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UNIVERSITY OF ALBERTA

STUDIES ON POXVIRUS PATHOGENESIS AND VIRUS-HOST INTERACTIONS

BY

JOANNE LESLIE MACEN

A Thesis

Submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Biochemistry

Edmonton, Alberta

Fall, 1995

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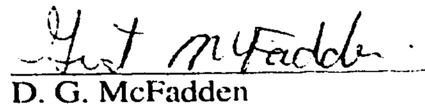

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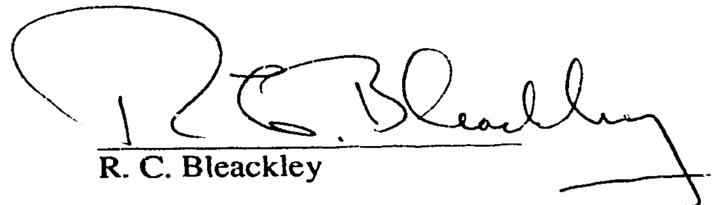

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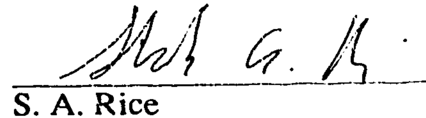

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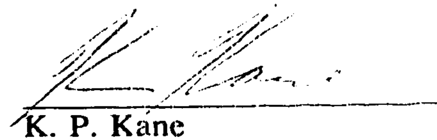

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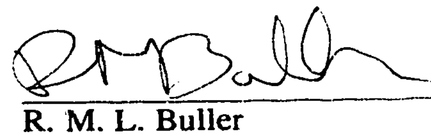

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If we open our eyes, if we open our minds, if we open our hearts, we will find that this world is a magical place. It is magical not because it tricks us or changes unexpectedly into something else, but because it can *be*, so vividly and brilliantly.

-Chögyam Trungpa

It is enough if one tries every day to comprehend a little of this mystery.

-Albert Einstein

For Shaman,

who is so much more

than what he seems

ABSTRACT

The poxviruses are a large family of complex DNA viruses that are ubiquitous in nature, and cause a diverse array of diseases that range from benign, localized infections to severe generalized infections that may be fatal. The extreme virulence exhibited by certain poxviruses is mediated by specific viral gene products that function to circumvent numerous host anti-viral defenses. The identification and analysis of these viral proteins is important for our understanding of both viral determinants of pathogenesis and virulence as well as the host anti-viral response.

Myxoma virus is a Leporipoxvirus that causes the highly lethal generalized infection in the European rabbit known as myxomatosis. Viruses containing targetted gene disruption mutations were constructed and used to show that the myxoma virus-encoded serine proteinase inhibitor, SERP1, and a secreted homologue of the receptor for tumour necrosis factor, T2, are important virulence factors for virus infection *in vivo*. Further studies of the SERP1 protein are presented, indicating that SERP1 is expressed from a late gene as a secreted glycoprotein that functions to increase virulence through interfering with the host inflammatory response. Flow cytometric analyses of cell surface proteins were performed and suggest that myxoma virus infection may result in compromised immune recognition of virally infected cells, through the downregulation of class I MHC proteins. During the course of these experiments, it was discovered that the myxoma virus T2 and M11L proteins function to prevent apoptotic death of infected T lymphocytes, suggesting that the T2 protein has more than one function. In addition, the related leporipoxvirus SFV was also shown to induce apoptosis in infected lymphocytes, resulting in a non-productive infection. Finally an investigation of the roles of several poxviral serpins in the inhibition of cell-mediated cytolysis indicates that while the myxoma virus SERP1 protein does not

function to inhibit cell-mediated cytolysis, the Orthopoxvirus SPI-1 and SPI-2 proteins contribute to inhibition of cytolysis mediated both by signalling through the Fas cell surface receptor as well as though the activity of proteins contained within cytolytic granules, and as such may represent useful tools with which to dissect the cytolytic mechanisms used by cytotoxic T lymphocytes.

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LIST OF ABBREVIATIONS

AraC ....cytosine arabinoside
ATCC..............................American type culture collection
bp..................................base pairs
BUdR..............................bromo-deoxyuridine
CAM ....chorioalantoic membrane
CPV...............................cowpox virus
CrmA,B,C ....cytokine response modifier A, B, or C
CTL................................cytotoxic T lymphocytes
DMEM............................Dulbecco's modified Eagle's medium
DNA ....deoxyribonucleic acid
DPBS ....Dulbeco's phosphate buffered saline
E. coli.............................Escherichia coli
EDTA ....ethylene diamine tetraacetic acid
EEV ....extracellular enveloped virus
EGF...............................epidermal growth factor
ER ....endoplasmic reticulum
FBS ....fetal bovine serum
FDG...............................fluorescein-di-ß-D-galactopyranoside
FITC ....fluorescein
gpt.................................guanosine phosphoribosyl transferase
ICE................................interleukin-1-beta converting enzyme
IFN................................interferon
IFNα/β...........................interferon alpha/interferon beta
IFNγ ....interferon gamma
IL-1 ....interleukin-1
IL-1ß..............................interleukin-1-beta
IL-2 ....interleukin-2
INV ....intracellular enveloped virus
IPTG..............................isopropylthio-ß-D-galactoside
Kb.................................kilobases
mAb...............................monoclonal antibody
MGF ....myxoma growth factor
MHC..............................major histocompatibility complex

MOI	multiplicity of infection
MRV	malignant rabbit fibroma virus
NCS	newborn calf serum
NGFR	nerve growth factor receptor
NK	natural killer
ORF	open reading frame
p.i.	post-infection
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
PMSF	phenylmethlysulphonyl fluoride
RNA	ribonucleic acid
RPE	r-phycoerythrin
RPV	rabbitpox virus
SCID	severe combined immunodeficient
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
serpin	serine proteinase inhibitor (refers to proteins belonging to the superfamily)
SFGF	Shope fibroma growth factor
SFV	Shope fibroma virus
SPI-1,2,3	serine proteinase inhibitor-1, -2, -3 (serpins encoded by the orthopoxviruses)
ß2m	beta-2-microglobulin
TdT	terminal deoxynucleotidyl transferase
TGF-α	transforming growth factor alpha
TGF-ß	transforming growth factor-beta
TIR	terminal inverted repeat
TK	thymidine kinase
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
VCP	vaccinia complement control protein
VGF	vaccinia growth factor
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

LIST OF VIRUSES USED

Myxoma virus	strain Lausanne
vMyxlac	recombinant myxoma virus that expresses the E. coli LacZ gene from a non-disruptive intergenic site
SFV	Shope fibroma virus (strain Kasza)
MRV	malignant rabbit fibroma virus, a recombinant between myxoma virus and SFV (strains unknown)
MRV-SERP1$^-$	also referred to as MRV-S1, a recombinant in which the SERP1 gene is disrupted
vMyx-SERP1$^-$	a recombinant myxoma virus (Lausanne) in which both copies of the SERP1 gene are disrupted
vMyx-T2$^-$	also referred to as vMyxT2g, a recombinant myxoma virus in which both copies of the T2 gene are disrupted
vMyx-M11L$^-$	a recombinant myxoma virus (Lausanne) in which the M11L gene is disrupted
vaccinia virus	Western reserve (WR) strain
VV-S1	recombinant vaccinia virus (WR) in which the E. coli Lac Z gene and the myxoma virus SERP1 gene are expressed from viral promoters inserted into the vaccinia thymidine kinase gene
VV601	control recombinant vaccinia virus (WR) that expresses the E. coli Lac Z gene from a viral promoter inserted into the vaccinia thymidine kinase gene

RPV	rabbitpox virus (Utrecht strain)
RPVΔSPI-1	recombinant RPV with a deletion of the SPI-1 gene
RPVΔSPI-2	recombinant RPV with a deletion of the SPI-2 gene
RPVΔSPI-1/2	recombinant RPV with deletions of the SPI-1 and the SPI-2 genes
CPV	cowpox virus (Brighton red strain)
CPVΔSPI-1	recombinant CPV with a deletion of the SPI-1 gene
CPVΔSPI-2	recombinant CPV with a deletion of the SPI-2 gene

CHAPTER I

GENERAL INTRODUCTION

The inherent nature of viruses and other obligate intracellular parasites requires that they evolve in intimate association with the hosts they invade. It is therefore not surprising that, while higher organisms have evolved a complex and often bewildering array of defenses against invading pathogens, so too have their assailants developed a multitude of strategies by which they evade and counteract these defenses. Within the context of a host that is structurally and genetically thousands of times more complex than itself, the invading virus embarks on a daunting mission. Finding its way into cells for which it has adapted itself, the virus must avail itself of all necessary host functions and materials, carry out a complete replicative cycle and make its progeny available for introduction into a new host organism, all in the face of a variety of both specific and nonspecific antiviral challenges, and without causing the demise of the host before the mission is complete. In our attempts to discover and understand the mechanisms that viruses use to accomplish these tasks, we learn not only about the viruses themselves, but also gain insight into the awesome complexity of the host organisms that they infect.

The poxviruses are among the largest eukaryotic DNA viruses and infect a variety of both vertebrate and invertebrate hosts. In their animal hosts, poxvirus infection results in a wide array of disease pathologies which, depending on the virus, route of inoculation and immunological state of the host, range from mild, localized infections to severe generalized infections that may be fatal. Research conducted during the last decade suggests that the highly virulent nature of certain members of this virus family results from the combined effects of numerous specific viral gene products that function to effectively disarm the various facets of the host anti-viral machinery. Indeed, the size and diversity of the poxvirus family itself attests to the successful evolution of such strategies and moreover suggests that much can be learned about the host response to acute viral infection from the study of the interaction of poxviruses

with their hosts. The studies presented in this thesis represent an attempt to contribute to our understanding of poxvirus-host interactions at both the molecular and cellular levels.

In this chapter, the characteristics of the poxviruses are outlined, with particular emphasis on members of the leporipoxvirus genus, and on determinants of poxviral pathogenicity and virus-host interactions relevant to the studies presented in the following chapters.

Physical properties and classification of poxviruses

The largest of all animal viruses, the Poxviridae comprise a family of complex DNA viruses that are ubiquitous in nature, with members that infect a wide variety of both vertebrate and invertebrate hosts. The classification and properties of poxviruses have been reviewed extensively elsewhere (Moss, 1990a; Buller and Palumbo, 1991) and are only briefly outlined here. In general, members of the poxvirus family share the following physical properties: (a) Complex virion morphology: the virion consists of a rectangular or oval shaped particle 200-400 nm in length, with an outer lipoprotein membrane surrounding the viral core, which in turn consists of a biconcave structure containing the viral DNA complexed with a number of proteins including enzymes involved in early RNA synthesis. Generally, two electron dense structures of unknown function termed lateral bodies are found residing in the concavities of the core. (b) Genome structure: the poxvirus genome consists of a single linear, double stranded DNA molecule that is 130 to 300 kilobases in length, with covalently closed hairpin termini and terminal inverted repeats (TIRs) of variable length. (c) Replication site:

poxvirus replication and virus assembly occurs within the cytoplasm of the infected cell.

Original classification schemes used for poxviruses were largely based on gross pathology and disease symptomology, and were later revised to include the morphology of the virion and cytoplasmic inclusion bodies, and more recently genomic structure. The family is further subdivided into two subfamilies, namely the Chordopoxviridae (poxviruses of vertebrates) and the Entemopoxviridae (poxviruses of insects). The Chordopoxviridae have been subdivided into eight genera: Orthopoxvirus, Leporipoxvirus, Parapoxvirus, Avipoxvirus, Capripoxvirus, Suipoxvirus, Molluscipoxvirus and Yatapoxvirus. The Chordopoxviridae are listed in Table I-1. Individual viruses are classified into these genera on the basis of antigenicity, virion morphology, host range, and genomic DNA cross-hybridization.

Poxvirus replication and gene expression

The replication cycle of poxviruses has been extensively studied using vaccinia virus (for review, see Moss, 1990a) and essentially consists of virus entry, temporally regulated gene expression, DNA replication, virion assembly and dissemination of mature virus particles. Poxvirus replication is highly autonomous, with all stages of the virus replicative cycle taking place within the cytoplasm of the host cell utilizing enzymes that are thought to be almost entirely the products of viral genes (for reviews, see Moss, 1990a; Traktman, 1991). Relatively little information is available on the mechanism of virus entry, which is further complicated by the existence of two forms of infectious mature virus particles, the intracellular naked virus (INV) which has a single membrane, and the extracellular enveloped virus (EEV) which has a second,

distinct membrane. Although several viral membrane proteins have been implicated in virus adsorption and entry, no cellular receptors for poxviruses have been identified, and virus entry is thought to occur via the pH-independent fusion of the viral membrane with the plasma membrane of the cell (Dales and Pogo, 1981; Moss, 1990a). Once the virus gains entry into the cell, the particles are uncoated in two stages, with concomitant expression of early virus genes.

The temporal regulation of poxviral genes is complex, and has been extensively reviewed (Moss, 1990a; Moss, 1990b; Moss *et al.*, 1991). Early gene expression begins within the virion prior to the final stages of uncoating, and is carried out by virus core-associated enzymes that recognize specific early promoter sequences (Davison and Moss, 1989a). Products of viral early genes include viral DNA polymerase and transcription factors required for the replication of viral DNA and expression of intermediate viral genes. Although intermediate gene expression from transfected templates can occur in the presence of inhibitors of viral DNA replication, parental DNA does not serve as the template for intermediate gene transcription (Moss *et al.*, 1991). Recent evidence suggests that a cellular factor is required for the transcription of vaccinia virus intermediate stage genes, which may in part determine whether a given cell is permissive for infection (Rosales *et al.*, 1994). The products of intermediate genes include several proteins required for the expression of late viral genes, which can only be transcribed when viral DNA replication is allowed to occur (Moss *et al.*, 1991). Late genes are transcribed from conserved, distinct promoter sequences (Davison and Moss, 1989b), and encode the early transcription factors that are packaged in the virion as well as other virion structural components.

Following the onset of late gene expression, the complex process of viral morphogenesis takes place. It is estimated that the virion contains more than 100 distinct polypeptides (Moss, 1990a), and the precise mechanism of assembly has not

been fully elucidated. Several discrete structures corresponding to different stages of virion assembly can be discerned by electron microscopy as immature virus particles are assembled and acquire membranes. Finally, virus dissemination occurs with the release of EEV from infected cells. Although INV appears to be the more abundant form of particle produced, EEV is thought to be critical for the spread of virus in the infected animal, as neutralizing antibodies that provide protective immunity to vaccinia virus infection recognize EEV but not INV (reviewed in Buller and Palumbo, 1991).

The Leporipoxviruses as a model system for studies of viral pathogenesis

Shope fibroma virus

The natural host of Shope fibroma virus (SFV; also referred to as rabbit fibroma virus) is the wild cottontail rabbit (*Sylvilagus floridanus*), although the virus also infects rabbits of other genera including the European domestic rabbit (genus *Oryctolagus*). The normal route of infection with SFV is intradermal, with arthropod vectors being the primary agents of viral transmission in the wild. Infection of immunocompetent adult rabbits with SFV results a localized benign fibroma which was first observed by R.E. Shope (Shope, 1932b), who subsequently showed the causative agent to be a poxvirus closely related to the myxoma virus (Shope, 1932a). In the European rabbit, this tumour reaches its maximum size within two weeks and then regresses due to a vigorous cell-mediated immune response (Allison and Friedman, 1966; Scott *et al.*, 1981). Circulating cytotoxic antibodies are also found in infected and recovering animals (Singh *et al.*, 1972; Tompkins and Schultz, 1972; Scott *et al.*, 1981) and may also contribute to the resolution of the infection and provide protective

immunity against secondary virus challenge. Other studies have demonstrated that increased levels of interferon-γ could be detected in animals infected with SFV, suggesting a role for this cytokine in the anti-viral response (Pathak and Tompkins, 1974). The course of infection is similar in *Sylvilagus floridanus*, except that the tumor is slower to develop and may take up to several months to regress (Febvre, 1962). In rabbits that are immunosupressed (Allison and Friedman, 1966), newborn (Allison and Friedman, 1966; Smith *et al.*, 1973; Tompkins *et al.*, 1973; Sell and Scott, 1981), or γ–irradiated (Febvre, 1962), SFV infection results in a progressive systemic infection which is often fatal. Therefore in the context of an impaired or underdeveloped cellular immune response, infection with SFV is highly invasive, illustrating the importance of cell-mediated immune responses in the resolution and limitation of the infection.

The tumorigenic potential of SFV was the subject of much early research on the virus (reviewed in McFadden, 1988). However, SFV differs from other DNA tumour viruses (such as the adenoviruses, SV40/polyoma viruses and certain herpesviruses) in several important respects. The virus does not physically enter the host cell nucleus and does not permanently immortalize or transform cells (Febvre, 1962; Obom and Pogo, 1988). Although cells in culture can become persistently infected with SFV (Padgett and Walker, 1970) and can take on some of the characteristics of the transformed phenotype such as loss of contact inhibition (Hinze and Walker, 1964; Obom and Pogo, 1988; Pogo *et al.*, 1989), viral antigens are always found associated with SFV-induced fibromas (Sell and Scott, 1981) and tumors continually shed live virus.

More recent research on SFV has mainly concerned analysis of the viral genome and specific viral gene products. The genome has been physically mapped and cloned (Wills *et al.*, 1983; DeLange *et al.*, 1984) and consists of approximately 160 kilobases (Kb) of linear double stranded DNA with characteristic hairpin ends. The genome of SFV is closely related to that of myxoma virus and considerable cross hybridization has

been demonstrated under conditions of moderate stringency (Wills *et al.*, 1983; Block *et al.*, 1985). The entire TIR (12.4 Kb) and adjacent unique regions have been sequenced (Upton and McFadden, 1986b; Upton *et al.*, 1987a; Chang *et al.*, 1987) and transcriptionally mapped (Cabirac *et al.*, 1986; Macaulay *et al.*, 1987). There is significant homology within this region to myxoma virus DNA sequences, with conservation of most of the open reading frames, including those genes encoding a number of proteins that function in virus virulence (see Table I-2). An interesting exception is the region homologous to the myxoma virus SERP1 gene, which in SFV is a fragmented pseudogene (the SERP1 gene is discussed in more detail below and in Chapter II). Several open reading frames in the central region of the genome have been mapped and sequenced, including genes encoding a thymidine kinase (Upton and McFadden, 1986a) and a DNA topoisomerase (Upton *et al.*, 1990b). Comparison of the genomic organization of SFV with that of vaccinia virus and other Orthopoxviruses suggests a similar structure: nonessential genes often involved in virulence and host range functions are found near the genomic termini, whereas genes involved in such conserved functions such as replication and transcription are organized in the central regions of the genome. The close degree of genetic similarity between SFV and myxoma virus is intriguing given the differences in virus pathology and virulence in the infected animal (see below), and suggests that the identification of differences between SFV and myxoma virus may be important in the analysis of determinants of viral virulence.

Myxoma virus

Myxoma virus is a Leporipoxvirus that causes a relatively moderate disease syndrome in its natural hosts, the South American tropical forest rabbit (*Sylvilagus brasiliensis*) and the California brush rabbit (*Sylvilagus bachmani*). The infection is

characterized by the appearance of a local fibromatous lesion at the site of intradermal inoculation which is resolved without further signs of infection, not unlike the infections that SFV induces in its natural host. Indeed, early studies established a close relationship between myxoma virus and SFV, based on immunological cross-protection (Shope, 1938; Woodroofe and Fenner, 1965) and on the observation that heat-killed myxoma virus could be reactivated by SFV, both *in vivo* and in cell culture (Berry and Dedrick, 1936; Fenner *et al.*, 1959). Unlike SFV, however, infection of the European rabbit (*Oryctolagus cuniculus*) with myxoma virus results in the rapidly lethal generalized infection known as myxomatosis (Fenner and Ratcliffe, 1965; Fenner and Myers, 1978). Two strains of myxoma virus that are highly virulent in the European rabbit have been extensively characterized with respect to viral pathogenesis. These are the "standard laboratory" or "Moses" strain, and the "Lausanne" strain. Both strains are rapidly lethal when inoculated into the naive European rabbit, producing a disease characterized by virus multiplication and formation of a proliferative lesion at the site of intradermal inoculation, followed by multiplication in the regional lymph node, a cell-associated viremia, generalization in the skin, genital region and internal organs, development of Gram-negative infections of the nasal and conjunctival mucosa and death usually within fourteen days of inoculation (Fenner and Woodroofe, 1953; Fenner and Marshall, 1957). While the overall pathogenic profile for the two strains is quite similar, the standard laboratory strain differs somewhat from the Lausanne strain in the gross morphology of the lesions produced (Fenner and Marshall, 1957).

The highly virulent nature of the myxoma virus coupled with its narrow host range led to its deliberate dissemination in Australia in 1950 and in France in 1952, as an agent of biological control for the feral European rabbit population (reviewed in Fenner and Ratcliffe, 1965; Fenner and Myers, 1978). Although initially highly successful in reducing the rabbit population, extensive studies conducted following the

release of myxoma virus indicated a rapid emergence of both attenuated strains of myxoma virus (Fenner and Marshall, 1957) and resistant populations of rabbits (Fenner and Ratcliffe, 1965; Fenner and Myers, 1978). Thus the release of myxoma virus in Australia inadvertantly provided a unique opportunity to observe the evolution of a viral disease in a wild population. Various field strains of myxoma virus have been isolated that vary in pathogenicity, with the most successful field strains being those of intermediate virulence (Fenner and Marshall, 1957; Fenner and Ratcliffe, 1965). The genetic basis for these strain differences in myxoma virus has not been determined and it is only during the last decade that more detailed information on the molecular biology of the virus has become available..

The myxoma virus genome (Lausanne strain and two other field strains) has been analyzed by restriction mapping (Block *et al.*, 1985; Russell and Robbins, 1989) and has been cloned (Russell and Robbins, 1989). The genome consists of a characteristic linear double stranded DNA molecule of approximately 160 Kb, similar in size to that of SFV. In addition, sequencing studies have indicated a genomic organization very similar to that of SFV, with genes encoding proteins involved in replication and gene expression found near the centre of the genome (Jackson and Bults, 1990; Jackson and Bults, 1992), and nonessential genes involved in pathogenesis and virus-host interactions located within and near the TIR regions of the genome (Upton *et al.*, 1987b; Upton *et al.*, 1990a; Upton *et al.*, 1991; Upton *et al.*, 1992; Opgenorth *et al.*, 1992a).

Myxoma virus infection in the European rabbit is generally described as being accompanied by dysfunctional cellular immunity (McFadden, 1988; McFadden and Graham, 1994; Strayer, 1989), which may in part be related to the ability of the virus to replicate in lymphocytes and monocytes, resulting in altered cellular function. Myxoma virus antigens are found throughout the lymphoreticular system during infection,

including lymph nodes and spleen, leading to the hypothesis that virus spread within the infected animal may be in part mediated by infected lymphoid cells (McFadden, 1988; Strayer, 1989). Unlike myxoma virus, SFV does not productively infect lymphocytes, and therefore the ability to infect lymphoid cells is likely to be an important determinant of virus virulence. Recently, myxoma virus infection of a $CD4^+$ T lymphoma cell line has been found to result in significant decreases in the cell surface expression of class I MHC antigens (Chapter III, this thesis) and CD4 (Barry *et al.*, 1995), both of which are important cell surface proteins for the initiation and orchestration of the cellular immune response (see Chapter III). Thus, infection of lymphocytes and the dysregulation of lymphocyte function and/or immune recognition may be important mechanisms used by the virus to evade the cellular immune response to infection.

The ability of the virus to rapidly disseminate from the site of inoculation to distal secondary sites implies that, in addition to the dysregulation of cellular immunity, the virus is able to circumvent the numerous early, non-specific host responses to virus infection. Indeed, a number of myxoma virus proteins function to interfere with various aspects of the host antiviral response including inflammation, the action of cytokines (reviewed in McFadden and Graham, 1994; McFadden, 1994; McFadden *et al.*, 1995a), and apoptosis (Chapter IV, this thesis). These proteins, all of which are encoded within the TIR and adjacent unique sequences of the viral genome, have been shown by mutational analyses to be important determinants of myxoma virus virulence in the infected European rabbit (Table I-2). Therefore, although the cellular immune response plays an essential role in clearing poxviral infections (reviewed in Buller and Palumbo, 1991), the ability of the virus to counter early non-specific host defenses may be largely responsible for determining the virulence characteristics of the virus. Individual gene products that influence poxvirus virulence are discussed in more detail

below. A model is therefore emerging in which the virus effectively circumvents a number of early non-specific host defenses in order to replicate at the primary infection site and spread within the infected animal, and then evades the cellular immune response allowing further viral replication to occur, resulting in a highly invasive and virulent infection. Because the origins, natural history and epidemiology of myxoma virus are well understood, myxoma virus provides an excellent model system for the study of viral dysregulation of host responses.

Malignant rabbit fibroma virus

Malignant rabbit fibroma virus (MRV) was isolated from an uncloned stock of SFV that, when inoculated into European rabbits, produced a highly virulent generalized infection reminiscent of myxomatosis (Strayer *et al.*, 1983). Although described in the literature as a novel virus (reviewed in Strayer, 1989), MRV has been shown to be a natural recombinant between SFV and an unknown strain of myxoma virus (Block *et al.*, 1985). Sequencing studies indicated that the MRV genome consists predominantly of myxoma virus sequences, with 7978 bp of SFV DNA derived from the TIR and adjacent unique sequences replacing its myxoma counterpart sequences at the left end of the MRV genome, and a 4674 bp subset of this region replacing related sequences at the right end of the genome (Upton *et al.*, 1988). The integrity of the open reading frames that occur at the recombination junctions is maintained in the MRV genome (Upton *et al.*, 1988). The myxoma virus derived sequences in the genome of MRV identified in these studies are identical to the same regions of myxoma virus (strain Lausanne) that have been sequenced, and therefore although the identity of the progenitor strain of myxoma virus is unclear, MRV should be considered a derivative of a virulent strain of myxoma virus. Not surprisingly, the disease course in European

rabbits infected with MRV is very similar to that observed with the Lausanne strain of myxoma virus (Strayer, 1989).

MRV has been extensively characterized by Strayer and colleagues with respect to the ability of the virus to induce immunosuppression. In most of this work, immune dysfunction is described as an inability of splenocytes from infected animals to respond to mitogenic stimulation and secrete IL-2 (reviewed in Strayer, 1989), which appears to be largely due to viral replication in lymphocytes (Strayer *et al.*, 1985; Strayer *et al.*, 1987; Strayer *et al.*, 1988b). The production of a soluble virus-induced suppressor factor (Strayer *et al.*, 1988a) and anti-suppressor factor (Strayer and Dombrowski, 1988) have been reported; however, the nature of these putative factors and their source has never been defined.

Viral proteins implicated in poxvirus pathogenesis and subversion of the host anti-viral response

The host response to poxvirus infection

Although a detailed review of the immune response to poxvirus infection is beyond the scope of the present work, some important features are briefly outlined below. Specific immune responses, both cellular and humoral, play roles in the host response to poxvirus infections. Available evidence suggests that the cellular immune response, mediated primarily by $CD8^+$ T lymphocytes, is largely responsible for the resolution of primary acute poxviral infection (reviewed in Buller and Palumbo, 1991). In contrast, although specific antibody is produced during most poxvirus infections, evidence suggests that humoral immune responses are not sufficient to clear virus from infected animals, although anti-viral antibody is thought to play an important role

immunity to secondary challenge, possibly through the development of antibody-dependent cell-mediated cytotoxicity (ADCC) (Buller and Palumbo, 1991). Because poxvirus infection is generally acute in nature, and does not involve the establishment of persistent or latent states, recovery from primary infection and the overall pathogenic profile of the infection are also largely determined by non-specific anti-viral responses that operate early during infection. These responses include the development of an inflammatory response, the cytolytic action of natural killer (NK) cells, the anti-viral activities of cytokines, and programmed cell death (apoptosis). The significance of non-specific host responses to poxviral infection is evidenced by the identification of specific gene products encoded by a number of poxviruses that function to block, dysregulate or otherwise interfere with inflammation, complement, a variety of cytokines and apoptosis. Many of these specific poxviral gene products contribute to viral pathogenesis and are discussed in more detail in the following sections.

Poxvirus serpins

The serpins are a superfamily of related proteins, many of which function as inhibitors of serine proteinases (for reviews see Huber and Carrell, 1989; Carrell *et al.*, 1987). Inhibitory serpins function as pseudosubstrates for their cognate proteinases, forming 1:1 stable complexes that inhibit proteinase function by virtue of the slow dissociation of the inhibitor from the enzyme. The serpin becomes cleaved during this reaction, and therefore functions as a suicide inhibitor. Serpins are involved in the regulation of numerous physiological processes that depend on the activity of serine proteinases, including coagulation, fibrinolysis, inflammation, and complement activation. Several poxviruses encode members of the serpin family, many of which have been implicated in virus virulence (for a recent review of poxvirus serpins, see Turner *et al.*, 1995).

Vaccinia virus encodes three distinct serpin-like proteins designated SPI-1, SPI-2 (Kotwal and Moss, 1989; Smith *et al.*, 1989) and SPI-3 (Boursnell *et al.*, 1989; Smith *et al.*, 1989). Nearly identical serpins are encoded by the closely related Orthopoxviruses cowpox virus (CPV) (Pickup *et al.*, 1986; Kotwal and Moss, 1989; Turner and Moyer, 1992), rabbitpox virus (RPV) (Turner and Moyer, 1992) and variola virus (Massung *et al.*, 1993a). One serpin gene has been identified in the genome of myxoma virus and designated SERP1 (Upton *et al.*, 1990a). The myxoma virus SERP1 protein is not a homologue of the Orthopoxvirus SPI-1,2 and 3 proteins, and has no greater homology to these serpins than to other members of the serpin family (the myxoma virus SERP1 protein is discussed in detail in Chapter II). A gene with homology to serpin-like proteins has also been identified in the genome of fowlpox virus (Tomley *et al.*, 1988), as well as in swinepoxvirus (Massung *et al.*, 1993b).

The CPV SPI-2 protein (also referred to as the 38 kDa protein and crmA), is 92% identical to the vaccinia virus SPI-2 protein (Smith *et al.*, 1989), and was the first poxvirus serpin identified (Pickup *et al.*, 1986). Deletion of the CPV SPI-2 gene resulted in an attenuated virus that produced white pocks upon infection of the chick chorioalantoic membrane (CAM), instead of the red hemorrhagic pock usually observed following infection with wild type CPV (Brighton red strain) (Pickup *et al.*, 1986). Although the white pock phenotype was originally predicted to be the result of inhibition of hemorrhage in the lesion, subsequent studies showed that in fact the production of white pocks was the result of a massive infiltration of inflammatory cells into the site of infection, suggesting that the function of the SPI-2 protein was to block the production or activity of an important inflammatory chemotactic factor (Palumbo *et al.*, 1989; Chua *et al.*, 1990; Frederickson *et al.*, 1992). Indeed, the SPI-2 protein inhibits the interleukin-1ß (IL-1ß) converting enzyme (ICE) (Ray *et al.*, 1992) a

cysteine proteinase that cleaves pro-IL-1ß to active IL-1ß which in turn is an important mediator of the inflammatory response. More recently, the CPV SPI-2 protein has been shown to block the induction of apoptosis, presumably through its ability to inhibit a member of the ICE family of cysteine proteinase inhibitors that are involved in apoptosis (recently reviewed by Kumar, 1995). The role of SPI-2 in apoptosis is discussed in greater detail below and in Chapter V. Recent characterization of a SPI-2 mutant in vaccinia virus indicated that this gene does not affect the virulence of this virus in a murine model (Kettle *et al.*, 1995), suggesting that the role of SPI-2 in virus virulence may depend on the context of both the virus and the host.

The Orthopoxvirus SPI-1 genes encode proteins with approximately 50% amino acid identity to the SPI-2 proteins, although the reactive centre region is different suggesting a different inhibitory specificity. Although SPI-1 mutants (CPV, RPV and vaccinia virus) in murine models are not attenuated (Ali *et al.*, 1994; Thompson *et al.*, 1993; Kettle *et al.*, 1995), the RPV SPI-1 mutant is unable to plaque on some cell types which may result from the induction of apoptosis in the non-permissive cells (Turner *et al.*, 1995).

The Orthopoxviral SPI-3 genes have recently been shown to function in the control of cell-cell fusion (Turner and Moyer, 1992; Law and Smith, 1992; Zhou *et al.*, 1992). The significance of this effect with respect to virus infection *in vivo* is unclear, since SPI-3 deletion mutants are not attenuated in infected animals (Turner and Moyer, 1992; Law and Smith, 1992).

Poxvirus growth factors

A number of poxviruses encode homologues of epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α) that have been shown by mutational analyses to contribute to virus virulence (for a recent review, see McFadden *et al.*,

1995b). Members of the EGF family are structurally related and are involved in a number of diverse processes related to the regulation of cell proliferation and differentiation (reviewed in Carpenter and Wahl, 1991). Members of the EGF family, with the exception two members encoded by the Leporipoxviruses SFV and myxoma virus, are liberated from the cell by proteolytic cleavage of a larger membrane bound precursor protein (reviewed in Derynck, 1992). Both the soluble and membrane-bound forms of EGF are active, and act through a specific receptor tyrosine kinase (Derynck, 1992).

Viral homologues of EGF are encoded by a number of poxviruses, including vaccinia virus (Bloomquist *et al.*, 1984; Brown *et al.*, 1985; Reisner, 1985), SFV (Chang *et al.*, 1987), myxoma virus (Upton *et al.*, 1987b), Molluscum contagiosum (Porter and Archard, 1987) and variola virus (Massung *et al.*, 1993a). Although the role played by poxviral growth factors is not well defined, several have demonstrated roles in virus virulence (see below). Suggested functions for poxviral growth factors include the upregulation of cellular metabolism, which might provide a more favorable environment for viral replication, or the potentiation of virus spread through tissue remodeling or increased virus titres (McFadden *et al.*, 1995b). The vaccinia virus growth factor (VGF) is cleaved from a membrane bound precursor, competes with EGF for receptor binding and causes receptor autophosphorylation (Stroobant *et al.*, 1985; Twardzik *et al.*, 1985; King *et al.*, 1986). Studies of deletion mutants in the VGF gene of vaccinia virus demonstrated that VGF contributes to virus virulence (Buller *et al.*, 1988a) and is responsible for cellular proliferation observed in vaccinia virus lesions (Buller *et al.*, 1988b).

The only other poxviral growth factors that have been studied extensively are those encoded by SFV and myxoma virus, the Shope fibroma growth factor (SFGF) and myxoma virus growth factor (MGF), respectively. Both SFGF and MGF are

synthesized as mature proteins that are secreted from the infected cell, unlike other members of the EGF family that are processed from membrane bound precursors. MRV encodes the SFGF protein, the gene for MGF having been replaced with that encoding SFGF during the recombinational events giving rise to MRV (Upton *et al.*, 1988). MRV containing a deletion mutation in SFGF as well as myxoma virus containing a deletion mutation in MGF were significantly attenuated in infected rabbits, indicating that these growth factors are important virulence factors for Leporipoxvirus infection (Opgenorth *et al.*, 1992b; Opgenorth *et al.*, 1992a). Like VGF, these studies also indicated that the SFGF and MGF molecules function to induce proliferative responses in lesions, as metaplasia and hyperplasia in the squamous epithelia overlying lesions were reduced in animals infected with the mutant viruses as compared to the wild type viruses (Opgenorth *et al.*, 1992b; Opgenorth *et al.*, 1992a). In addition, gene swapping studies indicated that SFGF,VGF, EGF and TGF-α could all replace MGF in myxoma virus without phenotypic alterations, suggesting that these growth factors function similarly during infection (Opgenorth *et al.*, 1993).

Viral proteins that interfere with complement

The complement system is involved in antiviral responses at several different levels including enhancement of the inflammatory response, phagocytosis and lysis of virally-infected cells (Frank and Fries, 1989). One of the major secreted proteins of vaccinia virus, a 35 kDa protein (later renamed the vaccinia virus complement -control protein or VCP, Issacs *et al.*, 1992), is homologous to the human C4b binding protein (Kotwal and Moss, 1988), binds to C4b and C3b, and inhibits the classical complement cascade (Kotwal *et al.*, 1990). Deletion of the gene encoding VCP results in virus attenuation in mice (Kotwal *et al.*, 1990; Issacs *et al.*, 1992), possibly by

preventing antibody-dependent complement-enhanced neutralization of infectivity (Issacs *et al.*, 1992).

A second vaccinia virus gene B5R, encodes a 42 kDa membrane anchored glycoprotein that is a component of the envelope of EEV (reviewed in Smith, 1993), and is related by sequence homology to the family of complement control proteins (Takahashi-Nishimaki *et al.*, 1991; Smith *et al.*, 1991b). Although no interaction with complement components has been demonstrated, this protein has been shown to influence plaque size and host range (Takahashi-Nishimaki *et al.*, 1991) , is required for the formation of the EEV envelope and contributes to virus virulence (Engelstad *et al.*, 1992; Engelstad and Smith, 1993). A homologous gene, designated ps/hr, is encoded by rabbitpox virus (Martinez-Pomares *et al.*, 1993) and also influences plaque size and host range. Unlike the vaccinia B5R protein which is membrane bound, the rabbitpoxvirus ps/hr exists in both membrane associated and secreted forms (Martinez-Pomares *et al.*, 1993) suggesting that it may have other functions that have yet to be demonstrated.

Finally, two open reading frames identified in the genome of rabbitpox virus that influence virus virulence are also related to complement proteins C4 and C5 (Bloom *et al.*, 1991), although whether these proteins influence the complement system is not known.

Viral proteins that modulate cytokine responses

It is becoming quite clear that the ability of poxviruses to modulate host cytokine-mediated defenses is an important determinant of the pathogenic characteristics of a given viral infection. Because specific cell-mediated immune responses can take up to a week to develop, the ability of the virus to circumvent early host responses may determine the extent to which the virus can replicate prior to the onset of specific

cellular anti-viral responses. Many early, non-specific anti-viral responses are mediated and augmented by the action of cytokines that are produced in response to virus infection. Viruses can interfere with cytokine responses on a number of different levels, including inhibition of synthesis or post-translational processing of a cytokine precursor, inhibition of signal transduction through cytokine receptors, blocking the ability of the cytokine to interact with its cellular receptor, or the production of cytokine antagonists (Pickup, 1994). Poxviral anti-cytokine strategies have evolved to include a number of these mechanisms, and are the subject of several of recent reviews (Smith, 1993; McFadden and Graham, 1994; McFadden, 1994; Pickup, 1994; McFadden *et al.*, 1995a). Cytokines known to be targetted during poxviral infection include interleukin-1ß (IL-1ß), tumour necrosis factor (TNF) and interferon gamma (IFNγ), and the strategies poxviruses use to counter them are outlined briefly below.

Interleukin-1β: IL-1ß is an important cytokine that has a wide range of biological activities, including the regulation of inflammatory and immune responses as well as the induction of systemic effects such as the febrile and acute phase responses. IL-1ß is produced as in inactive precursor that requires proteolytic cleavage for activity. Vaccinia virus, cowpox virus and myxoma virus all encode soluble proteins that bind to IL-1ß. Encoded by the vaccinia virus B15R gene and a homologous gene from cowpox virus, the B15R protein is a secreted homologue of the cellular type II receptor for IL-1ß (Smith and Chan, 1991; Alcamí and Smith, 1992; Spriggs *et al.*, 1992). The B15R protein is specific for IL-1ß, and does not bind to IL-1α or to the IL-1 receptor antagonist (Alcamí and Smith, 1992). The role of B15R during vaccinia virus infection of mice has been studied using gene disruption mutants. Interestingly, while the mutant virus is attenuated when mice are inoculated intracranially (Spriggs *et al.*, 1992), intranasal inoculation actually resulted in enhanced virulence and accelerated death

(Alcamí and Smith, 1992). Furthermore, it has been determined that the B15R protein prevents the febrile response in infected mice (Alcamí and Smith, 1995a), and it has thus been hypothesized that the role of B15R is to prevent the development of fever, thereby prolonging the life of the infected animal to facilitate maximal viral replication (Alcamí and Smith, 1995a). Recent studies indicate that an IL-1ß binding protein is secreted from myxoma virus infected cells; however, the gene encoding this protein has not been mapped and the protein has not been characterized (McFadden and Graham, 1994).

As mentioned above in the discussion on poxviral serpins, the cowpox virus SPI-2 (crmA) protein has been shown to inhibit the activation of pro-IL-1ß to mature IL-1ß through its ability to inhibit the proteolytic activity of the IL-1ß converting enzyme. Presumably, the other Orthopoxvirus SPI-2 proteins will have this same activity. The evolution of two distinct mechanisms to control IL-1ß suggests that the regulation of this cytokine is an important feature of poxvirus infection.

Tumour necrosis factor: TNF is a pleiotropic cytokine that has proinflammatory and antiviral activities (Camussi *et al.*, 1991). TNF, along with the interferons, is produced early during infection with many pathogens and plays an important role in the early defense against viral infection (Wong and Goeddel, 1986; Wong *et al.*, 1992). TNF is expressed as a type II membrane protein, and soluble TNF is released from the cell by a proteolytic cleavage event. The effects of TNF are mediated by one of two receptors, designated the type I (p55) receptor and the type II (p75) receptor. (Schall *et al.*, 1990; Smith *et al.*, 1990). The first poxviral protein linked to the regulation of TNF activity was the SFV T2 protein, when sequencing of the type II receptor revealed significant homology with T2 (Smith *et al.*, 1990; Smith *et al.*, 1991a). A highly homologous gene was identified in the genome of myxoma virus (Upton *et al.*, 1991).

Homology to the cellular TNF receptor is limited to the N-terminal 160 amino acids of the T2 protein, a region that is similar to the ligand-binding domain of the receptor. The C-terminal 139 amino acids of the T2 protein do not share significant homology with any known cellular proteins. Mutation of the T2 gene in myxoma virus demonstrated that it contributes to virus virulence in the infected rabbit (Chapter IV, this thesis). While studies of the T2 protein have demonstrated that T2 inhibits cell lysis mediated by rabbit TNF-α (Schreiber and McFadden, 1994), recent data indicates that T2 may also function to inhibit apoptosis in infected lymphocytes (Chapter IV, this thesis) and therefore may interact with other members of the TNF family implicated in the induction of apoptosis, such as the ligand for the Fas receptor, or perhaps even the receptors themselves (see below and Chapter IV).

Homologues of the T2 protein have also been identified in a number of Orthopoxviruses (for a recent review, see Smith and Goodwin, 1995), including cowpox virus (termed the cyokine response modifier B or crmB protein, Hu *et al.*, 1994) and two strains of variola virus (Shchelkunov *et al.*, 1993; Massung *et al.*, 1993a). These proteins have significant amino acid homology with the SFV and myxoma virus T2 proteins, including both the ligand binding domain and the C-terminal domain. Vaccinia virus contains homologous open reading frames as well (Goebel *et al.*, 1990; Howard *et al.*, 1991), but in both the strains examined, frameshift and nonsense mutations occur within the coding sequence for the predicted ligand binding domain and therefore no TNF binding activity is encoded by vaccinia virus. These proteins are predicted to bind to TNF that is produced locally in response to infection, thereby preventing the TNF molecule from interacting with its cellular receptor.

Finally, a second TNF-binding protein is encoded by cowpox virus. This protein, termed crmC (cytokine response modifier C), is composed only of the

extracellular, ligand binding region of the TNF receptor (Pickup, 1994). The function of this second TNF binding protein is unclear.

Interferons: Interferon-γ (IFNγ) is a one of the major anti-viral cytokines produced in response to viral infection, and has both direct anti-viral activity as well as widespread effects within the immune system (reviewed in Farrar and Schreiber, 1993). IFNγ has long been thought to be an important factor in the host response to poxviral infections (Buller and Palumbo, 1991), a point underscored by the observation that, while immunodeficient mice normally succumb to vaccinia virus infection, a recombinant vaccinia virus that expresses IFNγ is cleared from these mice (Kohonen Corish *et al.*, 1990). In addition, transgenic mice bearing null mutations in the gene for the IFNγ receptor undergo systemic infections with vaccinia virus that are more severe than in normal mice (Huang *et al.*, 1993).

Given the importance of IFNγ in the host response to poxvirus infection, it is not surprising that IFNγ is also targetted for inhibition by specific poxviral gene products. Soluble versions of the receptor for IFNγ are encoded by probably all poxviruses. The first of these to be described was the T7 protein of myxoma virus (Upton *et al.*, 1992). One of the most abundant secreted proteins produced during myxoma virus infection, T7 has been shown to bind to and inhibit the anti-viral activity of IFNγ (Upton *et al.*, 1992; Mossman *et al.*, 1995b). The B8R gene of vaccinia virus has been shown to encode a related protein that also binds and inhibits IFNγ (Alcamí and Smith, 1995b), and similar binding activities have been identified in ectromelia virus, cowpox virus and camelpox virus (Mossman *et al.*, 1995a; Alcamí and Smith, 1995b). Recent evidence indicates that the disruption of the T7 gene in myxoma virus results in virus attenuation (K. Mossman, N. Nation, J. Macen and G.McFadden,

manuscript in preparation), suggesting that blocking IFNγ action is an important determinant of viral virulence.

In addition to the B8R gene, vaccinia virus also encodes a soluble receptor for the type I interferons, IFNα/β. Type I IFNs bind to a common cellular receptor that is distinct from the receptor for IFNγ, and both IFNα and IFNß have similar biological activities. Type I IFNs are known to have potent anti-viral activities in addition to regulating such diverse processes as cellular differentiation, proliferation and expression of major histocompatibility complex proteins (reviewed in Callard and Gearing, 1994) The importance of type I IFNs in the host response to vaccinia virus infection is evidenced by the increased virulence of vaccinia virus infection in mice that contain a homozygous null mutation in the gene encoding the IFNα/β receptor (Müller *et al.*, 1994). Recent studies indicate that the vaccinia virus (strain WR) B18R gene encodes a type I IFN binding protein that is able to counteract the anti-viral effects of type I IFNs (Symons *et al.*, 1995; Colamonici *et al.*, 1995). Interestingly, one group reports that this protein, in addition to being released into the medium as a soluble protein, is also found on the cell surface (Colamonici *et al.*, 1995). Deletion mutants in the B18R gene are attenuated in mice (Symons *et al.*, 1995), again indicative of the importance of the type I interferons in the host response to vaccinia virus infection.

