns each amino acid into one of the following categories- helix former, helix breaker, sheet former and sheet breaker. Secondary structure is predicted based on the clustering of amino acids in each group. The RG method examines neighbors of each amino acid and, as each amino acid exerts an effect on nearby amino acids, determines the probability that that amino acid will be part of a helix, sheet, turn or coil.

When examining the predicted secondary structure, by a combination of the CF and RG methods, there are five predicted helix regions separated by sheet regions (data not shown). Ankyrin repeats are a section of approximately 33 amino acids that form a β-hairpin followed by two α-helices. Therefore, the ankyrin repeats predicted in M005 probably contribute to the secondary structure predicted.

4.2.6 Sequence analysis of M152

4.2.6.1 Mutations in the M152 ORF

Identical M152 nucleotide sequences were obtained for SLS and Ur. However, there were four nucleotide differences in SLS and Ur compared to Lu. The first nucleotide difference was G_{196} in Lu, whereas in SLS and Ur, the nucleotide at this position was an adenine (Figure 4.12). The next nucleotide difference was T_{625} in Lu, which was C_{625} in SLS and Ur (Figure 4.13). This same difference (T to C) was also observed 684 nucleotides downstream of the translation start site (Figure 4.14). The fourth difference observed between Lu and SLS and Ur was in the region $T_{778\text{-}782}$. In Lu there were five Ts in this region but only four in SLS and Ur (Figure 4.15), suggesting either an

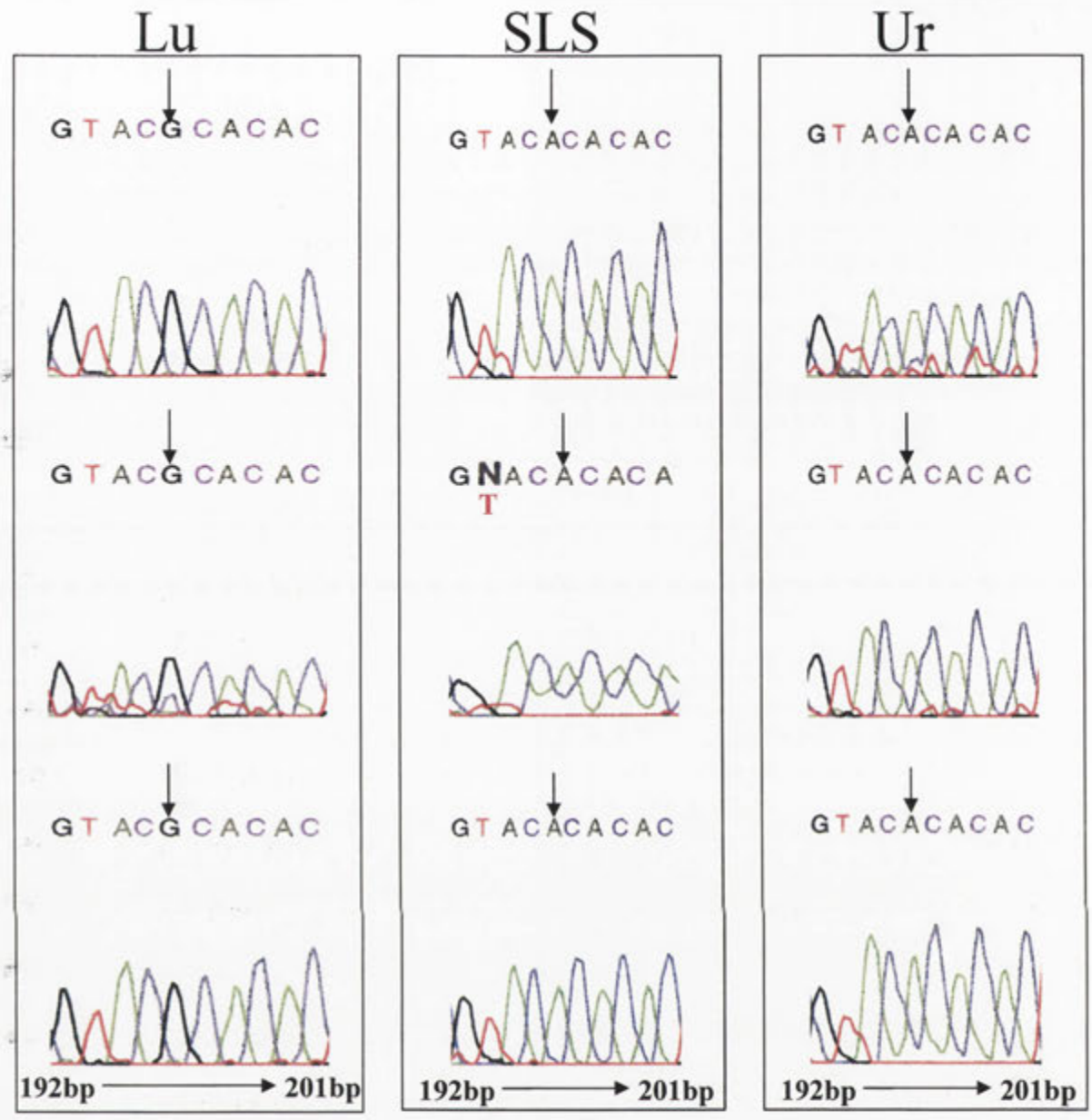


FIGURE 4.12. NUCLEOTIDE DIFFERENCES 196BP FROM THE FIRST ATG IN THE M152 ORF

Three sequence traces of the 192-201bp region of M152 ORF in Lu, SLS and Ur. The first difference in sequence between Lu and SLS and Ur was found 196 nucleotides from the start codon, with a G in Lu and an A in SLS and Ur (indicated by arrows). The M005 ORF was sequenced in all three virus strains from three independent PCR amplifications as shown.

insertion in the Lu M152 ORF or a deletion in SLS or Ur M152 ORF. The lack of this T in SLS and Ur induces a frameshift that abrogates the M152 stop codon. This results in the M152 ORF from SLS and Ur being 96 nucleotides longer than in Lu. The extension of the M152 ORF overlaps the first 63 nucleotides of the M153 ORF.

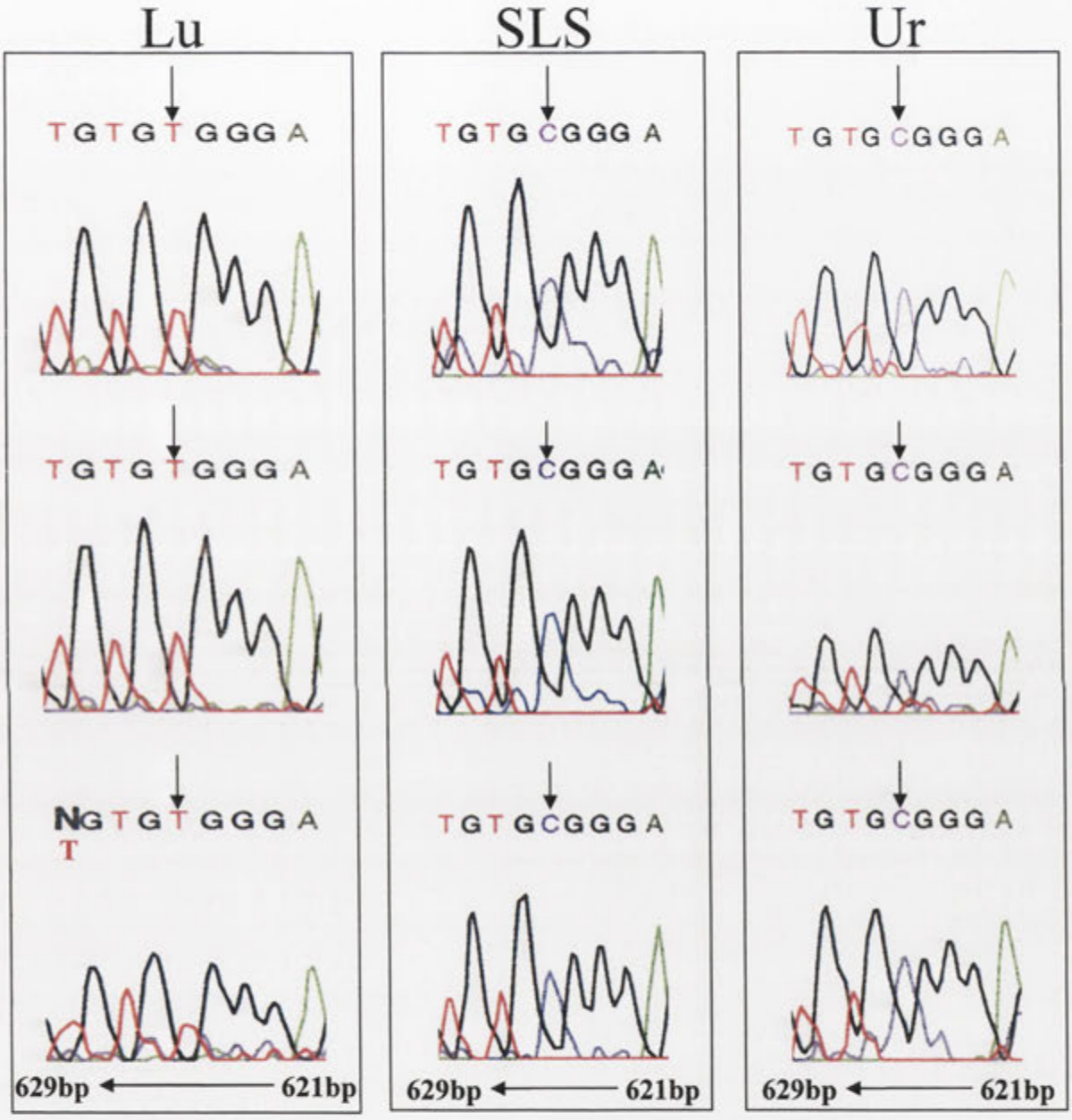


FIGURE 4.13. NUCLEOTIDE DIFFERENCES 625BP FROM THE FIRST ATG IN M152 ORF

Three sequence traces of the 621-629bp region of M152 ORF in Lu, SLS and Ur. The second difference in sequence Lu and SLS and Ur (indicated by an arrow) was 625 nucleotides from the start site. The sequence trace shows this region on the 3'→5' strand. In Lu this was a T and in SLS and Ur this was a C. The M152 ORF was sequenced in all three virus strains from three independent PCR amplifications as shown.

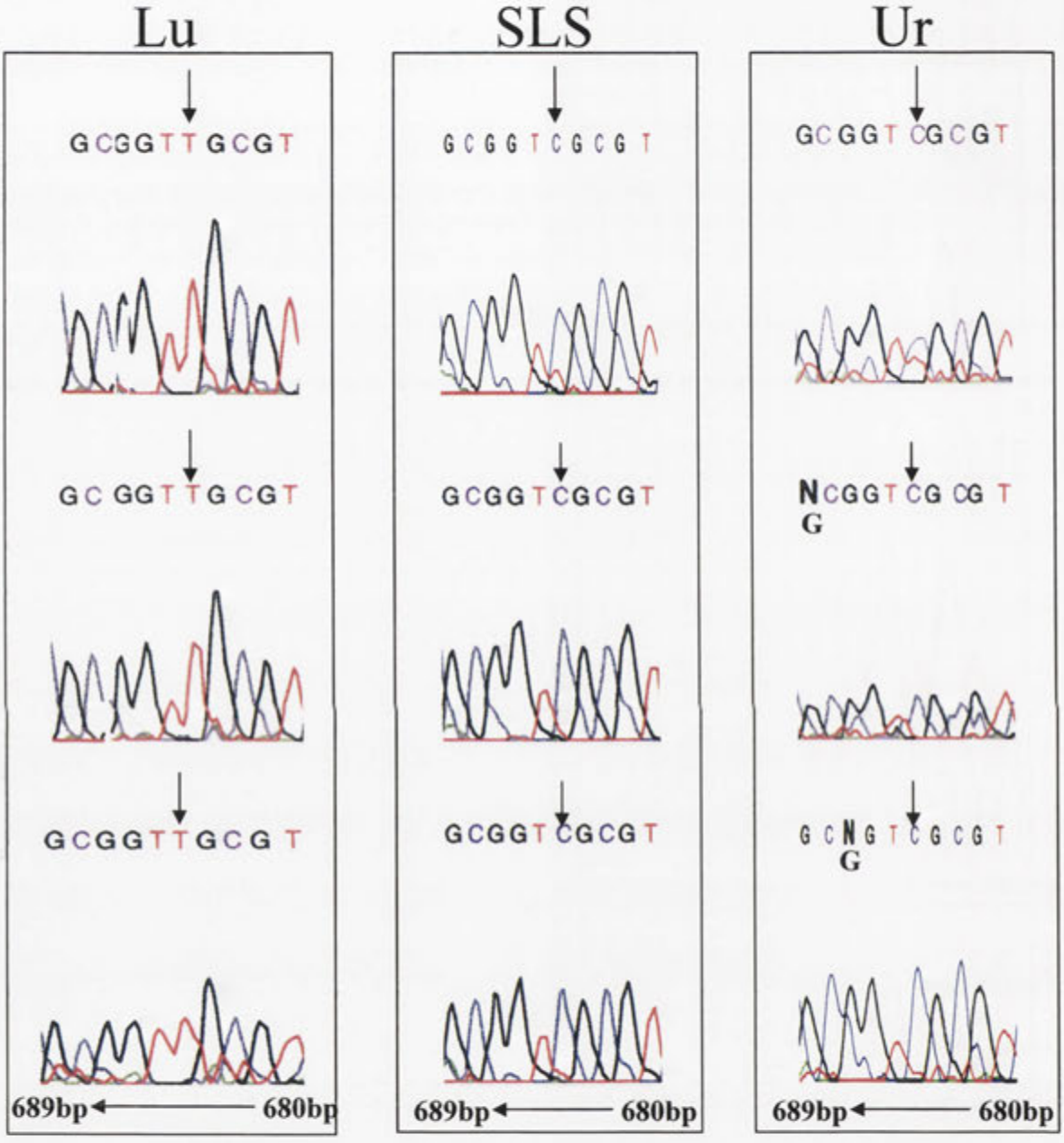


FIGURE 4.14. NUCLEOTIDE DIFFERENCES 684BP FROM THE FIRST ATG IN M152 ORF
Three sequence traces of the 680-689bp region of M152 ORF in Lu, SLS and Ur. A difference in nucleotide sequence Lu and SLS and Ur (indicated by an arrow) was found 684 nucleotides from the start codon. The sequence trace shows this region on the 3'→5' strand. In Lu this was a T and in SLS and Ur this was a C. The M152 ORF was sequenced in all three virus strains from three independent PCR amplifications as shown.

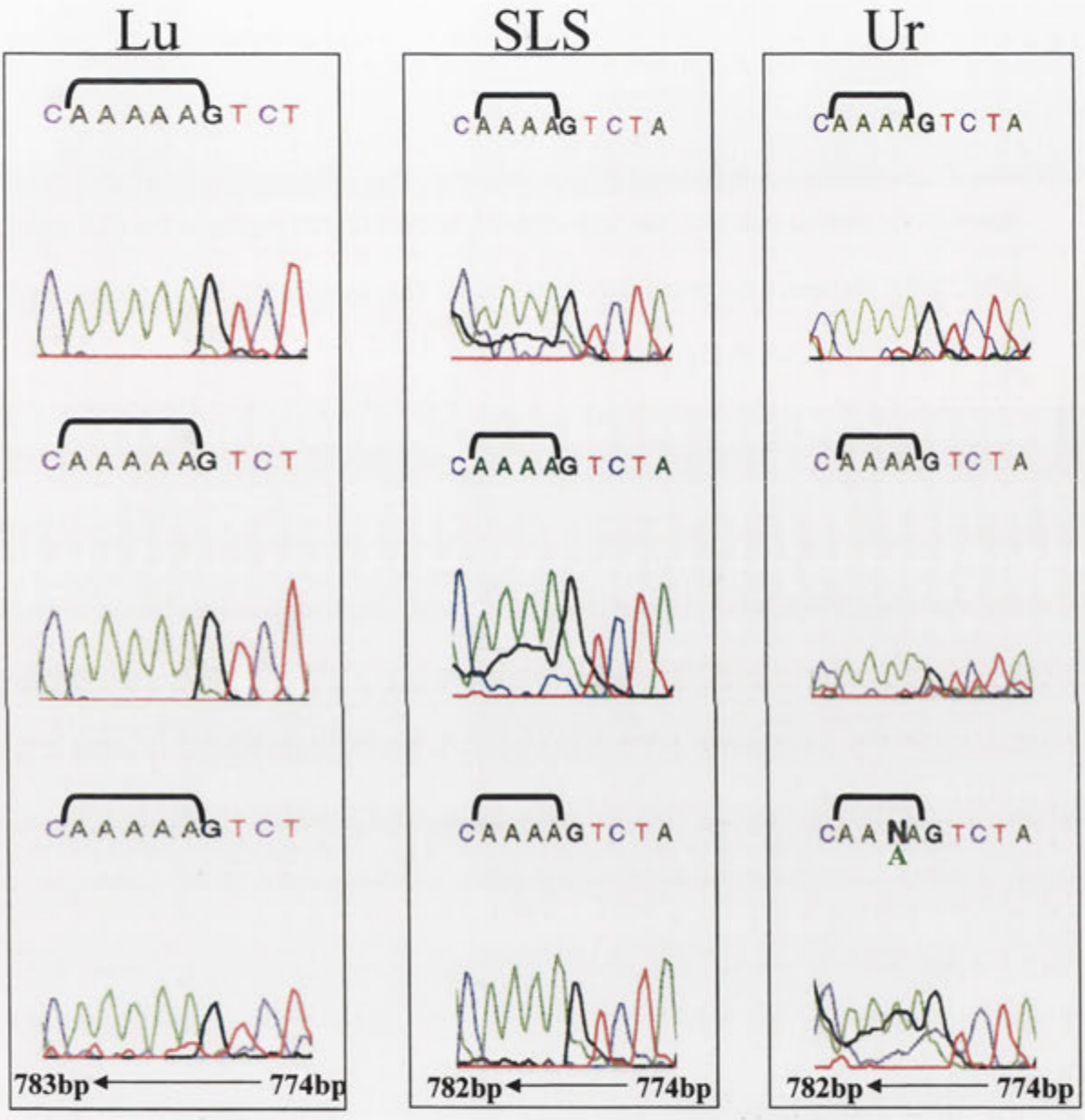


FIGURE 4.15. NUCLEOTIDE DIFFERENCES 778BP FROM THE FIRST ATG IN M152 ORF
The M152 ORF was sequenced three times in Lu, SLS and Ur. Differences were found in a region of T/A base pairs, 778-782 nucleotides from the start site in Lu. The sequence traces show this region (brackets) on the 3'→5' strand, where, in Lu, this region has five As whereas SLS and Ur has four As. The M152 ORF was sequenced in all three virus strains from three independent PCR amplifications as shown.

4.2.6.2 Implications of Different Nucleotide Sequences for M152 Protein Expression

The M152 nucleotide sequences from Lu, SLS and Ur, were translated and aligned (Figure 4.16). The nucleotide differences change the amino acids at positions 65, 209 and 261-266. In Lu these amino acids are A, T and LFMGKL respectively, whereas in SLS and Ur the amino acids at these positions are T, A and CLWGNS respectively. As stated in the previous section, the lack of the T nucleotide in the 778-782 region in the SLS and Ur M152 ORF abrogates the translation stop codon. This extends the M152 protein by 32 amino acids, as shown in Figure 4.16.

```
Lu    MDIFNHLNSINPRTRFCFSPVSVSYALSVCCTGNVPSDYVSSTVVVKNKVYINAFKQSPV:60
SLS   MDIFNHLNSINPRTRFCFSPVSVSYALSVCCTGNVPSDYVSSTVVVKNKVYINAFKQSPV:60
Ur    MDIFNHLNSINPRTRFCFSPVSVSYALSVCCTGNVPSDYVSSTVVVKNKVYINAFKQSPV:60

Lu    RGTSYAHFTNDVVHRLHKEVESYDVRFPIKSVNLDAVFLSINVTRVRLRWKIPLTEHNPY:120
SLS   RGTSYTHFTNDVVHRLHKEVESYDVRFPIKSVNLDAVFLSINVTRVRLRWKIPLTEHNPY:120
Ur    RGTSYTHFTNDVVHRLHKEVESYDVRFPIKSVNLDAVFLSINVTRVRLRWKIPLTEHNPY:120

Lu    QTAYGCSNDRLYSYNEYAFSHLKQDRVKIIELPCDDDYSVVLITHDSRSTITPDKVTGWL:180
SLS   QTAYGCSNDRLYSYNEYAFSHLKQDRVKIIELPCDDDYSVVLITHDSRSTITPDKVTGWL:180
Ur    QTAYGCSNDRLYSYNEYAFSHLKQDRVKIIELPCDDDYSVVLITHDSRSTITPDKVTGWL:180

Lu    RTTRLRYVNVSLPKGSTETGHNVTCLTPTHVNLCHRCRITITKTGVDATAFSCVDGDTCT:240
SLS   RTTRLRYVNVSLPKGSTETGHNVTCLTPAHVNLCHRCRITITKTGVDATAFSCVDGDTCT:240
Ur    RTTRLRYVNVSLPKGSTETGHNVTCLTPAHVNLCHRCRITITKTGVDATAFSCVDGDTCT:240

Lu    EHDTTASTCTIIIKTTGLDFLFMGKL.............................:266
SLS   EHDTTASTCTIIIKTTGLDFCLWGNSKKLSIKVNRLCKHGYCCKHGHCCKPGRCKPGG:298
Ur    EHDTTASTCTIIIKTTGLDFCLWGNSKKLSIKVNRLCKHGYCCKHGHCCKPGRCKPGG:298
```

FIGURE 4.16. ALIGNMENT OF M152 ORF TRANSLATIONS FROM LU, SLS AND UR

The M152 ORF was sequenced from the Lu, SLS and Ur strains of myxoma virus. The sequences were translated and aligned using Multalign, and shaded using BOXSHADE, based on amino acid identity.

4.2.6.3 Analysis of the Putative Protein Products from the M152 ORF

4.2.6.3.1 Amino Acid Composition

The differences in the nucleotide sequence of M152 between Lu, SLS and Ur, affects the expected molecular weight and pI of the M152 protein. The expected molecular weight of M152 from Lu is 30 kDa, whereas it increases to 33.5 kDa in SLS and Ur. The estimated pI for the M152 protein is 9.21 for Lu and 9.69 for SLS and Ur. The nucleotide differences do not affect the percent of non-polar, polar, acidic and basic amino acids encoded. Approximately 32% of the amino acids in M152 were non-polar compared to 42% polar, 9% acidic and 17% basic.

4.2.6.3.2 Protein Domains in M152

Table 4.6 lists each domain, its consensus sequence and function. M152 is predicted to have a zinc finger and RNA binding site (Cameron et al., 1999). In addition, PROSITE identifies the following non-specific sites in Lu M152; three N-glycosylation sites, one protein kinase C phosphorylation site, six casein kinase II phosphorylation sites, one tyrosine kinase phosphorylation site, four N-myristoylation sites, one microbodies C-terminal targeting signal and one threonine-rich region. The nucleotide difference at position 196, changing the amino acid from A in Lu to T in SLS and Ur, removes the predicted RNA binding site. The nucleotide differences at the C-terminus of Lu M152 remove the microbodies targeting signal. SLS and Ur also have a cAMP- cGMP-dependent protein kinase phosphorylation site, two extra protein kinase C phosphorylation sites, one extra N-myristoylation site and a cysteine-rich region created by the sequence changes compared to Lu. Figure 4.17 shows the main putative domains in M152.

TABLE 4.6. PUTATIVE PROTEIN DOMAINS IN M152

Domain*	Consensus Sequence	Function	Position in Lu protein sequence
N-glycosylation site	**N**-{P}-[ST]-{P} N = glycosylation site	· Site for addition of oligosaccharides	102-105 189-192 202-205
Eukaryotic RNA recognition motif	[RK]-G-{EDRKHPCG}-[AGSCI]-[FY]-[LIVA]-x-[FYLM]	· Binds ssRNA	· 61-68
Microbodies C-terminal targeting signal	[STAGCN]-[RKH]-[LIVMAFY]	· Target signal for import into microbodies, small single membrane organelles e.g. peroxisomes, glyoxysomes, glycosomes	· 264-266
Protein kinase C phosphorylation site	[**ST**]-x-[RK] S or T = phosphorylation site	· Regulation of protein function	· 182-184 · 266-268 · 270-272
Casein kinase II phosphorylation site	[**ST**]-x(2)-[DE] ST = phosphorylation site	· Regulation of protein function	· 133-136 · 224-227 · 232-235 · 238-241 · 240-243 · 256-259
Tyrosine kinase phosphorylation site	[RK]-x(2)-[DE]-x(3)-**Y** Y = phosphorylation site	· Regulation of protein function	· 75-83
N-myristoylation site	**G**-{EDRKHPFYW}-x(2)-[STAGCN]-{P} G = N-myristoylation site	· Site for addition of C14 fatty acid to protein	· 33-38 · 62-67 · 195-200 · 225-230 · 257-262
Threonine-rich region		· Regulation of protein function	· 170-256
cAMP- and cGMP-dependent protein kinase phosphorylation site	[RK](2)-x-[**ST**] S or T = phosphorylation site	· Regulation of protein function	· Not in Lu position 267-270 in SLS and Ur
Cysteine-rich region	C-x-[GNQ]-x(1,3)-G-x-C-x-C-x(2)-C-x-C	· Domain found in a variety of proteins, e.g. growth factors, cytokine receptors, apoptosis-related proteins, integrins	· Not in Lu position 277-294 in SLS and Ur

* Information on protein domains, their consensus sequence and function were obtained from PROSITE database

yellow shading indicates that this site is not present in SLS or Ur

green shading indicates that this site is not present in Lu

```
Lu  MDIFNHLNSINPRTRFCFSPVSVSYALSVCCTGNVPSDYVSSTVVVKNKVYINAFKQSPV:60
SLS MDIFNHLNSINPRTRFCFSPVSVSYALSVCCTGNVPSDYVSSTVVVKNKVYINAFKQSPV:60
Ur  MDIFNHLNSINPRTRFCFSPVSVSYALSVCCTGNVPSDYVSSTVVVKNKVYINAFKQSPV:60

Lu  RGTSYAHFTNDVVHRLHKEVESYDVRFPIKSVNLDAVFLSINVTRVRLRWKIPLTEHNPY:120
SLS RGTSYTHFTNDVVHRLHKEVESYDVRFPIKSVNLDAVFLSINVTRVRLRWKIPLTEHNPY:120
Ur  RGTSYTHFTNDVVHRLHKEVESYDVRFPIKSVNLDAVFLSINVTRVRLRWKIPLTEHNPY:120

Lu  QTAYGCSNDRLYSYNEYAFSHLKQDRVKIIELPCDDDYSVVLITHDSRSTITPDKVTGWL:180
SLS QTAYGCSNDRLYSYNEYAFSHLKQDRVKIIELPCDDDYSVVLITHDSRSTITPDKVTGWL:180
Ur  QTAYGCSNDRLYSYNEYAFSHLKQDRVKIIELPCDDDYSVVLITHDSRSTITPDKVTGWL:180

Lu  RTTRLRYVNVSLPKGSTETGHNVTCLTPTHVNLCHRCRITITKTGVDATAFSCVDGDTCT:240
SLS RTTRLRYVNVSLPKGSTETGHNVTCLTPAHVNLCHRCRITITKTGVDATAFSCVDGDTCT:240
Ur  RTTRLRYVNVSLPKGSTETGHNVTCLTPAHVNLCHRCRITITKTGVDATAFSCVDGDTCT:240

Lu  EHDTTASTCTIIIKTTGLDFLFMGKL.............................:266
SLS EHDTTASTCTIIIKTTGLDFCLWGNSKKLSIKVNRLCKHGYCCKHGHCCKPGRCKPGG:298
Ur  EHDTTASTCTIIIKTTGLDFCLWGNSKKLSIKVNRLCKHGYCCKHGHCCKPGRCKPGG:298
```

FIGURE 4.17. PUTATIVE PROTEIN DOMAINS OF M152
Putative domains of M152 in Lu, SLS and Ur were determined using PROSITE. Domains shown are eukaryotic RNA recognition motif (blue shading), microbodies C-terminal targeting signal (green line) and threonine-rich region (red text). The elongation of the M152 protein in SLS and Ur includes a cysteine-rich region (green shading). SLS and Ur do not have the eukaryotic RNA recognition motif the microbodies C-terminal targeting signal or the threonine-rich region.

4.2.6.3.3 Secondary Structure and Transmembrane Regions

Regardless of which secondary structure prediction method was used, M152 was predicted to have a predominantly β-sheet structure. Regions are similar in all virus strains. The extended C-terminus in SLS and Ur is predicted to be a turn region.

Lu M152 is predicted to be an intracellular cytoplasmic protein (Cameron et al., 1999). No transmembrane domains were predicted using "DAS"; Transmembrane Prediction server confirms this prediction (data not shown). There is no predicted transmembrane domain within the elongated C-terminal of M152 in SLS and Ur. Analysis of hydrophilicity shows the C-terminus of the M152 protein in SLS and Ur is hydrophilic.

4.2.7 Summary of Sequence Analysis

To identify if there was a genetic basis for the increase in proportion of non-viable cells in Ur-infected cell cultures detected in Section 4.2.1, ten genes with putative roles or known as having a role in apoptosis, were sequenced. M002, M004, M062, M063, M064, M128, M151 and M156 were identical between Ur and the published Lu sequence. Of the two that were not identical to the published Lu sequence, M005 and M152, only M005 was different between SLS and Ur. The nucleotide differences in M005 potentially results in translation of similar proteins in SLS and Lu, but a truncated or even absent protein in Ur. The nucleotide differences in M152 result in extension of the C-terminal end in SLS and Ur compared to Lu.

4.3 Discussion

This chapter examined the hypothesis that a difference existed between SLS and Ur in their ability to maintain lymphocyte viability after cell cycle arrest. It was found that Ur-infected cell cultures had significantly lower proportions of cells in G1/G0 compared with SLS-infected and mock-infected cell cultures at 72 h post infection. In addition SLS-infected cell cultures had significantly greater proportions of G1/G0 events than mock-infected cell cultures at this time. These results together suggest that after cell cycle arrest caused by virus infection, cell death is induced. Ur is not able to inhibit these pathways leading to cell death, whereas SLS is able to inhibit these pathways. This result clearly differentiates SLS from Ur.

Sequence analysis of ten myxoma virus genes with putative anti-apoptotic, host-range or immunomodulatory functions was undertaken in order to identify genetic differences between SLS and Ur that may affect the ability of Ur to maintain lymphocyte viability. Analysis showed the M005 ORF in the Ur strain of myxoma virus had an insertion of one cytosine at position 34 compared to the SLS strain. This either truncates the M005 protein or leads to its failure to be expressed. Failure to express M005 by myxoma virus has been previously implicated in lymphocyte apoptosis (Mossman et al., 1996) and so the difference in this ORF between SLS and Ur may contribute to the attenuation of Ur.

In addition, this chapter showed that cells in G2/M were more permissive for myxoma virus replication than cells in G1/G0. This may be due to the cell signaling pathways that are activated during cell division.

4.3.1 Cell Cycle Arrest and Induction of Apoptosis

Both SLS and Ur abrogate lymphocyte proliferation and therefore have the ability to arrest the cell cycle in lymphocytes (chapter 3). Results in chapter 3 showed the arrest was due to a blockage in transition from G1 to S stage of the cell cycle based on the failure of lymphocytes to incorporate ^{3}H-T and the inhibition of cell enlargement. The results in this chapter support this as the proportion of cells in G2/M did not increase over 72 h in virus-infected cultures, suggesting cells were not accumulating in this stage of the cell cycle. This is in contrast to MRV infection of RK13 cells where cells arrested in the G2/M stage of the cell cycle (Wali and Strayer 1996). This difference between myxoma virus and MRV will be discussed in more detail below. Even though cells in cultures infected with myxoma virus did not appear to accumulate in G2/M, a small number of events expressing high levels of viral protein and classified as G2/M were observed. The high levels of viral protein detected are presumably due to the enhanced virus replication that takes place in cells with activated cell signaling pathways, as would be the case when stimulated with mitogen.

Cell cycle progression involves numerous regulatory proteins and mechanisms that are highly conserved among eukaryotes (reviewed in Gregory 2001; Kohn 1999; Morgan 1997). Proliferation is largely controlled during the G1 stage of the cell cycle where cells must accumulate a specific level of protein synthesis to be able to transit from G1 to S stages. Cell division is inhibited if cell growth is halted, indicating there is a requirement for cell growth prior to DNA replication (Neufeld and Edgar 1998; Polymenis and Schmidt 1999). Cell growth is linked to DNA replication by coupling of the expression of cell cycle regulator(s), such as cyclins, to the rate of biosynthesis in a cell (reviewed in Neufeld and Edgar 1998). Cyclin E has been identified as the potential rate-limiting component of G1-S progression in eukaryotic cells (reviewed in Stocker

and Hafen 2000). Myxoma virus infection may inhibit the translation or increase the degradation of cell cycle regulators, such as cyclin E, in lymphocytes. This will lead to the failure of cell cycle regulators to accumulate in these cells and would arrest cells in the G1 stage of the cell cycle.

Cell cycle arrest can activate cell death pathways (Guo and Hay 1999) and proteins involved in cell cycle progression have been shown to mediate cell death (reviewed in Xaus et al., 1999). One example is p53, which can cause G1 cell cycle arrest when DNA is damaged and is involved in some apoptotic pathways (reviewed in Stewart and Pietenpol 2001). The level of p53 expression may determine the stage of the cell cycle in which arrest occurs or may determine whether cells remain viable or undergo apoptosis during virus infection. For example, one study with Epstein-Barr virus showed that low levels of p53 in infected cells led to cell cycle arrest at G2/M, medium levels of p53 led to G1 arrest but without apoptosis, and high levels of p53 during Epstein-Barr virus infection induced apoptosis (Chen et al., 1998).

p53 has also been shown to be up-regulated after infection of the RK13 rabbit kidney cell line with malignant rabbit virus (MRV). MRV abrogates proliferation of RK13 cells in the G2/M stage of the cell cycle (Wali and Strayer 1996). As discussed in the previous chapter, the close relationship between myxoma virus and MRV makes it reasonable to assume that SLS and Ur may also increase p53 expression. Results from this chapter showed that SLS and Ur abrogate proliferation in the G1/G0 stage of the cell cycle. While it is formally possible that myxoma virus may induce greater levels of p53 than MRV and thus induce G1/G0 arrest similarly to Epstein-Barr virus, it is likely that the stage of the cell cycle arrest may be a function of the cell type studied as G2/M

arrest was observed in infected RK13 cells, whereas this thesis examined rabbit lymphoid cells.

As Ur does not maintain lymphocyte viability late in infection, whereas SLS does, it may be postulated in a simple model, that both SLS and Ur infection increase p53 expression to levels that arrest the cell cycle in G1, but p53 builds up over time in Ur-infected cells such that by 72 h there is enough p53 to induce cell death. In contrast, SLS may be able to limit p53 levels or stop it from reaching its site of action, so that by 72 h post infection levels are not sufficient to induce apoptosis. This could be tested be examining p53 levels in lymphocytes infected with SLS or Ur.

4.3.2 Implication for Differences in Lymphocyte Viability *In Vivo*

The difference between SLS and Ur in maintaining lymphocyte viability and therefore inhibiting cell death pathways, clearly differentiated the virulent and attenuated strains of myxoma virus. SLS maintained the viability of lymphocytes significantly above that of mock-infected and Ur-infected cell cultures indicating that SLS can effectively inhibit cell death pathways in lymphocytes. The ability of SLS to inhibit lymphocyte death may help prolong the period SLS has available for replication *in vivo*. As lymphoid cells are predicted to be the major cell type involved in systemic dissemination of myxoma virus *in vivo* (Fenner and Woodroofe 1953; Best and Kerr 2000), maintaining the viability of these cells may enhance SLS spread. In contrast, Ur failed to inhibit cell death pathways late in infection. The inability to keep infected lymphocytes alive potentially decreases the time available for production of infectious progeny and would restrict the extent of virus dissemination *in vivo*. This will be discussed further in chapter 7.

4.3.3 Genetic Differences in the M005 ORF Between SLS and Ur

The difference in ability of SLS and Ur to maintain lymphocyte viability suggested that the genetic lesion in Ur was associated with anti-apoptotic or host-range genes. Ur was isolated in early 1953, two years after the release of SLS in the summer of 1950-1951. SLS was the only strain of myxoma virus released in Australia at this time, therefore Ur is derived genetically from SLS. The high degree of genetic conservation between SLS and Ur (Saint et al., 2001) indicates that the genetic differences between the virus strains are likely to be minor and in genes, or their regulatory sequences, that are associated with virulence in rabbits. The results in this chapter are the first demonstration of a difference associated with attenuation of Ur that is measurable *in vitro*.

The insertion of a C at position 34 in the M005 ORF and subsequent frameshift in Ur compared to SLS, may explain the different abilities of SLS and Ur to maintain lymphocyte viability. M005 is a critical virulence factor for myxoma virus and has been postulated to promote lymphocyte survival, as replication of a recombinant myxoma virus with M005 deleted was identical to wild type virus in RK13 cells but reduced in RL-5 cells and primary peripheral blood mononuclear leukocytes (Mossman et al., 1996). M005 inhibits apoptosis in infected lymphocytes by an unknown mechanism and, in the absence of M005, apoptosis occurs within 2 h of infection. In addition, host and viral protein synthesis is inhibited early after infection of RL-5 cells with the M005 deletion virus (Mossman et al., 1996).

As SLS is able to maintain lymphocyte viability, it is postulated that SLS has a complete set of functioning anti-apoptotic and host-range genes, including M005. However, the insertion of a C at position 34 in the SLS M005 ORF relative to the Lu

M005 ORF, leads to a frameshift in the ORF that will code for a truncated protein if the Lu M005 translation start codon is used. However, translation from a second AUG codon, 17 nucleotides downstream from the Lu M005 translation start codon, is predicted to produce a protein that is identical to Lu M005 except for the first five amino acids.

Leaky scanning by the eukaryote translation machinery (which consists of the 40S and 60S ribosomal subunits, tRNA molecule and initiation factors) may allow translation of SLS M005 to start from this second AUG codon. Leaky scanning involves the eukaryote translation machinery binding to the 5' mRNA cap and scanning the mRNA for an AUG translation start codon (reviewed in Pestova et al., 2001). For reasons, such as secondary RNA structures, the first AUG translation start codon may not be easily recognised and the machinery will skip to the second or third AUG translation start codon (reviewed in Pestova et al., 2001). If this second site is not used in SLS then a protein with homology to Lu M005 will not be produced. As M005 is essential for replication of myxoma virus in lymphocytes, the lack of this protein will lead to cell death. Even if only a small proportion of M005 transcripts are translated from the second AUG translation start codon, this may be enough to maintain lymphocyte viability. Because lymphocyte viability is maintained in SLS infections *in vitro* it must be assumed that either some functional M005 is being produced or that its function has been replaced by another protein.

It is interesting to note that the C insertion in SLS creates a run of five Cs at this position. This may indicate an area of increased mutation in the SLS genome as the machinery replicating the viral genome may have a tendency to stutter at this point. This

stutter may be the reason for the additional C at this site in Ur and may account for the generation of Ur in the field.

In Ur, the M005 ORF has an insertion of a C at position 34 compared to the M005 ORF in SLS, and an insertion of two Cs compared to the M005 ORF in Lu. These insertions produce a run of six Cs. The frameshift induced by these nucleotide insertions can be rectified 303 nucleotides downstream from the Lu M005 start codon. At this point there is an in-frame AUG translation start codon that will generate a protein with complete homology to the C-terminal sequence of Lu M005. It is presumed that Ur encodes at least a partial homolog of M005 as Ur infection does not lead to the high levels of apoptosis early in infection, observed in lymphocytes infected with the M005 deletion virus.

There are three potential scenarios for M005 expression in Ur infection: Firstly, that Ur expresses a truncated M005 protein that is partially functional and this serves to maintain lymphocyte viability at early times after infection but not beyond 48 h. A second possibility is that the C-terminal domain is sufficient for the anti-apoptotic action, but the protein is simply not produced in sufficient amounts from this downstream AUG to maintain cell viability at late time points after infection. Alternatively, it is also possible that the truncated protein is unstable and since M005 is produced as an early protein (Mossman et al., 1996) it does not survive in high enough concentrations to maintain cell viability beyond 48 h.

The third and, perhaps at first glance, most obvious possibility for M005 expression is that no M005 is produced during Ur infections. However, if this is assumed, then there must be some compensatory mechanism in the Ur genome that is not present in the Lu

genome, replacing M005 function sufficiently to maintain lymphocyte viability to 48 h. This logic stems from the fact that inactivation of M005 in Lu induced lymphocyte cell death within 2 h. In this scenario there is also no need for SLS to produce M005 because it would also have the compensatory mechanism as it is genetically similar. However, because SLS is able to maintain lymphocyte viability beyond 72 h we must hypothesize that Ur also has a mutation in the putative compensatory gene that leads to cell death in infected lymphocytes at late time points. At this point the argument becomes much more complex than either of the first two possibilities. Additionally, complete absence of M005 expression may have made Ur too attenuated to be transmitted in the field. The simplest conclusion is that it is more probable that the truncated M005 is produced but is not sufficient to maintain lymphocyte viability beyond 48 h.

Translation of the truncated Ur M005 may be explained by the mRNA transcripts being spliced or by the presence of internal ribosome entry sites (IRES) in the mRNA transcripts. Both these mechanisms would move ribosome binding sites spatially closer to the start codon. However, these scenarios are considered unlikely as poxvirus mRNA is precluded from the host cell splicing machinery which is housed in the nucleus (Moss 2001) and poxviruses have short 5' untranslated regions which would not permit formation of secondary RNA structures that are required for an IRES (reviewed in Martinez-Salas et al., 2001; Pestova et al., 2001). There is the possibility that translation may be able to start mid-way down the mRNA transcript due to a yet unidentified ribosome binding site, as happens in prokaryote polycistronic mRNA transcripts or in translation of *Caulimoviridae* virus gene products (Ryabova et al., 2002). In addition, leaky scanning may occur, but the probability that the eukaryote translation machinery will pick up a start codon 303 nucleotides downstream is unknown. Translation of both

SLS and Ur M005 will need to be confirmed by analysis of M005 expression in infected cells. This analysis was beyond the scope of this thesis.

The truncated form of M005 postulated for Ur will lack the N-terminal section of the ankyrin repeat region. Ankyrin domains are involved in protein-protein interactions and are generally associated with host-range. The absence or expression of a truncated M005 protein may abolish or disrupt protein-protein interactions between virus and host, leading to the inability of Ur to inhibit cell death pathways at late time points after infection. As postulated in Section 4.3.2, this may be due to a failed interaction between M005 and a host apoptotic factor, such as p53.

The M005 ORF is situated in the terminal inverted repeats (TIR) of the myxoma virus genome and so there are two copies of this gene. If disruption of the M005 ORF is the mechanism of Ur attenuation, then the mutation observed in the M005 gene must be present in both ORFs. As distinctive, clean peaks were observed in sequence traces from three independent PCR amplifications of the Ur M005 ORF, this suggests that both copies of the M005 ORF are identical. An alternative explanation to this is that mutations have occurred in one of the viral DNA binding sites used by the primer for PCR amplification of the M005 ORFs in one of the TIRs. This may result in either no PCR product being produced or PCR products of different sizes being produced. Similar to any postulated sequence changes in one copy of the M005 ORF, this is considered unlikely as, by definition, the mechanism of replication of poxvirus genomes leads to the TIRs being identical (reviewed in Moss 1996). There is also the formal possibility that the TIR boundaries are altered in Ur leading to rearrangements of the M005 genes, however, restriction mapping shows that there are no major genetic rearrangements between SLS and Ur including no change in the location of the TIR boundary (Russell

and Robbins 1989; Saint et al., 2001). Since the completion of this thesis, the sequences of M005 at both ends of the genome have been shown to be identical for Lu, SLS and Ur (Labudovic et al., unpublished data).

4.3.4 Genetic Changes in M152 (Serp-3)

The changes found in the M152 ORF were identical between SLS and Ur but different from Lu. The predicted M152 protein in SLS and Ur had two amino acid differences and a frameshift that abolishes the stop codon and results in the protein in SLS and Ur being extended by 32 amino acids.

M152 has been classified as a serpin as it contains conserved serpin motifs (Guerin et al., 2001). Serpins are involved in regulation of extracellular matrix remodeling, inflammatory responses, apoptosis, fibrinolysis, blood coagulation and complement activation (Rubin 1996; Church et al., 1997). A recombinant myxoma virus with M152 deleted has similar replicative ability both *in vivo* and *in vitro* to wild type virus (Guerin et al., 2001). In addition, deletion of M152 does not induce apoptosis *in vivo.* Hence, the changes in this ORF are not considered to be related to Ur attenuation. However, as the sequence in SLS and Ur are identical, but different from Lu, it may be postulated that M152 contributes to the different pathologies between the virus strains *in vivo.* The nucleotide changes in the SLS and Ur strains compared to the Lu strain do not affect the regions considered essential for serpin-like activity. They may, however, affect expression of M153 as the extension of the M152 ORF overlaps with the M153 ORF and, as both M152 and M153 are putatively expressed under the control of an early promotor (Cameron et al., 1999), the transcription machinery for M152 may interfere with the machinery for M153, or may interfere with M153 promotor or other regulatory elements. The function of M153 is not known (Cameron et al., 1999).

4.3.5 The Sub-population of Lymphoid Cells Permissive for Myxoma Virus Replication

This chapter showed that cells in G2/M could be considered more permissive for myxoma virus replication than cells in G1/G0, based on cells in G2/M had higher levels of late protein. Cells in G2/M would have activated signaling pathways as they are in the process of dividing. This supports the hypothesis from chapter 3 that activated T-cells were the main cell sub-population in rabbit lymphoid cell cultures that were permissive for myxoma virus infection. Further analysis of cell cycle stage with cell size showed that effectively all the cells in G2/M were large and the majority of cells in G1/G0 were large (data not shown). This indicates that cells in both G1/G0 and G2/M would have activated signaling pathways as they have responded to Con A. Cells in G2/M may be more permissive for virus replication than cells in G1/G0 because there may be more activated signaling pathways or the signaling that myxoma virus requires for replication may be increased or only active when cells are actively dividing. However, it must be remembered that infection of cells in G1/G0 would play a larger role in mxyoma virus pathogenesis as G1/G0 constitutes the major cell population in lymphoid cell cultures (~60%) compared to cells in G2/M (~5%).

Results from chapter 3 showed that myxoma virus infection abrogated replication of cellular DNA. In those experiments lymphoid cells were infected prior to mitogen stimulation. In contrast, the experiments discussed above were activated prior to infection, which allowed myxoma virus to infect lymphoid cells that were actively dividing.

