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POXVIRUS CHEMOKINE BINDING PROTEINS

by

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

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Fall 1999

University of Alberta

Library Release Form

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Title of Thesis: Poxvirus Chemokine Binding Proteins

Degree: Doctor of Philosophy

Year this Degree Granted: 1999

April 22/1999

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For my family

Sadrudin, Gulshan, Ashifa and Anisa

ABSTRACT

Poxviruses are large double-stranded DNA viruses that express a variety of proteins with activities aimed at disrupting host cytokine networks. Identification and characterization of these anti-cytokine strategies is crucial for understanding the mechanisms of viral pathogenesis and virulence as well as revealing key aspects of host antiviral immunity.

Myxoma virus is a member of the genus *Leporipoxvirus* that induces a systemic lethal disease known as myxomatosis in the European rabbit. The objective of this thesis was to identify myxoma virus soluble proteins involved in modulating the activities of chemoattractant cytokines or chemokines. Here we demonstrate that the purified myxoma virus soluble IFN-γ receptor homologue, M-T7, binds to a broad spectrum of C, CC, and CXC chemokines with sub-μM affinity in addition to binding to IFN-γ. Using a variety of truncated chemokine analogs, M-T7 was shown to interact with the conserved C-terminal heparin-binding domain, but not the variable N-terminal receptor binding region, found in a wide spectrum of chemokines. We propose that M-T7 is a multifunctional myxoma virus virulence factor that modulates leukocyte trafficking by competitively displacing chemokine-glycosaminoglycan interactions in virus-infected tissues.

In addition to M-T7, we have identified a second distinct soluble chemokine binding protein expressed by myxoma virus termed M-T1. Analogous proteins to M-T1, termed 'major secreted 35kDa' proteins, are also shown to be expressed by many orthopoxviruses. Although members of the T1/35kDa family of poxvirus chemokine binding proteins show variable homology amongst one another, their sequence is

unrelated to any known cellular proteins including chemokine receptors. Cross-competition and solid-phase binding analyses revealed that M-T1 binds CC chemokines such as RANTES with high affinity (K_d = 73 nM) and CXC chemokines such as IL-8 with lower affinity. M-T1 and several orthopoxvirus 35kDa proteins were purified to homogeneity and shown to potently inhibit (K_i = 0.07-1.02 nM) the biological activities of CC chemokines but not CXC chemokines *in vitro*. European rabbits infected with an M-T1-knockout virus revealed that M-T1 significantly inhibits the migration of CC chemokine-responsive leukocytes into infected tissues during the early phases of myxoma virus pathogenesis. M-T1/35kDa and M-T7 represent the first examples of secreted virus proteins that sequester chemokines as a novel virus strategy for circumventing the host inflammatory response to infection.

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ABBREVIATIONS

AIDS	acquired immune deficiency syndrome
BGMK	baby green monkey kidney
EEV	extracellular enveloped virus
EGF	epidermal growth factor
ELR	glutamate-leucine-arginine
fMLP	formyl-methionyl-leucyl-phenylalanine
GAG	glycosaminoglycan
GM-CSF	granulocyte macrophage-colony stimulating factor
GPT	guanosine phosphoribosyl transferase
GRO	growth related oncogene
GST	glutathione S transferase
HCMV	human cytomegalovirus
H&E	hematoxylin and eosin
HHV-	human herpesvirus-
HIV	human immunodeficiency virus
IC_{50}	50% inhibitory concentration
IL-	interleukin-
IMV	intracellular mature virus
IFN	interferon
kbp	kilobase pairs
K_d	dissociation constant
K_i	inhibition constant
kDa	kiloDalton
KS	Kaposi's sarcoma
Ltn	lymphotactin
MCMV	murine cytomegalovirus
MCP-	monocyte chemoattractant peptide-
MCV	molluscum contagiosum virus
MGF	myxoma growth factor
MIP-	macrophage inflammatory protein-
m.o.i	multiplicity of infection

NK	natural killer
ORF	open reading frame
PCR	polymerase chain reaction
PFU	plaque forming units
p.i.	post-infection
RANTES	regulated upon activation normal T cell expressed and secreted
RK-13	rabbit kidney-13
RPV	rabbitpox virus
SDF-	stromal cell derived factor-
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SFV	Shope fibroma virus
TGF	transforming growth factor
TIR	terminal inverted repeats
TNF	tumor necrosis factor
vCk	viral chemokine homologue
vCkBP	viral chemokine binding protein
vCkR	viral chemokine receptor homologue
VCP	vaccinia complement control protein
VEGF	vascular endothelial growth factor
VGF	vaccinia growth factor
VV	vaccinia virus
WR	Western reserve

CHAPTER 1

GENERAL INTRODUCTION

Viruses that successfully propagate within higher order eukaryotic hosts have had to adapt to survive the challenges imposed by the innate and acquired arms of the immune response to infection. By co-evolving with the immune system for untold millennia and under constant selective pressure, viruses have developed their own versions of defenses to counteract a broad variety of anti-viral immune effector molecules that would otherwise impede the successful propagation of the virus.

Large DNA viruses, such as poxviruses and herpesviruses, are particularly adept at subverting host defenses by capturing and modifying cellular genes that regulate the host immune response to infection. Included among these host-derived virus genes are modified versions of biological response modifiers including cytokines and cytokine receptors. Identification of such viral subversive molecules and elucidation of their function has provided enormous insight into the mechanisms of viral pathogenesis and of the complex immune circuitry involved in anti-viral host defenses. More recently, a number of viral proteins have also been identified that appear to modulate immune defenses but which do not share any sequence relationship to currently known cellular proteins. Characterization of these novel viral immunomodulatory proteins may potentially lead to the identification of novel cellular gene products or pathways involved in the host immune response.

The studies presented in this thesis attempt to further our understanding of virus-host interactions and mechanisms of viral pathogenesis. Using poxviruses as a model of pathogenesis, the dynamic interplay between viral determinants of

pathogenicity and host inflammatory molecules such as chemokines are characterized both at the molecular and cellular levels.

POXVIRUS BIOLOGY

General Properties and Classification of Poxviruses

Poxviruses are the largest and most complex family of eukaryotic viruses known. In general, members of the *Poxviridae* family share many common physical properties including a complex virion morphology, genomic structure and site of replication (for extensive reviews see 30, 129). Poxvirus virions are large, approximately 250-400 nm in length, and adopt a characteristic brick-shaped morphology. An outer lipoprotein coat surrounding the virion structure encases a biconcave core with two associated electron dense structures termed lateral bodies. While the function of the lateral bodies currently remains unknown, the core contains the virus genome complexed to virus-encoded proteins, many of which are enzymes involved in mRNA synthesis. All poxviruses possess a single, linear double-stranded DNA genome that is ~130-300 kilobase pairs (kbp) in length with covalently closed hairpin termini and terminal inverted repeats (TIRs). A typical poxvirus genome may encode over 200 polypeptides, many of which are involved in virion morphogenesis and factors required for viral gene expression and DNA replication (128, 129). In general, genes essential for virus structure, replication and gene expression are highly conserved amongst poxviruses and tend to be clustered in the central region of the genome. In contrast, genes that map toward the termini of poxvirus genomes display

considerable sequence variation, are often dispensable for growth in tissue culture, and encode factors involved in determining host-range and virus virulence. Poxviruses are unique amongst other eukaryotic DNA viruses in that viral macromolecular synthesis and replication occurs exclusively in the cytoplasm of infected cells.