Vaccinia virus has evolved an additional strategy for blocking the anti-viral activity of IFN. Interaction of interferon with its cellular receptor leads to the establishment of an antiviral state, through the activity of two IFN inducible enzymes, a dsRNA dependent protein kinase (PKR) and 2-5 A synthetase. Both of these enzymes are activated by dsRNA that is produced during viral infection and lead to inhibition of protein synthesis. Activated 2-5 A synthetase leads to the activation of a latent riboendonuclease which degrades viral RNA while activated PKR phosphorylates the translation initiation factor eIF-2α which is then incapable of initiating translation

(Farrar and Schreiber, 1993). The proteins encoded by the vaccinia virus K3L and E3L open reading frames are intracellular proteins that block the antiviral effects of both the type I IFNs and IFNγ by inhibiting the activity of PKR (Beattie *et al.*, 1991; Davies *et al.*, 1992; Chang *et al.*, 1992; Beattie *et al.*, 1995). Therefore vaccinia virus can abrogate both the direct anti-viral activity of the IFNs by virtue of the K3L and E3L proteins, as well as inhibit the effects of IFNγ and IFNα/β on other immune functions by virtue of the secreted B8R and B18R proteins.

Host range genes and the regulation of apoptosis

Programmed cell death, or apoptosis, is rapidly becoming accepted as yet another non-specific host antiviral response. Premature death of an infected cell would have obvious deleterious consequences for the production of viral progeny and therefore many viruses have evolved mechanisms to prevent apoptosis during infection (for a recent review, see Razvi and Weish, 1995). Among the poxviruses, several genes have been identified that can function to prevent or delay apoptosis in specific cell types. Among these are a number of genes described to have a host range phenotype, such that mutations in them render the virus unable to replicate in a given cell type. For example, the cowpox virus CHOhr gene (Ink *et al.*, 1995) and the vaccinia virus E3L gene (Lee and Esteban, 1994), are required in order to prevent or delay cell death by apoptosis and allow fully productive infection in Chinese hamster ovary cells and primate fibroblasts, respectively. Interestingly, the E3L gene is the same gene described as an inhibitor of the interferon-induced protein kinase discussed above. Other studies have indicated that this protein kinase may itself induce apoptosis in certain cell types (Lee and Esteban, 1994) and therefore E3L may function to block multiple antiviral effects mediated by this kinase.

The SPI-1 gene from rabbitpox virus has also been shown to function as a host range gene, possibly by blocking premature death of infected pig fibroblast or human A549 epithelial cells during infection (Ali *et al.*, 1994). As well, the SPI-2/crmA protein from cowpox virus has been shown to block apoptosis in a variety of cell types, presumably through its ability to inhibit an enzyme related to the interleukin-1β converting enzyme (Gagliardini *et al.*, 1994), which play central roles in apoptotic cell death.

Finally, recent studies have implicated two myxoma virus proteins, T2 and M11L, in the inhibition of apoptosis (Chapter IV, this thesis). The M11L gene encodes a unique membrane associated protein that functions to inhibit the host inflammatory response in viral lesions by an unknown mechanism (Opgenorth *et al.*, 1992a; Graham *et al.*, 1992). Lymphocytes infected with viruses bearing mutations in either the M11L or the T2 genes undergo apoptosis, while the same cells remain viable when infected with wild type virus (Chapter IV, this thesis), suggesting that these viral proteins also function to prevent apoptosis. Although the mechanism at work is unclear in either case, it is interesting that the TNF receptor family, of which T2 is a member, also includes the receptor Fas, through which cell death signals are transmitted by interaction with Fas ligand. Therefore the myxoma virus T2 protein and the vaccinia virus E3L protein provide interesting examples of how a single protein can interfere with both cytokine action as well as apoptotic cell death. Such proteins may also prove to be useful tools for the identification of common mediators shared by different host response pathways.

Thesis objective

The overall objective of the work presented in this thesis was to increase our understanding of poxvirus pathogenesis and virus-host interactions, through the study

of specific viral proteins, and their roles in virus pathogenesis, virulence, and virus-host interactions at the cellular level. Several studies were undertaken in order to achieve this objective:

(1) Studies on the myxoma virus serine proteinase inhibitor, SERP1: Because the SERP1 gene is present in myxoma virus but absent from SFV, we wished to determine whether the SERP1 protein was a determinant of myxoma virus virulence. Therefore, the role of the myxoma virus SERP1 protein in the virulence of both MRV and myxoma virus was investigated through the construction of viruses bearing targetted gene disruptions in the SERP1 open reading frame. In addition, the gene encoding SERP1 was analysed and the protein was characterized. Finally, a protocol for the purification of the SERP1 protein was developed. The results of these studies are presented in Chapter II.

(2) Studies on virus-induced downregulation of class I MHC proteins: Because myxoma virus infection is highly invasive and virulent, we wished to determine whether virus infection might compromise the recognition of virally-infected cells by cells of the immune system. As a first step toward addressing this issue, the effect of virus infection on the cell surface expression of class I proteins of the major histocompatibiliy complex was determined on both lymphoid and non-lymphoid cell lines. The results of these studies are presented in Chapter III.

(3) Studies on the myxoma virus T2 protein and the apoptotic response to Leporipoxvirus infection: The myxoma virus T2 gene encodes a homologue of the cellular receptor for tumour necrosis factor. The role of the T2 protein in virus virulence was investigated through the construction of a targetted gene disruption mutant. During the course of these studies and the studies presented in Chapter III, a number of viruses, including the myxoma virus T2 disruption mutant, a myxoma virus mutant in the M11L open reading frame, and SFV, were found to be unable to productively infect

a $CD4^+$ rabbit T lymphoma cell line. The basis for the nonproductive infection was then investigated. The results of these studies are presented in Chapter IV.

(4) Studies on the ability of poxviral serpins to inhibit cell death mediated by cytolytic lymphocytes: Cell death mediated by cytolytic lymphocytes involves the activity of a number of different proteinases. Studies were undertaken to determine whether the myxoma virus serpin, SERP1, could inhibit the cytolysis of infected cells by primary murine natural killer cells. In addition, the possibility that the myxoma virus SERP1 protein, or the Orthopoxvirus serpins SPI-1 and SPI-2 could inhibit the non-specific cytolysis of virus infected cells using cloned cytolytic effector cells that kill via two different cytolytic mechanisms was investigated. The results of these studies are presented in Chapter V.

TABLE I-1

POXVIRUSES OF VERTEBRATES
(Subfamily Chordopoxviridae)

Genus	Species
Orthopoxvirus	Buffalopox, camelpox, cowpox, ectromelia, monkeypox, rabbitpox, raccoon pox, tatera pox, vaccinia*, variola, and vole pox viruses
Leporipoxvirus	Hare fibroma, myxoma*, Shope (rabbit) fibroma, and squirrel fibroma viruses
Parapoxvirus	Chamois contagious ecthyma, Orf*, pseudocowpox, and stomatitis papulosa viruses
Avipoxvirus	Canary pox, fowlpox*, junco pox, pigeon pox, quail pox, sparrow pox, starling pox, and turkey pox viruses
Capripoxvirus	Goatpox, sheep pox*, and lumpy skin disease viruses
Suipoxvirus	Swinepox virus
Molluscipoxvirus	Molluscum contagiosum virus
Yatapoxvirus	Tanapox* and Yaba viruses

*Prototypical member

TABLE I-2

MYXOMA VIRUS PROTEINS THAT CONTRIBUTE TO VIRUS VIRULENCE

Gene	Cellular homologues	Function	References
MGF	TGF-α, EGF	Virulence factor; growth factor	Upton *et al.*, 1987; Opgenorth *et al.*, 1992a,b
SERP1	serpin family of serine proteinase inhibitors	Virulence factor ; secreted serine proteinase inhibitor; interferes with inflammation	This thesis; Upton *et al.*, 1990; Macen *et al.*, 1993
T2	Type I TNF receptor, Fas (ligand binding domains)	Virulence factor; binds and inhibits TNF; prevention of apoptosis in infected lymphoid cells	This thesis; Upton *et al.*, 1991; Schreiber and McFadden, 1994
T7	IFNγ receptor	Virulence factor[1] ; binds and inhibits IFNγ; binds and inhibits chemokines[2]	Upton *et al.*, 1992; Mossman *et al.*, 1995
M11L	?	Virulence factor; inhibits inflammation in viral lesions; prevention of apoptosis in lymphoid cells	Opgenorth *et al.*, 1992b; Graham *et al.*, 1992; This thesis

[1] K. Mossman, N. Nation, J. Macen and G. McFadden, manuscript in preparaton

[2] K. Graham, A. Lalani and G.McFadden, manuscript in preparation

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CHAPTER II

SERP1, A SECRETED SERINE PROTEINASE INHIBITOR ENCODED BY MYXOMA VIRUS AND MALIGNANT RABBIT FIBROMA VIRUS IS AN IMPORTANT VIRULENCE FACTOR THAT INTERFERES WITH INFLAMMATION[1]

[1]The material contained herein, with the exception of the purification of the SERP1 protein, has been published: Upton, C., Macen, J.L., Wishart, D. and McFadden, G. Virology, 179: 618-631, 1990; Macen J.L., Upton, C., Nation, N. and McFadden, G. Virology, 195: 348-368, 1993

INTRODUCTION

Myxoma virus is a *Leporipoxvirus* that causes a relatively moderate disease syndrome in its natural rabbit hosts (*Sylvilagus brasiliensis* in South America and *Sylvilagus bachmani* in North America), consisting of a local fibromatous lesion at the site of intradermal inoculation which is resolved without further signs of infection. In the European rabbit (*Oryctolagus cuniculus*) however, the same strains of myxoma virus cause a rapidly lethal generalized infection known as myxomatosis (reviewed in Fenner and Ratcliffe, 1965; Fenner and Myers, 1978). Intradermal inoculation of *O. cuniculus* with even very low doses of a virulent strain of myxoma, such as Lausanne, results in virus multiplication at the primary site to produce a raised lesion characterized by tissue degeneration and necrosis, followed by dissemination of the virus through the lymphoreticular system, generalization in the skin, genital region and internal organs, development of Gram negative infections of the nasal and conjunctival mucosa and death within fourteen days of infection (Fenner and Ratcliffe, 1965). Malignant rabbit fibroma virus (MRV) has been shown to be a recombinant between an unknown strain of myxoma virus and the closely related Leporipoxvirus Shope fibroma virus (Block *et al.*, 1985; Upton *et al.*, 1988). MRV infection of the European rabbit results in a rapidly lethal myxomatosis reminiscent of that caused by virulent strains of myxoma virus (Strayer, 1989). Perhaps the most interesting questions concerning the pathogenesis of myxomatosis revolve around how myxoma virus is able to effectively circumvent many facets of the *O. cuniculus* immune response to generate such a profoundly virulent systemic disease (reviewed in McFadden, 1988; McFadden, 1994; Strayer, 1989).

The poxviruses are among the largest eukaryotic DNA viruses and infect a variety of both vertebrate and invertebrate hosts. Depending on the virus, route of

inoculation and immunological state of the host, poxvirus infections result in a wide spectrum of disease pathologies ranging from relatively benign, localized infections to generalized and rapidly lethal infections (reviewed in Fenner, 1990; Buller and Palumbo, 1991; Turner and Moyer, 1990). Poxviruses share a number of common features including a large, complex virion and cytoplasmic site of replication (Moss, 1990). The poxvirus genome consists of a large (160-220 kb) double stranded DNA molecule with covalently closed hairpin termini, and terminal sequences which are repeated but in opposite orientation (terminal inverted repeats; TIRs). Sequencing studies have shown that the overall organization of open reading frames (ORFs) in myxoma virus DNA is similar to that of other poxviruses. Genes encoding proteins essential for virus replication and gene expression are located in the central region of the genome (Jackson and Bults, 1990; Jackson and Bults, 1992) whereas those encoding nonessential factors that determine virus virulence and host range tend to be located within and near the terminal regions of the genome (Upton *et al.*, 1987a; Upton *et al.*, 1990; Upton *et al.*, 1991; Upton *et al.*, 1992; Opgenorth *et al.*, 1992). Mutational analysis within and near the TIR region of the myxoma virus genome has revealed a number of genes that function in virus virulence, some of which have significant homologies to host proteins such as growth factors, cytokine receptors and regulatory proteins. These include secreted homologues of epidermal growth factor (Upton *et al.*, 1987a; Opgenorth *et al.*, 1992), the tumour necrosis factor receptor (Upton *et al.*, 1991) the interferon gamma receptor (Upton *et al.*, 1992) and serine proteinase inhibitors (Upton *et al.*, 1990) as well as a transmembrane protein designated M11L (Opgenorth *et al.*, 1992; Graham *et al.*, 1992). Most of the ORFs encoded within and near the TIRs of myxoma virus are also encoded and expressed by the closely related *Leporipoxvirus* Shope fibroma virus (Upton, 1986; Upton *et al.*, 1987b; Macaulay *et al.*, 1987). SFV, however, causes only a localized infection in the European rabbit

which is cleared by a vigorous cellular immune response (Shope, 1932; Allison and Friedman, 1966). It was therefore of significant interest that one particular ORF encoded within the myxoma virus TIR, designated *SERP1* because it had significant amino acid similarity to the serpin family of serine proteinase inhibitors (Upton *et al.*, 1990), was present as only a fragmented and discontinuous ORF in SFV. The identical ORF is present as a single copy in the genome of MRV (Upton *et al.*, 1990).

The serpins are a superfamily of related proteins, many of which are inhibitors of serine proteinases (Carrell *et al.*, 1987). Serpins are involved in the regulation of numerous physiological processes, including fibrinolysis, inflammation, and complement activation. It has recently been shown that the myxoma virus SERP1 protein binds to and inhibits the proteolytic activities of the human proteinases plasmin, urokinase, plasminogen activator and C1s (Lomas *et al.*, 1993) and as such is the first poxviral serpin homologue to have been demonstrated to function as an actual inhibitor of serine proteinases. Because the SERP1 gene has been maintained as an intact ORF in both myxoma virus and MRV, but not in SFV, it seemed likely that the this gene might encode a protein that functions in virus virulence. Therefore, a mutation was constructed in the single copy of the SERP1 gene in MRV, and the virulence of the resulting recombinant virus in *O. cunniculus* was determined. Because the strain of myxoma virus from which MRV is derived is unknown, and because MRV encodes only one copy of the SERP1 gene as compared to two copies in wild type myxoma virus, the SERP1 gene was further characterized in a well characterized strain of myxoma virus (Lausanne). Both copies of the myxoma virus SERP1 gene were disrupted by homologous recombination, and the virulence of the resulting virus was characterized in *O. cunniculus*. Furthermore, the expression of the gene and the protein it encodes were characterized. Finally, a method was developed for the purification of

the SERP1 protein from cells infected with a recombinant vaccinia virus that overexpresses the SERP1 protein.

These studies show that the disruption of the SERP1 gene in either MRV or myxoma virus results in a significant attenuation of the virus in infected rabbits, indicating an important role for the SERP1 gene in virus virulence *in vivo*. Histological analysis of lesions from animals infected with the myxoma virus SERP1 disruption mutant indicated that this attenuation was likely to be due in part to the generation of a more effective inflammatory response in the absence of the SERP1 protein. In addition, the SERP1 gene was found to be expressed late during virus infection, and encodes a glycosylated protein that is secreted from the infected cell.

MATERIALS AND METHODS

Cells and viruses

The sources of SFV, MRV and myxoma virus have been described (Block *et al.*, 1985). vMyxlac, a derivative of myxoma virus expressing the *E. coli lac Z* gene inserted between the myxoma growth factor and M9 genes and which has a wild type phenotype, has been described (Opgenorth *et al.*, 1992). The construction of MRV-SERP1$^-$, vMyx-SERP1$^-$ and VV-S1 are described below. Viruses were propagated in the monkey cell line BGMK (obtained from Dr. S. Dales, University of Western Ontario) grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (NCS; Gibco). Vaccinia virus (strain WR) was obtained from the ATCC, and propagated either in BGMK cells or in HeLa S3 cells (provided by Dr. V. Paetkau) maintained as suspension cultures in Joklik modified Eagle's medium (Flow Laboratories) supplemented with 10% horse serum (Gibco). TK$^-$ vaccinia virus

recombinants were selected and plaque purified on TK- H143 cells (obtained from Dr. D. Panicali).

RNA purification and S1-nuclease mapping

Semiconfluent BGMK cells were adsorbed with virus for one hour at a multiplicity of infection (MOI) of 10 pfu/cell, fresh medium was added and the infection allowed to proceed at 37°C . At indicated times post infection (p.i.), RNA was harvested as described (Macaulay *et al.*, 1987). Briefly, the monolayers were dissolved in 4*M* guanidium isothiocyanate, 5m*M* sodium citrate (pH7.0), 0.1*M* ß-mercaptoethanol and 0.5% sodium *N*-laurylsarcosine, the solution was homogenized by passing it several times through a 21 guage needle and total cellular RNA was collected by sedimentation through CsCl. The pellet was dissolved in 10 m*M* Tris (pH 8.0), 1m*M* EDTA, 0.1% SDS, extracted with phenol:chloroform:isoamyl alcohol (49:49:2), and precipitated with ethanol. The RNA was resuspended in diethyl pyrocarbonate-treated water.

S1-nuclease mapping was performed as described (Macaulay *et al.*, 1987), using a single stranded 5'-end labelled BamHI/AvaI fragment corresponding to the first 624bp of the myxoma BamHI U3 fragment (Russell and Robbins, 1989) as probe (Figure1).

Interruption of the SERP1 gene in MRV to produce the MRV-SERP1- mutant virus

The SERP1 deletion mutant in MRV was constructed using a clone, H^C-18-/21, a deletion in an M13 clone constructed during the sequencing of the MRV H^C fragment (see Upton *et al.*, 1990). This fragment consists entirely of myxoma virus DNA, and corresponds to nucleotides 1-1234 of the myxoma virus Bam HI U3 fragment (Russell

and Robbins, 1989; Upton *et al.*, 1990). The Bam HI and Hind III sites within the multiple cloning site of the M13 vector were used to excise the myxoma virus DNA and subclone it into Bam HI and Hind III digested pUC 8. This clone was then digested at a unique SstI site (corresponding to nucleotide 797 of the Bam HI U3 fragment), treated briefly with the exonuclease Bal 31 to generate a deletion within the ORF, followed by T4 DNA polymerase to generate blunt ends. This DNA was then ligated to phosphorylated BglII linkers to generate the plasmid pMDB-6. Electrophoretic analysis of this clone indicated a small (approximately 65 base pairs) bidirectional deletion in the ORF, with a unique Bgl II site replacing the original Sst I site. A 3.2 Kb Bgl II fragment was isolated from the plasmid pSC20 (Buller *et al.*, 1988), containing the VV p11 late promoter driving expression of the *E. coli* ß-galactosidase gene. This fragment was ligated to Bgl II digested, dephosphorylated pMDB-6. The resulting plasmid, pAPPZ-9, contained the p11-lacZ cassette inserted into the deletion in the SERP1 coding sequences such that it would be transcribed in the same direction as the SERP1 gene.

Recombinant MRV was constructed as follows. Semi-confluent BGMK cells were infected with wild type MRV at a multiplicity of infection (MOI) of 0.05 pfu/cell, and incubated at 37°C for two hours. Calcium phosphate/DNA precipitates were prepared and added to the cells (5µg carrier MRV DNA + 400ng Hind III digested (linearized) pAPPZ-9 DNA per 10^6 cells). After 6 hours at 37°C, the medium was replaced, and the virus was harvested after a further 18 hours of incubation. Recombinant virus expressing ß-galactosidase was selected by overlaying infected monolayers with 1% low melting point agarose in DMEM containing 500µg/mL 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal). Following incubation at 37°C for approximately 12 hours, blue foci, indicative of virus expressing ß-galactosidase, were picked using a Pasteur pipette. Recombinant virus was plaque

purified three times using this proceedure, and high titre virus stocks were prepared on BGMK cells. Several virus clones prepared in this way were analysed by Southern blotting of the viral DNA to confirm that the p11-lac Z cassette interupted the SERP1 ORF in these recombinant viruses, and that there was no contaminating wild type MRV present.

Interruption of both copies of the SERP1 gene in MYX to produce the vMyx-SERP1$^-$ mutant virus

Both copies of the SERP1 gene of myxoma virus were interrupted by homologous recombination leading to the insertion of a dominant selectable marker, the *E. coli* guanosine phosphoribosyl transferase (*gpt*) gene driven by the vaccinia 7.5K promoter, into the ORF. A 0.95 kb cassette consisting of the vaccinia virus 7.5K promoter driving the expression of the *E. coli gpt* gene (described in Upton *et al.*, 1991) was inserted with Bgl II linkers into the Bgl II site of the plasmid pMDB-6, described above. The resulting plasmid, pS1gptA, contained the *E. coli gpt* gene inserted into the deletion in the SERP1 ORF such that it would be transcribed in the same direction as the SERP1 gene.

Recombinant myxoma viruses were constructed essentially as described above, except that vMyxlac was used as the parent virus. Recombinants were selected in gpt selection medium (DMEM supplemented with 10% NCS and containing 25 μg/ml mycophenolic acid, 250 μg/ml xanthine and 15 μg/ml hypoxanthine). Recombinant viruses were picked from monolayers infected at low MOI and overlaid with 1% low melting point agarose in gpt selection medium containing 500μg/ml X-gal (to visualize plaques formed by viruses expressing ß-galactosidase) and plaque purified three times. The presence of the insertion into both copies of the SERP1 ORF was confirmed using

PCR, and the absence of detectable SERP1 protein expression by the recombinant (designated vMyx-SERP1$^-$) was shown by immunoblot analysis.

Infection of rabbits with MRV and MRV-SERP1$^-$

All animal experiments were performed using level III biocontainment following the guidelines provided by the Canadian Council on Animal Care. Male New Zealand White rabbits (*Oryctolagus cuniculus*; 12-16 weeks old) were obtained from a local supplier. The rabbits were observed for three days to ensure they were healthy. Each rabbit was given a single intradermal injection of either MRV or MRV-SERP1$^-$ (25 or 250 p.f.u. virus in 0.1mL DMEM), or mock infected with a single injection of DMEM alone. All rabbits were examined daily, and signs of infection were recorded. When rabbits appeared to experience difficulty in breathing due to supervening Gram negative infection of the nasal mucosa, which often preceeds death during MRV infection, they were sacrificed immediately by intravenous injection of euthanol.

Infection of rabbits with vMyxlac and vMyx-SERP1$^-$ and histological analysis

Male New Zealand White rabbits were obtained from a local supplier. All rabbits were observed for one week prior to infection to ensure they were healthy. Rabbits were inoculated intradermally with 10^3 pfu of either vMyxlac, or vMyx-SERP1$^-$ (two inoculations of 500 pfu per animal) in a volume of 0.1 mL and observed daily for the development of symptoms. Rabbits that became moribund with the infection were euthanized by intravenous injection of euthanol. All animals were subjected to a complete post-mortem examination, and various tissue samples were taken and stored in 10% neutral buffered formalin for future analysis. For histological studies, six rabbits were inoculated as described above with vMyxlac and six were

inoculated with an identical dose of vMyx-SERP1$^-$. At 4, 7 and 10 days p.i., two animals from each group were euthanized by intravenous euthanol injection, a complete post-mortem examination was conducted and samples of injection site (primary site) and ear, nasolabium, eyelid and nictitating membrane (secondary sites) were removed and placed in 10% neutral buffered formalin. Following fixation, tissues were trimmed, processed routinely into paraffin blocks, sectioned at 4 microns and stained with hematoxylin and eosin for microscopic examination. Photomicrographs were taken using TMAX 100 black and white film (Kodak) using a Nikon FX 35DX camera body on a Nikon AFXDX Labophot photomicroscope.

Expression of SERP1 from *E. coli* and generation of polyclonal antiserum

A 1131bp HhaI fragment containing the SERP1 ORF (missing the first 40bp) was treated briefly with Exonucleases III and VII and ligated into the BamHI site of pUC19 using BamHI linkers. A clone containing the BamHI site in the correct reading frame was identified by sequencing and the resultant BamHI fragment was inserted into the BamHI site of the expression vector pET-3c (Studier *et al.*, 1990) and used to transform *E. coli* HB101. The resultant clone, pET-M6-5, contained the SERP1 ORF expressed such that the first 14 amino acids are replaced by 11 residues from the phage T7 gene 10 protein followed by three residues derived from the engineered cloning site. Expression in *E. coli* was induced with IPTG (Studier *et al.*, 1990) and the cells were harvested and lysed using a French press. Inclusion bodies were isolated by low speed centrifugation as described (Harlow and Lane, 1988). When the total lysates and inclusion body protein were analyzed by SDS-PAGE, SERP1 was found as the major component of the inclusion bodies (data not shown).

Antiserum was prepared by intradermally injecting inclusion bodies into rabbits, using standard procedures (Harlow and Lane, 1988). Immunoblots confirmed that the polyclonal antiserum obtained contained antibody that recognized SERP1 expressed both from *E. coli* and from myxoma virus infected BGMK cells. The reactive species corresponding to the SERP1 protein were not present in extracts from uninduced *E. coli* or from uninfected BGMK cells.

High level expression of SERP1 from recombinant vaccinia virus

BV12-10a, an M13 clone used in sequencing the myxoma virus BamHI U3 fragment (Upton *et al.*, 1990) which contains the intact SERP1 ORF was grown in *E. coli* CJ236 *dut*$^-$, *ung*$^-$; (Kunkel *et al.*, 1987). Oligonucleotide directed mutagenesis was performed as described (Kunkel *et al.*, 1987) in order to insert a BamHI site directly 5' to the SERP1 initiation codon (GGATCCATG). The resultant phage was propagated in *E. coli* JM103. A 1301bp BamHI/HindIII fragment from this phage, containing the intact SERP1 ORF, was subcloned into pMTL22 (Chambers *et al.*, 1988). A 1344 BamHI/BglII fragment was then ligated into the BamHI site of the vaccinia expression plasmid pMJ601 (Davison and Moss, 1990) allowing SERP1 to be inserted into the TK gene of vaccinia virus under the control of a strong, synthetic late promoter. Recombinant vaccinia virus (strain WR) was selected on TK$^-$ H143 cells in the presence of 25 μg/mL BUdR, and plaque purified three times. Expression of the SERP1 protein from the recombinant virus, designated VV-S1, was confirmed by immunoblotting using anti-SERP1 antiserum. Control virus (VV-601) was prepared by generating TK$^-$ recombinants of vaccinia WR using the parental pMJ601 plasmid.

Protein labelling, SDS-PAGE and immunoblotting

To detect SERP1 produced from virus-infected BGMK cells, semi-confluent monolayers were adsorbed with virus at a MOI of 10-30 pfu/cell for one hour, DMEM+10% NCS was added and the infection allowed to proceed at 37°C for varying lengths of time. Cell-associated proteins were obtained by lysing monolayers with SDS gel loading buffer (50mM Tris-Cl pH6.8, 100m*M* DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) or with Triton X-100 lysis buffer (50m*M* Tris-HCl pH7.4, 0.15*M* NaCl, 2m*M* EDTA, 1% Triton X-100, 100μg/mL phenylmethylsulphonyl fluoride (PMSF)). To obtain secreted proteins, infected monolayers were washed with PBS and then incubated with serum free DMEM for an appropriate length of time after which the medium was collected. Secreted proteins were concentrated either by precipitation with 80% ammonium sulfate or using Centriprep 10 filters (Amicon). For ^{35}S protein labelling, infected monolayers in 100mm plates were washed with PBS, incubated in serum-free, Met-free DMEM containing 100 mCi ^{35}S Trans-label (ICN Biochemicals) for 1-2 hrs, washed twice with PBS and then incubated with serum-free DMEM for the times indicated. Proteins were analyzed by SDS-PAGE on either 12% gels or 7-15% gradient gels. For immunoblotting, proteins were transferred to nitrocellulose in Tris-glycine transfer buffer (48m*M* Tris pH9.2, 39m*M* glycine, 1.3m*M* SDS, 20% methanol) using a semi-dry transfer apparatus (Tyler Industries). Blots were blocked in Blotto/Tween (5% non-fat skim milk powder, 0.2% Tween-20 in PBS) for 1-3 hrs, incubated with a 1:500 dilution of anti-SERP1 antiserum for 6-16 hrs followed by alkaline phosphatase-conjugated goat anti-rabbit antibody (BioRad) for 1-2hrs, and then developed according to the manufacturer's instructions.

Tunicamycin treatment and peptide-N-glycosidase F digestion

BGMK cell monolayers were infected at a MOI of 15 pfu/cell, incubated for 6 hours at 37°C, washed with PBS, and then incubated in serum-free DMEM containing either no drug, 1 mg/mL or 5 mg/mL tunicamycin (Sigma). The monolayers and medium were harvested at 11.5 hrs post-infection, and analyzed by SDS-PAGE and immunoblotting as described above. For peptide N-glycosidase F (PNGase F; Boehringer Mannheim) digestions, serum-free medium was collected from cells infected for 24 hrs with either VV-S1 or the control virus VV-601, and precipitated with four volumes of methanol:acetone (1:1) at 0°C. Proteins were resuspended in 100 m*M* sodium phosphate pH 7.0, 1% SDS, boiled for 10 minutes, and then diluted 10 fold with 100 m*M* sodium phosphate pH 7.0, 20 m*M* EDTA, 1% Triton X-100, and 1.1 m*M* DTT. One unit of PNGase F was added, and the reaction was incubated overnight at 37°C. Reaction products were precipitated with 80% ammonium sulfate, analysed by 12% SDS-PAGE and immunoblotted as described above.

Purification of the SERP1 protein

BGMK cell monolayers in roller bottles (approximately $2x10^8$cells per bottle) were adsorbed with VV-S1 at a MOI of 1 pfu/cell for 3 hours in 15 mL of DMEM containing 10% NCS at 37° C. The inoculum was removed, and the cells were washed once with 100 mL and twice with 50 mL sterile PBS to remove the medium and serum proteins. 15 mL of serum-free DMEM was added to each bottle and the infection was allowed to proceed at 37° C for 20-24 hours. The medium was collected, spun at 3000 rpm for 10 minutes to pellet any cells, and the supernatants were pooled and either processed immediately or stored at -20°C until ready to use. The medium containing secreted viral proteins was concentrated approximately 25-fold using Centriprep 10 concentrators (Amicon), and then dialysed against 25 mM Tris pH 8.0 at 4°C using

Spectrapore dialysis tubing. The diaysed samples were loaded onto a MonoQ anion exchange column (Pharmacia), and protein was eluted using a linear salt gradient (0-300mM NaCl in 25mM Tris pH 8.0). Protein containing fractions were analysed by SDS-PAGE, and fractions containing the SERP1 protein were identified by immunoblotting using polyclonal anti-SERP1 antiserum. SERP1-containing fractions were pooled and concentrated using Centriprep 10 concentrators (Amicon) The total protein concentration in the sample was estimated by a Bradford assay. This material was then loaded onto a Superdex 75 gel filtration column (Pharmacia) equilibrated with 25 mM Tris pH8.0, 150 mM NaCl. Fractions were collected and analysed for SERP1 by SDS-PAGE and silver staining and immunoblotting using anti-SERP1 antiserum..

RESULTS

Expression of the myxoma virus SERP1 gene

The region immediately upstream of the SERP1 gene (Upton *et al.*, 1990) contains sequence motifs that are characteristic of the consensus late promoter of vaccinia virus (Davison and Moss, 1989). The 20 bases immediately upstream of the SERP1 gene are AT rich (70% AT) and include the sequence TAAATG at the predicted initiating ATG separated by six nucleotides from an upstream run of eight thymidine residues. Northern blot analysis of RNA extracted from myxoma virus-infected BGMK cells (MOI=10 pfu/cell) detected RNA of heterogeneous length that hybridized to a SERP1 probe, which first appeared between 4 and 6 hours p.i. and reached maximal levels at 8-10 hours p.i. (data not shown). In order to map the actual RNA start site for the SERP1 ORF in relation to the induction kinetics, S1-nuclease protection analysis was performed on total RNA extracted from infected BGMK cells at various times p.i.

(Figure II-1a). The RNA was hybridized to a single stranded 5'-end labelled BamHI/AvaI fragment corresponding to the first 624bp of the myxoma virus BamHI U3 fragment (Russell and Robbins, 1989) that overlaps with the 5' end of the SERP1 ORF. As shown in Figure II-1a (lanes 4-6) starting at 6 hrs p.i. three species of RNA were protected from S1 digestion following hybridization to this fragment, one of which (designated as C) corresponds to a transcript originating at the predicted start site (TAAATG). The remaining two protected fragments, A and B, correspond to late mRNA initiation events approximately 25 and 140bp further upstream of the predicted translation initiation codon but are not associated with consensus late promoter sequences, and their significance with respect to the translation of the SERP1 protein is unknown. Within this upstream region there are no translation initiation codons that correspond to any other predicted ORFs, and SERP1 could be translated from any of the three RNA species. We conclude that at least some of the late mRNA containing the SERP1 gene is regulated by a late promoter which is similar in sequence to the consensus late promoter of vaccinia virus (Davison and Moss, 1989). Although other Leporipoxvirus late promoters have not been examined, this result, coupled with the observation that vaccinia virus late promoters such as the p11 promoter function normally in Leporipoxvirus infected cells, suggests that myxoma virus late promoters are similar to consensus vaccinia virus late promoters.

Effect of SERP1 on MRV virulence in rabbits

In order to determine whether SERP1 functions as a virulence factor for myxoma virus infection, mutant virus was constructed in which the SERP1 gene was inactivated. Because the disease syndrome induced by MRV is very similar to that induced by myxoma virus, and because MRV encodes only a single copy of the SERP1 gene which is identical to that encoded by myxoma virus (Upton *et al.*, 1990), a

SERP1 mutation was originally constructed in MRV. A schematic representation of the relevant regions of the genomes of myxoma virus and MRV, showing the locations of the SERP1 gene, is given in Figure II-2. The SERP1 gene was inactivated as described above by homologous recombination resulting in the insertion of the *E. coli lac Z* gene driven by the vaccinia virus late promoter p11, which functions correctly in the Leporipoxvirus genome. The expression of ß-galactosidase allowed easy detection and plaque purification of recombinant virus. The structure of the plaque purified recombinant virus, here designated MRV-SERP1$^-$, was confirmed by Southern blot analysis of DNA extracted from MRV infected cells (data not shown). MRV-SERP1$^-$ was indistinguishable from wild type MRV with respect to titer, cytopathic effect or plaque morphology when grown on BGMK cells. Similarly, MRV-SERP1$^-$ grew normally in cultures of primary mixed splenocytes, both in the presence and absence of the T cell mitogen concanavalin A (J.L.Macen and D.S.Strayer, unpublished results). In addition, virus titres from splenocytes prepared from rabbits infected intradermally ($2x10^3$ pfu per rabbit) with either wild type MRV or MRV-SERP1$^-$ at days 4, 7 or 11 post-infection were comparable. Together, these data suggest that the SERP1 gene is dispensable for MRV replication.

The role of SERP1 in virus virulence was assessed in two preliminary experiments in which eighteen rabbits were infected with either wild type MRV, or with MRV-SERP1$^-$. New Zealand White rabbits were given an intradermal injection of either 25 or 250 pfu MRV or MRV-SERP1$^-$, and the animals were examined daily for signs of infection. A comparison of the disease profiles of MRV and MRV-SERP1$^-$ is presented in Table II-1. Animals infected with the wild type MRV displayed the expected disease profile (Strayer *et al.*, 1983). Large, tumour-like lesions developed at the site of intradermal inoculation, and were easily observed by three days post-infection. By seven days post-infection, secondary lesions at sites distal to the

inoculation site were observed, along with the development of bacterial infection of the nasal and conjunctival mucosa. By 10 days post infection with MRV, these bacterial infections had become severe, such that the animals began to experience difficulty in breathing. At this time, both the size and number of secondary lesions had increased substantially, and the animals appeared lethargic and largely unresponsive. Because others had shown that infection of European rabbits with MRV is uniformly lethal (reviewed in Strayer, 1989), and all animals appeared to undergo the expected disease syndrome, all MRV-infected rabbits were sacrificed by intravenous injection of euthanol prior to 14 days post-infection, due to the increasing severity of the disease. In addition, rabbits given either 25 or 250 pfu of virus were indistinguishable, suggesting that even very low doses of MRV are uniformly lethal.

Initially, there was little overall difference between animals infected with wild type MRV and those infected with MRV-SERP1$^-$. By three days post-infection, all animals, regardless of the virus dose administered, displayed a protuberant lesion at the inoculation site. By seven days post-infection, the signs were again very similar: secondary lesions were visible at sights distal from the primary inoculation site, and the animals had signs of bacterial infections of the nasal and conjunctival mucosa. At the later stages of the infection, however, differences between the animals infected with the wild type and mutant viruses becames apparent. Between days 7 and 10 post-infection, the bacterial infections in the rabbits infected with MRV-SERP1$^-$ appeared to progress more slowly. Although several animals developed severe bacterial infections and became moribund like their MRV-infected counterparts, more than half of the MRV-SERP1$^-$-infected rabbits underwent an attenuated disease course. The severity of the symptoms in six of the ten MRV-SERP1$^-$-infected animals was reduced, and the rabbits were able to completely recover from the infection. In the surviving rabbits, the viral lesions became hemmorhagic by 2 weeks post-infection. By three weeks post-

infection, the lesions had become necrotic and eventually were resolved to leave a relatively minor scar. The surviving animals were were subsequently challenged with wild type MRV, and all were found to be immune.

The following conclusions can be drawn from these results. First, although the SERP1 gene is not essential for MRV replication in tissue culture, it clearly plays an important role in virus virulence *in vivo*. Second, the SERP1 gene is not required for virus infection or spread of virus to sites distal from the inoculation site. MRV-SERP1⁻ produced primary lesions that were, at a gross level, indistinguishable from those produced by the parental MRV and the virus formed secondary lesions with similar kinetics and gross morphology to those formed during infection with the parental MRV. Thus, it seemed likely that the attenuated phenotype of MRV-SERP1⁻ is the result of an altered host response to the infection, and not of an alteration in the ability of the virus to infect tissues and spread to secondary sites.

Effect of SERP1 on myxoma virus virulence in rabbits

The results above show that the single copy of the SERP1 gene encoded by the recombinant MRV is important for virus virulence. Since the strain of myxoma virus from which MRV is derived is unknown, and in view of the fact that myxoma virus encodes two copies of SERP1 (Figure II-2), the role of SERP1 in virus virulence was further investigated in a well characterized strain of myxoma virus (strain Lausanne) by studying the effect of mutation of both copies of the SERP1 ORF.

We originally attempted to disrupt both copies of the SERP1 ORF by homologous recombination leading to insertion of the *E. coli lac Z* gene driven by the vaccinia p11 promoter, using the same procedure used to obtain the single insertion mutation of SERP1 in MRV. Although blue plaques indicative of insertion of the *lac Z* gene could be detected during the first round of infection following the transfection

procedure, recombinants with both copies of the ORF disrupted could not be propagated presumably due to the repair of single insertions by recombination with the wild type TIR at the other end of the genome. We therefore chose instead the *E. coli gpt* gene as a dominant selectable marker, since this has been shown to be useful for selecting poxvirus recombinants (Falkner and Moss, 1988). Recombinant virus in which the *gpt* gene, driven by the vaccinia virus 7.5K promoter, was inserted into the SERP1 ORF was constructed as described in Materials and Methods and outlined in Figure II-3. Plaque purified recombinant viruses were tested by PCR and immunoblotting to ensure that both copies of the ORF were disrupted by insertion of the *gpt* cassette and that no detectable SERP1 protein was made (data not shown). Many of the recombinant viruses tested contained only recombinant virus with insertions into both copies of the ORF, although some virus clones consisted of what appeared to be mixtures of wild type and recombinant virus.

This SERP1 mutant, designated vMyx-SERP1$^-$, is indistinguishable from wild type vMyxlac with respect to titre, cytopathic effect (CPE) and plaque morphology when grown on either BGMK or RK-13 cells (data not shown), indicating that the SERP1 protein is dispensable for virus replication in tissue culture. We next examined the effect of the mutation on the course of myxomatosis in the rabbit. The results of this experiment are summarized in Table II-2. There was very little difference between vMyxlac and vMyx-SERP1$^-$ at the gross level during the early stages of the infection. All animals developed a raised primary lesion at the site of inoculation which appeared at approximately Day 3. This lesion was identical in appearance for vMyxlac and vMyx-SERP1$^-$ recipients. By Day 7, all animals that received vMyxlac and 6 of 8 animals that received vMyx-SERP1$^-$ displayed a reddening of the conjunctiva and had initial signs of Gram negative infection of the conjunctiva and nasal mucosa. Secondary skin lesions were visible on the ears and face of all rabbits indicating that the mutation

does not have a significant effect on the ability of the virus to disseminate from the site of inoculation. By day 10 all vMyxlac recipients had severe Gram negative infections of the eyes and nose, all were experiencing difficulty in breathing and showed a lack of responsiveness to stimulation symptomatic of acute myxomatosis. In contrast, five of eight vMyx-SERP1$^-$-infected animals had less severe symptoms as indicated by more moderate Gram negative infections and a greater degree of responsiveness, and went on to fully recover from the infection. By Day 21 there were no signs of Gram negative infections in the surviving vMyx-SERP1$^-$-infected rabbits and by Day 28 the primary skin lesions had healed completely, as had many of the secondary lesions. As expected, none of the rabbits that received vMyxlac showed any signs of recovery from the infection and all were sacrificed prior to day 14. Recovered vMyx-SERP1$^-$-infected rabbits were immune to subsequent challenge with the virulent vMyxlac. Therefore SERP1 clearly plays an important role in the virulence of myxoma virus, since the majority of infected animals are able to recover from the infection in its absence.

Histological analysis of lesions from vMyxlac and vMyx-SERP1$^-$ infected rabbits

In order to determine whether there were any differences between the lesions produced by the wild type vMyxlac and the SERP1 mutant vMyx-SERP1$^-$, we performed a histological analysis of both primary and secondary infection sites taken from rabbits at different times during the course of the infection. For this experiment, six rabbits were inoculated with vMyxlac and six with vMyx-SERP1$^-$ on day 0 as described above. Two rabbits from each group were sacrificed on days 4, 7 and 10 p.i., and tissue from the inoculation site (primary sites) or from the ear, nasolabium, eyelid and nictitating membrane (secondary sites) was harvested for histological examination. The results of this experiment are summarized in Table II-3.

At day 4 p.i., the primary sites of vMyxlac injection demonstrated edema, hemorrhage and infiltration of a mixed population of heterophils and mononuclear inflammatory cells throughout the dermis and extending deep to the cutaneous trunci muscle. The primary sites of vMyx-SERP1$^-$ injection showed edema as well, but no hemorrhage was evident. The inflammatory response was qualitatively the same as for the wild type virus, but was less extensive, being confined to the dermal connective tissue and not extending beyond the cutaneous trunci muscle. Secondary sites were not involved for either virus.

By day 7 p.i. there was a well established inflammatory response to vMyxlac in the skin of the primary sites (Figure II-4a). This was most severe at the basal cell layer of the epithelium, and in the immediately underlying connective tissue. There was an interface dermatitis (Figure II-4a) with a focal variation of intensity characterized by subbasilar cleft formation, microthrombi in capillaries and focal hemorrhage and edema (Figure II-4c). Inflammation also extended throughout the dermis, with edema at this time being most severe in the connective tissue immediately superficial to the cutaneous trunci muscle. The cutaneous trunci was involved in the inflammation and there was also an intense inflammatory reaction immediately deep to the muscle layer. By this time, the the virus had clearly spread to secondary sites, where the reaction was characterized by mild to moderate edema, hemorrhage and a mixed mononuclear cell reaction in the dermis. By day 10 p.i. the reaction at the primary sites had progressed to include generalized edema of connective tissues, and an ongoing intense inflammatory reaction within and in the immediately superficial and deep connective tissues around the cutaneous trunci muscle. The primary lesion from one animal had progressed to formation of a central necrotic core (Figure II-5a) composed of epidermis and dermis down to, but not including, the cutaneous trunci muscle. This core was delineated by a zone of accumulating mononuclear inflammatory cells around its lateral, and to a lesser

extent, its deep edges. In the other vMyxlac recipient, the primary lesion had not resolved as well and still displayed an active interface dermatitis without necrosis, as well as considerable edema with inflammatory cells around its lateral and, to a lesser extent, its deep edges. The epidermal surface exhibited extensive areas of epithelial vacuolation. Secondary sites in both vMyxlac recipients exhibited extensive areas of edema with a mixed mononuclear cell and heterophil inflammatory response (Figure II-5d).

In contrast, by day 7 the inflammatory reaction in the primary sites of vMyx-SERP1$^-$ recipients was more advanced, similar to a stage in between that of wild type virus at days 7 and 10. The interface dermatitis was more severe and epithelial damage was evident as widespread cytoplasmic vacuolation (Figure II-4b,d). The mononuclear cell component of the dermal inflammation predominated, and was resolving into large foci of cells within which lymphocytes accounted for approximately half. Secondary sites were characterized by minimal edema, hemorrhage and an infiltration of mononuclear inflammatory cells which was occasionally focally more intense. By day 10 p.i., the differences between vMyx-SERP1$^-$ and vMyxlac recipients were the most pronounced (Figure II-5). At this time both vMyx-SERP1$^-$ recipients had primary sites with necrotic central cores that were well demarcated by broad zones of mononuclear inflammatory cells (Figure II-5b) in which, unlike for vMyxlac recipients, lymphocytes predominated. Secondary sites (Figure II-5c) revealed a pattern of necrosis of the central area which was clearly demarcated from surrounding viable skin by a zone of mononuclear cells.

From these observations we conclude that although the inflammatory reaction seen for both viruses was qualitatively similar, in the animals inoculated with vMyxlac there was greater tissue damage over a larger area while with vMyx-SERP1$^-$ tissue damage was more localized and the inflammatory reaction progressed more rapidly to

resolution. It therefore appears that the deletion of the SERP1 protein allows a more efficient and more localized inflammatory response within infected skin sites, and suggests that the SERP1 protein functions to somehow retard the progression of the inflammatory response, resulting in more tissue damage over a greater area.