4.3.6 Conclusion

The mutation in the M005 ORF of Ur is potentially the first identified naturally occurring mechanism of myxoma virus attenuation. Genetic analysis presented in this chapter showed an insertion in the Ur M005 ORF that induces a frameshift relative to both SLS and Lu M005 ORF and either truncates the M005 protein or abolishes expression of M005. M005 has been implicated as critical for lymphocyte survival as deletion of M005 leads to lymphocyte apoptosis (Mossman et al., 1996). If the genetic change in the Ur strain of myxoma virus reduces M005 translation or generates a truncated protein it may account for the lymphocyte death observed in Ur-infected cell cultures late in infection. This can be tested by insertion of an intact copy of the Lu M005 gene into Ur. Expression of a functional M005 protein by SLS, even though the M005 ORF has an insertion relative to Lu M005 ORF, may account for the maintenance of cell viability observed in SLS-infected cell cultures. The maintenance in viability of SLS-infected lymphocytes may aid *in vivo* amplification and dissemination and thus contribute to its greater virulence compared to the Ur strain of myxoma virus.

As the proportion of viable cells for each infection was similar between laboratory and wild rabbit cell cultures irrespective of mitogen stimulation, resistance to myxoma virus does not appear to be related to the ability of cells from resistant rabbits to alter kinetics of virus-induced cell death. The mechanism of resistance to myxoma virus will be investigated further in chapter 5 by examining the difference in expression of cell surface proteins involved in T-cell activation and immune responses.

Chapter 5

Expression of Cell Surface Proteins Following Infection of Primary Lymphoid Cell cultures with Myxoma Virus

5.1 INTRODUCTION

Earlier results in this thesis have shown that the activation state of lymphoid cells plays an important role in myxoma virus infection. This was because lymphocytes stimulated with Con A were significantly more permissive for myxoma virus replication than unstimulated lymphocytes. Results also showed that myxoma virus preferentially infected activated T-cells and that myxoma virus infection abrogated critical aspects of lymphocyte activation, such as cell proliferation and the increase in cell size. When considering the significance of these results in an *in vivo* context, it may be postulated that myxoma virus replication and subsequent propagation would be enhanced during an immune response as lymphocytes would be activated. However, the replication of myxoma virus will suppress the activity of infected lymphocytes leading to suppression of the anti-viral immune response, which in turn would aid survival of myxoma virus *in vivo*.

A key part of lymphocyte activation is the up-regulation of specific cell surface markers, such as MHC-I (Nussenzweig and Allison 1997). It has previously been shown that infection of the rabbit $CD4^+$ RL-5 T-cell line with the Lu strain of myxoma virus caused down-regulation of the surface expression of MHC-I and CD4 (Barry et al., 1995). In addition, infection of other cell types, such as BGMK cells, with myxoma virus also induced down-regulation of MHC-I from the cell surface (Boshkov et al., 1992). Based on these results in cell lines and on the fact that myxoma virus abrogated the activation of infected lymphocytes as shown in chapter 3 of this thesis, it may be possible that infection with myxoma virus has altered expression of some of these markers on the surface of infected primary lymphocytes. This chapter examines the expression of particular cell surface proteins in primary rabbit lymphoid cell cultures during myxoma virus infection.

The differential expression of cell surface proteins may contribute to rabbit resistance to myxoma virus. For example, resistance of rabbits to myxomatosis may be associated with lymphocytes from wild rabbits expressing higher levels of proteins critical for the anti-viral immune response or may be associated with the ability of lymphocytes from wild rabbits to prevent myxoma virus-induced down-regulation of some of these critical proteins. Higher levels of these critical cell surface proteins could enable the resistant rabbit immune response to function sufficiently to control and clear the virus. In susceptible rabbits, on the other hand, the lower levels of these critical proteins could prevent an effective or efficient immune response leading to the inability of the susceptible rabbit to control and clear the virus.

Similarly, attenuation of myxoma virus may reflect an altered ability to modify expressed levels of specific cell surface markers, For example, if an attenuated strain of myxoma virus is unable to induce down-regulation of key immunoregulatory proteins from the surface of myxoma virus-infected cells, the infected rabbit may be able to more efficiently and effectively control the virus compared to a virus strain that is able to down-regulate expression of key immunoregulatory proteins.

In this chapter, expression of CD4 and MHC-I on the surface of infected primary rabbit lymphoid cells was examined to determine whether the down-regulation observed in RL-5 and BGMK cell lines also occurred in primary rabbit lymphocytes. Unlike immortalized cell lines, primary lymphoid cells can be activated and so the expression of these cell surface proteins can be examined in both unstimulated and Con A-stimulated cell cultures. Previous chapters have shown that myxoma virus abrogated proliferation induced by Con A and so other cell surface proteins involved in T-cell activation were also investigated. CD25, the IL-2 receptor α-chain, is inducibly

expressed after T-cell activation and is essential for T-cell proliferation, and so the expression of CD25 was examined to determine whether virus inhibition of lymphocyte proliferation involved altered levels of this cell surface protein. The anti-rabbit MHC-II antibody, which recognizes activated T-cells in addition to B-cells (reviewed in Mage 1998), and the KEN-5 antibody, which recognizes an unidentified rabbit T-cell specific marker (called KEN-5 in this chapter and reviewed in Mage 1998), was used to examine the effects of myxoma virus on rabbit lymphocytes. The expression of CD43 and CD45 on primary lymphoid cells were used as control markers as it has been shown that expression of these proteins on the surface of RL-5 cells is not altered by myxoma virus infection (Barry et al., 1995).

Specifically, this chapter investigates the following questions:

1) Do differences exist between unstimulated and Con A-stimulated lymphocytes from susceptible and resistant rabbits in the cell surface expression of CD25, CD4, CD43, CD45, KEN-5, MHC-I or MHC-II?
2) Does infection with the virulent SLS or attenuated Ur strains of myxoma virus alter the expression of CD25, CD4, CD43, CD45, KEN-5, MHC-I or MHC-II on the surface of unstimulated or Con A-stimulated lymphocytes from susceptible and resistant rabbits?

To address these questions, lymphoid cell cultures from laboratory and wild rabbits were mock-infected or infected with SLS or Ur and were examined for expression of CD25, CD4, CD43, CD45, KEN-5, MHC-I and MHC-II using flow cytometry. The choice of proteins examined was restricted by the limited range of antibodies specific for rabbit cell surface markers. Cultures were incubated in the presence or absence of

Con A. For comparison, expression of these proteins on the surface of RL-5 cells infected with SLS or Ur was also examined.

5.2 RESULTS

5.2.1 Characteristics of the Cell Surface Markers Examined

Table 5.1 lists the cell surface proteins examined in this chapter, the cell type that expresses them, and their function and natural ligand.

TABLE 5.1. CELL SURFACE PROTEINS

Cell Surface Protein	Common Name(s)	Cell Type in rabbits	Protein Function	Natural Ligand
CD25	-IL-2 receptor α-chain	-activated T-cells -pre-B-cells	-involved in T-cell growth and proliferation	IL-2
CD4	-	-subset of T-cells ($CD4^+$)	-co-stimulatory protein involved in antigen recognition and presentation	MHC-II
CD43	-Leukosialin -gp115	-T-cells -thymocytes -progenitor B-cells	-hematopoietic progenitor cell proliferation	ICAM-I (CD54)
CD45	-Leukocyte common antigen	-leukocytes	-protein phosphatase -accessory molecule involved in cell signaling in T- and B-cells	CD22
KEN-5	-	-T-cells	-unknown	unknown
MHC-I	-	-all nucleated cells	-presentation of antigen derived from intracellular environment	TCR complex specific for antigen in association with CD8
MHC-II	-	-B-cells -macrophages -monocytes -subset of activated T-cells	-presentation of antigen derived from extracellular environment	TCR complex specific for antigen in association with CD4

Information obtained from Kuby (1997).

5.2.2 Method used to Examine Cell Surface Marker Expression

In this chapter, expression of cell surface proteins is measured using flow cytometry and antibodies specific for each cell surface protein. The results are presented in three ways, each giving different information about the data. The first is density plots of cell size

versus protein expression. Plots were divided into 'low' (fluorescence intensity less than 10^2 units) and 'high' (fluorescence intensity greater than 10^2 units) antibody binding and the mean proportion of cells in each sub-population from cultures from three laboratory and three wild rabbits was determined using CellQuest software. These plots are used to show the proportions of cells expressing high and low levels of protein and to show the relationship between cell size and the specific protein. This latter relationship will be examined in Section 5.2.4. In the second method, results are presented as histogram overlays of plots of number of cells versus protein expression. This enables direct comparison between infections with SLS and Ur.

The third way cell surface proteins are examined is by calculation of the mean median fluorescence intensity. This was calculated using CellQuest software from three replicate experiments. As previously stated, the mean median fluorescence intensity is a quantitative measure of protein expression on individual cells and is not likely to be skewed by very high or low antibody binding in a minor cell sub-population. Mean median fluorescence intensity is presented in column graphs. The first two ways of data presentation show results for individual rabbits whereas the third way shows mean results from lymphoid cell cultures from three laboratory and three wild rabbits. Statistical analysis was only undertaken on the mean median fluorescence intensity data because this data was considered the most accurate.

Preliminary experiments were undertaken to optimize antibody binding to CD43 and MHC-I, as representative cell surface proteins. Expression of these markers was examined in mock-infected lymphoid cell cultures after 24 and 48 h of Con A stimulation. Figure 5.1 shows CD43 and MHC-I antibody binding as histogram plots and calculated median fluorescence intensity for each plot. The greater level of antibody

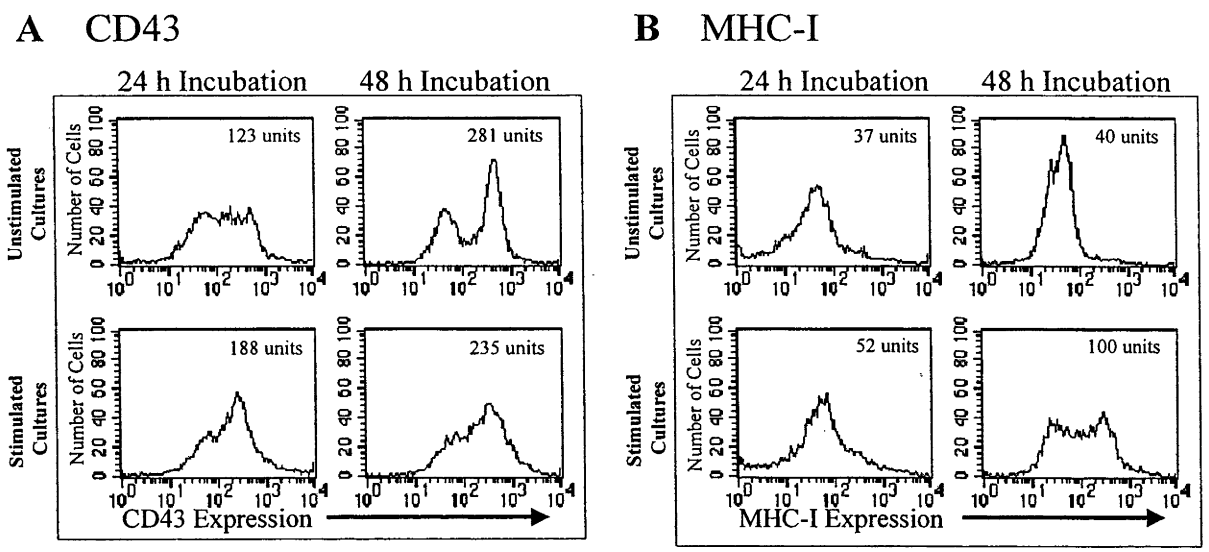


FIGURE 5.1. EXPRESSION OF CD43 AND MHC-I ON RABBIT LYMPHOCYTES
Preliminary experiments to determine the incubation time for analysis of antibody binding to cell surface proteins was undertaken. Lymphoid cell cultures from wild rabbits were incubated for 24 or 48 h with and without Con A stimulation. The cell surface expression of (A) CD43 and (B) MHC-I was analysed using flow cytometry. Histogram plots of lymphoid cells from one rabbit are shown, but are typical of those obtained. Numbers within the histogram plot indicate units of median fluorescence intensity for each sample.

binding for both markers was observed after 48 h, hence all subsequent experiments of primary lymphoid cells were analysed after 48 h incubation. Due to innate differences in the level of the cell surface expression of particular proteins and the differences in affinity of the monoclonal antibodies for their cognate antigen, different flow cytometer thresholds were used for each antibody to obtain a clear peak. This means that the absolute values of mean median fluorescence intensity cannot be compared between different cell surface markers.

5.2.3 Cell Surface Protein Expression in Unstimulated and Con A-Stimulated Mock-Infected Rabbit Lymphoid Cell Cultures

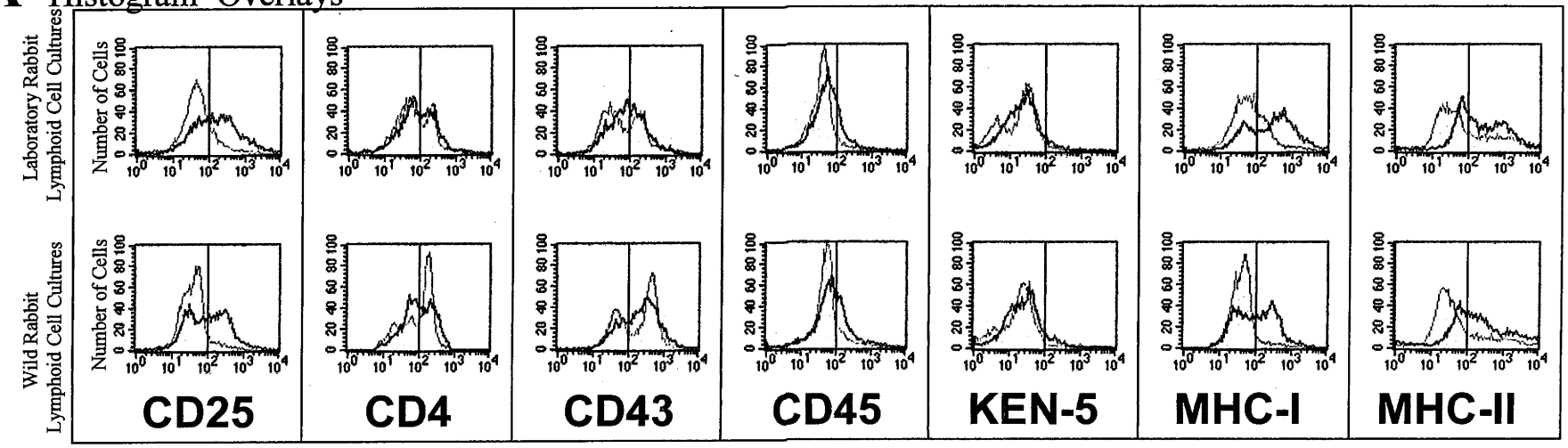
The expression of CD25, CD4, CD43, CD45, KEN-5, MHC-I and MHC-II on the uninfected rabbit lymphocyte cell surface was first examined using mock-infected rabbit lymphoid cells. This was done using histogram overlays of antibody binding for

unstimulated and Con A-stimulated cells and by calculating the median fluorescence intensity using CellQuest software. Each experiment was repeated three times and the mean median fluorescence intensity of the three replicates was calculated. Statistical analysis of cell surface marker expression was undertaken by ANOVA, with a p value <0.05 deemed significant.

Figure 5.2A shows histogram overlays of each cell surface marker in both unstimulated and stimulated lymphoid cell cultures. An increase in expression of the marker is seen as a shift to the right of the curve. These results were typical of those seen. Figure 5.2B shows the calculated mean median fluorescence intensity for the three replicates and statistical significance of the increased expression was calculated using ANOVA. Con A stimulation significantly increased the expression of CD25, MHC-I and MHC-II in mock-infected cell cultures (ANOVA, $p<0.05$). For example, the mean median fluorescence intensity for CD25 in lymphoid cell cultures from laboratory rabbits increased from 29 units to 133 units with Con A stimulation (Figure 5.2B).

The increase in CD25 and MHC-II expression observed when cell cultures were stimulated was expected as CD25 is the IL-2 receptor α-chain and is inducibly expressed on T-cell activation and activated rabbit T-cells have been shown to express MHC-II (reviewed in Mage 1998). The increase in MHC-I expression has not been reported before. It is interesting to note that the mean median fluorescence intensity of MHC-I in stimulated lymphoid cell cultures from wild rabbits, although not statistically significant at the 95% CI, tended to be higher than that in cell cultures from laboratory rabbits. This may reflect an important difference in the immune responses of these rabbits and will be explored in the discussion to this chapter.

A Histogram Overlays



B Mean Median Fluorescence Intensity

	CD25		CD4		CD43		CD45		KEN-5		MHC-I		MHC-II	
	Unstimulated Cultures	Stimulated Cultures	Unstimulated Cultures	Stimulated Cultures	Unstimulated Cultures	Stimulated Cultures	Unstimulated Cultures	Stimulated Cultures	Unstimulated Cultures	Stimulated Cultures	Unstimulated Cultures	Stimulated Cultures	Unstimulated Cultures	Stimulated Cultures
Laboratory Rabbit Lymphoid Cell Cultures	29 ±4.2	133* ±10.8	87 ±17.7	113 ±27.2	103▲ ±36.4	92 ±23.3	45 ±8.8	56 ±9.1	38 ±9.1	38 ±9.7	155 ±72.5	369* ±48.9	51 ±12.3	109 ±12.6
Wild Rabbit Lymphoid Cell Cultures	29 ±7.8	168* ±26.0	133 ±12.7	111 ±16.7	295 ±31.2	190 ±28.2	46 ±5.0	54 ±10.0	35 ±7.3	30 ±5.8	114 ±39.1	614 ±366.9	27 ±2.1	100* ±15.5

FIGURE 5.2. THE EFFECTS OF CON A STIMULATION ON CELL SURFACE EXPRESSION OF VARIOUS PROTEINS IN MOCK-INFECTED LYMPHOID CELL CULTURES FROM LABORATORY AND WILD RABBITS

(A) Expression of CD25, CD4, CD43, CD45, KEN-5, MHC-I and MHC-II on the surface of lymphocytes in mock-infected lymphoid cell cultures from laboratory and wild rabbits was analysed, with (line) and without (shaded region) Con A stimulation. Antibody binding was analysed using flow cytometry and the above histogram overlays generated using CellQuest software. A line has been drawn at a fluorescence intensity of 10^2 units as a visual aid. Each experiment was repeated three times using lymphoid cell cultures prepared from three laboratory and three wild rabbits (typical results from one rabbit are shown). (B) The median fluorescence intensity corresponding to binding of each antibody was calculated using CellQuest software. The numbers shown are the mean of the three replicates. Values were compared between stimulated and unstimulated cultures using ANOVA; * indicates $p<0.05$. Values were compared between laboratory and wild rabbit cultures using ANOVA; ▲ indicates $p<0.05$.

There was a biphasic distribution of CD4 and CD43 expression on cells in unstimulated mock-infected rabbit lymphoid cell cultures (shaded regions Figure 5.2A). CD4 expression tended to be higher in unstimulated lymphoid cell cultures from wild rabbits than unstimulated cultures from laboratory rabbits with a mean median fluorescence intensity of 133 and 87 units respectively, but this was not statistically significant at the 95% CI (Figure 5.2B). However, the differences in CD4 expression may have some biological significance (see the Discussion to this chapter). The greater levels of CD4 expression on the surface of wild rabbit lymphocytes may improve MHC-II engagement and signaling through the T-cell receptor after encountering antigen *in vivo.* The difference in CD4 expression between lymphoid cell cultures from laboratory and wild rabbits was not evident after Con A stimulation with a mean median fluorescence intensity of 113 and 111 respectively (Figure 5.2B).

CD43 expression was significantly higher overall in unstimulated lymphoid cell cultures from wild rabbits compared to laboratory rabbits, with mean median fluorescence intensities of 295 and 103 units respectively ($p<0.05$) (Figure 5.2B). The greater levels of CD43 may enhance T-cell activation *in vivo* in wild rabbits by stabilizing the interaction between T-cells and antigen presenting cells (reviewed in Ostberg et al., 1998). CD43 expression fell after stimulation in mock-infected lymphoid cell cultures from both laboratory and wild rabbits (Figure 5.2B). The decrease in CD43 expression was almost 10 fold greater in cell cultures from wild rabbits than those from laboratory rabbits, but was not statistically significant at a 95% CI.

CD45 and KEN-5 expression were not significantly altered by Con A stimulation with the mean median fluorescence intensity remaining between 30-38 units for the KEN-5 antibody and between 45-56 units for the CD45 antibody. No difference was observed

in the levels of CD45 or KEN-5 between lymphoid cell cultures from laboratory and wild rabbits.

5.2.3.1 Summary of Cell Surface Protein Expression in Mock-Infected Lymphoid Cell Cultures

Con A stimulation significantly increased the expression of CD25, MHC-I and MHC-II in mock-infected cell cultures, but did not alter the expression of KEN-5 or CD45. Lymphoid cells from wild rabbits had significantly greater expression of CD43 on the cell surface compared to lymphocytes from laboratory rabbits in the absence of Con A. Lymphoid cells from wild rabbits also had greater CD4 expression, although the difference was not statistically significant. When cultures were stimulated, there were greater levels of MHC-I and CD43 on the surface of lymphoid cells from wild rabbits compared to laboratory rabbits.

5.2.4 Expression of Cell Surface Proteins in Unstimulated and Con A-Stimulated Rabbit Lymphoid Cell Cultures Infected with Myxoma Virus

To determine the effect of myxoma virus infection on the expression of proteins on the surface of lymphocytes, cell cultures were infected with SLS or Ur at a moi of 0.1 and 3 and the expression of CD25, CD4, CD43, CD45, KEN-5, MHC-I and MHC-II was examined. Cell cultures were incubated in the presence or absence of Con A and were analysed 48 h post infection. The experiments with mock-infected cell cultures described in Section 5.2.2 were undertaken in parallel with myxoma virus infected-cell cultures, and will be compared in this section to virus-infected cell cultures. In some cases, a different individual rabbit is shown in the histogram overlays to the one shown

in Figure 5.2. However, the proportions of the cells expressing high and low levels of the cell surface markers shown represent the mean value calculated from cultures from all three laboratory or three wild rabbits. For some proteins, changes in the level of expression of some proteins are not statistically significant. However, they are discussed as it was considered that the changes, although small, may have some biological significance.

5.2.4.1 Expression of CD25

Figure 5.3 shows CD25 expression in unstimulated and Con A-stimulated lymphoid cell cultures from laboratory and wild rabbits that were either mock-infected or infected with SLS or Ur. The previous section showed Con A stimulation significantly increased CD25 expression on rabbit lymphoid cells ($p<0.05$). This can also be seen in the density plots (Figure 5.3A), where only 8% of cells in mock-infected cell cultures from laboratory rabbits expressed high levels of CD25 in the absence of stimulation, but 55% of cells expressed high levels of CD25 after stimulation. These density plots also show that cells expressing high levels of CD25 are those with a large cell size. The differential expression of cell surface proteins on large and small cells will be examined in detail in Section 5.2.4.

In infected cell cultures from laboratory rabbits, 15% of cells in cultures infected with SLS at a moi of 3, and 18% infected with Ur at a moi of 3, had high CD25 antibody binding after stimulation with Con A. The proportion of cells with high CD25 expression was significantly less in stimulated virus-infected cultures compared to stimulated mock-infected cultures ($p<0.05$). The inhibition of the Con A-induced increase in CD25 expression by both SLS and Ur is also shown in the histogram plots (Figure 5.3B) and the graph of mean median fluorescence intensity (Figure 5.3C).

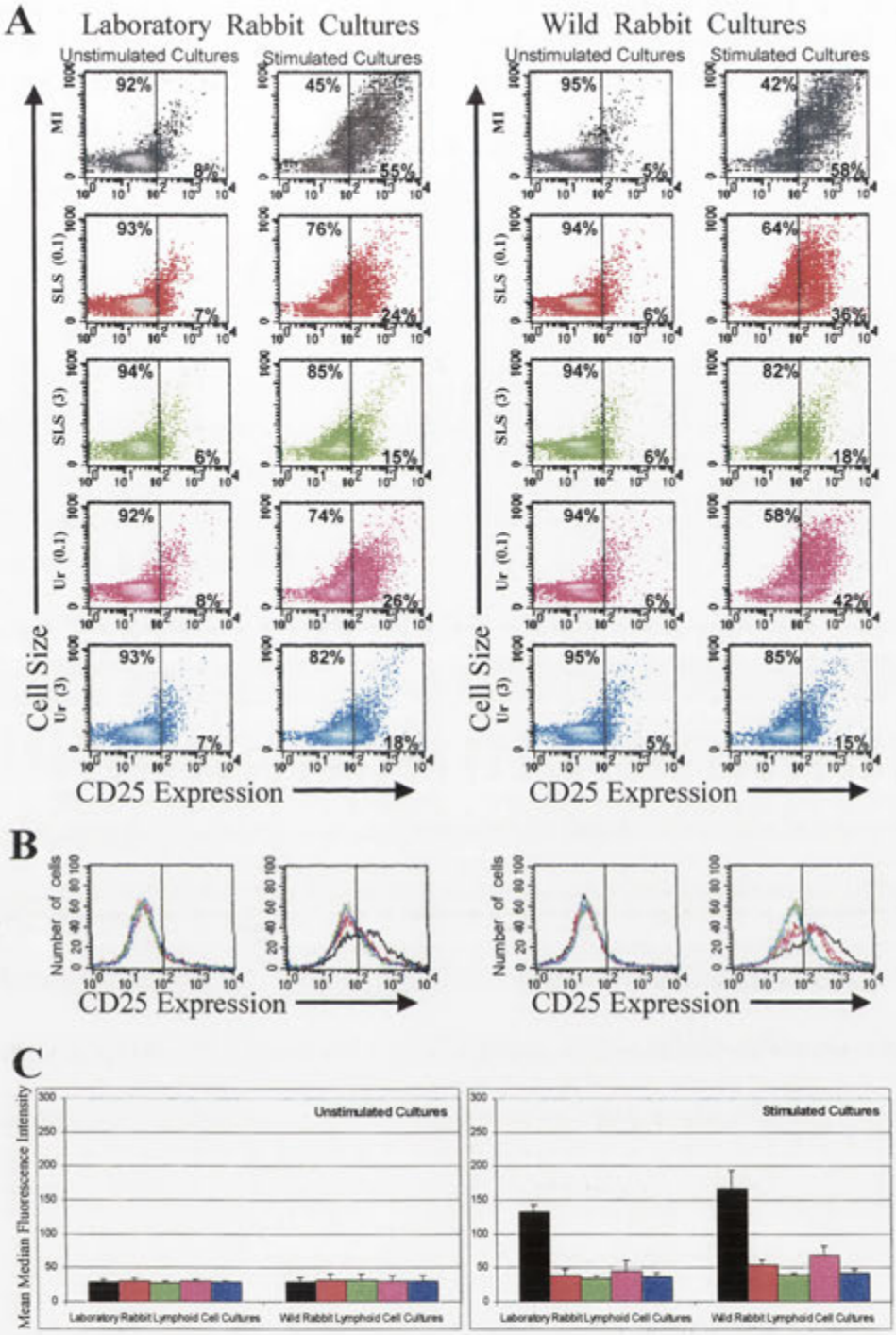


FIGURE 5.3. EXPRESSION OF CD25 IN RABBIT LYMPHOID CELL CULTURES INFECTED WITH SLS OR UR, WITH AND WITHOUT CON A STIMULATION

Expression of CD25 was analysed in lymphoid cell cultures from laboratory and wild rabbits and is presented as (A) density plots of CD25 antibody binding versus cell size and (B) histogram overlays of CD25 antibody binding. Cultures were mock-infected (MI)■ or infected with SLS, at a moi of 0.1■ or 3■, or infected with Ur, at a moi of 0.1■ or 3■. Infections were undertaken in parallel with and without Con A stimulation. The experiment was repeated three times with only one typical replicate shown here. Plots were divided such that cells with a fluorescence intensity greater than 10^2 units were classified as having high CD25 expression and cells with a fluorescence intensity less than 10^2 units were classified as having low CD25 expression. Numbers in the density plots represent the mean percentage of cells in each sub-population from three experiments. (C) The mean median fluorescence intensity corresponding to CD25 expression in lymphoid cultures from three laboratory and three wild rabbits was calculated and plotted on a column graph. Error bars represent one standard error.

A similar significant inhibition of the CD25 increase was observed in lymphoid cell cultures from wild rabbits ($p<0.05$). Neither strain of myxoma virus significantly altered CD25 expression in unstimulated rabbit lymphoid cell cultures.

The inhibition of the Con A-induced increase in CD25 expression by myxoma virus was moi-dependent, as 24% and 26% of lymphoid cell cultures from laboratory rabbits bound high levels of CD25 antibody after Con A stimulation when infected with SLS or Ur respectively at a moi of 0.1. This was also significantly lower than the 55% of cells in mock-infected lymphoid cell cultures from laboratory rabbits that expressed high levels of CD25 ($p<0.05$). The difference between moi can also be seen in the histogram plots of lymphoid cell cultures from wild rabbits, where the curves for the low moi are between those of mock-infected cell cultures and cell cultures infected with a moi of 3 (Figure 5.3B) and in mean median fluorescence intensity graphs (Figure 5.3C). The observation that CD25 expression was inhibited by myxoma virus in a moi-dependent manner when cell cultures were stimulated directly contrasts with the inhibition of cell proliferation observed in chapter 3, which did not appear to be moi-dependent.

5.2.4.2 Expression of CD4

Neither strain of myxoma virus significantly altered CD4 expression in unstimulated cell cultures ($p<0.05$), with the proportion of cells with high CD4 expression between 41% and 46% for a moi of 0.1 and 3 in lymphoid cell cultures from laboratory rabbits irrespective of virus strain and between 47% and 52% in lymphoid cell cultures from wild rabbits (Figure 5.4). However, when cell cultures were stimulated with Con A, cell cultures that were infected with either SLS or Ur at a moi of 3 had a lower proportion of cells binding high levels of CD4 antibody than those in mock-infected cell cultures. For example, the proportion of cells binding high levels of CD4 antibody was 53% in

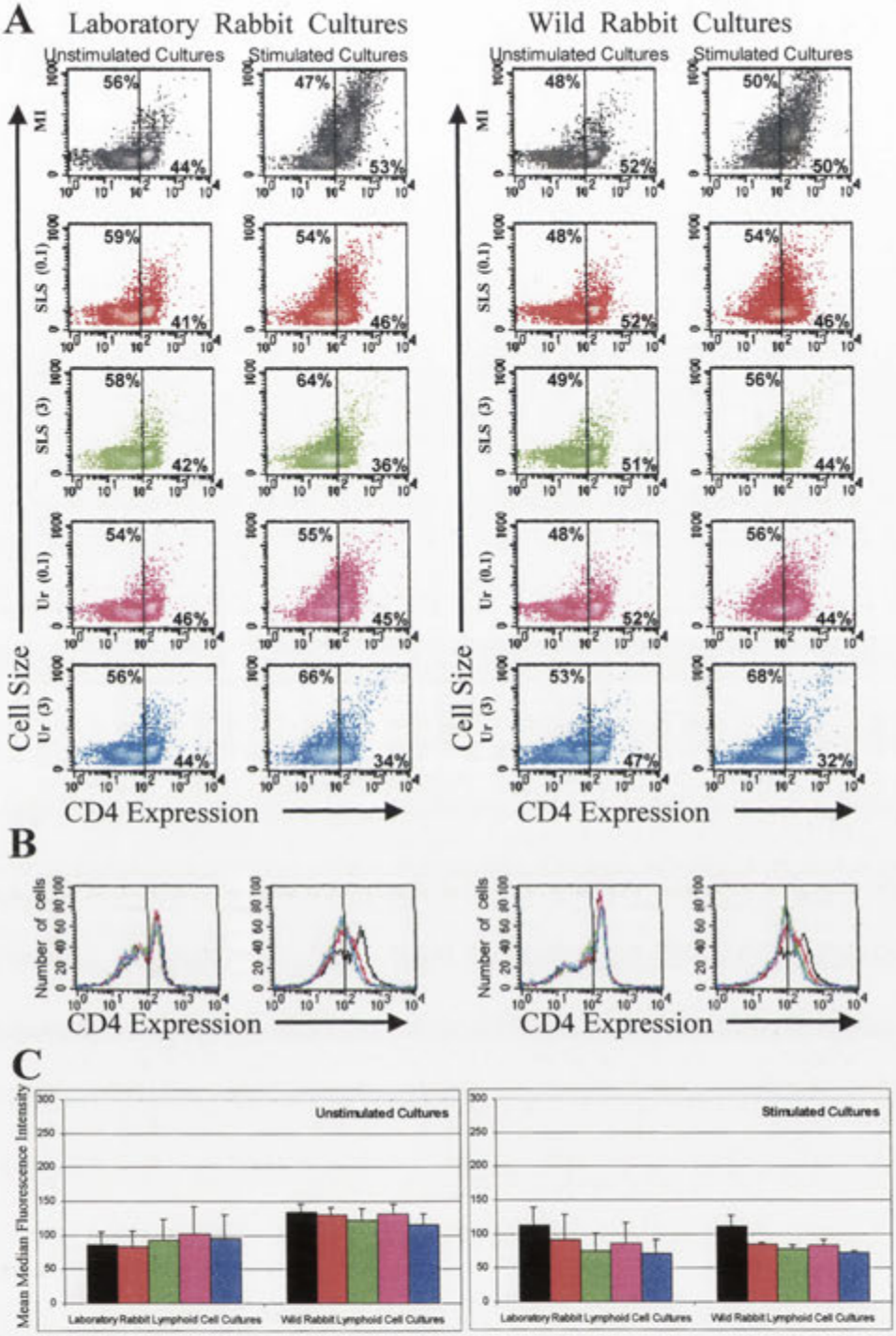


FIGURE 5.4. EXPRESSION OF CD4 IN RABBIT LYMPHOID CELL CULTURES INFECTED WITH SLS OR UR, WITH AND WITHOUT CON A STIMULATION

Expression of CD4 was analysed in lymphoid cell cultures from laboratory and wild rabbits and is presented as (A) density plots of CD4 antibody binding versus cell size and (B) histogram overlays of CD4 antibody binding. Cultures were mock-infected (MI)■ or infected with SLS, at a moi of 0.1■ or 3■, or infected with Ur, at a moi of 0.1■ or 3■. Infections were undertaken in parallel with and without Con A stimulation. The experiment was repeated three times with only one typical replicate shown here. Plots were divided such that cells with a fluorescence intensity greater than 10^2 units were classified as having high CD4 expression and cells with a fluorescence intensity less than 10^2 units were classified as having low CD4 expression. Numbers in the density plots represent the mean percentage of cells in each sub-population from three experiments. (C) The mean median fluorescence intensity corresponding to CD4 expression in lymphoid cultures from three laboratory and three wild rabbits was calculated and plotted on a column graph. Error bars represent one standard error.

stimulated mock-infected lymphoid cell cultures from laboratory rabbits and 36% and 34% when infected with SLS or Ur at a moi of 3 (Figure 5.4A). These proportions were even lower than that in unstimulated mock-infected cell cultures, which had 44% of cells binding high levels of CD4. This result suggests either myxoma virus infection can induce removal of pre-existing CD4 molecules off the surface of the cell, can inhibit CD4 transport to the cell membrane or can decrease the population of cells expressing high levels of CD4 (i.e. by inducing apoptosis). Statistical analysis of mean median fluorescence intensity showed that the decrease in levels caused by both SLS and Ur was significant in lymphocytes from wild rabbits but not in those from laboratory rabbits ($p<0.05$).

The down-regulation of CD4 appeared to be dependent on moi when analysed on the density and histogram plots. In Figure 5.4A, there were 46% and 45% of cells expressing high levels of CD4 when stimulated lymphoid cell cultures from laboratory rabbits were first infected with SLS or Ur respectively, at a moi of 0.1 compared to 36% and 34% when infected with a moi of 3. However, a difference between moi was not observed when mean median fluorescence intensity was examined. One explanation for this could be that CD4 down-regulation is linked to myxoma virus replication and in low moi myxoma virus may not have replicated to titres sufficient for down-regulating CD4 to similar levels as higher moi. However, it was shown in chapter 3 that by 48 h virus in low moi had replicated to similar proportions as virus in high moi. Alternatively, reducing CD4 expression may not be dependent on virus replication per se, but may be due to infection with the lower moi not abrogating activation to as great an extent as infection with the high moi. This would lead to a greater proportion of activated cells and thus a greater expression of CD4. Similar results were obtained for lymphoid cell cultures from wild rabbits, particularly with Ur-infected cells.

5.2.4.3 Expression of CD43

CD43 expression in unstimulated or Con A-stimulated lymphoid cell cultures from laboratory rabbits was not significantly altered by myxoma virus infection when examined on a density plot of cell size versus protein expression (95% CI). For example, in lymphoid cell cultures from laboratory rabbits, the mean proportion of cells expressing high levels of CD43 ranged between 36-49% without Con A stimulation and between 44-52% with Con A stimulation (Figure 5.5A). In lymphoid cell cultures from wild rabbits, the proportion of cells expressing high levels of CD43 was also not significantly altered by myxoma virus infection (95% CI). The apparent exception to this was in lymphoid cell cultures from wild rabbits that were infected with Ur at a moi of 3 and stimulated with Con A. In these cell cultures, only 51% of cells bound high levels of CD43 antibody compared to 68% in mock-infected cell cultures. This result is due to the small but distinct sub-population of cells, which can be seen in histogram plots with the peak in median fluorescence intensity at approximately 10^1 units. When this sub-population was further examined, it corresponded to the non-viable cell sub-population (data not shown). This sub-population was not present in the other replicates of this experiment but was observed after 48 h in some samples from experiments investigating cell viability during myxoma virus infection (chapter 4).

The mean median fluorescence intensity did not change significantly but a decrease in CD43 expression in virus-infected unstimulated lymphoid cell cultures from wild rabbits was observed (Figure 5.5C). This decrease was not observed in stimulated cultures from wild rabbits, or in lymphoid cell cultures from laboratory rabbits. A decrease in mean median fluorescence intensity suggests CD43 expression is decreased on individual cells.

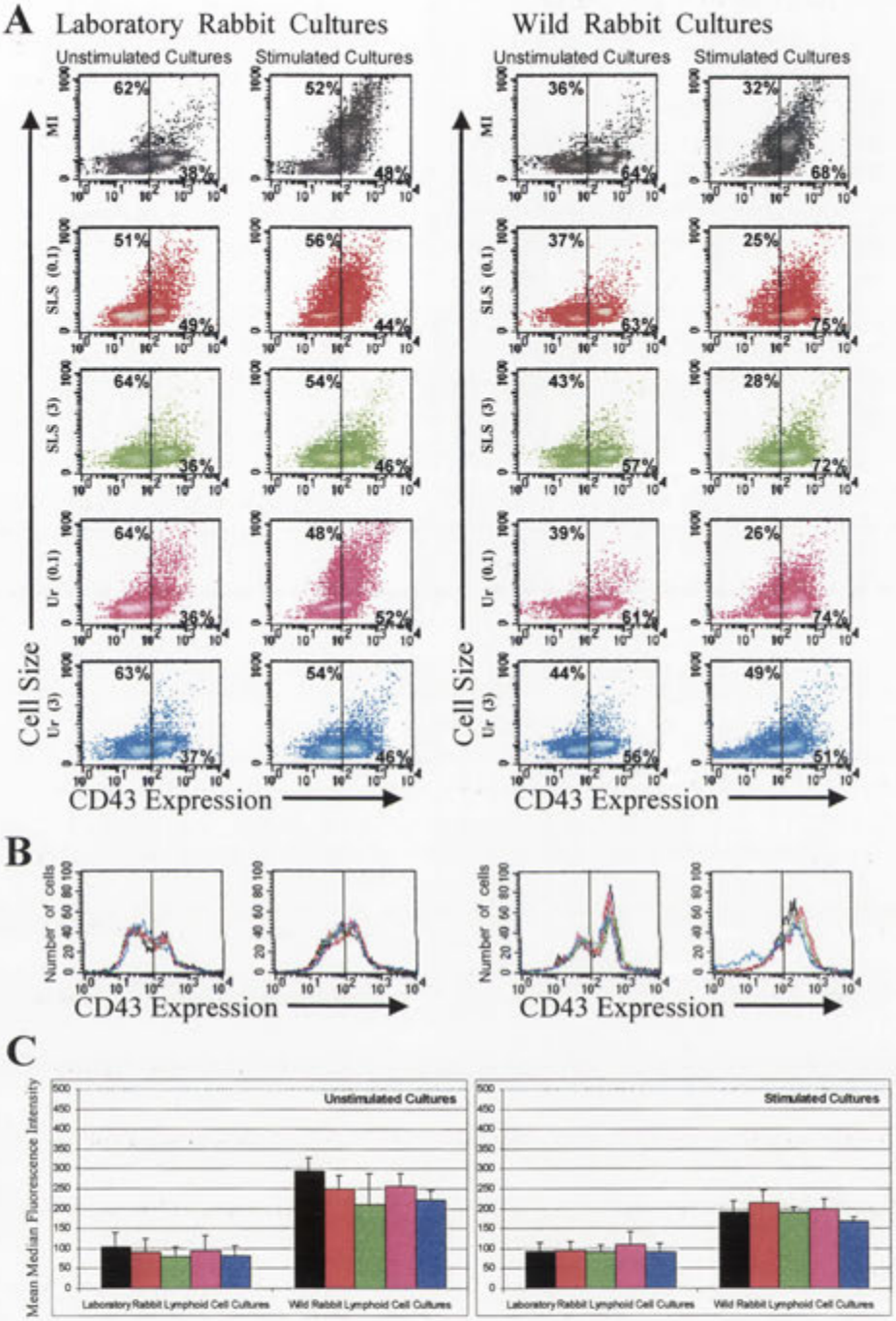


FIGURE 5.5. EXPRESSION OF CD43 IN RABBIT LYMPHOID CELL CULTURES INFECTED WITH SLS OR UR, WITH AND WITHOUT CON A STIMULATION

Expression of CD43 was analysed in lymphoid cell cultures from laboratory and wild rabbits and is presented as (A) density plots of CD43 antibody binding versus cell size and (B) histogram overlays of CD43 antibody binding. Cultures were mock-infected (MI)■ or infected with SLS, at a moi of 0.1■ or 3■, or infected with Ur, at a moi of 0.1■ or 3■. Infections were undertaken in parallel with and without Con A stimulation. The experiment was repeated three times with only one typical replicate shown here. Plots were divided such that cells with a fluorescence intensity greater than 10^2 units were classified as having high CD43 expression and cells with a fluorescence intensity less than 10^2 units were classified as having low CD43 expression. Numbers in the density plots represent the mean percentage of cells in each sub-population from three experiments. (C) The mean median fluorescence intensity corresponding to CD43 expression in lymphoid cultures from three laboratory and three wild rabbits was calculated and plotted on a column graph. Error bars represent one standard error.

5.2.4.4 Expression of CD45

Myxoma virus infection did not significantly alter CD45 expression in unstimulated lymphocyte cultures (95% CI) (Figure 5.6). However, cell cultures stimulated with Con A, generally had a lower proportion of lymphocytes expressing high levels of CD45 in myxoma virus-infected cultures than mock-infected cultures (Figure 5.6A). For example, 17% of cells in mock-infected cultures expressed high levels of CD45 compared to 10% in cultures infected with either SLS or Ur at a moi of 3. The difference in CD45 expression on individual cells between stimulated mock-infected and stimulated myxoma virus-infected cultures was not significant at the 95% CI (Figure 5.6C). In addition, the mean median fluorescence intensity in myxoma virus-infected cell cultures stimulated with Con A was similar to that in mock-infected cell cultures that had not been stimulated. This suggests that the lower CD45 expression in stimulated cell cultures infected with SLS or Ur was due to the virus inhibiting lymphocyte activation. This is further supported by closer examination of the density plots (Figure 5.6A), where the cells expressing high levels of CD45 are predominantly the larger cells in the culture. It has previously been shown that myxoma virus inhibits the increase in cell size associated with Con A activation. Hence, it may be postulated that cells in myxoma virus-infected cultures generally have a lower level of CD43 expression because the virus inhibits the increase in cell size associated with lymphocyte activation.

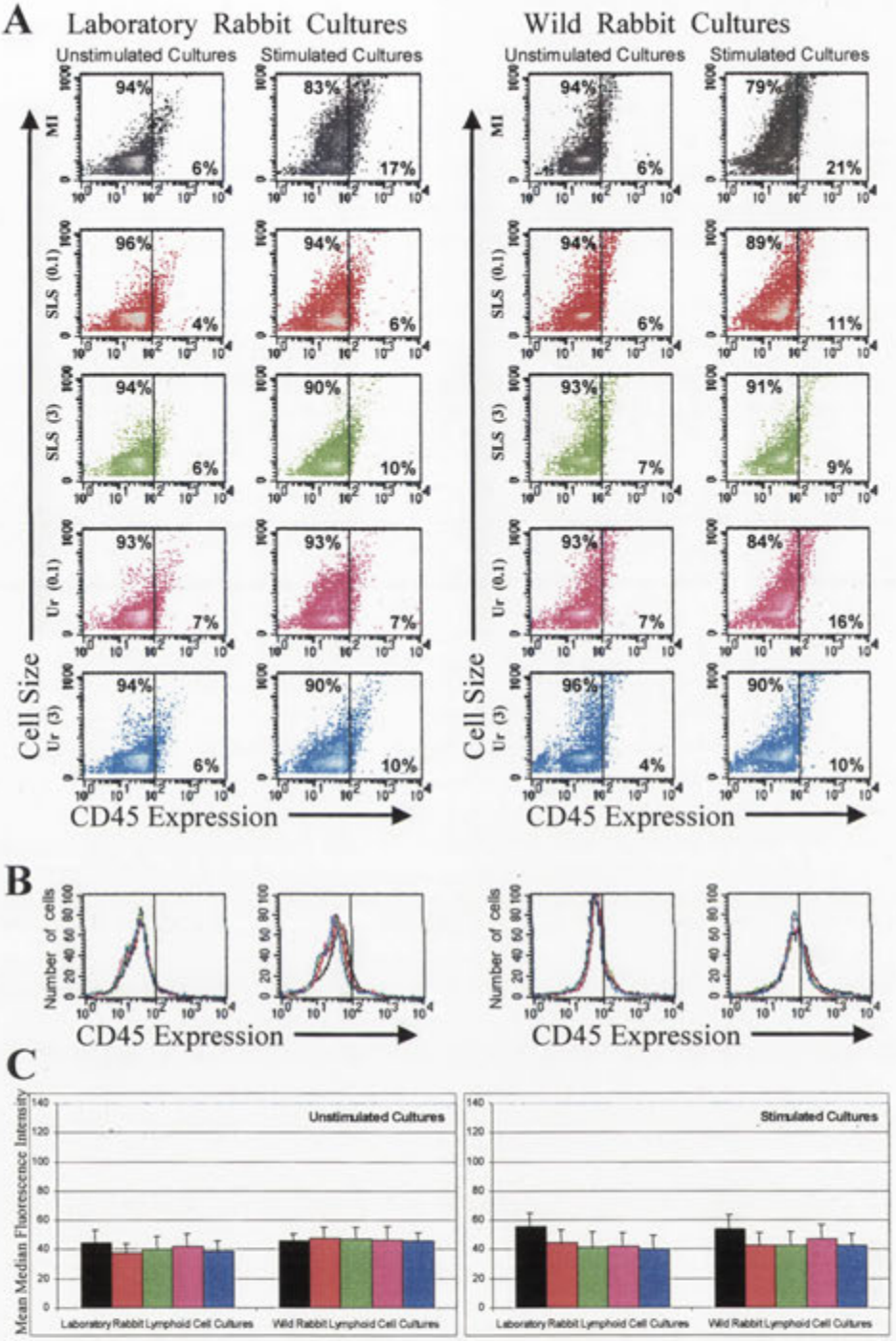


FIGURE 5.6. EXPRESSION OF CD45 IN RABBIT LYMPHOID CELL CULTURES INFECTED WITH SLS OR UR, WITH AND WITHOUT CON A STIMULATION

Expression of CD45 was analysed in lymphoid cell cultures from laboratory and wild rabbits and is presented as (A) density plots of CD45 antibody binding versus cell size and (B) histogram overlays of CD45 antibody binding. Cultures were mock-infected (MI)■ or infected with SLS, at a moi of 0.1■ or 3■, or infected with Ur, at a moi of 0.1■ or 3■. Infections were undertaken in parallel with and without Con A stimulation. The experiment was repeated three times with only one typical replicate shown here. Plots were divided such that cells with a fluorescence intensity greater than 10^2 units were classified as having high CD45 expression and cells with a fluorescence intensity less than 10^2 units were classified as having low CD45 expression. Numbers in the density plots represent the mean percentage of cells in each sub-population from three experiments. (C) The mean median fluorescence intensity corresponding to CD45 expression in lymphoid cultures from three laboratory and three wild rabbits was calculated and plotted on a column graph. Error bars represent one standard error.