Poxviruses infect a broad range of vertebrates and invertebrates hosts and are divided into two major subfamilies: the *Chordopoxvirinae* (vertebrate poxviruses) and the *Entomopoxvirinae* (insect poxviruses). Based on similar antigenicity, virion morphology, host range, disease phenotypes and DNA cross-hybridization, the *Chordopoxvirinae* subfamily are further divided into the following eight genera: *Orthopoxvirus*, *Leporipoxvirus*, *Yatapoxvirus*, *Molluscipoxvirus*, *Parapoxvirus*, *Avipoxvirus*, *Capripoxvirus* and *Suipoxvirus* (summarized in Table I-1).

Poxvirus Replication and Gene Expression

Much of our understanding of poxvirus replication, gene expression, and morphogenesis has come from extensive studies using vaccinia virus, a member of the genus *Orthopoxvirus* (reviewed in (127-129, 176, 190). Poxvirus replication occurs strictly in the cytoplasm and is largely autonomous; the virus genome encodes its own transcriptional and DNA replication machinery (21, 128, 129, 191). The replication cycle of poxviruses consists of three phases: (1) virus entry and uncoating; (2) regulated gene expression and DNA replication; and (3) assembly, maturation and release of mature virion particles. The cell surface receptor(s) required for poxvirus entry currently remains unknown, although some evidence exists for the role of cell surface proteoglycans in the specific attachment of vaccinia virus particles during

infection (38, 77). Shortly after entry, virion particles undergo an initial stage of uncoating followed by the transcription of early virus genes that is facilitated by virus-core associated enzymes. Poxvirus gene expression is temporally regulated with three classes of genes (early, intermediate and late) transcribed in a strictly regulated cascade (127, 128, 191). The products of early viral gene expression result in the secondary and complete uncoating of the virus core, the synthesis of enzymes for DNA replication and nucleotide biosynthesis, and the expression of transcription factors required for expression of the intermediate viral genes. DNA replication occurs in discrete cytosolic type "B" inclusion bodies, termed "viral factories", as early as 2 hours post-infection and is required for the expression of the intermediate and late viral genes. The intermediate genes encode several transcription factors needed for expression of the late genes, while the late genes encode the structural proteins of new virus particles and enzymes/early transcription factors that are packaged in the virion.

The complex process of virus morphogenesis ensues shortly after late gene expression and involves extensive wrapping with cellular membranes (179). Although discrete stages of virus assembly in the cytoplasm can be discerned by electron microscopy, the precise mechanism of poxvirus morphogenesis has not been fully determined. Vaccinia virus produces two morphologically distinct types of infectious particles, termed intracellular mature virus (IMV) and extracellular enveloped virus (EEV) (178). IMV represents the majority of infectious particles produced and is only released upon cell lysis. EEV particles arise from intracellular virions that are wrapped by cisternae derived from the trans-Golgi network to form intracellular enveloped virus (IEV) (164). Polymerization of actin tails propels IEV to the cell surface where it

fuses with the plasma membrane (46, 47). As the actin tail continues to grow, the newly formed EEV particle is pushed out of the cell on the tip of specialized microvilli that are easily visible by scanning electron microscopy (46, 47). Although EEV is usually much less abundant than IMV, depending on the virus strain, it is more important for virus dissemination and is the target for neutralizing antibodies that confer protective immunity to vaccinia virus infection (176).

ORTHOPOXVIRUSES

Members belonging to the *Orthopoxvirus* genus include variola, vaccinia, cowpox and rabbitpox viruses (62). Variola virus was a strictly human pathogen and the causative agent of smallpox, one of the most devastating diseases known to mankind (57). An intensive eradication effort by the World Health Organization resulted in the elimination of variola virus from the global population by 1979. However, the destruction of several remaining stocks of variola virus still continues to be a controversial issue (76). The successful eradication of smallpox is largely owed to the seminal work of Edward Jenner who introduced cowpox virus as a smallpox vaccine in 1796 (17). Although cowpox virus was first isolated from cattle, the virus is not presently enzootic in cattle. Despite causing sporadic infections in cows, humans and domestic cats, the natural reservoir of cowpox virus is thought to be wild rodents (19, 62). In the 20th century, live vaccinia virus replaced cowpox virus as the vaccination strain against variola (smallpox). Vaccinia virus is not considered a natural human pathogen and is transmitted poorly between humans. The precise origin

of vaccinia virus remains unclear and its natural host is unknown. Comparative analysis of the genomic sequences and organization of vaccinia, variola and cowpox viruses reveal that vaccinia and variola virus may have evolved independently from a common ancestor such as cowpox virus (171, 172). Given the unknown origins of vaccinia virus, no suitable animal models for investigating the natural infection and pathogenesis of vaccinia virus currently exist. Current methods of investigating vaccinia virus virulence are limited to intracranial and intranasal virus inoculations in mice. Like vaccinia virus, rabbitpox virus displays a broad host range and its natural host currently remains unknown. Rabbitpox virus causes a generalized, disseminated infection that is lethal in mice and rabbits, and thus serves as a viable model for studying virus-host interactions (22, 52, 112).

LEPORIPOXVIRUSES

The leporipoxviruses are generally species-specific and are restricted to rabbits, hares and squirrels (52, 112, 113). Members belonging to the genus *Leporipoxvirus* include myxoma virus, Shope fibroma virus, malignant rabbit fibroma virus (a natural recombinant between myxoma and Shope fibroma viruses), squirrel fibroma virus and hare fibroma virus. All the viruses in this genus appear to be closely related by serology, immunodiffusion and antibody assays.

Shope Fibroma Virus

Shope fibroma virus was first isolated in 1932 from its natural host, the eastern U.S.A. cottontail rabbit (*Sylvilagus floridanus*) (112, 113). In the cottontail rabbit,

virus infection causes a localized benign fibroma that can persist for many months with little evidence of disease. In immunocompetent domestic European rabbits (*Oryctoloagus cuniculus*), the primary fibroblastic lesion spontaneously regresses, again without any notable disease symptoms. The principle mode of transmission for leporipoxviruses is through intradermal inoculation by arthropod vectors such as mosquitoes, fleas and ticks (52). Replication of Shope fibroma virus occurs at local dermal sites and the virus rarely disseminates to secondary sites. The complete sequencing of Shope fibroma virus reveals a genome size of ~160 kbp with TIRs of 10-13 kbp (D. Evans, personal communication). Comparisons of the genomic organization between Shope fibroma virus and myxoma virus (next section) reveal that these two leporipoxviruses are significantly similar (G. McFadden, personal communication).