Localization of the SERP1 protein

As a first step toward determining the function of the SERP1 protein, we wished to examine the expression and localization of the SERP1 protein during infection of cultured cells. Figure II-6a shows a time course of ^{35}S-labelled proteins secreted from myxoma virus-infected BGMK cells, demonstrating the spectrum of virus-induced proteins that are secreted at both early and late times during the infection. Some of these proteins, such as the virus encoded homologues of the TNF receptor (Upton *et al.*, 1991) and the γ-interferon receptor (Upton *et al.*, 1992) have been characterized elsewhere. One of these secreted proteins, a diffuse 55 kDa species produced at late times (arrow, Figure II-6a), was identified as SERP1 by Western blotting of the same time course (Figure II-6b) using a polyclonal antiserum that was raised to SERP1 expressed from an *E. coli* expression vector (see Materials and Methods). This band was not present in medium from cells infected with either vMyx-SERP1$^-$ or the SERP1 disruption mutant in MRV, MRV-SERP1$^-$ (data not shown). The data confirmed that the SERP1 protein is indeed synthesized at late times and is secreted from the infected cell as a 55 kDa species.

A Western blot of lysates from myxoma virus infected cells prepared at the same times during the infection and probed with the anti-SERP1 antiserum indicates that a portion of the SERP1 protein remains cell-associated, and that this intracellular form migrates at a significantly lower apparent molecular weight than the secreted form (Figure II-7). This band did not correspond to a different, cross-reacting protein since it

was not detected in cells infected with vMyx-SERP1⁻ (data not shown). A minor species, migrating around 32kDa also reacts with the anti-SERP1 antiserum and was always present in lysates from cells infected with the wild type virus but not in cells infected with the SERP1 mutant virus. Although this species has not been characterized, we speculate that it may represent a cleavage product of the SERP1 protein. With the exception of some minor cross-reacting bands, protein reactive with the anti-SERP1 antiserum is absent from the mock, SFV and MRV-SERP1⁻-infected cells, as expected. This data indicates that the SERP1 protein is modified post-translationally to a higher molecular weight form before it is secreted from the cell.

Glycosylation of the SERP1 protein

The discrepancy in molecular weight between the secreted and cell-associated forms of SERP1 could be explained by a post-translational modification. The SERP1 sequence contains three potential N-linked glycosylation sites and therefore was predicted to be glycosylated prior to being secreted from the cell. In order to address this question, cells were infected with myxoma virus in the presence of tunicamycin, an inhibitor of N-linked glycosylation. In the presence of 1 μg/mL or 5 μg/mL tunicamycin, no SERP1 protein could be detected in the medium taken from infected cells (Figure II-8a lanes 1,2), even when the secreted proteins were concentrated by precipitation. In addition, very little SERP1 could be detected in lysates from cells infected in the presence of tunicamycin (Figure II-8b lanes 2,3) indicating that it is either unstable or inefficiently synthesized under these conditions.

To confirm whether SERP1 itself is glycosylated, enzymatic deglycosylation was carried out. In order to conveniently obtain sufficient quantities of the SERP1 protein for these and other studies, we cloned the SERP1 ORF into the vector pMJ601 (Davison and Moss, 1990), which directs the insertion of the ORF into the vaccinia

virus genome by homologous recombination into the thymidine kinase gene where it is expressed under the control of a synthetic late promoter. The SERP1 protein was expressed from this vaccinia virus construct, VV-S1, as a secreted protein which migrates at the same molecular weight as that secreted from myxoma virus-infected cells. The protein was expressed from VV-S1 at approximately 10-fold higher levels than from myxoma virus, and was as sensitive to tunicamycin treatment as was the SERP1 from myxoma-infected cells (Figure II-8b, lanes 4-6). Protein secreted from VV-S1-infected cells was enzymatically reacted with peptide N-glycosidase F (PNGaseF), blotted to nitrocellulose and probed with anti-SERP1 antiserum. As shown in Figure II-9, digestion of the secreted form of SERP1 with PNGaseF reduced its mobility to that predicted from the amino acid sequence (lane 4), and comparable to the mobility of the SERP1 protein as expressed from *E. coli* (lane 2). The small difference in mobility between the deglycosylated SERP1 and that expressed from *E. coli* may result either from the alterations in the 14 N-terminal amino acids produced during the cloning of the ORF into the bacterial expression vector (see Materials and Methods), or from possible signal sequence cleavage from the native SERP1 protein. We conclude that the SERP1 protein is N-glycosylated before it is secreted from the cell, and only the glycosylated form is detectable extracellularly.

Purification of the SERP1 protein

The above studies indicated that the SERP1 protein was an important virulence factor for myxoma virus infection, possibly due to its ability to alter the inflammatory response to virus infection. In order to further characterise the functional properties of the SERP1 protein and to facilitate the identification of cellular or viral proteins with which SERP1 may interact, a protocol was developed for the purification of the SERP1 protein.

Purification of the SERP1 protein was simplified by the fact that it is secreted from the infected cell. In order to generate sufficient SERP1 which was likely to be in active form the recombinant vaccinia virus, VV-S1, was used to overexpress the SERP1 protein. Serum-free medium was collected from approximately $5x10^9$ BGMK cells infected with VV-S1 at a multiplicity of infection of 1.0 pfu/cell and concentrated as described in Materials and Methods. This material contained a complex mixture of proteins as shown in Figure II-10 (lane 1). The presence of SERP1 in this mixture was confirmed by immunoblotting using the anti-SERP1 antiserum (not shown). The mixture was then dialysed against a low salt buffer at pH 8.0, sufficiently above the predicted isoelectric point of the protein that it would adhere to an anion exchange resin. The protein mixture was then separated by FPLC. First, the sample was applied to a MonoQ anion exchange column, and proteins were eluted with a linear salt gradient. Column fractions containing SERP1 were identified by immunoblotting (not shown) and pooled. Silver staining of SDS polyacrylamide gels indicated that the pooled MonoQ fractions were considerably less complex than the starting mixture, with the SERP1 protein being the predominant species, migrating as a diffuse band of approximately 55 kDa (Figure II-10, lane 2). The resulting mixture was then concentrated and separated by gel filtration chromatography on a Superdex 75 column. SERP1 containing fractions were again identified by SDS-PAGE and immunoblotting using anti-SERP1 antiserum (not shown). Figure II-10 (lane 3) indicates that the SERP1 protein was purified to apparent homogeneity by this protocol. The resulting protein has been shown to function as an inhibitor of plasmin in an *in vitro* assay (P.D.Nash and G. McFadden, unbublished observation), in accord with observations made with unpurified SERP1 (Lomas *et al.*, 1993). These results therefore define a rapid and simple protocol for the ready purification of active SERP1 protein in its native form.

DISCUSSION

Members of the serpin family of proteins are encoded by several poxviruses (recently reviewed in Turner *et al.*, 1995). Vaccinia virus, the prototype Orthopoxvirus, encodes three evolutionarily distinct serpin-like proteins designated SPI-1, SPI-2 (Kotwal and Moss, 1989; Smith *et al.*, 1989) and SPI-3 (Boursnell *et al.*, 1989; Smith *et al.*, 1989). Nearly identical serpins are encoded by the closely related Orthopoxviruses cowpox virus (Pickup *et al.*, 1986; Kotwal and Moss, 1989; Turner and Moyer, 1992) and rabbitpox virus (Turner and Moyer, 1992). In addition, a gene encoding serpin-like protein is also found in fowlpox virus (Tomley *et al.*, 1988). Until very recently, little information was available concerning the functions of these viral serpins. The first poxviral serpin for which any function was described and about which the most is known is the CPV 38K protein, which is 92% identical to the vaccinia SPI-2 protein (Smith *et al.*, 1989). Deletion of the 38K gene resulted in an attenuated CPV that produced white pocks upon infection of the chick chorioalantoic membrane (CAM), instead of the red hemorrhagic pock usually observed following infection with wild type CPV (Pickup *et al.*, 1986). The white pock phenotype results from a massive infiltration of inflammatory cells into the site of infection, suggesting that the function of the 38K protein is to block the production or activity of an important inflammatory chemotactic factor (Palumbo *et al.*, 1989; Chua *et al.*, 1990; Frederickson *et al.*, 1992). Indeed, the 38K protein inhibits the interleukin-1ß (IL-1ß) converting enzyme (Ray *et al.*, 1992) a cysteine proteinase that cleaves pro-IL-1ß to active IL-1ß which in turn is an important mediator of the inflammatory response. The Orthopoxviral SPI-3 genes have recently been shown to function in the control of cell-cell fusion, since deletion of this ORF from the viruses results in the formation of polykaryocytes (Turner and Moyer, 1992; Law and Smith, 1992; Zhou *et al.*, 1992).

The significance of this effect with respect to virus infection *in vivo* is unclear, since SPI-3 deletion mutants are not attenuated in infected animals (Turner and Moyer, 1992; Law and Smith, 1992). These observations suggest that poxviral serpinlike proteins have evolved multiple functions, making the elucidation of their roles during infection *in vivo* difficult.

In this communication we report the the characterization of a myxoma virus encoded serpin, SERP1, and show that it has several features that make it unique among the known poxviral serpins. The SERP1 gene is the only serpin localized within the TIR of the viral genome, and the SERP1 amino acid sequence does not bear any striking homology to any of the other poxviral serpins (Upton *et al.*, 1990). The nearest viral homologue is the SPI-3 gene of vaccinia virus, which shares only 28% amino acid identity with the myxoma SERP1 protein. There is no evidence that SERP1 has any functional relationship to this or any other poxviral protein described to date. Unlike all of the other poxviral serpins, SERP1 is expressed as a late protein. The transcription of the SERP1 gene is directed at least in part by a late promoter that resembles the consensus late promoter of vaccinia virus (Davison and Moss, 1989). In addition, we have shown that SERP1 is a glycoprotein that is secreted from the infected cell with an apparent molecular weight of 55 kDa. A detectable amount of the myxoma SERP1 protein does remain associated with the cell as a 42 kDa species, but the fact that a significant proportion of this species becomes glycosylated and is secreted suggests that SERP1 interacts with an extracellular target(s). Other nonviral serpins are known to display a similar secretion profile. For example, a large pool of nonglycosylated plasminogen activator inhibitor 2 is retained intracellularly, and only the glycosylated form is secreted from the cell (Kruithof, 1988). Since no SERP1 protein is detectable in the presence of tunicamycin, it may be unstable or poorly synthesized under these conditions.

In order to assess the contribution that SERP1 makes to the pathology associated with myxomatosis in rabbits, two mutant viruses were constructed: MRV-SERP1⁻, in which the single copy of the SERP1 gene was disrupted by insertion of the *E. coli Lac Z* gene, and vMyx-SERP1⁻, in which both copies of the SERP1 gene were disrupted by the insertion of the *E. coli gpt* gene, a dominant selectable marker. Mutation of the single copy of the SERP1 gene in MRV resulted in a significant attenuation of the virus in the rabbit, indicating that the SERP1 gene plays an important role in MRV virulence in the rabbit. Because the strain of myoxma virus from which MRV is derived is unknown, it was important to examine the role of SERP1 in the disease pathology induced by a virulent strain of myxoma virus that is better characterised both biologically and at the molecular level. Further studies of the SERP1 gene were therefore conducted using the Lausanne strain of myxoma virus. In agreement with the results obtained with MRV, disruption of both copies of the myxoma virus SERP1 gene resulted in a significant attenuation of the virus in the rabbit, again emphasizing the important role that SERP1 plays in virus virulence. The observed attenuation is not likely to be the result of a reduction in virus replication, since both mutant viruses replicated well in tissue culture, in both BGMK and RK-13 cells, with iden[illegible]PE and plaque morphology to the parental viruses from which they were derived. Both mutant viruses were able to disseminate from the site of inoculation with similar kinetics to that of the wild type virus, again suggesting that the defect is not in virus replication *per se*. Nevertheless, we cannot exclude the possibility that the absence of the SERP1 protein impairs virus replication in a certain subset of target tissues or cell types *in vivo* .

The results of preliminary histological analysis of both primary and secondary lesions taken from animals inoculated with vMyx-SERP1⁻ or vMyxlac provide some clues concerning the function of the SERP1 protein. Although the inflammatory

reactions occurring in lesions from animals infected with either virus were qualitatively similar, the reaction in the SERP1 mutant infected animals was less extensive, although focally more intense, and appeared to progress at a higher rate. At early stages (day 4, see Table II-3) there was less edema and infiltration during vMyx-SERP1$^-$ infection as compared to that seen for vMyxlac. Nevertheless, the inflammatory response progressed with faster kinetics for vMyx-SERP1$^-$ such that by day 7 the reaction was significantly more advanced. By day 10 p.i., healing was underway in the lesions from animals given vMyx-SERP1$^-$, but not vMyxlac, implying that the host response to vMyx-SERP1$^-$ infection was more effective.

Although a complete understanding how the SERP1 protein acts to affect the inflammatory response awaits a more detailed functional analysis of the SERP1 protein, some recent observations allow some predictions to be made. It has been shown that SERP1 forms inhibitory complexes with the human plasma serine proteinases plasmin, tissue-type plasminogen activator (t-PA), urokinase and the complement control protein C1s (Lomas *et al.*, 1993). Although these reactions occur with relatively slow second order association rates (approximately 3×10^3 M^{-1} s^{-1} for C1s and 4×10^4 M^{-1} s^{-1} for plasmin, urokinase and t-PA) they are still within the range of physiologically significant interactions. In addition, these studies were performed using human proteins as substrates because the counterpart rabbit proteins are not readily available. It is expected that SERP1 would interact more avidly with the rabbit equivalents of these proteins because other secreted myxoma virus proteins examined that interact with host proteins, such as the viral T2 protein (Schreiber and McFadden, 1994) and the T7 protein (Mossman *et al.*, 1995), bind with a greater affinity to target proteins from the rabbit that to homologous proteins from other species. It will therefore be important to assess the ability of SERP1 to interact with these rabbit proteinases in order to make any firm conclusions concerning its target *in vivo*. Nevertheless, the SERP1 protein is

the first of the poxviral serpins to have been shown to actually possess the ability to function as an inhibitor of a serine proteinase.

Further characterisation of the SERP1 protein and its role in inflammation are complicated by fact that myxoma virus encodes a plethora of molecules that function to subvert numerous host anti-viral responses (McFadden *et al.*, 1995; McFadden and Graham, 1994). Therefore, a protocol was developed by which the SERP1 protein may be readily prurified in active form. The availability of purified SERP1 protein will allow both its characterisation as a proteinase inhibitor, as well as providing pure material for use in various biological assays of inflammation. For example, the purified SERP1 protein has recently been shown to function as a potent anti-inflammatory agent in animal models of restenosis (Lucas *et al.*, 1995), providing further support for anti-inflammatory properties of this molecule as well as highlighting important potential clinical applications for viral anti-inflammatory proteins.

Important questions concerning the role of SERP1 in myxomatosis remain to be addressed. These include: 1) Does SERP1 actually interact with rabbit plasma proteinases *in vivo*? and 2) Is the inhibition of these proteinases responsible for the biological differences between vMyxlac and vMyx-SERP1^{-}? Inhibition of the complement cascade via inhibition of C1s would obviously have important consequences in terms of combatting the viral infection. The complement system is involved in antiviral responses at several different levels including enhancement of the inflammatory response and lysis of virally-infected cells (Frank and Fries, 1989). One of the major secreted proteins of vaccinia virus binds to C4b and inhibits the classical complement cascade (Kotwal *et al.*, 1990). Deletion of the gene encoding this vaccinia complement control protein results in virus attenuation in mice (Kotwal *et al.*, 1990; Issacs *et al.*, 1992), possibly by preventing antibody-dependent complement-enhanced neutralization of infectivity (Issacs *et al.*, 1992). The complement cascade is therefore

likely to be important to the host defense against poxvirus infection. This is further supported by the identification of two genes in RPV related to complement control proteins that also function in virus virulence (Bloom *et al.*, 1991). Inhibition of proteinases such as plasmin, t-PA and urokinase could also be predicted to influence the antiviral inflammatory response, but the situation is complicated by the fact that these proteinases function in multiple pathways whose regulation is complex (Andreasen *et al.*, 1990). For example, plasmin can be generated by the cleavage of plasminogen by t-PA or urokinase, and plasmin itself functions in fibrinolysis to degrade fibrin to soluble degradation products which can act as chemotactic factors for inflammatory cells. It has been suggested that plasmin may also function as a physiological activator of transforming growth factor ß (TGF-ß) (Lyons *et al.*, 1990), although the significance of plasmin activation of TGF-ß is controversial (Huber *et al.*, 1991). This issue is important to consider because TGF-ß can in turn act as a chemotactic factor for inflammatory cells (Adams *et al.*, 1991; Brandes *et al.*, 1991). Furthermore, urokinase itself has been found to be a potent chemotactic factor for polymorphonuclear leukocytes, an activity that requires its enzymatic activity (Boyle *et al.*, 1987). The dissection of how SERP1 influences myxoma virus virulence *in vivo* may also further our understanding of the roles that various host proteinases may play during the host response to virus infections in general.

TABLE II-1

COMPARISON OF WILD TYPE MRV WITH SERP1 DISRUPTION MUTANT

DAY	MRV (wild type)	MRV-SERP1$^-$ (SERP1 mutant)
0	8 adult rabbits inoculated intradermally with 25 or 250 pfu	10 adult rabbits inoculated intradermally with 25 or 250 pfu
3	Raised primary lesion at inoculation site	Raised primary lesion at inoculation site
7	Mild bacterial infection of the conjunctiva and nasal mucosa; Growing lesion at inoculation site (2cm); Small lesions visible at secondary sites	Mild bacterial infection of the conjunctiva and nasal mucosa; Growing lesion at inoculation site (2cm); Small lesions visible at secondary sites
10	Severe bacterial infection of the conjunctiva and nasal mucosa; Large (2.5-3cm) lesion at inoculation site; Numerous lesions at secondary sites	Moderate to severe bacterial infection of the conjunctiva and nasal mucosa; Large (2.5-3cm) lesion at inoculation site; Numerous lesions at secondary sites
14	No surviving rabbits	Three rabbits sacrificed due to increasing severity of symptoms; Other animals show little change from day 10
21		Six rabbits recovering from the infection; Lesions necrotic and scabbing over; Severity of bacterial infection decreasing
28		Six rabbits surviving, with most fully recovered; Lesions completely healed; Little or no sign of bacterial infections

TABLE II-2

COMPARISON OF WILD TYPE MYXOMA VIRUS WITH SERP1 DISRUPTION MUTANT

	vMyxlac (wild type)	vMyx-SERP1⁻ (SERP1 mutant)
Virus growth in cultured primate or rabbit cells	Normal titres and plaque morphology	Normal titres and plaque morphology
Course of myxomatosis (2x500 pfu intradermally)	Day 0: Four rabbits inoculated intradermally with 2x500 pfu vMyxlac.	Day 0: Eight rabbits inoculated intradermally with 2x500 pfu vMyxS1.
	Day 3: Appearance of raised primary lesion at inoculation site.	Day 3: Appearance of raised primary lesion at inoculation site.
	Day 7: Evidence of Gram negative infection of conjunctiva and nasal mucosa of all rabbits. Appearance of secondary skin lesions on face and ears	Day 7: Evidence of Gram negative infection of conjunctiva and nasal mucosa of four of eight rabbits. Appearance of secondary skin lesions on face and ears
	Day 10: Large primary lesion (approx. 3cm diameter) Severe gram negative infection of conjunctival and nasal mucosa Numerous secondary skin lesions on ears and face. Low responsiveness to stimuli	Day 10: Large primary lesion (approx. 3cm diameter) Mild to moderate (5 rabbits) or severe (three rabbits) Gram negative infection of conjunctival and nasal mucosa Numerous secondary skin lesions on ears and face. Low (three rabbits) to moderate (5 rabbits) responsiveness to stimuli
	Day 14: No surviving animals	Day 14: 5 of 8 animals with decreased severity of symptoms; 3 rabbits sacrificed due to increased severity of symptoms
		Day 28: 5 animals partially or completely recovered from disease
Rabbit survival	$< 1\%$	60%

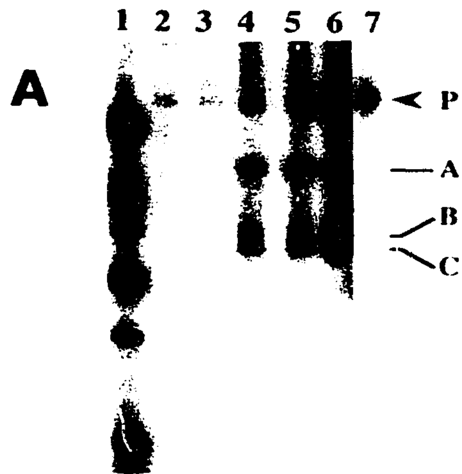
TABLE II-3

SUMMARY OF HISTOLOGICAL OBSERVATIONS OF LESIONS FROM INFECTED RABBITS[a]

	vMyxlac (wild type)	vMyx-SERP1⁻ (SERP1 mutant)
DAY 4: Primary sites[b]	Edema Hemorrhage Infiltration with heterophils and mononuclear inflammatory cells	Edema No hemorrhage observed Less extensive inflammatory infiltrate
Secondary sites[c]	No detectable reaction	No detectable reaction
DAY 7: Primary sites[b]	Edema Focal hemorrhage Interface dermatitis Well established dermal inflammation with mononuclear cell infiltrate	Edema Hemorrhage Interface dermatitis more severe Mononuclear cell infiltrate resolving into large foci of cells containing lymphocytes Epithelial damage
Secondary sites[c]	Mild to moderate edema Hemorrhage Mixed mononuclear cell infiltrate	Mild edema Hemorrhage Mononuclear cell infiltrate focally more intense
DAY 10: Primary sites[b]	Generalized edema Active interface dermatitis Intense inflammatory reaction Some necrosis Epithelial degeneration	Necrotic central cores, well demarcated by mononuclear cells, predominantly lymphocytes
Secondary sites[c]	Ongoing edema Mixed mononuclear cell and heterophil infiltrate	Necrosis of central area of lesion, demarcated by mononuclear cells

[a]Rabbits were given two intradermal injections of 500 pfu vMyxlac or vMyx-SERP1⁻ on Day 0
[b]Samples taken from lesion at inoculation site
[c]Samples taken from lesions on ear, nasolabium, eyelid and/or nictitating membrane

Figure II-1. S1-nuclease analysis of the myxoma virus SERP1 gene region. (A) S1-nuclease protection analysis of total RNA from myxoma virus-infected BGMK cells harvested at 2,4,6,8 and 12 hrs post-infection (lanes 2-6) or from mock-infected cells (lane 7). Lane 1, HinfI digested pBR322 (517, 506, 396, 344, 298, 221/220,154, 75 bp). P denotes the position of migration of the labelled BamHI/AvaI probe, and A, B and C indicate the position of the three major species protected by this probe. (B) Schematic representation of the results shown in (A). The bold line represents the 624 nucleotide BamHI/AvaI subfragment of the BamHI U3 fragment of myxoma DNA, * indicates position of ^{32}P-end label. The dashed arrow indicates the putative start site of the small ORF situated upstream from SERP1 (Upton *et al.*, 1990) and the open circle indicates the end of this ORF. The position of the predicted translational start codon for SERP1 is shown by the bold arrow, and the position of the RNA species A, B and C protected from S1-nuclease digestion are shown below.


A
1
2
3
4
5
6
7
P
A
B
C

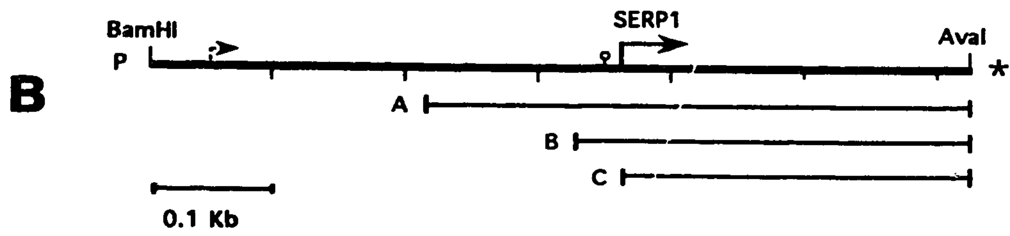
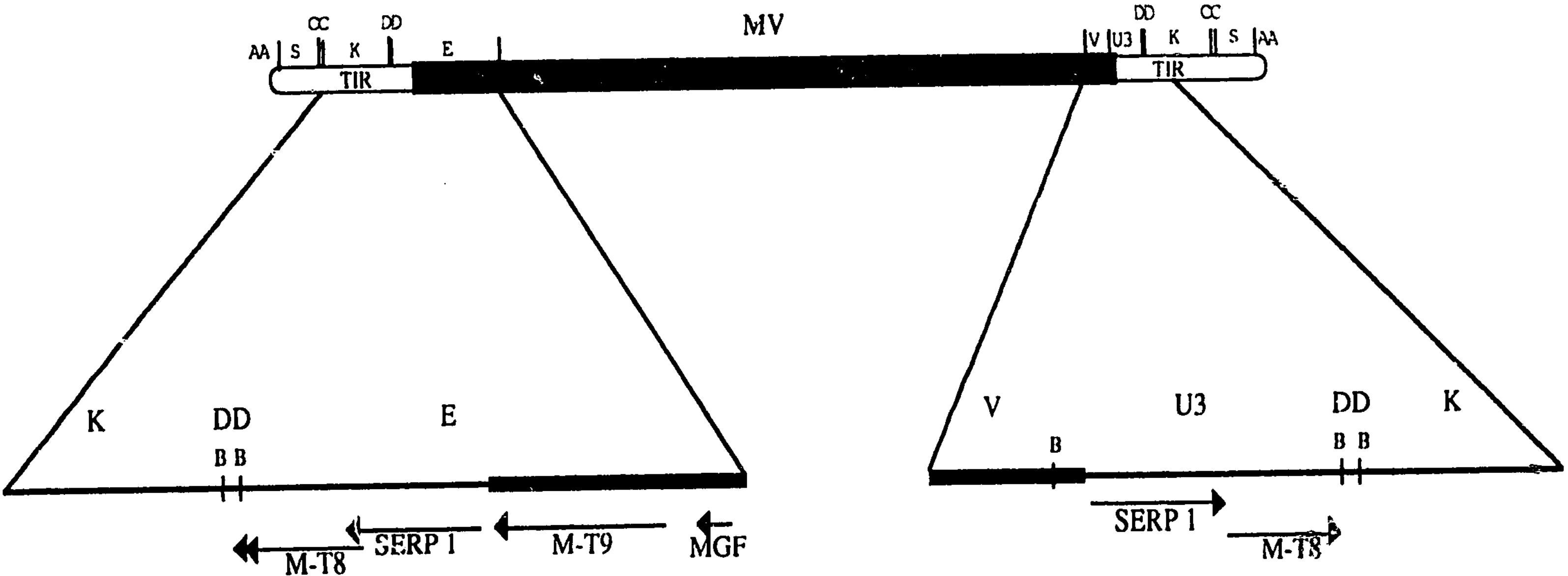
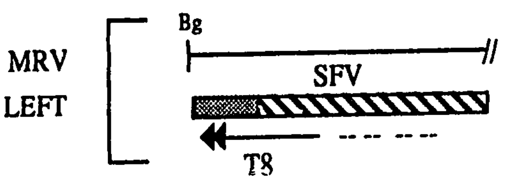

B
BamHI
P
SERP1
AvaI
*
A
B
C
0.1 Kb

Figure II-2. Schematic representation of the myxoma virus and MRV genomes illustrating the locations of the SERP1 gene. Partial BamHI restriction map of the myxoma virus genome (Russell and Robins, 1989). Solid bars represent the unique region of the genome while the open ends represent the terminal inverted repeat (TIR) regions. BamHI sites and fragments are indicated, labelled according to Russell and Robins (1989). Arrows represent open reading frames. The equivalent regions of the MRV genome are illustrated below. Striped bars represent DNA sequences derived from SFV, while shaded bars represent sequences derived from myxoma virus. Note that the left end of this region of the MRV genome consists entirely of SFV-derived sequences and does not encode a SERP1 open reading frame. MGF and SFGF refer to the myxoma virus and Shope fibroma virus growth factor genes, respectively.





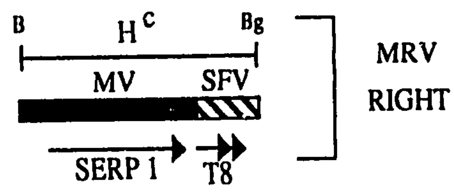
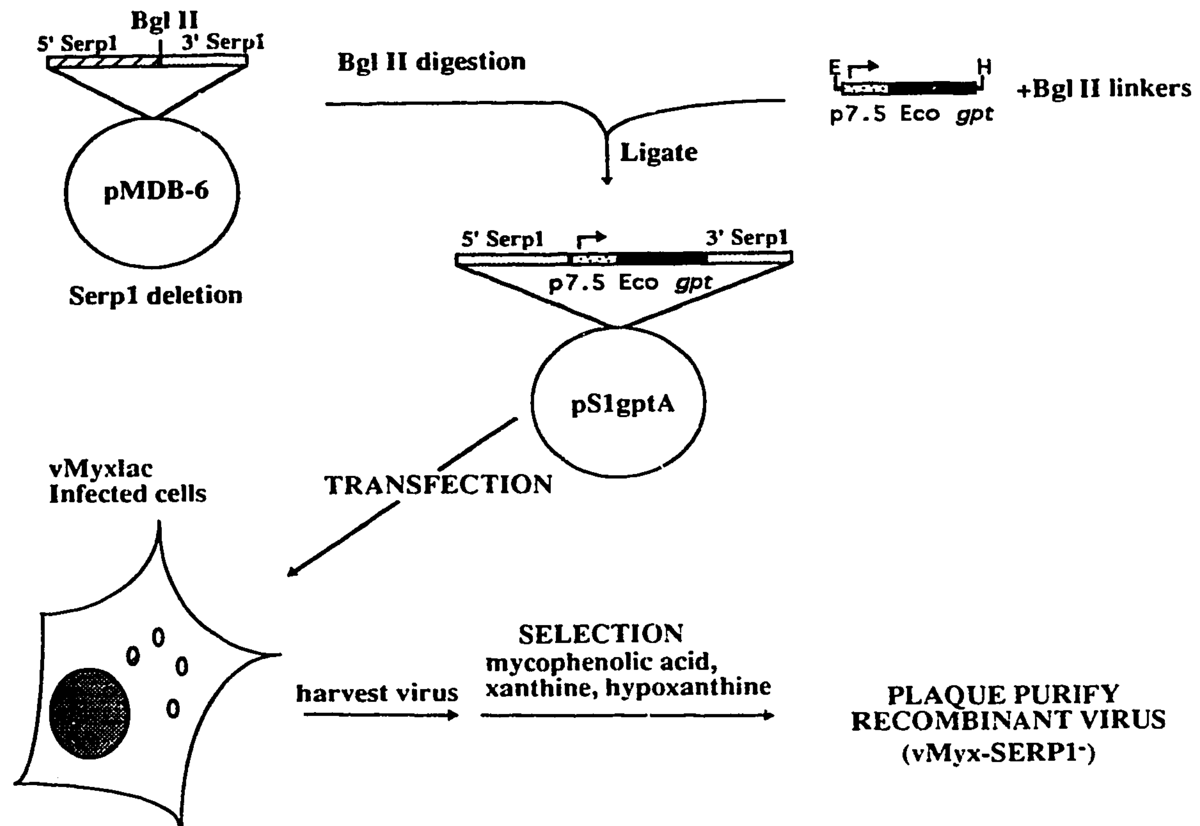


FIGURE II-2

Figure II-3. Construction of the SERP1 deletion mutant in myxoma virus. (A) The plasmid pMDB-6, which contains the SERP1 ORF with a small central deletion engineered into a BglII site (Upton *et al.*, 1990) was digested with BglII and ligated to a 0.9Kb fragment consisting of the *E. coli gpt* gene driven by the vaccinia p7.5 promoter using BglII linkers. The resultant plasmid, pS1gptA, contained the *gpt* gene such that it would be transcribed in the same direction as the SERP1 gene. pS1gptA was transfected into vMyxlac infected BGMK cells (MOI=0.05), virus was harvested and recombinants were selected as described under Materials and Methods. (B) Schematic representation of the vMyx-SERP1^{-} genome at the SERP1 locus in the right terminal inverted repeat of the myxoma genome, showing insertion of the p7.5 gpt cassette. The arrows indicate the start sites for the SERP1 and the *gpt* ORFs. The SERP1 coding sequence (dark bars), contains a 65 bp deletion into which the p7.5 gpt cassette (striped bar) has been inserted using Bgl II linkers. Open bars represent the BamHI U3 fragment 5' and 3' of the SERP1 ORF. The insertion is identical in the other copy of the SERP1 gene (not shown), located in the Bam HI E fragment (Upton *et al.*, 1990). Bm, Bam HI; Bg, Bgl II.

A



B

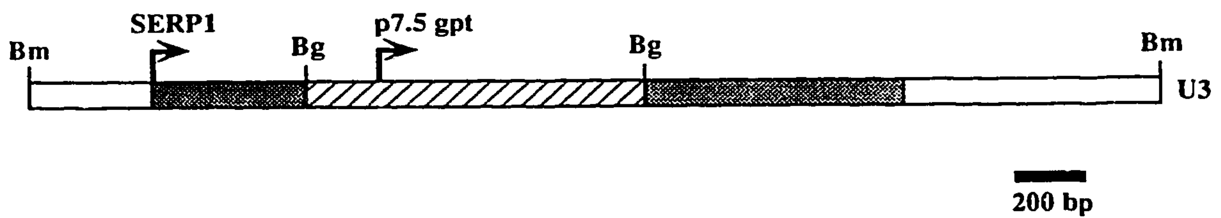
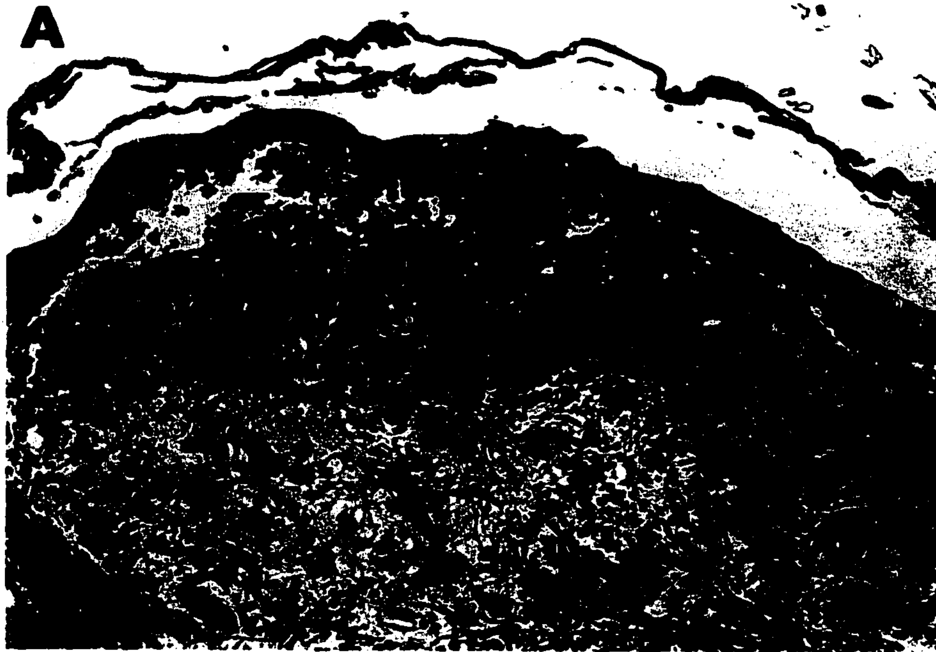


FIGURE II-3

Figure II-4. Primary lesions from vMyxlac and vMyx-SERP1^{-}-infected rabbits at day 7 post-infection. Tissue samples harvested from infected animals were fixed in neutral buffered formalin, embedded in paraffin, sectioned at 4μm and stained with hematoxylin and eosin. (A) Inoculation site from a vMyxlac-infected rabbit, showing interface dermatitis (arrows) and tissue damage in the lesion. Bar= 0.1 mm. (B) Inoculation site from a vMyx-SERP1^{-}-infected rabbit, showing more severe interface dermatitis and less severe tissure damage as compared to the lesion from vMyxlac recipient. Bar=0.1 mm. (C) Inoculation site from a vMyxlac-infected rabbit, showing edema seen as widespread separation of connective tissue and epithelial separation (arrows). Bar=1.0 mm. (D) Inoculation site from a vMyx-SERP1^{-}-infected rabbit. Note less extensive edema as compared to (C). Bar=1.0 mm.

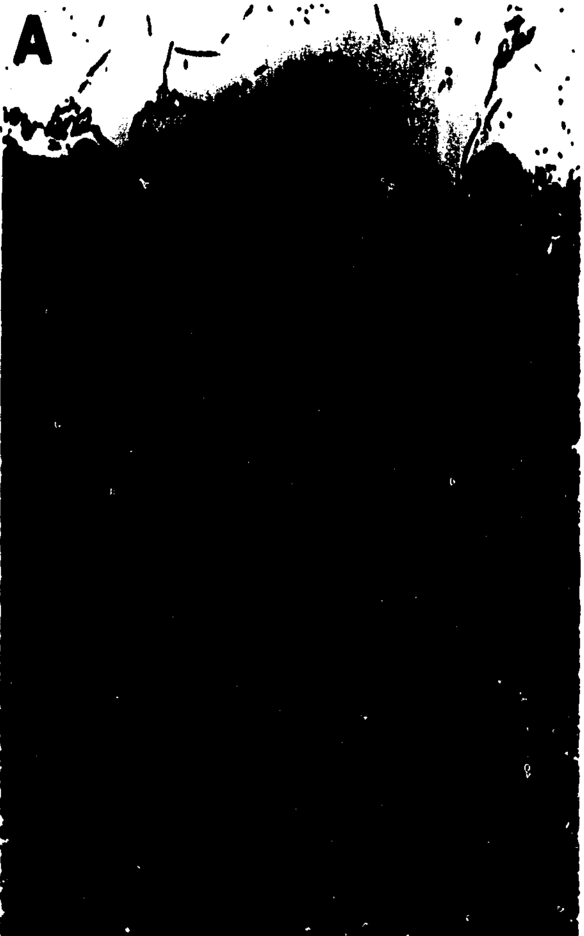

A

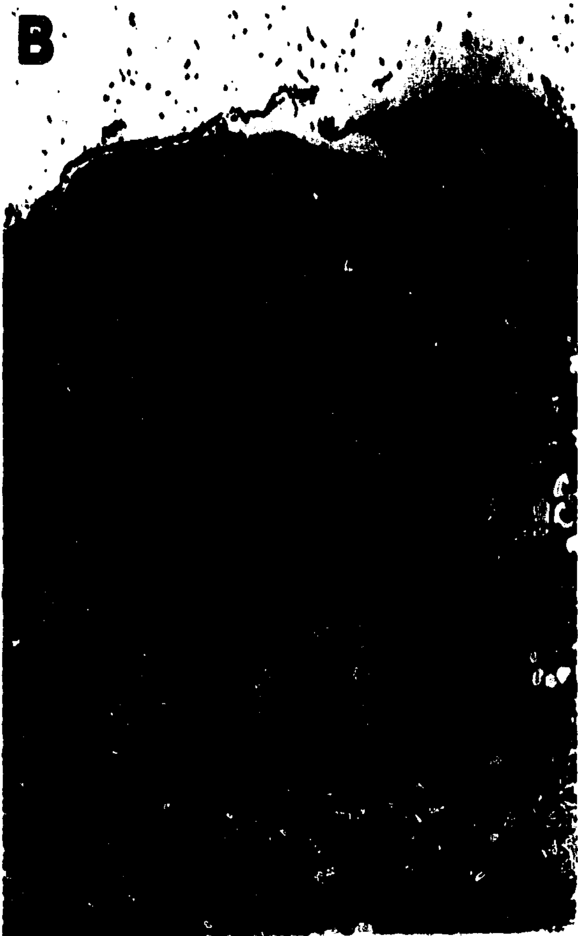
B

C

D

Figure II-5. Primary and secondary lesions from vMyxlac and vMyx-SERP1^{-}-infected rabbits at day 10 post-infection. Tissue samples were processed as described in the legend to Figure 3. (A) Inoculation site from a vMyxlac-infected rabbit. (B) Inoculation site from a vMyx-SERP1^{-}-infected rabbit. Note that the edge of the lesion is well demarcated (arrows) as compared to that from the vMyxlac recipient shown in (A). (C) Secondary lesion from the eyelid of a vMyx-SERP1^{-}-infected rabbit, showing a similar state of resolution to the primary site illustrated in (B). (D) Secondary lesion from the eyelid of a vMyxlac-infected rabbit. Note that the lesion is not as well resolved as that from the vMyx-SERP1^{-} recipient (compare to panel C). A-D, Bar=1.0 mm.

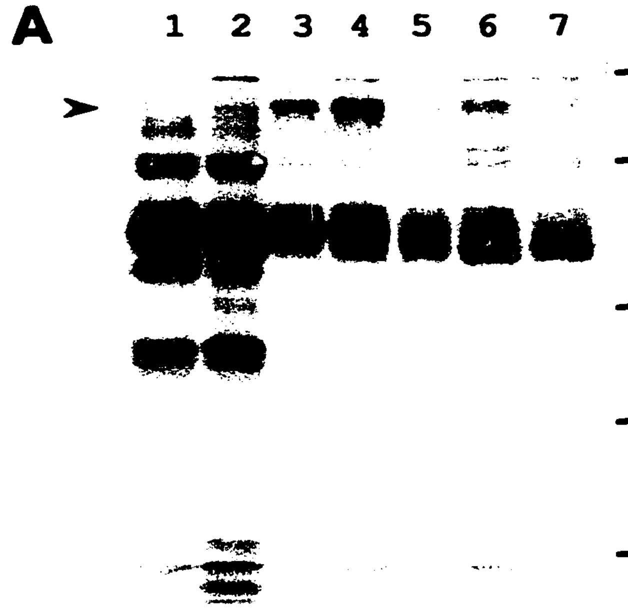

A


B

C

D

Figure II-6. Secretion of SERP1 from infected cells at late times during infection. (A) Secretion of ^{35}S-labelled proteins from myxoma virus infected cells. Cells were pulsed with ^{35}S-Met/Cys for one hour at various times post-infection and the medium harvested one hour later and analysed by electrophoresis in an SDS-polyacrylamide gel; harvest times were: 3,5,7,9.5,11.5,13.75 and 15.5 hrs post-infection, lanes 1-7, respectively (the sample in lane 4 was harvested 1.5 hrs after labelling instead of one hour). The arrow indicates the position of migration of the SERP1 protein, which appears between 5 and 7 hrs post-infection. Dashes indicate the positions of molecular weight markers (66.2, 45.0, 31.0, 21.5, 14.4 kDa). (B) Western blot of the samples shown in (A), using anti-SERP1 antiserum. MW, molecular weight markers (97.4, 66.2, 45.0, 31.0, 21.5, 14.4 kDa).


A
1 2 3 4 5 6 7

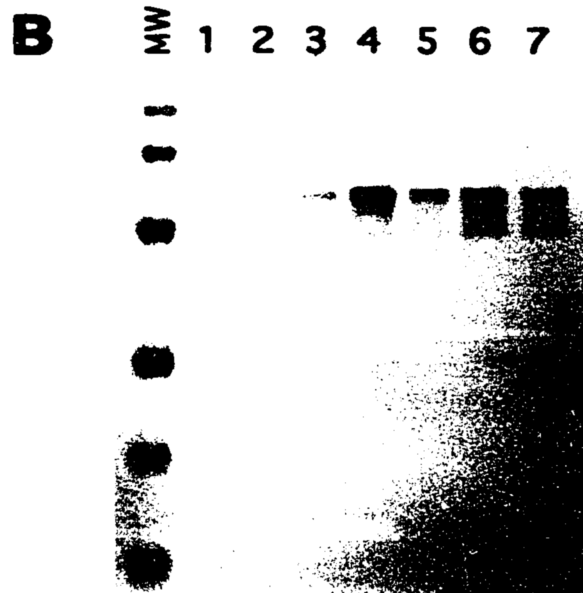

B
MW 1 2 3 4 5 6 7

Figure II-7. Comparison of secreted and intracellular forms of myxoma SERP1. Myxoma virus-infected monolayers were harvested, lysed in SDS gel loading buffer and samples were resolved by electrophoresis on a 6-18% polyacrylamide gradient gel and subjected to Western blotting using anti-SERP1 antiserum. S, protein secreted from myxoma-infected BGMK cells harvested at 16 hrs post-infection; MW, molecular weight markers (see Fig.5 legend); lanes 1-7, lysates of myxoma-infected cells taken at 3,5,7,9.5,11.5,13.75 and 15.5 hrs post-infection; lanes 8-11, lysates of mock, MRV, SFV and MRV-SERP1$^-$-infected cells, respectively, harvested at 12 hrs post-infection.