5.2.4.5 Expression of KEN-5

The protein that the KEN-5 antibody recognizes is unknown, but the antibody is T-cell specific (reviewed in Mage 1998). The proportion of cells binding either high or low levels of KEN-5 antibody remained effectively unchanged, ranging from 3-14%, regardless of Con A stimulation or infection with myxoma virus infection (Figure 5.7). This is supported by the mean median fluorescence intensity of KEN-5 (Figure 5.7C) where the mean median fluorescence intensity in cultures infected with myxoma virus were not significantly different to that in mock-infected cultures (95% CI).

5.2.4.6 Expression of MHC-I

Figure 5.8A shows that in mock-infected laboratory rabbit lymphoid cell cultures stimulated with Con A, 75% of cells bound high levels of MHC-I antibody. These cells also had increased cell size. If these cell cultures were first infected with SLS or Ur at a moi of 3 prior to stimulation, only a mean of 53% and 52% of cells respectively, bound high levels of MHC-I antibody. This is a similar proportion to unstimulated mock-infected lymphoid cell cultures (Figure 5.8A) and therefore suggests that infection with either SLS or Ur inhibits the MHC-I up-regulation induced by Con A stimulation. The inhibition of MHC-I expression by myxoma virus was dependent on moi as 63% and 62% of lymphoid cell cultures from laboratory rabbits infected with SLS or Ur respectively at a moi of 0.1 bound high levels of MHC-I antibody. Similar results were observed after analysis using histogram plots (Figure 5.8B). The mean median fluorescence intensity analysis also shows that both SLS and Ur abrogated the increase in MHC-I expression associated with Con A stimulation as the levels of mean median fluorescence intensity were similar to those from unstimulated mock-infected cultures (Figure 5.8C). However, statistical analysis shows that the decrease in mean median fluorescence intensity in MHC-I antibody binding between mock-infected and myxoma

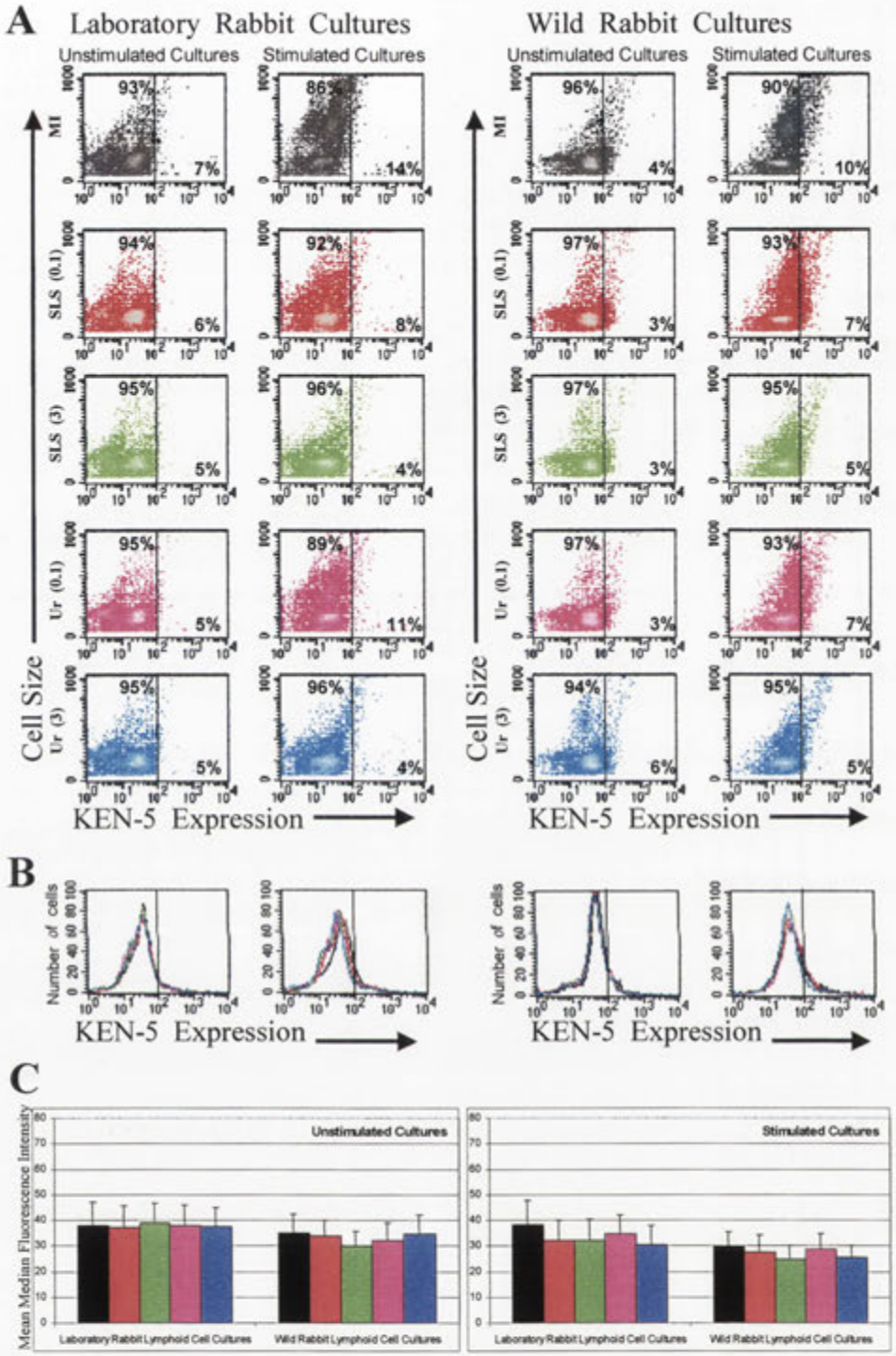


FIGURE 5.7. EXPRESSION OF KEN-5 IN RABBIT LYMPHOID CELL CULTURES INFECTED WITH SLS OR UR, WITH AND WITHOUT CON A STIMULATION

Expression of KEN-5 was analysed in lymphoid cell cultures from laboratory and wild rabbits and is presented as (A) density plots of KEN-5 antibody binding versus cell size and (B) histogram overlays of KEN-5 antibody binding. Cultures were mock-infected (MI)■ or infected with SLS, at a moi of 0.1■ or 3■, or infected with Ur, at a moi of 0.1■ or 3■. Infections were undertaken in parallel with and without Con A stimulation. The experiment was repeated three times with only one typical replicate shown here. Plots were divided such that cells with a fluorescence intensity greater than 10^2 units were classified as having high KEN-5 expression and cells with a fluorescence intensity less than 10^2 units were classified as having low KEN-5 expression. Numbers in the density plots represent the mean percentage of cells in each sub-population from three experiments. (C) The mean median fluorescence intensity corresponding to KEN-5 expression in lymphoid cultures from three laboratory and three wild rabbits was calculated and plotted on a column graph. Error bars represent one standard error.

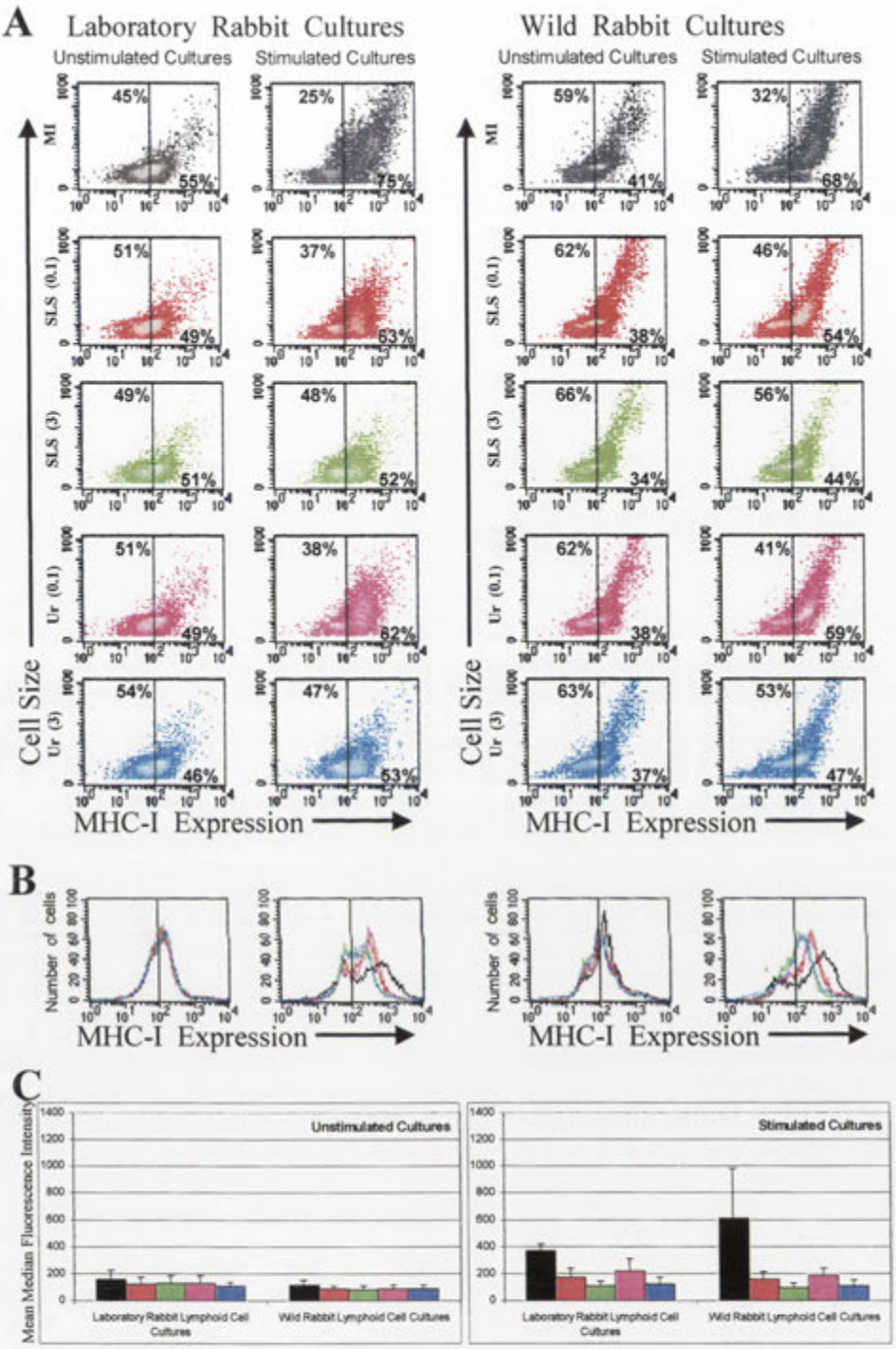


FIGURE 5.8. EXPRESSION OF MHC-I IN RABBIT LYMPHOID CELL CULTURES INFECTED WITH SLS OR UR, WITH AND WITHOUT CON A STIMULATION

Expression of MHC-I was analysed in lymphoid cell cultures from laboratory and wild rabbits and is presented as (A) density plots of MHC-I antibody binding versus cell size and (B) histogram overlays of MHC-I antibody binding. Cultures were mock-infected (MI)■ or infected with SLS, at a moi of 0.1■ or 3■, or infected with Ur, at a moi of 0.1■ or 3■. Infections were undertaken in parallel with and without Con A stimulation. The experiment was repeated three times with only one typical replicate shown here. Plots were divided such that cells with a fluorescence intensity greater than 10^2 units were classified as having high MHC-I expression and cells with a fluorescence intensity less than 10^2 units were classified as having low MHC-I expression. Numbers in the density plots represent the mean percentage of cells in each sub-population from three experiments. (C) The mean median fluorescence intensity corresponding to MHC-I expression in lymphoid cultures from three laboratory and three wild rabbits was calculated and plotted on a column graph. Error bars represent one standard error.

virus-infected stimulated cultures was not significant. The reason for this is probably the large standard errors for some of the samples. Similar results were also observed in lymphoid cell cultures from wild rabbits. Thus, overall myxoma virus down-regulated MHC-I expression.

5.2.4.7 Expression of MHC-II

Neither SLS nor Ur infection significantly altered the proportion of cells expressing high levels of MHC-II in unstimulated lymphoid cell cultures from laboratory or wild rabbits when examined by any of the three methods used ($p<0.05$) (Figure 5.9).

Based on the density plots, myxoma virus infection did not alter MHC-II expression in stimulated lymphoid cultures from laboratory rabbits (Figure 5.9A). In stimulated lymphoid cultures from wild rabbits, cultures infected with Ur at a moi of 3 had a lower proportion of cells expressing high levels of MHC-II compared to mock-infected and SLS-infected cultures (Figure 5.9A). However, detailed examination of MHC-II expression per cell by using mean median fluorescence intensity, showed that both SLS and Ur infection decreased MHC-II expression in stimulated lymphocytes from both rabbit types, though this was only significant at the 95% CI for Ur infection of lymphoid cells from wild rabbits (Figure 5.9C). This apparent contradiction can be readily resolved by the fact that MHC-II is not only expressed on rabbit B-cells but also on activated T-cells (reviewed in Mage 1998) and thus levels of antibody binding will increase in lymphoid cell cultures when T-cells are activated. Hence, the down-regulation of MHC-II in cultures infected with myxoma virus is most likely due to inhibition of T-cell activation, which would decrease the proportion of activated cells and therefore the proportion of cell expressing high levels of MHC-II.

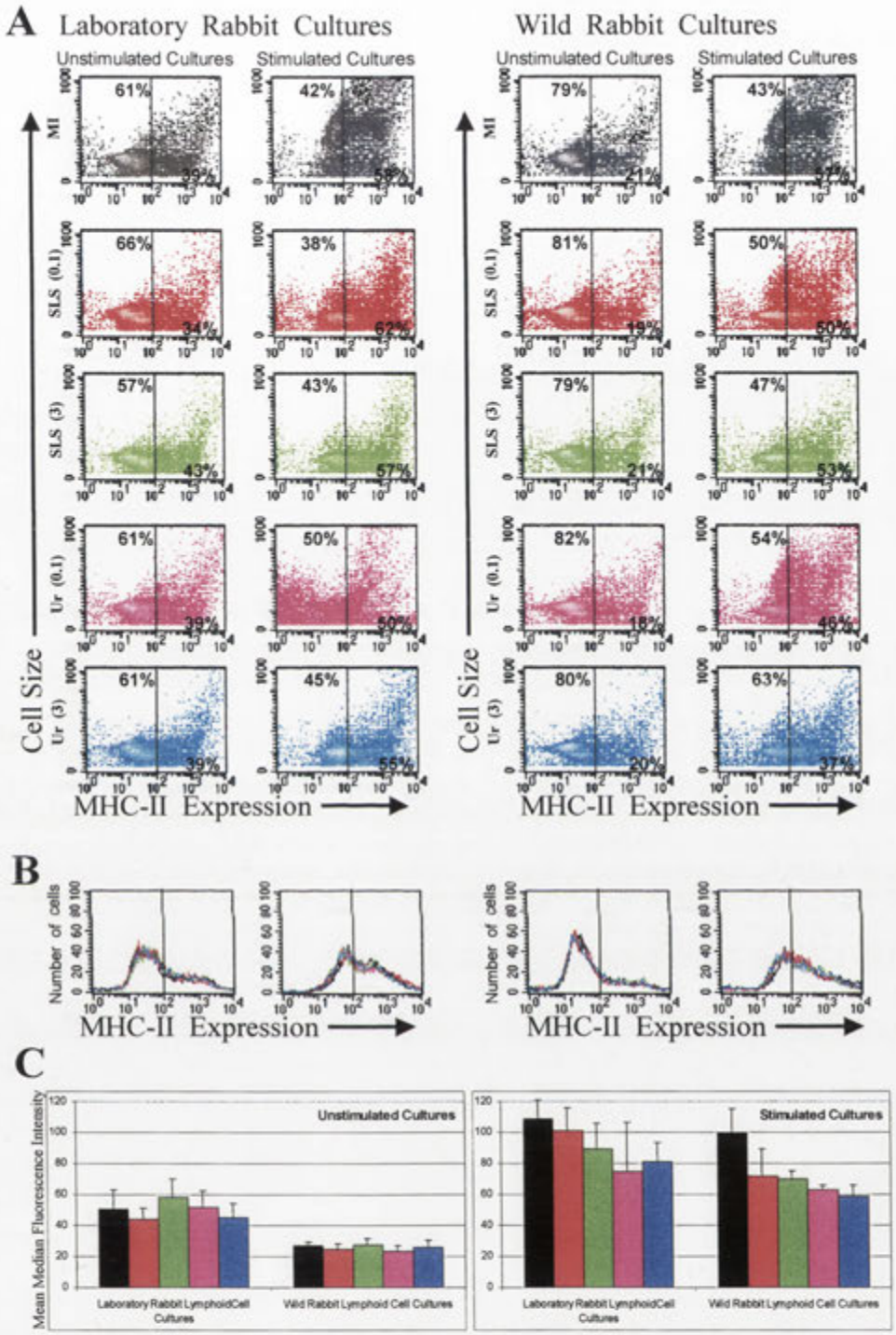


FIGURE 5.9. EXPRESSION OF MHC-II IN RABBIT LYMPHOID CELL CULTURES INFECTED WITH SLS OR UR, WITH AND WITHOUT CON A STIMULATION

Expression of MHC-II was analysed in lymphoid cell cultures from laboratory and wild rabbits and is presented as (A) density plots of MHC-II antibody binding versus cell size and (B) histogram overlays of MHC-II antibody binding. Cultures were mock-infected (MI)■ or infected with SLS, at a moi of 0.1■ or 3■, or infected with Ur, at a moi of 0.1■ or 3■. Infections were undertaken in parallel with and without Con A stimulation. The experiment was repeated three times with only one typical replicate shown here. Plots were divided such that cells with a fluorescence intensity greater than 10^2 units were classified as having high MHC-II expression and cells with a fluorescence intensity less than 10^2 units were classified as having low MHC-II expression. Numbers in the density plots represent the mean percentage of cells in each sub-population from three experiments. (C) The mean median fluorescence intensity corresponding to MHC-II expression in lymphoid cultures from three laboratory and three wild rabbits was calculated and plotted on a column graph. Error bars represent one standard error.

5.2.4.8 Summary of Expression of Cell Surface Proteins In Cultures Infected with SLS or Ur

The major results from Section 5.2.3 which will be discussed further in Section 5.3 are as follows:

1. SLS and Ur inhibited the Con A-induced increase in CD25 and appeared to inhibit MHC-I expression, though this was not statistically significant.
2. SLS and Ur generally down-regulated CD4 and MHC-II molecules from the lymphocyte surface in cultures that were stimulated with Con A.
3. CD43, CD45 and KEN-5 expression were not substantially altered by myxoma virus infection.

5.2.5 Expression of Cell Surface Proteins in Large and Small Cell Sub-Populations

The previous sections have shown that myxoma virus infection can alter the expression of some proteins on the surface of lymphocytes from cultures stimulated with Con A. Addition of Con A to primary rabbit lymphoid cell cultures was shown in chapter 3 to induce a sub-population of activated T-cells which could be distinguished from the rest of the cell culture by an increase in cell size as determined by FSC. The generation of this activated T-cell sub-population was inhibited by myxoma virus infection, with approximately 15% of cells classified as large at the 48 h time point compared to approximately 50-60% in mock-infected cultures. Activated T-cells have been shown to express different levels of cell surface proteins to quiescent T-cells (Nussenzweig and Allison 1997). Thus, it may be hypothesised that myxoma virus infection inhibits the overall expression in the lymphocyte culture of specific cell surface proteins by inhibiting T-cell activation. This hypothesis can be investigated by examining the

median fluorescence intensity of large and small cell sub-populations. For some proteins, changes in the level of expression of some proteins are not statistically significant. However, they are discussed as it was considered that the changes, although small, may have some biological significance.

Consider the following hypothetical example where the entire stimulated culture (large and small cells combined) has a median fluorescence intensity of a particular protein of 75 units, 50% of cells in the stimulated culture are large and have a median fluorescence intensity of 100 units and the small cell sub-population has a median fluorescence intensity of 50 units. If the cells are infected with myxoma virus prior to stimulation then the large cell sub-population may only contain 10% of the cells in the culture, as it has been documented in previous chapters that myxoma virus inhibits the increase in lymphocyte size associated with Con A stimulation. Virus infection may result in the median fluorescence intensity of the entire culture dropping to 55 units as there are a greater proportion of small cells. Without examining the median fluorescence intensity in discrete sub-populations it may be assumed that myxoma virus down-regulates cell surface expression of the particular protein in stimulated cultures. However, myxoma virus may just be decreasing the proportion of cells in the large sub-population, which reduces the overall median fluorescence intensity.

Examination of the median fluorescence intensity in large and small cell sub-populations will determine whether myxoma virus is modulating expression of the particular proteins either directly or by inhibiting T-cell activation. Continuing with the hypothetical example started above, if the median fluorescence intensity of the gated large cell sub-population in infected cultures is also 100 units, then it can be concluded that myxoma virus does not alter the level of protein expression, rather just the

proportion of cells in that sub-population. However, if the median fluorescence intensity of the large cell sub-population is 50 units then it can be concluded that myxoma virus down-regulates expression of that protein on the cell surface.

Therefore if the mean median fluorescence intensity of antibody binding in the large (or small) cell sub-population is the same both before and after myxoma virus infection, then it can be concluded that myxoma virus inhibits the expression of specific cell surface proteins by inhibiting T-cell activation. Alternatively, if the mean median fluorescence intensity of antibody binding is different in discrete cell sub-populations before and after infection then it can be concluded that myxoma virus infection is directly affecting cell surface protein expression.

This section re-examines the data from Section 5.2.3 by gating large and small cell sub-populations in cell cultures mock-infected or infected with myxoma virus, determining the median fluorescence intensity for each rabbit and then calculating the mean median fluorescence intensity of antibody binding for the three rabbits in each group as before. Figure 10 shows how this was done, using CD25 expression as an example.

5.2.5.1 Protein Expression on Large and Small Cells

5.2.5.1.1 Expression of CD25 and MHC-I in Large and Small Cell Sub-Populations

Figure 5.11 and Figure 5.12 show that mock-infected large cells expressed greater amounts of CD25 and MHC-I than did small cells. This can be seen as a greater mean median fluorescence intensity in the large cell sub-population compared to the small cell sub-population (Figure 5.11 and Figure 5.12). Although not statistically significant at

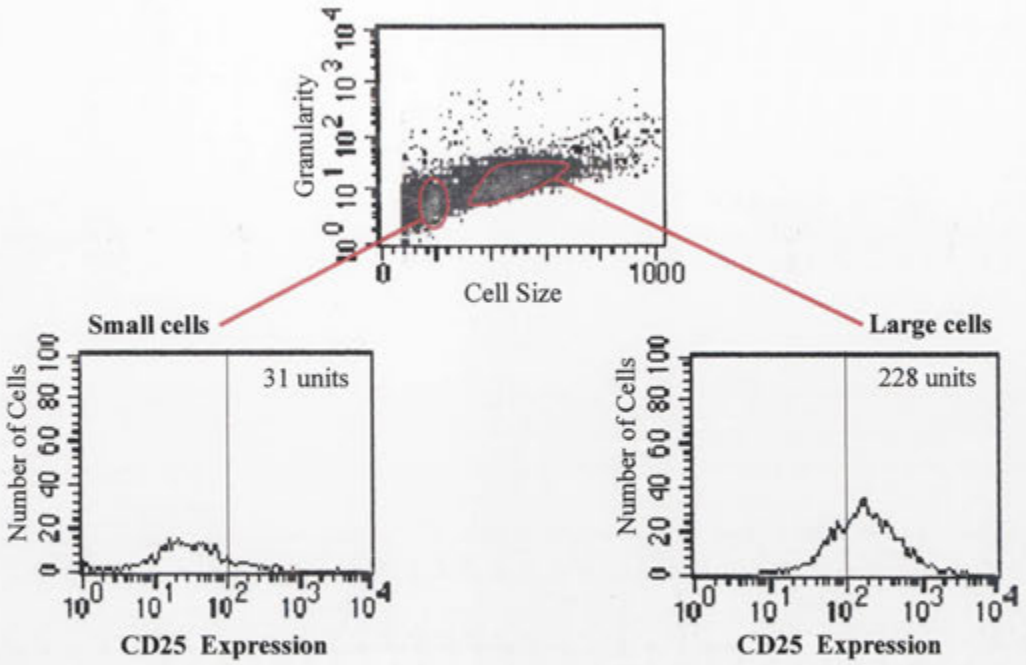


FIGURE 5.10. FLOW CYTOMETRIC ANALYSIS OF EXPRESSION OF VARIOUS PROTEINS ON THE SURFACE OF LARGE AND SMALL CELLS IN RABBIT LYMPHOID CELL CULTURES

The cell surface expression of CD25 was examined on unstimulated and Con A-stimulated laboratory rabbit lymphoid cells that had been mock-infected and analysed 48 h later. The large and small cell sub-populations were gated on density plots of cell size versus granularity and plotted as histograms of protein expression. The median fluorescence intensity for each cell sub-population was determined (numbers in histograms) and in subsequent figures is plotted on a column graph as a mean of three experiments.

the 95% CI, Con A stimulation increased the mean median fluorescence intensity of CD25 and MHC-I in the large cell sub-population, indicating that generally the expression of CD25 and MHC-I on each large cell was increased and not just the proportion of large cells. In the small cell sub-population, Con A stimulation did not significantly alter the mean median fluorescence intensity of CD25 or MHC-I (95% CI).

Infection with either SLS or Ur inhibited the Con A-induced increase in expression on large cells of CD25 (Figure 5.11) and MHC-I (Figure 5.12). The mean median fluorescence intensity of CD25 and MHC-I in infected stimulated cultures was similar to that in mock-infected unstimulated cultures, suggesting that that myxoma virus

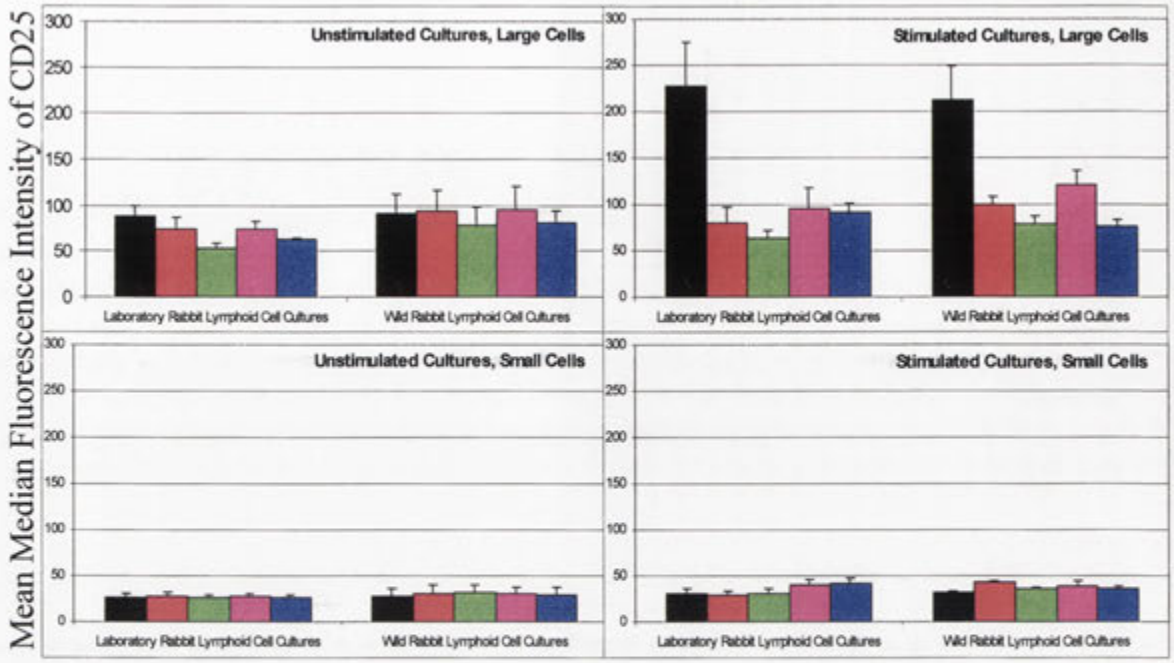


FIGURE 5.11. CD25 EXPRESSION ON LARGE AND SMALL CELLS IN RABBIT LYMPHOID CELL CULTURES

The mean median fluorescence intensity corresponding to CD25 antibody binding was determined in lymphoid cell cultures from three laboratory and three wild rabbits. Cultures were mock-infected■ or infected with either SLS, at a moi of 0.1■ or 3■, or Ur, at a moi of 0.1■ or 3■. Con A was added after cultures were infected. CD25 expression was analyzed in large and small cell sub-populations as outlined in Figure 5.10. Error bars represent one standard error.

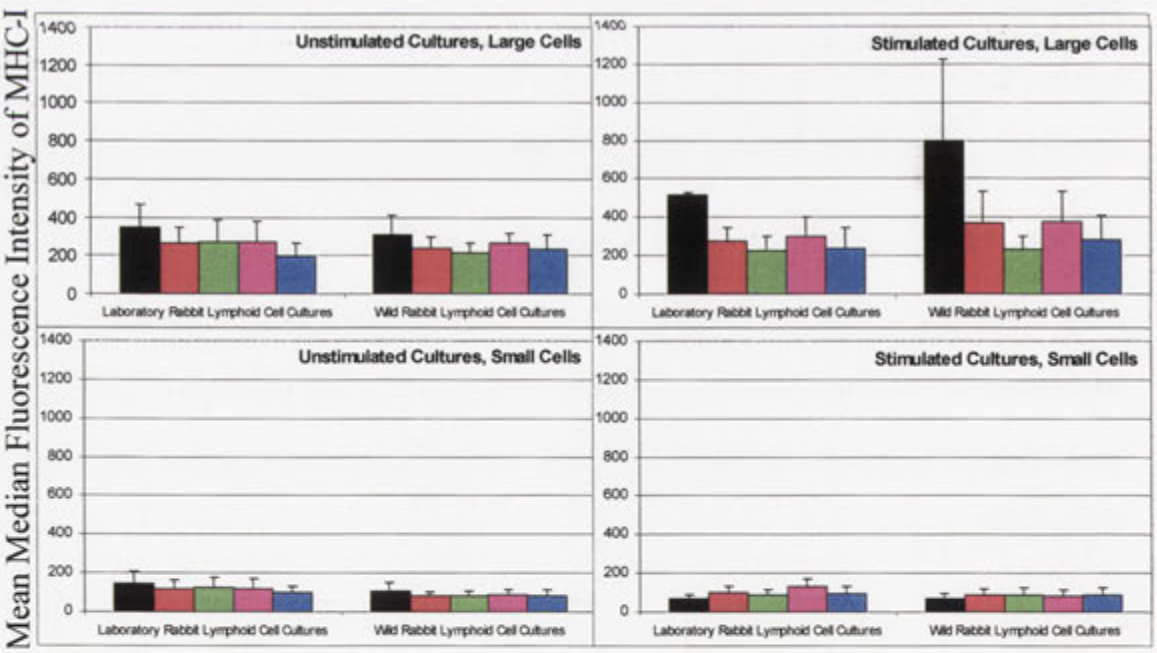


FIGURE 5.12. MHC-I EXPRESSION ON LARGE AND SMALL CELLS IN RABBIT LYMPHOID CELL CULTURES

The mean median fluorescence intensity corresponding to MHC-I antibody binding was determined in lymphoid cell cultures from three laboratory and three wild rabbits. Cultures were mock-infected■ or infected with either SLS, at a moi of 0.1■ or 3■, or Ur, at a moi of 0.1■ or 3■. Con A was added after cultures were infected. MHC-I expression was analyzed in large and small cell sub-populations as outlined in Figure 5.10. Error bars represent one standard error.

infection is down-regulating CD25 and MHC-I expression on large cells by inhibiting T-cell activation. Small cells bound low levels of CD25 and MHC-I antibodies. These levels were not significantly altered by infection with myxoma virus or by Con A stimulation.

5.2.5.1.2 Expression of CD4 in Large and Small Cell Sub-Populations

CD4 was expressed at higher levels on mock-infected large cells compared with small cells (Figure 5.13). CD4 expression was higher overall, but not significantly so, in unstimulated mock-infected large lymphocytes from wild rabbits (mean median fluorescence intensity of 214 units) compared to those from laboratory rabbits (mean median fluorescence intensity of 162 units) (Figure 5.13). In stimulated mock-infected lymphoid cell cultures from wild rabbits there were lower levels of CD4 expression on the surface of small and large cells compared to unstimulated cells. This was not statistically significant at the 95% CI. Stimulation did not significantly change CD4 expression in mock-infected large cells from laboratory rabbits at the 95% CI.

Myxoma virus infection did not significantly alter mean median fluorescence intensity of CD4 on large or small lymphoid cells from laboratory rabbits or wild rabbits in the absence of Con A (95% CI) (Figure 5.13). For small cells, the mean median fluorescence intensity ranged from a minimum of 76 units to a maximum of 94 units in lymphoid cell cultures from laboratory rabbits, and ranged from a minimum of 115 units and a maximum of 131 units in lymphoid cell cultures from wild rabbits (Figure 5.13).

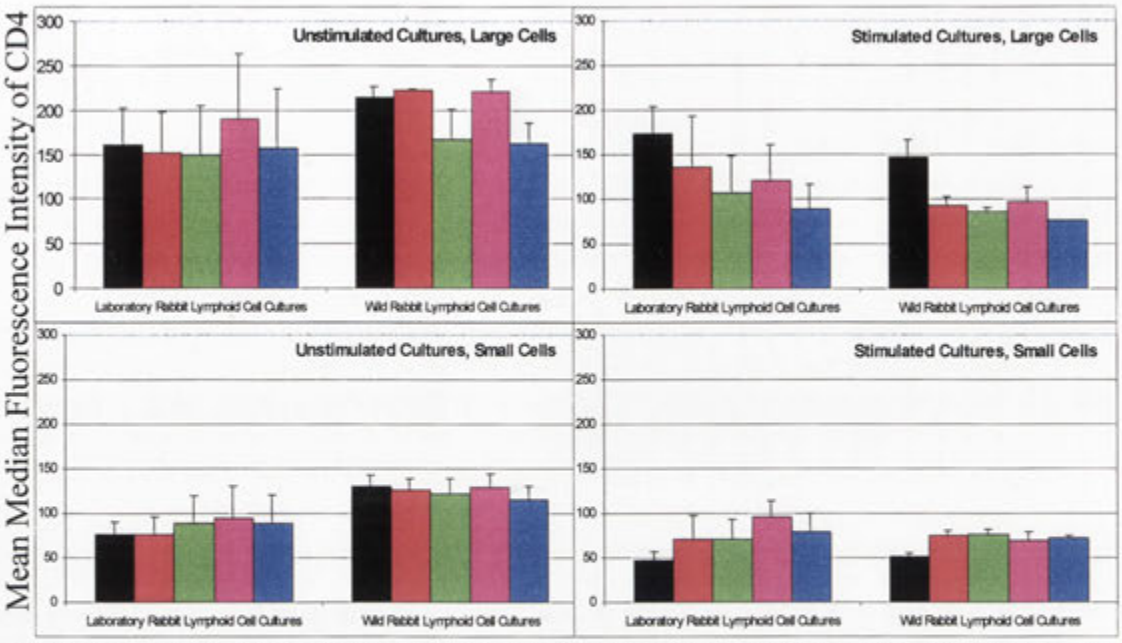


FIGURE 5.13. CD4 EXPRESSION ON LARGE AND SMALL CELLS IN RABBIT LYMPHOID CELL CULTURES

The mean median fluorescence intensity corresponding to CD4 antibody binding was determined in lymphoid cell cultures from three laboratory and three wild rabbits. Cultures were mock-infected■ or infected with either SLS, at a moi of 0.1■ or 3■, or Ur, at a moi of 0.1■ or 3■. Con A was added after cultures were infected. CD4 expression was analyzed in large and small cell sub-populations as outlined in Figure 5.10. Error bars represent one standard error.

However, infection with either strain of virus generally decreased CD4 expression on large cells in stimulated lymphoid cell cultures from both rabbit types (Figure 5.13). This decrease, although not statistically significant at the 95% CI, was apparent even at low moi and levels were lower than in unstimulated mock-infected large cells. This supports the hypothesis that CD4 is either actively down-regulated from the surface of infected, stimulated cells or that CD4 transport to the cell surface is inhibited by myxoma virus infection. The mean median fluorescence intensity of CD4 in small cells in stimulated lymphoid cell cultures from both rabbit types was greater in cultures infected with myxoma virus compared to mock-infected cultures. These results show that myxoma virus infection induced CD4 down-regulation only on large cells.

5.2.5.1.3 Expression of CD43 in Large and Small Cell Sub-Populations

Large cells in mock-infected cultures expressed greater levels of CD43 than small cells (Figure 5.14). Con A stimulation decreased CD43 expression on large and small cells in cultures from both rabbit types. This was significant only in small cells in lymphoid cell cultures from wild rabbits ($p<0.05$). Myxoma virus infection down-regulated CD43 expression on large cells in unstimulated lymphoid cell cultures from laboratory and wild rabbits (Figure 5.14). This decrease in CD43 expression, was not statistically significant at the 95% CI but inversely correlated with moi and was more apparent in lymphoid cell cultures from wild rabbits. The mean median fluorescence intensity of CD43 in the small cell sub-population was not significantly altered by myxoma virus infection in unstimulated lymphoid cell cultures from laboratory rabbits. In unstimulated mock-infected lymphoid cell cultures from wild rabbits, the mean median fluorescence intensity of CD43 expression was 292 units. In cultures infected with SLS or Ur the mean median fluorescence intensity was 212 and 222 units respectively (Figure 5.14).

Con A stimulation of cell cultures infected with SLS or Ur, led to a significant increase in CD43 expression in the small cell sub-population compared to levels in stimulated mock-infected cultures ($p<0.05$) (Figure 5.14). There was no statistically significant difference in CD43 expression on large cells between mock-infected and myxoma virus-infected cultures in either rabbit type. However, CD43 expression was increased by approximately 50 units by SLS infection in large cells from stimulated cultures from wild rabbits.

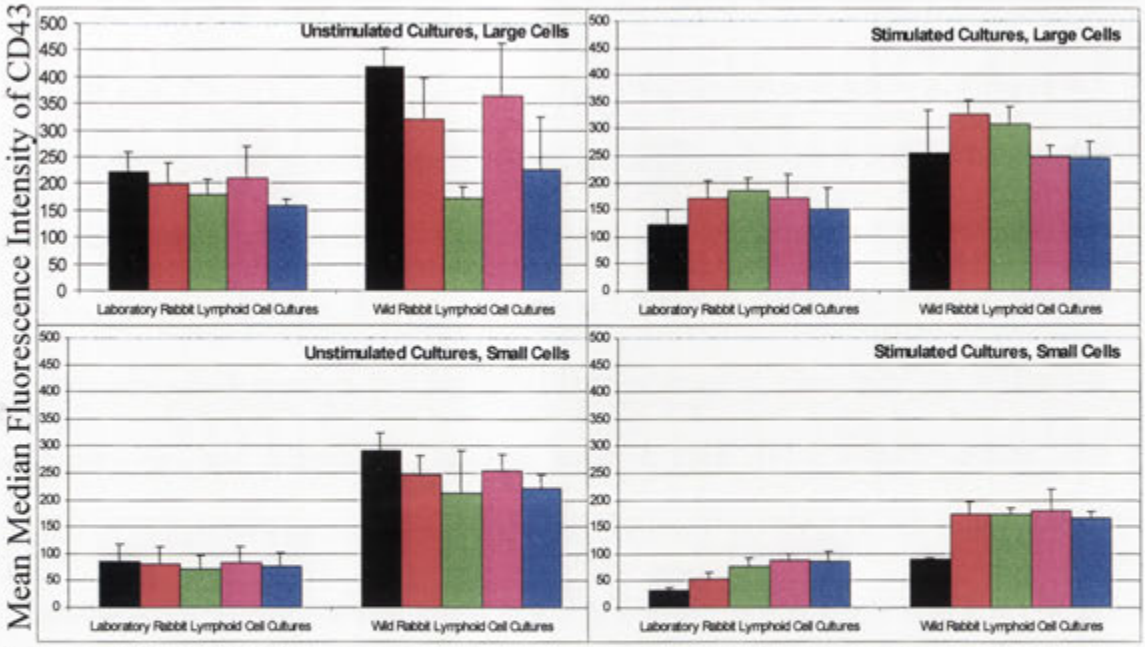


FIGURE 5.14. CD43 EXPRESSION ON LARGE AND SMALL CELLS IN RABBIT LYMPHOID CELL CULTURES

The mean median fluorescence intensity corresponding to CD43 antibody binding was determined in lymphoid cell cultures from three laboratory and three wild rabbits. Cultures were mock-infected■ or infected with either SLS, at a moi of 0.1■ or 3■, or Ur, at a moi of 0.1■ or 3■. Con A was added after cultures were infected. CD43 expression was analyzed in large and small cell sub-populations as outlined in Figure 5.10. Error bars represent one standard error.

The results presented in this section are in contrast to the results shown for the mean median fluorescence intensity in the entire cell culture, which did not show any difference in CD43 expression between mock-infected and virus-infected cell cultures (Section 5.2.3.3). However, it must be remembered that examination of large and small cell sub-populations is more sensitive than examination of the entire cell culture as the large and small cell sub-populations represent defined subsets of cells and that large and small cells constitute different proportions of the total culture.

5.2.5.1.4 Expression of CD45 in Large and Small Cell Sub-Populations

Large cell sub-populations had a greater mean median fluorescence intensity of CD45 antibody binding compared to small cell sub-populations (Figure 5.15). Infection of cell

cultures with either SLS or Ur did not significantly alter CD45 expression on cell sub-populations in unstimulated cell cultures (95% CI) (Figure 5.15). Stimulation of infected cell cultures with Con A did not significantly alter CD45 expression on large and small cells in lymphoid cell cultures from laboratory or wild rabbits compared to stimulated mock-infected cell cultures (95% CI) (Figure 5.15).

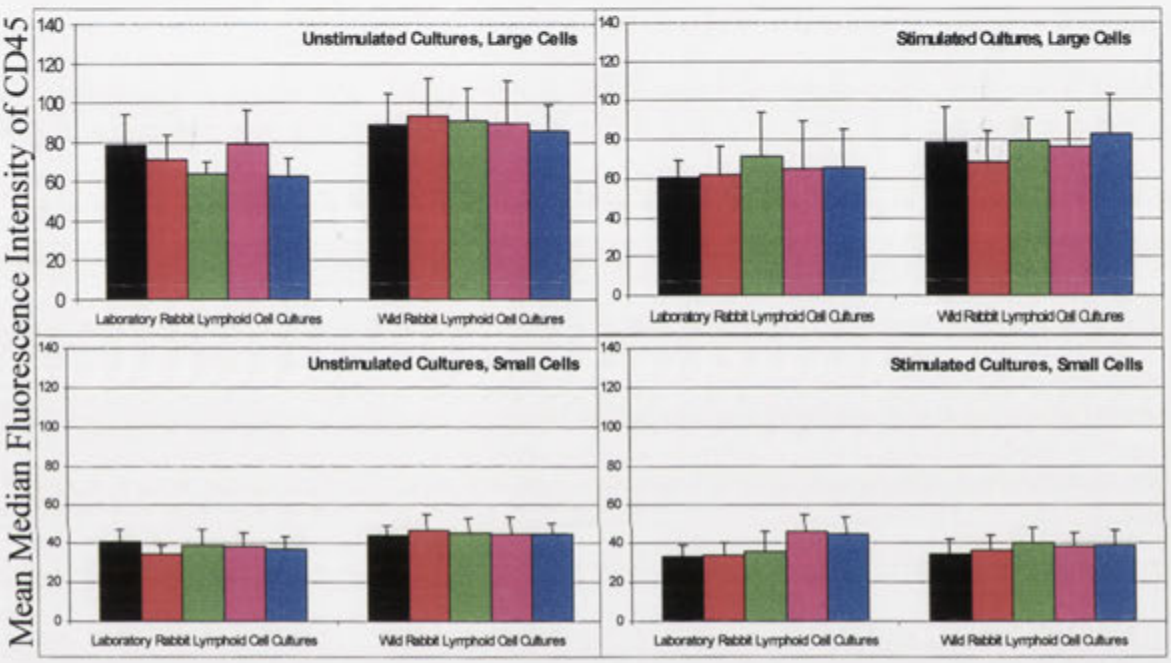


FIGURE 5.15. CD45 EXPRESSION ON LARGE AND SMALL CELLS IN RABBIT LYMPHOID CELL CULTURES

The mean median fluorescence intensity corresponding to CD45 antibody binding was determined in lymphoid cell cultures from three laboratory and three wild rabbits. Cultures were mock-infected■ or infected with either SLS, at a moi of 0.1■ or 3■, or Ur, at a moi of 0.1■ or 3■. Con A was added after cultures were infected. CD45 expression was analyzed in large and small cell sub-populations as outlined in Figure 5.10. Error bars represent one standard error.