Myxoma Virus and Myxomatosis

Myxoma virus was first identified in 1896 in Uruguay as the causative agent of a new virulent disease (now known as myxomatosis) amongst imported laboratory European rabbits (*Oryctolagus cuniculus*) (52, 61, 112). The natural hosts of myxoma virus are the Tapeti or South American tropical forest rabbit (*Sylvilagus brasiliensis*) and the North American brush rabbit (*Sylvilagus bachmani*) (61). In their native hosts, the virus causes a benign cutaneous lesion that may persist for several months without any further disease symptoms. In contrast to the trivial symptomology of myxoma virus infection in these indigenous hosts, infection of European (*Oryctolagus*) rabbits results in a highly systemic, lethal disease syndrome known as myxomatosis (52, 60, 61, 82, 112). Myxomatosis is characterized by large protuberant dermal lesions at the

primary site of infection that within days after the primary infection become hemorraghic and necrotic. The virus effectively disseminates to secondary sites via the host afferent lymphoreticular system causing extensive internal and external secondary lesions that become visibly evident around the eyes, nares and ears. A generalized dysfunction of the host cell-mediated immune response facilitates supervening Gram-negative bacterial infections of the nasal and conjunctival mucosa and upper respiratory tract. Within two weeks of infection, rabbits invariably experience dyspnea and succumb to death. Mortality in myxoma virus infected European rabbits is usually >99%.

The lethal nature of myxomatosis in European rabbits prompted the use of myxoma virus as the first biological control agent to control the unmanageable feral rabbit populations in Australia (60, 61). The deliberate introduction of myxoma virus into Southern Australia in 1950 caused an excess of 99% mortality in the wild rabbit populations. However, the resistance to myxoma virus quickly resulted by the emergence of attenuated field strains of the virus and the survival of moderately resistant rabbits (60, 61, 82). Myxoma virus is currently endemic in Australia with rabbit populations reaching similar levels to those found prior to the initial release of the virus. Little is known of the mechanism of resistance, and current studies are underway to determine the genetic basis of resistance among surviving rabbits and the molecular basis that may influence the loss of pathogenicity in certain field isolates (82). Nonetheless, the release of myxoma virus in European rabbit populations in Australia has provided us with one of the best-documented examples of host-virus co-evolution.

The myxoma virus model is ideal for the study of viral pathogenesis and virus-host interactions for a variety of reasons. Firstly, the evolutionary history of both the virus and its native and susceptible rabbit hosts are well documented. Secondly, the virus causes an extensively described pathogenic profile in a tractable animal host. Thirdly, the host and virus are each amenable to analysis in the laboratory. And finally, a collection of naturally selected attenuated variants of the virus and resistant rabbit strains are available for investigating the relationship between virus pathogenesis and evolutionary pressures that confer virulence.

The genome of the prototypic (Lausanne) strain of myxoma virus has been cloned, and analyzed by restriction mapping (26, 159). Figure I-1 illustrates a *Bam*H1 restriction map of the myxoma virus genome indicating the location of several virulence factors that have been analyzed to date. Recently, the entire myxoma virus DNA genome was sequenced and shown to be approximately ~161 kbp in size and contains over 160 potential open reading frames (ORFs) (G. McFadden, personal communication). Similar to other poxviruses, myxoma virus contains many genes clustered near the central regions of the genome that are involved in housekeeping functions such as viral replication and gene expression. In addition, a variety of distinct ORFs are found within or near the genomic termini whose gene products modulate the host immune response or are involved in contributing to viral pathogenicity (116, 118, 138).

POXVIRUS IMMUNE EVASION STRATEGIES

It has become increasingly evident that poxviruses have evolved a multitude of strategies to subvert the innate and acquired immune responses of their host animals. The use of virus encoded anti-immune proteins to evade, block or modulate immune effector molecules has been the subject of many extensive recent reviews (114, 117, 152, 177, 182). Such mechanisms for immune evasion directed by poxviruses include interference with antigen presentation (120), inhibition of apoptosis (115, 192), blockade of complement cascades (79, 87), and modulation of cytokine networks (8, 118, 122, 181). In general, poxvirus immunomodulatory proteins can be grouped into three major classes, *Viroceptors*, *Virokines*, and *Viromitigators*, based on their targets and mechanisms of action. Viroceptors are soluble or membrane-bound virus-encoded cytokine receptor homologues that generally function by binding and sequestering cytokine ligands away from their cognate cellular receptors. *Virokines* are often secreted mimics of cytokines, growth factors or extracellular immune regulators. *Viromitigators* are viral intracellular proteins that generally disrupt specific signal transduction pathways or inhibit apoptosis cascades of infected cells. Examples of poxvirus immune subversive proteins within each class can be found in Table I-2. In certain cases, it is apparent that these viral proteins were clearly acquired or "hijacked" from cellular genes during the co-evolution of the virus with its infected host. However, several unique viral immunomodulatory proteins have been identified with no known cellular homologues and whose origins remain obscure. Although a comprehensive review of poxvirus immunoregulatory proteins is beyond the scope of this chapter, the following section provides several prototypic examples of major

poxvirus virokines and viroceptors that are involved in modulating host cytokine networks.

Virokines

Poxvirus Complement Regulatory Proteins

Complement is a major non-specific host defense against microorganisms and consists of a cascade of reactions that lead to the formation of a membrane attack complex that damages the membranes of enveloped viruses or infected cells (157). Vaccinia virus encodes a soluble 35 kiloDalton (kDa) protein, termed vaccinia complement control protein (VCP), with structural and functional similarity to the family of complement binding proteins (79, 88, 89). Highly conserved homologues of VCP have been also found in variola and cowpox viruses (125). Purified VCP has been shown to bind to C3b and C4 more potently than its cellular counterparts and block the activation of both the alternative and classical complement pathways at multiple sites (88, 123). *In vivo*, both VCP and its homologue in cowpox virus, termed IMP, have been demonstrated to function as important virulence factors by protecting the virus from antibody-dependent complement neutralization and complement-mediated host inflammation during infection (78, 79, 125).