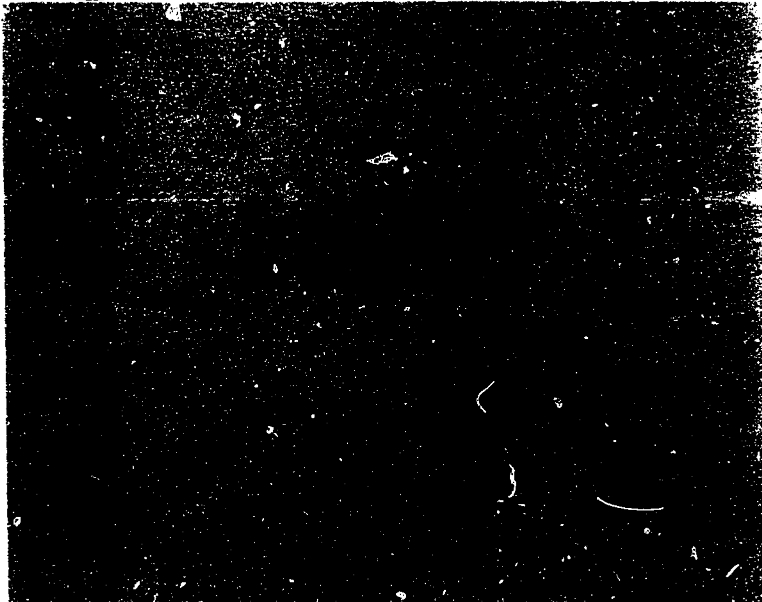
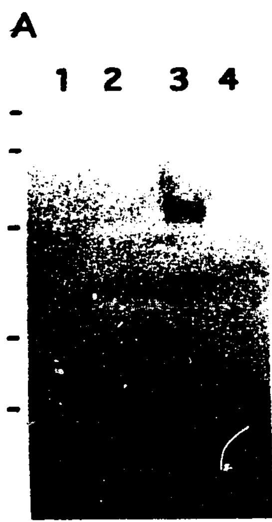

S MW 1 2 3 4 5 6 7 8 9 10 11

Figure II-8. Effect of tunicamycin treatment on the expression of the SERP1 protein. Western blots using anti-SERP1 antiserum of: (A) proteins secreted from cells infected with vMyxlac in the presence of 1 or 5 µg/mL tunicamycin (lanes 1,2), in the absence of tunicamycin (lane 3) or mock infected cells (lane4), or (B) lysates from cells infected in the presence of 0, 1 or 5 µg/mL tunicamycin with vMyxlac (lanes 1,2,3), VV-S1(lanes 4,5,6), or mock infected (lanes 7,8,9). Samples were harvested at 11.5 hours post-infection as described in Materials and Methods. Arrow indcates the position of the 42kDa intracellular SERP1 protein. Dashes indicate the positions of molecular weight markers (see Figure II-6 legend)


A
1 2 3 4

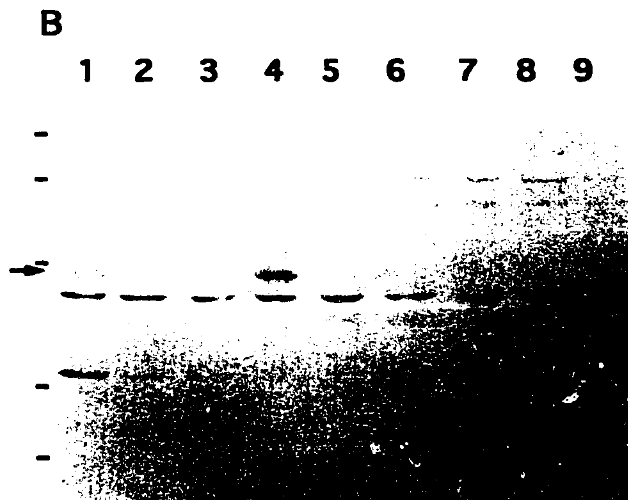

B
1 2 3 4 5 6 7 8 9

Figure II-9. Analysis of N-linked glycosylation of SERP1. Western blots using anti-SERP1 antiserum of protein secreted from VV-S1-infected BGMK cells before (lane3) and after (lane4) digestion with PNGaseF. Lane2, SERP1 expressed from *E. coli*. Lane 1, molecular weight markers (see Figure II-6 legend).

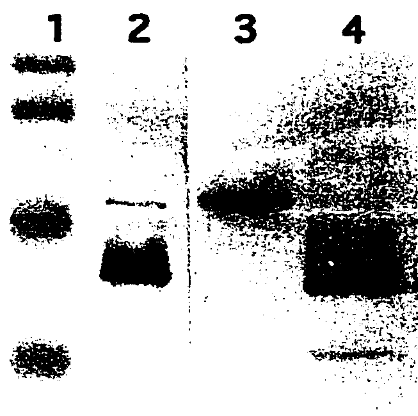

1
2
3
4

FIGURE 11-10. Purification of the SERP1 protein. Silver stained SDS-polyacrylamide gel showing the stages of purification of the SERP1 protein. Lane 1, crude supernatant from VV-S1 infected cells. Lane 2, pooled fractions recovered from anion exchange chomatography on a MonoQ column. Lane 3, SERP1 fraction recovered from gel filtration chromatography on a Superdex 75 column. Dashes indicate molecular weight markers (see Figure II-6 legend).

1 2 3

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Brandes, M. E., Mai, U. E. H., Ohura, K., and Wahl, S. M. (1991). Type I transforming growth factor-ß receptors on neutrophils mediate chemotaxis to transforming growth factor-ß. *J. Immunol.* **147**, 1600-1606.

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CHAPTER III

VIRUS-INDUCED LOSS OF CLASS I MHC ANTIGENS FROM THE SURFACE OF CELLS INFECTED WITH MYXOMA VIRUS AND MALIGNANT RABBIT FIBROMA VIRUS[1]

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[1]All data contained herein pertaining to analysis of class I MHC antigens on BGMK cells has been published: Boshkov, L.K., Macen, J.L. and McFadden, G. *J. Immunol.* **148**: 881-887. 1992

INTRODUCTION

Viral and other intracellular protein antigens are recognized by $CD8^+$ cytotoxic T lymphocytes (CTL) in association with polymorphic cell surface class I glycoproteins encoded within the host major histocompatibility complex (MHC) (Zinkernagel and Doherty, 1979). Class I MHC glycoproteins are heterodimers consisting of a polymorphic membrane spanning 44 kDa heavy chain encoded within the MHC locus noncovalently associated with the nonpolymorphic, non-MHC-encoded 14 kDa light chain, β_2-microglobulin (β_2m) (reviewed in Bjorkman and Parham, 1990). The process whereby antigens associate with MHC proteins for recognition at the cell surface is referred to as antigen presentation. Evidence generated during the late 1980s suggested that endogenously synthesized viral antigens are recognized as short peptide fragments presented to the CTL by class I molecules (reviewed in Yewdell and Bennink, 1990). From these studies, it was predicted that peptides bind to class I proteins, forming a complex that is displayed at the cell surface where it may be recognized by receptors on the surface of CTL. It is now known that peptides are required for the stable association of class I heavy and light chains, and for the transport of the complex to the cell surface (Townsend *et al.*, 1989). Furthermore, inhibitors of the exocytic pathway such as brefeldin A (Nuchern *et al.*, 1989; Yewdell and Bennik, 1989) or viral proteins that bind to class I molecules in the endoplasmic reticulum (Cox *et al.*, 1990) inhibit antigen presentation, indicating that transport of newly synthesized MHC proteins from the ER to the cell surface is required. In addition, it has been demonstrated that antigenic peptides associate with class I proteins within the ER (Cox *et al.*, 1990), and it is the resultant trimolecular complex that is subsequently transported to the cell surface.

An effective antiviral immune response requires the specific recognition and elimination of virally-infected cells by host CTL. Class I MHC proteins bearing antigenic peptides are recognized by the T cell receptor on $CD8^+$ T lymphocytes.

Engagement of the T cell receptor results in the stimulation of cytolytic activity of the T cell, resulting in the destruction of virus-infected cells. In addition to direct cytotoxicity, stimulation of $CD8^+$ T lymphocytes through interaction with antigen bearing class I MHC proteins can also result in the production of cytokines, such as IFNγ, which themselves have anti-viral activities. Therefore the interruption of this critical antigen presentation pathway represents a powerful strategy by which intracellular pathogens might subvert immune recognition, thereby gaining a replicative advantage in the infected host. Indeed, the disruption of normal MHC antigen expression is a strategy that has been adopted by a number of viruses as a mechanism to avoid destruction by the host anti-viral immune response (reviewed in McFadden and Kane, 1994; Rinaldo, 1994).

The poxviruses have evolved multiple strategies for the evasion of the host antiviral immune and inflammatory responses, including the interruption of cytokine responses and the complement cascade, inhibition of inflammation and apoptosis, and modulation of antigen presentation to CTLs (reviewed in Turner and Moyer, 1990; Buller and Palumbo, 1991; McFadden and Graham, 1994; McFadden *et al.*, 1995; Smith, 1993, 1994; Pickup, 1994). Much evidence has accumulated implicating the cellular immune response, and in particular $CD8^+$ T lymphocytes, as critical effectors in the clearance of poxviral infections in the infected host (Buller and Palumbo, 1991). That dysregulation of class I MHC antigens may be a mechanism used by poxviruses to evade recognition by $CD8^+$ T cells is supported by the observation that a number of poxvirus infections result in reduced cell surface expression of class I MHC antigens (Koszinowski and Ertl, 1975; Gardner *et al.*, 1975; Lakdhar and Senik, 1982; Boshkov *et al.*, 1992; McFadden and Kane, 1994).

Myxoma virus is a natural pathogen of rabbits that causes a relatively benign disease in its natural hosts (*Sylvilagus brasiliensis* and *Sylvilagus bachmani*). Infection

of the European rabbit (*Oryctolagus cuniculus*) however, results in a highly lethal generalized infection known as rabbit myxomatosis (Fenner and Ratcliffe, 1965). Infection of the European rabbit with virulent strains of myxoma virus is characterized by virus multiplication and formation of a raised lesion at the site of intradermal inoculation, followed by multiplication in the regional lymph nodes, a cell-associated viremia, generalization in the skin, genital region and internal organs, development of Gram-negative infections of the nasal and conjuctival mucosa and death within fourteen days of inoculation (Fenner and Ratcliffe, 1965; McFadden, 1988, 1994b). The highly virulent nature of this infection is believed to result from the ability of the virus to circumvent numerous aspects of the host anti-viral response(McFadden, 1988, 1994b, 1994a; McFadden and Graham, 1994; McFadden *et al.*, 1995), and may be related to the ability of the virus to replicate in both resting and stimulated lymphocytes (Tompkins *et al.*, 1975; Strayer *et al.*, 1985). In contrast to myxoma virus, infection of the European rabbit with Shope fibroma virus (SFV) a closely related leporipoxvirus results in a benign, self-limiting infection that is cleared by a vigorous cellular immune response (Shope, 1932; Allison, 1966; Allison and Friedman, 1966a; Scott *et al.*, 1981). In immunosupressed (Allison and Friedman, 1966b), newborn (Allison, 1966; Smith *et al.*, 1973; Tompkins *et al.*, 1973), or γ–irradiated (Febvre, 1962) rabbits, SFV induces progressive systemic infections which are often fatal. Presumably, the invasive nature of these infections results from an impaired or underdeveloped cellular immune response, and highlights the importance of cell-mediated immune responses in the limitation of the infection. Nevertheless, relatively little is known at the molecular and cellular levels concerning immune recognition of leporipoxviruses during infection. Studies of the immune response to leporipoxvirus infection have been complicated by the lack of inbred rabbit strains and rabbit cell lines with which immunological investigations could be undertaken.

The studies described here were undertaken to explore the possibility that highly virulent leporipoxviruses, such as myxoma virus and the closely related myxoma virus recombinant designated malignant rabbit fibroma virus (MRV) (Strayer *et al.*, 1983), may evade immune recognition through the dysregulation of class I MHC antigens. Flow cytometry was used to monitor cell surface levels of class I MHC proteins during infection with myxoma virus, MRV, SFV and the orthopoxvirus vaccinia. Because antibodies specific for rabbit MHC proteins were not commercially available until recently, initial studies were conducted using the primate cells line BGMK, which, unlike murine or human cells, is permissive for leporipoxvirus infection. Using monoclonal antibodies specific for human class I MHC proteins, these studies demonstrated that infection with myxoma virus and MRV, but not SFV and vaccinia, resulted in a rapid and extensive loss of cell surface class I MHC proteins (Boshkov *et al.*, 1992). When monoclonal antibodies specific for rabbit immunological markers, including class I MHC, became available, further studies were conducted using the rabbit T cell lymphoma RL-5. These studies confirmed and extended the initial observation that viral infection results in the downregulation of cell surface class I MHC antigens, and suggested that viral downregulation of class I proteins may be the result of disruption of the normal recycling of cell surface proteins.

MATERIALS AND METHODS

Cells and Viruses

The rabbit T cell lymphoma cell line, RL-5, was obtained from the National Institutes of Health: AIDS Reference Reagent Program, and cultured in RPMI 1640 (Gibco BRL Life Technologies, Inc.) supplemented with 10% fetal bovine serum

(FBS; Gibco BRL Life Technologies, Inc.). The primate cell line BGMK (obtained from S. Dales, University of Western Ontario) was maintained in DMEM supplemented with 10% newborn calf serum (Gibco BRL Life Technologies, Inc.). MRV is a recombinant between Shope fibroma virus and an unknown strain of myxoma virus, the structure and source of which has been described (Block *et al.*, 1985; Upton *et al.*, 1988). Shope fibroma virus (SFV; strain Kasza) was obtained from the American Type Culture Collection (ATCC) and engineered to express the *Escherichia coli lac Z* gene under the control of the vaccinia virus p11 promoter, inserted in the intergenic region between the S-T9 and the Shope fibroma growth factor open reading frames (J.L. Macen, M.Schreiber and G.McFadden, unpublished); vMyxlac, a derivative of myxoma virus (strain Lausanne), which expresses the *E. coli lac Z* gene from an intergenic site and has a wild type phenotype, has been described (Opgenorth *et al.*, 1992a). Wild type vaccinia virus (strain WR) was obtained from the ATCC (Rockville MD, USA). VV-601 is a TK^- derivative of vaccinia virus (strain WR) that has been described (Chapter 2, this thesis, and Macen *et al.*, 1993). All viruses were propagated and titred on BGMK cells.

Rabbit spleen cell cultures

All procedures involving animals were carried out under approval of the University of Alberta Animal Welfare Committee, following guidlines provided by the Canadian Council on Animal Care. Adult New Zealand White rabbits were either mock infected or given two intradermal injections of 1 x 10^3 pfu vMyxlac on day 0. On day 7, the rabbits had developed characteristic signs of myxomatosis, and spleen cell cultures were prepared as described (Opgenorth *et al.*, 1992b). Cell viability was monitored by trypan blue exclusion. Cells were either washed immediately and prepared for flow cytometric analysis as described below, or cultured in 100mm plates

at a density of 0.5-1.0 x 10^6 cells/mL in RPMI 1640 containing antibiotics and 10% FBS at 37°C for the indicated times and then prepared for flow cytometric analysis as described below.

Monoclonal antibodies and antisera

The monoclonal antibody (mAb) W6/32 recognizes a monomorphic determinant of the human HLA-A,B,C, H chains in association with β_2m (Parham *et al.*, 1979) and was a gift from Dr. P. Halloran (University of Alberta, Edmonton, Alberta). mAb IOT2, which recognizes a monomorphic determinant of the human HLA-A,B,C H chains in association with β_2m (Malissen *et al.*, 1982); and IOT9, which recognizes the human transferrin receptor (Brown *et al.*, 1981) were obtained from AMAC/Bio/Can (Missisauga, Ontario). Anti-HLA-ABC (H chain) recognizes an SDS-stable framework determinant on the H chain of the class I MHC antigen (Bushkin *et al.*, 1986) and was obtained from Olympus Immunochemicals (Lake Success, NY, USA). mAb MOPC 141 is an isotype control for murine IgG2b that was obtained from the Sigma Chemical Co. (St. Louis, MO, USA). Polyclonal affinity purified FITC-conjugated F(ab')2 goat anti-mouse Ig was obtained from AMAC/Bio/Can. The mAb 9H.1 recognizes human β_2m (George *et al.*, 1986) and was a gift from Dr. A.R.E.Shaw (University of Alberta). Murine monoclonal antibodies specific for rabbit CD18, CD43, CD45, and MHC class I H chain (Wilkinson *et al.*, 1984; De Smet *et al.*, 1983; Galea-Lauri *et al.*, 1993; Wilkinson *et al.*, 1992a; Wilkinson *et al.*, 1992b) were all obtained from Spring Valley Laboratories (Sykesville, MD, USA). Goat polyclonal anti-rabbit β_2m antiserum was a kind gift from Dr. T.J. Kindt (National Institutes of Health, USA). The hybridoma cell line L11/135 (murine anti-rabbit CD43) was obtained from the ATCC; hybridoma supernatants were prepared by standard techniques as described (Harlow and Lane, 1988). R-phycoerythrin (RPE)-conjugated F(ab)2' goat anti-mouse IgG was

obtained from Dako, Dimension Laboratories, and FITC-conjugated streptavidin was obtained from AMAC/Bio/Can.

Detection of infected cells by the FACS-GAL technique

An ImaGene green™ C_2-C_{16} FDG lacZ gene expression sample kit (Molecular Probes, Eugene, OR) containing lipophilic fluorescein digalactoside substrates, C_n-fluorescein-di-ß-D-galactopyranoside (C_nFDG where n = 2,4,8,12, or 16) was used to determine whether cells infected with viruses that express the *E.coli lac Z* gene could be stained and quantitated by flow cytometry. Cells were stained essentially as described (Nolan *et al.*, 1988; Roederer *et al.*, 1991). Cell concentrations were adjusted to 5 x 10^6 cells/mL in Dulbecco's phosphate buffered saline (DPBS) containing 2% FBS and 0.1% sodium azide, pH 7.4. 100µL aliquots were warmed for 5 min. at 37 °C in a water bath. A 20mM stock solution of C_nFDG in dimethly sulfoxide was diluted in DPBS/2%FBS to 100 µM, and warmed for 5 min. at 37 °C. Equal volumes (100 µL each) of prewarmed cell suspension and C_nFDG solution were mixed and incubated at 37 °C for varying time periods (10 min to 2 hours) to determine optimal values for n and incubation times for staining. Following incubation, 1 mM phenylethyl-ß-D-thiogalactopyranoside was added to stop ß-galactosidase activity. The samples were washed in DBPS, stained with antibodies, fixed with 1% paraformaldehyde, and subjected to flow cytometric analysis as described below.

Virus infection of cell lines

Semi-confluent BGMK cells were either mock-infected or adsorbed with virus for 1 hour at 37°C at a multiplicity of infection (MOI) of 10 plaque forming units (pfu) per cell in DMEM supplemented with 10% NBS, fresh medium was added, and the infection was allowed to proceed for the indicated period of time. RL-5 cells were either

mock-infected or adsorbed with virus at 37°C at a MOI of 10 pfu/cell at a density of 2-5 x 10^7 cells/mL in RPMI 1640 supplemented with 10% FBS, the cells were diluted to 5 x 10^5 cells/mL in RPMI 1640/10%FBS and the infection was allowed to proceed at 37°C for the indicated time. Where indicated, the infection was carried out in the presence of cycloheximide (100 µg/mL), cytosine arabinoside (Ara C; 40 µg/mL), or brefeldin A (5 µg/mL) (all obtained from the Sigma Chemical Co.), added immediately following virus adsorption.

Infected or mock-infected BGMK cells were harvested by aspirating the medium and then incubating the cells in 1X SSC (0.15M NaCl, 0.015M sodium citrate) at 37 °C for 15 minutes. RL-5 cells were harvested by centrifugation and any adhering cells were harvested with 1X SSC as described above.

Antibody binding and flow cytometric analysis

BGMK cells: Cells were harvested as described above, washed twice in binding buffer (137 mM NaCl, 12 mM $NaHCO_3$, 2.6 mM KCl, 2 mM $MgCl_2$, 5.6 mM glucose, pH 7.4), and filtered through 52 µm Spectra/Mesh nylon mesh filters (Fisher Scientific, Boston, MA, USA) to remove any cell clumps. Cells were counted in a hemacytometer, and cell viability was determined by trypan blue exclusion.

Antibody binding: 5 x 10^5 cells were incubated with 20 µg/mL mAb in 120 µL. (This concentration of antibody was determined to be in excess of saturating antibody concentrations in preliminary experiments). Samples were incubated on ice for 30 minutes, 10 µL FITC-conjugated goat anti-mouse antibody was added, and the mixture was incubated for a further 20 minutes on ice. In some experiments, 0.2% BSA was added to the incubation; however, it was determined that neither this nor an additional wash step before the addition of the secondary antibody affected specific nor nonspecific antibody binding. Following incubation with antibodies, the cells were

fixed in fresh 2% paraformaldehyde in binding buffer for 15 minutes, and the fixation was quenched by addition of an equal volume of 0.05 M Tris/0.1 M glycine in binding buffer. Neither fixation nor quenching affected the observed levels of antibody binding to uninfected cells during flow cytometric analysis. Following fixation/quenching, the cells were resuspended in 0.4 mL binding buffer and subjected to flow cytometric analysis.

Flow cytometry: Samples were analysed on a FACScan flow cytometer (Becton Dickson Immunocytometry Systems, Mountain View, CA, USA) using a 15 mW argon laser, an excitation wavelenth of 488 nm with a 530 nm bandpass filter. Data was acquired ungated for 10,000 cells per sample, light scatter signals were acquired at linear gain and fluorescence signals at logarithmic gain. Debris of small size was gated out before final analysis of data, with a minimum of 7000 events remaining per sample and the data were analyzed using FACScan software. Results are expressed as histograms of cell number vs log fluorescence intensitiy. Analysis with all viruses and antibodies used were replicated in a minimum of three independent experiments.

RL-5 cells: Cells were harvested as described above, and washed 3 times in phosphate buffered saline (PBS) containing 1% FBS. The cells were counted in a hemacytometer, and cell viability was monitored by trypan blue exclusion.

Antibody binding: Cell concentrations were adjusted to 1.25×10^7 cells/mL in Dulbecco's PBS (DPBS) containing 0.2% (w/v) bovine serum albumin and 0.1% (w/v) sodium azide, pH 7.4 (DPBS binding buffer). All monoclonal antibodies and antiserum was used at concentrations in excess of predetermined saturating concentrations. Two colour staining was performed as follows: 5×10^5 cells were incubated with primary monoclonal antibodies (20-50 μg/mL) for 20 min. on ice, washed with DPBS and resuspended in 100 μL DPBS binding buffer. 5 μL of RPE-conjugated $F(ab)'_2$ goat anti-mouse IgG was added and the cells were incubated for 20

min. on ice. The cells were washed in DPBS and excess mouse IgG (Zymed, Dimension Laboratories, Mississauga, Ontario) was added to block the remaining binding sites (10 min. on ice) and then cells were incubated for 20 min. on ice with 5 μL biotinylated primary antibody (25 μg/mL). The samples were then incubated with FITC-conjugated streptavidin on ice for 20 minutes, washed in DPBS and fixed with 1% (w/v) paraformaldehyde in PBS, washed again and subjected to flow cytometric analysis.

Flow cytometry: Flow cytometric analysis was carried out on a FACScan flow cytometer essentially as described above, with data collected on 10,000 cells/sample, light scatter set at linear gain and flourescence set at logarithmic gain. The optical filter assembly used for these experiments was 530/30 bandpass, 585/42 bandpass and 650 long pass filter. Fluorescence compensation was set to eliminate any spectral overlap of the emitted signal.

RESULTS

Downregulation of cell surface class I MHC antigens on BGMK cells infected with myxoma virus and malignant rabbit fibroma virus

Because myxoma virus and the myxoma virus recombinant termed malignant rabbit fibroma virus (MRV) have long been described as capable of supressing or evading the host anti-viral immune response (Fenner and Ratcliffe, 1965; McFadden, 1988; Strayer, 1989; McFadden, 1994b), it was of interest to examine whether the virus is able to escape immune recognition through the downregulation of cell surface proteins that are essential for the recognition and elimination of virally infected cells. Studies were therefore initiated to examine the effect of virus infection on cell surface

levels of class I MHC proteins. At the time these studies were initiated, reagents specific for rabbit immunological markers were not available, and therefore preliminary studies were conducted using the primate fibroblast cell line BGMK. Although the host range of the leporipoxviruses is generally restricted to cells derived from rabbits and other leporids, and the viruses are not able to productively infect cells of human or murine origin, myxoma virus, MRV and SFV are all able to productively infect BGMK cells (Block *et al.*, 1985). The similarity between primate MHC proteins and those of human origin permitted the use of anti-human monoclonal antibodies for these studies.

Uninfected BGMK cells were found to show strong positive cell surface staining with three different monoclonal antibodies that recognize monomorphic determinants on the heavy chain of the class I MHC proteins HLA-A,B, and C (Figure III-1a-d) as well as a monoclonal antibody specific for human β_2m (Figure III-1d). When the cells were infected for 24 hours with either myxoma virus or MRV, however, there was a dramatic and reproducible decrease in the level of staining, with the mean fluorescence intensity being a full log lower than that obtained for uninfected cells, and nearly decreased to the levels of nonspecific antibody binding (Figure III-1a-d). As indicated in Figure III-1a, the levels of class I MHC proteins at 24 hours post infection were decreased almost to the level of nonspecific antibody binding (Figure III-1a). In all experiments, the results for MRV-infected cells were indistinguishable from those obtained for myxoma virus-infected cells. In order to determine whether this decrease represented an overall perturbation of cell surface glycoproteins, the levels of other cell surface antigens were examined. The data shown in Figure III-1e indicate that the levels of the transferrin receptor, an unrelated cell surface glycoprotein, were unaffected by virus infection. In addition, flourescence due to nonspecific antibody binding fell only very slightly during infection, and did not reflect the observed decreases in specific antibody staining.

Analysis of cells at various times post-infection indicated that the level of class I MHC antigens decreases progressively over time (Figure III-2). Decreased cell surface class I MHC antigen staining was observed as early as 4 hours post-infection, and was maximal by 16 to 24 hours post-infection. It therefore appeared as though the decrease in class I antigen expression was the result of some early event during the infection.

Effect of SFV and vaccinia virus infection on the level of cell surface class I MHC antigens on BGMK cells

Unlike myxoma virus, both SFV and vaccinia virus are cleared from the infected host by an efficient cell-mediated immune response. To determine whether the decrease in class I MHC proteins correlates with pathogenicity, class I levels were also measured on cells infected with SFV and vaccinia virus. Figure III-3 indicates that infection with SFV also results in decreased class I MHC levels, but this effect was demonstrably less dramatic than the decrease observed during infection with myxoma virus, even when the infection was allowed to proceed for longer periods of time, up to 36 hours (data not shown). In addition, infection with vaccinia virus (wild type strain WR) was also found to result in a decrease in class I levels comparable to that induced by SFV (Figure III-3). Since SFV and vaccinia virus are both cleared in infected animals by a cellular immune response, there is a correlation between pathogenicity and downregulation of cell surface class I MHC proteins. However, whether the observed decrease in class I levels results in impaired recognition of myxoma virus infected cells by $CD8^+$ T lymphocytes awaits further experimentation.

Requirement for protein synthesis and viral gene expression for downregulation of class I MHC antigens on BGMK cells

The following experiments were performed in order to determine whether decreased MHC antigens levels could be explained by a virus-mediated shutdown in cellular protein synthesis and whether viral gene expression was required. Class I MHC levels were monitored on BGMK cells infected in the presence of either the protein synthesis inhibitor cycloheximide, or the inhibitor of virus DNA replication and therefore of late viral gene expression, cytosine arabinoside (AraC). BGMK cells were incubated with 100μg/mL cycloheximide for 24 hours and the cell surface levels of class I proteins were determined. The data shown in Figure III-4 indicate that inhibition of protein synthesis in uninfected cells results in a moderate decrease in class I MHC antigen levels that is less dramatic than that observed during infection with myxoma virus, and comparable to that observed during infection with vaccinia virus (Figure III-3,4). This result may reflect the turnover of a portion of class I proteins from the cell surface, but also suggests that the class I proteins are relatively stable during the time course in which the experiments were conducted, since a proportion still remain at the cell surface. Although the half life of MHC proteins in BGMK cells was not determined, the result suggests that the decrease in class I MHC antigen levels observed during myxoma virus infection can not be explained by viral shutdown of host cell protein synthesis and turnover of class I proteins. A viable explanation that remains to be tested experimentally is that viral infection interferes with the recycling of class I antigens from the cell surface or the transport of class I antigens from the ER/Golgi to the cell surface.

In order to further examine the role of protein synthesis and viral gene expression in the downregulation of class I MHC antigen levels, BGMK cells were infected with myxoma virus or MRV in the presece of either cycloheximide, to inhibit

all protein synthesis, or AraC, to inhibit viral late gene expression. Again, the results obtained with myxoma virus were indistinguishable from those obtained with MRV, and only the results with MRV are shown here. If no new protein synthesis was required for virus-mediated loss of class I MHC antigens, then infection in the presence or absence of cycloheximide should not differ with respect to MHC antigen levels. The results shown in Figure III-5 indicate that in the presence of cycloheximide class I MHC protein levels do not drop beyond that observed when uninfected cells are treated with cycloheximide alone for the same time period. This result can be interpreted in two ways: either viral gene expression and protein synthesis are required in order for downregulation of class I proteins to occur, or alternatively, active cellular protein synthesis is required to mediate the effect. These possibilities cannot be distinguished on the basis of these data alone.

Treatment of uninfected BGMK cells with AraC (40 µg/mL) for up to 24 hours did not result in any decrease in the levels of class I MHC antigens on the cell surface (data not shown). If late viral protein synthesis is required to mediate the downregulation of class I antigens, then infection in the presence of AraC should abrogate the observed decrease. If only early viral gene expression is required, then infection in the presence or absence of AraC should be indistinguishable. The data presented in Figure III-5 indicated that when cells were infected in the presence of AraC, there was a moderate decrease in MHC antigen levels. However, even over longer incubation times (not shown), cells infected in the presence of AraC never lost class I antigens to the extent observed when cells were infected with myxoma virus in the absence of the drug. This result suggests that the downregulation of class I MHC antigens from the surface of cells infected with myxoma virus and MRV involves both early and late gene expression, since some downregulation occurs in the absence of late gene expression, but complete downregulation does not.

Downregulation of class I MHC on infected BGMK cells is not due to variations in infected cell viability

Poxvirus infection is accompanied by characteristic cytopathic effects and can result in decreases in cell viability. The integrity of infected cells was monitored by trypan blue exclusion prior to antibody staining. The percentage of cells excluding trypan blue was in excess of 90% for uninfected BGMK cells, 50-70% for cells infected with myxoma virus or MRV for 24 hours, 60-75% for cells infected with SFV for 24 hours, and 30-50% for cells infected with vaccinia virus for 24 hours. However, a unimodal distribution of staining with all anti-class I MHC mAbs used was obtained, indicating that decreased class I expression was occurring on the infected cells regardless of their ability to exclude typan blue. When cells were infected at a low MOI, a bimodal distribution of cells with high and low staining for class I MHC was observed, reflecting the proportion of infected cells (data not shown). Therefore, the decreased class I MHC detected on infected cells is not a secondary effect of a loss in cell viability during infection.

Downregulation of cell surface class I MHC antigens during myxoma virus infection of rabbit lymphocytes

Because myxoma virus is a natural pathogen of rabbits, it was important to determine whether viral infection resulted in the downregulation of cell surface class I proteins on cells of rabbit origin. Therefore, when monoclonal antibodies to rabbit immunological markers became available, studies were initiated to examine the effect of virus infection on a number of rabbit cell surface proteins of immunological relevance, including class I MHC. Since myxoma virus is able to infect lymphocytes, and circulating infected lymphocytes are thought to enable the virus to spread within the

infected animal (McFadden, 1988, 1994b), it is of interest to ask whether virus infection results in altered cell surface class I MHC expression on rabbit lymphocytes. In addition, an important question to address is whether the downregulation phenomenon is relevant to myxoma virus infection of the rabbit.

i. Detection of myxoma virus infected lymphocytes by the FACS-GAL technique and analysis of rabbit splenocytes

The following experiments were performed in an attempt to determine whether the levels of class I MHC proteins were altered on rabbit lymphocytes taken from myxoma virus-infected rabbits. Because significant virus titres can be obtained from spleens harvested from myxoma virus infected rabbits at approximately seven days post-infection (for example, Opgenorth *et al.*, 1992b), rabbit splenocytes harvested from infected rabbits at 7 days post-infection were analysed for both virus infection and class I MHC antigen levels.

A sensitive method to detect myxoma virus-infected cells was required for these studies. Using the recombinant myxoma virus, vMyxlac, which expresses the *E.coli lacZ* gene from a non-disruptive intergenic site (Opgenorth *et al.*, 1992a), a sensitive fluorogenic method for detecting ß-galactosidase activity in intact cells (Nolan *et al.*, 1988; Roederer *et al.*, 1991) was adapted for detection of infected cells. In preliminary experiments, the rabbit T lymphoma cell line RL-5 was infected with vMyxlac at a MOI of 10 pfu/cell, and the infected cells were loaded with lipophilic fluorescein digalactoside substrates, C_n-fluorescein-di-ß-D-galactopyranoside (C_nFDG where n = 2,4,8,12, or 16). If cells expressing ß-galacosidase are able to take up the C_nFDG, enzymatic cleavage of the substrate liberates fluorescein, which can be detected by flow cytometry. Preliminary assays (data not shown) indicated that the C_{12}FDG substrate was optimally taken up by the cells and gave a strong positive flourescence when used

to stain vMyxlac-infected cells but not mock-infected cells. Thus, subsequent studies were performed using the $C_{12}FDG$ substrate.

Dual staining of RL-5 cells either mock-infected or infected at 10 pfu/cell for 24 hours with vMyxlac using $C_{12}FDG$ and an anti-rabbit class I MHC mAb is illustrated in Figure III-6. Whereas mock-infected RL-5 cells stained brightly for MHC I, no fluorescence due to flourescein is detectable in these cells (Figure III-6a), indicating that the $C_{12}FDG$ substrate is not cleaved in uninfected cells. When cells were infected with vMyxlac, however, there was a significant positive increase in fluorescence intensity (Figure III-6b) indicating that cleavage of the substrate had occurred, and therefore that infected cells could be readily detected using this methodology. In addition, there was a moderate decrease in the mean fluorescence intensity corresponding to staining for class I MHC antigens suggesting that infection resulted in reduced class I levels. In subsequent experiments (see below) decreases in class I MHC antigen levels on RL-5 cells were analysed in more detail. Finally, in dilution experiments, in which known proportions of infected and uninfected RL-5 cells were mixed and then stained using $C_{12}FDG$, it was determined that the percentage of positively staining cells gave a reasonable estimate of the percentage of infected cells (data not shown).

Preliminary experiments were then performed in order to determine whether splenocytes from myxoma virus infected rabbits contained detectable virus-infected cells, and whether altered levels of class I MHC antigens could be observed. Spleens were harvested from a total of four rabbits either mock-infected or infected with vMyxlac for 7 days, and splenocytes were subjected to dual staining with the anti-rabbit class I MHC mAb and $C_{12}FDG$. Representative data given in Table III-1 indicates that class I MHC and CD45 were readily detectable on splenocytes. However, although the animals showed characteristic signs of myxomatosis and virus spread, no infected cells were detectable using this method. When the splenocytes were incubated in culture for

1 to 3 days, which results in increased virus titres (Opgenorth *et al.*, 1992b), subsequent staining was still unable to detect virus-infected cells (Table III-1). It therefore seemed likely that the percentage of total splenocytes harbouring virus was too low to be detected, and thus this approach was impractical.

ii. Downregulation of cell surface class I MHC antigens on RL-5 cells infected with myxoma virus

Experiments were then undertaken to determine whether infection of rabbit cell lines with myxoma virus resulted in altered cell surface expression of class I MHC proteins. RL-5 cells, a rabbit $CD4^+$ T lymphoma cell line and RK-13 cells, a rabbit kidney cell line with similar characteristics to BGMK cells, were chosen for these studies. Unfortunately, no useful data could be obtained for RK-13 cells, which showed high levels of autofluorescence during flow cytometric analysis (data not shown). RL-5 cells, however, showed strong positive cell surface staining with a monoclonal antibody directed to rabbit class I MHC proteins, with low background fluorescence. Infection of RL-5 cells with myxoma virus resulted in a reproducible decrease in the levels of class I MHC antigens (Figure III-7), similar to that observed on infected BGMK cells. As seen on BGMK cells, infection of RL-5 cells with myxoma virus resulted in a progressive decrease in cell surface staining with a monoclonal anti-rabbit class I antibody that was detectable as early as 4 hours post-infection and maximal by 24 hours post-infection (Figure III-7). Similarly, flow cytometric analysis using a polyclonal anti-rabbit β_2m antiserum indicated a similar decrease (Figure III-8), suggesting that the observed decrease in class I antigen levels is not the result of the masking of an epitope at the cell surface. Again, cell integrity was monitored by trypan blue exclusion and usually to exceeded 90% for RL-5 cells infected with myxoma virus for 24 hours, suggesting that the results are not a secondary effect of a loss in cell viability during infection.

In addition to class I MHC antigens, the levels of a number of other RL-5 cell surface proteins were monitored by flow cytometry. Data shown in Figure III-9 indicates that the cell surface levels of CD18, CD43, and CD45 are not altered on infected RL-5 cells. Therefore, the downregulation of class I MHC proteins on RL-5 cells is not the result of an overall perturbation of cell surface glycoproteins. Similar results have been obtained when myxoma virus infected RL-5 cells are analysed for cell surface CD4 antigens (Barry *et al.*, 1995), suggesting that virus infection may result in a selective decrease in certain cell surface proteins.

Infection of RL-5 cells with Shope fibroma virus and vaccinia virus results in a comparable loss of cell surface class I MHC antigens

Cell surface levels of class I MHC antigens were then determined on RL-5 cells infected with either vaccinia virus (VV601) or SFV. In contrast to the results obtained using BGMK cells (Figure III-3), infection of RL-5 cells with either SFV or vaccinia resulted in a comparable loss in cell surface staining for class I MHC antigens to that observed during infection with myxoma virus (Figure III-10). An interesting difference between myxoma virus and SFV infected RL-5 cells was observed during these studies: while typically 90% of myxoma virus infected cells excluded trypan blue at 24 hours post-infection, SFV-infected cells were largely non-viable, with only approximately 30% of cells able to exclude trypan blue. Vaccinia virus infected RL-5 cells also showed decreased membrane integrity as compared to myxoma virus infected cells, but the loss in integrity was not as dramatic as that observed during infection with SFV. This loss in viability is also reflected in the staining profiles obtained during flow cytometric analysis, with SFV and vaccinia infected cells having lower peak heights corresponding to the decreased number of viable cells. In addition, flow cytometric analysis of RL-5 cells infected with SFV indicated a distinct population of cells with

decreased cell size, as indicated by altered light scattering properties of the cells. When this phenomenon was further analyzed, it was discovered that the cells infected with SFV undergo apoptotic cell death. This phenomenon is examined and discussed in greater detail in Chapter IV. This result makes it difficult to rigorously compare class I MHC regulation in myxoma-infected cells with that in cells infected with SFV and vaccinia virus.

Requirement for protein synthesis and viral gene expression for downregulation of class I MHC antigens on RL-5 cells

Similar experiments to those performed with BGMK cells were then undertaken to examine the requirements for protein synthesis and viral gene expression for downregulation of class I MHC antigens on RL-5 cells. In contrast to BGMK cells, when RL-5 cells are treated with the protein synthesis inhibitor cycloheximide for 24 hours, the levels of cell surface class I proteins decrease to a comparable extent to that observed during myxoma virus infection (Figure III-11). In addition, as seen during infection of RL-5 cells with SFV and vaccinia virus, treatment of uninfected RL-5 cells with cycloheximide resulted in a loss in cell viability that other studies (Chapter 4) have shown to be at least in part due to the induction of apoptosis. Furthermore, when RL-5 cells were infected with myxoma virus in the presence of cycloheximide, there was a striking increase in the proportion of cells undergoing apoptosis (see Chapter 4 for a more detailed discussion).

Experiments performed using the viral DNA replication inhibitor AraC gave similar results to those obtained for BGMK cells: the levels of class I MHC antigens on uninfected cells were unaltered, while infection of RL-5 cells in the presence of AraC resulted in a decrease in cell surface class I MHC antigens that is less extensive than that observed during infection with myxoma virus in the absence of the drug (Figure

III-12). This results again suggests that the downregulation of cell surface class I MHC antigens involves late viral gene expression. However, further experimentation is required to determine whether class I MHC antigens are specifically targetted for downregulation during infection or whether the data reflects a non-specific effect of viral infection on a particular class of membrane proteins.

Effect of brefeldin A on RL-5 cell surface class I MHC antigens.

In order to determine whether inhibition of the exocytic pathway would result in a loss of class I MHC proteins from the cell surface, RL-5 cells were treated with the drug brefeldin A, a fungal metabolite that inhibits the egress of proteins from the endoplasmic reticulum (ER) and causes redistribution of proteins from the Golgi to the ER, thereby blocking the transport of proteins to the cell surface (Doms *et al.*, 1989; Lippencott-Schwartz *et al.*, 1989). Figure III-13 illustrates that there is a progressive loss in cell surface staining for class I MHC proteins when the cells are incubated with brefeldin A, such that by 24 hours there is a similar decrease in MHC class I levels to that which occurs during infection with myxoma virus (compare Figure III-7). Comparable results were obtained in another study in which the levels of CD4 on RL-5 cells were monitored (Barry *et al.*, 1995). This suggests that there is some turnover of class I MHC proteins from the surface of RL-5 cells and that when newly synthesized class I (and CD4) proteins are unable to be transported to the cell surface, the cell surface levels progressively drop. In contrast, when RL-5 cells were treated with brefeldin A for 24 hours and then stained for CD18, CD43 or CD45, no differences were observed in the levels of staining (Figure III-14), indicating that these proteins are stable and do not turn over from the cell surface during the time course of these experiments. Taken together, the results suggest that virus infection specifically targets

proteins that are turned over from the plasma membrane, resulting in decreased cell surface levels.

DISCUSSION

The data presented here demonstrate that infection with myoma virus results in a rapid and significant decrease in the normal expression of cell surface class I MHC antigens on cells of both lymphoid (RL-5) and non-lymphoid (BGMK) origin. Many viruses have adopted strategies for evasion of immune recognition that involve the downregulation of cell surface class I MHC proteins (for recent reviews, see McFadden and Kane, 1994; Rinaldo, 1994). This appears to be a particularly common feature among large DNA viruses, and the variety of mechanisms used to modulate class I expression are a testament to the importance of class I MHC antigen presentation in host defense against intracellular pathogens. For example, adenoviruses can downregulate class I MHC proteins both by interfering with the transcription of class I MHC genes, in the case of adenovirus 12 (Schrier *et al.*, 1983; Eager *et al.*, 1989; Shemesh *et al.*, 1991), as well by interfering with the transport of class I proteins to the cell surface by virtue of the E3 19K protein, expressed by adenoviruses 2-5,9,11,19 and 34, that functions to bind and anchor class I proteins in the ER (Kvist *et al.*, 1978; Burgert and Kvist, 1985; Andersson *et al.*, 1985; Paabo *et al.*, 1986; Burgert *et al.*, 1987; Korner and Burgert, 1994; Wold *et al.*, 1994). Infection with several Herpesviruses also results in decreased class I surface expression. Herpes simplex virus can downregulate class I surface levels (Jennings *et al.*, 1985; Hill *et al.*, 1995; Fruh *et al.*, 1995) as do both human (Browne *et al.*, 1990; Barnes and Grundy, 1992; Beersma *et al.*, 1993; Yamashita *et al.*, 1993) and murine (Del Val *et al.*, 1992;

Campbell and Slater, 1994) cytomegaloviruses, the latter two interfering with class I expression by causing degradation of class I proteins in the ER (Yamashita and Shimokata, 1994) or by reducing class I synthesis and posttranslational events (Campbell and Slater, 1994).

Evidence for poxvirus-mediated downregulation of class I MHC proteins remains limited. Several early studies demonstrated a reduction in cell surface class I expression during infection of L929 cells with vaccinia virus (Koszinowski and Ertl, 1975; Lakdhar and Senik, 1982). Significantly, the observed reduction in the expression of H-2^k class I antigens correlated with reduced lysis of infected H-2^k target cells by CTL (Koszinowski and Ertl, 1975). A similar study indicated reduced cell-surface class I expression during infection with ectromelia virus (Gardner *et al.*, 1975). More recently, decreased levels of H-$2K^k$ and D^k class I levels have been demonstrated to correlate with a loss in susceptibility of vaccinia virus-infected cells to cytolysis by allospecific CTL, with a concommitant increase in sensitivity to lysis by natural killer (NK) cells (Brutkiewicz *et al.*, 1992). Interestingly, the decreased susceptibility to lysis by CTL was limited to allospecific effectors and did not extend to CTL-mediated cytolysis by virus-specific CTL, suggesting that CTL recognition of infected cells may not reflect only overall levels of class I molecules but also the origin of peptides (viral or cellular) with which they are associated (Brutkiewicz *et al.*, 1992). Nevertheless, poxvirus-specific $CD8^+$ T cell populations have been shown to be responsible for reductions in virus titres in internal organs (for example, Blanden, 1977; Zinkernagel and Althage, 1977), which may in part be mediated by the production of antiviral cytokines such as interferon gamma (Kohonen-Corish *et al.*, 1990; Ruby and Ramshaw, 1991). Therefore the interpretation of the potential impact of downregulation of class I antigens remains complicated, especially in view of the

fact that virus-mediated reductions in class I MHC proteins are often accompanied by altered sensitivity to NK cells (Brutkiewicz and Welsh, 1995).

Virulent strains of myxoma virus cause a highly invasive, rapidly fatal infection in the European rabbit. This disease is characterized by the rapid dissemination of the infection from the primary inoculation site to distal secondary sites, and is thought to involve depressed cellular immune functions (Fenner and Ratcliffe, 1965; McFadden, 1988; Strayer, 1989). The highly virulent and invasive nature of the infection suggests that myxoma virus may have evolved mechanisms to circumvent the anti-viral host response and avoid immune surveillance. Indeed, myxoma virus encodes a number of soluble proteins that function to interfere with inflammation and cytokine responses, each of which have been demonstrated to contribute to viral virulence (reviewed in McFadden and Graham, 1994; McFadden *et al.*, 1995). Nevertheless, evidence for altered cellular immune responses to myxoma virus infected cells remains circumstantial, and little is known concerning the recognition of virally infected cells during infection *in vivo*. The restricted host range of myxoma virus coupled with the relative lack of information and reagents which would facilitate immunological experimentation in the rabbit present formidable obstacles to the study of the cellular immune response to myxoma virus infection.