5.2.5.1.5 Expression of KEN-5 in Large and Small Cell Sub-Populations

Large cells in rabbit lymphoid cell cultures generally expressed higher levels of KEN-5 than small cells (Figure 5.16). The level of KEN-5 expression in the large or small cell sub-population was not altered significantly between rabbit types or stimulation.

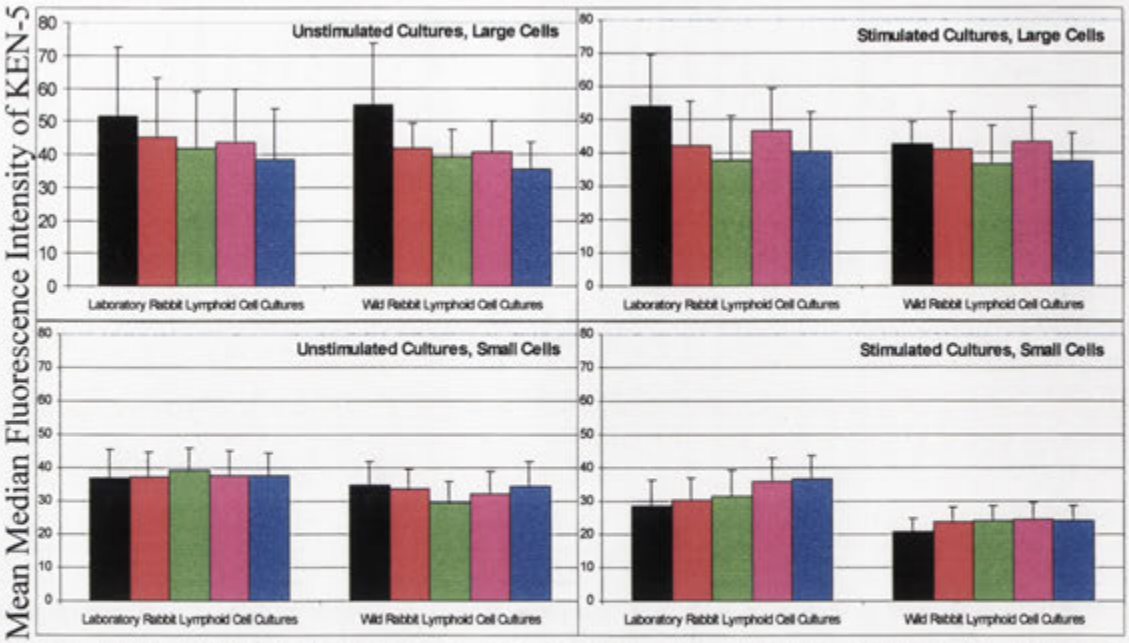


FIGURE 5.16. KEN-5 EXPRESSION ON LARGE AND SMALL CELLS IN RABBIT LYMPHOID CELL CULTURES

The mean median fluorescence intensity corresponding to KEN-5 antibody binding was determined in lymphoid cell cultures from three laboratory and three wild rabbits. Cultures were mock-infected■ or infected with either SLS, at a moi of 0.1■ or 3■, or Ur, at a moi of 0.1■ or 3■. Con A was added after cultures were infected. KEN-5 expression was analyzed in large and small cell sub-populations as outlined in Figure 5.10. Error bars represent one standard error.

5.2.5.1.6 Expression of MHC-II in Large and Small Cell Sub-Populations

Examination of mean median fluorescence intensity in mock-infected large and small cell sub-populations showed large cells had significantly higher expression of MHC-II than small cells ($p<0.05$) (Figure 5.17) For comparison, the mean median fluorescence intensity graphs for the entire cell culture (shown in Figure 5.9) are also shown with the same scale. This gives an indication that MHC-II expression on individual cells may not impact greatly on events within the entire lymphoid cell cultures.

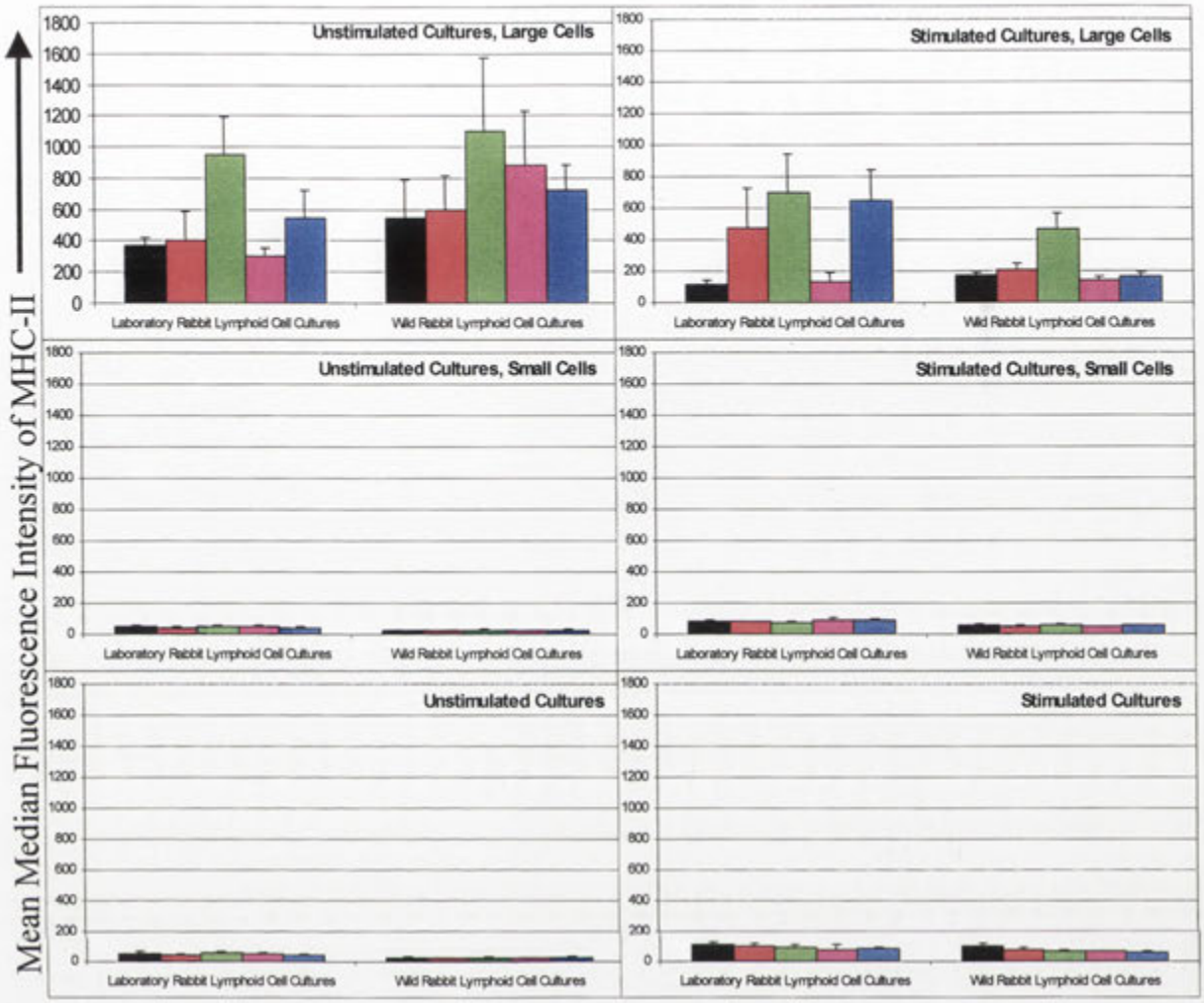


FIGURE 5.17. MHC-II EXPRESSION ON LARGE AND SMALL CELLS IN RABBIT LYMPHOID CELL CULTURES

The mean median fluorescence intensity corresponding to MHC-II antibody binding was determined in lymphoid cell cultures from three laboratory and three wild rabbits. Cultures were mock-infected■ or infected with either SLS, at a moi of 0.1■ or 3■, or Ur, at a moi of 0.1■ or 3■. Con A was added after cultures were infected. MHC-II expression was analyzed in large and small cell sub-populations as outlined in Figure 5.10. The mean median fluorescence intensity in the entire culture from Figure 5.9C are also plotted with the same scale as for large and small cell sub-populations. Error bars represent one standard error.

Infection of rabbit lymphoid cell cultures with SLS or Ur at a moi of 3 increased MHC-II levels on large cells in unstimulated cell cultures relative to mock-infected cell cultures (Figure 5.17). For instance, the mean median fluorescence intensity of MHC-II expression in mock-infected cultures from laboratory rabbits was 370 units. This increased to 956 and 543 units for SLS and Ur respectively. This was not statistically significant due to the wide variation in MHC-II expression levels in cultures infected

with myxoma virus. Similar results were observed in lymphoid cell cultures from wild rabbits.

Con A stimulation of these cell cultures partially inhibited the increase in MHC-II expression in infected cell cultures, however MHC-II expression was still greater than that in mock-infected cell cultures as Con A reduced MHC-II expression on large cells. The increase in MHC-II expression tended to be greater in cell cultures infected with SLS compared to cell cultures infected with Ur. The mean median fluorescence intensity in small cell sub-populations was not greater than 90 units in any infection.

The discrepancy between MHC-II levels in the entire cell culture and on large cells can be accounted for by the small proportion of large cells (approximately 15%). When the entire cell culture is considered, this minor population will not greatly affect results when a median is calculated.

5.2.5.1.7 Summary of Expression of Cell Surface Proteins in Large and Small Cell Sub-populations

The main results in this section are:

1. SLS and Ur inhibited the Con A-induced increase in CD25 and MHC-I expression on large cells.
2. SLS and Ur did not significantly alter CD4 expression on large or small cells in unstimulated cultures. CD4 expression on large cells was down-regulated by both virus strains when cultures were stimulated.
3. CD43 expression was down-regulated on large cells in the absence of stimulation. There was no change in CD43 expression on large cells when

cultures were stimulated, however, CD43 expression was significantly increased in the small cell sub-population when cultures were stimulated.

4. Overall, CD45 and KEN-5 expression was not altered substantially in either cell sub-population when cultures were infected with myxoma virus.
5. MHC-II expression on large cells increased dramatically with myxoma virus infection.

5.2.6 Expression of Cell Surface Proteins in Rabbit Lymphoid Cell Cultures Stimulated with Con A Prior to Infection with Myxoma Virus

Chapter 3 showed that myxoma virus inhibited lymphocyte proliferation induced by Con A. During the anti-viral immune response, however, some cells would be stimulated prior to their being infected with virus. As chapter 3 showed that cells already stimulated are the predominant sub-population infected with myxoma virus, the ability of the virus to alter cell surface expression of specific proteins in cell cultures activated prior to infection was examined. To determine whether myxoma virus could alter the activation state of lymphoid cells by altering cell surface expression of CD25 or MHC-I, cell cultures were stimulated with Con A for 4, 24 or 48 h and then mock-infected or infected with SLS or Ur at a moi of 3. These experiments also allow better comparison with previously published studies examining the expression of cell surface markers during myxoma virus infection of constantly replicating cell lines. Cell cultures were examined 24 and 48 h after infection. These are the same samples used in chapter 3 for analysis of cell size. Samples were analysed by density plots of cell size versus protein expression and histogram overlays of protein expression. Cell cultures stimulated with Con A for 24 h prior to infection are not shown as these results are

intermediate between results from cultures stimulated for 4 or 48 h prior to infection. Similarly, results from cultures infected for 24 h after Con A stimulation for 4 or 48 h are not shown, because results from virus-infected cultures can not be as clearly distinguished from mock-infected cultures as that in cultures infected for 48 h.

CD25 and MHC-I expression were decreased at 24 and 48 h after infection in rabbit lymphoid cell cultures that had been stimulated with Con A for 4 h prior to infection with SLS or Ur (Figure 5.18 and 5.19). This was more apparent after 48 h of infection compared to 24 h post infection (data not shown).

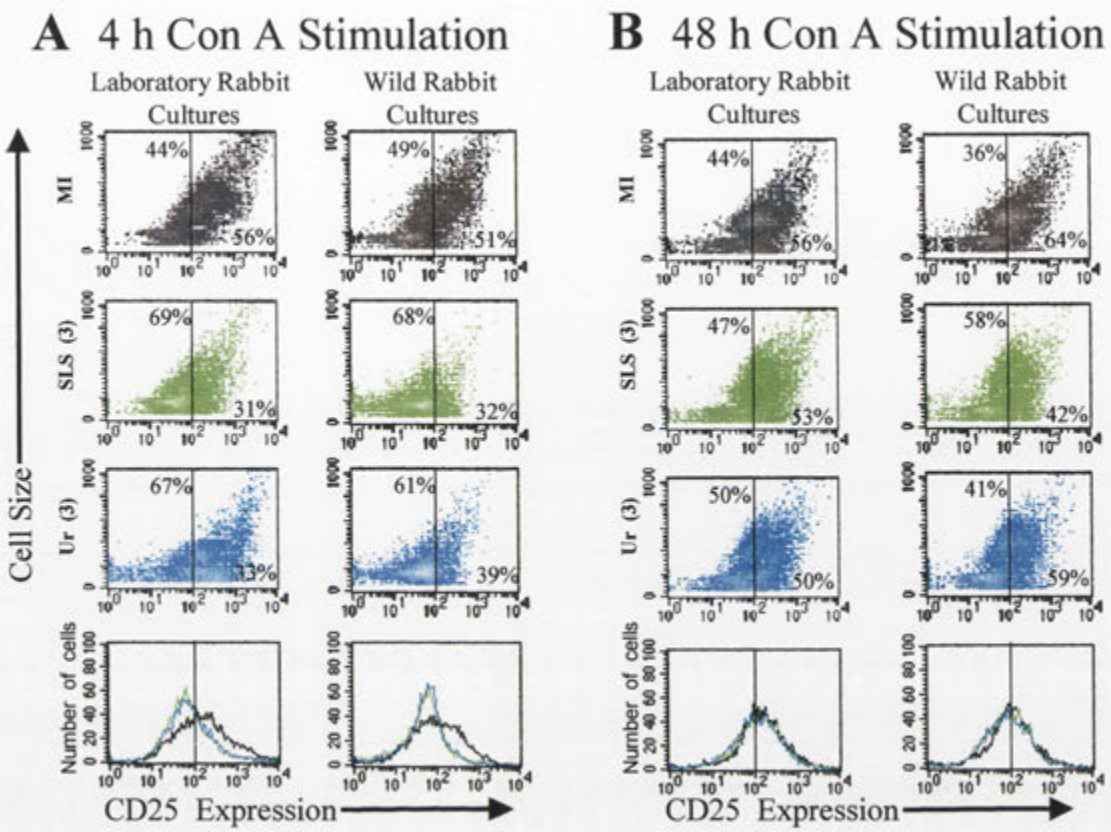


FIGURE 5.18. CELL SURFACE EXPRESSION OF CD25 IN RABBIT LYMPHOID CELL CULTURES STIMULATED WITH CON A PRIOR TO INFECTION WITH SLS OR UR

Lymphoid cell cultures from laboratory and wild rabbits were incubated with Con A for (A) 4 h or (B) 48 h and then were either mock-infected (MI)■ or infected with SLS■ or Ur■, at a moi of 3, for an additional 48 h. CD25 expression was analysed by flow cytometry. The experiment was undertaken using lymphoid cells isolated from three laboratory and three wild rabbits (one shown here). Data is presented as density plots of cell size versus CD25 expression, and histogram overlays of CD25 expression. Cells with a fluorescence intensity greater than 10^2 units were classified as expressing high levels of CD25, whereas cells with a fluorescence intensity less than 10^2 units were classified as expressing low levels of CD25. Numbers are mean proportions of cells in sub-populations expressing high and low CD25 levels from the three experiments.

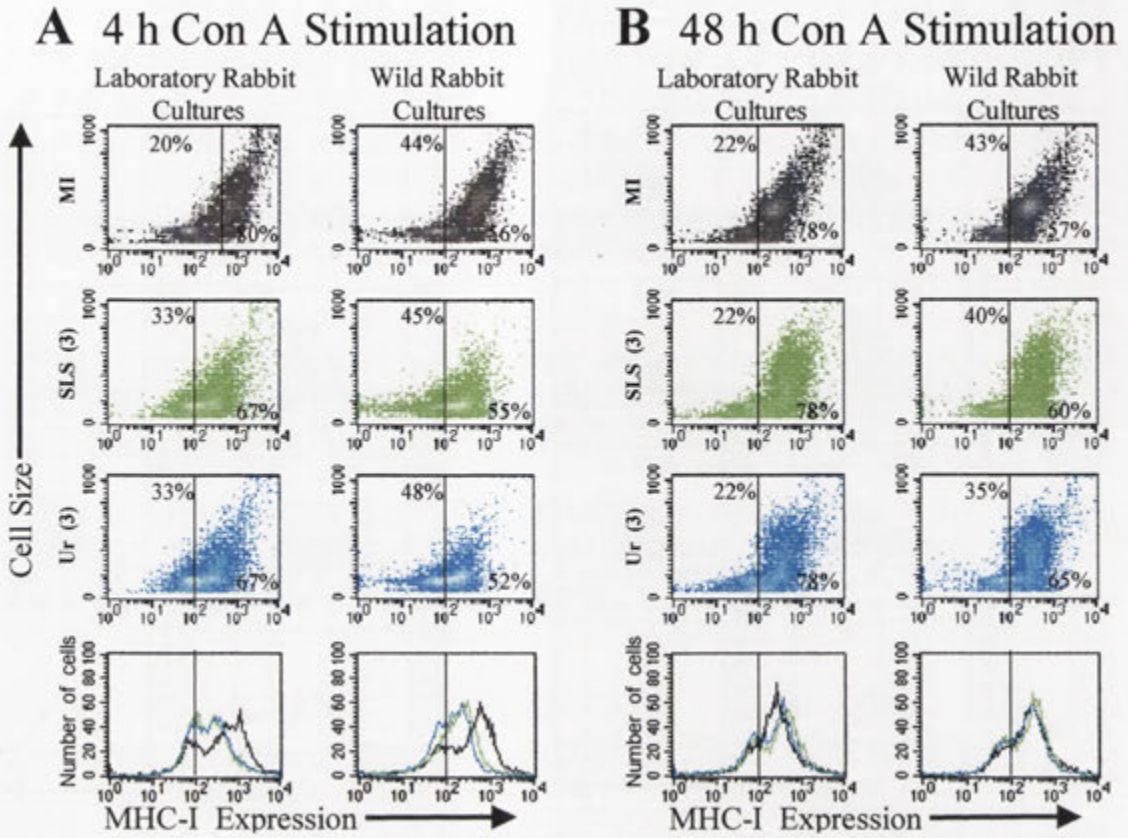


FIGURE 5.19. CELL SURFACE EXPRESSION OF MHC-I IN RABBIT LYMPHOID CELL CULTURES STIMULATED WITH CON A PRIOR TO INFECTION WITH SLS OR UR

Lymphoid cell cultures from laboratory and wild rabbits were incubated with Con A for (A) 4 h or (B) 48 h and then were either mock-infected (MI)■ or infected with SLS■ or Ur■, at a moi of 3, for an additional 48 h. MHC-I expression was analysed by flow cytometry. The experiment was undertaken using lymphocytes isolated from three laboratory and three wild rabbits (one shown here). Data is presented as density plots of cell size versus MHC-I expression, and histogram overlays of MHC-I expression. Cells with a fluorescence intensity greater than 10^2 units were classified as expressing high levels of MHC-I, whereas cells with a fluorescence intensity less than 10^2 units were classified as expressing low levels of MHC-I. Numbers are mean proportions of cells in sub-populations expressing high and low MHC-I levels from the three experiments.

The longer cell cultures were stimulated with Con A prior to infection, the less the decrease in CD25 and MHC-I expression following infection. This can be seen by the similar proportions of cells from laboratory rabbits expressing high levels of CD25 (Figure 5.18) or MHC-I (Figure 5.19) between mock-infected and myxoma virus-infected cell cultures when stimulated for 48 h prior to infection and in the histogram overlays where similar curves were observed in mock-infected and virus-infected

cultures stimulated for 48 h prior to infection. Similar results were observed in wild rabbit cultures.

These results suggest that myxoma virus inhibits CD25 and MHC-I expression by inhibiting cell activation because CD25 and MHC-I expression were not affected if cells were activated prior to infection. This hypothesis is supported by analysis of mean median fluorescence intensity which shows the ability of myxoma virus to decrease CD25 (Figure 5.20) and MHC-I (Figure 5.21) antibody binding is reduced the longer cell cultures were stimulated prior to infection. Mock-infected cultures stimulated with Con A for 48 h had lower levels of MHC-I expression compared to cultures stimulated for shorter periods, suggesting that in cultures stimulated for 48 h, MHC-I was either actively taken off the surface of the cell or the production of new molecules was reduced. The expression of CD25 and MHC-I on small cells was not altered by Con A stimulation or by myxoma virus infection in cell cultures from either type of rabbit.

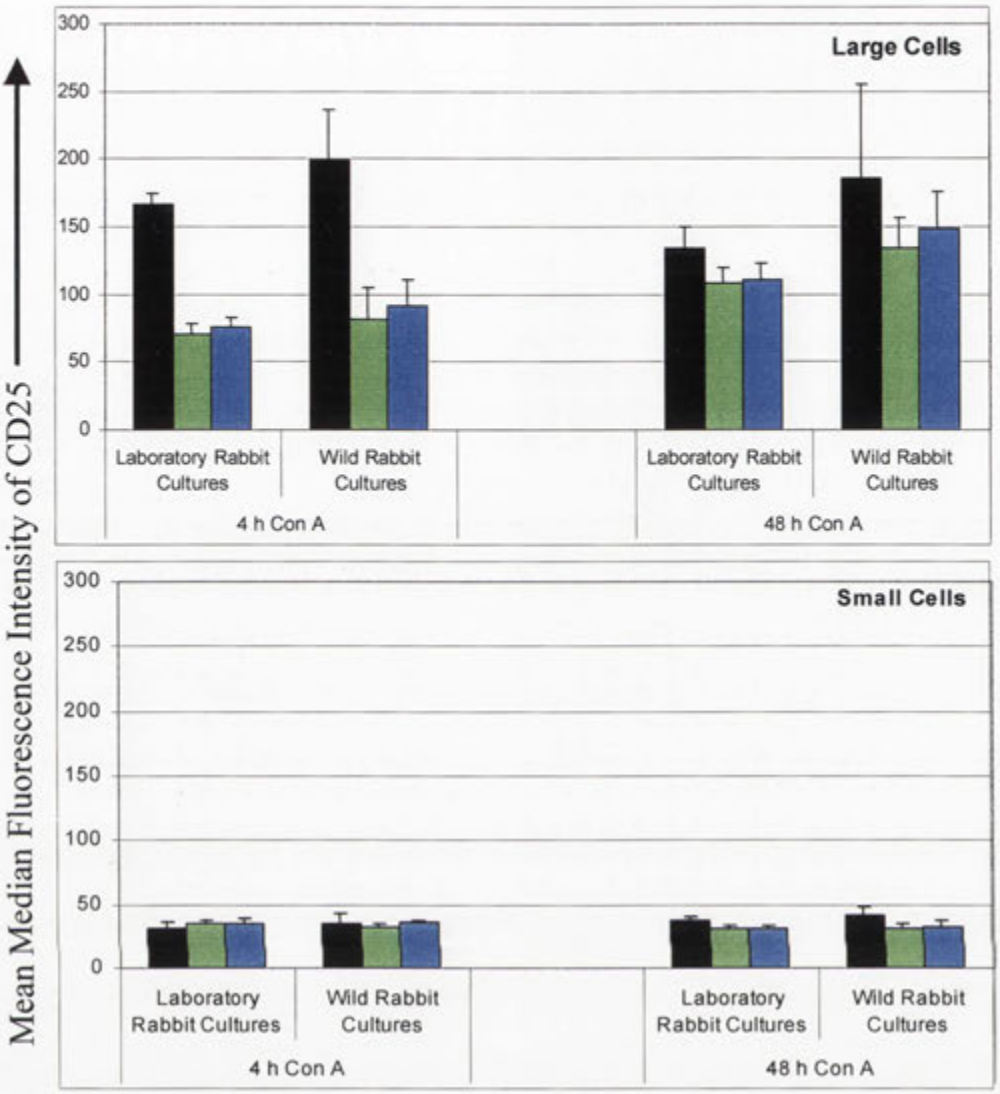


FIGURE 5.20. MEAN MEDIAN FLUORESCENCE INTENSITY CORRESPONDING TO CD25 EXPRESSION IN RABBIT LYMPHOID CELL CULTURES STIMULATED WITH CON A PRIOR TO INFECTION WITH SLS OR UR

The mean median fluorescence intensity corresponding to CD25 expression was determined in cell cultures stimulated with Con A for 4 or 48h prior to infection for 48h. CD25 expression was analysed in large and small cell sub-populations in lymphoid cell cultures from laboratory and wild rabbits. Cultures were mock-infected■ or infected with SLS■ or Ur■ at a moi of 3. Error bars represent one standard error.

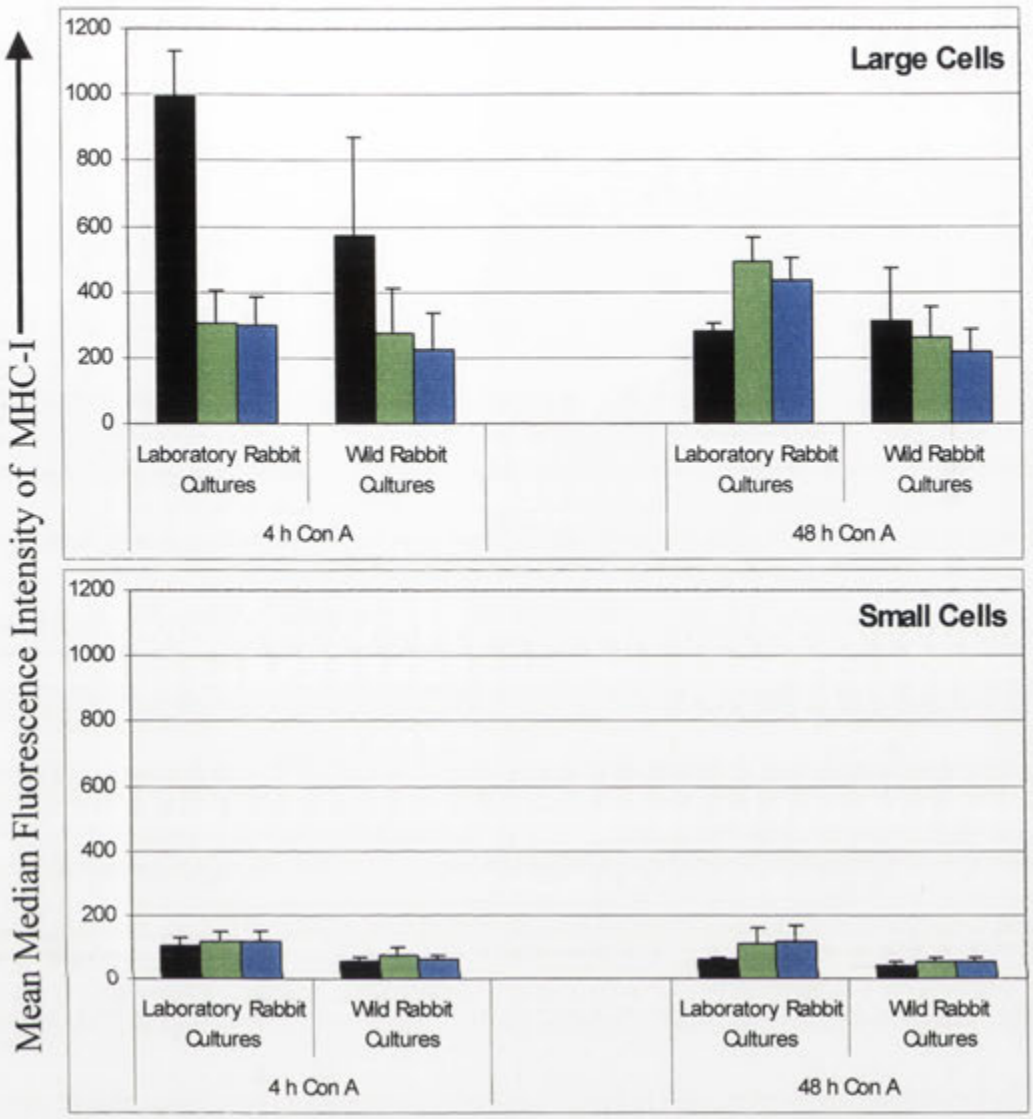


FIGURE 5.21. MEAN MEDIAN FLUORESCENCE INTENSITY CORRESPONDING TO MHC-I EXPRESSION IN RABBIT LYMPHOID CELL CULTURES STIMULATED WITH CON A PRIOR TO INFECTION WITH SLS OR UR

The mean median fluorescence intensity corresponding to MHC-I expression was determined in cell cultures stimulated with Con A for 4 or 48h prior to infection for 48h. MHC-I expression was analysed in large and small cell sub-populations in lymphoid cell cultures from laboratory and wild rabbits. Cultures were mock-infected■ or infected with SLS■ or Ur■ at a moi of 3. Error bars represent one standard error.

5.2.7 Expression of Cell Surface Proteins on RL-5 Cells Infected with SLS or Ur

Infection with myxoma virus has previously been shown to decrease CD4 and MHC-I expression on RL-5 cells, but not affect CD43 or CD45 expression . Myxoma virus infection has also been shown to decrease MHC-I on the primate kidney cell line, BGMK (Boshkov et al., 1992). These experiments were undertaken using the Lu strain of myxoma virus. To investigate if SLS and Ur could also decrease CD4 and MHC-I expression on RL-5 cells, analysis of cell surface protein expression on RL-5 cells infected with SLS and Ur was undertaken in a similar manner to that done for primary lymphocytes. The effects of SLS and Ur infection on expression of the other cell surface proteins examined in this chapter were also investigated. Protein expression is presented as density plots of cell size versus protein expression, histogram overlays and mean median fluorescence intensity. MHC-II expression was not analyzed as preliminary examination of MHC-II expression, by staining cells with normal mouse ascites fluid or with anti-MHC-II monoclonal antibody, showed RL-5 cells did not express MHC-II (data not shown).

Results presented here confirm and extend the results of Barry et al. (1995) in that myxoma virus caused a decrease in CD4 and MHC-I expression and no change in CD43 and CD45 expression on RL-5 cells. Infection with either SLS or Ur decreased CD4 expression, with 90% of cells expressing high levels of CD4 in mock-infected cultures, but only 73% and 75% in cultures infected with SLS or Ur respectively (Figure 5.22). The concurrent decrease in mean median fluorescence intensity was not statistically significant at the 95% CI. Similarly, a decrease in the proportion of cells expressing high levels of MHC-I was observed (Figure 5.23); the concurrent decrease in mean

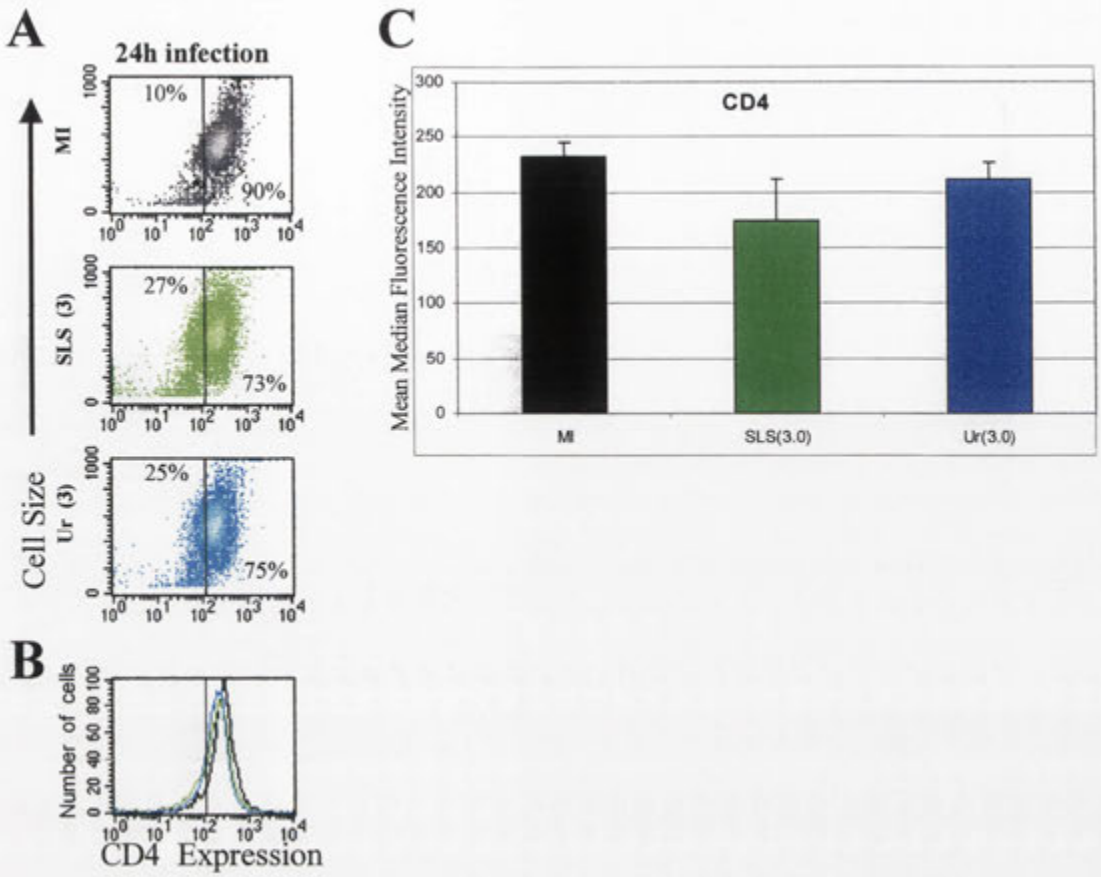


FIGURE 5.22. EXPRESSION OF CD4 ON THE SURFACE OF RL-5 CELLS INFECTED WITH SLS OR UR

RL-5 cells were mock-infected (MI)■ or infected with SLS■ or Ur■ at a moi of 3, for 24 h. Expression of CD4 was analysed by flow cytometry. The experiment was undertaken on three separate occasions (one shown here). Data is presented as (A) density plots of cell size versus protein expression and (B) histogram overlays of protein expression. A black line has been drawn to distinguish sub-populations expressing high and low levels of CD4. (C) The mean median fluorescence intensity corresponding to CD4 for each infection was calculated for the three separate occasions.

median fluorescence intensity was greater, decreasing from 205 units in mock-infected cultures to 110 and 140 units in SLS- and Ur-infected cultures respectively.

Neither SLS nor Ur infection significantly altered CD43 (Figure 5.24) or CD45 (Figure 5.25) expression with differences in mean median fluorescence intensity between mock-infected and myxoma virus-infected cell cultures less than 10 units.

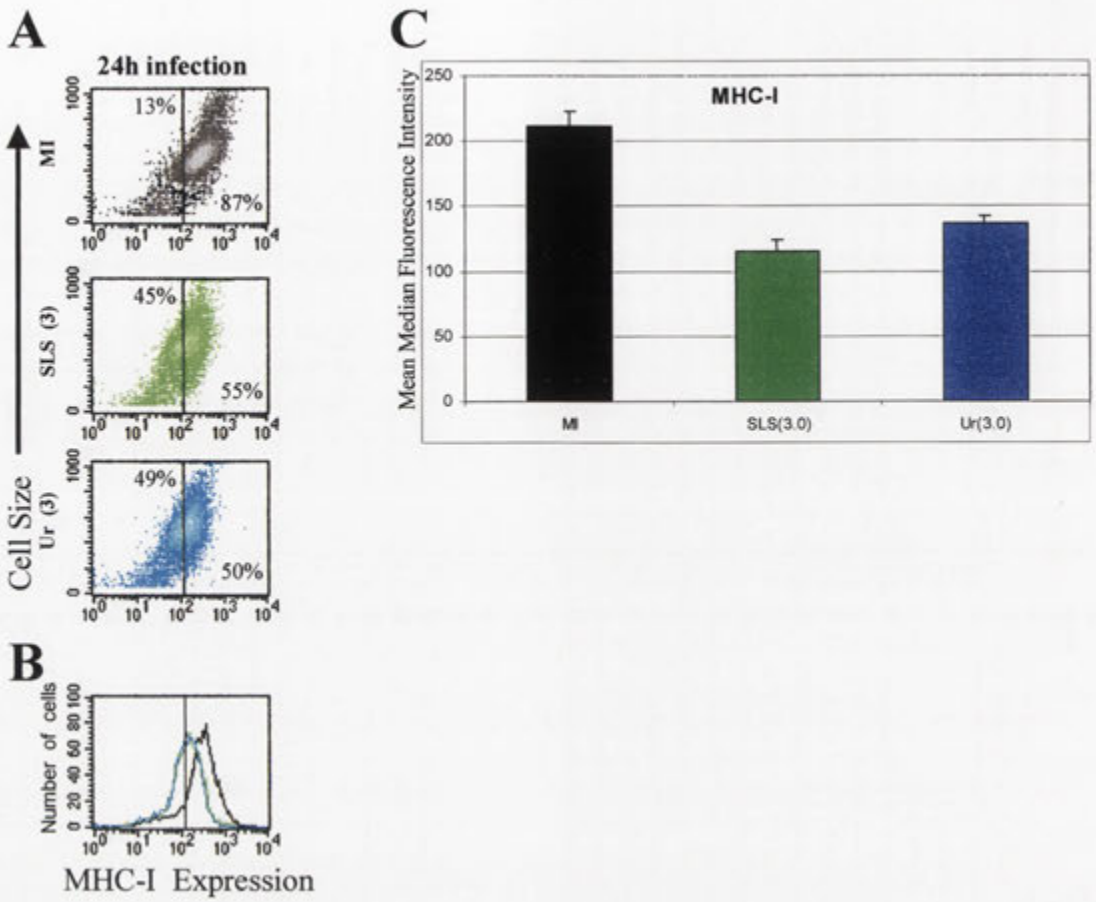


FIGURE 5.23. EXPRESSION OF MHC-I ON THE SURFACE OF RL-5 CELLS INFECTED WITH SLS OR UR

RL-5 cells were mock-infected (MI)■ or infected with SLS■ or Ur■ at a moi of 3, for 24 h. Expression of MHC-I was analysed by flow cytometry. The experiment was undertaken on three separate occasions (one shown here). Data is presented as (A) density plots of cell size versus protein expression and (B) histogram overlays of protein expression. A black line has been drawn to distinguish sub-populations expressing high and low levels of MHC-I. (C) The mean median fluorescence intensity corresponding to MHC-I for each infection was calculated for the three separate occasions.

Neither expression of CD25 nor expression of the KEN-5 marker has been previously examined in cell lines infected with myxoma virus. Infection with SLS or Ur decreased CD25 expression on the surface of RL-5 cells (Figure 5.26). This can be seen in the density plots for CD25 expression, where 51% of mock-infected RL-5 cells express high levels of CD25 compared to 30% and 34% of RL-5 cells infected with SLS or Ur respectively. The decrease in CD25 expression on RL-5 cells was also observed on the histogram plots and after analysis of mean median fluorescence intensity (Figure 5.26B and 5.26C), but was not statistically significant at the 95% CI.

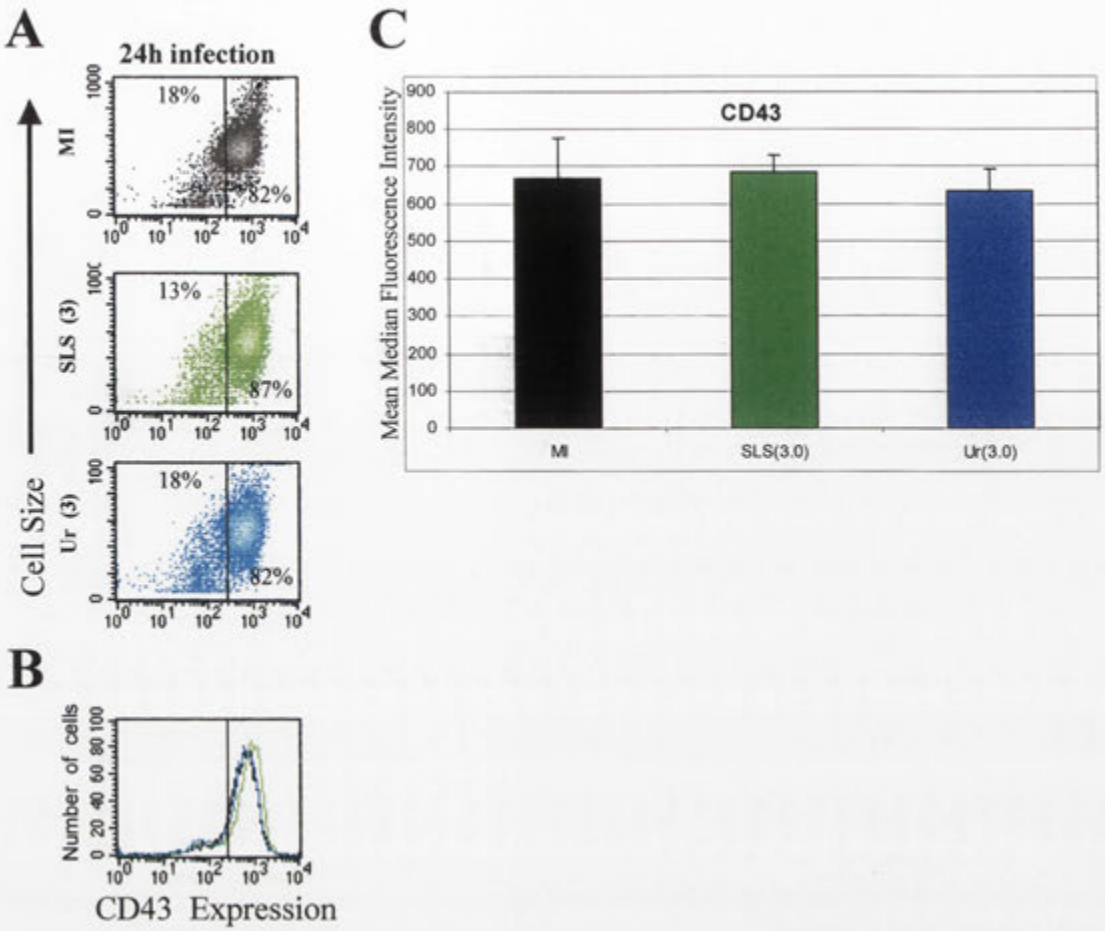


FIGURE 5.24. EXPRESSION OF CD43 ON THE SURFACE OF RL-5 CELLS INFECTED WITH SLS OR UR

RL-5 cells were mock-infected (MI)■ or infected with SLS■ or Ur■ at a moi of 3, for 24 h. Expression of CD43 was analysed by flow cytometry. The experiment was undertaken on three separate occasions (one shown here). Data is presented as (A) density plots of cell size versus protein expression and (B) histogram overlays of protein expression. A black line has been drawn to distinguish sub-populations expressing high and low levels of CD43. (C) The mean median fluorescence intensity corresponding to CD43 for each infection was calculated for the three separate occasions.

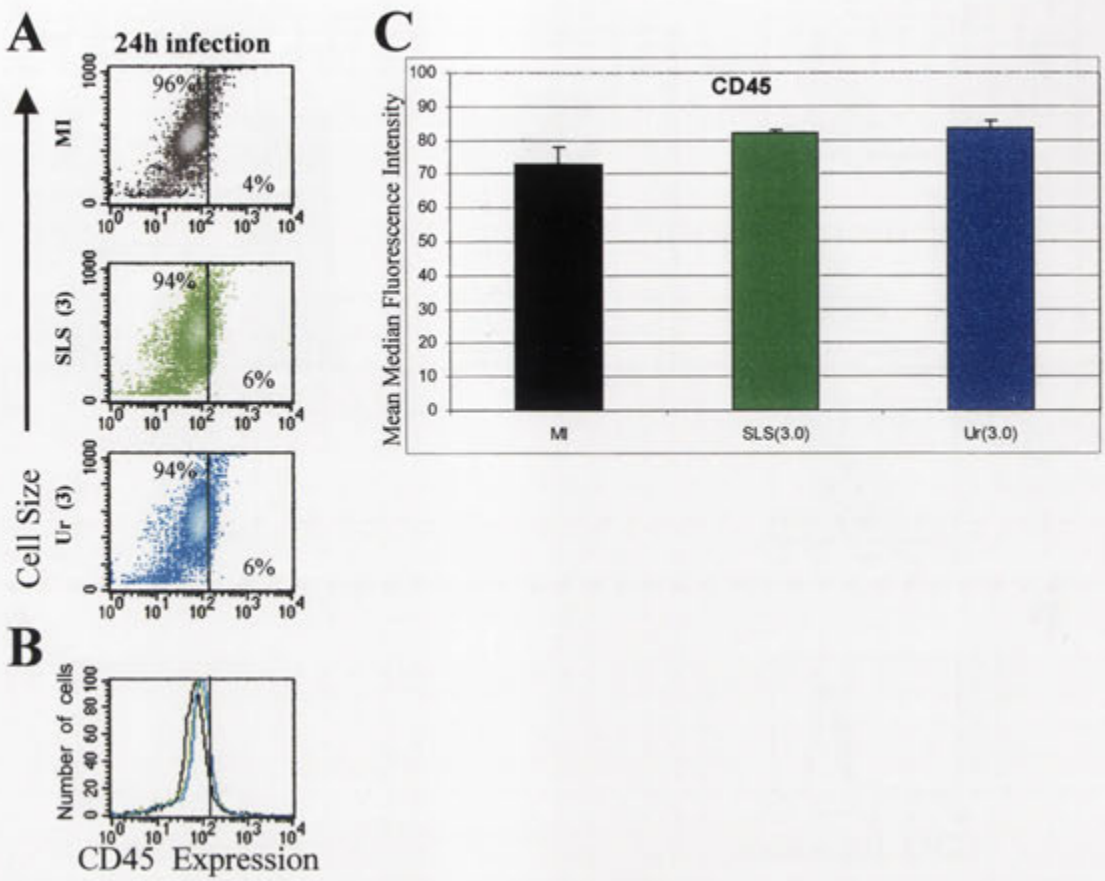


FIGURE 5.25. EXPRESSION OF CD45 ON THE SURFACE OF RL-5 CELLS INFECTED WITH SLS OR UR

RL-5 cells were mock-infected (MI)■ or infected with SLS■ or Ur■ at a moi of 3, for 24 h. Expression of CD45 was analysed by flow cytometry. The experiment was undertaken on three separate occasions (one shown here). Data is presented as (A) density plots of cell size versus protein expression and (B) histogram overlays of protein expression. A black line has been drawn to distinguish sub-populations expressing high and low levels of CD45. (C) The mean median fluorescence intensity corresponding to CD45 for each infection was calculated for the three separate occasions.