Poxvirus Growth Factors

A variety of poxviruses including myxoma, Shope fibroma, vaccinia, variola and cowpox viruses encode growth factors with striking homology to epidermal growth factor (EGF) and transforming growth factor-α ligands (TGFα) (119). Prototypic members include the vaccinia growth factor (VGF) and the myxoma

growth factor (MGF). These poxvirus EGF/TGFα homologues are secreted glycoproteins and have been shown to be *bona fide* ligands for cellular receptors belonging to the Erb-B receptor superfamily (83, 96, 185, 194, 195). Binding of the viral growth factor to cellular Erb-B receptors stimulates receptor signal transduction pathways and causes mitogenesis in certain cell types (83, 96, 185, 194). Poxviral growth factors appear to be dispensable for viral replication since their deletion does not appear to effect virus replication in culture cells (31, 147). However, deletion of the VGF gene from vaccinia virus resulted in a significant reduction of disease in infected mice indicating that VGF is an important virulence factor *in vivo* (31). Similarly, a myxoma MGF deletion mutant virus was significantly attenuated when compared to wild-type myxoma virus in infected European rabbits (147). Moreover, cassette replacement experiments demonstrate that TGF-α, VGF and Shope fibroma virus growth factor (SFGF) can all functionally replace MGF in myxoma virus (146). It has been proposed that the biological role of the poxvirus growth factors is to cause mitogenic stimulation of quiescent cell populations within infected tissues so as to provide a more favorable environment for virus replication (119).

Orf virus, a member of the *Parapoxvirus* genus, causes a contagious pustular dermatitis in infected sheep and goats. Orf lesions appear to have excessive proliferation of vascular vessels and dermal swelling. Interestingly, Orf virus expresses a functional vascular endothelial growth factor (VEGF) ligand homologue which has been demonstrated to induce angiogenesis *in vivo* (73, 103, 124).

Poxvirus Serpins

Serine proteinase inhibitors, or serpins, are a superfamily of related proteins that regulate complex proteinase-dependent cascades in tissue remodeling, inflammatory responses and vascular coagulation (39). Although eight poxviral genes with homology to serpins have been identified to date (139, 193), SERP-1 of myxoma virus is the only known virus-encoded serpin that is secreted from infected cells (140). SERP-1 is expressed as a 55-50 kDa soluble glycoprotein during late times of myxoma virus infection and can form stable inhibitory complexes with a variety of extracellular serine proteinases such as tissue plasminogen activator, plasmin, and urokinase *in vitro* (140). Although the precise biological serine proteinase target of SERP-1 during infection remains to be identified, SERP-1 appears to be a potent biologically active virokine *in vivo*. Deletion of the SERP-1 gene from myxoma virus results in a significant attenuation of myxomatosis in infected European rabbits (106). Moreover, the anti-inflammatory properties of SERP-1 have been demonstrated in several animal models of inflammation. In a coronary restenosis model following balloon angioplasty, SERP-1 was highly efficacious in reducing the development of atherosclerotic plaque (101). In addition, articular injections of purified SERP-1 were shown to reduce cellular hyperplasia, chronic inflammatory infiltration and cartilage erosion in an animal model of antigen-induced arthritis (109).

Poxvirus Chemokines

The complete DNA sequencing of molluscum contagiosum virus revealed an ORF, designated MC148R, with high homology to CC chemokines (170). Subsequent studies demonstrated that MC148R functions as a broad spectrum chemokine

antagonist *in vitro* (49, 90). A more detailed description of MC148 is given below in the section *Virus-Encoded Chemokine Ligand Homologues.*

Viroceptors

Poxvirus Soluble IL-1β Receptors

Interleukin-1 (IL-1) is a pleiotropic cytokine that plays a critical role in mediating the host immune and inflammatory responses to tissue injury and infection (53). Vaccinia virus (strain WR) encodes an ORF, termed B15R, that possesses characteristic structural immunoglobulin domains and shares ~30% amino acid similarity with the extracellular region of cellular type II IL-1 receptors. Parallel studies have demonstrated that the vaccinia (and related cowpox) virus B15R gene product is a secreted glycoprotein capable of binding IL-1β, but not IL-1α or IL-1RA (the naturally occurring receptor antagonist) with high affinity (6, 183). Consistent with the proposed function as a *bona fide* viroceptor, soluble B15R binds IL-1β and prevents this cytokine from interacting with its cognate cellular receptors. Moreover, expression of B15R from vaccinia virus was shown capable of inhibiting the biological activity of IL-1β-induced lymphocyte proliferation *in vitro* (6, 183).

Given that the virus has targeted IL-1β, but not the other IL-1 isoforms, suggests that IL-1β may play a more crucial role in mediating the inflammatory response to virus infection. Conflicting evidence remains regarding the contribution of B15R in poxvirus virulence. B15R-null vaccinia virus recombinants cause more pronounced disease (increased morbidity) in mice but no alteration in host mortality when the virus is administered intranasally (6). In contrast, intracranial infection of

mice with the B15R-deficient virus resulted in a marked decrease in lethality compared to infection with wild-type virus supporting the notion that this viral decoy receptor contributes to virus virulence *in vivo* (183). Recent studies have shown that B15R plays a pivotal role during poxvirus infection in modulating the febrile response during vaccinia virus infection (4). Mice infected intranasally with B15R-deficient virus demonstrated an increased fever response, thus accounting for the observed accelerated disease phenotype when compared to a wild-type vaccinia infection (4).

Poxvirus Soluble TNF Receptors

The first example of a viroceptor encoded by a DNA virus was the S-T2 gene product of Shope fibroma virus that showed homology to the extracellular ligand-binding domain of cellular tumor necrosis factor (TNF) receptors (174). Related genes have subsequently been identified in other poxviruses including the M-T2 ORF of myxoma virus (169, 175, 207). Similar to cellular TNF receptors, M-T2 contains characteristic cysteine rich domains and a signal sequence, but the lack of a transmembrane domain suggests that this viral protein is soluble and binds extracellular TNF. M-T2 is a secreted glycoprotein that is expressed early during virus infection as both a 80 kDa disulfide linked dimer and a 40 kDa monomer (166, 167). Purified M-T2 has been demonstrated to bind TNFα in a species-specific manner, with an affinity comparable to its cellular counterparts (166, 167). M-T2 effectively sequesters TNF from receptor engagement and thus neutralizes the biological anti-viral activities of this cytokine (165-168). The expression of M-T2 is not required for virus replication *in vitro*, but is necessary for myxoma virus pathogenesis *in vivo* as determined by recombinant knockout virus analysis in infected European rabbits

(197). In addition to protecting virus-infected cells from TNF-induced cytolysis, M-T2 appears to have an intracellular function in blocking apoptosis. The productive infection of $CD4^+$ lymphocytes is thought to be crucial for effective virus dissemination during infection. Whereas wild-type myxoma virus productively infects and replicates in $CD4^+$ T-lymphocytes, M-T2-deficient myxoma virus recombinants cause infected $CD4^+$ T-lymphocytes to undergo premature apoptosis (105, 168). Extracellular M-T2 protein was shown incapable of rescuing M-T2 knockout virus from inducing this phenotype in infected cells, suggesting that the apoptosis protective functions of M-T2 are intracellular. Current studies are underway to determine the molecular mechanism of how intracellular M-T2 protects lymphocytes from undergoing programmed cell death. Thus, M-T2 is a unique viroceptor that has dual functions: extracellular cytokine inhibition and intracellular apoptosis inhibition.