The studies described in this chapter were undertaken in an attempt to ascertain whether myxoma virus infection results in altered class I MHC antigen expression, and therefore whether impaired class I antigen presentation represents a potential mechanism for evasion of the host response to infection. The analysis of class I MHC antigens on both BGMK cells and RL-5 cells indicated that myxoma virus infection indeed results in a dramatic reduction in cell surface class I antigen expression. Although the mechanism by which this downregulation occurs is unclear, the extent of downregulation, at least on BGMK cells, appears to exceed that which results from the

inhibition of synthesis of new class I proteins. Furthermore, infection of both BGMK cells and RL-5 cells with myxoma virus in the presence of AraC indicates that late gene expression plays a role in the observed downregulation. Whether class I MHC proteins are specifically targetted for downregulation or whether the loss of cell surface class I proteins represents a non-specific effect of virus infection on particular classes of cell surface proteins remains to be determined. Brefeldin A, an inhibitor of the exocytic pathway, caused a similar progressive loss of class I MHC proteins from the surface of RL-5 cells, but not CD18, CD43, or CD45, suggesting a correlation between downregulation during virus infection and protein turnover from the cell membrane. Whether myxoma virus indeed interferes with normal transport of class I proteins from the ER/Golgi to the cell surface and/or recycling of class I proteins awaits further experimentation.

The most important question that remains to be addressed is whether the observed decrease in class I MHC antigens during myxoma virus infection results in altered recognition of virally infected cells by cytolytic effectors including both $CD8^+$ CTL and NK cells. Although the results presented here indicate an extensive drop in class I antigen levels during infection, it is difficult to predict the physiological impact of such a reduction. One study suggested that as few as 200 class I complexes per cell were sufficient for sensitization of EL4 cells to lysis by CTL (Christinck *et al.*, 1991). Therefore the absolute level of class I proteins on the cell surface is likely to be of less importance than the peptide content of the class I complexes available for recognition. Thus the balance between virus and cell derived peptides displayed on the cell surface will be an important factor in determining the fate of the infected cell. A model in which myxoma virus infection results in a rapid inhibition of class I MHC transport to the cell surface would predict that viral peptides generated during infection that associate with

new or recycled class I proteins would not be available for recognition at the cell surface. Again, such a model awaits experimental verification.

Both SFV and vaccinia virus infection of BGMK cells resulted in moderate decreases in cell surface class I MHC antigens that were less extensive than that observed during myxoma virus infection. Measurement of class I MHC antigens on RL-5 cells was complicated by the observation that SFV infection resulted in the rapid induction of apoptotic cell death (see Chapter 4), and therefore this data is difficult to interpret. It is tempting to draw a correlation between the degree of class I MHC downregulation and viral virulence in the infected host. Thus the more virulent myxoma virus causes a more extensive loss of class I antigens from the cell surface. In contrast to SFV, myxoma virus can productively infect lymphoid cells (Strayer *et al.*, 1985; Tompkins *et al.*, 1975), and virus spread within the infected animal has been hypothesized to occur in part via infected lymphocytes (McFadden, 1988). Although experimental evidence in support of this idea is lacking, the ability of myxoma virus to downregulate class I MHC antigens on T lymphocytes makes this model even more attractive, since viral dissemination via infected lymphocytes would be facilitated by the disruption of normal immune recognition.

In summary, myxoma virus infection of both lymphoid and non-lymphoid cells resulted in a rapid and dramatic decrease in cell surface class I MHC proteins. Although the mechanism by which viral infection leads to downregulation of class I proteins remains to be determined, these studies indicate that the modulation of class I antigen presentation may represent yet another important viral strategy for the subversion of the host response to infection, and suggest that further experimentation directed to understanding immune recognition of myxoma virus infected cells is warranted.

TABLE III-1

Dual staining for viral infection and cell surface antigens on splenocytes from myxoma virus infected rabbits

			% Positive[3]		Mean Fluorescence[4]	
Infection	**Marker[1]**	**Day[2]**	**FL1**	**FL2**	**FL1**	**FL2**
Mock	C_{12}FDG+ MHC I	0	0	76	165	631
		1	0	98	129	592
		2	0	95	123	539
		3	0	97	129	573
	C_{12}FDG+ CD45	0	0	91	178	714
		1	0	100	133	647
		2	0	100	134	635
		3	0	100	134	618
vMYXLAC	C_{12}FDG+ MHC I	0	0	99	124	600
		1	0	100	114	631
		2	0	99	102	556
		3	0	100	104	551
	C_{12}FDG+ CD45	0	0	100	134	669
		1	0	100	107	606
		2	0	100	109	598
		3	0	100	113	615

[1]Splenocytes were dual stained with C_{12}FDG to stain virus-infected cells and mAb specific for either rabbit class I MHC or rabbit CD45, as described in Materials and Methods.

[2]Splenocytes were stained immediately following harvest from infected animals (Day 0) or cultured in RPMI 1640 containing antibiotics and 10% FBS at 37°C for 1 to 3 days prior to staining.

[3]Percentage of cells that fluoresce above the level of non-specific antibody binding. FL1 indicates percentage of cells fluorescing due to staining with C_{12}FDG and FL2 indicates percentage of cells fluorescing due to staining with either anti-MHC I or anti-CD45 mAbs.

[4]Mean fluorescence intensity in arbitrary units for staining with C_{12}FDG (FL1) or either anti-MHC I or anti-CD45 mAbs (FL2).

Figure III-1. Flow cytometric analysis of cell surface antigens on BGMK cells infected with myxoma virus and MRV for 24 hours. Data are shown as plots of frequency (cell number) vs. log fluorescence intensity. *A*, Mock-, myxoma (MYX)- and MRV-infected BGMK cells stained with the anti-HLA mAb W6/32. For comparison, staining with the nonspecific control mAb, *MOPC* 141 on myxoma virus-infected BGMK cells is shown (NSB). *B*, Mock- and myxoma-infected BGMK cells stained with the anti-HLA mAb IOT2. *C*, Mock- and myxoma-infected BGMK cells stained with the mAb designated anti-HLA. *D*, Mock- and myxoma-infected BGMK cells stained with the anti-$ß_2$m mAb 9H.1. *E*, Mock- and myxoma-infected BGMK cells stained with the anti-trasferrin receptor mAb IOT9.

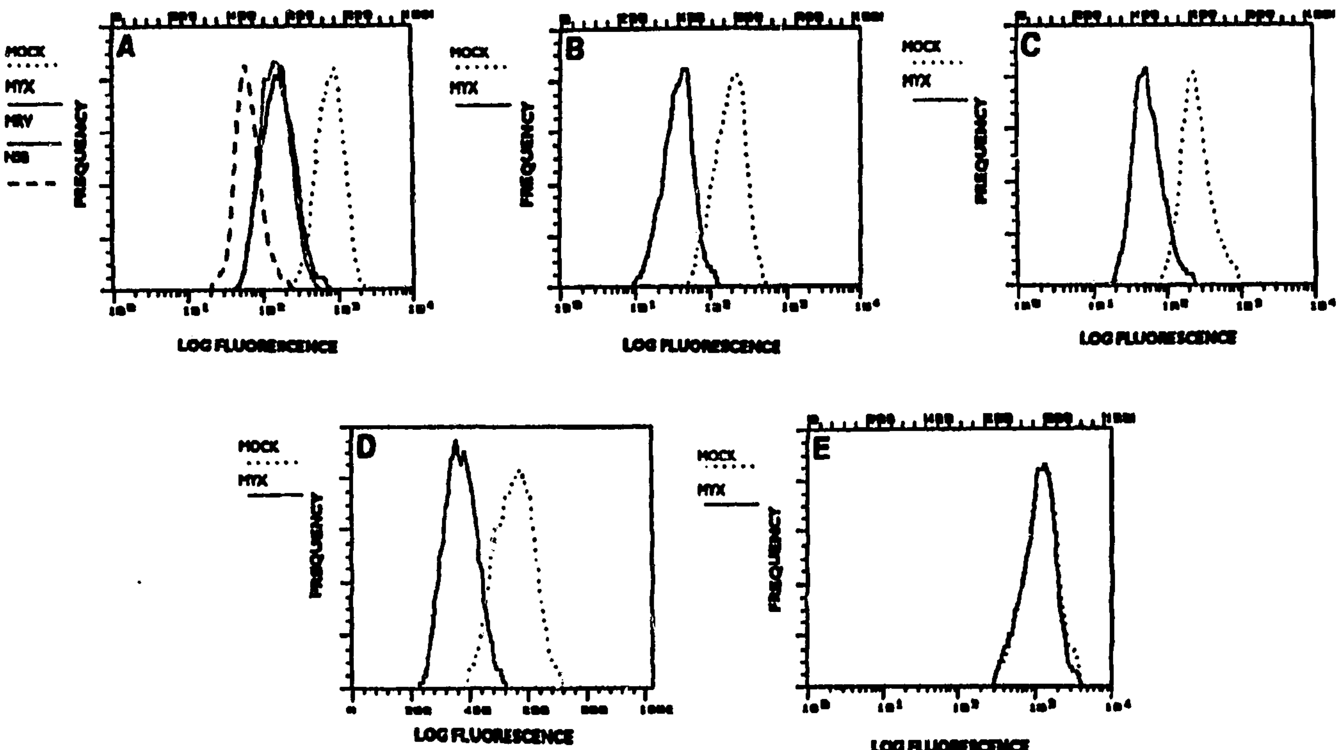

FIGURE III-1

Figure III-2. Flow cytometric analysis of cell surface class I MHC antigens on MRV-infected BGMK cells. Cells were either mock-infected or infected with MRV for 4, 12, and 24 hours. Cells were stained using the anti-HLA mAb W6/32.

Figure III-3. Effect of SFV and vaccinia virus infection on cell surface levels of class I MHC antigens on BGMK cells. Flow cytometric analysis of cell surface class I MHC antigens on BGMK cells mock-infected or infected with myxoma virus (MYX), Shope fibroma virus (SFV), or vaccinia virus (VV) at a MOI of 10pfu/cell. Cells were stained with mAb W6/32 24 hours post-infection and analysed by flow cytometry.

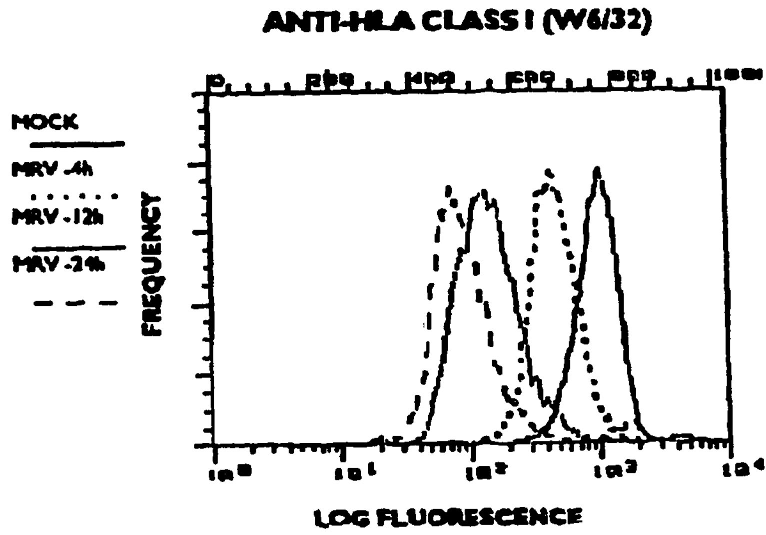

FIGURE III-2

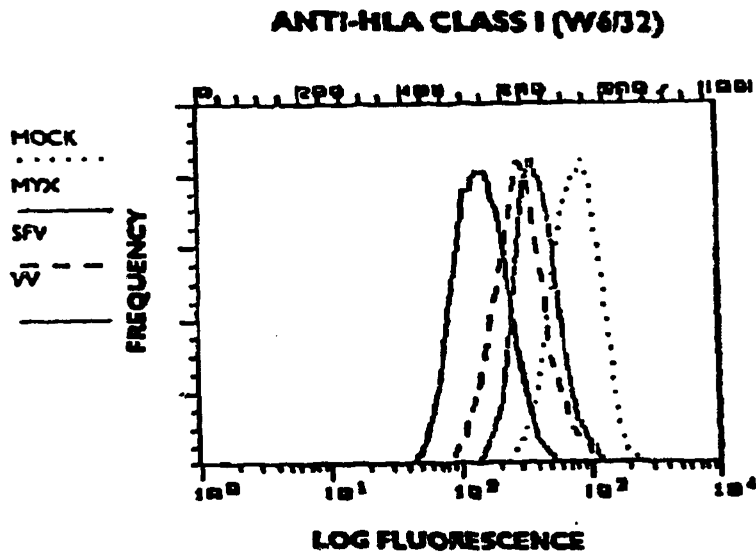

FIGURE III-3

Figure III-4. Effect of cycloheximide on cell surface class I MHC antigens on BGMK cells. Cells were either mock-infected (MOCK), infected with 10pfu/cell myxoma virus (MYX) or 10pfu/cell vaccinia virus (VV) for 24 hours, or treated with the protein synthesis inhibitor cycloheximide for 24 hours (Chx), stained with the anti-HLA mAb W6/32 and analysed by flow cytometry.

Figure III-5. Effect of AraC on viral downregulation of class I MHC antigens on BGMK cells. BGMK cells were either mock-infected or infected with MRV for 24 hours in the presence of cytosine arabinoside (Ara-C) or Cycloheximide (Chx), stained with the anti-HLA mAb W6/32 and analysed by flow cytometry.

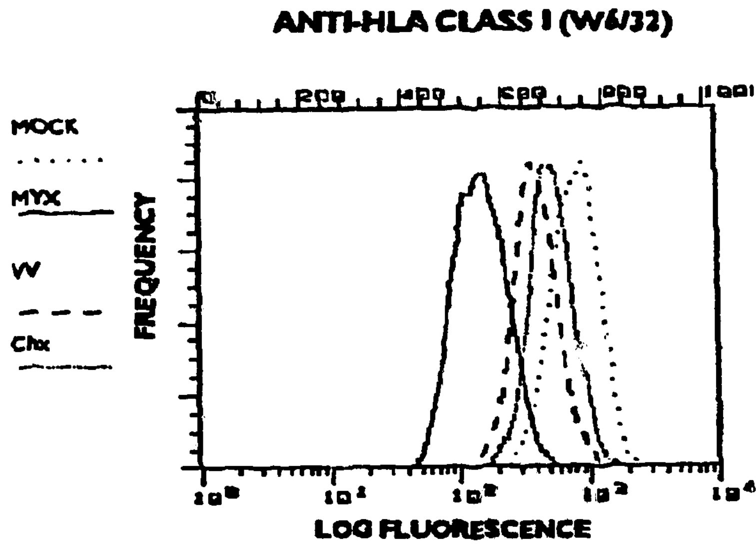

FIGURE III-4

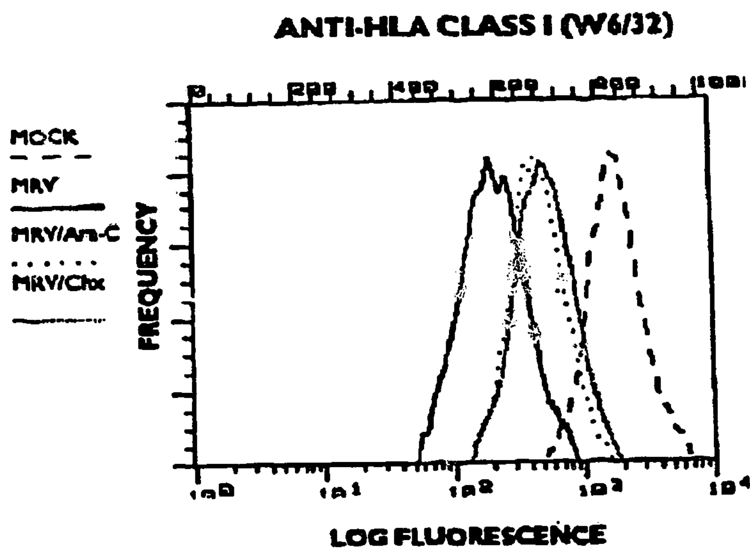

FIGURE III-5

Figure III-6. Simultaneous detection of virally infected cells and cell surface class I MHC antigens by flow cytometry. RL-5 cells were either mock-infected (panel A) or infected with vMyxlac for 24 hours (panel B), and stained with $C_{12}FDG$ followed by anti-rabbit class I MHC mAb. Dot plots of FL2 (corresponding to staining of class I MHC antigens using a phycoerythrin-conjugated secondary antibody) vs. FL1 (corresponding to fluorescein generated by cleavage of $C_{12}FDG$) are shown.

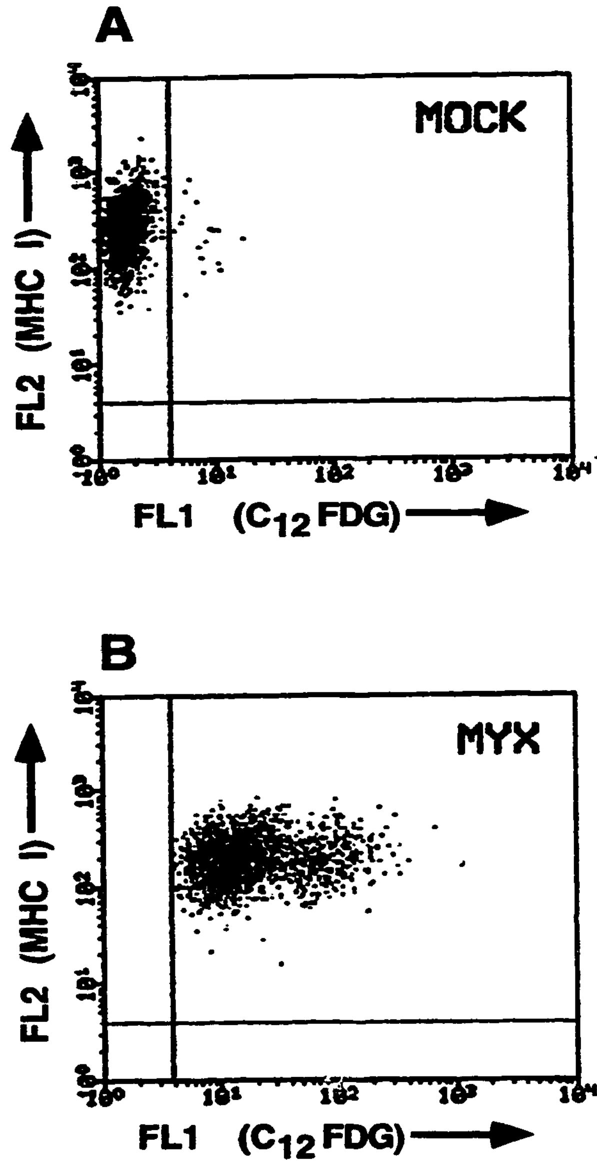

FIGURE III-6

Figure III-7. Flow cytometric analysis of cell surface class I MHC antigens on RL-5 cells. Cells were either mock-infected (open histograms) or infected with myxoma virus (vMyxlac) (shaded histograms). Cells were harvested and stained at *A*, 4 hours, *B*, 12 hours or *C*, 24 hours post-infection with anti-rabbit class I MHC mAb.

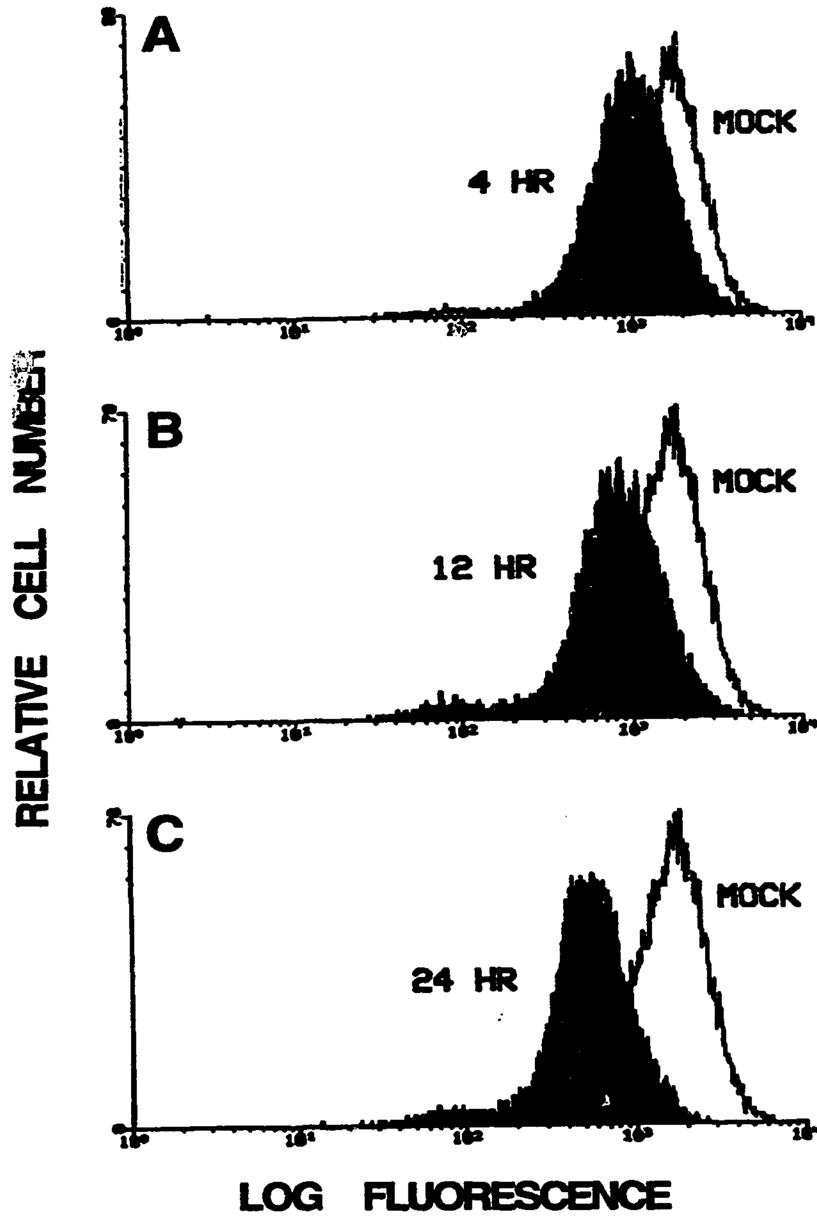

FIGURE III-7

Figure III-8. Flow cytometric analysis of cell surface ß2-microglobulin on RL-5 cells. Cells were either mock infected or infected with myxoma virus (MYX) for 24 hours, stained with polyclonal anti-rabbit ß2m antiserum and analysed by flow cytometry.

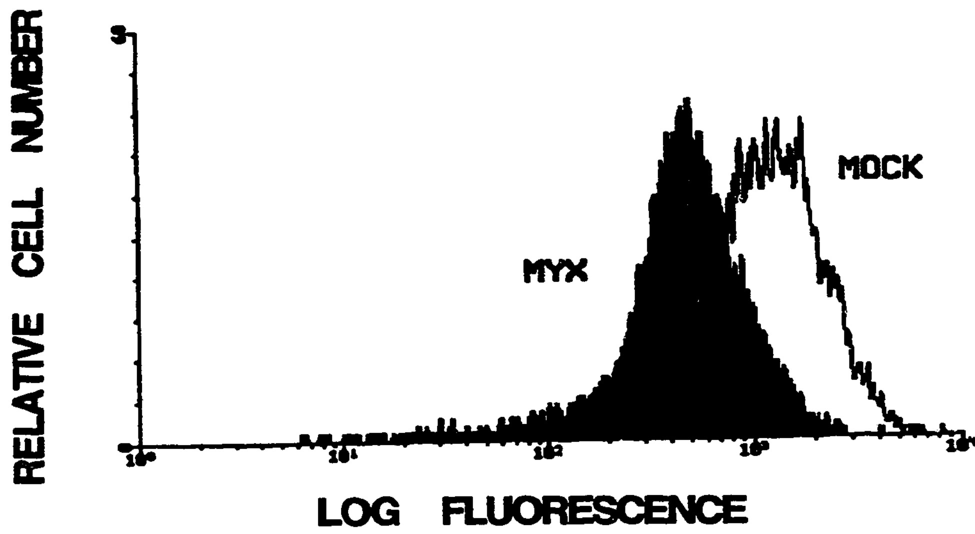

FIGURE III-8

Figure III-9. Flow cytometric analysis of CD18, CD43 and CD45 on RL-5 cells. RL-5 cells were either mock infected or infected with myxoma virus (vMyxlac) for 24 hours. Cells were harvested and stained with monoclonal antibodies: *A*, anti-rabbit CD18; *B*, anti-rabbit CD43; *C*, anti-rabbit CD45. Histograms corresponding to myxoma virus infected cells are shaded.

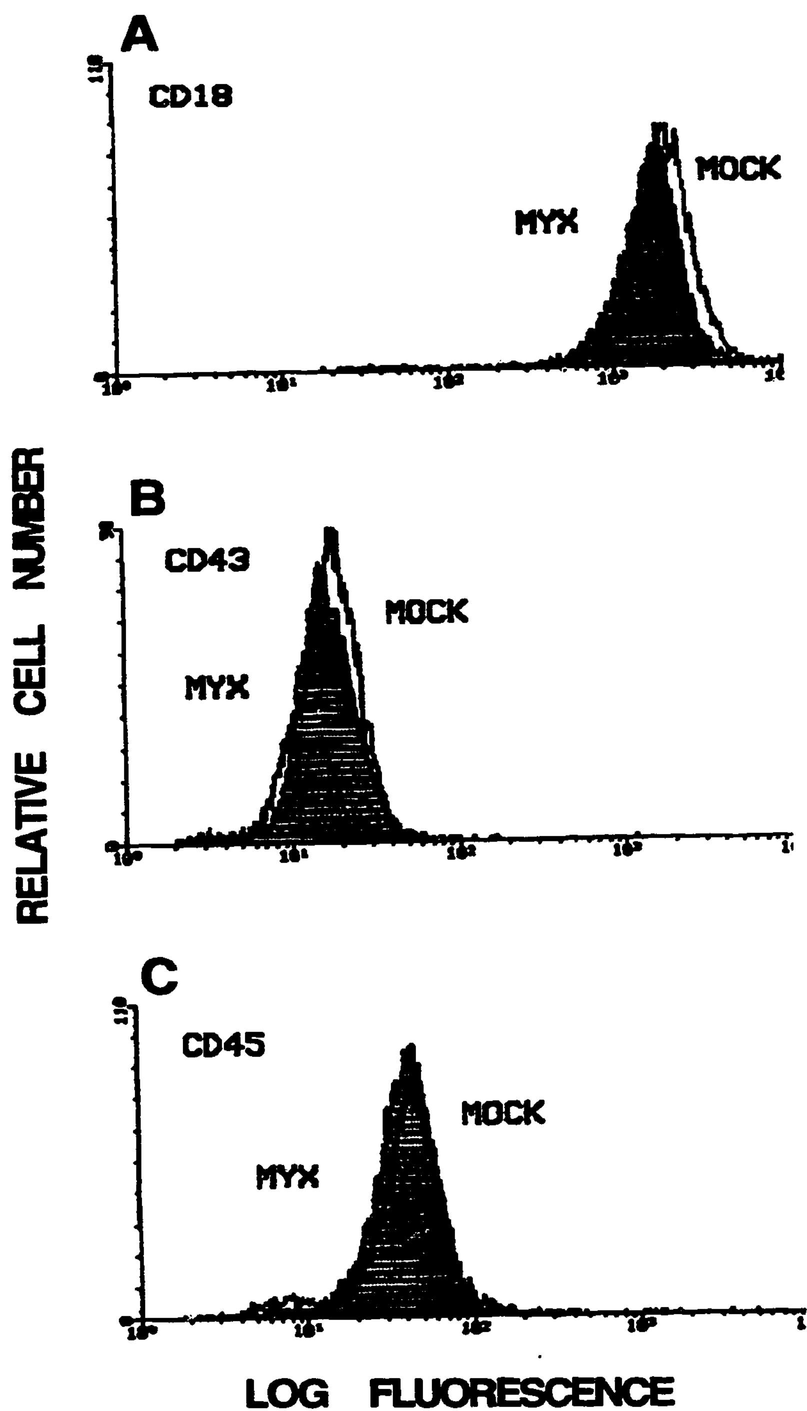

FIGURE III-9

Figure III-10. Comparison of cell surface class I MHC antigen levels on RL-5 cells infected with myxoma virus, SFV or vaccinia virus. Cells were either mock infected or infected with *A*, myxoma virus, *B*, SFV (Kasza), or *C*, vaccinia virus (VV601) and stained with anti-rabbit class I MHC mAb. Histograms corresponding to virus infected cells are shaded.

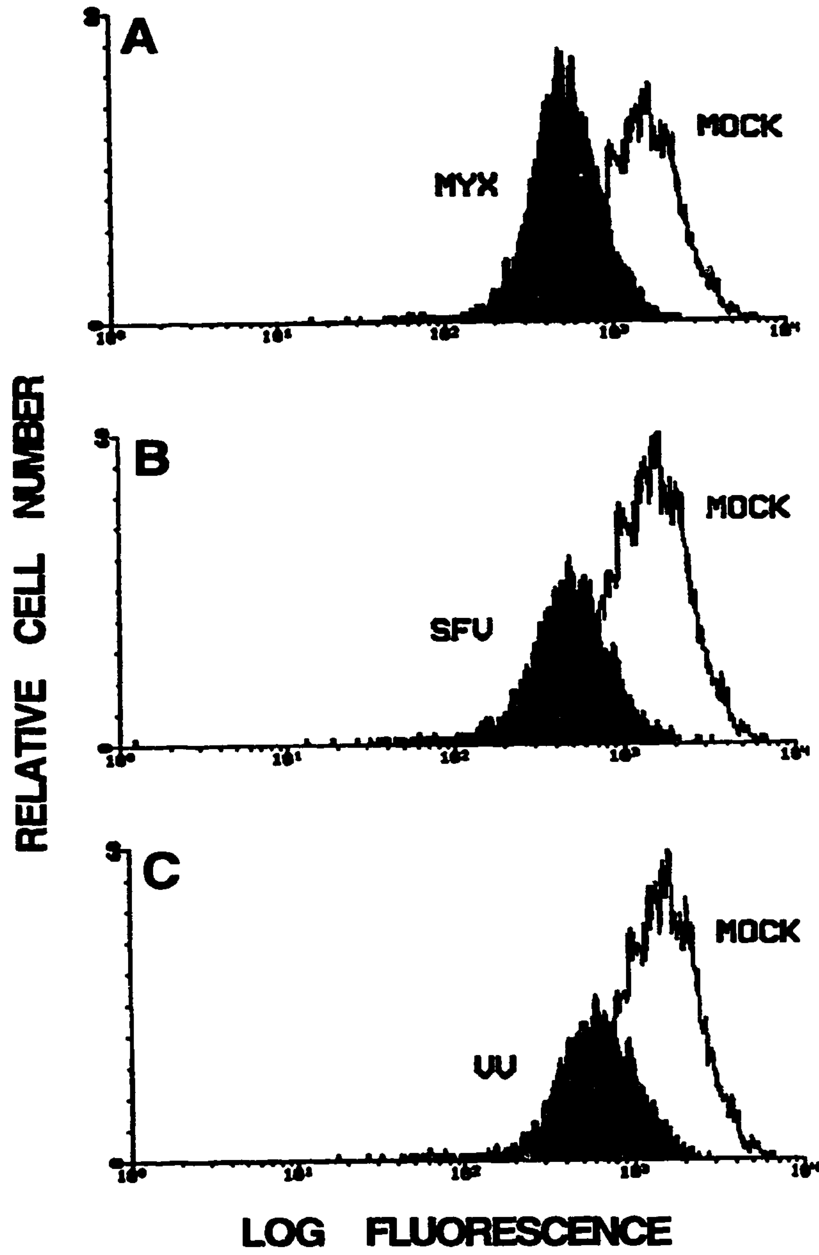

FIGURE III-10

Figure III-11. Effect of cycloheximide on cell surface class I MHC antigens on RL-5 cells. Mock infected cells were treated for 24 hours with cycloheximide (CHX; 100μg/mL) and stained with anti-rabbit class I MHC (panel A). Histogram corresponding to cycloheximide-treated cells is shaded. For comparison, panel B shows RL-5 cells infected with myxoma virus (vMyxlac) for 24 hours, and stained with anti-rabbit class I MHC.

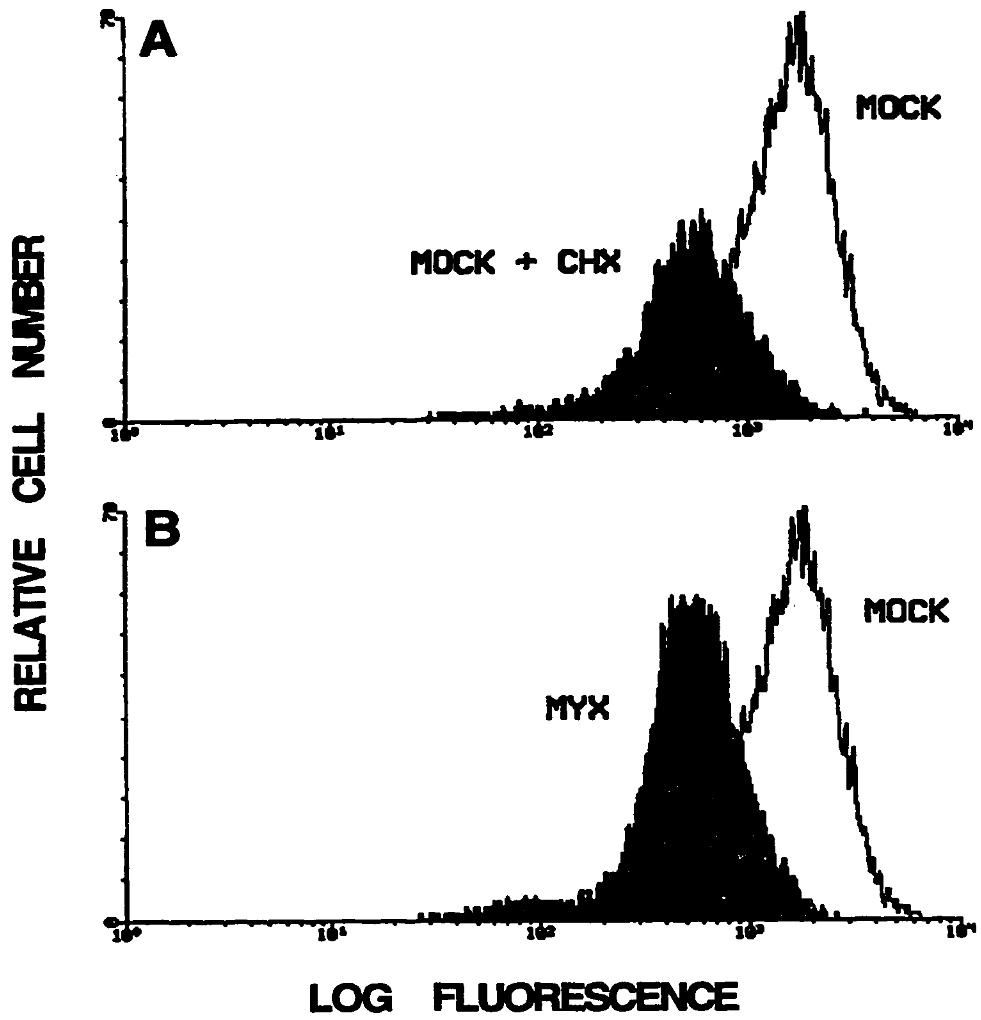

FIGURE III-11

Figure III-12. Effect of cytosine arabinoside treatment on cell surface class I MHC levels on infected and uninfected RL-5 cells. A, mock infected RL-5 cells were either untreated or incubated with AraC (40μg/mL) for 24 hours (shaded); B, untreated, mock infected RL-5 cells compared to cells infected with myxoma virus (vMyxlac) for 24 hours in the presence of AraC (MYX + AraC) (shaded); for comparison, panel C shows RL-5 cells mock infected or infected with myxoma virus (vMyxlac) for 24 hours (shaded). All cells were stained with anti-rabbit class I MHC and analysed by flow cytometry.

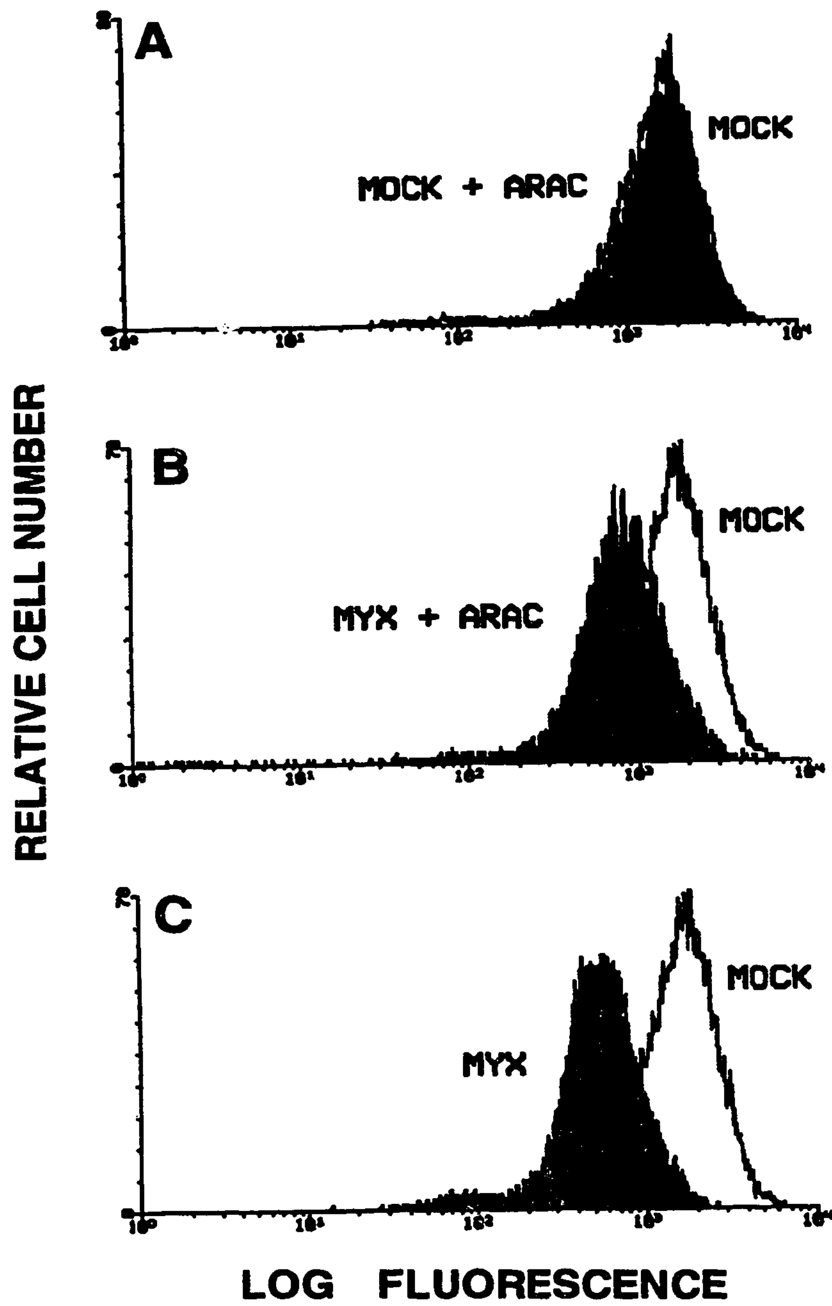

FIGURE III-12

Figure III-13. Flow cytometric analysis of cell surface class I MHC antigens on RL-5 cells treated with brefeldin A. RL-5 cells were either untreated (MOCK) or incubated in the presence of brefeldin A (5μg/mL) (shaded) for 3, 12, or 24 hours (panels A, B and C, respectively). Cells were stained with anti-rabbit class I MHC and analysed by flow cytometry.

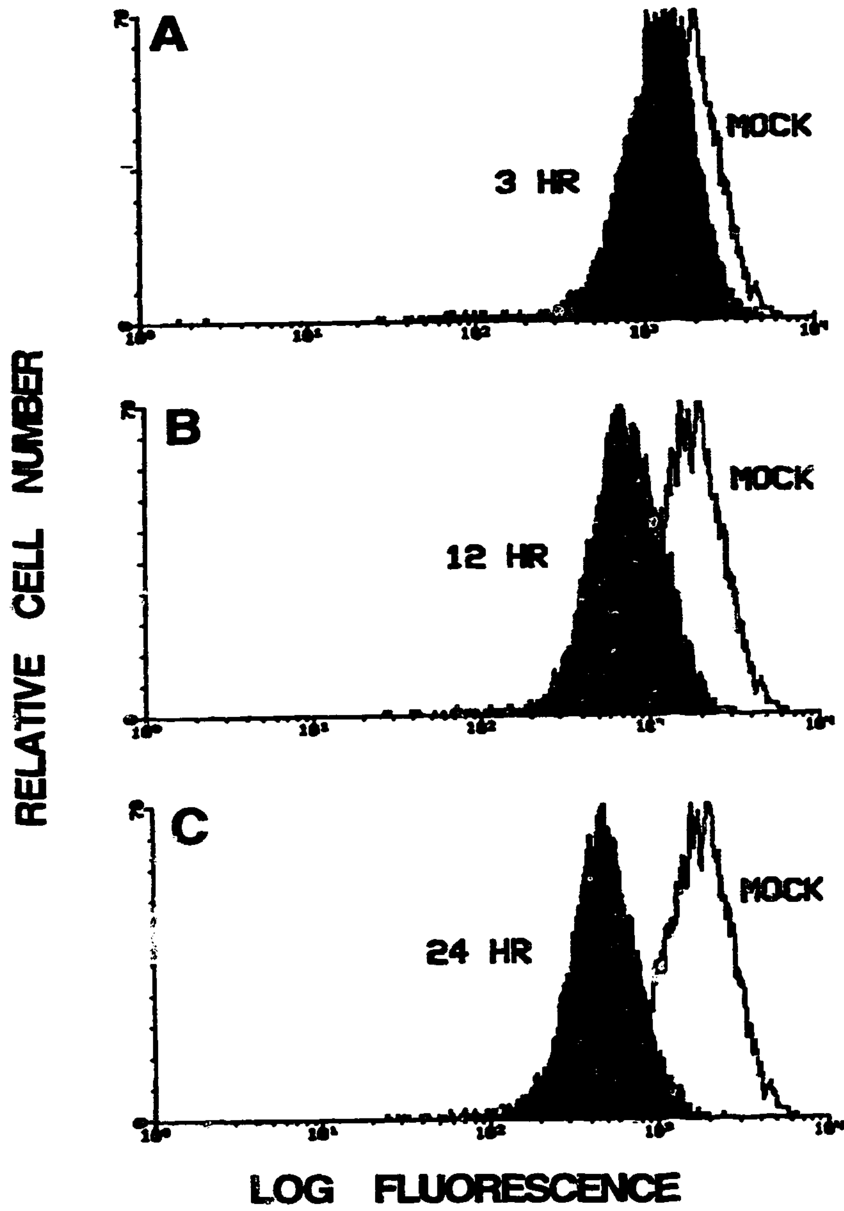

FIGURE III-13

Figure III-14. Flow cytometric analysis of CD18, CD43 and CD45 on RL-5 cells treated with brefeldin A. Cells were either untreated (mock) or treated with brefeldin A (5µg/mL) for 24 hours (shaded). Cells were harvested and stained with monoclonal antibodies specific for CD18, CD43 and CD45 (panels A, B and C, respectively) and analysed by flow cytometry.

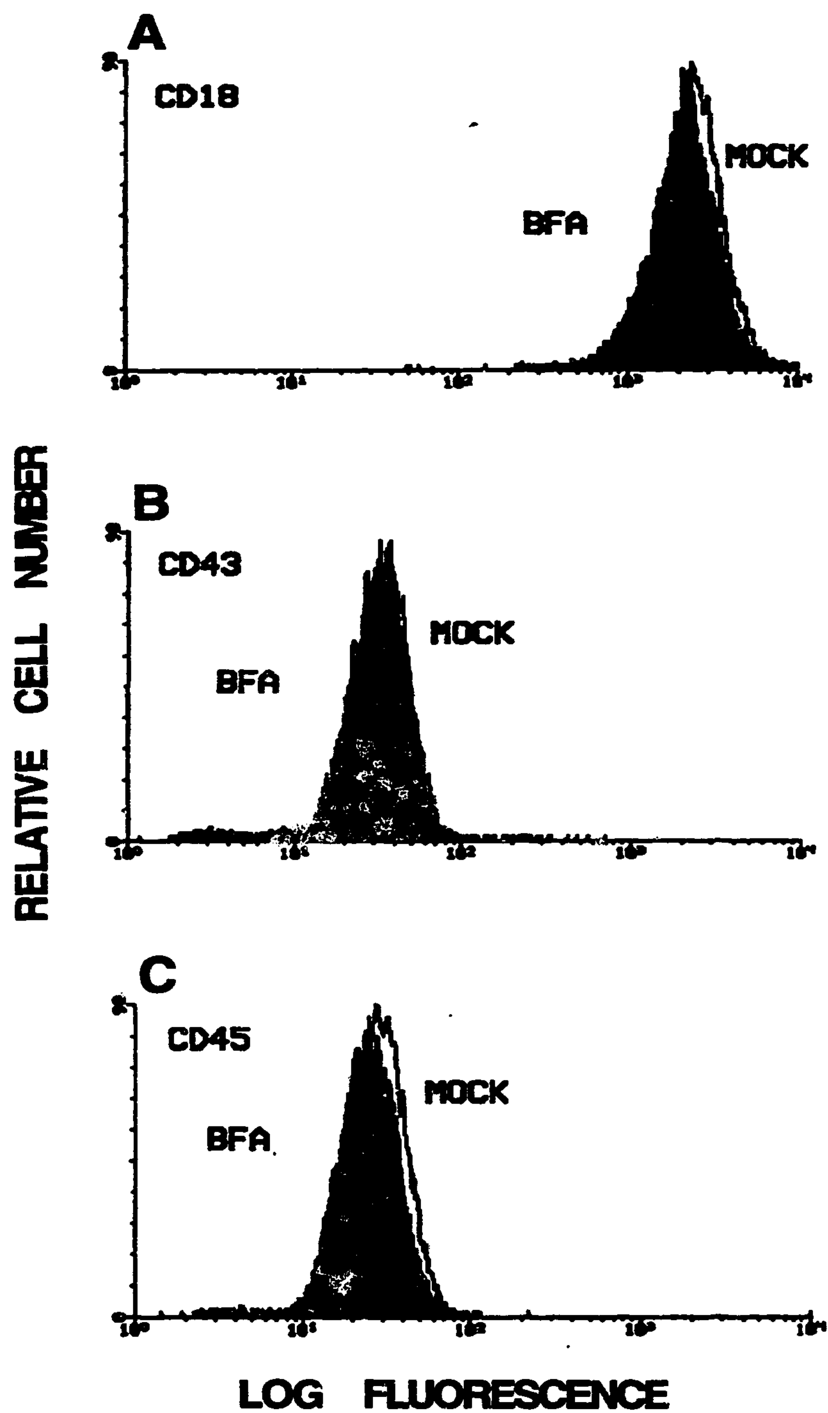

FIGURE III-14

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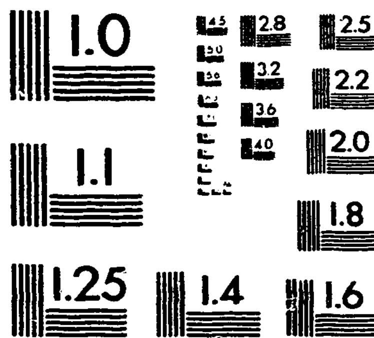
CHAPTER IV

EXPRESSION OF THE MYXOMA VIRUS TUMOR NECROSIS FACTOR RECEPTOR HOMOLOGUE (T2) AND M11L GENES IS REQUIRED TO PREVENT VIRUS-INDUCED APOPTOSIS IN INFECTED RABBIT T LYMPHOCYTES[1]

[1]A version of this chapter has been submitted for publication: Macen, J.L. Lee, S.F., Graham, K.A., Schreiber, M., Boshkov, L. and McFadden, G. Expression of the myxoma virus tumor necrosis factor receptor homologue (T2) and M11L genes is required to prevent virus-induced apoptosis in infected rabbit T lymphocytes. Submitted to *Virology*, July, 1995.
Data presented in Figure IV-1 and Table IV-1 have been published: Upton, C., Macen, J.L., Schreiber, M. and McFadden, G. (1991) 184:370-382.