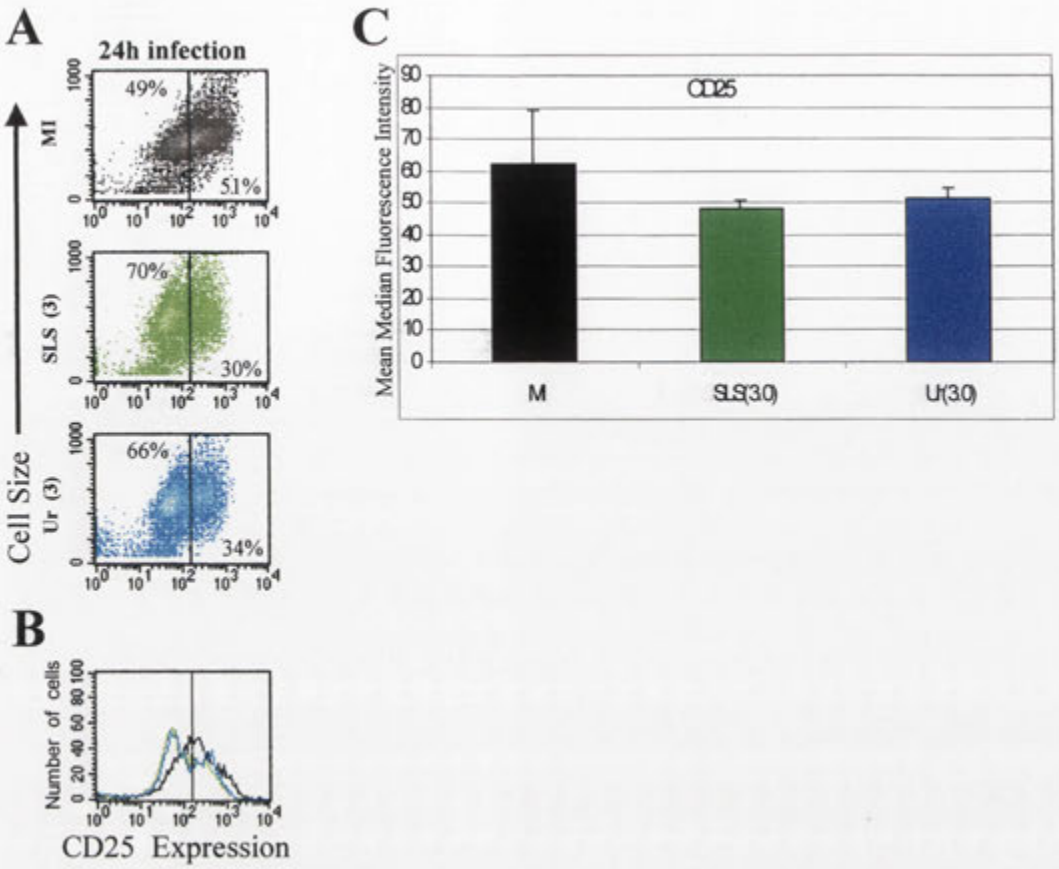


FIGURE 5.26. EXPRESSION OF CD25 ON THE SURFACE OF RL-5 CELLS INFECTED WITH SLS OR UR

RL-5 cells were mock-infected (MI)■ or infected with SLS■ or Ur■ at a moi of 3, for 24 h. Expression of CD25 was analysed by flow cytometry. The experiment was undertaken on three separate occasions (one shown here). Data is presented as (A) density plots of cell size versus protein expression and (B) histogram overlays of protein expression. A black line has been drawn to distinguish sub-populations expressing high and low levels of CD25. (C) The mean median fluorescence intensity corresponding to CD25 for each infection was calculated for the three separate occasions.

A decrease in KEN-5 expression on the surface of RL-5 cells by SLS and Ur infection was only evident when mean median fluorescence intensity was calculated (Figure 5.27). However, this decrease was not significant at the 95% CI.

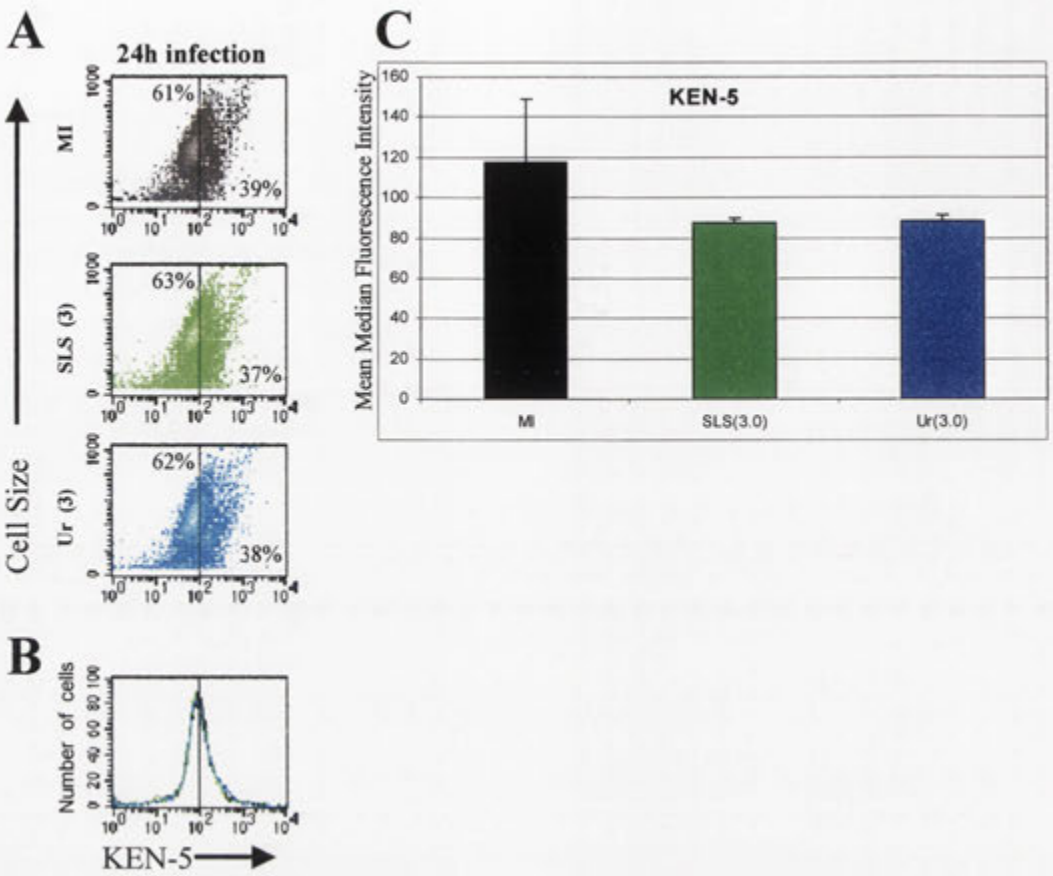


FIGURE 5.27. EXPRESSION OF KEN-5 ON THE SURFACE OF RL-5 CELLS INFECTED WITH SLS OR UR

RL-5 cells were mock-infected (MI)■ or infected with SLS■ or Ur■ at a moi of 3, for 24 h. Expression of KEN-5 was analysed by flow cytometry. The experiment was undertaken on three separate occasions (one shown here). Data is presented as (A) density plots of cell size versus protein expression and (B) histogram overlays of protein expression. A black line has been drawn to distinguish sub-populations expressing high and low levels of KEN-5. (C) The mean median fluorescence intensity corresponding to KEN-5 for each infection was calculated for the three separate occasions.

5.2.8 Sub-population of Lymphoid Cells Responding to Con A

Chapter 3 showed distinct large and small cell sub-populations after Con A stimulation. Section 5.2.1 and Section 5.2.4 showed the large and small cell sub-populations expressed different levels of the one protein. However, the analysis in this thesis so far has not considered how the sub-population of lymphoid cells in a culture change with Con A activation. Therefore cell surface protein expression in large and small cell sub-populations was investigated in mock-infected cell cultures to determine the sub-

population that responded to Con A. Density plots of cell size versus protein expression for lymphoid cell cultures are shown in Figure 5.28. The results obtained were similar between lymphoid cell cultures from laboratory and wild rabbits and are only shown for laboratory rabbits.

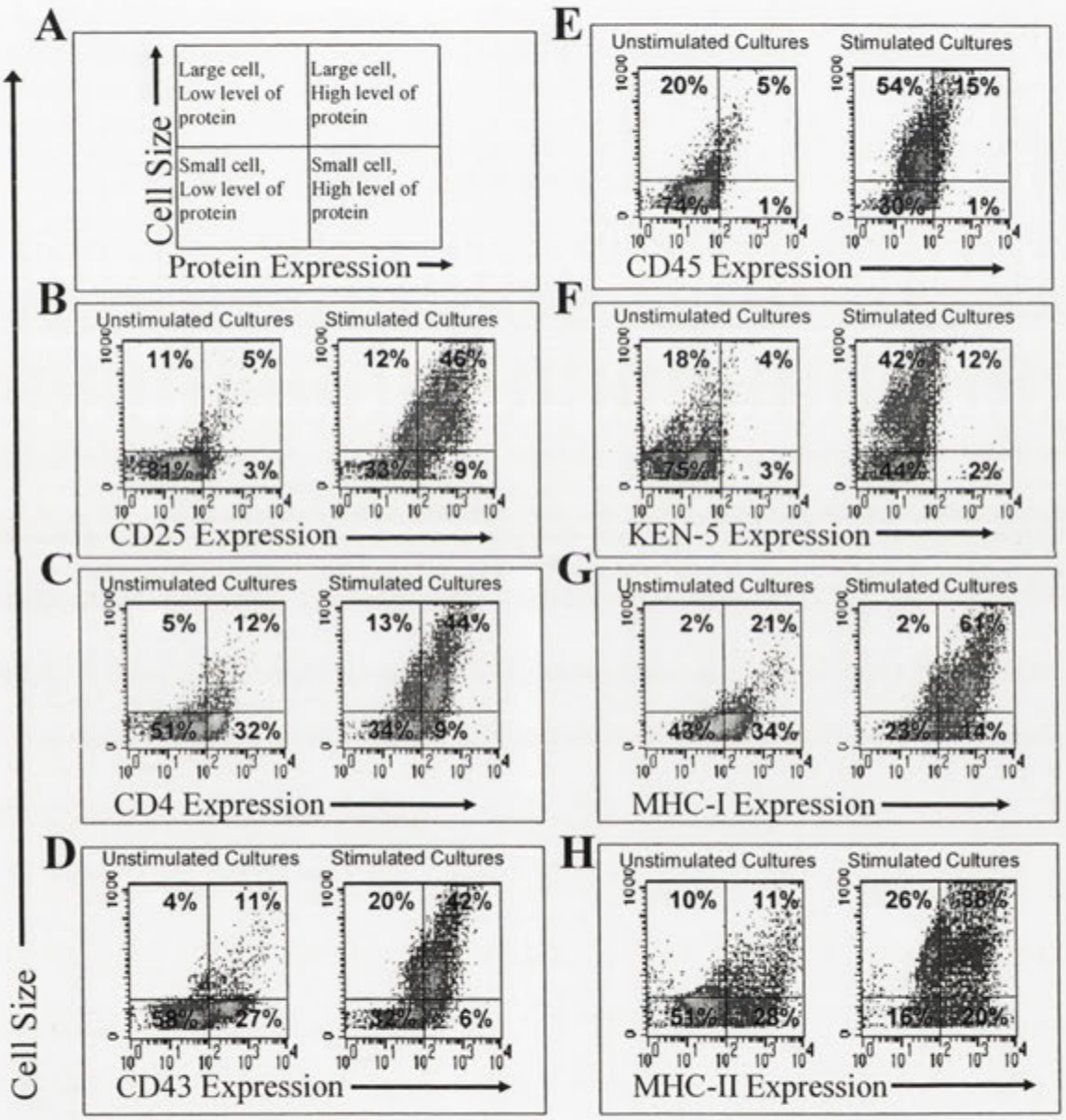


FIGURE 5.28. SUB-POPULATION OF LYMPHOID CELLS THAT RESPONDED TO CON A
Lymphoid cell cultures from laboratory rabbits were mock-infected and incubated with or without Con A stimulation. (A) Samples were divided into quadrants based on cell size and level of cell surface protein expression. Cell size was plotted against binding of (B) CD25, (C) CD4, (D) CD43, (E) CD45, (F) KEN-5, (G) MHC-I and (H) MHC-II antibodies. Expression was analysed through flow cytometry and the plots generated using CellQuest software. The experiment was repeated three times with one replicate shown here. Numbers represent the mean percentage of cells in each quadrant from the three experiments.

Identical quadrants were drawn on density plots of cell size versus protein expression, to define four sub-populations; (1) large cells expressing high levels of protein, (2) large cells expressing low levels of protein, (3) small cells expressing high levels of protein and (4) small cells expressing low levels of protein (Figure 5.28A). For protein expression, cells with a fluorescence intensity greater than 10^2 units were classified as expressing high levels of protein and cells with a fluorescence intensity less than 10^2 units were classified as expressing low levels of protein. For cell size, the cut off for large and small cells was set at 250 units. These gates appeared to reasonably divide the four populations.

From these results it could be hypothesized that the large cell sub-population following Con A stimulation of lymphoid cell cultures is derived from small cells expressing high levels of CD4 and CD43. The proportion of all cells with low levels of CD4 expression in unstimulated cell cultures (56%) was similar to that when cultures were stimulated (47%) (Figure 5.28C). Likewise, the proportion of all cells with low CD43 expression was 62% in unstimulated cultures and 52% in stimulated cultures. Overall, the proportion of cells expressing high levels of CD4 was not greatly altered by Con A stimulation with 44% and 53% of cells expressing high levels of CD4 in unstimulated and stimulated cultures respectively. This indicates that cells with high levels of CD4 expression are not altering the level of CD4 expression, rather they are just altering their size. In a similar manner, an argument for cells with high levels of CD43 expression being the sub-population that responded to Con A can be made (Figure 5.28D). This indicates that the large cells have higher levels of CD4 and CD43 compared to the small cells and that the large cell sub-population induced by Con A stimulation is mostly derived from the small cell sub-population that expresses high levels of CD4 and CD43,

although some of these cells are also derived from cells in the small sub-population that express low levels of CD4.

As shown previously, the large, and thus high CD4 and CD43 expressing cells, also had increased expression of CD25 (Figure 5.28B) and MHC-I (Figure 5.28G). CD45 and KEN-5 were not expressed on any specific sub-population in mock-infected cell cultures. However, cells in cultures examined for CD45 or KEN-5 expression did respond to Con A as shown by the increase in cell size in these samples (Figure 5.28E and 5.28F).

Antibodies specific for rabbit MHC-II recognize B-cells and activated T-cells (reviewed in Mage 1998). B-cells were not removed from the primary lymph node preparations used for the experiments and are not expected to respond to Con A. It is therefore reasonable to assume the small, high MHC-II expressing cells (lower right quadrant) in unstimulated cell cultures are most likely B-cells. The proportion of cells in this sub-population was similar both before (28%) and after (20%) Con A stimulation but was less than the estimated proportion of B-cells in rabbit lymph node preparations of 37% (Mage 1998). The large cell sub-population, which increased in number with the addition of the T-cell specific mitogen, Con A, was expected to be predominantly activated T-cells. The density plot of cell size versus MHC-II expression showed that approximately 38% of cells are large and bind high levels of the MHC-II antibody (Figure 5.28H).

In conclusion, these results thus suggest that small cells expressing high levels of CD4 and CD43 respond to Con A by increasing their cell size and expression of CD25, MHC-I and MHC-II.

5.3 Discussion

This chapter describes the expression of cell surface proteins in primary rabbit lymphocytes infected with myxoma virus. The first aim of this chapter was to investigate if differences existed in the cell surface expression of CD25, CD4, CD43, CD45, KEN-5, MHC-I and MHC-II between unstimulated and stimulated lymphocytes from susceptible and resistant rabbits. Unstimulated lymphocytes from wild rabbits had greater expression of CD4 and CD43 than those from laboratory rabbits and stimulated lymphocytes from wild rabbits had greater MHC-I expression than those from laboratory rabbits. This differentiated lymphocytes from the two rabbit types. When mock-infected cell cultures were stimulated with Con A, expression of CD25 (lymphocytes from both rabbit types), MHC-I (lymphocytes from laboratory rabbits), and MHC-II (lymphocytes from wild rabbits) was significantly increased on the surface of activated lymphocytes.

The second aim was to examine the effects of myxoma virus infection on expression of these cell surface proteins. Myxoma virus infection down-regulated CD25 and MHC-I expression in primary rabbit lymphoid cell cultures and in RL-5 cell cultures. Further analysis showed that this was significant on large cells in primary cultures. Myxoma virus also down-regulated pre-existing CD4 molecules on large cells when cultures were stimulated with Con A and on the surface of RL-5 cells. Infection with myxoma virus did not alter expression of CD25 or MHC-I on the surface of lymphocytes activated prior to infection. There was no difference between SLS and Ur in their ability to alter cell surface marker expression.

5.3.1 Experimental Approach

Three methods of analysis were used to examine cell surface protein expression in rabbit lymphocytes. The first of these was density plots of cell size versus protein expression. These plots were useful as they allowed a clear overview of the broad sub-populations that exist within lymphoid cell cultures, with respect to cell size and protein expression. The second method of analysis was by histogram overlays. Overlaying histograms of protein expression from different infections on the one graph allowed direct comparison of the different infections. An advantage of this method of analysis was being able to immediately see that both SLS and Ur had similar effects on the expression levels of proteins.

The final method for analysis of cell surface proteins was mean median fluorescence intensity. The median fluorescence intensity gives an estimate of protein expression on individual cells. This method has been essential for determining expression levels in cell sub-populations that only constituted a minor proportion of the entire culture. Unlike the first two methods, this analysis averages results over a group of three rabbits. By combining these three methods a thorough examination of protein expression on the cell surface was undertaken.

5.3.2 Expression of Specific Markers on the Lymphocyte Surface

Lymphocytes can be differentiated based on cell surface expression of specific proteins. Experiments in this thesis utilized lymphoid cells prepared from rabbit lymph nodes. Rabbit lymph nodes contain approximately 57% T-cells and 37% B-cells (reviewed in Mage 1998). Cultures prepared from lymph nodes also contain phagocytic cells as well

as some stromal and endothelial cells. However, in this thesis, the majority of these cells were removed from the lymphoid cell cultures prior to all experiments. Thus, the cell preparations used in experiments were primarily composed of T- and B-cells.

Cells that responded to the T-cell mitogen, Con A, as shown by an increase in cell size, were those that initially had high levels of CD4 and CD43. CD4 expression defines a subset of T-cells and CD43 is expressed on T-cells and thymocytes (reviewed in Mage 1998). Thus, the fact that cells with high expression levels of these proteins responded to the T-cell mitogen, Con A, was not unexpected. The proportion of cells in activated cultures expressing high levels of CD4 (53%) was not too dissimilar to the expected proportion of T-cells (57%). This suggests that most of the T-cells in popliteal lymph node preparations may be $CD4^+$ T-cells as opposed to $CD8^+$ T-cells. Alternatively, some $CD8^+$ T-cells may also express high levels of CD4, although current hypotheses of T-cell differentiation deem this unlikely (reviewed in Germain 2002), or more likely the proportion of B-cells in the culture may not be as high as the previous estimates of 37%. From this thesis, if all cells in unstimulated lymphoid cell cultures expressing MHC-II are presumed to be B-cells, then B-cells constituted approximately 28% of the culture.

A subset of activated T-cells in the cell culture also had increased expression of CD25, MHC-I and MHC-II. CD25, the IL-2 receptor α-chain, is one of the key markers of T-cell activation. The MHC-II antigen is also expressed on activated T-cells (reviewed in Mage 1998). Hence, it was not surprising to find high levels of CD25 and MHC-II expression on the activated T-cell sub-population. Small cells did not have substantially altered cell surface marker expression irrespective of stimulation or virus infection.

The increase in MHC-I expression when cells were stimulated may be postulated to lead to enhanced antigen presentation of intracellular-derived antigen simply due to a greater numbers of MHC-I molecules on the cell surface. This may lead to greater killing of infected lymphocytes and thus less chance for virus to replicate and disseminate. Alternatively, it may be postulated that the higher levels of MHC-I may be required to prevent NK cell targeting. In a simple model, NK cells trigger apoptosis in cells that express abnormally low MHC-I levels (reviewed in Parkin and Cohen 2001). A quiescent T-cell will express a particular level of MHC-I. If this cell is activated then it will increase its size. For the MHC-I levels to remain constant per unit volume and thereby minimizing the chance of NK cell attack, then expression of MHC-I must also be increased. In this model, the infected cell will not be targeted for killing and will thus be able to disseminate the virus. Obviously in this model there will be other considerations, for example, the involvement of membrane rafts, which may not distribute MHC-I molecules across the lymphocyte surface (Carrie Miceli et al., 2001; Goot and Harder 2001).

5.3.3 Implications of Different Levels of Lymphocyte Surface Proteins for the Immune Response

The mechanism of T-cell activation is different depending on the history of the particular T-cell. For example, naïve T-cells (T-cells that have not been exposed to a specific pathogen) require high levels of antigen, sustained signaling and co-stimulation for effective activation (reviewed in Lanzavecchia and Sallusto 2000; Guermonprez et al., 2002). In contrast, T-cells that have previously been exposed to antigen require only short periods of stimulation and low levels of antigen. The sustained signaling needed to prime naïve T-cells requires continuous engagement of the T-cell receptor and MHC/peptide complex (reviewed in Lanzavecchia and Sallusto 2000; Guermonprez et

al., 2002). The time available for sustained signaling is dependent on adhesion molecules and co-stimulatory interactions. If adhesion is increased between the cells then a stable interaction will be formed leading to a potentially longer signaling time. Similarly, an increase in co-stimulation will decrease the time required for activation.

The higher levels of cell surface expression of CD4, CD43, in the absence of Con A, and MHC-I in the presence of Con A, in lymphoid cell cultures from wild rabbits when compared to cultures from laboratory rabbits, may have important consequences for activation of naïve T-cells *in vivo*. For instance, CD4 is a co-stimulatory molecule and an increase in its expression increases the sensitivity of T-cells to antigen presented in association with MHC-II (reviewed in van Parjis and Abbas 1998). An elevated level of CD4 expression may thus help reduce the time required for priming of naïve T-cells by increasing the opportunities for co-stimulation. CD43, on the other hand, is involved in cell adhesion during the interaction between T-cells and antigen presenting cells (reviewed in Ostberg et al., 1998). Increased CD43 levels on the surface of T-cells may help the formation of stable cell-cell interactions, again decreasing the time required for T-cell activation and increasing the probability of effective and sufficient signaling.

Taken together, the increase in expression of CD4 and CD43 in lymphoid cell cultures from wild rabbits compared to laboratory rabbits may allow a more effective and efficient immune response against myxoma virus. The greater expression of MHC-I on the surface of activated lymphocytes from wild rabbits may increase presentation of viral antigen on the surface of infected cells. These results may contribute to resistance of rabbits against myxoma virus.

5.3.4 CD25 Expression in Myxoma Virus Infection

Previous chapters have shown myxoma virus inhibited T-cell activation in two ways: inhibition of the increase in cell size and inhibition of cell proliferation by G1/G0 cell cycle arrest. Signaling through the IL-2 receptor is essential for T-cell proliferation and activation and IL-2 receptor signaling mediates the transition from G1/G0 into S phase (Cantrell and Smith 1984). Therefore, the down-regulation of CD25 expression in primary cultures, and RL-5 cells, infected with myxoma virus may curtail signaling through the IL-2 receptor leading to the cell cycle arrest observed. Thus down-regulation of CD25 may be a key mechanism by which myxoma virus inhibits T-cell activation.

5.3.5 Cell Surface Protein Expression During Myxoma Virus Infection

This thesis examined the differences between cell surface marker expression in the RL-5 cell line and in primary lymphoid cell cultures. Myxoma virus has been previously shown to down-regulate CD4 and MHC-I on RL-5 cells (Barry et al., 1995) and MHC-I on BGMK cells (Boshkov et al., 1992). These cell lines are continuously activated and proliferating. Results from this chapter show myxoma virus can also down-regulate CD4 and MHC-I proteins in primary rabbit lymphoid cell cultures. CD4 expression on large cells was only down-regulated when cell cultures were activated, suggesting that activated cell signaling pathways and their down-stream effects may be essential for this process. Alternatively, enhanced myxoma virus replication, which is increased after activation of T-cells, may be responsible for the CD4 down-regulation. The experiments undertaken in this thesis were not able to differentiate between these possibilities.

The down-regulation of CD4 on large cells in primary cell cultures may have important consequences *in vivo.* For instance, high levels of CD4 expression on activated T-cells will enable more effective co-stimulation to occur as it will increase the sensitivity of T-cells to antigen presented in association with MHC-II (van Parjis and Abbas 1998). It has previously been postulated that the down-regulation of CD4 on lymphocytes infected with myxoma virus would cause the T-cells to become less capable of responding to exogenously produced antigen (Barry et al., 1995). This may suppress the anti-viral immune response.

Recently, a model has been proposed for the myxoma virus infection-induced down-regulation of CD4 and MHC-I (Mansouri et al., 2003). CD4 and MHC-I are synthesised in the endoplasmic reticulum and travel through the Golgi apparatus to be presented on the cell surface. However, in myxoma virus-infected cells, once these proteins are at the cell surface they are internalised and sorted to intracellular vesicles. This internalisation was proposed to be induced by the myxoma virus protein, MV-LAP (Mansouri et al., 2003). This is contrary to the results observed in primary cells in this thesis, where SLS and Ur inhibited the up-regulation of MHC-I induced by Con A stimulation, but could not down-regulate pre-existing MHC-I molecules if the cells had been stimulated prior to infection.

As both SLS and Ur were shown in chapter 4 to have nucleotide sequences for MV-LAP (M153 ORF) identical to the published Lu sequence, one explanation for the difference is that experiments in this thesis were not sensitive enough to detect down-regulation of MHC-I by SLS and Ur. Alternatively, the differences may be due to the activation state of the cells. For instance, stimulation of primary cells was required for the down-regulation of CD4. Furthermore, in agreement with published results (Barry et

al., 1995), down-regulation of CD4 and MHC-I was detected in RL-5 cells, which are continuously proliferating. This thesis also provides evidence that cell lines act differently to primary cells. For instance, RL-5 cells maintain a uniform size, whereas primary cells increase their size depending on their activation state. The findings of Mansouri et al. (2003) were based on studies using cell lines, which may account for the differences observed.

The results presented in this chapter have demonstrated that myxoma virus modulates cell surface expression of lymphocyte proteins critical for T-cell activation and the immune response and is the first demonstration of this in primary rabbit lymphocytes. Changing the expression levels of cell surface proteins could significantly disrupt or enhance the anti-viral immune response and may be involved in resistance of rabbits to myxomatosis. Another way in which myxoma virus may modulate the immune response is through altering the profile of cytokine expression. The effects of myxoma virus infection on the cytokine expression will be further examined in chapter 6.

Chapter 6

Production and Characterization of Antibodies Specific for Rabbit Cytokines

6.1 INTRODUCTION

The results in the previous chapters have shown that myxoma virus can alter the activation of lymphocytes, including inhibition of lymphocyte proliferation and modulation of the cell surface expression of proteins involved in regulation of the immune response. These actions will potentially disrupt T-cell function. T-cells have been shown to be critical in the immune response to other poxvirus infections. For example, in mice infected with ectromelia virus, both $CD4^+$ and $CD8^+$ T cells are critical for effective control and clearance of virus (Karupiah et al., 1996; Karupiah 1998). In ectromelia virus infection, induction of an effective cell-mediated immune response depended on the profile of cytokines produced and in particular, expression of the T_H1-inducing cytokines, IFNγ and IL-2. Expression of the T_H2-inducing cytokines, such as IL-4 without the co-expression of T_H1 cytokines, was associated with a failure to clear virus infection in this model (Karupiah 1998).

One common method used to measure cytokine levels is to examine mRNA expression. Analysis of mRNA expression is particularly useful in tissues, and is possible in rabbits as the cytokines have been cloned and sequenced. However, Karupiah (1998) reported that mRNA levels of IL-2, IFNγ and TNFα in lymphoid organs did not reflect the levels of protein seen using immunocytochemistry. This suggested that post-transcriptional regulation of these cytokines occurs *in vivo*, and so mRNA levels are not representative of changes in cytokine expression in mice. Post-translational regulation of other cytokines, for example IL-12 (Carra et al., 2000), has also been shown previously.

The alternative to measurement of cytokine mRNA expression is to assay cytokine protein levels. To do this, antibodies that are specific for the cytokine under

examination are required. Unfortunately, antibodies directed to rabbit cytokines are not commercially available and so a similar analysis can not be readily undertaken.

The aim of the work described in this chapter is to produce and characterize antibodies to rabbit IL-2 and IFNγ, as representative T_H1 cytokines, and rabbit IL-4, IL-6 and IL-10, as representative T_H2 cytokines. The cDNAs for these rabbit cytokines have been cloned and sequenced (Perkins et al., 2000) and so can be used to design synthetic peptides for antibody production or, alternatively, cloned into protein expression vectors for production of protein for use in antibody preparation. In this study, synthetic peptides corresponding to potential epitopes on these cytokines, were designed from the published sequence data. Rats (and chickens for the IL-2 synthetic peptides) were inoculated with the synthetic peptides conjugated to the immunogenic carrier protein mariculture keyhole limpet hemocyanin (mcKLH). The specificity of the anti-peptide sera generated was examined by immunofluorescence and immunoblot assays, using rabbit cytokines produced in a mammalian expression system as antigen. In addition, recombinant myxoma viruses expressing rabbit cytokines previously constructed in this laboratory were tested to confirm cytokine production from these viruses.

The application of the antibodies produced for analysis of cellular localization of cytokine expression during myxoma virus infection was investigated by flow cytometric analysis of RL-5 cells and by immunohistochemical analysis of lymph node sections from laboratory and wild rabbits infected with myxoma virus. The results reported here demonstrate that the antibodies generated in this work provide the basis for more extensive and detailed studies characterizing cytokine expression during myxoma virus infection of rabbits.

6.2 RESULTS

6.2.1 Production of Anti-Peptide Sera Specific for Rabbit Cytokines

6.2.1.1 Identification of Peptides with Potential Immunogenicity

Cloning and sequencing of rabbit IL-2, IL-4, IL-6, IL-10 and IFNγ (accession numbers gi:2804256, gi:4432961, gi:230694) have been previously described (Perkins et al., 2000). The predicted rabbit amino acid sequence was compared to cytokine sequences from other species and the 3-dimensional human model for each cytokine was examined in order to identify at least two potentially immunogenic regions (12-20 amino acids) for each cytokine (Dr H. Perkins, School of Biochemistry and Molecular Biology, ANU). These regions were selected so as to have as little homology as possible to the amino acid sequence of other species. Potential immunogenic regions were either linear surface exposed regions of the protein, such as a loop, or the surface of an exposed helix that would be likely to have its secondary structure retained in the peptide.

Figure 6.1A shows the amino acid alignment of rabbit, rat, chicken and human IL-2 and the regions corresponding to the selected peptide sequences. The predicted 3-dimensional model of human IL-2 is shown (Figure 6.1B) with the locations of the helix B and C/D loop synthetic peptides identified. The peptides are both exposed regions on the molecule. Similarly, alignments of rabbit, rat and human cytokine amino acid sequence and modeling of the synthetic peptide sequence on 3-dimensional models of human IL-4 (Figure 6.2), IL-6 (Figure 6.3), IL-10 (Figure 6.4) and IFNγ (Figure 6.5) were undertaken. A list of the synthetic peptide sequences selected for synthesis and antibody production is shown in Table 6.1.

A

```
Rabbit      -MYKVQLLSCIALTLALLTSSAPTSSS---TKETQEQLDQLLLDLQVLLKGVNDYKNSKL 56
Rat         -MYSMQLASCVALTLVLLVNSAPTSSP---AKETQQHLEQLLLDLQVLLRGIDNYKNLKL 56
Chicken     MMCKVLIFGCISVAMLMTTAYGASLSS-----AKRKPLQTLIKDLEILEN---------I 46
Human       -MYRMQLLSCIALILALVTNSAPTSSSTKKTKKTQLQLEHLLLDLQMILNGINNYKNPKL 59

Rabbit      SRMLTFKFYMPKKVTELKH--LQCLEEELKPLEEVLNLAQGKNSHGGNTRESISNINVTV 114
Rat         PMMLTFKFYLPKQATELKH--LQCLENELGALQRVLDLTQSKSFHLEDAGNFISNIRVTV 114
Chicken     KNKIHLELYTPTETQECTQQTLQCYLGEVVTLKKETEDDTEIKEEFVTAIQNIEKNLKSL 106
Human       TRMLTFKFYMPKKATELKQ--LQCLEEELKPLEEVLNLAQSKNFHLR-PRDLISNINVIV 116

Rabbit      LKLKGSE-TFMCEY-DETVTIVEFLNRWITFCQSIISASSS 153
Rat         VKLKGSENKFECQFDDEPATVVEFLRRWIAICQSIISTMTQ 155
Chicken     TGLNHTGSECKICEANNKKKFPDFLHELTNFVRYLQK---- 143
Human       LELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLT- 156
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B

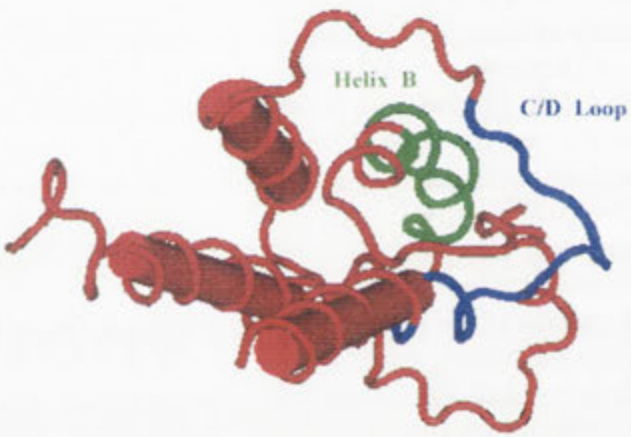

FIGURE 6.1. SEQUENCE ALIGNMENT AND MODEL OF IL-2
(A) The amino acid sequence alignment of rabbit, rat, chicken and human IL-2, with the helix B peptide sequence in green and the C/D loop peptide sequence in blue. (B) A 3D model of human IL-2 shows the location of these peptides, with the helix B peptide in green and the C/D loop peptide in blue. Sequences and model were obtained from Genbank.

A

```
Rabbit      MGLPAQLPVTLLCLLAGTAHFIQGRRGDIILPEVIKTLNILTERKTPCTKLMIADALAVP 60
Rat         MGLSPHLAVTLFCFLICTGNGIHGCN-DSPLREIINTLNQVTEKGTPCTEMFVPDVLTAT 59
Human       MGLTSQLLPPLFFLLACAGNFVHGHKCDITLQEIIKTLNSLTEQKTLCTELTVTDIFAAS 60

Rabbit      KNTTEREAVCRAATALRQFYLHHK--------VSWCFKEHGELGDLRLLRGLDRNLCSMA 112
Rat         RNTTENELICRASRVLRKFYFPRD--------VPPCLKNKSGV--LGELRKLCRGVSGLN 109
Human       KNTTEKETFCRAATVLRQFYSHHEKDTRCLGATAQQFHRHKQL--IRFLKRLDRNLWGLA 118

Rabbit      KLSNCPGKEARQTTLEDFLDRLKTAMQEKYSK-RQS-- 147
Rat         SLRSCTVNESTLTTLKDFLESLKSILRGKYLQSCTSMS 147
Human       GLNSCPVKEANQSTLENFLERLKTIMREKYSK-CSS-- 153
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B

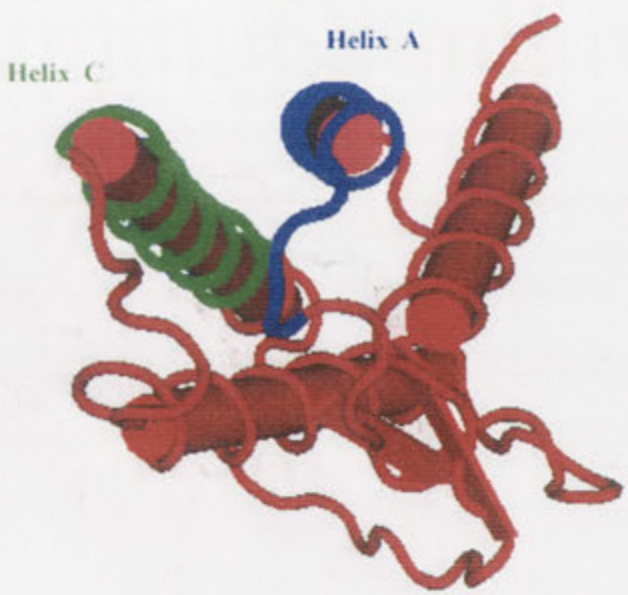

FIGURE 6.2. SEQUENCE ALIGNMENT AND MODEL OF IL-4

(A) An amino acid sequence alignment of rabbit, rat, and human IL-4. The helix C peptide sequence is shown in green and the helix A peptide sequence in blue. (B) A 3D model of human IL-4 shows the location of these peptides. Sequences and model were obtained from Genbank.

A

```
Rabbit          MNSFTSALRPGPLGCSLALLLVVATAFPTSAPVREDSNTKAS--PDKTLTPPGRTIESIR 58
Rat             MKFLS-ARDFQPVAF-LGLMLLTATAFPTSQVRRGDFTEDTT--HNRPVYTTSQVGGLIT 56
Human           MNSFS-TSAFGPVAFSLGLLLVLPAAFP-APVPPGEDSKDVAAPHRQPLTSSERIDKQIR 58

Rabbit          SILETIKELRKEMCDHDVNCMNRKEALAEVNLHLPRLIEEDGCFPPAVNNETCLLRITSG 118
Rat             YVLREILEMRKELCNGNSDCMNSDDALSENNLKLPEIQRNDGCFQTGYNQEICLLKICSG 116
Human           YILDGISALRKETCNKSNMCESSKEALAENNLNLPKMAEKDGCFQSGFNEETCLVKIITG 118

Rabbit          LMEFRMYLEHLQAKFRSDEENTRVSMVLKNIQHLIKTLRPKVKNLNEEATLKPAVAVSLM 178
Rat             LLEFRFYLEFVKNNLQ-DNKKDKARVIQSNTETLVHIFKQEIKDSYKIVLPTPTSNALLM 175
Human           LLEFEVYLEYLQNRFE-SSE-EQARAVQMSTKVLIQFLQKKAKNLDAITTPDPTTNASLL 176

Rabbit          ENLQQKNQWLKTTTIHFILRGLTNFLEFTLRAVDLMECGCPCLRNFMGSASHGQNTPSCP 238
Rat             EKLESQKEWLRTKTIQLILKALEEFLKVTMRSTRQT---------------------- 211
Human           TKLQAQNQWLQDMTTHLILRSFKEFLQSSLRALRQM---------------------- 212

Rabbit          LD 240
Rat             --
Human           --
```

B

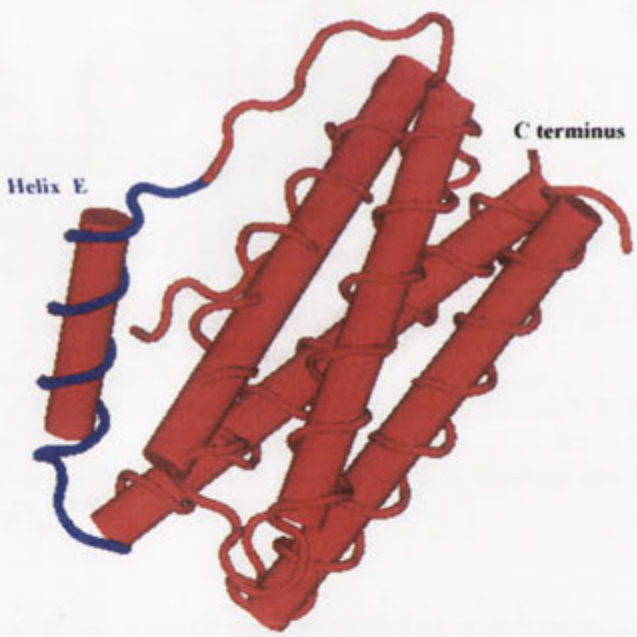

FIGURE 6.3. SEQUENCE ALIGNMENT AND MODEL OF IL-6

(A) An amino acid sequence alignment of rabbit, rat and human IL-6. The helix E peptide sequence is shown in blue and the tail peptide sequence is shown in green. (B) A 3D model of human IL-6 shows the location of the helix E peptide. The tail peptide (green) is not shown as this is contained in a region of the C-terminus of rabbit IL-6 that is not present in human IL-6. Sequences and model were obtained from Genbank.