Poxvirus Soluble IFN-α/β Receptors

Interferons (IFN) are a group of related cytokines that were originally identified by their ability to induce cellular resistance to viral infection (69). In addition to their antiviral effects, IFNs exhibit multiple proinflammatory and immunomodulatory activities. There are two major types of IFNs: type I IFN (IFN-α and IFN-β) and type II IFN (IFN-γ). Type I IFN bind to a common cellular receptor, IFN-α/β receptor, whereas, type II IFN (IFN-γ) binds to a distinct IFN-γ receptor (151). Although both types of IFN possess potent antiviral activities, IFN-γ serves as a major immune regulator by stimulating cellular (T_H1) immune responses such as the production of cytotoxic T-lymphocytes that are crucial for the destruction of virus-infected cells.

Vaccinia virus (strain WR) expresses a soluble glycoprotein, termed B18R, that binds type I IFNs (IFN-α and IFN-β) with high affinity (44, 186). The IFN-α/β binding activity of this viral protein was unexpected since B18R possesses amino acid sequence motifs characteristic of the immunoglobulin superfamily, but little sequence similarity to cellular IFN-α/β receptors. Binding of soluble B18R to type I IFNs effectively blocks the ability of these cytokines from engaging their appropriate cellular receptors and inducing intracellular signalling (44, 186). B18R exhibits a broad species-specificity and has been demonstrated to bind to multiple type I IFN isoforms with high affinity (97). Homologous B18R genes have also been detected in other orthopoxviruses including cowpox and ectromelia viruses. The expression of B18R appears to be important for vaccinia virus virulence. Vaccinia virus recombinants lacking a B18R gene are attenuated in mice infected by intracranial or respiratory routes and have restricted viral replication and dissemination in tissues of infected mice (186).

Poxvirus Soluble IFN-γ Receptors

The identification of the myxoma virus M-T7 gene product as an IFN-γ receptor homologue gave the first indication that viruses produce soluble decoy receptors to neutralize the activities of IFNs (198). M-T7, the gene product of the seventh ORF from the termini of the myxoma virus genome, shares approximately 30% homology to the extracellular ligand-binding domain of cellular IFN-γ receptors including the strict conservation of eight structurally critical cysteine residues. Interestingly, the carboxyl terminus of M-T7 possesses a stretch of 60 amino acids that does not share any sequence identity to IFN-γ receptors or other cellular proteins.

Consistent with its proposed role as a soluble viral IFN-γ receptor, M-T7 was subsequently shown to bind to IFN-γ with high affinity (K_d = 1.2 nM) and inhibit its antiviral activities (133, 198). A variety of orthopoxviruses have also been demonstrated to express M-T7 homologues, but unlike M-T7 which strictly binds and inhibits rabbit IFN-γ, orthopoxvirus soluble IFN-γ receptors display a broad ligand-binding species specificity (5, 7, 130, 132). In myxoma virus, M-T7 is the most abundantly secreted virus protein from infected cells (198). M-T7 is secreted as a 37 kDa glycoprotein early during myxoma virus infection and accumulates (10^7 molecules/cell/hour) in the extracellular milieu as a stable protein throughout the course of virus infection (133).

M-T7 is a crucial myxoma virus virulence factor for the progression of myxomatosis in European rabbits. Rabbits infected with a myxoma virus in which both copies of the M-T7 ORF had been disrupted recovered fully and displayed a vigorous and effective cellular response to infection (131). In particular, the absence of M-T7 expression resulted in an elevation of reactive leukocyte levels in secondary lymphoid organs, contributing to efficient virus clearance. Furthermore, dermal tissue sites surrounding virus infection showed a striking disruption of the inflammatory cell migration, while tissues from a rabbit with an M-T7 deletion virus infection showed this apparent blockade of leukocyte infiltration to be relieved (131). It was hypothesized, therefore, that M-T7 may perform additional biological functions such as modulating the directional signals required for trafficking of leukocytes. Astonishingly, purified M-T7 was subsequently shown to also bind to multiple members of the chemokine superfamily (see Chapter II) (93). Thus, we propose that

M-T7 represents a multi-functional immunosubversive protein targeted toward neutralizing the biological activities of both IFN-γ and members of the chemokine superfamily within infected tissues.

Other Soluble Poxvirus Cytokine Binding Proteins

Recently, several novel cytokine-binding proteins have shown to be expressed by poxviruses. A secreted 35 kDa glycoprotein expressed from tanapoxvirus has been shown to be capable of interacting with three distinct cytokines, IL-2, IL-5, and IFN-γ (58). The gene encoding this multi-functional viral protein has yet to be formally mapped. A 58 kDa protein soluble protein expressed by Orf virus was demonstrated to bind and inhibit the activity of ovine granulocyte macrophage-colony stimulating factor (GM-CSF) (72). The significance of this protein in Orf infection and its gene identification has also yet to be determined. A naturally occurring cellular IL-18 binding protein (IL-18BP) was recently identified and shown to bind and neutralize the activities of IL-18, but its sequence is unrelated to cellular IL-18 receptors (145). Sequence analysis of IL-18BP showed it to possess significant homology to genes found in certain poxviruses including molluscum contagiosum, swinepox, cowpox, variola and ectromelia viruses (145). Future studies aimed at the characterization of poxvirus IL-18 binding proteins during infection will be of significant interest and may further our understanding in the role that this newly discovered T_H1 cytokine plays in host defense.

CHEMOKINES AND CHEMOKINE RECEPTORS

A critical feature involved in the early host inflammatory response to an initial virus challenge is the accumulation of immune cells at the site of infection or tissue damage which help initiate the earliest phases of anti-viral immune activation (141, 208). Neutrophils, monocytes/macrophages and natural killer (NK) cells in particular, participate in the first wave of cellular infiltration but require directional signals to mobilize them to infected tissues for localized combat with the invading pathogen (23, 184). The selective activation and recruitment of inflammatory cells to sites of virus infection is largely orchestrated by a growing superfamily of low-molecular-weight chemoattractant cytokines known collectively as chemokines (reviewed extensively in (11, 13, 99, 102, 161, 162, 188). This superfamily of proinflammatory molecules is divided into four subfamilies (C, CC, CXC, and CX_3C) based on the number and spatial arrangements of conserved cysteine residues within their peptide sequences. These designations also loosely correlate with the target cell populations to which they impart their biological activity. For example, CXC chemokines such as interleukin-8 (IL-8) and growth-related oncogene-α (GROα) are generally potent activators and chemoattractants of neutrophils but not monocytes (14). In contrast, CC chemokines such as macrophage inflammatory protein-1α (MIP-1α), MIP-1β, monocyte chemoattractant peptide-1 (MCP-1) and regulated upon activation normal T-cell expressed and secreted (RANTES), have been shown to attract and activate monocytes/macrophages, T-lymphocytes, NK cells, granulocytes and dendritic cells (12, 154). Lymphotactin (Ltn), the sole member of the C subfamily to date, appears to be a T-lymphocyte-specific chemoattractant (80). Fractalkline, the only CX_3C chemokine member, is the only membrane-bound chemokine that is known (20).