3

PM-1 3½"x4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT



PRECISION℠ RESOLUTION TARGETS

INTRODUCTION

It is becoming increasingly clear that programmed cell death, or apoptosis, functions not only to regulate development and homeostasis in higher organisms, but also serves as an effective antiviral mechanism. Because premature death of infected cells can potentially limit the production of progeny virus, it is not surprising that evolution has endowed many viruses with genes encoding proteins that protect cells against programmed cell death (Birnbaum *et al.*, 1994; Clem *et al.*, 1991; Crook *et al.*, 1993; Gagliardini *et al.*, 1994; Henderson *et al.*, 1993; Rao *et al.*, 1992; Tarodi *et al.*, 1994; Henderson *et al.*, 1991). These viral anti-apoptosis genes have been shown to intersect the programmed cell death pathway at a variety of junctures (Vaux *et al.*, 1994), thereby providing the virus with a selective advantage by allowing for replication in a greater spectrum of cell types. Examples of viral proteins that have been shown to block apoptosis include the baculovirus p35 and iap proteins (Clem *et al.*, 1991; Crook *et al.*, 1993; Birnbaum *et al.*, 1994), the adenovirus E1B 19-kDa protein (Rao *et al.*, 1992; Henderson *et al.*, 1991), the Epstein-Barr Virus BHRF1 and LMP-1 proteins (Henderson *et al.*, 1993; Tarodi *et al.*, 1994) and the human immunodeficiency virus type 1 Tat protein (Zauli *et al.*, 1993). In addition, the African swine fever virus *LMW5-HL* gene (Neilan *et al.*, 1993), the herpes simplex virus 1 *γ134.5* gene (Chou and Roizman, 1994) and the poliovirus A2 protein (Tolskaya *et al.*, 1995) have also been implicated in the prevention of virus-induced apoptosis. Among the poxviruses several genes have been identified that can function to prevent or delay apoptosis in specific cell types. These include the host range genes *CHOhr* from cowpox virus (Ink *et al.*, 1995) and *E3L* from vaccinia virus (Lee and Esteban, 1994), which are required in order to prevent or delay cell death by apoptosis and allow fully productive infection in Chinese hamster ovary cells and primate fibroblasts, respectively. The *SPI-1* gene from rabbitpox virus has also been shown to function as a host range gene, possibly by

blocking premature death of infected pig fibroblast or human A549 epithelial cells during infection (Ali *et al.*, 1994). As well, the SPI-2/crmA protein from cowpox virus has been shown to block apoptosis in a variety of cell types, presumably through its ability to inhibit an enzyme related to the interleukin-1β converting enzyme (Ray *et al.*, 1992; Gagliardini *et al.*, 1994).

Myxoma virus is a Leporipoxvirus and the causative agent of a highly lethal, virulent disease in the European rabbit (*Oryctolagus cuniculus*) known as myxomatosis (Fenner and Ratcliffe, 1965). The ability to productively infect lymphocytes is an important aspect of myxoma virus pathogenesis in terms of both the disruption of normal lymphocyte function and the spread of virus to secondary sites within infected lymphocytes (reviewed in McFadden, 1988, 1994). Shope fibroma virus (SFV) is a closely related Leporipoxvirus that, unlike myxoma virus, induces a benign self-limiting infection in *O. cuniculus* and does not productively infect lymphocytes (McFadden, 1988, 1994). To evaluate this distinction in lymphotropism between myxoma virus and SFV we have investigated the infection of T lymphocytes using the rabbit $CD4^+$ T cell lymphoma cell line RL-5. Wild type myxoma virus was found to productively infect RL-5 cells, whereas SFV underwent only an abortive infection in these cells. In addition, we investigated the infection of RL-5 cells with several myxoma virus mutants containing targetted gene disruptions. Unexpectedly, we observed that infection of RL-5 cells with myxoma virus mutants in either the T2 or M11L open reading frames resulted in a rapid and dramatic loss of cell integrity and a concomitant reduction in the production of progeny virus. Cell death was due to the rapid induction of apoptosis in RL-5 cells infected with the T2 mutant (vMyx-$T2^-$) or the M11L mutant (vMyx-$M11L^-$), but was not observed with a comparably attenuated mutant in the SERP1 gene.

The myxoma virus T2 open reading frame was mutated by homologous recombination leading to the insertion of the Escerichia coli guanosine phosphoribosyl transferase (Ecogpt) gene into both copies of the open reading frame to produce the virus vMyx-T2$^-$. To assess the contribution of T2 to virus infection *in vivo*, rabbits (*Oryctolagus cunniculus*) were inoculated with vMyx-T2$^-$ and the course of infection observed. vMyx-T2$^-$ was found to be significantly attenuated in rabbits, such that over 60% of the infected rabbits completely recovered from the infection. In contrast, the parental virus vMyxlac was uniformly lethal, as previously demonstrated (Opgenorth *et al.*, 1992). The T2 gene encodes a secreted protein with homology to the ligand binding domains of members of the tumour necrosis factor receptor superfamily (Upton *et al.*, 1991). Other studies have shown that the myxoma virus T2 protein binds to and inhibits the cytocidal activity of rabbit tumour necrosis factor alpha (TNF-α) (Schreiber and McFadden, 1994). This, together with the results presented here, demonstrate that the myxoma virus T2 protein is an important virulence factor for myxoma virus infection *in vivo*, and may serve to block two related yet distinct host anti-viral responses: the anti-viral effects of soluble TNF that would be expected to be produced in response to virus infection, as well as virus-induced apoptosis of T lymphocytes.

The M11L gene has also been shown to be an important virulence factor for myxoma virus infection in rabbits (Opgenorth *et al.*, 1992; Graham *et al.*, 1992). M11L encodes a novel type II transmembrane protein that is expressed at the cell surface of infected BGMK cells and functions to prevent the infiltration of inflammatory cells into infected sites *in vivo* (Graham *et al.*, 1992; Opgenorth *et al.*, 1992). No significant sequence homology between M11L and any other known cellular protein sequence has been found to date. The apparent requirement for expression of both of these viral genes in RL-5 cells in order to prevent myxoma virus-induced apoptotic cell death indicates that T2 and M11L represent novel anti-death genes. T2 and M11L are thus the

first examples of poxvirus-encoded proteins that function to block apoptosis in virus-infected T lymphocytes.

MATERIALS AND METHODS

Cells and viruses

The rabbit T cell lymphoma cell line, RL-5, was obtained from the NIH AIDS Reference Reagent Program, and cultured in RPMI 1640 (Gibco BRL Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco BRL Life Technologies, Inc.). RK-13 (rabbit kidney) cells were obtained from the ATCC, and propagated in DMEM (Gibco BRL Life Technologies, Inc.) supplemented with 10% FBS. The primate cell line BGMK (obtained from S. Dales, University of Western Ontario) was maintained in DMEM supplemented with 10% newborn calf serum (Gibco BRL Life Technologies, Inc.). The Leporipoxviruses used in this study are as follows: Shope fibroma virus (SFV; strain Kasza) was obtained from the ATCC and engineered to express the *Escherichia coli Lac Z* gene under the control of the vaccinia p11 promoter, inserted in the intergenic region between the S-T9 and the Shope fibroma growth factor open reading frames (unpublished); vMyxlac, a derivative of myxoma virus (strain Lausanne), which expresses the *E. coli lac Z* gene from an intergenic site and has a wild type phenotype, has been described (Opgenorth *et al.*, 1992). vMyx-T2$^-$ (previously designated vMyxT2gpt Upton *et al.*, 1991) is described below. vMyx-M11L$^-$ and vMyx-SERP1$^-$ have been described (Opgenorth *et al.*, 1992; Macen *et al.*, 1993). Recombinant vaccinia viruses (strain WR) used in this study were constructed using the vectors pMJ601 or pMJ602 (Davison and Moss, 1990) which direct insertion of foreign genes within the vaccinia virus thymidine kinase gene where

they are expressed from a synthetic late promoter: VV-T2 over-expresses the myxoma virus T2 gene (Schreiber and McFadden, 1994); and VV-M11L over-expresses the myxoma virus M11L gene (Graham *et al.*, 1992); VV-601 is a control TK⁻ derivative of vaccinia virus that has been described (Macen *et al.*, 1993). All viruses were propagated and titred on BGMK cells.

Construction of the myxoma virus T2 deletion mutant vMyx-T2⁻

The construction of the plasmid pMT2G, used for the inactivation of both copies of the T2 gene within the myxoma virus terminal inverted repeats is outlined in Figure IV-1. A 1.8 Kb KpnI/HindIII fragment of the myxoma virus BamHI S fragment (Russell and Robbins, 1989) was ligated to KpnI/HindIII digested pUC19 to generate the plasmid pMT2KH. pMT2KH was restricted with SmaI, which generated a deletion of 30 nucleotides within the T2 coding sequences near the 5' end of the open reading frame. BglII linkers were used to insert the *Escerichia coli* guanosine phosphoribosyltransferase (*Eco-gpt*) dominant selectable marker, under the control of the vaccinia virus 7.5K promoter (Falkner and Moss, 1988), into SmaI digested pMT2KH to create the plasmid pMT2G. The fusion of the 7.5K promoter, as a 289 bp *Eco*RI fragment from pVV5.1 (gift of D. Hruby), to the *Eco-gpt* coding sequence, as a 650 bp *Bgl*II/*Dde*I fragment from pSV2-gpt (gift of R. Godbout), was acheived by cloning these cassettes into the *Sma*I and *Bam*HI sites of pUC19, respectively, to create p7.5gptA (C. Macaulay, unpublished). The p7.5-gpt fusion fragment was released by digestion with *Eco*RI and *Hind*III, blunt-ended and ligated to *Bgl*II linkers for insertion into pMT2KH.

To facilitate indentification of recombinant virus, a parent virus (vMyxlac) was chosen that had been engineered to express β-galactosidase (see above). The construction and selection of recombinant virus was achieved as follows. vMyxlac-

infected BGMK cells were transfected with pMT2G using standared protocols. After 24 hours, virus was harvested and plated onto BGMK cell monolayers in the presence of gpt selection medium (DMEM containing 10% NCS, 25 μg/ml mycophenolic acid, 250 μg/ml xanthine, 15 μg/ml hypoxanthine). Viruses were twice plaque purified in gpt selection medium, and individual viral plaques were then tested by polymerase chain reaction using appropriate primers for the insertion of the *Eco-gpt* DNA into the T2 gene. Stocks of two different gpt-positive viruses, here designated vMyx-T2$^-$, were prepared and tested by Southern blotting to confirm that both copies of the T2 gene had been disrupted by insertion of the *Eco-gpt* DNA (data not shown).

Infection of rabbits with vMyxlac and vMyx-T2$^-$

All animal experiments were conducted in level III biocontainment facilities under the guidelines of the Canadian Council on Animal Care and the University of Alberta Animal Welfare Committee. Adult female New Zealand White rabbits (*Oryctolagus cuniculus*) were obtained from a local supplier. The rabbits were observed for 7 days to ensure they were healthy, and then injected intradermally on each flank with 500 pfu of virus in 0.1ml of DMEM. The rabbits were monitored daily for signs of myxomatosis (reviewed in Fenner and Ratcliffe, 1965), and observations were recorded. If any animal displayed signs of severe distress or difficulty in breathing due to supervening Gram negative bacterial infections which frequenly preceeds death, they were sacrificed immediately by intravenous injection of euthanol.

RL-5 cell infection and determination of cell viability

RL-5 cells were either mock-infected or adsorbed with virus at 37° C for one hour at the indicated multiplicity of infection (MOI), at a cell density of 2-5x10^7 cells/mL. Following adsorption, cells were diluted to 2-5x10^5/mL with RPMI 1640

containing 10% FBS and the infection was allowed to proceed for the indicated period of time at 37°C. Non adherent cells were harvested by centrifugation, and any adhering cells were removed using 1X SSC (0.15M NaCl, 15mM Sodium citrate, pH 7.0). Cell viability was determined by trypan blue exclusion and cell counting using a hemacytometer.

Virus growth in RL-5 cells

Single step growth curves were obtained in duplicate on either RL-5 cells or RK-13 cells as follows: cells were adsorbed with virus at MOI=3 pfu/cell for one hour at 37°C; the inoculum was removed, the cells washed and the infection allowed to proceed at 37°C in the appropriate medium. Cultures were harvested either by centrifugation (non adherent cells) or by removal of cells using 1X SSC (adherent cells) at various times post infection. Virus titres were determined in duplicate by plaque assay on BGMK cells stained with 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside as described (Opgenorth *et al.*, 1992).

Quantitation of apoptotic cells by flow cytometry and fluorescence microscopy

RL-5 cells were either mock-infected or infected with virus at a multiplicity of infection 10 pfu/cell, and harvested for staining at the indicated times. When infections were carried out in the presence of exogenous homogenously purified myxoma T2 protein (M. Schreiber and G. McFadden, unpublished), T2 was added to the medium at a final concentration of 5 µg/ml and was present throughout the entire course of infection. DNA fragmentation in apoptotic cells was measured by incorporation of fluorescein-12-dUTP (Boehringer Mannheim) into DNA using terminal deoxynucleotidyl transferase (TdT; Boehringer Mannheim), which provides a direct

measure of both the amount of DNA fragmentation as well as the proportion of cells containing fragmented DNA, as described (Sgonc *et al.*, 1994). Briefly, 2×10^6 cells were washed once in phosphate buffered saline containing 1% FBS, fixed in 2% paraformaldehyde for 30 min and washed once in phosphate buffered saline+1% FBS. The fixed cells were permeabilized by incubating in cold 0.1% Triton X-100, 0.1% sodium citrate on ice for 2 min, washed twice in phosphate buffered saline+1% FBS, and incubated in TdT reaction mixture as described (Sgonc *et al.*, 1994). Nonspecific uptake of label was determined by reacting the cells in the absence of TdT. Reactions were stopped in 20 mM EDTA, and the cells were subjected to flow cytometric analysis or observed by microscopy.

Flow cytometric analysis was performed on a FACScan flow cytometer (Becton Dickenson, Mountain View, CA) equipped with an argon-ion laser with 15 mW of excitation at 488nm. Data was acquired on 10,000 cells/sample with light scatter signals at linear gain and fluorescence signals at logarithmic gain. Results shown are representative data acquired in at least three independent experiments. Photomicrographs were obtained using a Zeiss Axioskop 20 fluorescence microscope. Equivalent exposures were taken using a MC100 camera and TMAX 400 ASA film (Kodak).

RESULTS

Construction of the myxoma virus T2 disruption mutant vMyx-T2⁻

In order to assess the contribution that the T2 gene makes to both myxoma virus replication in tissue culture and virus virulence in the European rabbit, a mutant myxoma virus was constructed in which both copies of the T2 gene were disrupted. In previous experiments in which both copies of the myxoma virus SERP1 gene, also

located within the TIR, were disrupted by homologous recombination it was found that recombinant virus containing insertions within the TIR regions could only be successfully generated under dominant selection pressure (Macen *et al.*, 1993). Therefore the *E. coli* guanine phosphoriboslyltransferase (Eco-gpt) gene, previously found to be useful in the selection of vaccinia virus recombinants (Falkner and Moss, 1988; Boyle and Coupar, 1988) was used to construct the the insertion vector pMT2G (Figure IV-1) in which the Eco-gpt gene driven by the vaccinia virus p7.5 promoter interrupts the myxoma T2 coding sequence. In order to more readily detect virus both in tissue culture and infected rabbit tissues the parent virus chosen for inactivation of the T2 gene was vMyxlac, which expresses the *E. coli Lac Z* gene from an intergenic site and which has a wild type phenotype (Opgenorth *et al.*, 1992). As described in Materials and Methods, recombinant virus was selected and propagated in the presence of mycophenolic acid, which prevents the replication of virus that does not express the Eco-gpt gene. Two recombinant virus clones were grown up and tested for the insertion of the p7.5-gpt cassette into both copies of the T2 gene by both PCR and Southern blotting (data not shown). One clone, here designated vMyx-T2⁻, was shown to fail to secrete a 55kDa protein (Upton *et al.*, 1991), and was used in the experiments described below.

The T2⁻ myxoma virus, vMyx-T2⁻, is significantly attenuated in rabbits

In order to ascertain the effect of the deletion of the myxoma virus T2 gene on the course of myxomatosis in the European rabbit (*O. cuniculus*), adult New Zealand White rabbits were injected intradermally at two sites per rabbit with either vMxy-T2⁻ or vMyxlac (500 pfu per site). Infected animals were monitored daily for the well-established clinical signs of myxomatosis. The results are summarized in Table IV-1. Rabbits infected with the vMyxlac parental virus routinely succumb within 10-11 days

to the progressive viral infection at multiple secondary sites, both internal and external, as has been described for virulent strains of myxoma virus (Fenner and Ratcliffe, 1965). Because this syndrome has been well characterized, both for wild type myxoma virus and the parental vMyxlac, only two rabbits were infected with vMyxlac for this experiment. As expected, both of these rabbits showed typical signs of myxomatosis (Table IV-1). In contrast, the eight rabbits infected with vMyx-T2$^-$ manifested considerably moderated signs of infection, including consistently smaller primary and secondary skin lesions as well as less extensive supervening Gram negative bacterial infections of the nasal and conjunctival mucosa (Table IV-1). Although three of these rabbits showed increasing severity of infection and were therefore euthanized 10 days post-infection, the five remaining rabbits were only moderately afflicted and were able to completely clear the infection. These survivors had totally recovered within 4 weeks, and were found to be immune to challenge by wild type myxoma virus. The reasons underlying the ability of rabbits infected with the T2$^-$ virus to recover from the infection await detailed immunopathological and histological analyses. However, it is clear that the results reflect an important role for TNF or related molecules in the host response to myxoma virus infection. We therefore conclude that the T2 gene can be classified as an important virulence factor for myxoma virus infection.

Loss of RL-5 cell integrity during infection with either SFV or myxoma virus deletion mutants in the T2 or M11L genes.

Because infection of lymphocytes plays such a pivitol role in the pathogenesis of myxoma virus infection, we wished to investigate whether there was any correlation between virus virulence and the ability to infect lymphocytes. We have investigated the infection of rabbit T lymphocytes using the rabbit CD4$^+$ T cell lymphoma cell line RL-5. In the following experiments, infection of RL-5 cells was studied for the parental

myxoma virus, vMyxlac, and the T2$^-$ myxoma virus derrivative, vMyx-T2$^-$. For comparison, infection of RL-5 cells was also examined using several other attenuated viruses. The viruses examined were an attenuated mutant myxoma virus containing a disruption mutation in the SERP1 open reading frame (vMyx-SERP1$^-$), as well as vMyx-M11L$^-$ and SFV, both of which are highly attenuated compared to wild type myxoma virus and both of which have been shown to be defective in replication in cultures of primary mixed splenocytes (Strayer *et al.*, 1985; Opgenorth *et al.*, 1992).

During the course of these studies, it was observed that infection of RL-5 cells with either vMyx-T2$^-$, vMyx-M11L$^-$ or SFV resulted in a dramatic loss of cell integrity as determined by trypan blue exclusion, such that by 24 hours post infection only 25-35% of infected cells were able to exclude trypan blue (Figure IV-2). In contrast, cells that were either mock infected or infected with the parental derivative of myxoma virus (vMyxlac) continued to exclude trypan blue over the same period (Figure IV-2). No comparable loss in cell integrity was noted in RL-5 cells infected with an attenuated myxoma virus containing a disruption mutation in the SERP1 gene, or in non-lymphoid cell lines such as BGMK or RK-13 upon infection with any of these viruses (data not shown). Therefore, the maintenance of RL-5 cell integrity during myxoma virus infection appears to rely upon the expression of both the T2 and M11L open reading frames, since deletion of either from the virus resulted in the loss of cell integrity. We next sought to determine the basis for this apparent distinction between RL-5 cells infected with SFV or the T2$^-$ and M11L$^-$ myxoma virus mutants and comparable infection with the parental myxoma virus.

Induction of apoptosis in RL-5 cells infected with myxoma virus mutants in the T2 or M11L open reading frames or with SFV.

In general, cell death may occur by one of two mechanisms: necrosis or programmed cell death (apoptosis). Characteristic features of apoptosis include a rapid induction of nuclear DNA condensation and fragmentation, as well as cell shrinkage and plasma membrane blebbing. We measured DNA fragmentation in RL-5 cells at various times post-infection by quantitating the TdT-mediated incorporation of fluorescein-12-dUTP into DNA using flow cytometric analysis and fluorescence microscopy. In addition, changes in cell morphology were monitored by examination of the light scattering properties of the cells by flow cytometry as well as by microscopic examination.

Flow cytometric analysis of infected RL-5 cells revealed a significant population of cells undergoing DNA fragmentation among cells infected with vMyx-T2$^-$, vMyx-M11L$^-$ and SFV but not among mock-infected cells or cells infected with myxoma virus (Figure IV-3) or vMyx-SERP1$^-$ (data not shown). By 16 hours post-infection, 35-40% of cells infected with vMyx-T2$^-$, vMyx-M11L$^-$ and SFV contained highly fragmented DNA as measured by flow cytometry (Figure IV-3 and Table IV-2). The light scattering properties of the cells were also examined. Plots of side scatter vs. forward scatter shown in Figure IV-3 clearly indicated the emergence of a population of cells that were smaller in size, seen as a shift to the left in the forward scatter profile, also indicative of cells undergoing apoptosis. In contrast, neither mock-infected cells nor cells infected with the parental myxoma virus contained significant amounts of fragmented DNA or any significant population of cells with altered cell morphology (Figure IV-3). Microscopic examination of infected cells, shown in Figure IV-4, confirmed these observations. Cells infected with vMyx-T2$^-$, vMyx-M11L$^-$ and SFV manifested membrane blebbing, cell shrinkage and the condensation and compartmentalization of nuclei characteristic of apoptotic cells (Fig.IV-4, panels E-J), whereas neither mock-infected nor myxoma virus infected cells had this appearance (Fig.IV-4, panels A-D).

DNA fragmentation in infected RL-5 cells was observed very early during virus infection. As indicated in Table IV-2, by 3 hours post-infection, before viral DNA replication has begun, significant nuclear DNA fragmentation was observed in cells infected with vMyx-T2$^-$, vMyx-M11L$^-$ and SFV. The early induction of DNA fragmentation was most dramatic during infection with vMyx-T2$^-$ and SFV. By 6 hours post-infection with these viruses more than 40% of the cells were undergoing extensive DNA fragmentation (Table IV-2). DNA fragmentation was significant but less extensive in cells infected with vMyx-M11L$^-$ at this time (Table IV-2). At 10 hours post-infection with vMyx-T2$^-$ or SFV, the proportion of cells containing fragmented DNA was reproducibly less than that observed at 6 hours post infection (see Table IV-2), suggesting that at least some apoptotic cells observed at 6 hours post-infection have been physically lost as intact cells from the infected cell population by this time, and/or that the induction of apoptosis did not occur synchronously during the course of the infection. By 16 hours post infection approximately 40% of the cells infected with either vMyx-T2$^-$, vMyx-M11L$^-$ or SFV contained extensively fragmented DNA. We conclude from these observations that infection of RL-5 cells with vMyx-T2$^-$, vMyx-M11L$^-$ and SFV, but not myxoma virus or vMyx-SERP1$^-$, resulted in the rapid induction of DNA fragmentation and cell death by apoptosis.

Non-productive infection of RL-5 cells with vMyx-T2$^-$, vMyx-M11L$^-$ and SFV.

In order to determine whether the induction of apoptosis in RL-5 cells functions to limit the production of progeny virus, we examined the growth of the different viruses in RL-5 cells. As indicated in Figure IV-5, the yield of progeny virus from RL-5 cells infected with either vMyx-T2$^-$, vMyx-M11L$^-$ or SFV is greatly reduced in comparison to the parental myxoma virus or vMyx-SERP1$^-$. Therefore, the induction

of apoptosis in RL-5 cells functions to severely limit the production of virus progeny. In addition, since vMyx-SERP1⁻ does not induce apoptosis in RL-5 cells and replicates to wild type levels (Figure IV-5), we conclude that reduced viral yields are not the result of the expression of the *lac Z* or *Ecogpt* genes from the recombinant viruses and that the inability to replicate in RL-5 cells is not a consequence of virus attenuation *per se*. In contrast, we observed no striking differences in the production of viral progeny with any of these viruses in RK-13 cells, which do not undergo apoptosis during infection (data not shown). Therefore we conclude that viral growth is severely compromised by the induction of apoptosis during infection of RL-5 cells, and that M11L and T2 can be formally classified as host range genes for the replication of myxoma virus in rabbit T lymphocytes.

Effects of expression of T2 and M11L from recombinant vaccinia virus.

We next examined RL-5 cells infected with recombinant vaccinia viruses for the induction of apoptosis in RL-5 cells. We wished to determine whether vaccinia virus infection also induced apoptosis in these cells, and whether this induction could be prevented by the expression of either the T2 or M11L genes from recombinant vaccinia viruses. The results presented in Table IV-2 indicated that DNA fragmentation could be observed in vaccinia virus infected RL-5 cells within 6 hours post-infection. By 16 hours post-infection, apoptosis was observed in 20% of vaccinia virus infected cells (Table IV-2). Expression of myxoma virus M11L from a synthetic late promoter in the recombinant vaccinia virus VV-M11L resulted in a partial reduction in the number of apoptotic cells observed at 16 hours post infection (Table IV-2). To our surprise, the expression of the myxoma virus T2 gene from a synthetic late promoter in the recombinant vaccinia virus VV-MT2 resulted in a reproducible and dramatic increase in the amount of apoptosis observed, such that by 16 hours post infection there were over

twice as many apoptotic cells in the population as compared to cells infected with the control virus VV-601 (Table IV-2). In contrast, infection with VV-MT2 does not result in increased cell death of non-lymphoid cells such as RK-13 or BGMK cells (data not shown). Thus while the T2 protein appears to play a protective role when expressed early during myxoma virus infection of RL-5 cells, it also appears to be capable of exacerbating the apoptotic response in T cells when expressed as a late protein in vaccinia virus infected cells.

Exogenous purified T2 protein does not rescue cells infected with vMyx-T2⁻ from apoptosis.

The myxoma virus T2 protein has been shown to bind to and inhibit the cytocidal activity of rabbit TNF-α *in vitro* (Schreiber and McFadden, 1994). Because TNF-α can induce cell death by a mechanism that resembles apoptosis, it was therefore possible that the T2 protein was acting to inhibit the activity of extracellular TNF-α produced by RL-5 cells during myxoma virus infection. Two observations suggest that this is not the case. Firstly, data provided in Table IV-3 shows that the addition of exogenous, purified myxoma virus T2 protein to the medium (5μg/ml) throughout infection of RL-5 cells with vMyx-T2⁻ did not rescue the cells from undergoing apoptosis. The concentration of T2 protein added into the medium in this experiment was in excess of the amount of T2 secreted into the medium during infection with wild type myxoma virus, and has been shown to be sufficient to inhibit the cytocidal activity of rabbit TNF-α on L929 cells (M.S. and G.M., unpublished observations). It is therefore highly unlikely that the T2 protein could be functioning to protect the cells from apoptotic cell death by binding to TNF-α in the medium. Secondly, RL-5 cells themselves are not sensitive to cytolysis or apoptotsis by rabbit TNF-α (M.S. and G.M., unpublished observation). Therefore, we conclude that the T2 protein must be

synthesized *de novo* during myxoma virus infection in order to confer protection from apoptosis; that the T2 protein most likely exerts its anti-apoptotic effects in a cell-associated form, possibly from an intracellular or cell surface location; and that the ability of the T2 protein to protect RL-5 cells from apoptosis is distinct from its previously described role as an extracellular inhibitor of TNF-α (Schreiber and McFadden, 1994).

Effects of inhibition of protein synthesis on the induction of apoptosis in RL-5 cells.

Although apoptosis was originally described as a process that requires cellular protein synthesis, some cells undergo apoptosis in response to protein synthesis inhibitors (reviewed in Schwartzman and Cidlowski, 1993). We next examined RL-5 cells treated with the protein synthesis inhibitor cycloheximide for the presence of apoptotic cells by the TdT-mediated incorporation of fluorescein-12-dUTP. Treatment of RL-5 cells for 16 hours with cycloheximide resulted in the induction of apoptosis in 20% of the cells (Table IV-3), suggesting that the inhibition of cellular protein synthesis could potentially contribute to the apoptotic response to virus infection. Interestingly, the infection of RL-5 cells with wild type myxoma virus in the presence of cycloheximide resulted in a dramatic increase in the proportion of apoptotic cells (38%, Table IV-3), indicating that myxoma virus is a potent inducer of apoptosis in these cells when both viral and cellular protein synthesis is inhibited, and that viral protein synthesis is required for the protection of RL-5 cells from virus-induced apoptosis.

DISCUSSION

Previous studies have indicated that myxoma virus is able to productively infect primary cultures of rabbit splenocytes (Opgenorth *et al.*, 1992). In contrast, neither SFV nor vMyx-M11L⁻ are able to replicate efficiently in unstimulated primary spleen cell cultures (Strayer *et al.*, 1985; Opgenorth *et al.*, 1992). The results reported here suggest that the observed defect in viral replication in primary splenocytes undoubtedly results from the rapid induction of apoptosis in rabbit lymphoid cells and that the attenuated disease course in rabbits observed upon infection with vMyx-M11L⁻ (Opgenorth *et al.*, 1992) or vMyx-T2⁻ may be related to impaired virus replication in rabbit T lymphocytes or other relevant leukocytes. The infection of the rabbit T cell line RL-5 with myxoma virus mutants in either the T2 or M11L genes resulted in the rapid induction of cellular DNA fragmentation, followed by morphological changes and loss in cell integrity characteristic of cell death by apoptosis. However, there is no simple correlation between virus attenuation and failure to inhibit apoptosis in RL-5 cells, since vMyx-SERP1⁻, which is comparably attenuated in rabbits (Chapter II, this thesis and Macen *et al.*, 1993) does not induce apoptosis and replicates to the same extent as the parental myxoma virus (Figure IV-5). Interestingly, infection of RL-5 cells with SFV also resulted in a nonproductive infection characterized by extensive cell death by apoptosis, even though SFV encodes homologues of the myxoma virus T2 and M11L proteins (Upton *et al.*, 1987; Smith *et al.*, 1990; Upton *et al.*, 1988) that share 76% and 73% amino acid identity, respectively, with their myxoma virus counterparts. It is therefore entirely possible that there are other genes, present in myxoma virus but functionally absent from SFV, which are involved in blocking the apoptotic response of T cells to virus infection. Another possible explanation could be that there are functional differences between the SFV and myxoma virus versions of the T2 and/or M11L

proteins, although this seems less likely due to the high degree of identity between these proteins. Alternatively, it is possible that the induction of apoptosis during SFV infection may proceed along a different pathway from that induced by myxoma virus, or that SFV may encode some factor that acts to induce apoptosis downstream of the point at which either T2 or M11L intersect the apoptotic pathway. In either case, the expression of T2 or M11L would be insufficient to block apoptosis. The identification of the cellular and/or viral components with which T2 and M11L interact, as well as the elucidation of the mechanism by which these viruses induce apoptosis in rabbit T lymphocytes will be required to resolve this issue.

The SFV and myxoma virus T2 genes encode secreted proteins that are homologous to the ligand binding domain of the receptors for the multifunctional cytokines TNF-α/β (Smith *et al.*, 1990; Upton *et al.*, 1991). Disruption of both copies of the myxoma virus T2 gene resulted in virus attenuation in infected rabbits. The myxoma virus T2 protein is secreted from infected cells early during infection and has been shown to inhibit the cytocidal activity of rabbit TNF-α *in vitro* (Schreiber and McFadden, 1994), presumably binding to TNF-α and thereby precluding ligand-receptor interaction. Nevertheless, the results presented here indicate that binding and inhibition of soluble extracellular TNF-α cannot explain the requirement for T2 expression early during RL-5 cell infection, since the addition of exogenous purified T2 was completely unable to rescue vMyx-T2$^-$ infected cells from apoptosis. Although the intracellular distribution of the T2 protein has yet to be determined, these results suggest that T2 may have other functions, in addition to TNF-α binding, that require a cell-associated form of the protein. For example, it has been demonstrated that self-association of the intracellular "death domains" of the p55 TNF receptor and Fas/APO1 can prompt ligand-independent signalling leading to cell death and that the association of the extracellular domains of these receptors can exert an inhibitory effect on receptor

signalling (Boldin *et al.*, 1995). Thus one could envision a scenario in which the T2 protein forms heterodimers with the ligand binding domain of Fas, resulting in a molecule than cannot transmit a signal. Alternatively, since T2 is homologous to the extracellular ligand binding domains of these receptors, one possibility is that the nascent T2 protein associates intracellularly with the Fas ligand and is transported to the cell surface as a complex that cannot recognize the Fas receptor, thereby preventing "fratricide" of neighbouring cells that may otherwise be triggered by viral infection. The possibility that the Fas/APO1 or related signalling pathways are involved in the apoptotic response to virus infection is appealing in light of the observation that in the absence of protein synthesis myxoma virus is a potent inducer of RL-5 cell apoptosis. Other studies have shown that while the apoptotic response generally requires new protein synthesis, T cells activated by TNF or anti-Fas antibodies undergo apoptosis in a protein synthesis independent manner (reviewed in Gill *et al.*, 1994).

Somewhat surprising was the observation that the recombinant vaccinia virus VV-MT2, in which the myxoma virus T2 protein is expressed from a synthetic late viral promoter, consistently acted to exacerbate RL-5 cell apoptosis in response to vaccinia virus infection. Because the induction of DNA fragmentation was found to be well underway by three hours post infection, it is likely that the protective effect of the T2 protein is highly dependent upon the time of expression during infection of RL-5 cells. T2 is normally expressed early during myxoma virus infection, and this early synthesis may very well be critical for the inhibition of the apoptotic response in RL-5 cells. In addition, it is also likely that the nature of the apoptotic signal, as well as the cellular context, are important in determining whether the myxoma T2 protein is able to function to block apoptosis. Constructs in which the myxoma T2 protein is expressed from an early promoter in the recombinant vaccinia virus system will be useful in

determining whether the results in RL-5 cells can be explained simply by a requirement for early expression.

The observed requirement for expression of the M11L protein in addition to T2 for productive RL-5 cell infection by myxoma virus suggests that the inhibition of apoptosis during viral infection may involve components of more than one cell death pathway. In contrast to VV-MT2, the expression of M11L in a recombinant vaccinia virus resulted in a partial protection of the cells from apoptosis. Like T2, M11L is expressed from a late promoter in this vaccinia construct, whereas in myxoma virus M11L is expressed as an early gene (Graham *et al.*, 1992). It is therefore possible that expression of M11L is also required early during viral infection in order to confer complete protection from apoptosis or that the M11L protein alone is insufficient to block T cell apoptosis.

The M11L gene encodes a unique protein, predicted to be a type II transmembrane protein, that is expressed on the surface of BGMK cells early during infection (Graham *et al.*, 1992). With the exception of the predicted C10L protein encoded by swine pox virus (Massung *et al.*, 1993) which shares 27% identity with the M11L sequence, the M11L protein and its SFV homologue share no significant homology with other known proteins and contain no motifs that allow prediction of a biochemical function. Previous studies showed that a mutation which prevents cell surface localization of the M11L protein resulted in marked attenuation of the virus in infected rabbits and induced a disease syndrome characterized by extensive inflammatory cell migration into infected sites (Opgenorth *et al.*, 1992; Graham *et al.*, 1992). Whether the extensive inflammation observed during infection of rabbits with vMyx-M11L$^-$ is related to the induction of apoptosis in particular classes of infected cells remains to be determined. However, it has been suggested that the apoptotic death of certain leukocytes, particularly of the monocytic lineage, actually stimulates

macrophage chemotaxis and induces increased inflammation (Kornbluth, 1994). Experiments designed to determine which cell types, *in vivo*, become infected with both wild type and mutant viruses, as well as which cell types are induced to undergo apoptosis during viral infection will allow a more complete understanding of the relationship between virus-induced apoptosis and pathogenesis.

Although further experimentation is clearly required in order to determine the functions of the myxoma virus T2 and M11L proteins during lymphocyte infection, our results suggest that these two viral proteins act as "anti-death" factors that permit myxoma virus replication in T lymphocytes. Investigation of the mechanisms by which T2 and M11L function to block programmed T cell death will provide useful information concerning both the regulation of apoptosis in lymphoid cell populations as well as the role that induction and inhibition of apoptosis plays in the pathogenesis of lymphotropic virus infections.

TABLE IV-1

OBSERVATIONS OF MYXOMA AND MYXOMA $T2^-$ INFECTED RABBITS

DAY	MYXOMA (wild type)	$vMYX\text{-}T2^-$
0	Adult (3 Kg) female NZW rabbits inoculated i.d. in two sites with $5x10^2$ pfu/site myxoma virus.	8 adult (3 Kg) female NZW rabbits inoculated i.d. in two sites with $5x10^2$ pfu/site Myx-T2gpt virus.
4	Primary lesions at inoculation sites, approx 1.5cm, soft, flat; no other symptoms.	Primary lesions at inoculation sites, approx 0.5-1.0cm, hard, raised; no other symtoms.
7	Primary lesions approx 3cm, soft in centre, flat, hemorrhagic; multiple secondary lesions; swollen eyes; moderate bacterial infection of nasal and conjunctival mucosa.	Primary lesions 1-2cm, hard, raised, slightly hemorrhagic and flattening in centre. Some secondary lesions; eyes somewhat swollen; slight to moderate bacterial infection of nasal and conjuctival mucosa.
10	Primary lesions approx 4cm, necrotic, flat; multiple secondary lesion; moderate to severe bacterial infections. Animals sacrificed due to increasing severity of symptoms.	Primary lesions approx 2cm, raised, hard, necrotic; multiple secondary tumors on 50% of animals; mild to severe bacterial infections. Three animals sacrificed due to increased severity of symptoms.
14		Five rabbits recovering: Primary and secondary lesions drying up and scabbing over; bacterial infections moderate to absent.
21		Five rabbits recovering: All lesions scabbed over; all animals recovered from bacterial infections.
31		Five rabbits completely recovered: Small scars remain from primary and secondary lesions; no further signs of infection remain.

Table IV-2. Quantitation of apoptotic cells during viral infection of RL-5 cells

Hours post infection	PERCENTAGE APOPTOTIC CELLS							
	MOCK	vMyxlac	vMyx-M11L$^-$	vMyx-T2$^-$	SFV	VV-601	VV-M11L	VV-MT2
3	2	2	10	20	20	n.d.	n.d.	n.d.
6	2	4	22	45	42	9	6	21
10	2	2	19	31	26	10	9	28
16	4	6	38	41	37	20	13	45

RL-5 cells were mock infected or infected at a multiplicity of infection of 10 plaque forming units per cell and harvested at the indicated times. The percentage of apoptotic cells was determined by incorporation of fluorescein-12-dUTP by TdT as described in text. Numbers represent the percentage of postively staining cells as determined by FACS analysis. n.d., not determined.

Table IV-3. Quantitation of apoptotic cells during infection of RL-5 cells in the presence of purified T2 protein or cycloheximide.

	PERCENTAGE APOPTOTIC CELLS			
	Purified T2 (5μg/ml)		Cycloheximide (40 μg/ml)	
	-	+	-	+
Mock	2	2	4	20
vMyxlac	1	4	4	38
vMyx-T2⁻	39	36	n.d.	n.d.

RL-5 cells were mock infected or infected at a multiplicity of infection of 10 plaque forming units per cell in the presence or absence of either purified T2 protein or cycloheximide. Cells were harvested at 12 hours (T2 treatment) or 16 hours (cycloheximide treatment) post infection . The percentage of apoptotic cells was determined by incorporation of fluorescein-12-dUTP by TdT as described in text. Numbers represent the percentage of postively staining cells as determined by FACS analysis. n.d., not determined.

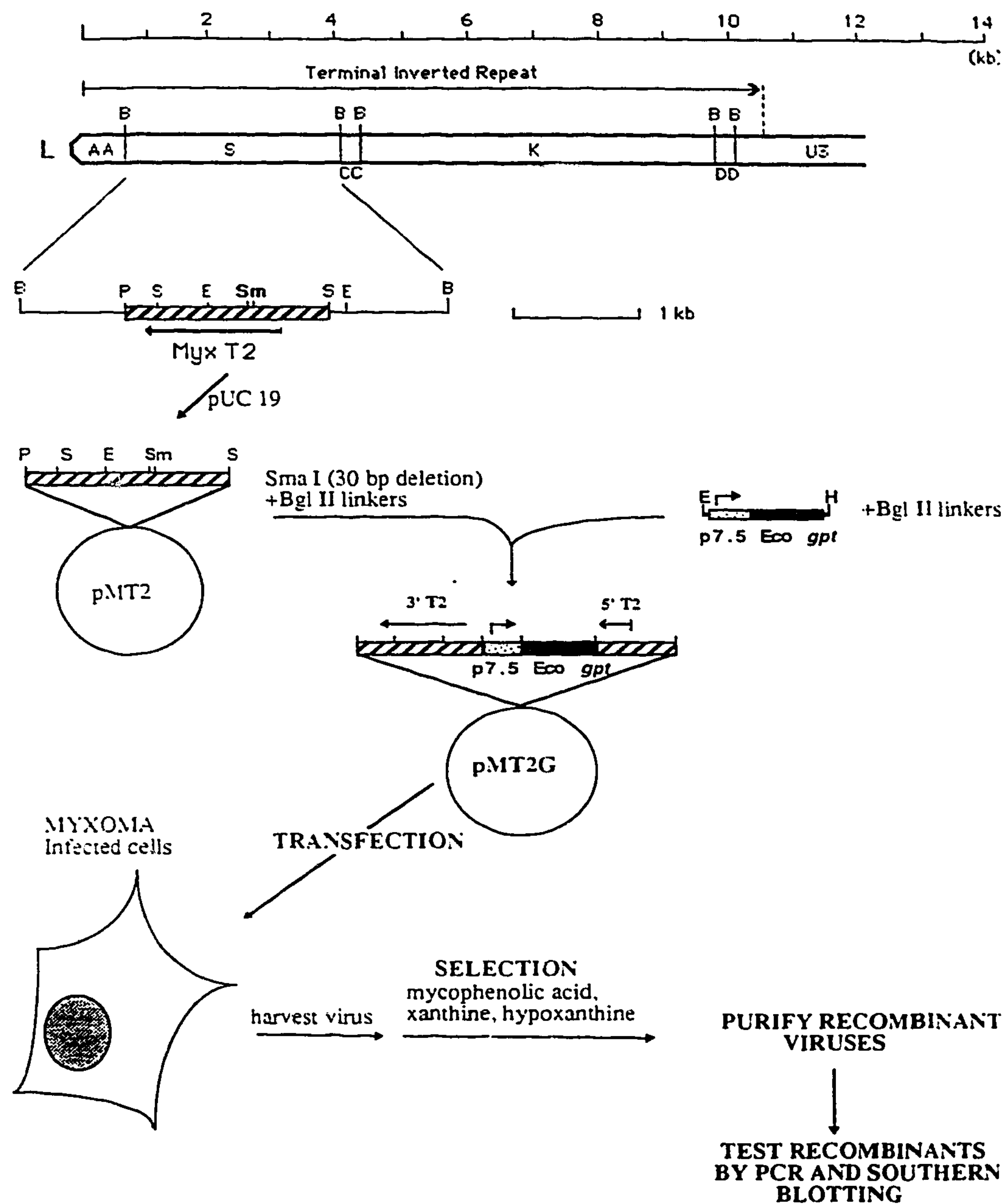


FIGURE IV-1. Construction of the myxoma virus T2 gene disruption mutant

FIGURE IV-2. Measurement of RL-5 cell integrity during viral infection by trypan blue exclusion. RL-5 cells were either mock infected or infected at an MOI of 10 pfu/cell with the indicated virus and uptake of trypan blue was determined at 3, 6, 12 and 24 hours post infection by cell counting using a hemacytometer. Results are expressed as percent viability, representing the percentage of cells excluding trypan blue.