A

```
Rabbit          MLSSALLCCLVFLGGTGASRGQDTPAENSCIHFPGGLPHMLRELRAAFGRVKTFFQSKDQ 60
Rat             MLGSALLCCLLLLAGVKTSKGHSIRGDNNCTHFPVSQTHMLRELRAAFSQVKTFFQKKDQ 60
Human           MHSSALLCCLVLLTGVRASPGQGTQSENSCTHFPGNLPNMLRDLRDAFSRVKTFFQMKDQ 60

Rabbit          LNSMLLTESLLEDLKGYLGCQALSEMIQFYLKDVMPQAENHSPAIREHVNSLGENLKTLR 120
rat             LDNIVLTDSLLQDFKGYLGCQALSEMIKFYLVEVMPQAENHGPEIKEHLNSLGEKLKTLW 120
Human           LDNLLLKESLLEDFKGYLGCQALSEMIQFYLEEVMPQAENQDPDIKAHVNSLGENLKTLR 120

Rabbit          LRLRQCHRFLPCENKSKAVEQVKSAFSKLQEEGVYKAMSEFDIFINYIETYMTMKIKS 178
Rat             IQLRRCHRFLPCENKSKAVEQVKNDFNKLQDKGVYKAMNEFDIFINCIEAYVTLKMKN 178
Human           LRLRRCHRFLPCENKSKAVEQVKNAFNKLQEKGIYKAMSEFDIFINYIEAYMTMKIRN 178
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B

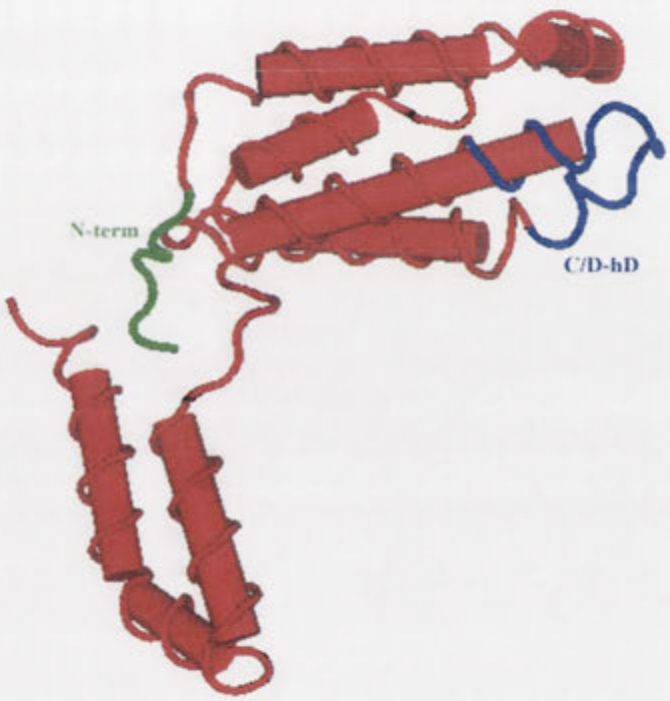


FIGURE 6.4. SEQUENCE ALIGNMENT AND MODEL OF IL-10

(A) The amino acid sequence alignment of rabbit, rat and human IL-10. The N-term peptide sequence is shown in green and the C/D-hD peptide sequence in blue. (B) A 3D model of human IL-10 shows the location of these peptides. Sequences and model were obtained from Genbank.

A

```
Rabbit          ---MSYTSYILAFQLCLILGSYGCYCQDTLTRETEHLKAYLKANTSDVANGGPLFLNILR 57
Rat             ---MSATRRVLVLQLCLMALS-GCYCQGTLIESLESLKNYFNSSSMDAMEGKSLLLDIWR 56
Human           ---MKYTSYILAFQLCIVLGSLGCYCQDPYVKEAENLKKYFNAGHSDVADNGTLFLGILK 57

Rabbit          NWKEESDNKIIQSQIVSFYFKLFDNLKDHEVIKKSMESIKEDIFVKFFNSNLTKMDDFQN 117
Rat             NWQKDGNTKILESQIISFYLRLFEVLKDNQAISNNISVIESHLITNFFSNSKAKKDAFMS 116
Human           NWKEESDRKIMQSQIVSFYFKLFKNFKDDQSIQKSVETIKEDMNVKFFNSNKKKRDDFEK 117