Unlike other chemokines, fractalkline contains a mucin-like stalk in addition to a transmembrane domain at the COOH- terminus of the protein that facilitates both cell-adhesion and migration of lymphocytes (20). In addition to playing a key role in mediating leukocyte influx during inflammatory responses, chemokines impart multiple biological effects in a variety of cell types by binding to specialized receptors that belong to the seven-transmembrane G protein-coupled receptor superfamily (for several excellent reviews see 81, 99, 135, 136, 153). Over 40 chemokines and 15 chemokine receptors have been characterized with many additional candidates currently under investigation (13, 107). A current list of representative human chemokines and their target cell surface receptors is shown in Table I-3.

Biological Activities of Chemokines

The most salient biological activity of chemokines is their ability to stimulate the unidirectional migration (chemotaxis) of leukocytes. In addition, chemokines induce many cellular functions including cytoskeletal reorganization, lymphocyte activation, granule release and oxidative burst, upregulation and activation of cell adhesion molecules, cell growth and differentiation, and release of inflammatory cytokines (11, 14, 161). Recent studies have also shown a role of chemokines in leukocyte maturation and development of lymphoid tissues (11).

Since chemokines play a critical role in the generation of cellular inflammation, it is not surprising that the dysregulation of chemokine and chemokine receptor expression contributes to the pathophysiological processes of many inflammatory disease states (102). For example, chemokines have been implicated in

asthma, atherosclerosis, allergic reactions, rheumatoid arthritis, inflammatory bowel disease, psoriasis, and pulmonary disorders (1, 12, 71, 91, 102, 134, 188, 189).

Direct evidence for the anti-viral properties of chemokines comes from several studies on chemokine knockout mice, human immunodeficiency virus-1 (HIV-1) pathogenesis, and recombinant chemokine-expressing vaccinia viruses. Mice bearing a homozygous deletion in the MIP-1α gene have a severely impaired inflammatory response to either coxsackie or influenza virus (45). Furthermore, the CC chemokines MIP-1α, MIP-1β, and RANTES, have recently been shown to suppress HIV-1 infection *in vitro* and *in vivo* (43, 149). Studies on long-term asymptomatic HIV-infected individuals illustrate that chemokines may contribute significantly to the outcome of viral disease progression and pathogenesis (84). The role of chemokines in mediating host defense against poxviruses has also recently been addressed. Low-dose infection of recombinant vaccinia viruses expressing the murine CXC chemokines, monokine induced by gamma interferon (MIG) and cytokine responsive gene-2 (CRG-2), resulted in marked attenuation and reduced pathogenicity in athymic nude mice in contrast to a generalized lethal infection caused by a control vaccinia virus (108). The antiviral effects of these chemokines *in vivo* appear to be partially mediated by an enhanced cytolytic activity of infiltrating NK cells at sites of vaccinia virus replication (108). These findings, taken together with the identification of a number of virus-encoded chemokine modulators (described below), underscore the importance of chemokines in the host immune response against virus infections.

Structure Function Studies of Chemokines

Chemokines are small (8-10 kDa), disulfide-linked and highly basic polypeptides. Although they show limited (20-80%) overall sequence homology at the amino acid level, structural studies demonstrate that members of the chemokine superfamily adopt a similar tertiary structure; a variable flexible NH_2-terminal loop, three antiparallel β-strands and a COOH-terminal α-helix (16, 37, 40, 42, 100, 155, 156, 173). Studies using ala-scan or truncation mutants demonstrate that distinct residues found within the NH_2-terminus of chemokines are critical for high-affinity receptor binding or receptor activation (40, 41, 65-67, 75, 148, 203). Determinants that dictate selectivity of chemokine receptor binding have also been localized to the NH_2-terminus (40, 148, 203). In addition to binding to chemokine receptors, chemokines bind glycosaminoglycans (GAGs) such as heparin with lower affinity through their COOH-terminus (206). The COOH-terminal α-helix of most chemokines possess a stretch of surface-exposed positively charged amino acids which is called the GAG- or heparin-binding domain (70, 86, 180, 202). It has been proposed that the interaction of chemokines with GAGs, either at the surface of endothelial cells or within the extracellular matrix, allows a solid-phase chemokine gradient to be established and may allow for effective presentation to their target leukocyte receptors *in vivo* (158, 187, 202, 206). Some evidence also suggests that interactions of certain CXC chemokines to GAGs may potentiate their activities (202). Furthermore, the high affinity receptor-binding and lower affinity GAG-binding domains of some chemokines may be functionally interdependent. Mutations in the GAG-binding sites

of MIP-1α were shown to abrogate several functional activities ascribed to the high-affinity interaction of this chemokine to its cognate receptors (68).

Chemokine Receptors

Chemokine receptors are seven-transmembrane-spanning, G protein-coupled receptors that are expressed on a variety of leukocytes (135, 136, 153). This subfamily of receptors are all structurally related, have a single polypeptide chain and have 25-80% amino acid sequence identity to each other. Over a dozen, distinct cellular chemokine receptors have now been identified (Table I-3) (11, 99, 204). In general, most chemokine receptors bind multiple chemokines within a given chemokine subfamily with nanomolar affinity. Some receptors have a restricted pattern of expression (i.e. CXCR1 is expressed predominantly on neutrophils), whereas others are more widely expressed (i.e. CCR2 is expressed on T-lymphocytes, monocytes, NK cells and dendritic cells) (102). Furthermore, certain chemokine receptors appear to be constitutively expressed whereas some are induced upon cellular activation. For example, CCR5 is expressed preferentially in activated T-lymphocytes belonging to the T_H1 phenotype. In contrast, CCR3 and CCR4 appear to be expressed on activated T_H2-type lymphocytes (160, 201).

Site-directed mutagenesis and domain swapping experiments reveal that the highly variable extracellular NH_2-terminus of chemokine receptors, such as CXCR1, appear to be critical in chemokine binding and determining ligand specificity (2, 74, 94, 95). In addition, residues within the second and third extracellular loops of CXCR1 have also been found to be important for high affinity binding to IL-8. The intracellular COOH-terminus of chemokine receptors interacts with heterotrimeric

guanine nucleotide-binding proteins (G proteins) (28). Receptor engagement elicits a series of intracellular signals involving phospholipases and inositol triphosphate which culminates in the release of intracellular Ca^{2+} and activation of protein kinase C. Chemokine signalling pathways are sensitive to *Bordetella pertussis* toxin, suggesting that chemokine receptors are linked primarily to the G_i-type of heterotrimeric G proteins (137). Elucidation of chemokine-mediated signalling pathways is still in its infancy and continues to be an active area of investigation.