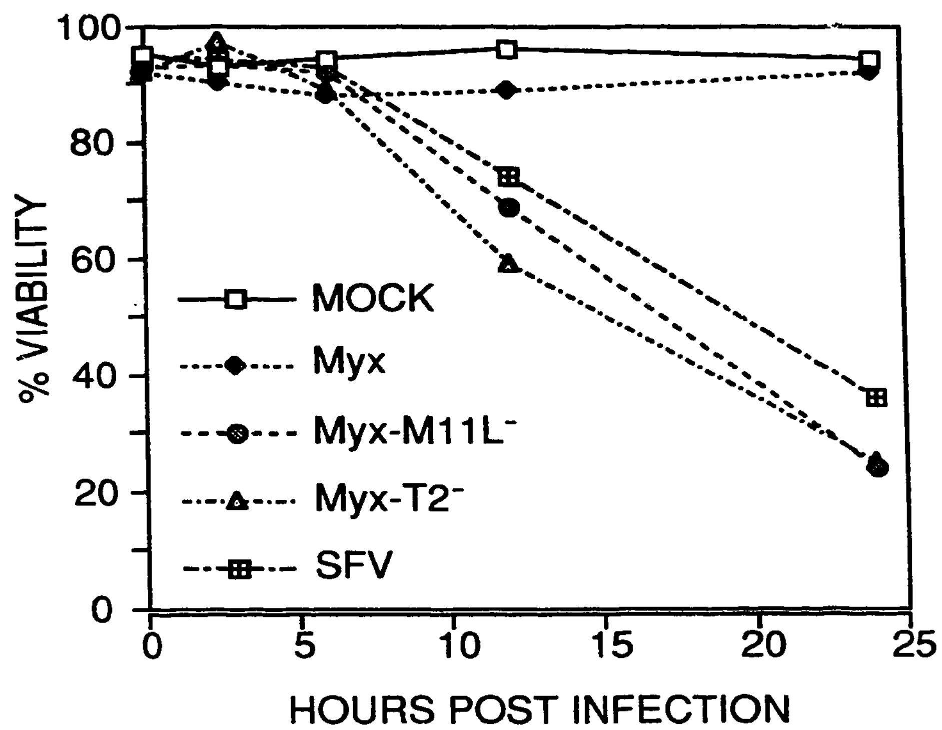


FIGURE IV-2

FIGURE IV-3. Flow cytometric analysis of infected RL-5 cells demonstrating virus-induced alteration in cell morphology and DNA fragmentation. Cells were either mock infected (panels A and B) or infected at an MOI of 10 pfu/cell with vMyxlac (panels C and D), vMyx-M11L- (panels E and F), vMyx-T2⁻ (panels G and H) or SFV (panels I and J). At 16 hours post infection cells were fixed, permeabilized and reacted with TdT and fluorescein-12-dUTP to stain fragmented DNA, and subjected to flow cytometric analysis. Data are shown as plots of side scatter vs forward scatter (panels A,C,E,G,I) and as histograms showing relative cell number vs log flourescence intensity in arbitrary units (panels B,D,F,H,J).

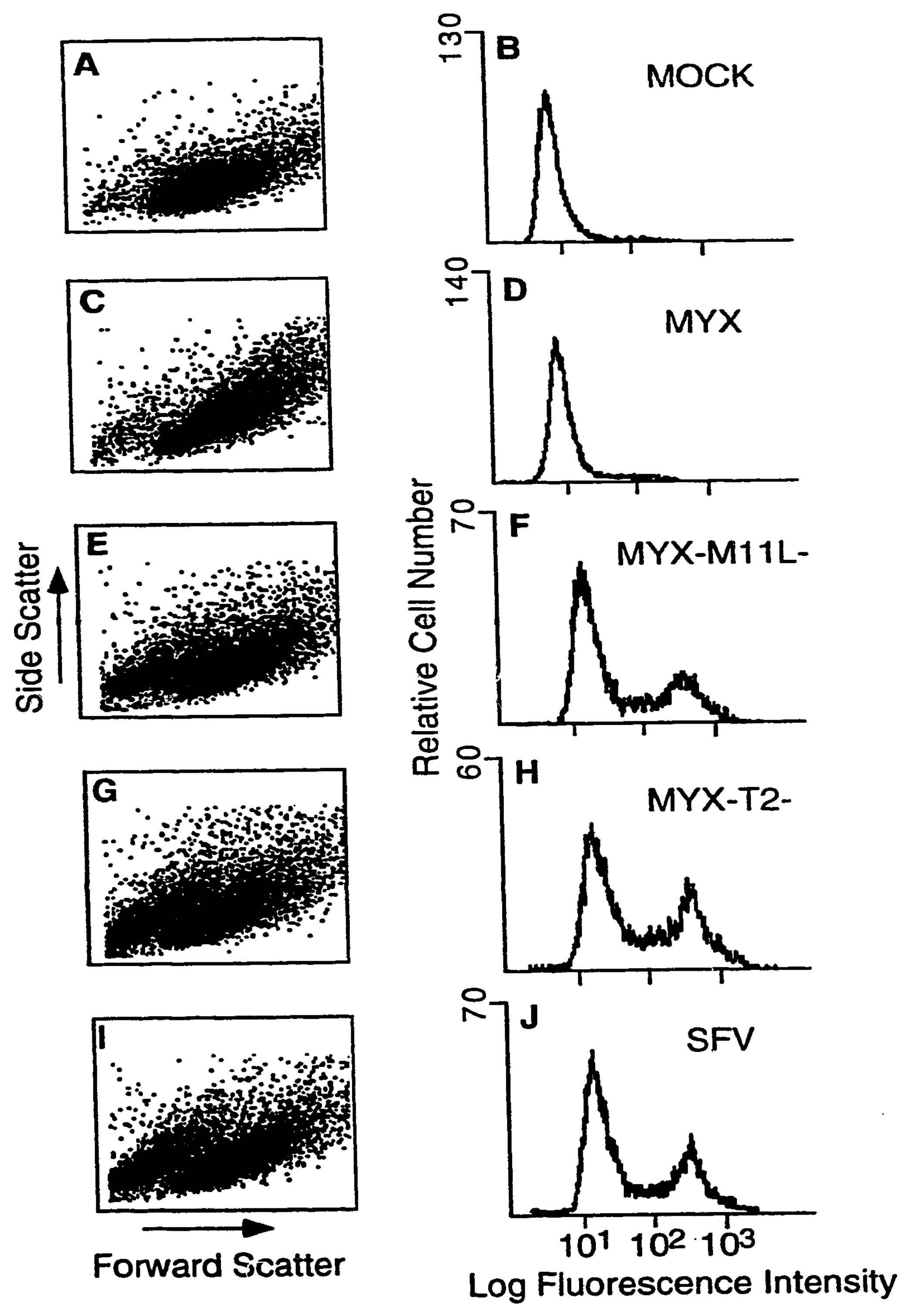
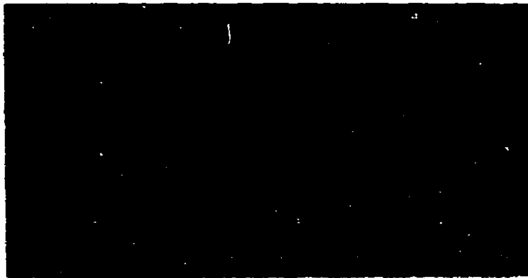

A
C
E
G
I
Side Scatter
Forward Scatter
130
B
MOCK
140
D
MYX
70
F
MYX-M11L-
60
H
MYX-T2-
70
J
SFV
Relative Cell Number
10¹ 10² 10³
Log Fluorescence Intensity

FIGURE IV-4. Microscopic examination of infected RL-5 cells. Cells were either mock infected (panels A and B) or infected at an MOI of 10 pfu/cell with vMyxlac (panels C and D), vMyx-M11L$^-$ (panels E and F), vMyx-T2$^-$ (panels G and H) or SFV (panels I and J). At 16 hours post infection cells were fixed, permeabilized and reacted with TdT and fluorescien-12-dUTP to stain fragmented DNA. Identical fields were photographed under fluorescence (panels A,C,E,G,I) or phase contrast (panels B,D,F,H,I) microscopy.



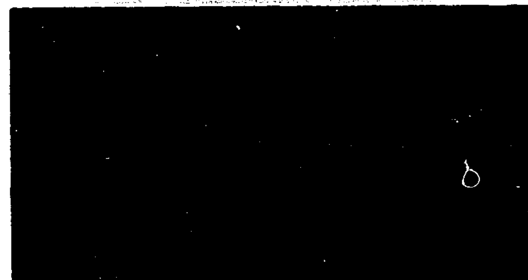
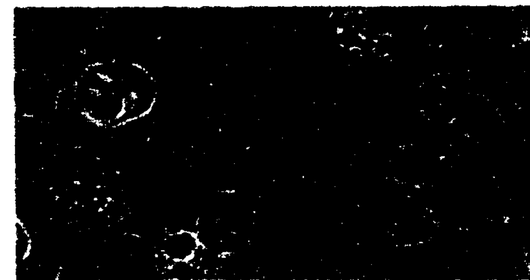
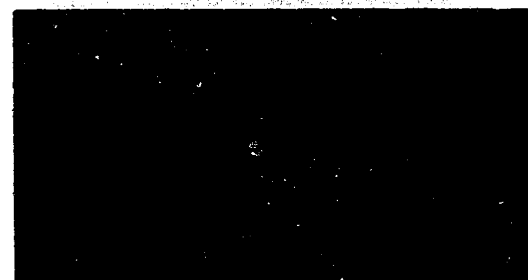
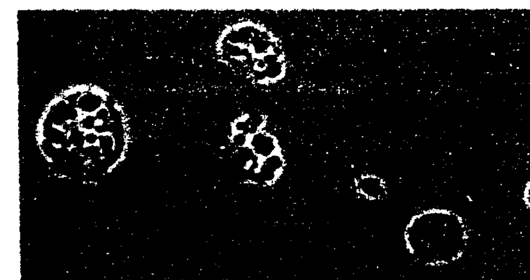
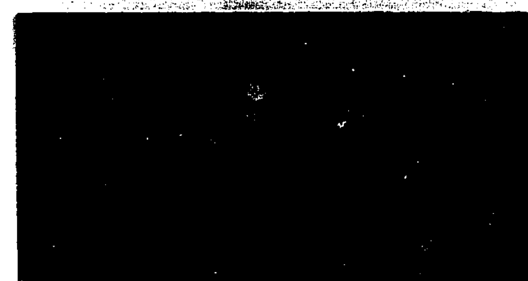






FIGURE IV-5. Replication of SFV, myxoma virus, and gene disruption derivatives of myxoma in RL-5 cells. RL-5 cells were infected at MOI of 3 pfu/cell, and harvested at 24, 48, 72, and 96 hours post infection. Virus titres were determined in duplicate on BGMK cells. Data is expressed as total plaque forming units produced at each time point. The viruses tested were SFV, vMyxlac (Myx), and myxoma virus derivatives with disruptions in the SERP1 (Myx-SERP1⁻), M11L (Myx-M11L⁻) and T2 (Mxy-T2⁻) genes.

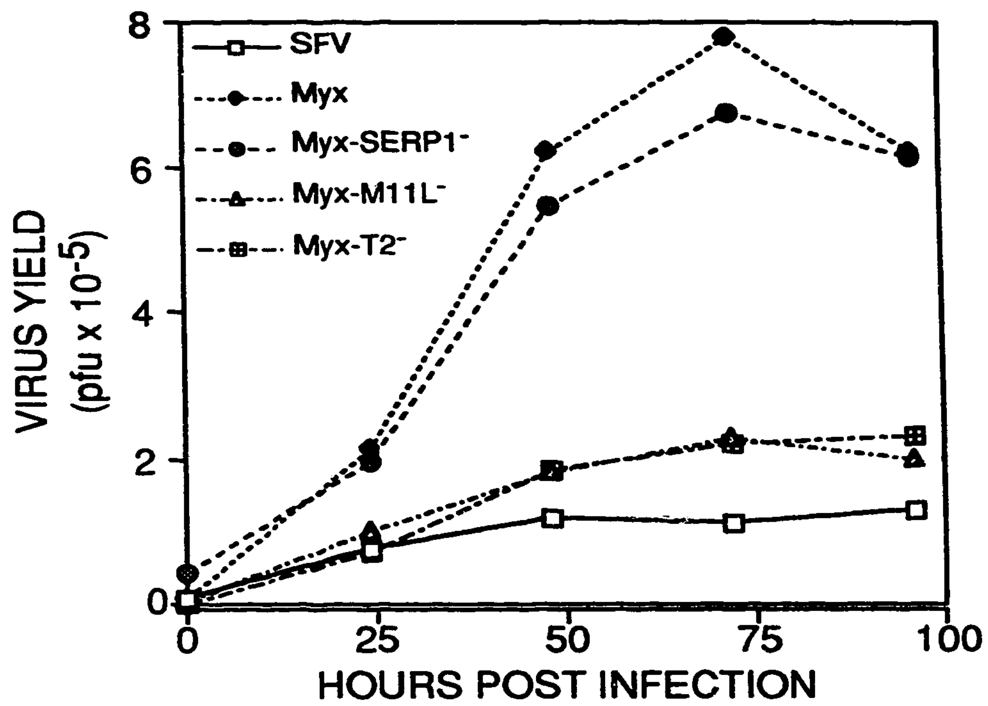


FIGURE IV-5

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CHAPTER V

DIFFERENTIAL INHIBITION OF THE FAS AND GRANULE-MEDIATED CYTOLYSIS PATHWAYS BY THE ORTHOPOXVIRUS CRMA/SPI-2 AND SPI-1 PROTEINS[1]

[1] A version of this chapter has been submitted for publication. Macen, J.L., Garner, R.S., Musy, P.Y., Brooks. M.A., Turner, P.T., McFadden, G., Moyer, R.W. and Bleackley, R.C. Differential inhibition of the Fas and granule-mediated cytolysis pathways by the orthopoxvirus crmA/SPI-2 and SPI-1 proteins. Submitted August, 1995

INTRODUCTION

Cytotoxic T lymphocytes (CTL) are important effectors of antiviral immunity and can affect target cell lysis by one of two pathways, namely granule exocytosis and signalling through the Fas cell surface receptor (for recent reviews, see Berke, 1994; Nagata and Golstein, 1995; Squier and Cohen, 1994). The degranulation pathway is generally considered to be the primary mechanism utilized by CTL as well as natural killer (NK) cells for the elimination of virally-infected and malignant cells. Target cell cytolysis by this mechanism involves the recognition and conjugation of the target cell by the effector cell, followed by a Ca^{2+}-dependent exocytosis of electron-dense cytoplasmic granules which contain a number of proteins that facilitate the demise of the target. Among these are perforin, which in the presence of Ca^{2+} polymerizes to form a pore in the target cell membrane (for review see Liu *et al.*, 1995), and the granzymes (also referred to as cytotoxic cell proteinases or fragmentins), a family of serine proteinases (for review see Smyth and Trapani, 1995).

Although the roles played by all of the granzymes have not been elucidated, studies with transgenic mice bearing a homozygous null mutation in the gene encoding granzyme B indicated a requirement for this protein for the rapid induction of DNA fragmentation and apoptosis in susceptible target cells by both CTL and NK cells (Heusel *et al.*, 1994; Shresta *et al.*, 1995) Others have suggested that in the presence of perforin, a second granule proteinase, granzyme A, can also induce DNA fragmentation in target cells (Hayes *et al.*, 1989; Shiver *et al.*, 1992) and contributes to the lytic activity of CTL (Talento *et al.*, 1992). The generation of transgenic mice deficient in perforin confirmed a central role for this molecule in cytolysis by both CTL and NK cells (Kagi *et al.*, 1994a; Lowin *et al.*, 1994a). Nevertheless, a number of earlier studies indicated that CTL can also lyse some target cells in the absence of calcium and

therefore independent of degranulation and perforin polymerization (MacLennan *et al.*, 1980; Ostergaard and Clark, 1987; Ostergaard *et al.*, 1987; Tirosh and Berke, 1985; Trenn *et al.*, 1987). It was therefore not surprising when other studies with perforin knock-out mice demonstrated that an alternate lytic pathway is also utilized by CTL, involving signalling through the Fas cell surface receptor (Kagi *et al.*, 1994b; Kojima *et al.*, 1994; Lowin *et al.*, 1994b; Walsh *et al.*, 1994).

The Fas antigen (also referred to as APO-1 and CD95) was originally discovered as the target antigen for monoclonal antibodies that caused a rapid induction of apoptotic death in a number of mammalian cells (Trauth *et al.*, 1989; Yonehara *et al.*, 1989). The Fas (APO-1) antigen was subsequently found to be a cell surface protein that belongs to the tumour necrosis factor receptor (TNFR)/nerve growth factor receptor (NGFR) family (Itoh *et al.*, 1991; Oehm *et al.*, 1992). This growing receptor family also includes a number of other receptors found on lymphoid cells, such as CD40 (Stamenkovic *et al.*, 1989), CD30 (Durkop *et al.*, 1992), OX40 (Mallett *et al.*, 1990), 4-1BB (Kwon and Weissman, 1989) and CD27 (Camerini *et al.*, 1991), as well as the Shope fibroma virus and myxoma virus soluble T2 proteins (Smith *et al.*, 1990; Upton *et al.*, 1991). Homology within this family is generally restricted to the extracellular domains of the proteins, which are characterized by three to six cysteine rich subdomains. Limited homology exists within the cytoplasmic domains of two members of the family that transmit cell death signals, Fas and the type I TNFR, in regions termed "death domains", defined by mutational analysis as sequences required for the transmission of a cell death signal (Itoh and Nagata, 1993; Tartaglia *et al.*, 1993). Given the homology between the extracellular domains of the members of this family of receptors, it is not surprising that the ligands for FAS and TNFR also belong to a family of structurally related proteins. Thus, Fas ligand is a type II transmembrane protein that bears significant homology to tumour necrosis factor (Suda *et al.*, 1993), as

well as the ligands for CD40, CD27, CD30 and 4-1BB (Armitage *et al.*, 1992; Goodwin *et al.*, 1993a; Goodwin *et al.*, 1993b; Smith *et al.*, 1993).

Although apoptosis induced by signalling through the Fas cell surface receptor has been the subject of intense investigation, relatively little is known concerning the molecular events involved in the transmission of the cell death signal. Receptor clustering resulting from ligation with either ligand or antibody is thought to initiate the signal, through the interaction between neighboring "death domains" (Boldin *et al.*, 1995). The importance of the death domain for induction of the cell death programme is highlighted by the recent discovery that two novel proteins, FADD and RIP, both of which contain homologous death domains, interact with the death domain of Fas, and result in apoptotic cell death when overexpressed in transfected cells (Chinnaiyan *et al.*, 1995; Stanger *et al.*, 1995). Recent evidence indicates that proteinase activity related to that of the cysteine proteinase interleukin-1ß converting enzyme (ICE), one member of an emerging family of proteinases that are capable of inducing cell death (recently reviewed by Kumar, 1995), is also involved in Fas-mediated apoptosis (Enari *et al.*, 1995; Los *et al.*, 1995; Tewari and Dixit, 1995). Two groups recently reported the identification of a candidate for this apoptosis-inducing proteinase, an ICE-like enzyme designated CPP32 or Yama (Nicholson *et al.*, 1995; Tewari *et al.*, 1995). Thus, while it is not known whether the granule-mediated and Fas-mediated cytolytic mechanisms utilized by cytolytic lymphocytes share common components, proteinase activity has been implicated as an important feature of both pathways. Interestingly, enzymes of the ICE family share a common substrate specificity with granzyme B, cleaving after an Asp residue in the P1 position, suggesting that there may be common substrates involved in cell death induced by the Fas and degranulation pathways.

Among the plethora of proteins encoded by poxviruses that function to interfere with host anti-viral defenses are members of the serpin family of serine proteinase

inhibitors (recently reviewed in Turner *et al.*, 1995). As discussed in detail in Chapter 2, myxoma virus encodes one such serpin, designated SERP1, that is an important virulence factor for virus infection *in vivo* (Macen *et al.*, 1993; Upton *et al.*, 1990). Although the SERP1 protein has been shown to inhibit several human proteinases *in vitro*, the proteinase targeted by SERP1 *in vivo* remains to be identified. The orthopoxviruses, including cowpoxvirus (CPV), rabbitpoxvirus (RPV), and vaccinia virus, each encode three members of the serpin family of proteinase inhibitors, designated SPI-1, SPI-2 and SPI-3 (Turner *et al.*, 1995). Of these, SPI-2 (also called crmA in CPV) has been shown to inhibit the proteolytic activity of ICE (Komiyama *et al.*, 1994; Ray *et al.*, 1992) and ICE-like enzymes such as Yama/CPP32 (Tewari *et al.*, 1995) as well as granzyme B (Quan *et al.*, 1995). Furthermore, CPV SPI-2 can block apoptosis induced by different stimuli including signalling through Fas or the type 1 TNFR (Enari *et al.*, 1995; Los *et al.*, 1995; Tewari and Dixit, 1995). SPI-1, a serpin that is approximately 45% identical to SPI-2, has also been implicated in the inhibition of apoptosis, since certain cells infected with RPV SPI-1 mutants undergo apoptotic cell death (Turner *et al.*, 1995). Because serine proteinase inhibitors have been shown to block cell killing by cytolytic lymphocytes, a possible function for poxvirus-encoded serpins may be the inhibition of cell-mediated cytotoxicity. The studies presented in this chapter were undertaken to address the following questions: 1) Do the poxvirus serpins SERP1, SPI-1 and SPI-2 function to inhibit either granule-mediated or Fas-mediated cytolysis of infected cells by cytolytic lymphocytes? and 2) Can these viral serpins be used as tools with which to probe the mechanisms of either degranulation- or Fas-dependent killing?

In order to address these questions, the cytolysis of a variety of poxvirus-infected target cells was examined using primary murine NK cells or cloned murine cytotoxic T cell lines that kill via either the granule-mediated or the Fas-mediated

pathways. The myxoma virus SERP1 protein, expressed from a recombinant vaccinia virus, was unable to inhibit cytolysis by either NK cells or CTL that kill by either granule-mediated or Fas-mediated cytolytic pathways. In contrast, cells infected with wild type CPV (Brighton red strain) or RPV (Utrecht strain) exhibited resistance to cytolysis by either cytolytic mechanism utilized by CTL. Whereas mutation of the crmA/SPI-2 gene was sufficient to relieve inhibition of Fas-mediated cytolysis, in some cell types mutation of SPI-1, in addition to SPI-2, was necessary to completely abrogate inhibition. In contrast, viral inhibition of granule-mediated killing was unaffected by mutation SPI-2 alone, and was relieved only when both the SPI-2 and SPI-1 genes were inactivated. These results suggest that an ICE-like enzymatic activity is involved in both killing mechanisms and indicate that two viral proteins, SPI-1 and SPI-2, are sufficient to inhibit both cytolysis pathways.

MATERIALS AND METHODS

Cells and viruses

Cytolytic effector cells: Primary cultures of murine NK cells were prepared as described below. CTL21.9 is a cloned murine $H2^k$ cytotoxic T cell line (Havele *et al.*, 1986) that is non-adherent and IL-2 dependent (obtained from R.C. Bleackley, University of Alberta), and requires either antigenic stimulation (irradiated $H2^d$ BALB/c splenocytes) or treatment with anti-CD3 monoclonal antibody for activation of cytolytic activity. CTL21.9 were cultured in RPMI 1640 (Gibco) containing 10% fetal bovine serum (FBS; Gibco) and 10^{-4}M ß-mercaptoethanol. PMM-1 is a BALB/c derived murine CTL hybridoma and MD90 is a mixed lymphocyte culture hybridoma (both obtained from G. Berke, Weizmann Institute of Science, Rehovot, Israel) which

have been described previously (Kaufmann *et al.*, 1981). Cells were maintained in RPMI 1640 (Gibco) containing 10% Fetal Bovine Serum (FCS; Gibco) and 10^{-4}M ß-mercaptoethanol.

Target cells: YAC-1 cells are a murine lymphoma cell line derived from A/sn mice. EL4 cells are a murine ($H2^b$; C57BL/6N) lymphocytic leukemia cell line. L1210 cells are a murine T lymphoma derived from DBA/2 mice and L1210-FAS is an L1210 cell transfectant that expresses the entire murine Fas antigen (Rouvier *et al.*, 1993, both obtained from Dr. Pierre Golstein, CNRS, Marseilles, France). All target cells were maintained in RPMI 1640 containing 5% FBS, and 10^{-4}M ß-mercaptoethanol.

Viruses: Recombinant vaccinia virus VV-S1, expressing the myxoma virus SERP1 protein, and the vector control virus VV601, are both derived from the WR strain and have been described previously (Chapter 2, this thesis and Macen *et al.*, 1993). Cowpox virus (CPV; Brighton Red strain) and rabbitpoxvirus (RPV; Utrecht strain) were both obtained from R.W. Moyer, University of Florida, Gainesville, FL, USA. CPV mutant in the SPI-1 gene (CPVΔSPI-1) has been described (Thompson *et al.*, 1993). RPV mutants in the SPI-1 or SPI-2 genes (RPVΔSPI-1 and RPVΔSPI-2, respectively) and CPV mutant in the SPI-2 gene (CPVΔSPI-2) have been described (Ali *et al.*, 1994). RPV bearing mutations in both the SPI-1 and the SPI-2 genes (RPVΔSPI-1/2) was constructed from RPVΔSPI-1 by homologous recombination leading to replacement of a 447 bp region of the SPI-2 gene with the E.coli lacZ gene driven by the vaccinia virus p11 late promoter (Brooks, M.A., Stern, R.J. and Moyer, R.W., personal communication). All SPI-1 and SPI-2 mutant derivatives of CPV and RPV were obtained from R.W. Moyer (University of Florida, Gainesville, FL, USA).

Preparation of NK cells and NK cell cytolysis assays

NK cells were prepared in the laboratory of Dr. K. Kane (University of Alberta) from either C57BL/6 mice or from SCID mice as follows. Mouse spleens were removed from freshly killed mice and ground in RPMI 1640 containing 3% FBS (RPMI/FBS) to disperse cells. Cells were washed in RPMI/FBS, red blood cells were osmotically lysed and the cells passed over a nylon wool column (Julius *et al.*, 1973) to remove B cells. Eluted cells were washed in RPMI 1640 containing 5% FCS and then incubated at 2 x 10^6 cells/mL in RPMI 1640 containing 10% FBS, 50µM ß-mercaptoethanol and 800 units/mL IL-2 (NK medium) for 72 hours at 37°C. Non-adherent cells were removed by aspiration, and adherent cells were removed from tissue culture dishes with 0.2% (w/v) EDTA, washed in RPMI/FBS, resuspended at 5 x 10^5 cells/mL in NK medium and incubated a further 72 hours at 37°C. Cells were then removed, washed, and incubated in fresh NK medium for 48 to 72 hours and then used in assays as described below.

Cytolysis was measured by ^{51}Cr release assays as follows. YAC-1 cells were adsorbed with virus at a multiplicity of infection (MOI) of 10 plaque forming units (pfu) per cell at a cell density of 5 x 10^6 to 1 x 10^7 cells/mL in RPMI 1640 containing 5% FBS and 10^{-4}M ß-mercaptoethanol (RPMI/5% FBS) for one hour at 37°C with occasional mixing. The cells were diluted to 5 x 10^5 cells/mL in RPMI/5%FBS and incubated for 12 hours at 37°C. Cells were mock-infected by incubating cells in an identical fashion in the absence of virus. The cells were labelled with ^{51}Cr as follows: 5-10 x 10^6 cells were resuspended in ~85µL FBS, 100µL $Na_2{}^{51}CrO_4$ (100µCi; Dupont NEN) was added and the cells were incubated at 37°C for 1 hour with occasional mixing. Labelled cells were washed five times in RPMI/FBS, diluted to 1 x 10^5 cells/mL in RPMI 1640 containing 5% FBS, and added to 95-well V-bottom plates (50µL/well). Where indicated, purified SERP1 protein, prepared as described (Chapter 2, this thesis) was then added to labelled cells such that the final SERP1 concentration

was 2.5 µg/mL. Finally, NK cells were added at an effector to target ratio of either 12:1 or 20:1. The final volume in each well was 200µL. Each sample was assayed in trriplicate. The plates were spun for 3 minutes at 1000rpm in an IEC centrifuge and then incubated at 37°C for 5 hours in a humidified CO_2 incubator. ^{51}Cr release was determined by removing 100 µL of culture medium from wells (sample) or by resuspending cells and then removing 100µL of suspension (total) and counting in a gamma counter. Spontaneous ^{51}Cr release was determined by incubating labelled target cells for 5 hours in the absence of NK effector cells in 200µL/well and then removing 100µL of culture medium for counting. % specific lysis was calculated as follows: %specific lysis=(cpm sample-cpm spontaneous release)/(cpm total-cpm spontaneous release)x100.

Activation of cytolytic T lymphocytes and cytolysis assays

CTL21.9 cells were stimulated by incubating the cells for 24 hours at 37°C with a 1/250 dilution of 2C11 hybridoma supernatant (anti CD3 monoclonal antibody, Leo *et al.*, 1987) in RPMI 1640 containing 10% FBS, 10^{-4}M ß-mercaptoethanol and 60 u/mL IL-2, at a density of 5 x 10^5 cells/mL. MD90 and PMM-1 cells were stimulated for 3 hours prior to assay by incubating the cells in phorbol-12-myristate-13-acid (PMA; 10 ng/mL; Sigma) and ionomycin (3 µg/mL; Sigma) at 37°C.

Cytolysis of infected target cells was measured by ^{51}Cr release assays as follows. Target cells (EL4, L1210, L1210-Fas, or YAC-1) were either mock-infected or infected with the indicated virus at a MOI of 10 pfu/cell, as described above, in RPMI/5% FBS and 10^{-4}M ß-mercaptoethanol, for 12 hours. Target cells were labelled with ^{51}Cr as follows: approximately 2 x 10^6 cells were pelleted by centrifugation, resuspended in 100 µL (100 µCi) $Na_2{}^{51}CrO_4$ (Dupont NEN) and incubated at 37°C for 90 minutes. Cells were pelleted, resuspended in 10 mL RPMI/5% FBS and incubated

at 37°C for 30 minutes and then washed twice with phosphate buffered saline. Cells were resuspended at a density of 10^5cells/mL in RPMI/5% FBS, and added to 96-well V-bottom plates (1×10^4 cells/well) in triplicate. Where indicated, purified SERP1 protein (Chapter 2, this thesis) was added to the wells such that the final SERP1 concentration was 1 µg/mL. Effector cells (5×10^4 cells/well) were added to each well and the final volume/well was 200 µL. In some assays, concanavalin A was added at a final concentration of 2 µg/mL to facilitate effector cell-target cell interactions. Spontaneous ^{51}Cr release was determined by incubating target cells in the absence of effector cells.The plates were centrifuged at 500 rpm for 5 minutes, and then incubated at 37°C for 4 hours. The plates were then centrifuged at 1200 rpm for 5 minutes to pellet cells and samples were harvested as described above for the NK cell cytolysis assays. % specific lysis was determined as described above.

RESULTS

The myxoma virus SERP1 protein does not inhibit NK cell-mediated cytolysis of YAC-1 cells

Because NK cells lyse their targets by a granule-mediated mechanism that is inhibitable by proteinase inhibitors, studies were initiated to determine whether the myxoma virus SERP1 protein was able to inhibit NK cell-mediated cytolysis. Cytolysis of YAC-1 cells by NK effector cells can be mimicked by incubating YAC-1 cells with purified fragmentin 2 (granzyme B) and cytolysin (perforin) (Shi *et al.*, 1992a; Shi *et al.*, 1992b). When purified SERP1 protein (Chapter II, this thesis) was preincubated with purified cytolysin and purified fragmentin 2 and then added to YAC-1 cells, cytolysis was effectively inhibited (A.Greenberg and L.Shi, personal communication).

This result suggested that it would be worthwhile to directly test whether the myxoma virus SERP1 protein might function to inhibit NK cell-mediated killing of infected cells. Therefore, experiments were conducted to determine whether the SERP1 protein could inhibit cytolysis in a more physiologically relevant system, using intact NK cells as cytolytic effectors and virus-infected YAC-1 cells as targets.

Because myxoma virus is not able to productively infect murine cells, the recombinant vaccinia virus, VV-S1 (Chapter II, this thesis) was used to express the SERP1 protein in YAC-1 cells. NK cells were prepared from either C57BL/6 mice, or from B6 SCID mice. YAC-1 cells were either mock infected or infected with VV-S1 or the vector control virus VV601 (Chapter II, this thesis) for 12 hours at a MOI of 10 pfu/cell in the presence or absence of exogenous purified SERP1 protein. The cells were then harvested, and assayed for cytolysis by NK cells in standard ^{51}Cr release assays as described in Materials and Methods. The results of two such assays, given in Table V-1, clearly indicate that the myxoma virus SERP1 protein is completely unable to inhibit NK cell mediated cytolysis of YAC-1 cells under these conditions. While as little as 400 ng/mL of purified SERP1 protein was able to almost completely inhibit cytolysis of YAC-1 cells by purified cytolysin and fragmentin 2 (A. Greenberg and L.Shi, personal communication), incubation of either infected or uninfected YAC-1 cells with 2.5 µg/mL of purified SERP1 protein had no effect on YAC-1 cell cytolysis using intact NK cells derived from either C57BL/6 or SCID mice as cytolytic effectors. Therefore the SERP1 protein does not appear to function to inhibit NK cell mediated cytolysis of infected cells.

The myxoma virus SERP1 protein does not inhibit either granule-mediated or Fas-mediated cytolysis of infected target cells by cloned CTL

The following experiments were then performed in order to determine whether the myxoma virus SERP1 protein could inhibit CTL-mediated cytolysis of infected cells. Three cloned murine CTL lines were used as effector cells in these experiments: CTL21.9, PMM-1 and MD90. CTL21.9 is a cytotoxic T cell line (Havele *et al.*, 1986) that is IL-2 dependent and requires stimulation (either irradiated BALB/c splenocytes or anti-CD3 monoclonal antibody 2C11(Leo *et al.*, 1987)) for cytotoxic activity. It has previously been demonstrated that murine L1210 and EL4 cells are efficiently lysed by stimulated CTL21.9 cells in a calcium-dependent reaction involving the secretion of cytoplasmic granules (Garner *et al.*, 1994). In contrast, both MD90, derived from a mixed lymphocyte culture, and PMM-1, derived from primary peritoneal exudate lymphocytes, are cytolytic hybridomas (both described in Kaufmann *et al.*, 1981) that do not contain cytoplasmic granules and do not express either perforin or granzymes (Helgason *et al.*, 1992), and lyse cells via the Fas lytic pathway (Garner *et al.*, 1994).

To determine whether the SERP1 protein had any effect on the granule-mediated cytolysis of target cells, EL4 and L1210 cells were either mock infected or infected at a MOI of 10 pfu/cell with the recombinant vaccinia virus, VV-S1, that expresses the SERP1 protein, or VV601, the vector control virus, in the presence or absence of purified SERP1 protein (1 µg/mL). The cells were harvested at 12 hours post infection and assayed in triplicate by ^{51}Cr release as described in Materials and Methods. Representative results from a single experiment are given in Table V-2. The data clearly indicate that SERP1 has no inhibitory effect on cytolysis of infected cells. In the case of EL4 cells, neither vaccinia virus infection itself, nor the presence of purified SERP1 protein had any effect on the levels of cytolysis. When L1210 cells were used as targets, a small but reproducible reduction in the level of cytolysis was observed when the cells were infected with either VV601 or VV-S1. However, the SERP1 protein, either expressed from the virus or added exogenously, had no effect on

target cell cytolysis. Therefore, as seen for NK cell-mediated cytolysis, the myxoma virus SERP1 protein is unable to inhibit granule-mediated cytolysis by CTL21.9 effector cells.

In addition to the granule exocytosis pathway, CTL are also capable of lysing target cells via the Fas lytic pathway. Because proteinase activity is also required for Fas-mediated cytolysis, experiments were undertaken to determine whether the SERP1 protein could inhibit this killing pathway. Three different Fas expressing cell lines were used as target cells in these experiments: EL4, YAC-1, and L1210-Fas (Rouvier *et al.*, 1993, an L1210 cell transfectant that expresses the entire murine Fas protein), all of which have been demonstrated to be killed efficiently by either MD90 or PMM-1 effector CTLs (Garner *et al.*, 1994). In addition, L1210 cells were used as targets to demonstrate the dependence on Fas expression for cytolysis by both agranular effector cell lines. Again, target cells were either mock-infected or infected at a MOI of 10 pfu/cell with the recombinant vaccinia viruses VV-S1 or VV601, and assayed for cytolysis by ^{51}Cr release 12 hours post infection. Representative data are shown in Tables V-3 (PMM-1 effectors) and V-4 (MD90 effectors). As expected, mock-infected L1210-Fas cells were lysed to a much higher level than L1210 cells, confirming a Fas-dependent killing mechanism. A reproducible reduction in cytolysis of all target cell lines tested was observed upon infection of the cells with either the control vaccinia virus, VV601, or the SERP1 expressing vaccinia virus, VV-S1. Therefore infection with vaccinia virus itself appears to result in an inhibition of Fas-mediated cytolysis by approximately 50%. Expression of the SERP1 protein again had no effect on the observed levels of cytolysis of any of the target cells tested. Taken together, these results suggest that the SERP1 protein does not function to inhibit cell-mediated cytolysis of infected cells.

Infection with either RPV or CPV results in a partial inhibition of granule-mediated cytolysis by CTL21.9 effector cells

The orthopoxviruses cowpoxvirus (CPV), and rabbitpox virus (RPV) each encode three members of the serpin family of proteinase inhibitors. In order to determine whether the expression of the viral SPI-1 and/or SPI-2 proteins impairs the function of cytolytic lymphocytes, the cytolysis of a variety of target cells infected with wild type CPV and RPV or CPV and RPV bearing specific mutations in the SPI-1 and SPI-2 genes was examined using the cloned CTL lines, described above, that kill cells by either the granule-mediated or the Fas-mediated pathways.

The first question addressed was whether virus-infected cells were resistant to granule-mediated cytolysis by CTL. EL4 cells and L1210 cells were infected at a MOI of 10 pfu per cell for 12 hours with either wild type CPV (Brighton Red strain) or RPV (Utrecht strain) and then assayed for cytolysis by CTL21.9 effector cells in standard ^{51}Cr release assays. Infection of L1210 cells with wild type CPV resulted an a dramatic and reproducible inhibition of cytolysis by CTL21.9 (Figure V-1a) compared to mock-infected cells, while infection with RPV resulted in only a moderate inhibition, which was nevertheless reproducible. Infection of EL4 cells with either CPV or RPV resulted in a reduction in cytolysis by CTL21.9 of approximately 50% (Figure V-1b). Cytolysis was abrogated when the assays were carried out in the presence of EGTA, confirming a calcium-dependent killing mechanism (data not shown).

Infection with either RPV or CPV results in a partial inhibition of Fas-mediated cytolysis by PMM-1 effector cells

Similar experiments were then performed to determine whether infection with CPV or RPV interfered with Fas mediated cytolysis. PMM-1 cells were used as cytolytic effectors in these experiments. Three different Fas expressing target cell lines

were used: EL4, L1210-Fas, and YAC-1. The results shown in Figure V-2 demonstrated that CPV and RPV both reproducibly inhibited the Fas-mediated cytolysis of infected L1210-Fas, EL4 and YAC-1 cells, with RPV infected cells showing the greatest inhibition (Figure V-2a,b,c). The dependence on Fas expression for cytolysis by PMM-1 is again demonstrated in Figure V-2d, in which L1210-Fas cells were lysed efficiently, while the parental L1210 cells were not. In addition, killing by PMM-1 effectors was unaffected by the presence of EGTA (data not shown), confirming a calcium-independent killing mechanism.

Effect of mutation of the CPV and RPV SPI-1 and SPI-2 genes on viral inhibition of Fas-mediated cytolysis by PMM-1 effector cells

The CPV SPI-2 protein can block Fas-mediated cytolysis of transfected cells, and inhibits the proteolytic activity of both ICE and granzyme B in vitro (Komiyama *et al.*, 1994; Quan *et al.*, 1995). In addition to SPI-2, the orthopoxviruses encode two other members of the serpin family of proteinase inhibitors. Of interest is the viral SPI-1 gene, which encodes a protein that shares approximately 45% amino acid identity with SPI-2, but for which no biochemical activity has yet been described. CPV and RPV containing specific disruption mutations in the SPI-1 and SPI-2 genes were used to ask whether these proteinase inhibitors mediated the observed inhibition of cell-mediated cytolysis. Cells infected with CPV containing a mutation in the SPI-2 gene were lysed by PMM-1 effectors similarly to mock-infected cells (Figure V-3a,b,c) whereas cells infected with a CPV mutant in the SPI-1 gene showed comparable inhibition to wild type CPV-infected cells. Similarly, mutation of the RPV SPI-2 gene relieved virus-mediated inhibition of cytolysis of infected L1210-FAS cells (Figure V-3d) almost completely, but only partially in EL4 and YAC-1 cells (Figure V-3e,f). As observed for CPV-infected cells, mutation of the SPI-1 gene alone did not alter virus-

mediated inhibition of cytolysis. However, mutation of both the SPI-1 and SPI-2 genes completely abrogated RPV-mediated inhibition of cytolysis in EL4 and YAC-1 cells (Figure V-3e,f). Western blotting of lysates prepared from the same target cells used in these experiments, using antisera specific for the viral SPI-1 and SPI-2 proteins, confirmed that these viral proteins are synthesized in these cells (R.W. Moyer, personal communication). Together, these results suggest that, in agreement with results reported by others using cells transfected with the SPI-2 (crmA) gene (Enari *et al.*, 1995; Los *et al.*, 1995; Tewari and Dixit, 1995), SPI-2 is able to inhibit Fas-mediated apoptosis. In addition, the results obtained with the RPV serpin gene mutants indicate that SPI-1 contributes to viral inhibition of Fas-mediated killing in certain cell types (EL4 and YAC-1) and suggests that the expression of both the SPI-1 and the SPI-2 genes are sufficient to block Fas-mediated cytolysis of infected cells.

Effect of mutation of the CPV and RPV SPI-1 and SPI-2 genes on viral inhibition of granule-mediated cytolysis by CTL21.9 effector cells

In contrast to the results obtained using PMM-1 effector cells, mutations in the viral SPI-2 genes alone did not relieve the observed virus-mediated inhibition of granule-mediated cytolysis by CTL21.9 effector cells (Figure V-4a-d). Similarly, mutations in the viral SPI-1 genes alone also had no effect (Figure V-4). However, in both EL4 and L1210 cells infected with RPV bearing mutations in both the SPI-1 and the SPI-2 genes, no inhibition of cytolysis was observed (Figure V-4c,d). Since the overall levels of inhibition of CTL21.9-mediated cytolysis are higher for CPV than for RPV, it would be interesting to examine a double mutant in the CPV SPI-1 and SPI-2 genes. Unfortunately, a double mutant in CPV was not available for study. Nevertheless, these results suggest that, while inhibition of Fas-mediated cytolysis can be relieved by mutation of the SPI-2 gene alone, loss of inhibition of granule-mediated

cytolysis by CTL21.9 effector cells requires mutation of both the SPI-1 and SPI-2 genes.

DISCUSSION

The myxoma virus encoded serpin, SERP1, has been shown to be an important virulence factor for virus infection *in vivo* (Chapter 2, this thesis and Macen *et al.*, 1993; Upton *et al.*, 1990). Although the SERP1 protein has been shown to be a *bona fide* serine proteinase inhibitor (Lomas *et al.*, 1993), whether it exerts its effect on virus virulence *in vivo* through the inhibition of one or more serine proteinases, and the identity of these proteinases, remain to be determined. Studies conducted using purified SERP1 protein and purified cytolysin and fragmentin 2 initially suggested that SERP1 could inhibit YAC-1 cell cytolysis induced by the NK cell granule proteins cytolysin and fragmentin 2 (L. Shi and A. Greenberg, personal communication). It was therefore of interest to determine whether the SERP1 protein could function to block NK cell-mediated cytolysis of virus-infected cells. The studies presented here indicate that the SERP1 protein, expressed from the recombinant vaccinia virus VV-S1, was unable to prevent NK cell mediated cytolysis of YAC-1 cells. Purified SERP1 protein added to either infected or uninfected YAC-1 target cells prior to the addition of effector cells, at a concentration in excess of that found to inhibit cytolysis of YAC-1 cells by purified cytolysin and fragmentin 2, was also unable to inhibit NK cell-mediated cytolysis. The basis for the inhibition of YAC-1 cell cytolysis using purified proteins remains to be determined. Preliminary experiments using purified SERP1 protein indicated that SERP1 is unable to bind to or inhibit the proteolytic activity of purified fragmentin 2 (J.L. Macen, R.C.Bleackley and G. McFadden, unpublished observations). Together, these data support the conclusion that the SERP1 protein does not function as an

inhibitor of NK cell-mediated cytolysis. Furthermore, these results suggest that the ability of purified SERP1 to inhibit cytolysis of YAC-1 cells by purified cytolysin and fragmentin 2 is not likely to be of physiological relevance.

Because serine proteinase inhibitors can block CTL-mediated cytolysis of target cells, experiments were conducted in order to determine whether the myxoma virus SERP1 protein could block CTL-mediated lysis of infected target cells using cloned murine CTL lines that kill target cells by either the degranulation pathway or the Fas-dependent pathway. The results presented here indicate that the SERP1 protein, either expressed from vaccinia virus or added exogenously as purified protein, is unable to inhibit cytolysis by these effector cells. When granule-containing CTL (CTL21.9) were used as cytolytic effectors, a very slight, but reproducible, reduction in the CTL21.9-mediated cytolysis of L1210 cells was observed when cells were infected with vaccinia virus itself, but again, the SERP1 protein had no effect on the observed levels of cytolysis. When Fas-dependent effector cells (MD90 or PMM-1) were used as cytolytic effectors, the level of cytolysis of infected cells was approximately 50% of the level observed for mock-infected cells, regardless of the effector cell examined (EL4, L1210-Fas, or YAC-1). However, as observed for the other effector cells used, neither the expression of the SERP1 protein from vaccinia virus nor the addition of exogenous purified SERP1 protein had any effect on cytolysis. Therefore, although vaccinia virus infection itself may result in partial inhibition of cytolysis with some effector cell-target cell combinations, the SERP1 protein is unable to inhibit cell-mediated cytolysis in the assays used. Since vaccinia virus itself encodes homologues of the SPI-1 and SPI-2 proteins, the observed inhibition of cytolysis during vaccinia virus infection is likely to be the result of the expression of these proteinase inhibitors.