Rabbit          LTRISVDDRLVQRKAVSELSNVLNFLSPKSNLKKRKRSQTLFRGRRASKY 167
Rat             IAKFEVNNPQIQHKAVNELIRVIHQLSPESSLRKRKRSRC---------- 156
Human           LTNYSVTDLNVQRKAIHELIQVMAELSPAAKTGKRKRSQMLFRGRRASQ- 166
```

B

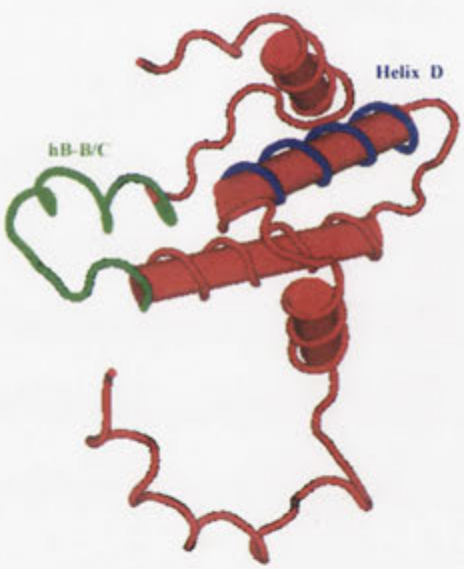


FIGURE 6.5. SEQUENCE ALIGNMENT AND MODEL OF IFNγ

(A) The amino acid sequence alignment of rabbit, rat and human IFNγ, with the hB-B/C peptide sequence in green and the helix D peptide sequence in blue. (B) A 3D model of human IFNγ shows the location of these peptides. Sequences and model were obtained from Genbank.

TABLE 6.1. SYNTHETIC PEPTIDE SEQUENCES

Synthetic Peptide	Sequence
IL-2 helix B	CLEEELKPLEEVLN
IL-2 C/D loop	CEYDETVTIVEFLN
IL-4 helix A	LPEVIKTLNILTERKTPC
IL-4 helix C	CFKEHGELGDLRLLRGLDRN
IL-6 helix E	LKPAVAVSLMENLQQKNQW
IL-6 tail	LRNFMGSASHGQNTPSC
IL-10 C/D-hD	PQAENHSPAIREHVNS
IL-10 Nterm	SRGQDTPAENSC
IFNγ helix D	KKSMESIKEDIFVK
IFNγ hb-B/C	LFLNILRNWKEESDNK

6.2.1.2 Conjugation of Synthetic Peptides to mcKLH

Each synthetic peptide was obtained as a dry powder from the Biomolecular Resource Facility (JCSMR, Canberra, Australia). For conjugation to the immunogenic carrier protein mcKLH, the peptides needed to be solubilised and so various solvents were tested. IL-6 tail, IL-10 C/D-hD, IL-10 N-term and IFNγ helix D peptides were soluble in the conjugation buffer supplied with the Imject® Maleimide Activated mcKLH kit. The IL-2 helix B and IFNγ hb-B/C peptides were dissolved in a 1:10 dilution of 7 M guanidine in conjugation buffer. The IL-6 helix E and IL-2 C/D loop peptides were partially dissolved in 7 M guanidine and the IL-4 helix A peptide were dissolved in 5 M guanidine. The IL-4 helix C peptide was only soluble in 20% DMSO.

The peptide solutions were conjugated to mcKLH and rats were inoculated with each conjugate. Sera was collected from all rats except those inoculated with the IL-4 helix C peptide conjugate that was fatal for inoculated rats. This may have been due to traces of DMSO in the inoculation.

6.2.1.3 Testing Rat Sera for Cytokine Specific Antibodies

The presence of cytokine-specific antibodies in the rat sera was tested by immunofluorescence assay of Vero cell monolayers transfected with pcDNA3.1 expressing each specific rabbit cytokine. Expression of the cytokine gene from pcDNA3.1 was under the control of the IE1 cytomegalovirus promotor, which allows expression of genes that have been put under the control of this promoter, in mammalian tissue culture systems. Each cytokine had been cloned into the plasmid in frame with a six Histidine tag (His-tag) at the C-terminus. Detection of the His-tag, using an anti-His monoclonal antibody, confirmed that the cytokines were being expressed in the tissue culture system.

Figure 6.6 shows the results of immunofluorescence assays screening for the presence of anti-rabbit cytokine antibodies. All pictures were taken with the same exposure time. Fluorescence was observed for sera from all rats inoculated with IL-4 helix A, IL-6 helix E, IL-6 tail, IFNγ helix D or IFNγ hb-B/C, indicating the sera contained cytokine-specific antibodies (only serum from one rat for each synthetic peptide is shown in Figure 6.6). Only one rat inoculated with IL-10 C/D-hD tested positive for rabbit IL-10-specific antibodies. No antibodies were detected in rats immunized with either of the IL-2 peptide conjugates or with the IL-10 N-term peptide conjugate. Variation was observed between transfections of pcDNA3.1 expressing different cytokines. To allow for this during testing of the rat sera the binding of each sera was compared to the anti-His positive control for that transfection. Anti-His positive controls were done for every transfection, but only the positive control for pcDNA3.1 IL-6 is shown in Figure 6.6.

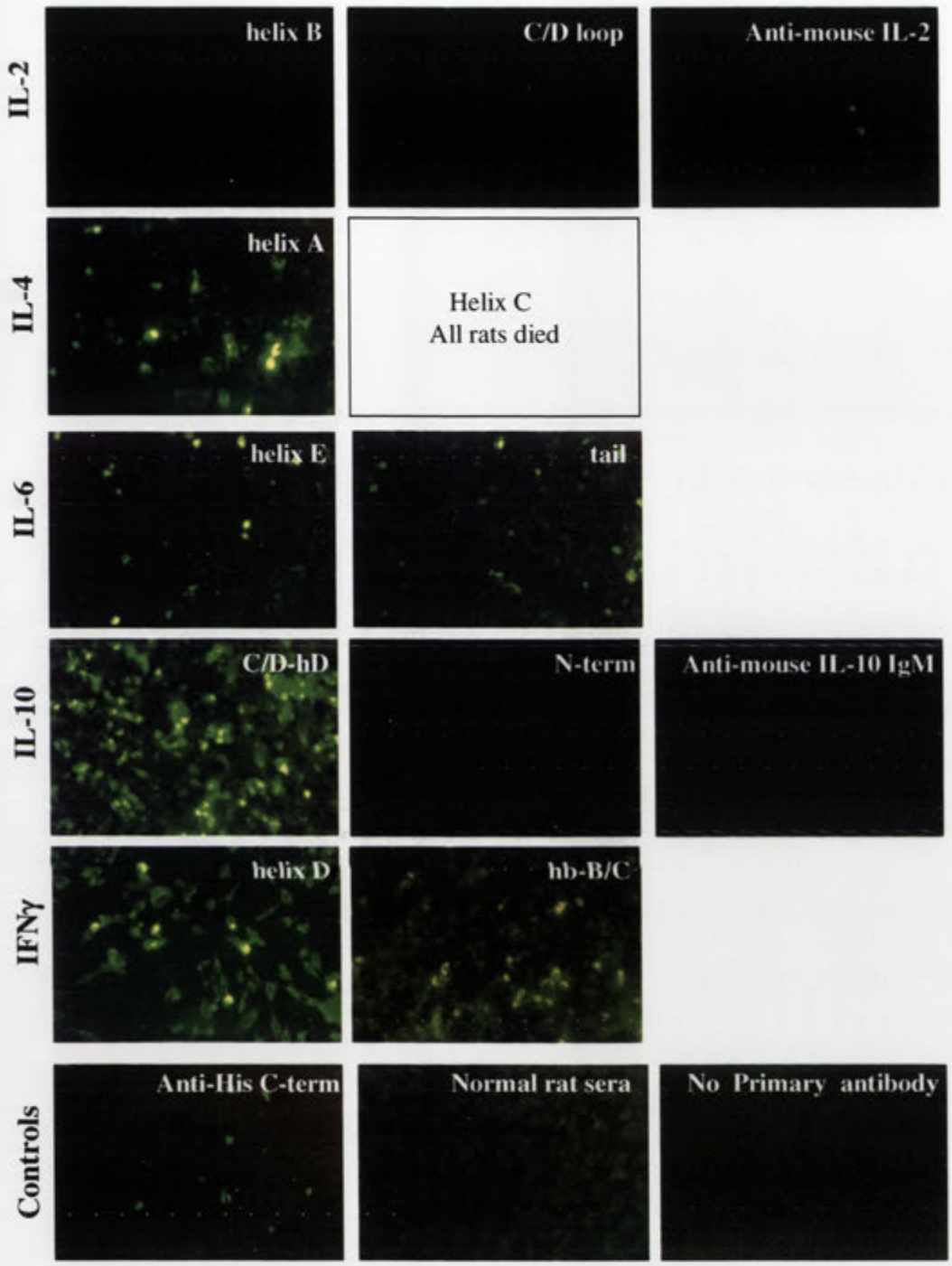


FIGURE 6.6. DETECTION BY IMMUNOFLUORESCENCE OF RABBIT CYTOKINE-SPECIFIC ANTIBODIES IN RAT SERA

Sera from rats immunised with the IL-2 helix B, IL-2 C/D loop, IL-4 helix A, IL-6 helix E, IL-6 tail, IL-10 C/D-hD, IL-10 N-term, IFNγ helix and IFNγ hb-B/C were tested for antibodies to the specific cytokine from which the peptide was derived, by immunofluorescence assay on Vero cell monolayers transfected with plasmids expressing each rabbit cytokine. Anti-His C-terminal antibody was used as a positive control in pCDNA3.1-IL-6 transfected cells. Anti-rat Ig-FITC (no primary antibody) and normal rat sera were used to determine non-specific binding. Anti-mouse IL-2 and anti-mouse IL-10 were used to determine species cross-reactivity of the anti-sera. Binding of antibodies was detected by a secondary antibody conjugated to FITC (anti-rat Ig-FITC). Pictures were taken using the 40× objective.

Monoclonal antibodies to mouse IL-2 and IL-10 were obtained from Dr A. Ramsay, (JCSMR, Canberra, Australia) to test for cross reactivity to rabbit cytokines. Anti-mouse IL-2 and anti-mouse IgM IL-10 (Figure 6.6) and anti-mouse IgG IL-10 (data not shown) did not show fluorescence with rabbit IL-2 and IL-10. As no cross-reactivity was seen for IL-2 and IL-10, which are the cytokines with the most conserved amino acid sequences, antibodies raised to cytokines from other species were not tested on the rabbit cytokines.

6.2.1.4 Inoculation of Chickens with IL-2 Peptide/KLH Conjugate and Testing Sera for Anti-Peptide Antibodies

One potential reason for the sera from rats inoculated with either of the IL-2 peptide conjugates being negative for IL-2-specific antibodies was that the synthetic peptide is too similar to the same region of rat IL-2. In this scenario, the rat immune system will not recognize the rabbit IL-2 peptides as foreign and will not generate an immune response against the peptides. Rat IL-2 has 63% amino acid identity to rabbit IL-2 (53% and 57% identity over the IL-2 C/D loop and helix B peptide regions respectively). In contrast, chicken IL-2 only shares 22% amino acid identity to rabbit IL-2 (approximately 27% and 21% identity over the IL-2 C/D loop and helix B peptide regions respectively). Therefore, in an attempt to produce antibodies to rabbit IL-2, chickens were inoculated with the conjugated synthetic peptides.

Chicken sera was tested for the presence of anti-rabbit IL-2 antibodies by immunofluorescence on Vero cell monolayers infected with a recombinant myxoma virus expressing rabbit IL-2. The recombinant virus was prepared by Mr S. Junankar (School of Biochemistry and Molecular Biology, ANU). The pattern of cells infected with the recombinant myxoma virus can be clearly seen in the positive control cells,

under UV light only and UV together with visible light (Figure 6.7A and 6.7B). No antibodies that recognised rabbit IL-2 were detected in chicken sera (Figure 6.7E and 6.7F). The cells incubated with the test chicken sera show the fluorescence is generalized and spread across the entire field of view. The immunofluorescence observed in chickens inoculated with IL-2 C/D loop conjugate is associated with cell debris and not infected cells.

6.2.1.5 DNA Immunisation of Rats with pMagicIL-2

As immunization of either rats or chickens with IL-2 synthetic peptides did not generate IL-2-specific antibodies, rats were immunized with DNA vaccine expressing IL-2 and boosted with the IL-2 peptides in a further attempt to raise antibodies to rabbit IL-2. Analysis by immunofluorescence of sera from rats immunized with pMagic-IL-2 did not detect any rabbit IL-2-specific antibodies (data not shown).

6.2.2 Characterisation of Anti-Peptide Sera Specific for Rabbit Cytokines

6.2.2.1 Titration of Anti-Peptide Rat Sera on Cells Transfected with Plasmids Expressing Rabbit Cytokines

Rat sera were titrated on Vero cell monolayers transfected with pcDNA3.1 expressing the appropriate rabbit cytokine genes under control of the CMV IE1 promotor. Sera were tested at 1:20, 1:40, 1:100 and 1:500 dilutions.

Sera from rats inoculated with IL-4 helix A showed strong fluorescence at a dilution of 1:500 (Figure 6.8). Both the IL-6 tail sera (Figure 6.9) and the IL-6 helix E sera (Figure 6.10) had fluorescence at the 1:100 dilutions, but not at the 1:500 dilutions. Only one rat

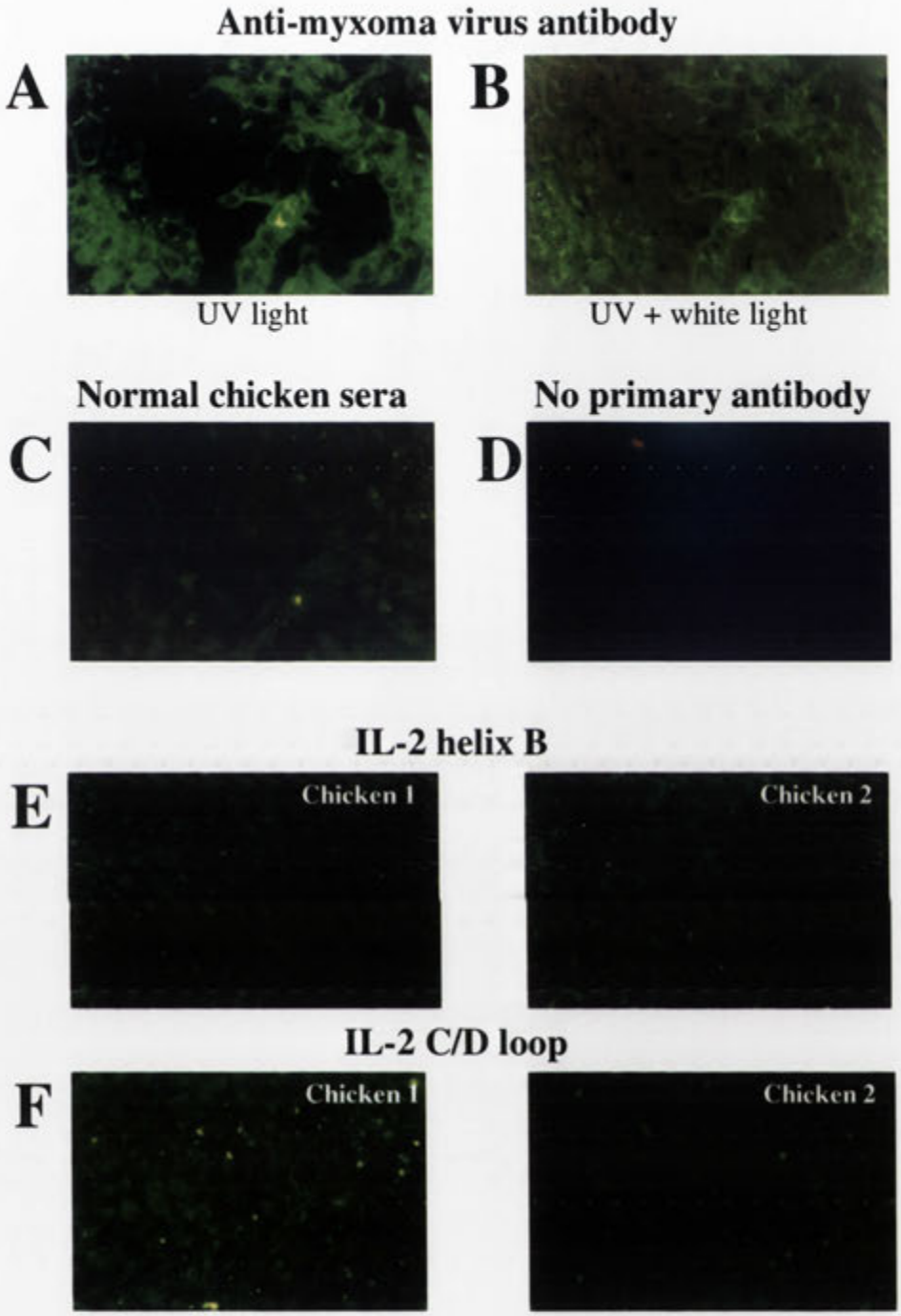


FIGURE 6.7. DETECTION BY IMMUNOFLUORESCENCE OF RABBIT IL-2-SPECIFIC ANTIBODIES IN CHICKEN SERA

Sera from chickens were tested for antibodies to rabbit IL-2by immunofluorescence of Vero cell monolayers infected with recombinant myxoma virus expressing rabbit IL-2,. (A, B) Anti-myxoma virus antibody was used as a positive control and (C) normal chicken sera and (D) anti-chicken Ig-FITC were used to determine non-specific binding. Chickens were immunised with (E) IL-2 helix B or (F) IL-2 C/D loop. Binding of antibodies was detected by a secondary antibody conjugated to FITC. Pictures were taken at 40× magnification.

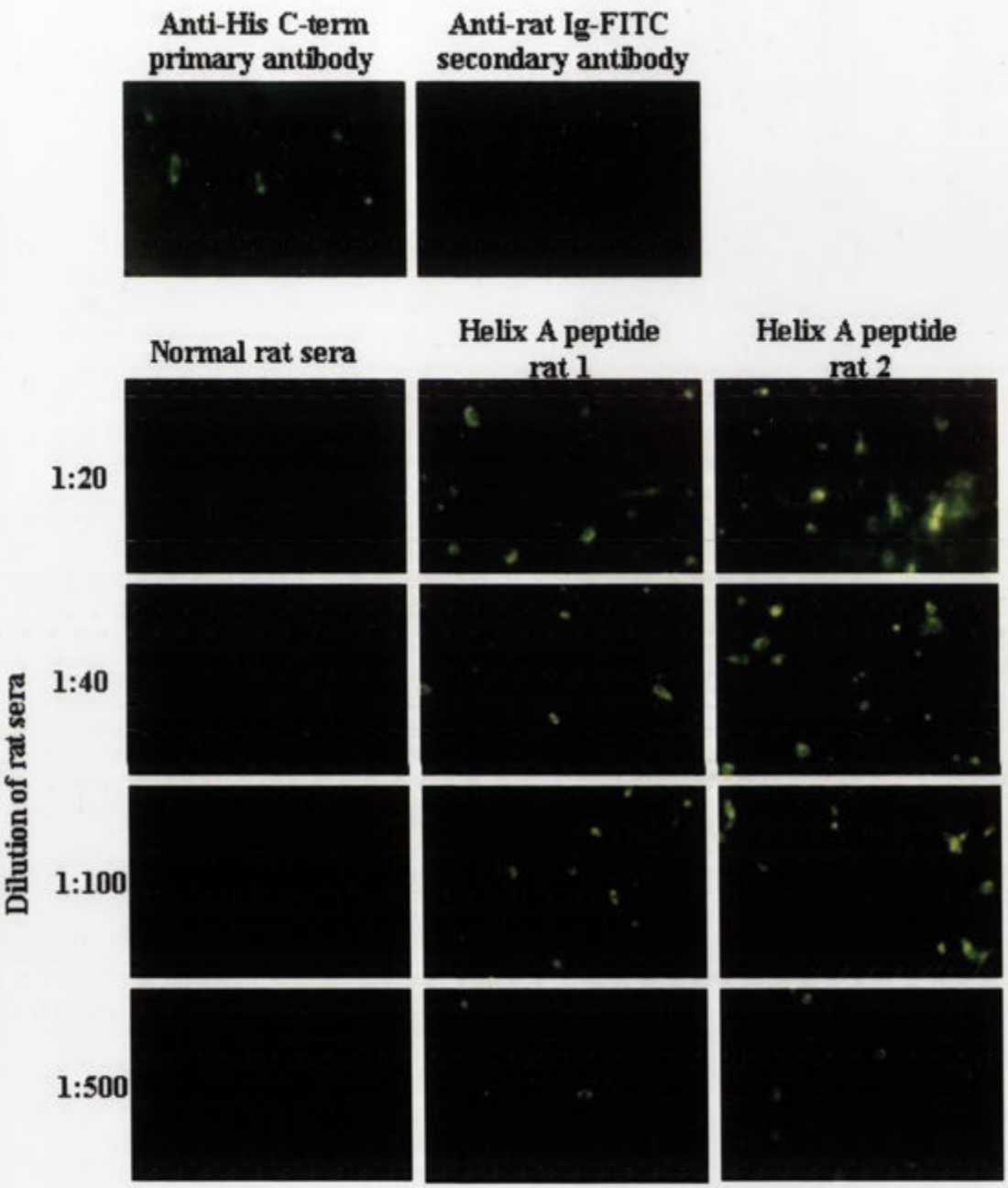


FIGURE 6.8. TITRATION OF ANTI-IL-4 (HELIX A) RAT SERA

Sera from rats immunised with the IL-4 helix A peptide were titrated at 1:20, 1:40, 1:100 and 1:500 against Vero cell monolayers transfected with pcDNA3.1 plasmid expressing rabbit IL-4. Results from each of the two rats immunised with the helix A peptide are shown. Anti-His C-terminal antibody was used as a positive control, and anti-rat Ig-FITC in the absence of primary antibody, and normal rat sera as the primary antibody, were used to determine non-specific binding. Pictures were taken at 40× magnification.

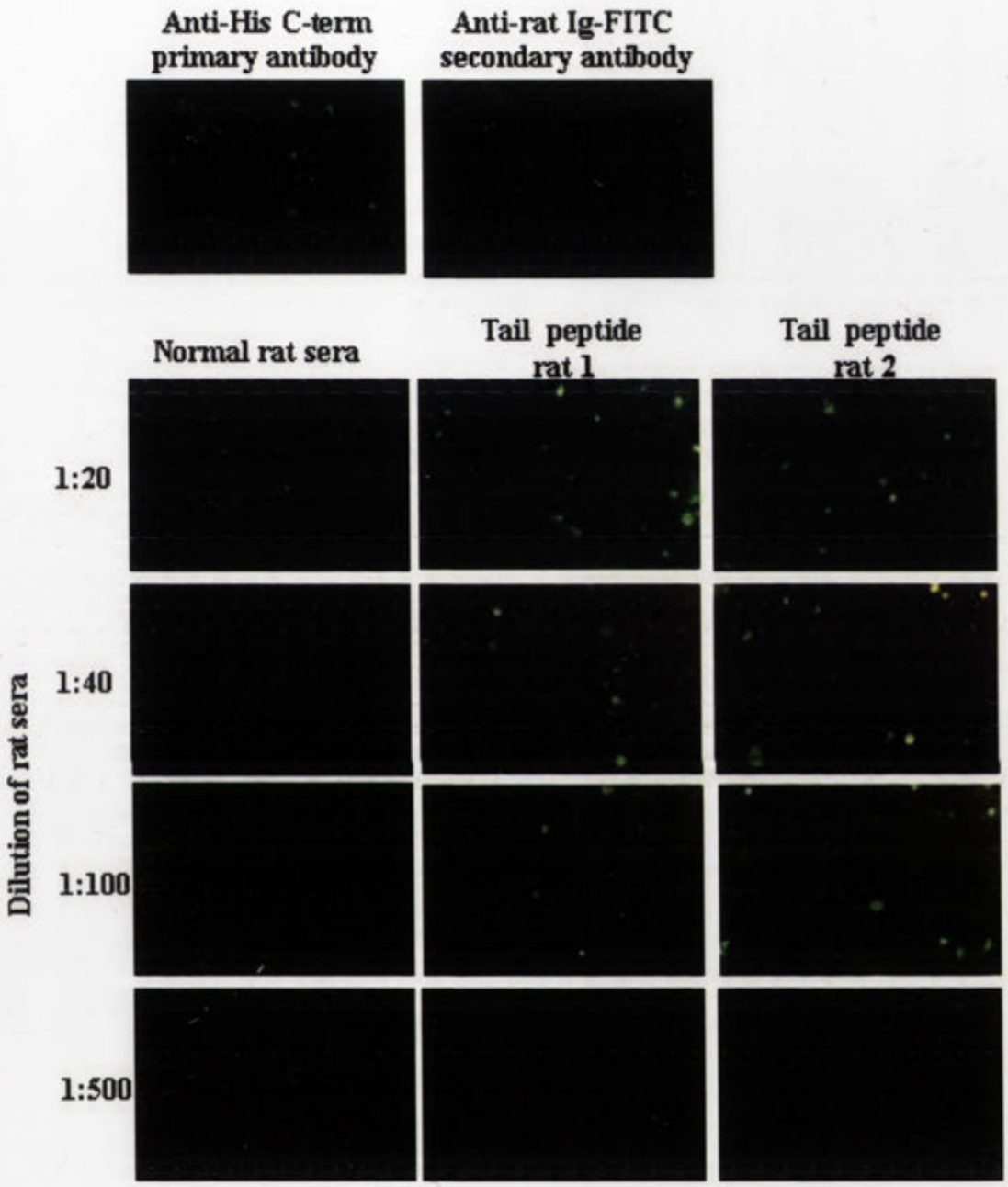


FIGURE 6.9. TITRATION OF ANTI-IL-6 (TAIL) RAT SERA

Sera from rats immunised with the IL-6 tail peptide were titrated at 1:20, 1:40, 1:100 and 1:500 against Vero cell monolayers transfected with pcDNA3.1 plasmid expressing rabbit IL-6. Results from each of the two rats immunised with the IL-6 tail peptide are shown. Anti-His C-terminal antibody was used as a positive control, and anti-rat Ig-FITC in the absence of primary antibody, and normal rat sera as the primary antibody, were used to determine non-specific binding. Pictures were taken at 40× magnification.

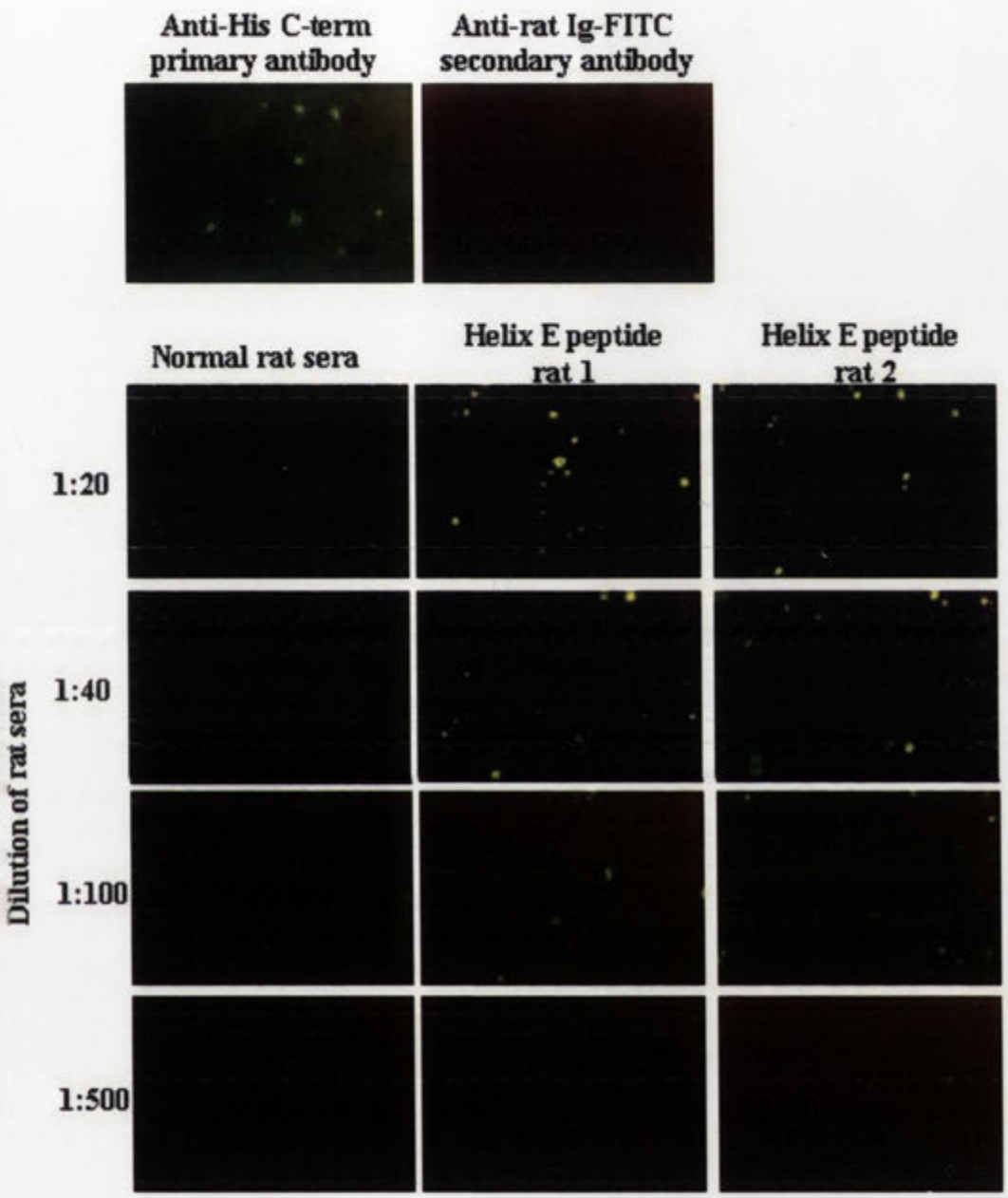


FIGURE 6.10. TITRATION OF ANTI-IL-6 (HELIX E) RAT SERA

Sera from rats immunised with the IL-6 helix E peptide were titrated at 1:20, 1:40, 1:100 and 1:500 against Vero cell monolayers transfected with pcDNA3.1 plasmid expressing rabbit IL-6. Results from each of the two rats immunised with the helix E peptide are shown. Anti-His C-terminal antibody was used as a positive control, and anti-rat Ig-FITC in the absence of primary antibody, and normal rat sera as the primary antibody, were used to determine non-specific binding. Pictures were taken at 40× magnification.

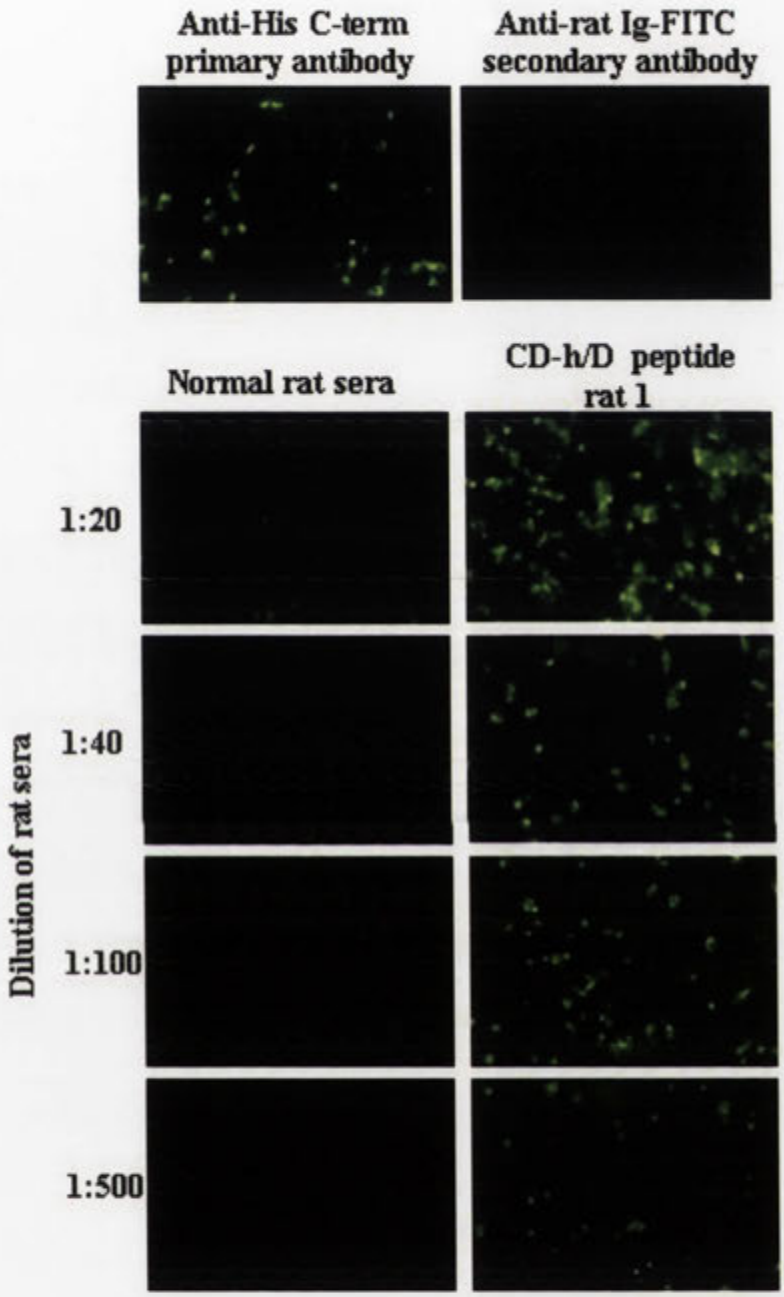


FIGURE 6.11. TITRATION OF ANTI-IL-10 (CD-H/D) RAT SERA

Sera from rats immunised with the IL-10 CD-h/D peptide were titrated at 1:20, 1:40, 1:100 and 1:500 against Vero cell monolayers transfected with pcDNA3.1 plasmid expressing rabbit IL-10. Only serum from one rat had IL-10 specific antibodies, thus only results from that rat are shown. Anti-His C-terminal antibody was used as a positive control, and anti-rat Ig-FITC in the absence of primary antibody, and normal rat sera as the primary antibody, were used to determine non-specific binding. Pictures were taken at 40× magnification.

Sera from rats inoculated with synthetic peptides derived from IFNγ showed fluorescence at a dilution of 1:100 (Figure 6.12 and Figure 6.13) and one rat inoculated with IFNγ hb-B/C showed fluorescence at 1:500 dilution (Figure 6.12). Non-specific binding was not observed at these dilutions as shown by the cells stained with normal rat serum and the cells stained in the absence of primary antibody. However, some non-specific background binding was present at 1:20 and 1:40 dilutions. As in the initial screening, the anti-His C-terminus antibody was used as the positive control and showed that the cytokine protein was expressed from the pcDNA3.1 constructs in all transfections.

6.2.2.2 Immunoblot Analysis of Anti-Peptide Rat Sera

The rat sera were tested to see if they bound the appropriate cytokine by immunoblot. Antigen samples were prepared as either secreted cytokines from recombinant myxoma viruses expressing one of the cytokines in mammalian cell culture and in some cases, expression of cytokines from plasmid DNA transfected into mammalian cells.

6.2.2.2.1 Analysis of Samples from Virus-Infected Cells

RK13 cells were infected with recombinant myxoma virus expressing one of rabbit IL-4, IL-6, IL-10 or IFNγ (obtained from Dr H. Perkins and Ms B. Inglis, School of Biochemistry and Molecular Biology, ANU). Supernatants or cell extracts were collected, supernatants were concentrated by acetone precipitation, and run on a SDS-PAGE gel under reducing or non-reducing conditions. Gels were either Coomassie blue stained and silver stained to visualize total protein in the gel, or used for transfer of protein to a PDVF membrane and subsequent analysis by immunoblotting using enhanced chemiluminescence (ECL) detection.

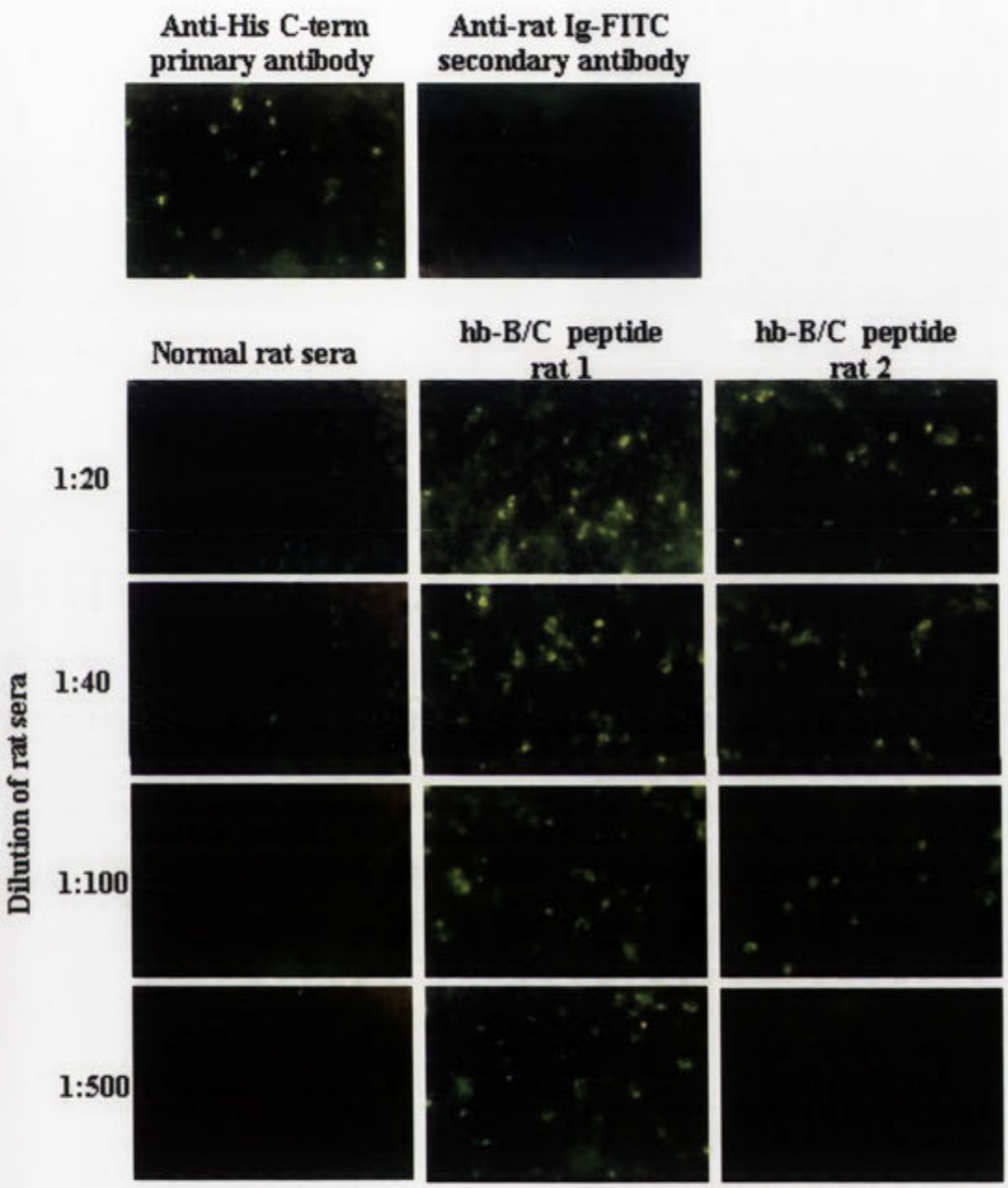


FIGURE 6.12. TITRATION OF ANTI-IFNγ (HB-B/C) RAT SERA

Sera from rats immunised with the IFNγ hb-B/C peptide were titrated at 1:20, 1:40, 1:100 and 1:500 against Vero cell monolayers transfected with pcDNA3.1 plasmid expressing rabbit IFNγ. Results from each of the two rats immunised with the IFNγ hb-B/C peptide are shown. Anti-His C-terminal antibody was used as a positive control, and anti-rat Ig-FITC in the absence of primary antibody, and normal rat sera as the primary antibody, were used to determine non-specific binding. Pictures were taken at 40× magnification.

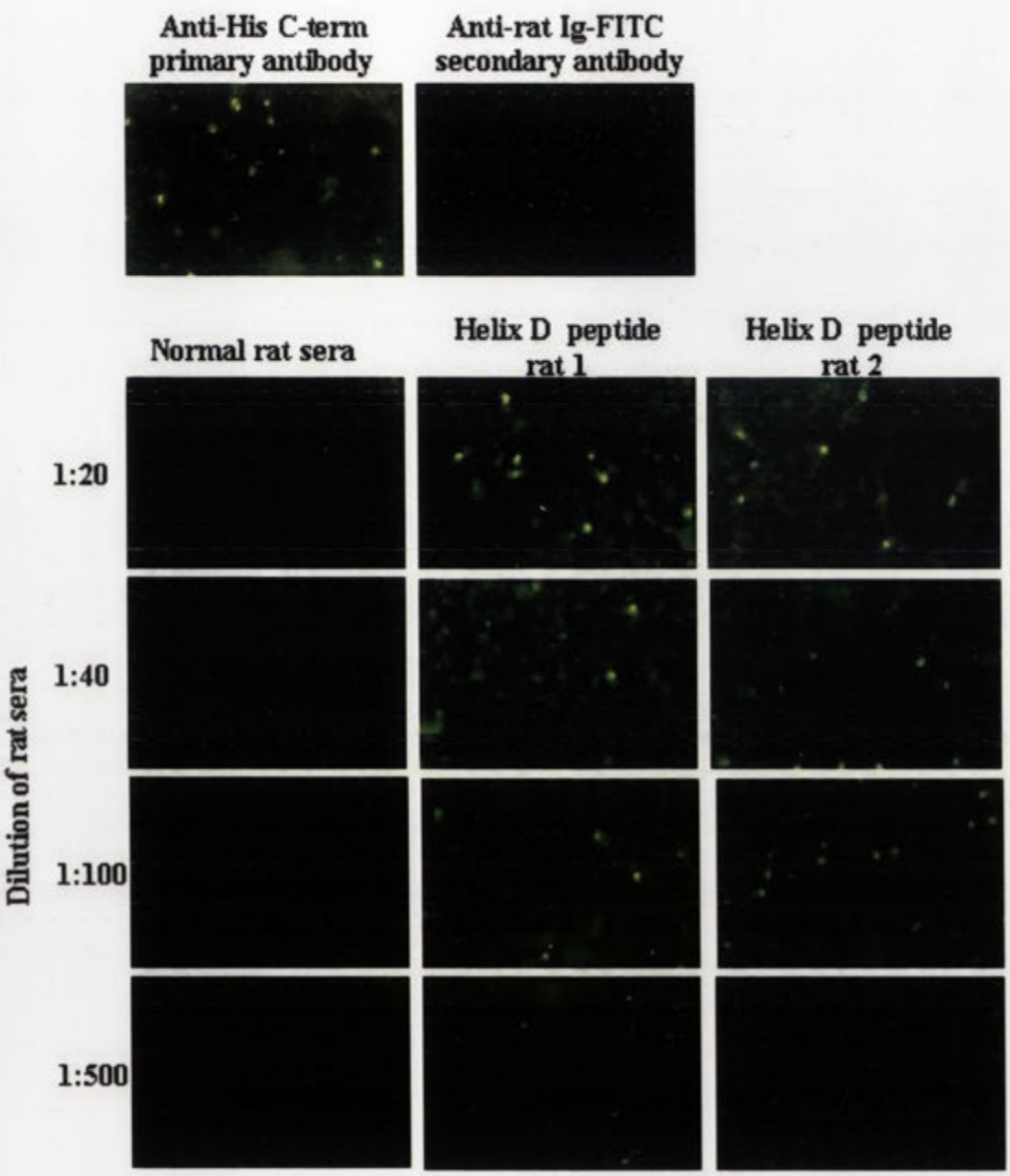


FIGURE 6.13. TITRATION OF ANTI-IFNγ (HELIX D) RAT SERA

Sera from rats immunised with the IFNγ helix D peptide were titrated at 1:20, 1:40, 1:100 and 1:500 against Vero cell monolayers transfected with pcDNA3.1 plasmid expressing rabbit IFNγ. Results from both rats immunised with the helix D peptide are shown. Anti-His C-terminal antibody was used as a positive control, and anti-rat Ig-FITC in the absence of primary antibody, and normal rat sera as the primary antibody, were used to determine non-specific binding. Pictures were taken at 40× magnification.

More proteins were visualized on silver stained reduced gels than Coomassie stained reduced gels due to the increased sensitivity of the former staining method (Figure 6.14A and B). No bands corresponding to the particular cytokine, based on molecular weight, were observed by staining with Coomassie blue or silver stain. Detection of viral proteins by the anti-myxoma polyclonal serum (Figure 6.14C) showed that the cells were successfully infected.

The IL-4 helix A rat sera was able to detect IL-4 (molecular weight of approximately 16 kDa) in the supernatant of cells infected with myxoma virus expressing IL-4 when samples were run on a reduced gel (Figure 6.14D). Interestingly, the IL-10 C/D-hD rat sera was able to detect IL-10 (molecular weight of approximately 20 kDa) in both the supernatant and in the cell extract of cells infected with myxoma virus expressing IL-10 (Figure 6.14G). No IL-6 or IFNγ was detected in supernatants or cell extracts from cells infected with myxoma virus expressing IL-6 and IFNγ. The background using ECL was high when IL-6 (Figure 6.14E and H) or IFNγ helix D (Figure 6.14I) rat sera were used as the primary antibody. This may have masked specific IL-6 or IFNγ antibody binding. Further analysis with shorter exposure times, nitrocellulose membrane and different detection methods (e.g. DAB/NiCl) did not decrease this background (data not shown).

Rat sera non-specifically bound to cellular and/or viral proteins. This non-specific binding can be seen in blots probed with IL-4 helix A sera, IL-10 C/D-hD sera and IFNγ hb-B/C sera (Figure 6.14D, G and H). In these blots, the non-specific binding is observed in lanes containing cell extracts from cells infected with any of the recombinant viruses.

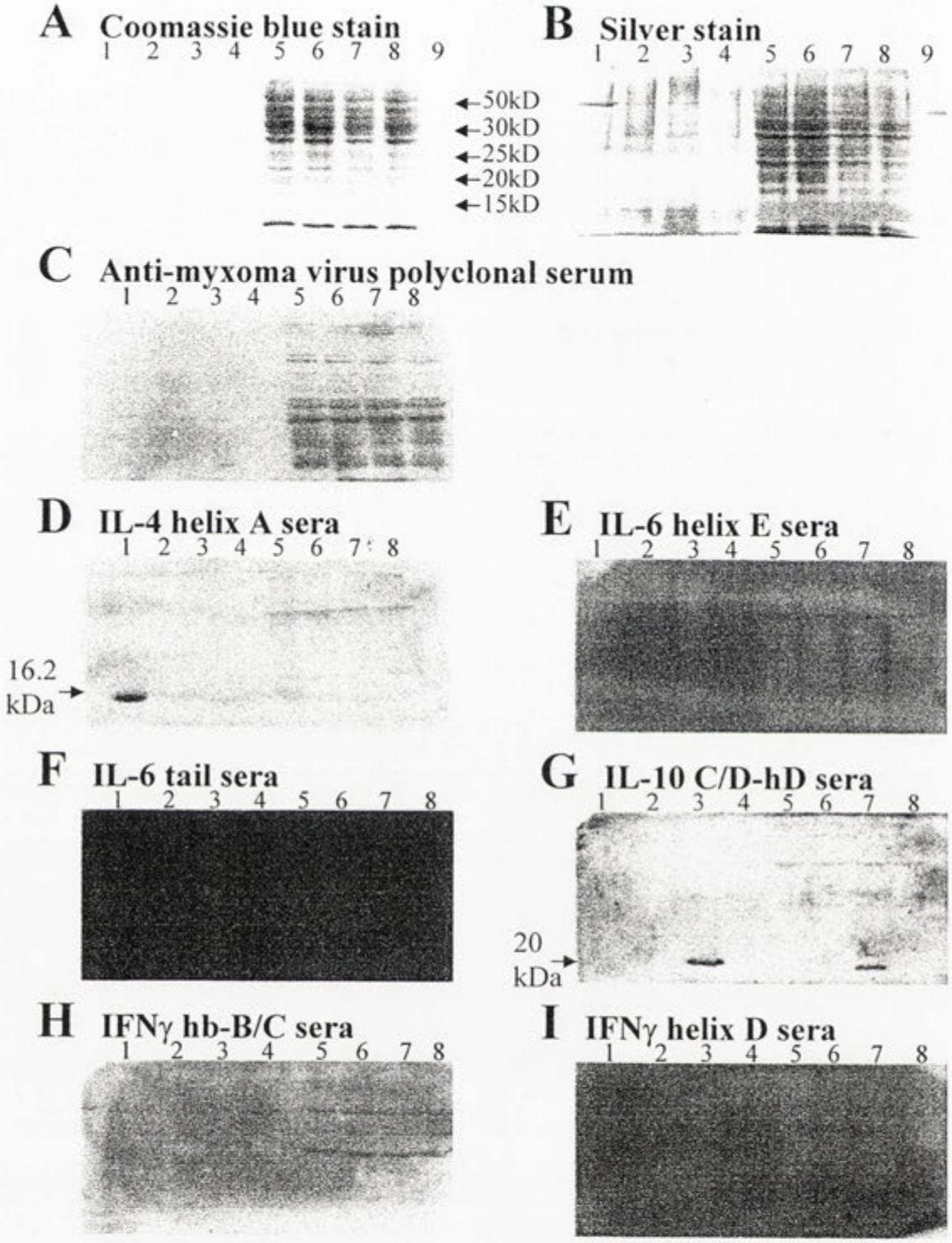


FIGURE 6.14. IMMUNOBLOT ANALYSIS OF RAT SERA SPECIFIC FOR RABBIT CYTOKINES ON REDUCING SDS-PAGE

Supernatants and cell extracts from cells infected with recombinant myxoma viruses expressing one of rabbit IL-4, IL-6, IL-10 or IFNγ were run on a SDS-PAGE gel under reducing conditions in the following lane order; supernatants from myxoma virus expressing (1) IL-4, (2) IL-6, (3) IL-10 or (4) IFNγ and cell extracts from myxoma virus expressing (5) IL-4, (6) IL-6, (7) IL-10 or (8) IFNγ and (9) molecular weight markers. Gels were stained with (A) Coomassie or (B) silver stain or used for analysis by immunoblot. The primary antibodies using in immunoblot analysis were (C) polyclonal anti-myxoma virus serum, (D) IL-4 helix A rat sera, (E) IL-6 helix E rat sera, (F) IL-6 tail rat sera, (G) IL-10 C/D-hD rat sera, (H) IFNγ hb-B/C rat sera or (I) IFNγ helix D rat sera. Bands corresponding to IL-4 (16.2 kDa) and IL-10 (20 kDa) are indicated in panel (D) and (G) respectively.

A possible reason why bands were not seen for IL-6 and IFNγ is that reducing conditions break disulphide bonds and therefore may disrupt the secondary protein structure. The secondary structure may be important for the antibody to recognize the native protein. To try and maintain the secondary protein structure, some of the samples were analyzed under non-reducing conditions (Figure 6.15). Interestingly, IL-4 was not detected with the IL-4 helix A rat sera (Figure 6.15D), in spite of IL-4 being able to be detected under reducing conditions. IL-6 and IFNγ were also not detected by any rat sera under non-reduced conditions (data not shown). Analysis of IL-10 C/D-hD under non-reducing conditions was not performed.

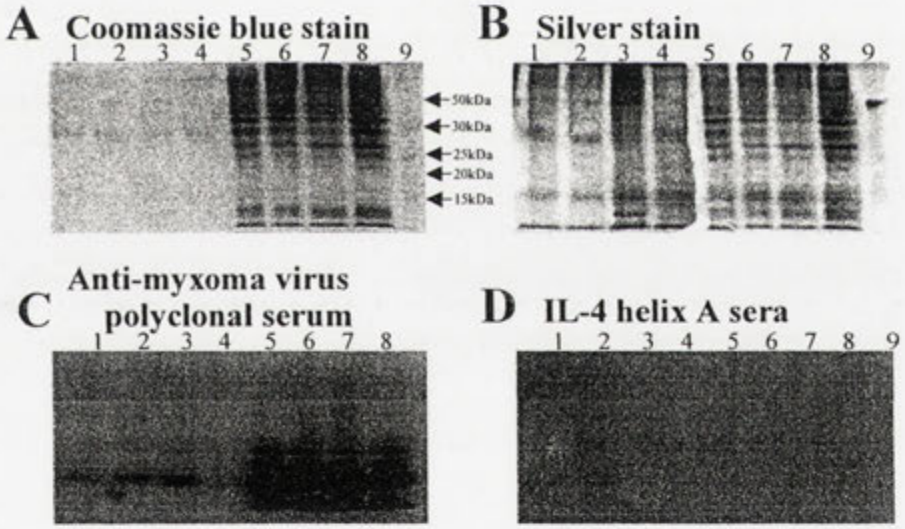


FIGURE 6.15. IMMUNOBLOT ANALYSIS OF RAT SERA SPECIFIC FOR RABBIT CYTOKINES ON NON-REDUCING SDS-PAGE

Supernatants and cell extracts from cells infected with recombinant myxoma virus expressing one of rabbit IL-4, IL-6, IL-10 or IFNγ were run on a SDS-PAGE gel under non-reducing conditions in the following lane order; supernatants from myxoma virus expressing (1) IL-4, (2) IL-6, (3) IL-10 or (4) IFNγ and cell extracts from myxoma virus expressing (5) IL-4, (6) IL-6, (7) IL-10 or (8) IFNγ and molecular weight markers. Gels were stained with (A) Coomassie or (B) silver stain or used for analysis by immunoblot. The primary antibodies using in immunoblot analysis were (C) polyclonal anti-myxoma virus serum and (D) IL-4 helix A rat sera.

In conclusion, by immunoblot, the rat anti-rabbit IL-4 helix A anti-sera and IL-10 C/D-hD anti-sera were shown to bind their respective cytokines secreted by recombinant myxoma viruses. The IL-6 and IFNγ anti-sera did not detect cytokines secreted by recombinant myxoma virus-infected cells using immunoblotting.

To further examine whether the IL-6 and IFNγ anti-sera could detect their respective cytokines by immunoblotting, COS-7 cells, which are routinely used for expression of mammalian proteins, were transfected with pcDNA3.1 containing either rabbit IL-6 or IFNγ, and the supernatants and cell monolayers were tested for IL-6 and IFNγ expression. Immunofluorescence assay of transfected cells, using the anti-His antibody, showed that both these plasmids had successfully been transfected and were expressing protein (data not shown). However, expression of neither IL-6 nor IFNγ could be shown by immunoblotting of cell supernatants or cell extracts (data not shown). Analysis of samples concentrated using centricon units and large sample volumes did not change the lack of detection of the cytokines by the rat sera (data not shown). This suggests that either not enough protein was produced in the transfection for detection or that the rat sera do not recognize these proteins by immunoblot. As both IL-6 and IL-10 were detected using the anti-sera by immunofluorescence staining of cells transfected with pcDNA3.1 encoding these cytokines, the epitopes must be altered by the immunoblotting procedure. Interestingly, similar backgrounds to Section 6.2.2.2.1 were observed with ECL when rat sera with antibodies specific for IL-6 or IL-10 were used as the primary antibody. This suggests that a high background is a property of the sera itself rather then the source of antigen.

6.2.3 Use of Anti-Peptide Sera for Detection of IL-4 and IFNγ

Analysis of cytokine expression during myxoma virus infection was undertaken in two ways. Firstly, expression of IFNγ in RL-5 cells was examined by flow cytometry. RL-5 cells constitutively express IFNγ mRNA and presumably IFNγ protein (Seto et al., 1997). The rat sera specific for IFNγ hb-B/C were used to test for the expression of IFNγ by RL-5 cells. Secondly, IFNγ and IL-4 expression in tissue sections from rabbits infected with either SLS or Ur were examined by immunofluorescence (Section 6.2.3.2).

6.2.3.1 Antibody Detection of IFNγ by Flow Cytometry

6.2.3.1.1 Detection of IFNγ Expressed by RL-5 Cells

To determine if IFNγ protein was expressed by RL-5 cells, cells were incubated with Brefeldin A (Bref A) and examined by flow cytometry, for IFNγ expression using the rat sera specific for IFNγ,. Bref A interrupts secretion of proteins from the cell by interfering with the Golgi apparatus. As IFNγ is a secreted protein, Bref A was used to sequester protein inside the cell. Con A, although not affecting the proliferation of RL-5 cells, may activate cell signaling pathways involved with IFNγ expression. Hence, experiments were also done in the presence and absence of Con A.

Figure 6.16 shows that addition of Bref A to RL-5 cultures increased the detection of IFNγ compared to cells without Bref A. This indicates that Bref A was inducing sequestration of IFNγ and that the cells are secreting IFNγ in the absence of Bref A. No further increase in IFNγ expression was detected in cells stimulated with Con A. It is interesting to note that IFNγ could not be detected by immunoblot (Section 6.2.2), but

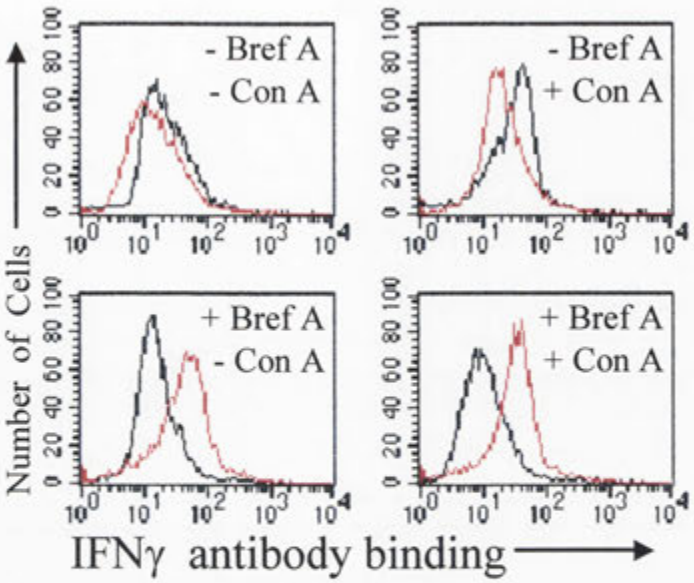


FIGURE 6.16. IFNγ EXPRESSION IN RL-5 CELLS
RL-5 cells were incubated with or without Bref A and with or without Con A. Binding of normal rat sera (black) or IFNγ hb-B/C rat sera (red) to RL-5 cells was detected by flow cytometry following incubation with anti-rat-FITC. Results are presented as histogram overlays of the number of cells versus IFNγ antibody binding.

could be detected in cells expressing IFNγ in culture. This supports the idea that IFNγ antibody epitopes are altered when cells are examined after immunoblotting.

6.2.3.1.2 Detection of IFNγ in RL-5 Cells Infected with SLS or Ur

RL-5 cells were infected with SLS or Ur at a moi of 3 to examine whether myxoma virus infection altered IFNγ expression. In this experiment, IFNγ was also detected in RL-5 cells in the absence of Bref A. However, this result was not repeatable in some experiments, hence subsequent experiments were undertaken using Bref A.

Infection with either strain of myxoma virus increased the non-specific binding of normal rat serum in RL-5 cultures such that fluorescence intensities were similar to those corresponding to IFNγ in mock-infected cell cultures (Figure 6.17). This was

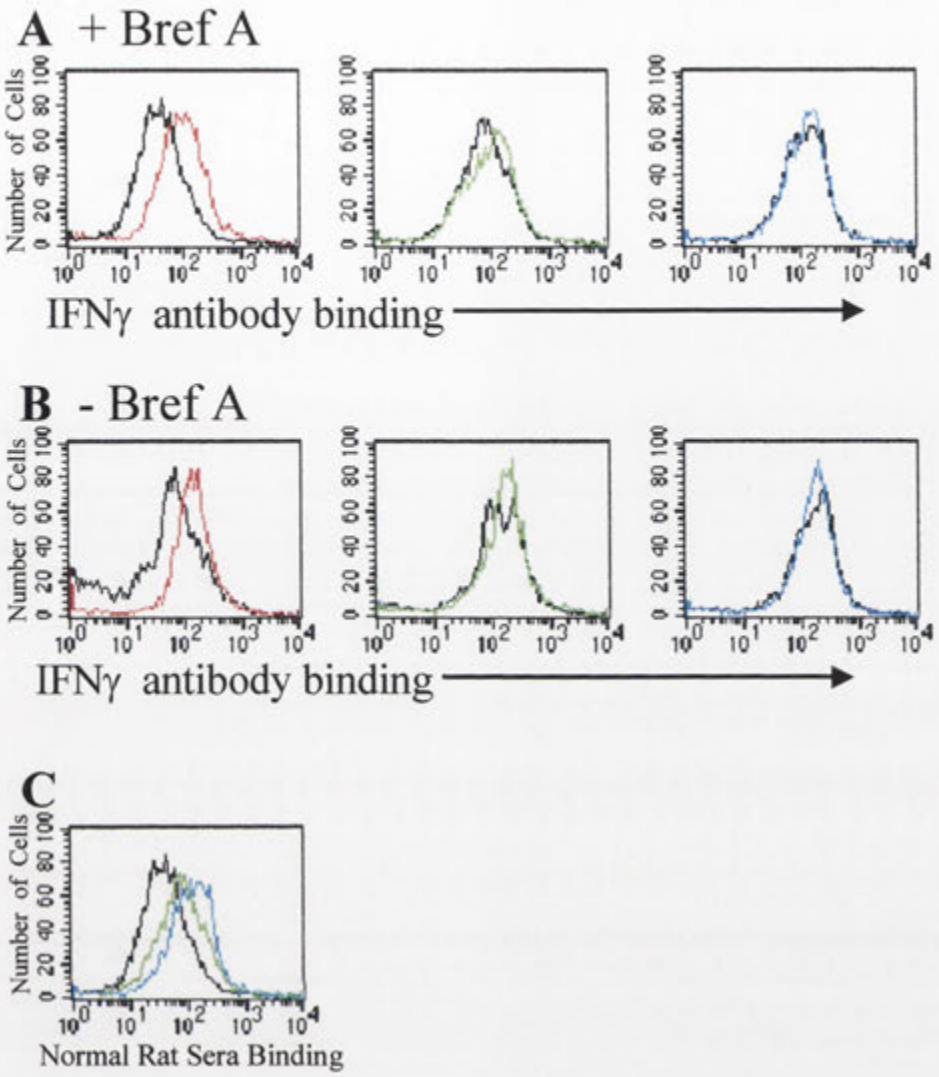


FIGURE 6.17. IFNγ EXPRESSION IN RL-5 CELLS INFECTED WITH SLS OR UR

RL-5 cells were infected with SLS or Ur at a moi of 3 and incubated (A) with or (B) without Bref A. Cells were analysed as histogram overlays of number of cells versus IFNγ hb-B/C antibody binding in mock-infected (red), SLS- (green) or Ur-infected (blue) cultures. Non-specific binding of normal rat sera in each infection is shown in black. (C) The fluorescence intensity due to normal rat sera in mock-infected (black), SLS-infected (green) and Ur-infected (blue) RL-5 cells were overlaid on the one histogram.

independent of addition of Bref A. The non-specific binding of normal rat serum precluded any comparison of the effects of infection with SLS and Ur. Attempts to overcome this problem by using different fixation solutions (Figure 6.18), such as ethanol and buffered formaldehyde acetone (BFA), and pre-incubating cells with fetal calf serum or BSA (Figure 6.19) to block non-specific binding sites, did not decrease non-specific binding. In fact, in uninfected cell cultures, fixation with ethanol or BFA.

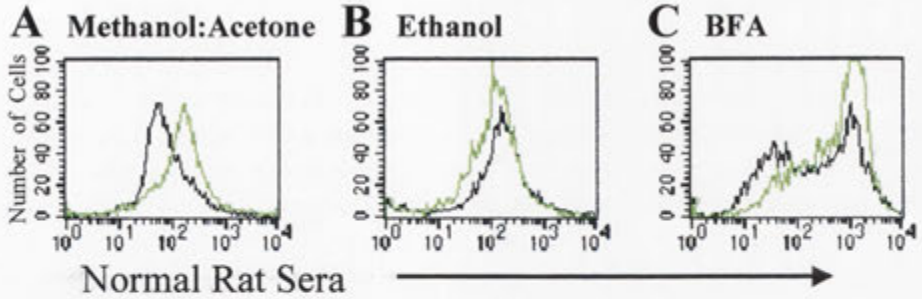


FIGURE 6.18. EXAMINATION OF PERMEABILISATION SOLUTIONS FOR IFNγ DETECTION IN INFECTED RL-5 CELLS

RL-5 cells were mock-infected (black) or infected with SLS (green) at a moi of 3 and incubated with Bref A. Cells were permeabilised by (A) methanol:acetone, (B) ethanol or (C) buffered formaldehyde acetone (BFA) and analysed as histogram overlays of number of cells versus binding of normal rat serum.

and the use of 3% BSA in cell cultures permeabilised with methanol:acetone, increased the non-specific binding of normal rat serum (Figure 6.19). To remove non-specific binding of antibodies, sera was adsorbed to infected cells prior to use in immunofluorescence but this also had no effect on non-specific binding (data not shown). In conclusion, IFNγ was detected in RL-5 cells by flow cytometry using anti-IFNγ anti-sera. However, problems with increased non-specific binding of normal rat serum to cells infected with myxoma virus hampered analysis of IFNγ expression during myxoma virus infection.

6.2.3.2 Immunostaining of Rabbit Tissue Sections for IFNγ and IL-4

To determine whether the anti-peptide sera could be used for staining tissue sections, frozen sections of lymph node from uninfected rabbits or four days post infection with either SLS or Ur, were tested with anti-IFNγ or anti-IL-4 rat sera. Detection of IL-4 and IFNγ was undertaken using IL-4 helix A and IFNγ hb-B/C rat sera respectively. The anti-rabbit-CD43 monoclonal antibody and normal rat serum were used as positive and

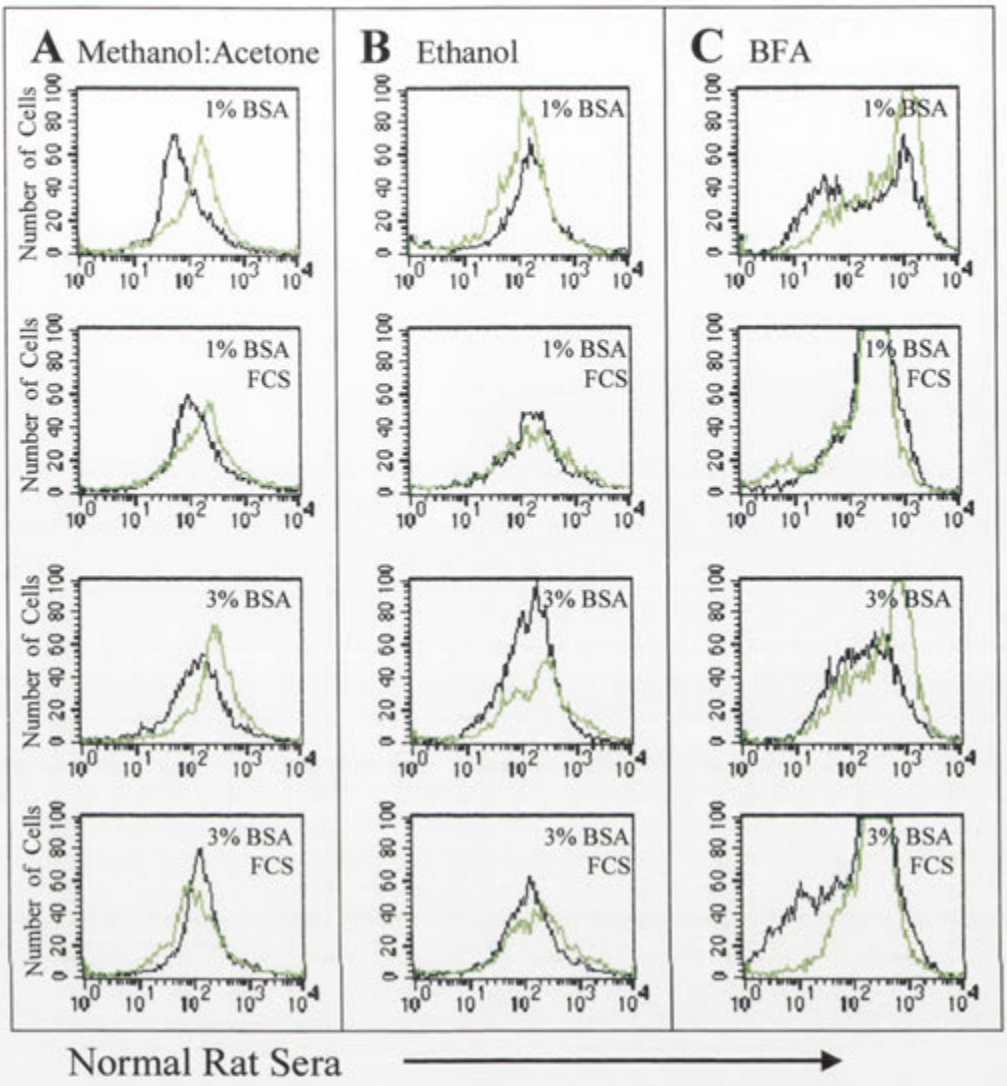


FIGURE 6.19. BLOCKING NON-SPECIFIC BINDING WITH FETAL CALF SERUM AND BSA
RL-5 cells were mock-infected (black) or infected with SLS (green) at a moi of 3 and incubated with Bref A. Cells were permeabilised by (A) methanol:acetone, (B) ethanol or (C) buffered formaldehyde acetone (BFA) and blocked with fetal calf serum (FCS). The percentage of BSA in all solutions was also used at 1% and 3%. Cells were analysed as histogram overlays of number of cells versus binding of normal rat sera.

negative controls respectively. CD43 is expressed on T-cells and thymocytes (Ostberg et al., 1998) and may cross-react with progenitor rabbit B-cells (reviewed in Mage 1998), thus it should bind to rabbit lymph node sections. Figure 6.20 shows the detection of CD43, IL-4 and IFNγ in lymph node sections from an uninfected rabbit. Fluorescence was observed with antibodies to each of these proteins.

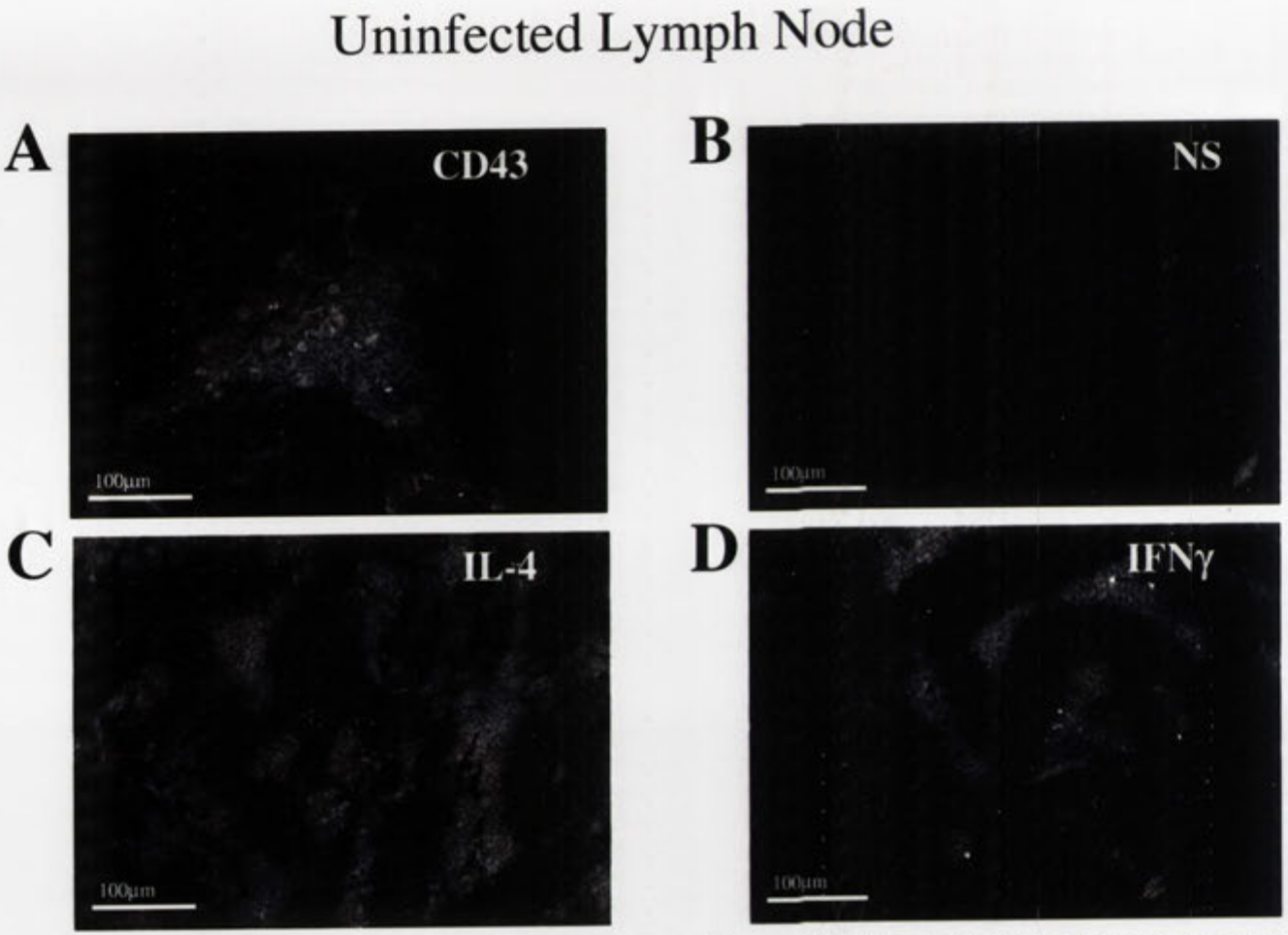


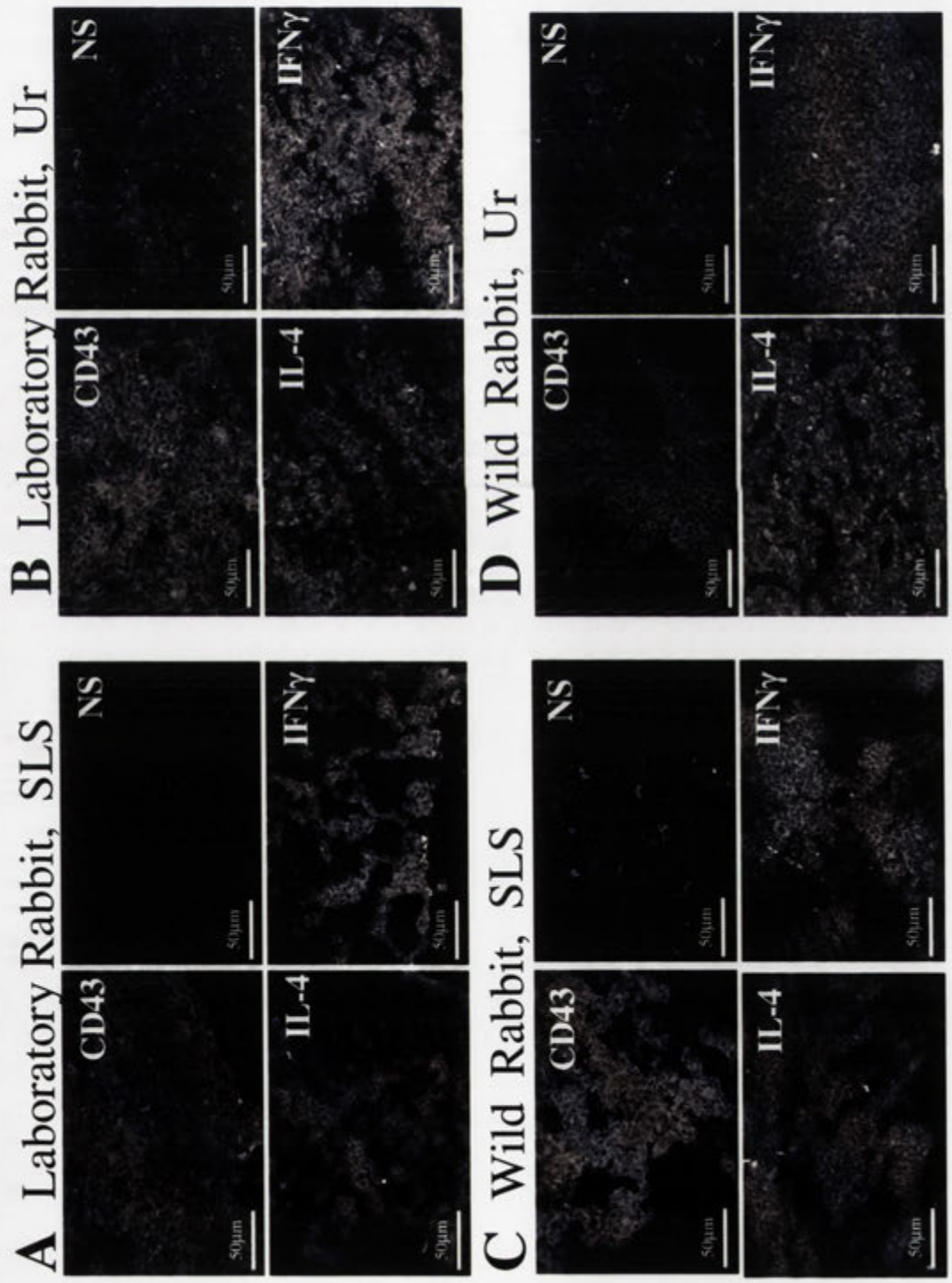
FIGURE 6.20. IMMUNOFLUORESCENCE OF RABBIT CYTOKINES IN TISSUE SECTIONS FROM THE LYMPH NODE OF AN UNINFECTED RABBIT
Frozen lymph nodes from uninfected rabbits were sectioned and analysed by immunofluorescence. (A) CD43 expression was analysed as a positive control and (B) normal rat sera (NS) was used as a negative control. Expression of (C) IL-4 (IL-4 helix A rat sera) and (D) IFNγ, (IFNγ hb-B/C rat sera) were also examined. Pictures were taken on a Confocal microscope. Magnification is shown as a 100µm bar on each picture.

The confocal images shown in Figure 6.21A and 6.21B suggest that both SLS and Ur infection could increase expression of IFNγ in the lymph node draining the inoculation site of both laboratory and wild rabbits compared to lymph nodes from uninfected rabbits as shown in Figure 6.20. IL-4 expression also appeared greater in rabbits infected with Ur than in rabbits infected with SLS (Figure 6.21B and 6.21D). However these results will need to be repeated. Interestingly, staining of virus-infected sections did not appear to increase non-specific background, suggesting the problem of non-specific binding seen in immunoblots does not occur on tissue sections.

Although a comprehensive study of cytokine expression was outside the scope of this thesis, the results obtained strongly suggest that the anti-IFNγ and anti-IL-4 anti-sera will be useful in future studies of detection of expression of these cytokines by immunofluorescence in tissue sections.

FIGURE 6.21. IMMUNOFLUORESCENCE OF RABBIT CYTOKINES IN TISSUE SECTIONS FROM THE LYMPH NODE OF A RABBIT INFECTED WITH SLS OR UR

The lymph node draining the inoculation site from a laboratory rabbit infected with (A) SLS or (B) Ur and a wild rabbit infected with (C) SLS or (D) Ur, 4 days post infection were sectioned and analysed by immunofluorescence for expression of IL-4 (IL-4 helix A rat sera) and IFNγ (IFNγ hb-B/C rat sera). CD43 expression was used as a positive control and normal rat sera were used as a negative control. Magnification is shown as a 50um bar on each picture.



6.3 Discussion

This chapter detailed the production and preliminary characterization of anti-peptide rat sera specific for rabbit IL-4, IL-6, IL-10 and IFNγ. Antibodies to IL-4, IL-6, IL-10 and IFNγ were generated after immunization of rats with synthetic peptides conjugated to an immunogenic carrier protein, mcKLH. Production of anti-peptide sera to rabbit IL-2 was attempted but was unsuccessful.

Each of the sera from rats (with the exception of IL-2) recognized the specific cytokine in mammalian cells transfected with pcDNA 3.1 containing the cytokine gene. In addition, anti-IL-10 sera detected IL-10 by immunoblotting, anti-IFNγ sera detected IFNγ in tissue sections and in uninfected cells transformed with pcDNA3.1 and anti-IL-4 sera detected IL-4 in immunoblotting and tissue sections. The different ability to detect cytokines under different experimental conditions indicates that some of the antibodies may be highly sensitive to a specific structure on the expressed protein.

The aim of producing antibodies to the rabbit T_H1 and T_H2 cytokines was to detect expression of these cytokines during myxoma virus infection, particularly infection of lymphoid cells *in vitro*. The antibodies bound specifically to their cognate cytokine expressed in mammalian cells and anti-IFNγ antibodies bound IFNγ in uninfected RL-5 cells. This indicates that the regions chosen for the synthetic peptides were immunogenic for the intact native protein. However, problems occurred with these antibodies when analysis of virus-infected cells was attempted. The major obstacle in the *in vitro* analysis was the non-specific binding of rat sera to cells that had been infected with myxoma virus. The normal rat sera bound strongly to viral plaques on cell monolayers (data not shown). This binding could not be blocked by pre-incubation with

fetal calf serum or by increasing the concentration of BSA. The non-specific binding of normal rat sera to cell monolayers increased when cells were infected with myxoma virus and was observed by immunoblot. The source of the non-specific binding by normal rat sera was not further investigated.

Examination of IL-4 and IFNγ expression *in vivo* in tissue sections from rabbits infected with myxoma virus did not produce high background when viewed by confocal microscopy. This indicated that these antibodies are likely to be useful as a tool for analyzing tissue expression of cytokines during the immune response to myxoma virus infection. At this stage, the use of IL-6 and IL-10 in tissue sections has not been examined.

The limitations of using synthetic peptides for antibody production were highlighted by the fact that antibodies specific for rabbit IL-2 were not able to be produced. The lack of IL-2 antibody production may be because the regions of IL-2 chosen for the synthetic peptide may not be immunogenic or may be too similar to the host cytokine amino acid sequence. It is interesting to note that DNA immunization of rats was also not successful in generation of IL-2 antibodies.

In conclusion, this chapter described the production and characterization of a group of antibodies specific for rabbit cytokines. The antibodies produced will enable future investigation of the role of cytokine expression in the attenuation of myxoma virus and genetic resistance to myxoma virus. Their most useful role is likely to be in immunostaining in tissue sections rather than in immunostaining of infected cultures.

CHAPTER 7

FINAL DISCUSSION

7.1 INTRODUCTION

The interaction between myxoma virus and the European rabbit is one of the best documented natural models of host/ virus co-evolution. However, in spite of extensive studies having been done documenting the emergence of rabbits resistant to myxomatosis and the development of attenuated myxoma virus strains in the field, the cellular and molecular mechanisms behind the co-evolution are not clear.

The interactions between myxoma virus and the host rabbit ultimately determine the outcome of virus infection. This interaction is complex and involves the host rabbit innate and adaptive immune responses and the many immunomodulatory proteins encoded by myxoma virus.

In this thesis, resistance and attenuation have been examined using the same experiments, but analysis of results has been from a different perspective. For instance, examination of resistance of rabbits to myxomatosis primarily focused on experimental differences between lymphoid cells from wild and laboratory rabbits, whereas examination of attenuation of myxoma virus primarily focused on experimental differences between SLS and Ur. Results from this thesis were also examined to determine whether the use of an attenuated strain of myxoma virus could be used to model rabbit resistance and conversely whether the use of rabbits with some resistance to myxomatosis could be use to model virus attenuation.

This thesis focused on the role of lymphoid cells in the interaction between myxoma virus and the European rabbit as these cells have been previously identified as important for myxoma virus pathogenesis and have been implicated in resistance of rabbits to