Role of Chemokine Receptors in HIV-1 entry

The relationship between chemokines and viruses has recently become an area of intense scientific investigation as a result of the seminal observation made by Lusso and colleagues that the CC chemokines, MIP-1α, MIP-β, and RANTES, potently suppress HIV-1 infection (43). Shortly after this remarkable discovery, several independent labs showed that certain chemokine receptors function as obligate coreceptors for HIV-1 entry into $CD4^+$ leukocytes (9, 35, 51, 55, 56, 59). CCR5 has been established as the major coreceptor for macrophage-tropic strains of HIV-1, whereas, CXCR4 is the major coreceptor for entry by T-cell tropic strains. Recently, several additional chemokine receptors including CCR2, CCR3, CCR8, and CX_3CR1, have been shown to function as HIV coreceptors but only for a limited number of virus strains (196). The role of chemokine receptors in HIV pathogenesis is further underscored by the finding that polymorphic variants of CCR5, CCR2 and the chemokine, SDF-1, confer resistance to HIV-1 infection *in vivo* or slow the rate of disease progression to AIDS (24). The fusion mechanism that governs HIV-1 entry into target cells has not yet been fully elucidated but is known to involve complex

interactions between multiple extracellular domains of chemokine receptors, the V3 loop of HIV-1 envelope glycoprotein 120, and CD4 (25, 36). Readers are encouraged to consult several excellent summaries that have been recently published on this topic (18, 24, 54, 98, 99).

ANTI-CHEMOKINE STRATEGIES EMPLOYED BY DNA VIRUSES

Given the central role of chemokines in mediating an inflammatory response for effective viral clearance (163), it is not surprising that some viruses have adopted cunning tricks for abrogating their biological activities. Over two dozen virus gene products have now been identified in poxviruses and herpesviruses which appear to regulate chemokines and chemokine receptors and this list is likely to grow in the coming years as more pathogens reveal their anti-chemokine strategems (Table I-4). There are currently three modes of chemokine modulation known to be used by these viral proteins: (1) virus-encoded secreted chemokine ligand homologues (vCk) which function as antagonists; (2) virus-encoded cell-surface chemokine receptor homologues (vCkR); and (3) virus-encoded secreted chemokine binding proteins (vCkBP) (this thesis) (Figure I-2) (48, 92, 150, 205).

Virus-Encoded Chemokine Ligand Homologues (vCk)

The complete sequencing of many herpesvirus and poxvirus genomes has revealed several viral ORFs with striking homology to chemokine ligands (Table I-4). Human herpesvirus-8 (HHV-8) is an oncogenic γ-herpesvirus etiologically linked to Kaposi's sarcoma (KS) and Castleman's disease. In addition to a number of other

virus-encoded immunomodulatory proteins, HHV-8 possesses three ORFs, termed K4/vMIP-II, K6/vMIP-I and BCK/vMIP-III, with 25-40% sequence identity to the CC chemokine, MIP-1α (126, 144).

K4/vMIP-II of HHV-8 is a potent antagonist for a wide range of chemokine receptors. By competition binding analysis, K4/vMIP-II was demonstrated to effectively displace chemokine binding to a number of different CC and CXC chemokine receptors with high affinity *in vitro* (85). Binding of this vCk to chemokine receptors did not induce cellular signal transduction suggesting that it functions as a competitive antagonist. Consistent with this, K4/vMIP-II potently blocked the chemoattractive effects of both CC and CX_3C chemokines *in vitro* (29, 34, 85). Moreover, K4/vMIP-II was shown to markedly reduce the influx of inflammatory leukocytes and attenuate the host inflammatory response in a rat model of glomerulonephritis (34).

In addition to blocking cellular influx, vCk's may also have secondary functions *in vivo*. The formation of new blood vessels (angiogenesis) is central in the pathology of KS and HHV-8 associated tumors demonstrate marked neovascularization. Although CC chemokines do not exhibit angiogenic activities *per se*, remarkably, both vMIP-I and v-MIP-II induced angiogenesis in a chick chorioallantoic membrane assay (29). Thus, although it seems likely that these viral proteins function to prevent leukocytic mobilization into infected tissues, HHV-8 vCk's may also play a role to some extent in contributing to the angioneoplasms of KS.

The human poxvirus, molluscum contagiosum virus (MCV), induces benign proliferative skin lesions that can cause serious clinical problems in immunocompromised hosts. MCV lesions are typically devoid of infiltrating inflammatory cells suggesting a possible alteration of local chemoattractant activities in dermal tissues during virus infection. Genomic sequencing of MCV permitted the identification of two candidate viral proteins, one of which designated MC148R, has direct anti-chemokine activity (170). MC148R is a 104 amino acid peptide that is structurally related (approximately 30% sequence identity) to CC chemokines. Interestingly, MC148R contains a short five amino acid deletion at its NH_2-terminus, a domain of CC chemokines that is known to be critical for receptor activation. Consequently, MC148R was predicted to bind chemokine receptors but to be unable to elicit chemotactic activity. Consistent with this, recombinant MC148R protein was shown to lack intrinsic chemoattractant properties, but effectively interfered with the chemotaxis of monocytes, lymphocytes and neutrophils in response to both CC and CXC chemokines (49, 90). Although direct binding of MC148R to chemokine receptors has not yet been demonstrated, competition receptor binding analysis suggests that MC148R interacts with multiple CC and CXC chemokine receptors with high affinity (49). Unfortunately, since the virus cannot be propagated in cell culture and no animal models of infection currently exist, the precise biological role of MC148R during MCV pathogenesis remains uncertain.

The molecular basis governing the broad-spectrum antagonistic properties of any vCk remains to be determined. For MC148R, the inhibitory properties may reside in a natural deletion of a receptor activation domain found at the NH_2-terminus of the

ligand. If this is the case, MC148R may be a highly illustrative example of how viruses not only pirate, but also modify host genes in an effort to combat the immune defenses. Researchers have begun using this same strategy to generate novel effective anti-inflammatory agents. A variety of multi-specific and high-affinity antagonists have been made by deleting or modifying selective residues within the NH_2-terminus of chemokine peptides (67, 121, 203). Without further mutational or structural studies, the antagonistic basis of HHV-8 vCk's remains obscure since the NH_2-terminal portion of the viral peptides is similar to that of their mammalian counterparts.

Human herpesvirus-6 and murine cytomegalovirus also possess ORFs with homology to CC chemokines (64, 104). Since they have yet to be characterized, it is not known whether these vCk's function as agonists or as antagonists.

Virus-Encoded Chemokine Receptor Homologues (vCkR)

A striking number of viral ORFs have been identified in herpesvirus and poxvirus genomes that resemble cellular chemokine receptors (Table I-4). Many of these viral chemokine receptors homologues (vCkR) have been shown to effectively engage chemokines and/or transmit intracellular signals suggesting that vCkR's may play a role in modulating the host response for the benefit of viral infection and propagation.