Others have shown that the CPV SPI-2 protein is capable of inhibiting apoptosis induced by a variety of signals in transfected mammalian cells (eg., Boudreau

et al., 1995; Enari *et al.*, 1995; Gagliardini *et al.*, 1994; Los *et al.*, 1995; Miura *et al.*, 1993; Tewari and Dixit, 1995; Tewari *et al.*, 1995). Because CPV SPI-2 has been demonstrated to function as an inhibitor of ICE and ICE-related proteinases (Komiyama *et al.*, 1994; Ray *et al.*, 1992; Tewari *et al.*, 1995), the data presented here support a model in which Fas-mediated apoptotic cell death induced by CTL requires the proteolytic activity of an ICE-related proteinase. EL4, L1210-Fas and YAC-1 cells infected with wild type CPV and RPV express the SPI-1 and SPI-2 proteins and suppress Fas-mediated cytolysis induced by CTL. Viruses bearing specific mutations in the SPI-2 gene show variable loss of this inhibition, depending on both the virus and target cell examined. Thus, the inhibition of Fas-mediated cytolysis during infection with RPV and CPV can be attributed to the SPI-2 protein for L1210-Fas, EL4 and YAC-1 cells infected with CPV, and for L1210-Fas cells infected with RPV. Unexpectedly, for both EL4 cells and YAC-1 cells infected with RPV, mutation of the SPI-2 protein did not relieve the observed viral inhibition of cytolysis. In these cells, inhibition was lost when the cells were infected with a double mutant in both the SPI-1 and SPI-2 genes. Therefore both the SPI-1 and SPI-2 genes contribute to the observed inhibition of Fas-mediated cytolysis. A possible explanation for the difference observed between CPV and RPV mutants in the SPI-2 gene with respect to cytolysis of infected EL4 and YAC-1 cells may lie in the expression levels of the viral SPI-1 genes. Although the expression of the SPI-1 protein in these cells was verified by immunoblotting (R.W.Moyer and J.L. Macen, unpublished observation) the amount of protein expressed was not quantitated. However, it has been observed in other cell types that the level of SPI-1 expression in CPV-infected cells is much lower than that in RPV-infected cells (R.W. Moyer, personal communication). Nevertheless, the results reported here indicate a role for the SPI-1 protein in the inhibition of Fas-mediated cytolysis that has not been previously described.

Studies of granule-mediated cytolysis by CTL21.9 effector cells indicated that, in contrast to Fas-mediated killing, the CPV SPI-2 protein alone was not responsible for the observed inhibition. Comparable results were obtained in studies of RPV SPI-2 mutants, although there was more variability in the overall levels of inhibition. When target cells were infected with RPV bearing mutations in both the SPI-1 and SPI-2 genes, however, cytolysis levels were comparable to mock-infected cells. Therefore the data indicate that both the SPI-1 and the SPI-2 genes contribute to inhibition of cytolysis in this system.

Taken together, the data presented here confirm a role for SPI-2 in the inhibition of Fas-mediated cell death and provide preliminary evidence that the orthopoxviral SPI-1 proteins may play a role in inhibition of apoptotic cell death induced by CTL that kill by either the degranulation or Fas-dependent pathways. Since others have shown that mutation of the RPV SPI-1 gene results in an inability of the virus to plaque on PK-15 or A549 cells (Ali *et al.*, 1994) due to the induction of apoptosis (Ali *et al.*, 1994; Turner *et al.*, 1995), the viral SPI-1 proteins may represent useful tools for the identification of additional proteolytic activities involved in apoptotic cell death. Furthermore, the data presented here provide evidence that the Fas-dependent and degranulation pathways of cell-mediated cytolysis share common components, and that two proteolytic activities, inhibitable by SPI-1 and SPI-2, may be sufficient to inhibit both cytolysis pathways. Nevertheless, a large body of evidence indicates that cells infected with recombinant vaccinia viruses expressing foreign antigens are efficiently recognized and lysed by both primary CTL and CTL lines (reviewed in Bennink and Yewdell, 1990), even though vaccinia virus itself encodes homologues of the SPI-1 and SPI-2 proteins. However, it is not possible to directly compare these studies with those reported here since the status of the SPI-1 and SPI-2 proteins in the recombinant vaccinia viruses is unknown. Furthermore, the ability of the SPI-1 and/or SPI-2

proteins to interfere with apoptotic death of infected cells may be dependent on the type of cell infected, the host species, and the evolutionary history of the virus. For example, the results reported here indicate differences in the ability of the SPI-1 and SPI-2 proteins to inhibit cytolysis depending upon both the virus and the target cells used. In addition, while mutation of either the SPI-1 or the SPI-2 genes in vaccinia virus does not result in virus attenuation in infected mice (Kettle *et al.*, 1995), mutation of the SPI-1 or SPI-2 genes of RPV does result in virus attenuation in infected rabbits, and the RPV SPI-1/SPI-2 double mutant is highly attenuated in infected rabbits compared to either single mutant (M.A.Brooks, R.J.Stern and R.W. Moyer, in preparation). This suggests that, at least in the case of RPV, the prevention of apoptotic cell death during infection may be a critical determinant of virus virulence and suggests a rationale for the evolution of the multiple anti-proteolytic activities expressed by the orthopoxviruses.

TABLE V-1

Effect of SERP1 on NK cell-mediated cytolysis of YAC-1 cells infected with recombinant vaccinia viruses*

	NK Cells from C57BL/6 mice E:T=12:1		NK Cells from SCID mice E:T=20:1	
SERP1 protein†	+	-	+	-
MOCK	24.3 +/- 3.1	27.8 +/- 2.4	43.0 +/- 2.0	40.9 +/- 0.9
VV601	27.4 +/- 2.3	33.3 +/- 1.2	50.7 +/- 2.5	47.0 +/- 2.4
VV-S1	35.5 +/- 1.6	35.4 +/- 2.9	50.4 +/- 0.6	42.5 +/- 3.3

*YAC-1 cells were either mock-infected or infected with recombinant vaccinia viruses at MOI=10pfu/cell, and harvested for labelling and assayed in triplicate by chromium release (see Materials and Methods) at 12 hours post-infection. Values represent mean % specific lysis and standard error, where % specific lysis = (sample - spontaneous release)/(total - spontaneous release)x100.

†Where indicated, purified SERP1 protein was added to the target cells immediately prior to addition of NK cells, at a concentration of 2.5 µg/mL.

TABLE V-2

Effect of SERP1 on CTL21.9 -mediated (granule-mediated) cytolysis of cells infected with recombinant vaccinia viruses‡

	EL4		L1210	
SERP1 protein§	+	-	+	-
MOCK	38 +/- 1.9	38 +/- 1.7	82 +/- 3.3	81 +/- 5.3
VV601	34 +/- 0.8	39 +/- 3.0	69 +/- 1.5	61 +/- 3.9
VV-S1	39 +/- 0.9	37 +/- 3.2	67 +/- 0.3	65 +/- 0.5

‡Cells were infected and assayed as described in Table V-1. Values represent mean % specific lysis and standard error.

§Where indicated, purified SERP1 protein was added to the target cells immediately prior to addition of effector cells, at a concentration of 1.0 µg/mL.

TABLE V-3

Effect of SERP1 on Fas-mediated cytolysis of cells infected with recombinant vaccinia viruses

	PMM-1 effector cells*			
	EL4	L1210	L1210-Fas	YAC-1
MOCK	28 +/- 0.9	10 +/- 1.0	75 +/- 2.1	46 +/- 1.7
VV601	18 +/- 1.0	3.4 +/- 0.5	44 +/- 0.8	21 +/- 1.7
VV-S1	15 +/- 0.3	4.7 +/- 0.6	51 +/- 0.7	25 +/- 1.3

*Cells were either mock-infected or infected with recombinant vaccinia viruses at MOI=10pfu/cell, and harvested for labelling and assayed in triplicate by chromium release (see Materials and Methods) at 12 hours post-infection. Values represent mean % specific lysis and standard error, where % specific lysis = (sample - spontaneous release)/(total - spontaneous release)x100.

TABLE V-4

Effect of SERP1 on Fas-mediated cytolysis of cells infected with recombinant vaccinia viruses

	MD90 effector cells†			
	EL4	L1210	L1210-Fas	YAC-1
MOCK	15 +/- 1.6	5.3 +/- 0.4	66 +/- 1.5	38 +/- 0.7
VV601	6.3 +/- 0.6	2.0 +/- 0.5	39 +/- 1.4	18 +/- 1.4
VV-S1	6.7 +/- 1.5	3.6 +/- 0.5	44 +/- 1.9	22 +/- 0.5

†Cells were infected and assayed as described in Table V-3. Values represent mean % specific lysis and standard error.

Figure V-1. Inhibition of granule-mediated cytolysis of cells infected with CPV or RPV. L1210 (*A*) or EL4 (*B* cells were either mock-infected or infected with CPV or RPV and cytolysis was assayed by incubation with stimulated CTL21.9 effector cells in a standard 4 hour chromium release assay (see below) at an effector to target ratio of 5:1. To facilitate comparison of multiple experiments, the values for %specific lysis for mock-infected cells were set to 100%, and other data are shown relative to mock infected cells. The range of values for % specific lysis in individual experiments was 67% to 75% for L1210 cells and 19% to 70% for EL4 cells. Each bar represents the mean and standard deviation calculated from 3-4 independent experiments, each performed in triplicate. Relative % specific lysis = mean % specific lysis (virus infected cells)/mean % specific lysis (mock infected cells).

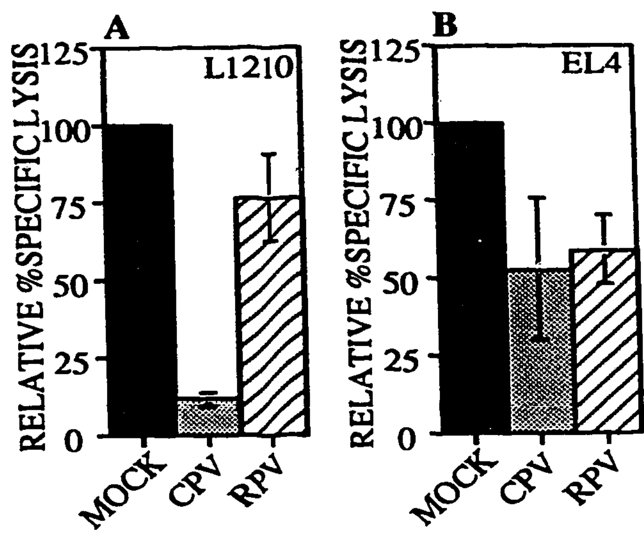

FIGURE V-1

Figure V-2. Inhibition of Fas-mediated cytolysis of virus-infected L1210-Fas, EL4 and YAC-1 cells. L1210-Fas (*A*), EL4 (*B*) and YAC-1 (*C*) cells were either mock-infected or infected with the indicated virus as described in Materials and Methods. Cytolysis of target cells was assayed by incubation with stimulated PMM-1 effector cells in a standard 4 hour chromium release assay at an effector to target ration of 5:1. To facilitate comparison of multiple experiments, the values for % specific lysis for mock-infected cells were set to 100%, and other data are shown relative to mock infected cells. The range of values for % specific lysis in individual experiments was 63% to 82% for mock-infected L1210-Fas cells, 7% to 18% for mock-infected EL4 cells, and 33% to 55% for mock-infected YAC-1 cells. Each bar represents the mean and standard deviation calculated from 3 independent experiments, each performed in triplicate. d, L1210-FAS and L1210 cells were either mock-infected or infected with CPV or RPV, and incubated with stimulated PMM-1 effector cells at an effector to target ratio of 5:1 and % specific chromium release was determined. Representative data from a single experiment performed in triplicate are shown.

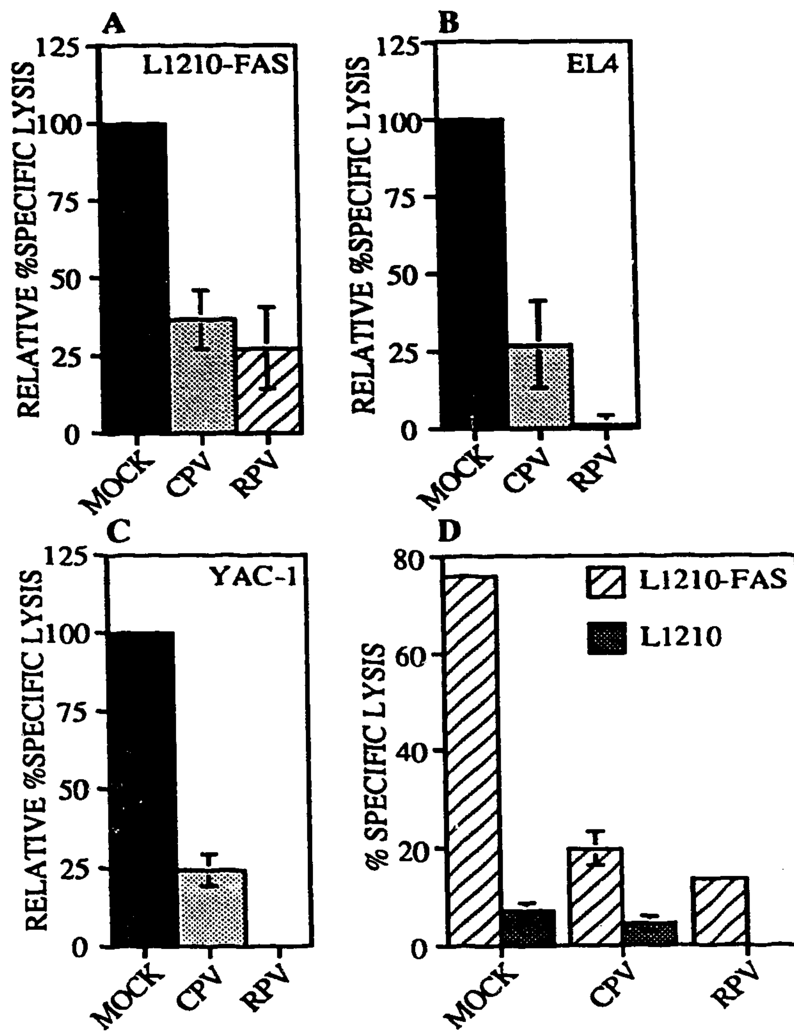

FIGURE V-2

Figure V-3. Fas-mediated cytolysis of target cells infected with virus mutants in the SPI-1 or SPI-2 genes. L1210-FAS (A,D), EL4 (B,E), or YAC-1 (C,F) were either mock-infected or infected with either wild type CPV, a CPV mutant in the SPI-1 gene (CPVΔSPI-1), a CPV mutant in the SPI-2 gene (CPVΔSPI-2), wild type RPV, or RPV mutants in the SPI-1, SPI-2, or both SPI-1 and SPI-2 genes (RPVΔSPI-1, RPVΔSPI-2, RPVΔSPI-1/2, respectively). Data are shown as %specific lysis relative to mock infected cells and are the mean and standard deviations calculated from three independent experiments.

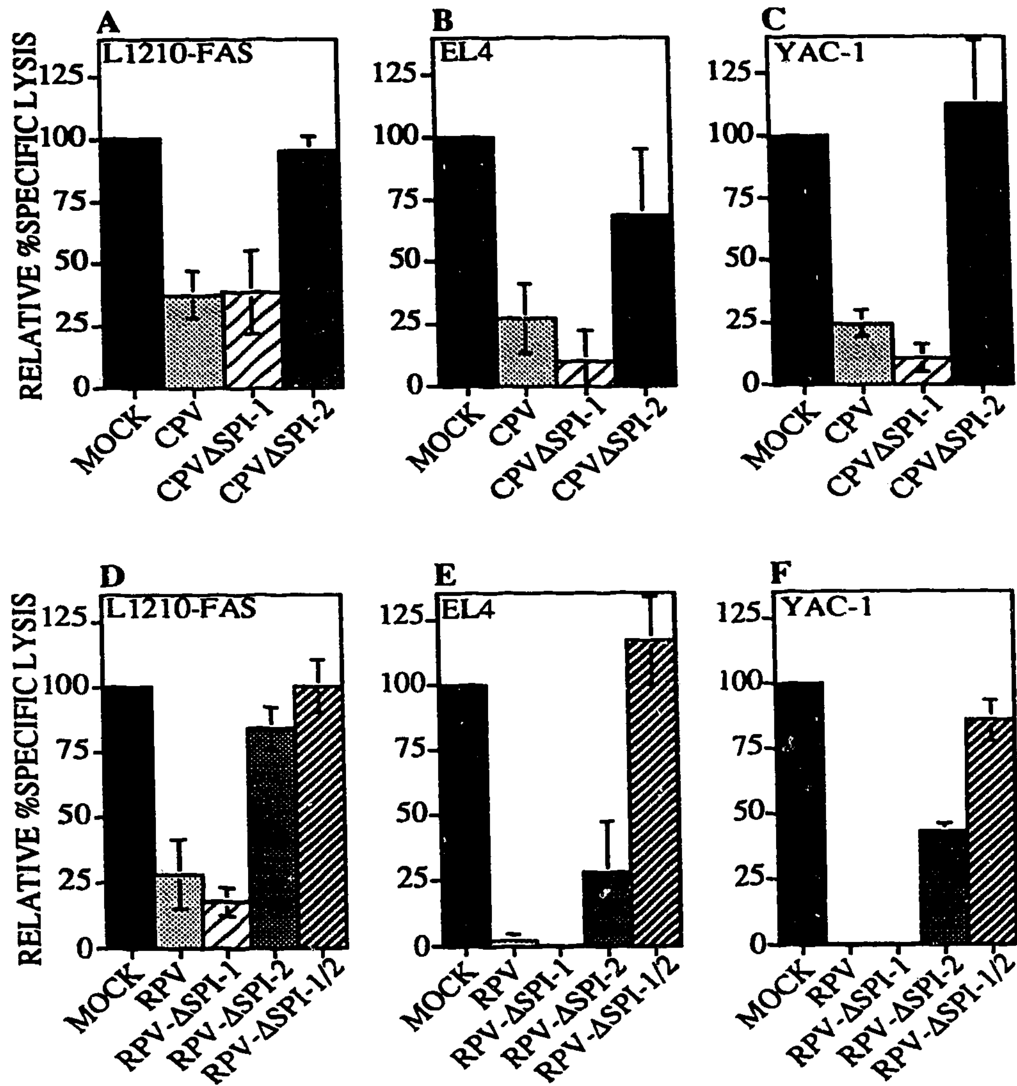

FIGURE V-3

Figure V-4. Granule-mediated cytolysis of target cells infected with virus mutants in the SPI-1 or SPI-2 genes. L1210 (A,C) or EL4 (B,D) cells were infected with wild type viruses or virus mutants in either the SPI-1 or SPI-2 genes, or with RPV containing mutations in both the SPI-1 and the SPI-2 genes (RPVΔSPI-1/2). Data are shown as % specific lysis relative to mock infected cells and represent the mean and standard deviations of three independent experiments.

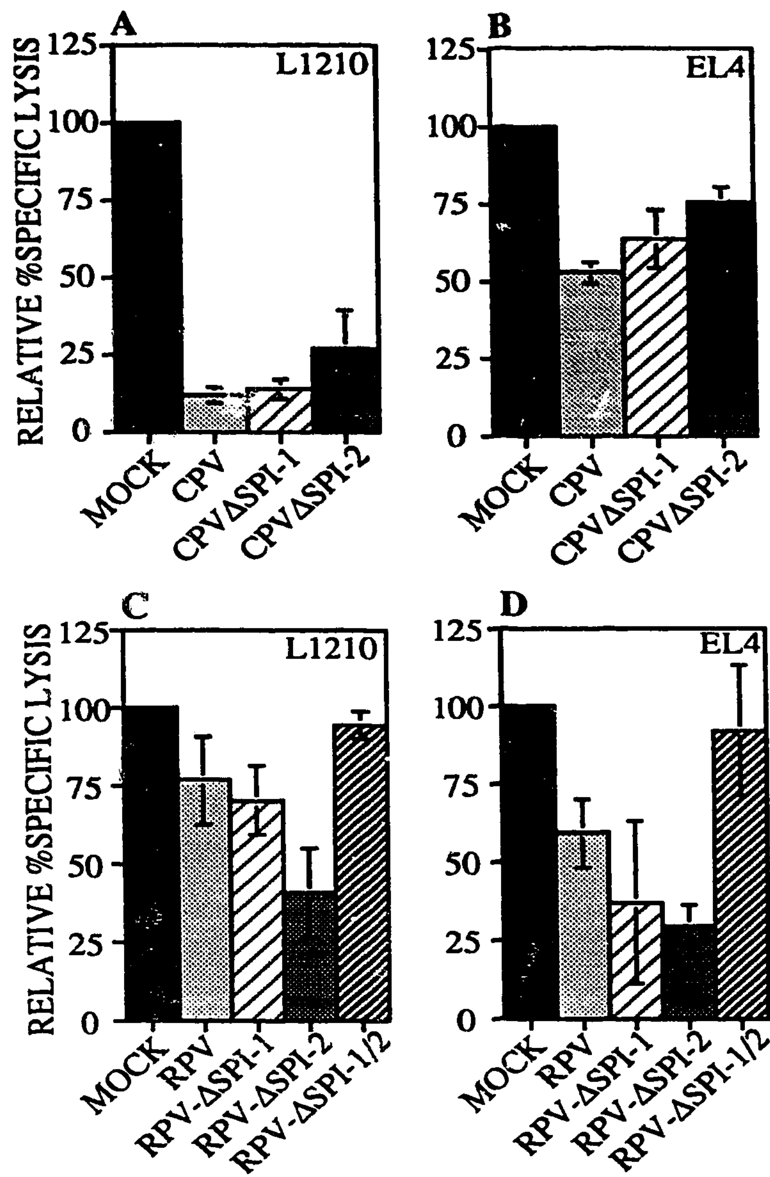

FIGURE V-4

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CHAPTER VI

GENERAL DISCUSSION AND CONCLUSIONS

The evolutionary process that ensures the successful propagation of complex viruses in nature has endowed them with an array of proteins that target critical aspects of the host anti-viral defenses. Within the TIR and adjacent unique sequences of the myxoma virus genome are numerous genes that encode proteins that, while generally non-essential for virus replication in tissue culture, are important in determining the virulence characteristics of the virus infection *in vivo*. As the functions of more of these gene products are determined, we begin to obtain a more complete understanding of factors that constitute important viral determinants of pathogenesis and virulence. At the same time, by learning how viruses counter the various host defenses, we gain important insight into the complex workings of the host organism itself. The work presented in this thesis not only provides information concerning the function of certain viral proteins and the interactions of poxviruses with the host cell, but also provides a foundation upon which future studies may be based. The important findings from these studies are summarized below, and the implications of this work for future study are outlined.

The data presented in Chapter II represent a detailed study of the myxoma virus SERP1 protein. The last open reading frame encoded within the TIR region of the myxoma virus genome, the SERP1 gene initially came to attention because of its similarity to the serpin superfamily of serine proteinase inhibitors. Early studies showed that the myxoma virus recombinant, MRV, encodes one copy of the SERP1 gene, while myxoma virus itself encodes two copies of the gene (Upton *et al.*, 1990). Of particular interest was the observation that the genome of SFV, while encoding complete functional versions of the myxoma virus open reading frames surrounding the SERP1 gene, does not possess an intact SERP1 gene. Because SFV and myxoma virus possess such vastly differing virulence characteristics, the question arose as to whether

the SERP1 gene encodes a protein that is an important virulence determinant for myxoma virus infection.

The experimental approach taken to address this question was to construct specific gene disruption mutations in the SERP1 gene and examine the virulence characteristics of the mutant viruses *in vivo*. While the SERP1 gene was non-essential for either MRV or myxoma virus replication in tissue culture, both viruses bearing mutations in the SERP1 gene were found to be significantly attenuated *in vivo*. Histological analyses suggested that the SERP1 protein interferes with the inflammatory response, since different patterns of infiltrating cells were observed in tissues examined from animals infected with wild type myxoma virus as compared to the SERP1 mutant virus. Analysis of the SERP1 gene revealed that it is a late gene, expressed from a late viral promoter that resembles the consensus late promoter sequence defined for vaccinia virus. Polyclonal antiserum raised to the SERP1 protein was used in immunoblotting to confirm its expression at late times during infection. and indicated that the SERP1 protein is secreted from the infected cell as a species somewhat larger than the size predicted from the gene sequence. Further analysis of the protein revealed that it was N-glycosylated, which accounted for its appearance as a diffuse band of 55 kDa on SDS polyacrylamide gels. Taking advantage of a vaccinia virus expression system, the SERP1 protein was produced in quantities sufficient to allow the development of a rapid and simple chromatographic purification scheme, by which the SERP1 protein may be readily purified to apparent homogeneity.

These studies confirmed the initial prediction that the SERP1 gene encodes a protein that is important for myxoma virus virulence in the infected rabbit. During the course of these studies, collaborators in Robin Carrell's group at Cambridge determined that the SERP1 protein indeed functions as an inhibitor of several human serine proteinases involved in the regulation of fibrinolysis (Lomas *et al.*, 1993).

However, it remains to be determined whether the SERP1 protein interacts with similar proteinases in the infected rabbit. Nevertheless the observation that the SERP1 protein asserts its effect on virus virulence by interfering with the host inflammatory response highlights the importance of inflammation as an antiviral mechanism. Furthermore, the successful use of the purified SERP1 protein in the inhibition of inflammation associated with restenosis following balloon angioplasty provides the first example of the potential clinical applications of poxviral proteins that function to regulate the host anti-viral response.

The identification of host proteins with which SERP1 interacts presents the most important challenge for future investigation. Because the SERP1 protein is an inhibitory serpin, its most likely target would be a serine proteinase. Serine proteinases are involved in numerous physiological processes, including coagulation and fibrinolysis, complement regulation, inflammation, target cell killing by certain classes of cytolytic lymphocytes, and the proteolytic activation of cytokines. Each of these processes can, either directly or indirectly, influence inflammation and therefore represent potential areas for investigation. Because serpins generally form tight complexes with their target proteinases, possible approaches to this problem might include the use of interactive cloning techniques, such as the yeast two-hybrid system, to identify potential cellular partners. In addition, the generation and study of point mutations in the SERP1 protein with respect to its influence on virus virulence and inflammation may also be useful approaches to understanding how SERP1 exerts its effects on inflammation. The inclusion of purified SERP1 protein in various assays of inflammation may also yield useful information concerning SERP1 function. However, it is important to use caution in extrapolating results from *in vitro* assays using purified proteins to physiological situations, a point well illustrated in Chapter V by the discrepancy between the ability of purified SERP1 to inhibit cytolysis by purified NK

cell proteins and the inability to inhibit the same process when intact cells are used as cytolytic effectors.

The downregulation of cell surface class I MHC proteins as a mechanism of immune evasion is a phenomenon common to a number of complex DNA viruses and can result in altered immune recognition (reviewed in McFadden and Kane, 1994; Rinaldo, 1994). Because the cellular immune response is critically important for the clearance of primary poxviral infections, viral downregulation of cellular components required for immune recognition could contribute to the extreme virulence of myxoma virus infection. Therefore the studies described in Chapter III were undertaken to determine whether the cell surface levels of class I antigens of the major histocompatibility complex were altered during myxoma virus infection. Flow cytometric analysis of cell surface class I MHC antigens, using a variety of antibodies recognizing both heavy and light chains of the class I molecule, indicated that myxoma virus infection of either the primate kidney cell line BGMK or the rabbit T lymphoma cell line RL-5 resulted in a significant decrease in the cell surface levels of class I MHC antigens. Analysis of class I antigens during infection of BGMK cells in the presence of inhibitors of protein synthesis suggested that the observed downregulation is not likely to result from viral inhibition of protein synthesis. In addition, when cells were infected in the presence of arabinosyl cytosine, and therefore in the absence of late viral gene expression, downregulation of class I MHC antigens was less extensive, suggesting that viral late protein synthesis may be required in part to mediate the effect. However, it is not possible to determine from these results whether the downregulation is the result of a viral function that specifically targets class I proteins, or whether it is a non-specific or indirect effect of virus infection. Nevertheless, the results indicate that the proteins that are downregulated during infection are those that turn over from the

membrane. Inhibition of transport of newly synthesized class I MHC proteins from the ER/Golgi in RL-5 cells using the drug brefeldin A suggests that class I proteins are not stable at the cell surface and must be continually transported. Therefore, a possible explanation is that myxoma virus infection may result in altered transport or recycling of certain classes of membrane proteins.

Although the mechanism by which class I antigen levels are reduced during myxoma virus infection cannot be discerned from these experiments, the results suggest that dysregulated class I MHC expression may be of significance and therefore warrants further investigation. The critical issue that must be addressed is whether this phenomenon is of physiological relevance. First, it will be important to determine whether the observed decrease in cell surface class I proteins results in reduced recognition of infected cells by $CD8^{+}$ T lymphocytes. Second, it will be important to determine whether this phenomenon is relevant to cells infected *in vivo*. Although complicated by a lack of inbred rabbit lines and cloned cell lines, it should be possible to carry out cytolysis assays using both target cells and effector cells from MHC-matched rabbits as they become available. In addition, the question remains as to whether altered immune recognition of infected lymphocytes is a feature of myxoma virus infection *in vivo*. Although myxoma virus clearly infects lymphoid cells, it is not known which subsets of lymphocytes are susceptible to infection. This can be determined by analysis of primary lymphocytes infected *ex vivo*. Dual staining for virus infected cells and cell surface markers specific for different lymphocyte subsets is possible using the FACS-GAL technique described here, along with various monoclonal antibodies that are now commercially available. The levels of class I MHC proteins can then be examined on subsets of cells that are susceptible to infection. While such experiments may not directly reflect the population of cells that become infected in the intact animal, they should provide an indication of whether altered

immune recognition of infected primary lymphoid cells can occur. This information will contribute to our understanding of the dissemination of the infection in the infected animal.

The T2 genes of the leporipoxviruses came to attention when homology between the ligand binding domain of the cloned type II receptor for tumour necrosis factor and the SFV T2 protein was discovered (Smith *et al.*, 1990; Smith *et al.*, 1991). Sequencing of myxoma virus DNA revealed the presence of a closely related gene in a similar location in the myxoma virus TIR (Upton *et al.*, 1991). This was the first gene encoded by myxoma virus with known homology to a cytokine receptor, and generated considerable interest. One of the first obvious questions to address was whether the expression of a cytokine receptor homologue contributed to the virulence of the virus infection *in vivo*. Studies described in Chapter IV were undertaken to address this question. The potential contribution to virus virulence was assessed by the construction of a recombinant myxoma virus in which the T2 gene was interrupted. This virus was found to be significantly attenuated in the infected European rabbit. Other experiments ascertained that the myxoma virus T2 protein was in fact capable of binding to and inhibiting the cytocidal activity of rabbit TNF (Schreiber and McFadden, 1994). It was therefore assumed that the T2 protein contributes to virus virulence by binding and sequestering TNF, thereby preventing the protein from interacting with its cellular receptor (Upton *et al.*, 1991; Schreiber and McFadden, 1994). However, recent results, also described in Chapter IV, suggest that inhibition of TNF action may be only part of the story.

During the experiments described in Chapter III, the observation was made that SFV was unable to productively infect the rabbit cell line RL-5. Previous studies had indicated that a myxoma virus mutant in the M11L gene was unable to grow in primary

cultures of mixed splenocytes (Opgenorth *et al.*, 1992). Because both of these viruses are highly attenuated compared to wild type myxoma virus, we asked whether there was a correlation between virus virulence and the ability to productively infect lymphocytes. Further studies described in Chapter IV indicated that neither SFV, nor myxoma viruses bearing mutations in the M11L or T2 open reading frames were able to productively infect the rabbit T lymphoma cell line, RL-5. Furthermore, flow cytometric analysis of infected RL-5 cells coupled with microscopic analysis indicated that cells infected with these viruses were undergoing apoptotic cell death. Therefore the myxoma virus M11L and T2 proteins appear to function to prevent apoptotic cell death of infected T lymphocytes, which in turn allows a productive infection. That the T2 protein can function extracellularly to block TNF action, as well as intracellularly to prevent apoptotic cell death is interesting, and further suggests that the function of a given viral protein cannot be discerned solely from its membership in a particular protein family.

Although the genome of SFV encodes counterparts of the T2 and M11L genes, RL-5 cells infected with SFV still underwent apoptosis. There are several possible explanations for this apparent discrepancy, which can be tested experimentally. First, the expression of the T2 and M11L proteins in SFV has not been rigorously characterized. The results with myxoma virus indicate that both the M11L and T2 genes are required to prevent apoptotic death of infected RL-5 cells, and therefore if either of the SFV versions of M11L or T2 are not expressed, or are expressed in insufficient quantities, apoptosis may ensue. Secondly, although the SFV homologues are very similar to their myxoma virus counterparts, they are not identical and therefore it is possible that SFV does not encode functional versions of one or another or both of these proteins. Gene swapping experiments might be useful to determine if the SFV genes can indeed function as the myxoma virus versions. Finally, it is possible that the

prevention of apoptosis results from the interaction of T2 and/or M11L with other viral proteins, which may not be encoded by SFV, and therefore the identification of proteins with which T2 and M11L interact will be important in determining the functions of these virus proteins.

The myxoma virus T2 protein is a member of the TNF receptor superfamily. Although the most significant homology exists between T2 and the type II receptor for TNF, the T2 protein is clearly related to other family members. Included among these is the cell surface receptor Fas, which shares with the TNF receptor the ability to transmit a signal leading to cell death upon interaction with its ligand (reviewed in Nagata and Golstein, 1995). The possibility therefore arises that the T2 protein not only interacts with TNF, but may also interact with ligands for other members of this receptor family. An interesting result is that the apoptotic death of RL-5 cells is not prevented by inclusion of soluble T2 protein in the medium. This result implies that the T2 protein functions to prevent apoptosis by some mechanism that requires its synthesis *de novo*. It is possible that the induction of apoptosis in RL-5 cells is the result of signalling through the Fas receptor on infected cells. The expression of T2 during infection may allow it to become complexed with Fas ligand, which would then appear on the cell surface as a complex with T2 that is unable to interact with Fas receptor that may exist on neighbouring cells. Another possibility is that, since members of the TNF receptor family can form heterodimers and heteromeric complexes (Boldin *et al.*, 1995), T2 may form heteromeric complexes involving the Fas receptor. Because the cell death signal requires the association of intracellular domains of the receptor molecule (the so called "death domains"), receptor complexes with T2 would be unable to transmit a death signal because T2 does not contain the "death domain". In addition, because the C-terminal domain of the T2 protein bears no significant homology to any other protein, it may interact with other components that mediate cell death that cannot be predicted from

the amino acid sequence. The analysis of deletion mutants will determine whether the TNF binding ability of the T2 protein and the ability to inhibit apoptosis are independent functions of different domains of the protein. Furthermore, expression of T2 from a virus-free system as well as the identification of proteins with which it interacts will allow the role of T2 in the prevention of apoptosis to be more clearly defined.

How M11L functions to prevent apoptosis is unclear. The M11L protein is predicted to be a type II transmembrane protein, and bears no significant homology to any cellular protein or any viral protein of known function. Of interest is the observation that lesions from rabbits infected with myxoma virus bearing mutations in the M11L gene contain massive inflammatory infiltrates (Opgenorth *et al.*, 1992). This raises the question of whether apoptosis of infected cells in the lesions is responsible for the induction of this enormous inflammatory infiltrate. It has been suggested that cells of the macrophage/monocyte class release pro-inflammatory mediators when undergoing cell death by apoptosis (Kornbluth, 1994). Again, it will be important to determine which subsets of lymphoid cells become infected *in vivo*, and then whether infection of these cells with viruses bearing mutations in the M11L (or T2) genes undergo apoptosis.

It is now generally accepted that cytolytic lymphocytes can affect target cell lysis by one of two pathways: granule exocytosis and signalling through the Fas cell surface receptor. Although both pathways result in cell death by apoptosis, and proteolytic activity has been shown to be involved in each cytolytic mechanism, it is not known whether the two pathways share common components (reviewed in Berke, 1994). The studies presented in Chapter V were initiated in order to determine whether the myxoma virus SERP1 protein was able to inhibit the non-specific cytolysis of infected target

cells by either primary murine NK cells or by cloned cytotoxic T cells that kill target by either the Fas-dependent or degranulation pathways.

Studies conducted using purified SERP1 and the purified NK cell granule proteins cytolysin and fragmentin 2, initially suggested that SERP1 was able to inhibit cytolysis of YAC-1 cells (A. Greenberg and L.Shi, personal communication). Therefore it was of interest to determine whether the SERP1 protein could inhibit cytolysis in a system of more physiological relevance, using intact primary NK cells as cytolytic effectors. YAC-1 cells were infected with the recombinant vaccinia virus, VV-S1, that expresses the SERP1 protein (Chapter II), in the presence or absence of additional exogenous purified SERP1, and then assayed for cytolysis in chromium release assays. Under these conditions, the SERP1 protein was completely unable to inhibit NK cell-mediated cytolysis. In similar experiments using several murine cell lines as target cells, SERP1 was also completely unable to inhibit cytolysis by cloned cytotoxic T cell lines that kill target cells via either the degranulation pathway or the Fas-dependent pathway. Together, these results indicate that the SERP1 protein does not function to inhibit cytolysis mediated by cytolytic lymphocytes. Furthermore, the discrepancy between the results obtained using purified proteins and those obtained using intact cells as cytolytic effectors suggests that it may not be possible to extrapolate results obtained with purified components to systems using whole cells.

Although SERP1 did not inhibit cell-mediated cytolysis, during the course of these experiments we observed that cells infected with vaccinia virus itself exhibited reduced levels of cytolysis by cloned CTL lines. Because the orthopoxviruses themselves encode several inhibitors of serine proteinases, it seemed possible that the reduced levels of killing observed in these experiments might be mediated by one or more of these proteins. Experiments were therefore conducted to address the question of whether the orthopoxviral SPI-1 and SPI-2 serpins were able to inhibit cytolysis by

cloned murine cytotoxic T cell lines that kill target cell via either the degranulation pathway or the Fas-dependent pathway. Because not all strains of vaccinia virus encode functional homologues of SPI-1 and SPI-2 (Goebel *et al.*, 1990), we decided to investigate the role of the SPI-1 and SPI-2 proteins encoded by two other orthopoxviruses, cowpox virus (CPV) and rabbitpox virus (RPV). The objective of these experiments was to determine whether cytolysis mediated by either effector cell type was inhibited by infections with CPV or RPV, and if so, whether the inhibition was mediated by either or both serpins. Furthermore, we wished to determine whether these viral serpins could be used as tools with which to study the cytolytic effector mechanisms used by the different effector cell types.

Experiments were conducted using a variety of murine cell lines infected with RPV or CPV, or derivatives of RPV and CPV bearing specific mutations in either the SPI-1 or SPI-2 genes, as target cells in cytolysis assays. In these assays, both CPV and RPV infection showed consistent, but somewhat variable inhibition of cytolysis using both effector cell types. Furthermore, the results obtained with viruses bearing mutations in the SPI-1 or SPI-2 genes confirm a role for the SPI-2 protein in the inhibition of Fas-mediated apoptosis, an observation others have made using cells transfected with the CPV SPI-2 gene (Enari *et al.*, 1995; Los *et al.*, 1995; Tewari and Dixit, 1995). Somewhat surprising was the result that for EL4 and YAC-1 target cells infected with RPV, mutation of the SPI-2 gene alone did not completely abrogate the observed inhibition of Fas-mediated cytolysis. However, inhibition was relieved by mutation of both the SPI-1 and the SPI-2 genes together. This result suggests that SPI-1 contributes to inhibition of Fas-mediated cytolysis of certain cell types. Similar experiments using granule-dependent effector cells indicated that viral infection again resulted in inhibition of cytolysis. However, unlike Fas-mediated killing, the inhibition was not relieved by mutation of the SPI-2 gene. A similar result was obtained when

target cells were infected with viruses containing deletions in the SPI-1 gene. However, when RPV containing mutations in both the SPI-1 and SPI-2 genes together was used to infect target cells, no inhibition was observed. Taken together, the results confirm a role for the SPI-2 protein in the inhibition of Fas-mediated apoptosis, and suggest that the orthopoxviral SPI-1 proteins may also play a role in the inhibition of apoptotic cell death mediated by cytolytic lymphocytes. The SPI-1 gene has been shown to function in the inhibition of apoptosis of RPV-infected A549 and PK-15 cells (Turner *et al.*, 1995). Thus, the SPI-1 protein may target some cellular component involved in apoptosis in certain cell lines.

Together, these data provide the first evidence that the Fas-mediated and granule-mediated cytolysis pathways share common components, and that the orthopoxviral SPI-1 and SPI-2 proteins are sufficient to inhibit both killing mechanisms. Therefore, both the SPI-1 and the SPI-2 proteins represent useful tools with which the mechanisms of cytolysis can be probed. It will be of interest to determine with which cellular proteins these viral serpins interact. Others have demonstrated that the CPV SPI-2 protein inhibits the proteolytic activity of granzyme B (Quan *et al.*, 1995), as well as a number of cysteine proteinases that belong to the ICE family of enzymes that are implicated in apoptotic cell death (Ray *et al.*, 1992; Miura *et al.*, 1993; Komiyama *et al.*, 1994; Boudreau *et al.*, 1995; Enari *et al.*, 1995; Los *et al.*, 1995; Tewari *et al.*, 1995; Quan *et al.*, 1995). To date, no proteins have been identified that interact with the viral SPI-1 protein. Although SPI-1 is nearly 50% identical to SPI-2 at the amino acid level, the predicted reactive centre differs significantly, suggesting that SPI-1 and SPI-2 interact with different target proteinases. Thus, the identification of proteinases with which SPI-1 interacts may provide valuable information concerning the cytolytic mechanisms employed by CTL. Finally, because vaccinia virus is widely used as a vector for the expression of foreign proteins for

immunological analyses (reviewed in Bennink and Yewdell, 1990), it may be important to consider the status of the SPI-1 and SPI-2 proteins in vaccinia virus vectors used in such experiments.

The studies presented in this thesis and elsewhere have illustrated the importance of non-specific host responses to infection, such as the inflammatory response, in the overall pathogenic profile of the virus infection. For example, the product of the myxoma virus M11L open reading frame (Opgenorth *et al.*, 1992) as well as the product of the SERP1 open reading frame (Chapter II, this thesis), influence both the inflammatory reaction within infected sites *in vivo*, as well as the degree of virulence of the infection. Similarly, the identification of viral gene products that target particular cytokines in turn informs us of the critical roles played by certain host proteins during viral infections. For example, the products of the T2 and T7 open reading frames target cytokines that have both direct anti-viral and immune regulatory functions. Again, disruption of these genes influences virus virulence (Chapter IV, this thesis; and K. Mossman, N. Nation, J. Macen and G. McFadden, manuscript in preparation), and therefore provides an indication of the roles played by such host factors in the defense against virus infection.

Perhaps even more informative is the observation that particular viral proteins may influence more than one host anti-viral strategy. This point is well illustrated by studies of the myxoma virus T2 protein, which both binds and inhibits rabbit TNF (Schreiber and McFadden, 1994) as well as preventing apoptosis of certain types of infected cells by a mechanism that is apparently independent of the inhibition of TNF (Chapter IV, this thesis). Therefore viral proteins that influence virus virulence may also represent extremely useful tools with which we may identify common or related components of distinct host pathways, as well as determine how certain viral effects on

individual cells may influence the histopathology observed within a tissue. For example, further study of proteins such as M11L may facilitate a greater understanding of the relationship between apoptosis in certain classes of cells and the induction and regulation of inflammation.

Similarly, viral proteins that interact with critical host functions may also be useful both for the study of particular cellular processes independent of the anti-viral response *per se*, and for the development of reagents with clinical applications. Poxvirus serpins have been found to influence apoptotic cell death, as exemplified by studies of the cowpox virus serpin, SPI-2 (crmA) (Gagliardini *et al.*, 1994; Tewari and Dixit, 1995; Enari *et al.*, 1995; Los *et al.*, 1995; Komiyama *et al.*, 1994). While it is not known whether the inhibition of apoptosis is a feature of cowpox virus infection *in vivo,* the study of this protein has been very useful in the identification of important factors that regulate apoptosis in the cell. In addition, the studies presented here indicate that both the orthopoxvirus SPI-1 and SPI-2 proteins may be useful tools for the study of cytolytic mechanisms employed by cytotoxic T lymphocytes (Chapter V, this thesis). Furthermore, the anti-inflammatory properties of the myxoma virus SERP1 protein have been successfully exploited in an animal model of restenosis following balloon angioplasty (Lucas *et al.*, 1995), indicating that such proteins may have important clinical applications.

In summary, the results presented in this thesis indicate that the myxoma virus SERP1 and T2 proteins are important virulence factors for virus infection *in vivo*. While the SERP1 protein functions to increase virulence through interfering with the host inflammatory response (this thesis), the T2 protein may function both in the inhibition of TNF (Schreiber and McFadden, 1994) as well as in the inhibition of apoptotic cell death (this thesis), allowing the virus to propagate in a wider spectrum of cell types. Myxoma virus infection may also result in compromised immune recognition

of virally infected cells, through the downregulation of class I MHC proteins. Finally, while the SERP1 protein does not function to inhibit cell-mediated cytolysis, the orthopoxvirus SPI-1 and SPI-2 proteins contribute to inhibition of cytolysis mediated both by signalling through the Fas cell surface receptor as well as though the activity of proteins contained within cytolytic granules, and as such may represent useful tools with which to probe the cytolytic mechanisms.

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