myxomatosis and in the pathogenesis of attenuated strains of myxoma virus (discussed in chapter 1). The interaction between myxoma virus and rabbit lymphoid cells *in vivo* is complicated by other factors, such as the immune response initiated in the skin on infection. To characterize the effects of myxoma virus infection on lymphocytes without other *in vivo* factors, impacting on the results, this study was performed *in vitro*. Lymphoid cells were isolated from lymph nodes from susceptible and resistant rabbits and infected *in vitro* with either SLS or Ur. Infection was compared both before and after activation of T-cells, to mimic the *in vivo* situation during the anti-myxoma virus immune response. Presumably *in vivo* some T-cells would be activated prior to infection or become activated after infection due to signals generated as a result of the anti-viral immune response. The results of the *in vitro* analysis have been discussed in the earlier chapters. This final discussion chapter will therefore extend the *in vitro* results by testing against an *in vivo* model. This chapter will also present models for rabbit resistance and attenuation of the Ur strain of myxoma virus.

7.2 Resistance to Myxomatosis

This thesis examined resistance of rabbits to myxomatosis by infecting primary rabbit lymphoid cells from resistant and susceptible rabbits with attenuated and virulent strains of myxoma virus. Differences in the response of lymphocytes from these two rabbit types identified potential mechanisms for rabbit resistance. Specifically, a small but statistically significant reduction was observed in the permissivity for myxoma virus replication in lymphocytes from resistant rabbits, compared to lymphocytes from susceptible rabbits (chapter 3). This was clearest when using an attenuated strain of myxoma virus. In addition, lymphocytes from resistant rabbits also had an increase in the expression of specific cell surface proteins that could enhance the early immune

response to the virus (chapter 5). These changes may enable resistant rabbits to more effectively control and clear myxoma virus infection compared to susceptible rabbits. Previously, resistance of rabbits to myxomatosis has been compared to the murine/ectromelia virus model (Best and Kerr 2000). Resistance of inbred mice to ectromelia virus infection has been primarily associated with an enhanced immune response generated by the host, with an increased CTL–mediated immune response (O'Neill and Brenan 1987) and early enhanced non-specific host defenses, involving NK cells and IFNα/β (Jacoby et al., 1989) correlating to resistance. Induction of a T_H1 cytokine response has also been shown to correlate with resistance of mice to ectromelia virus, whereas induction of T_H2 cytokine response in ectromelia virus infection leads to failure to control ectromelia virus replication and death of the infected mouse (Karupiah 1998).

The role of the immune response in resistance of rabbits to myxomatosis has not been examined in detail due to the paucity of rabbit-specific reagents. However, Best et al. (2000) showed that there were gross differences in pathology of the lymph node draining the inoculation site when rabbits were infected with virulent SLS. In lymph nodes from susceptible rabbits infected with SLS there was an absence of lymphocytes and an infiltration of polymorphs, whereas resistant rabbits infected with SLS, mostly retained a normal lymphocyte population in the lymph node draining the inoculation site (Best et al., 2000).

Studies by Best and Kerr (2000) also showed differences in virus pathogenesis between the two rabbit types as virus dissemination was delayed and replication was lower in distal tissues in resistant rabbits compared to susceptible rabbits. When this difference in virus replication was examined *in vitro*, no difference was observed in replication of

the virulent SLS strain in lymphocyte cultures from the two rabbit types, suggesting that there was no difference between lymphocytes from susceptible and resistant rabbits in their permissivity for myxoma virus replication (Best and Kerr 2000). There was also no difference observed in the ability of SLS to replicate in primary fibroblast cells isolated from resistant and susceptible rabbits. However, this study did not test the attenuated Ur strain for its ability to replicate *in vitro* and the lymphoid cells were not activated (Best and Kerr 2000). As shown in this thesis, the use of the attenuated strain of myxoma virus and lymphocyte activation did highlight differences between lymphocytes from resistant and susceptible rabbits. These results illustrated that an attenuated strain of myxoma virus could be used to model some resistance factors.

As well as the lack of difference in cell permissivity, Best and Kerr (2000) did not find any differences in antibody titres between susceptible and resistant rabbits, suggesting that antibodies were not crucial but that CTLs probably were. However, since differences in virus replication were observed early in infection *in vivo*, it was postulated that innate factors were also involved. From the results described above, and based on the model for resistance of mice to ectromelia virus infection, Best and Kerr (2000) hypothesized that resistant rabbits had an enhanced early innate immune response, compared to susceptible rabbits, and that this limited virus replication enough in key tissues for an effective, specific cell-mediated anti-viral immune response to develop.

This thesis challenges the notion that the permissivity of lymphocytes to myxoma virus replication does not contribute to resistance, as lymphocytes from resistant rabbits were significantly less permissive for myxoma virus replication than lymphocytes from susceptible rabbits. This was clearest in infection of activated lymphocytes from

resistant rabbits with the Ur strain of myxoma virus, where Ur did not reach a mean titre greater than 5 pfu/input cell over the 72 h. In comparison, Ur was able to replicate in activated lymphocytes from susceptible rabbits with greater than 10 pfu/input cell reached after 24 h (chapter 3).

Lymphocytes would be activated in the lymph node draining the virus inoculation site as an immune response against the virus will have been initiated by the rabbit on infection. Thus the observation that virus titres are reduced in activated cultures from resistant rabbits (chapter 3), particularly with infection using an attenuated myxoma virus strain, suggests that myxoma virus replication *in vivo* in the lymph node draining the inoculation site could also be reduced. It is therefore reasonable to assume that *in vivo,* myxoma virus replication in lymph nodes from resistant rabbits will not be as efficient as its replication in lymph nodes from susceptible rabbits.

Due to the dynamic interaction between host and virus, any small difference in virus replication may potentially have a great effect on the outcome of disease. The permissivity of lymphocytes for myxoma virus replication will be particularly important for myxoma virus pathogenesis as lymphocytes are considered one of the primary cells for *in vivo* amplification and dissemination. The reduced replication of myxoma virus in resistant rabbit lymphocytes will decrease the production of infectious progeny and limit virus-induced immunosuppression. This would enable greater control of the virus at the lymph node by the host, which will prevent virus dissemination and reduce pathology and may enable a specific cell-mediated anti-viral immune response to develop in sufficient time to control virus replication in distal tissues.

The reduced ability of myxoma virus to replicate in lymphoid cells from resistant

rabbits may also enable a greater number of lymphocytes to function appropriately during the anti-viral immune response. This, combined with the increased expression of proteins critical for the immune response such as CD4 and CD43 on resting lymphocytes from resistant rabbits and MHC-I on activated lymphocytes from resistant rabbits, would enable a more effective immune response in the resistant rabbit that would help control virus replication in distal tissues. This has been discussed in detail in chapter 5. Further examination of the rabbit immune response using rabbit cytokine-specific antibodies, similar to those described in chapter 6 of this thesis, will help elucidate whether the immune response generated after myxoma virus infection of resistant rabbits is more effective than that of susceptible rabbits.

The genetic basis for rabbit resistance to myxomatosis has not been investigated. However, resistance of mice to ectromelia virus is multigenic with the main genetic component in gene(s) within the natural killer gene complex (Delano and Brownstein 1995). It would not be unreasonable to assume that resistance to myxomatosis is also multigenic, especially in light of the results from this thesis that show resistant rabbit lymphocytes have increased cell surface expression of multiple proteins as well as decreased permissivity for virus replication, which may be discretely controlled.

7.2.1 Summary of Experimental Results Pertaining to Resistance

The two main results from this thesis that provide the basis for the model outlined in Section 7.2.2 are as follows:

1. Lymphoid cells from resistant rabbits were significantly less permissive for myxoma virus replication than lymphoid cells from susceptible rabbits. This was enhanced when cultures were infected with Ur and were activated; and

2. Resting lymphoid cells from resistant rabbits had greater expression of CD4 and CD43 and activated lymphoid cells had greater expression of MHC-I compared to lymphoid cells from susceptible rabbits.

7.2.2 Proposed Model for Resistance to Myxomatosis

Taking the results presented in this thesis together with the pathogenesis studies of Best and Kerr (2000) and Best et al. (2000) and the model proposed in these studies by the authors, the following model of resistance of rabbits to myxomatosis is presented (Figure 7.1). Myxoma virus infects susceptible and resistant rabbits and reaches the lymph node draining the inoculation site in susceptible rabbits at a similar time to that in resistant rabbits (Best and Kerr 2000). A non-specific anti-viral immune response in the skin is generated by the rabbit. To combat the anti-viral immune response, myxoma virus produces a number of immunomodulatory proteins. The interaction between the rabbit immune response induced in the skin and those viral immunomodulatory proteins results in soluble factors that activate T-cells in the lymph node draining the inoculation site.

From the skin, myxoma virus is transported to the lymph node draining the inoculation site by MHC-II$^+$ dendritic-like cells (Best and Kerr 2000). Once at the lymph node, myxoma virus targets T-cells, especially activated T-cells, for infection and replication. As reported in this thesis, myxoma virus abrogates the proliferation, and presumably subsequent activity, of infected T-cells. However, the T-cell activation signals that would be present as a result of the anti-viral immune response increases virus replication in these cells. T-cells from resistant rabbits have a lower permissivity for myxoma virus replication especially in the presence of stimulatory signals as shown in

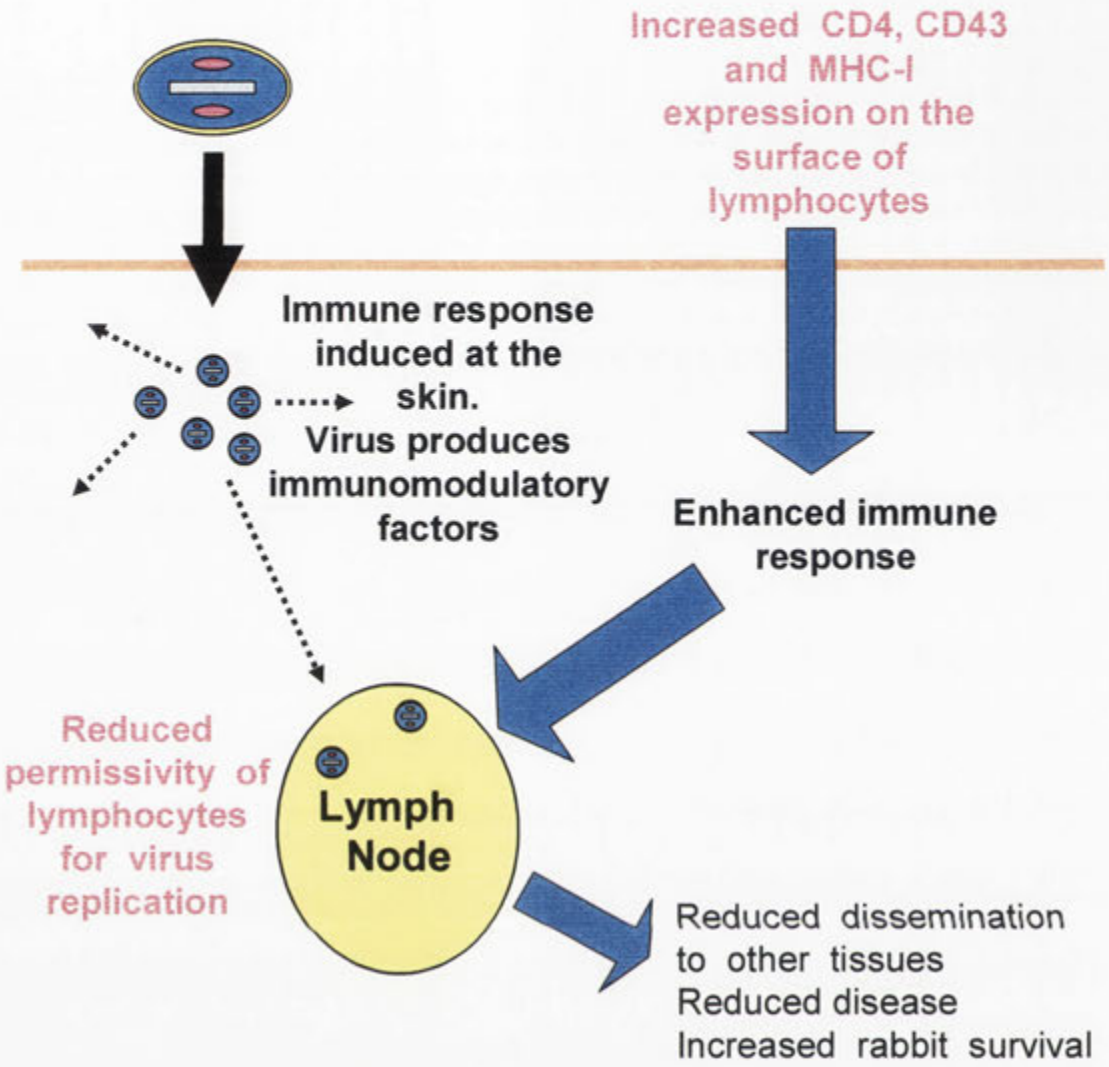


FIGURE 7.1. PROPOSED MODEL FOR RESISTANCE OF RABBITS TO MYXOMATOSIS

Results from this thesis are shown in red. Myxoma virus infects a resistant rabbit. Resistant rabbits have increased expression of CD4 and CD43 on the surface of resting lymphoid cells and MHC-I on the surface of activated lymphoid cells. This enables a more effective and efficient early immune response against the virus. The virus that reaches the lymph node draining the inoculation site is not able to replicate as efficiently in T-cells as these cells are less permissive for virus replication. This, along with the enhanced immune response, which would also be acting in the draining lymph node, reduces the production of infectious virus and limits virus dissemination to distal tissues. This reduces disease and prolongs rabbit survival time.

chapter 3 of this thesis. This leads to the reduced viral titres in lymphoid tissues compared to infected susceptible rabbits reported by Best and Kerr, (2000). The lower level of virus replication in resistant rabbits compared to susceptible rabbits, reduces

virus dissemination to distal tissues as previously shown (Best and Kerr 2000). The lower levels of myxoma virus replication in resistant rabbit lymphocytes also leads to reduced production of viral immunomodulatory proteins and therefore a lower ability of myxoma virus to suppress the immune response at the level of the lymph node. In addition to this, the increased cell surface expression of CD4 and CD43 on resting lymphocytes from resistant rabbits, as described in chapter 5, may generate a faster and more effective immune response early in infection. The immune response may be enhanced once the lymphocytes are activated by the increased expression of MHC-I on lymphocytes from resistant rabbits compared to lymphocytes from susceptible rabbits.

The lower virus replication and subsequent immunosuppression together with the increased ability of the resistant rabbit lymphocytes to participate in the anti-viral immune response (due to the increase in expression of certain cell surface proteins) enable more effective and efficient control and clearance of virus from the resistant rabbit, reduced clinical disease and increased rabbit survival when compared to susceptible rabbits. It must be noted that the resistant rabbit is not able to reduce the replication of virulent SLS to as great an extent as it is for the attenuated virus strains such as Ur, but is still able to reduce replication to levels lower than those in susceptible rabbits. This gives resistant rabbits an advantage over susceptible rabbits in clearing myxoma virus infection.

In susceptible rabbits, myxoma virus is able to effectively replicate in tissues distal to the skin. Lymphocytes from susceptible rabbits also have lower levels of CD4, CD43 and MHC-I on resting lymphocytes relative to resistant rabbits. It is therefore predicted that lymphocytes from susceptible rabbits are not able to respond to infection as quickly or effectively as lymphocytes from resistant rabbits. This leads to susceptible rabbits not

being able to control myxoma virus replication and dissemination and replication in distal tissues to as great an extent as in resistant rabbits, often resulting in death.

7.3 Attenuation of Myxoma Virus

This thesis is the first report of a potential mechanism for attenuation of a naturally-derived strain of myxoma virus. Attenuation of the Ur strain of myxoma virus was examined by comparing infection of lymphoid cells with the virulent SLS and attenuated Ur strain of myxoma virus. A difference between the two strains of myxoma virus was observed in that SLS was more able to maintain lymphocyte viability after cell cycle arrest than Ur (chapter 4). The genetic mechanism for this difference was investigated by sequencing genes whose products have putative anti-apoptotic or host-range function. A lesion was detected in the M005 ORF which is potentially responsible for the inability of Ur to maintain lymphocyte viability. The genetic mechanism of attenuation of naturally derived strains of myxoma virus has not previously been investigated.

Attenuation of a virus, in a particular host species, must be accompanied by changes in its genetic sequence. Most genetic studies of myxoma virus have examined the effects of experimentally deleting genes on myxoma virus pathogenesis. These studies have shown that the deletion of one of a number of genes (e.g. M011, M002, M004 and M005 ORFs) leads to an attenuated disease phenotype *in vivo* (Macen et al., 1996; Mossman et al., 1996; Opgenorth et al., 1992; Barry et al., 1997). However, it is unlikely that Ur attenuation is due to deletion of an entire gene as, in the field, the transmission of any myxoma virus strain that has a deletion of a virulence gene will most likely be compromised. The deletion mutants that are attenuated have limited

replication and are quickly controlled by the host immune response. This will result in the virus not being able to remain in the skin at titres sufficient for transmission. The virus strain will not be able to transmit to a new host and will rapidly die out (Fenner and Kerr 1994; Fenner and Ratcliffe 1965).

Experiments have shown the postulated mechanism of attenuation of these recombinant myxoma viruses with any one of M011, M002, M004 or M005 deleted is the induction of lymphocyte death, as has been demonstrated *in vitro* (Macen et al., 1996; Mossman et al., 1996; Opgenorth et al., 1992; Barry et al., 1997). Lymphocytes are important for virus amplification and dissemination *in vivo*, so the inability of the deletion viruses to replicate in lymphocytes will reduce virus replication and dissemination and enable effective control of the virus by the rabbit. Control of virus replication will reduce pathology and disease and enhance rabbit survival.

However, all of these deletion mutant viruses could replicate in fibroblast cells as efficiently as wild type virus. The ability of myxoma virus to replicate in fibroblast and other skin cells is essential for transfer of virus between hosts. This is because myxoma virus is passively transmitted between hosts on the mouthparts of biting arthropods. In its natural host, *S. brasiliensis*, myxoma virus replicates in the skin and so the virus can propagate between hosts. Virus does not infect lymphocytes and does not disseminate systemically where it could cause disease and potentially death of the host. Such a scenario would reduce the time period the virus has available to produce infectious progeny. Thus in its natural host, it could be considered that myxoma virus has evolved to have the maximum opportunity for replication.

In *O. cuniculus,* which was immunologically naïve to myxoma virus prior to the virus'

introduction into wild rabbit populations, myxoma virus can infect lymphocytes, does disseminate systemically and often causes death of the infected rabbit. This suggests that as well as the ability of myxoma virus to replicate in lymphocytes being critical for virulence it may also be critical in an evolutionary sense.

The pathogenesis of virulent and attenuated myxoma virus strains has previously been examined (Best and Kerr, 2000; Best et al, 2000). In wild rabbits Ur titres are reduced at the lymph node draining the inoculation site and dissemination to distal tissues from this site is delayed compared to SLS (Best and Kerr 2000). This supports the hypothesis in this thesis that the ability of specific naturally-attenuated strains of myxoma virus to replicate in lymphocytes is a determinant of myxoma virus virulence. Further work on other attenuated strains of myxoma virus is required to determine whether the inability to replicate in lymphoid cells is a common phenotype of these strains. To explain the reduced titres and delayed dissemination of Ur, Best and Kerr (2000) suggested three possibilities: either Ur disseminates less efficiently *in vivo* than SLS; the lymph node draining the inoculation site controls virus replication thereby preventing dissemination; or there is an enhanced immune response in rabbits infected with Ur that controls virus replication in distal tissues. These possibilities are potentially inter-linked.

Results from this thesis support all three of these hypotheses. Firstly, both strains of myxoma virus inhibited cell proliferation before S phase of the cell cycle (chapter 3). However, SLS and Ur differed in their ability to maintain lymphocyte viability after cell cycle arrest (chapter 4). For instance, in Ur-infected cultures there was a reduction in lymphocyte viability, presumably due to the failure of Ur to inhibit cell death pathways that were initiated on cell cycle arrest. In contrast, SLS was able to maintain lymphocyte viability. Genetic analysis undertaken in this thesis is the first identification of a genetic

lesion that may alter the effect of the virus on lymphocyte viability.

Sequencing of the M005 ORF in Ur found an insertion of a cytosine relative to the M005 ORF in SLS (chapter 4). The insertion of this cytosine induces a frameshift, potentially resulting in production of a truncated M005 protein. M005 has been previously implicated in lymphocyte survival (Mossman et al., 1996), so this lesion may be responsible for the reduction in lymphocyte viability observed in Ur-infected cultures at late time points (discussed in chapter 4). The failure of Ur to maintain lymphocyte viability may limit virus dissemination *in vivo* as Ur infection will kill the cell. However, it is unclear whether this lesion is responsible for the reduced ability of Ur to replicate in lymphocytes from wild rabbits compared to laboratory rabbits as observed in chapter 3. Whether the lesion in M005 is responsible for attenuation of Ur could be examined by creating a recombinant Ur strain of myxoma virus that has the SLS M005 gene inserted, and testing for its ability to maintain lymphocyte viability or whether its virulence in rabbits is increased.

Overall, activation of T-cells significantly increased both the viral titres produced in infected lymphoid cell cultures and the proportion of lymphocytes infected with myxoma virus (chapter 3). The increase in SLS titres observed after activation of rabbit lymphocytes was due to an increase in virus production in every infected cell. For Ur infection, however, the increase in titres observed with Con A stimulation, which was only detected in lymphoid cell cultures from laboratory rabbits, was due to an increase in the number of infected cells. These results illustrated that the use of resistant rabbits in experiments examining myxoma virus attenuation may highlight some factors involved in attenuation.

Lymphocytes will be activated during an immune response and are presumably activated in the lymph node draining the inoculation site due to the immune response being initiated in the skin. The results in this thesis suggest that Ur replication in activated lymphocytes in the rabbit lymph node will not be as efficient as SLS. The lower replication of Ur will enable greater control of the virus at the lymph node draining the inoculation site, which will reduce virus dissemination and immunosuppression and enhance virus clearance.

The third possibility presented by Best and Kerr (2000) was that there was an enhanced innate immune response in rabbits infected with Ur that controlled virus replication sufficiently for a T-cell response to develop. This T-cell response subsequently controls virus replication in distal tissues. This thesis showed that both strains of myxoma virus, although preferentially replicating in activated T-cells, completely abrogated features of T-cell activation, such as cell proliferation, increase in cell size and up-regulation of CD25, CD4 and MHC-I on the lymphocyte surface. This effect was observed in lymphoid cell cultures that were activated after myxoma virus infection, but not in cultures activated prior to infection.

The reduced ability of Ur to replicate in lymphoid cells may allow a greater number of lymphocytes to function appropriately *in vivo*. This, combined with the reduced dissemination of Ur and the lower ability of Ur to replicate in activated cells, as discussed in the previous paragraphs, will enable a more effective immune response that will help control virus replication in distal tissues. The greater number of lymphocytes that function appropriately may also account for the observation of Best et al. (2000) that the lymph nodes in susceptible rabbits infected with SLS are effectively depopulated of lymphocytes, but in Ur infection, there is no net loss of lymphocytes that

is visible histologically.

7.3.1 Summary of Experimental Results Pertaining to Attenuation

The main results from this thesis that provide the basis for the model outlined in Section 7.3.2 are as follows:

1. Ur was not able to maintain lymphocyte viability late in infection, whereas SLS was able to maintain lymphocyte viability late in infection;
2. Ur had a genetic lesion in M005 compared to SLS, which may be responsible for the inability of Ur to inhibit cell death pathways; and
3. Ur-infected lymphocytes generated lower titres of virus per cell when activated compared to lymphocytes infected with SLS.

7.3.2 Proposed Model for Ur Attenuation

In conclusion, this thesis proposes the following model for Ur attenuation (Figure 7.2). Infection of a rabbit with SLS or Ur induces an inflammatory response in the skin, which activates cells in the lymph node. Both SLS and Ur reach the lymph node draining the inoculation site at similar times and infect T-cells. Both strains abrogate T-cell proliferation as shown in this thesis. However, Ur cannot replicate as effectively as SLS as shown by the differences in viral titre per infected cell (chapter 3), and fails to maintain lymphocyte viability after cell cycle arrest (chapter 4). This may explain the decreased titres in the lymph node draining the inoculation site observed in wild rabbits by Best and Kerr (2000). Although Best and Kerr (2000) did not observe reduced Ur titres at the draining lymph node in susceptible rabbits, it is postulated, based on results from chapter 3 of this thesis that Ur will not replicate as efficiently as SLS in the lymph

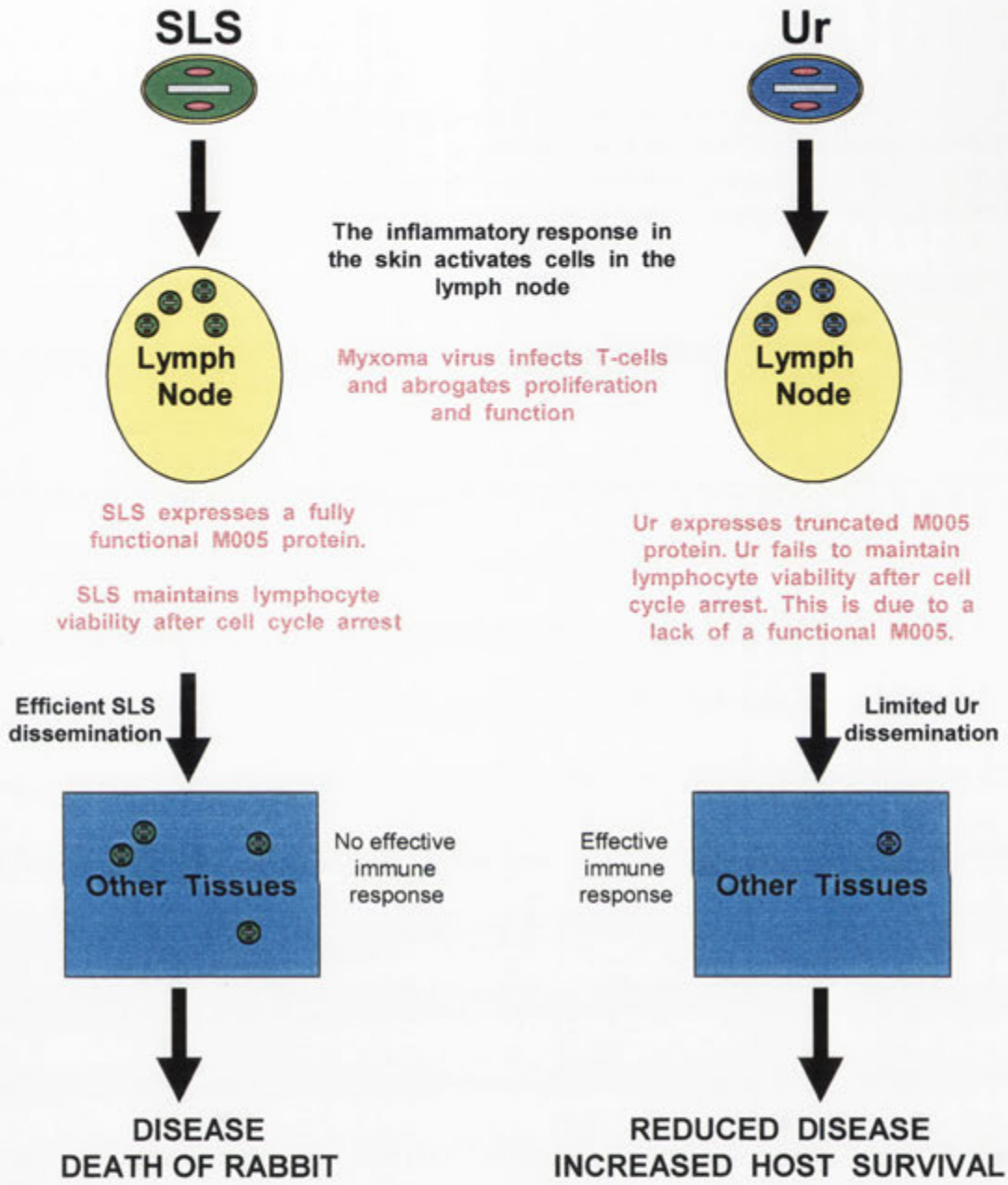


FIGURE 7.2. PROPOSED MODEL FOR ATTENUATION OF MYXOMA VIRUS

Results from this thesis are shown in red. Both SLS and Ur reach the lymph node draining the site of infection at similar times. Here they infect T-cells which, along with monocytes and macrophages, disseminate the virus to distal tissues. Ur can not maintain the viability of T-cells late in infection possibly due to the lesion in the M005 gene, so limited Ur dissemination occurs. This reduces Ur in distal tissues and therefore Ur replication in these tissues. The reduced dissemination and replication of Ur at these sites leads to reduced disease and pathology and increased rabbit survival. In contrast SLS is able to replicate and disseminate in T-cells. SLS also maintains lymphocyte viability late in infection. This may allow greater virus replication in the distal tissues causing pathology and severe clinical disease in the rabbit.

node draining the inoculation site in these rabbits.

The failure of Ur to maintain lymphocyte viability is potentially due to the mutation in the M005 ORF. A decrease in viability of infected lymphocytes may reduce the ability of Ur to disseminate to distal tissues. This would lead to lower levels of virus present in distal tissues and, coupled with the lower levels of Ur replication per cell in these tissues compared with SLS, would enable easier control and clearance of virus.

The lower levels of Ur replication would lead to reduced production of immunomodulatory proteins and lower ability of Ur to suppress the immune response which also favors development of an effective cell-mediated immune response. This, together with the enhance control and clearance due to reduced Ur titres would explain the decrease in the clinical disease in Ur-infected rabbits and increase in rabbit survival.

As SLS is able to maintain lymphocyte viability, SLS would be able to disseminate and would potentially have greater chance to replicate to high numbers. The increase in viral replication in lymphocytes and the maintenance of lymphocyte viability would contribute to the systemic dissemination, severe pathology and reduced rabbit survival observed with SLS infection.

It has been previously shown that SLS induces apoptosis in lymphocytes from the lymph node draining the inoculation site early in infection (Best et al., 2000). This was hypothesized to be due to either the lymphocytes being primed for apoptosis prior to expression of late viral proteins, significant external factors (cytokines or ligand interactions) that induce apoptosis in uninfected cells, partial activation of antigen-specific cells or the production of a virus-induced factor that induces apoptosis in uninfected cells. Results from this thesis are not able to determine which of these is the mechanism for the early apoptosis observed. However, experiments in this thesis

were done in isolation from external *in vivo* factors and found that lymphoid cell cultures infected with SLS had less cell death than those infected with Ur, suggesting that significant external factors generated outside the lymph node are required either directly or indirectly for the induction of bystander apoptosis.

7.4 CONCLUSION

This thesis has identified a putative mechanism for resistance of rabbits to myxomatosis. Based on results obtained, resistance to myxomatosis is postulated to be due to a combination of a reduced permissivity of lymphocytes from resistant rabbits for virus replication and an increase in expression on naïve T-cells of certain cell surface proteins critical for the immune response.

This thesis is also the first report of a naturally occurring mechanism for attenuation of myxoma virus. Based on flow cytometric analysis of infected lymphocyte cultures and of sequencing, attenuation of the Ur strain of myxoma virus is postulated to be due to a lesion in the M005 gene that reduces the ability of this strain to maintain lymphocyte viability after cell cycle arrest.

This thesis also showed that the use of an attenuated strain of myxoma virus could be used to model some aspects of rabbit resistance and conversely the use of rabbits with some resistance to myxomatosis could be use to model some aspects of virus attenuation.

In conclusion, this thesis indicates that the interactions between myxoma virus and T-cells may be critical for determining the outcome of infection and do indeed play a

major role in the pathogenesis of myxoma virus in resistant rabbits and of attenuated strains of virus. Future studies applying these *in vitro* findings to *in vivo* experiments will extend our understanding of the genetic mechanism of resistance of rabbits to myxomatosis and also of the attenuation of myxoma virus in the field.

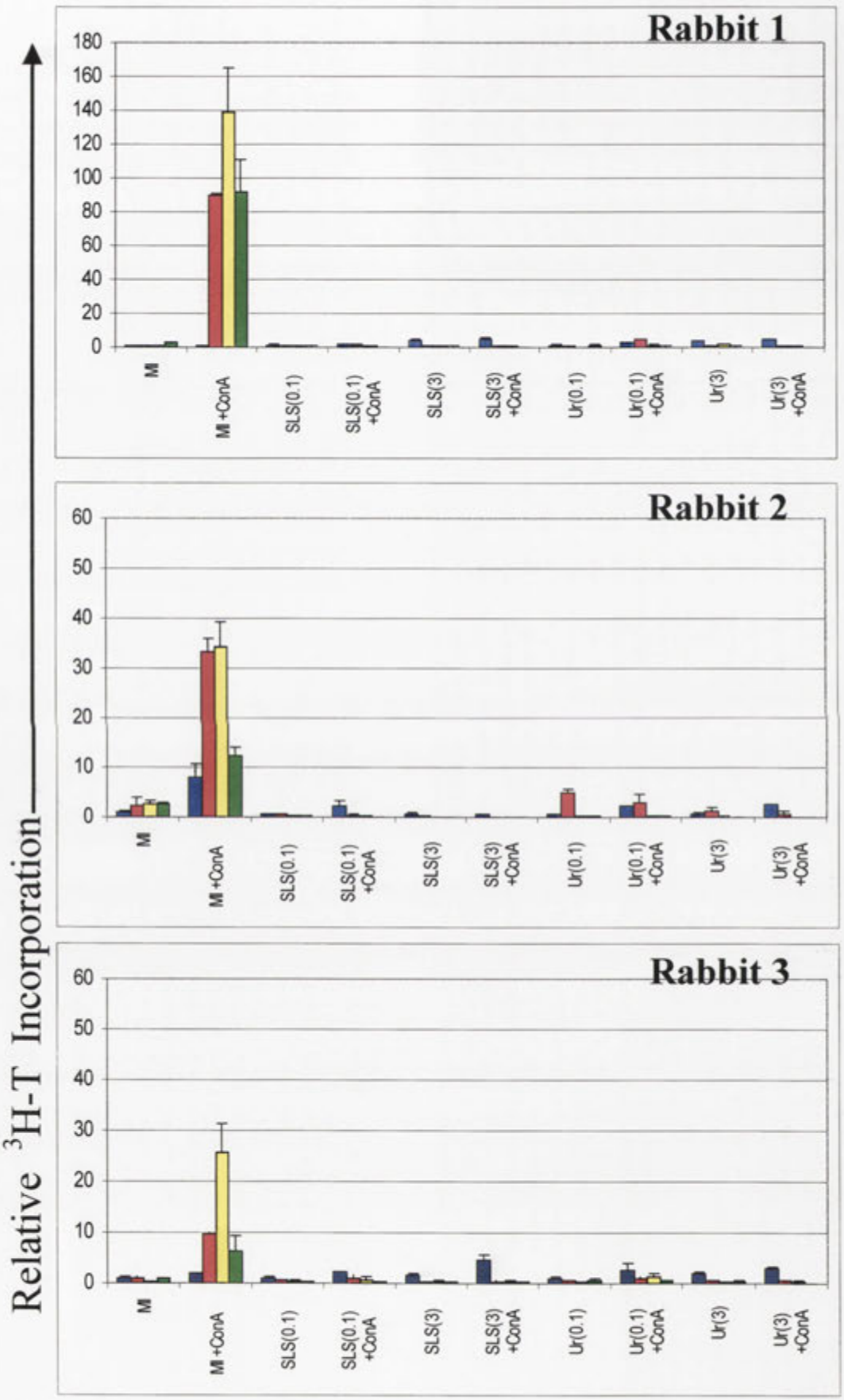
APPENDICES

APPENDIX 1. 3H-T INCORPORATION OF LYMPHOID CELLS FROM LABORATORY OR WILD RABBITS INFECTED WITH SLS OR UR

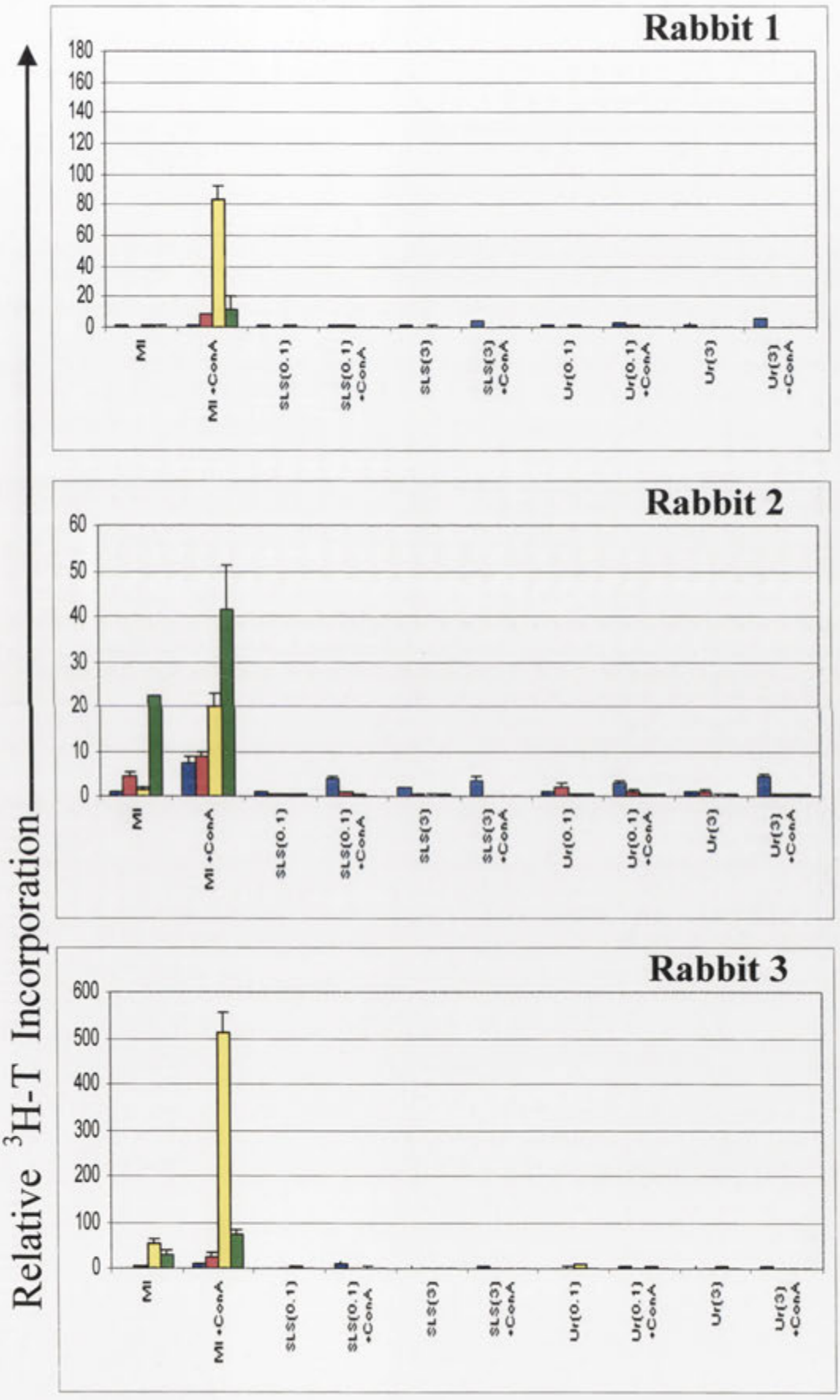
Lymphoid cell proliferation was measured by ^{3}H-T incorporation. Lymphoid cells were isolated from (A) laboratory and (B) wild rabbits and mock-infected (MI) or infected with SLS or Ur at a concentration of 0.1 or 3 pfu/input cell, for one hour prior to 0h time point. Con A was added at 0h. ^{3}H-T was added at 0■, 24■, 48 and 72h■ and the cells pulsed for 18h. Values were adjusted such that ^{3}H-T incorporation of MI cultures at 0h was equal to 1. Reactions were done in triplicate and repeated for three laboratory and three wild rabbits (labeled 1, 2 and 3). Error bars represent standard errors. Due to the variability observed with this technique, graphs have different scales.

Graphs from all rabbit cultures are shown here in addition to the graph of rabbit #1 which is shown in the body of the thesis (Figure 3.4).

A Laboratory Rabbit Lymphoid Cultures



B Wild Rabbit Lymphoid Cultures

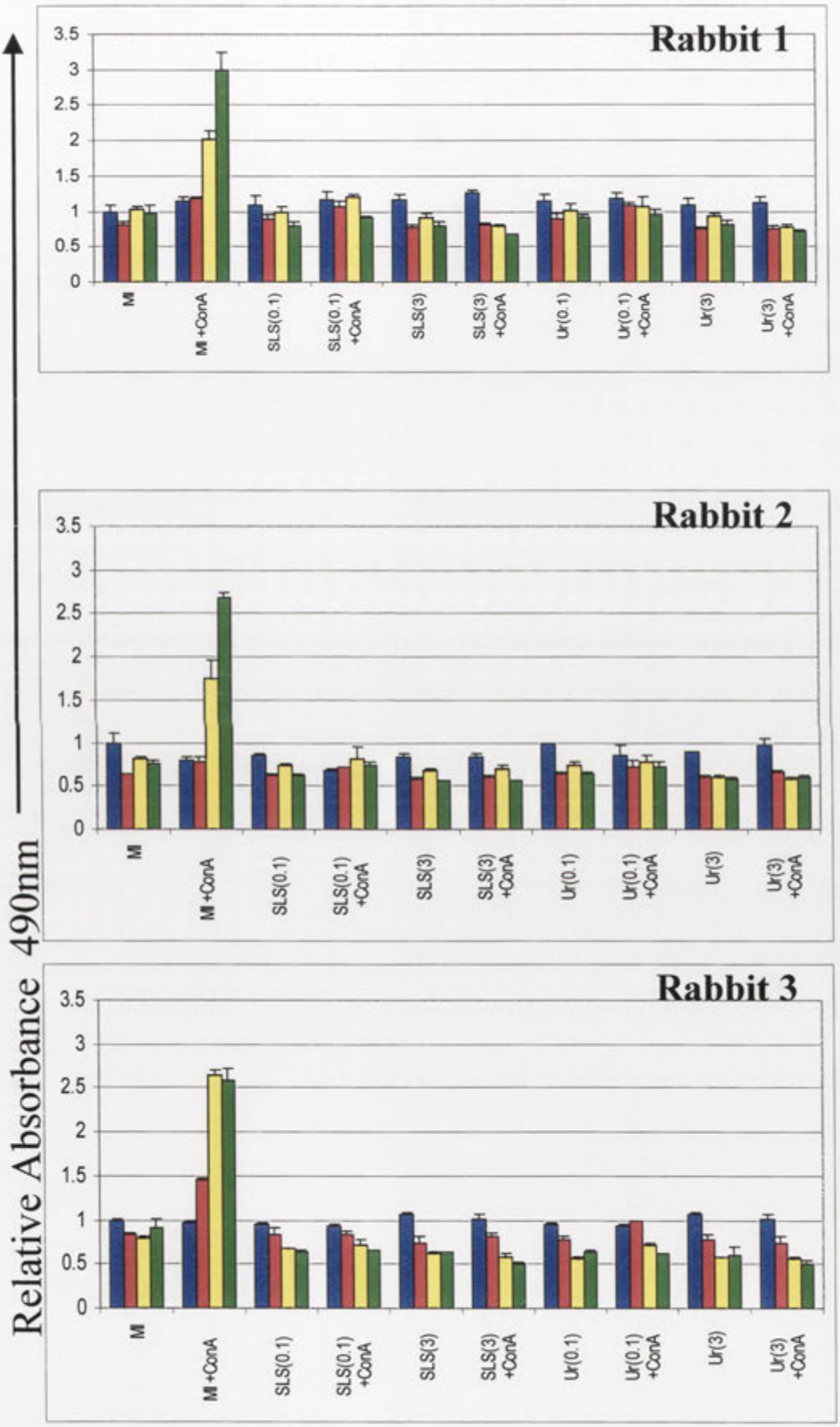


APPENDIX 2. PROLIFERATION OF RABBIT LYMPHOID CELLS FROM LABORATORY OR WILD RABBITS AND INFECTED WITH SLS OR UR

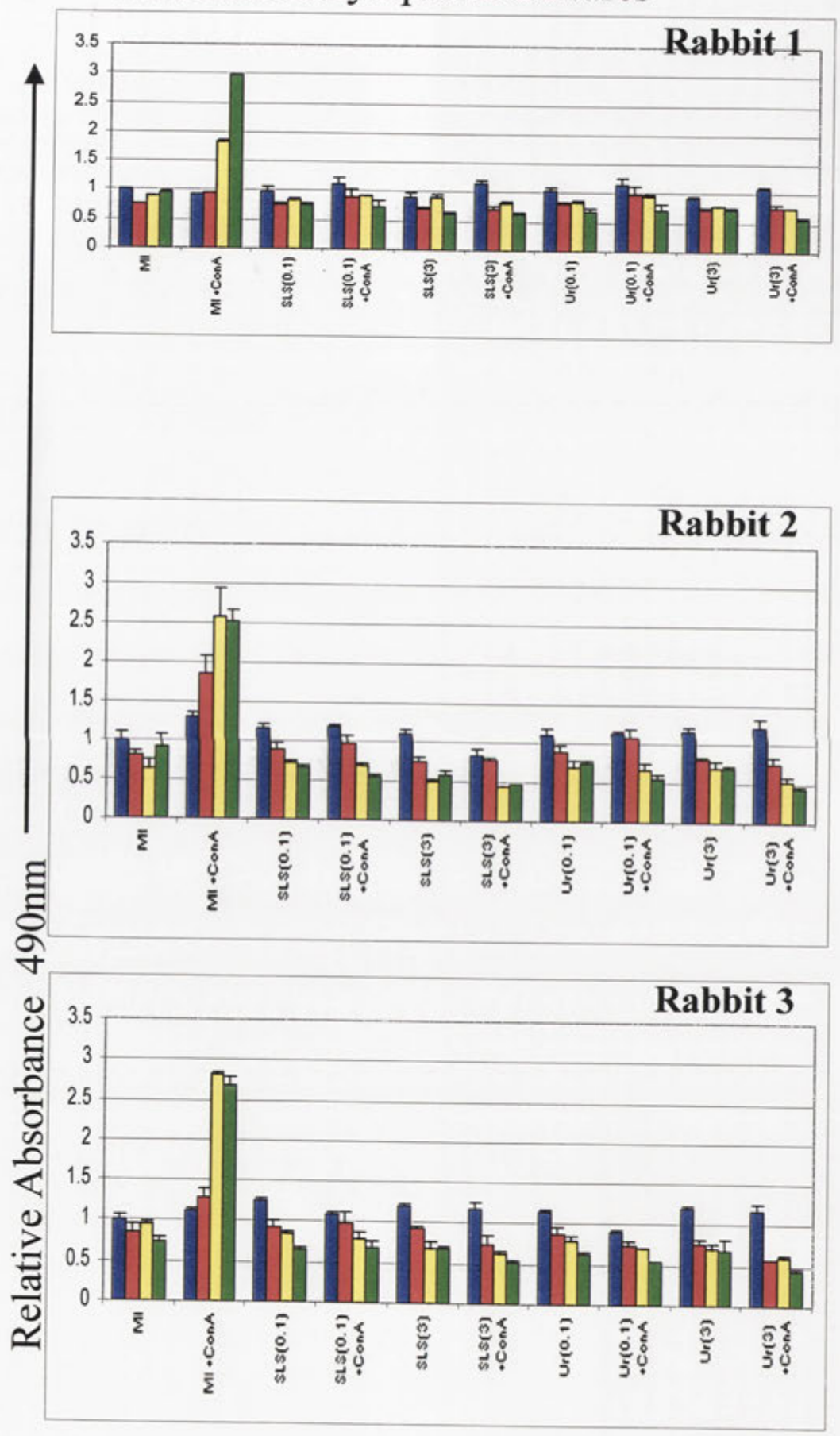
The proliferation of lymphoid cells from (A) laboratory and (B) wild rabbits was measured using the CellTitre 96® AQueous One Solution Cell Proliferation Assay using the MTS reagent. Lymphoid cells were mock-infected (MI) or infected with SLS or Ur at a moi of 0.1 or 3 pfu/input cell, for one hour prior to the 0h time point. Con A was added at 0h. The MTS reagent was added at 0■, 24■, 48 and 72h■ post infection. Absorbance was read at 490nm after a 4h incubation with the MTS reagent. Values were adjusted such that the absorbance of MI samples at 0h was equal to 1. Reactions were done in triplicate and repeated using cultures from three laboratory and three wild rabbits (labelled 1, 2 and 3). Error bars represent standard errors.

All graphs from all rabbits are shown here in addition to the graph of rabbit #1 which is shown in the body of the thesis (Figure 3.5).

A Laboratory Rabbit Lymphoid Cultures



B Wild Rabbit Lymphoid Cultures

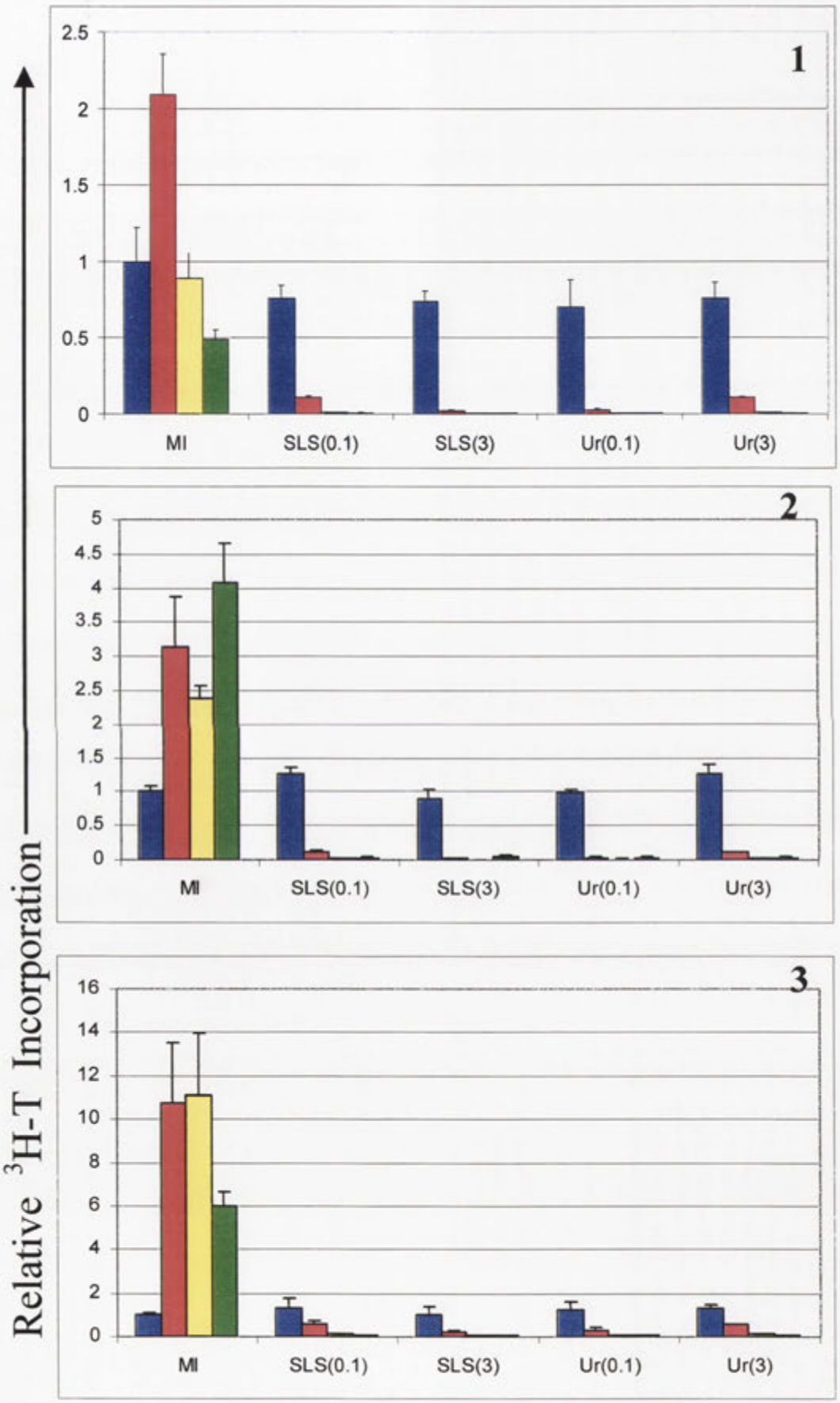


APPENDIX 3. PROLIFERATION OF THE RABBIT T-CELL LINE, RL-5, INFECTED WITH SLS OR UR

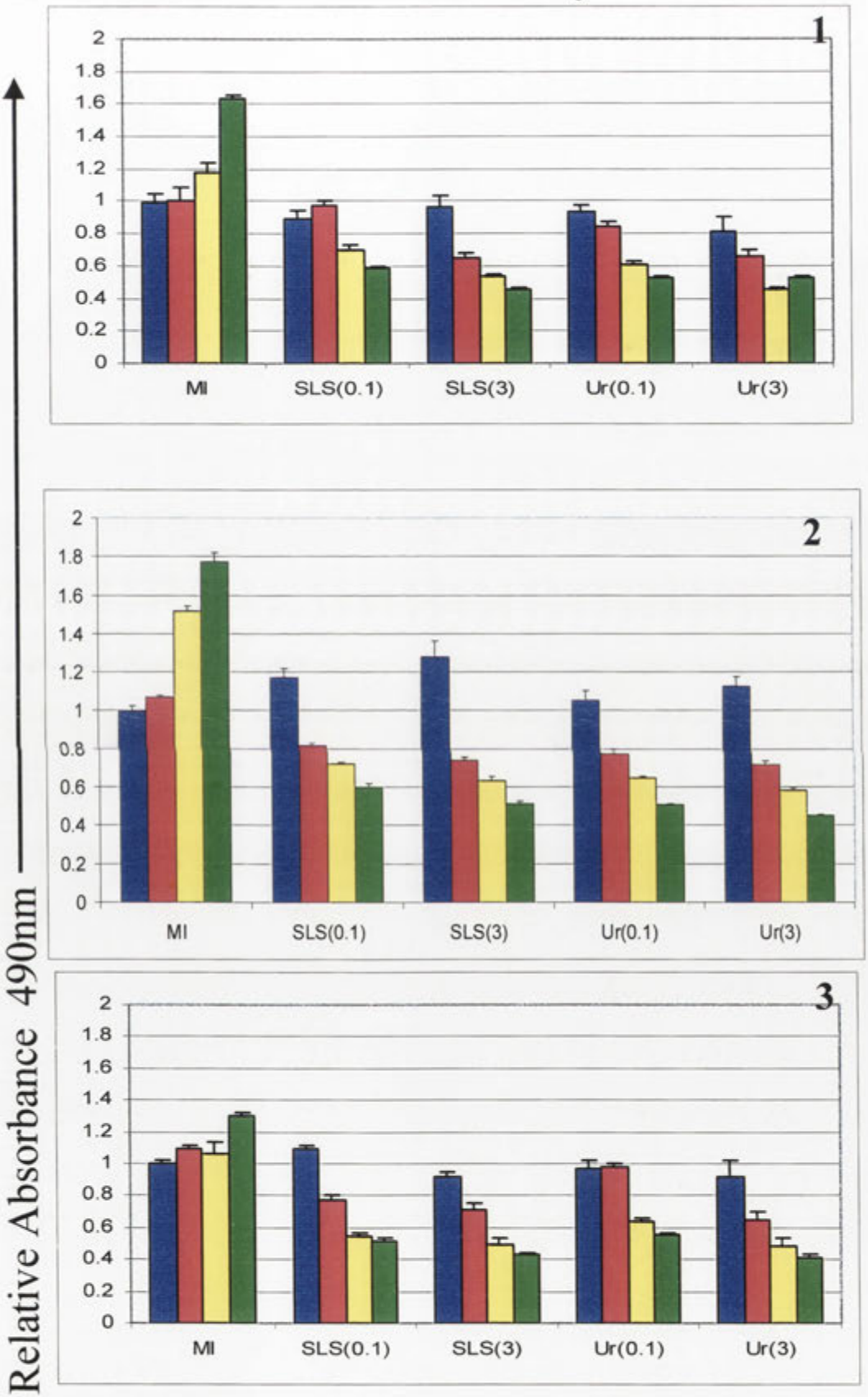
RL-5 proliferation was measured by tritiated thymidine incorporation and the CellTitre 96® AQueous One Solution Cell Proliferation Assay, using the MTS reagent. RL-5 cells were mock-infected (MI) or infected with SLS or Ur at a moi of 0.1 or 3 pfu/input cell, for one hour prior to 0h time point. 3H-T or MTS reagent was added at 0■, 24■, 48 and 72h■ post infection. The cells were pulsed for 4h with 3H-T or MTS reagent. Values were adjusted such that the 3H-T incorporation or absorbance of MI samples at 0h equaled 1. Reactions were done in triplicate and repeated three times (represented by 1, 2 and 3). Error bars represent standard errors. Due to the variability observed with this technique, graphs may have different scales.

All graphs are shown here in addition to the graphs shown in the body of the thesis (Figure 3.6).

A ^{3}H-T incorporation assay



B CellTitre Proliferation Assay



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