Sequence analysis of human cytomegalovirus (HCMV) predicts three ORFs, US27, US28 and UL33, that share conservation to chemokine receptors (33). Of these, the biochemical properties of US28 have been the best characterized. HCMV US28 most closely resembles CC chemokine receptors in the NH_2-terminal domain (63). Consequently, binding studies have demonstrated that US28 interacts with multiple

CC chemokines with high affinity but exhibits no affinity for CXC chemokines (63, 142). In addition, US28-transfected cells have been shown to be capable of intracellular calcium signalling in response to CC, but not CXC chemokines (63).

US28 is transcribed as an early gene from HCMV-infected cells and does not appear to be required for viral replication in cultured cells (199). US28 expressed from HCMV-infected cells was demonstrated to bind CC chemokines with high affinity and effectively depleted the amount of chemokines present in the extracellular milieu surrounding HCMV-infected cells *in vitro* (27, 199). However, given the intracellular signalling properties of this vCkR, it seems unlikely that US28 functions in evading the host immune response by direct sequestration of chemokines from the environment of virus-infected cells. Although the role of US28 during HCMV infection remains to be determined, it is intriguing to speculate whether US28 may promote dissemination of HCMV-infected cells in response to chemokine gradients *in vivo*. A similar role has also been postulated for the M33 gene product of murine cytomegalovirus (MCMV) which is 45% homologous and colinear with HCMV UL33 (50, 110). Both genes appear to be dispensable for virus replication *in vitro*. However, deletion of M33 from MCMV results in severe restriction of virus growth in the salivary gland, a major site of viral replication (50). Thus, certain herpesviruses may express a vCkR to promote the trafficking of infected leukocytes or transmission of virus to target organs *in vivo*.

A number of γ-herpesviruses have been shown to also express a functional vCkR. First identified in herpesvirus saimiri as the gene product of the ECRF3 gene, this vCkR is most closely related to the cellular IL-8 receptor, CXCR2 (143). Consistent with its predicted role as a CXC chemokine receptor, transfected ECRF3

was shown to be capable of eliciting intracellular Ca^{2+} signalling in response to a variety of CXC, but not CC chemokines (3). HHV-8 encodes a closely related vCkR, termed ORF74, which is homologous and colinear with HVS ECRF3. In contrast to ECRF3 however, HHV8-ORF74 displays a broader chemokine binding repertoire by interacting with a number of CXC and CC chemokines (10). Remarkably, and in contrast to other viral or cellular chemokine receptors currently known, HHV-8 ORF74 appears to be a constitutively activated receptor able to induce cellular signalling in transfected cells in the absence of chemokine stimulation (10). In transfected rat fibroblasts, HHV8 ORF74 induced cellular proliferation and tumorogenicity implicating its potential role as a cellular transformation factor, although no evidence exists for the role of this vCkR in contributing to the development of HHV-8 associated malignancies (15).

The role of ORF74 *in vivo* currently remains undefined due to the lack of an appropriate animal model for HHV-8. However, a mouse model for studying γ-herpesvirus pathogenesis has recently been developed using murine γherpesvirus-68. The genome sequencing of murine γherpesvirus-68 has recently been completed and shown to encode an ORF74 which has significant similarity to vCkRs encoded by HVS and HHV-8 (200). Deletion analysis of the murine γherpesvirus-68 ORF74 will be of significant interest for understanding the role of this vCkR in viral infection, persistence, cellular transformation and pathogenesis.

Two putative vCkRs have also been identified in members of the poxvirus family. Both K2R and the truncated C3L ORFs of swinepox and Q2/3L ORF of capripox viruses were identified as bearing sequence similarity and hydropathy

profiles similar to chemokine receptors (32, 111). To date, the functional properties of these viral ORFs have not been described.

Virus-Encoded Chemokine Binding Proteins (vCkBP)

The most recent examples of viral chemokine modulators are two novel classes of poxvirus-encoded soluble chemokine binding proteins, whose identification and characterization form the basis of this thesis. As will be described in the following chapters, myxoma virus expresses two distinct soluble glycoproteins, M-T1 and M-T7, that bind chemokines and modulate leukocyte influx *in vivo*. M-T1 and M-T7 represent the first examples of soluble chemokine scavengers as part of a novel viral strategy to prevent the infiltration of inflammatory cells into virus infected tissues.

THESIS OBJECTIVE

The purpose of this thesis is to further our knowledge in the areas of poxviral strategies to evade the host inflammatory response to infection. The studies presented herein describe the identification, biochemical and functional characterization of two distinct classes of poxvirus soluble glycoproteins that bind chemokines and modulate their proinflammatory activities. The identification and characterization of poxvirus soluble chemokine binding proteins not only increases our understanding of the mechanisms used by viruses to achieve virulence and induce pathogenesis in their infected hosts, but also sheds light on the role of chemokines as crucial effector molecules in the host defense against pathogens. Several studies were undertaken to achieve this objective:

(1) Previous studies from the McFadden laboratory suggested that the myxoma virus IFN-γ receptor homologue, M-T7, may have additional functions such as blocking leukocyte influx *in vivo* (131). Therefore, M-T7 was purified to homogeneity to further characterize the biochemical properties of this protein *in vitro*. Binding analyses revealed that purified M-T7, in addition to binding to IFN-γ, was capable of binding to a variety of chemokines with sub-μM affinity. A variety of binding studies using chemokine truncation mutants were performed to localize the conserved domains of chemokines necessary for M-T7 binding.

(2) Binding analysis using the myxoma M-T7 deletion virus revealed the presence of an additional secreted myxoma virus chemokine binding protein that was distinct from M-T7. Genetic loss/gain of function tests using recombinant poxviruses were used to identify the second class of poxvirus chemokine binding protein as the M-T1 gene product of myxoma virus and its homologous "major secreted 35kDa" proteins of many orthopoxviruses. Chemical cross-linking and solid-phase binding analyses were performed to assess the ligand specificity and affinity of the T1/35kDa viral proteins to chemokines. To determine whether members of the T1/35kDa family of proteins could modulate leukocyte chemotaxis *in vivo*, histological analysis of lesions from rabbits infected with a rabbitpox virus 35kDa-null mutant was performed.

(3) To determine whether members of the T1/35kDa family of proteins were functional inhibitors of chemokines *in vitro*, three representative members of the T1/35kDa family of proteins, namely M-T1, vaccinia virus-35kDa and rabbitpox virus-35kDa, were purified to homogeneity. Functional comparisons of all three

proteins were examined in their ability to inhibit the biological activities of CC and CXC chemokines using several *in vitro* assays.

(4) To determine the biological role of M-T1 during myxoma virus pathogenesis, European rabbits were infected with a recombinant myxoma M-T1-deletion mutant virus and assessed for differences in gross- and histo-pathological profiles.

Table I-1. Poxviruses of Vertebrates

(Family: *Poxvirinae* ; Subfamily: *Chordopoxvirinae*)

GENUS	SPECIES[†]
Orthopoxvirus	**Vaccinia**, variola (smallpox), rabbitpox, cowpox, ectromelia (mousepox), monkeypox, camelpox, buffalopox, raccoonpox, taterapox, and volepox viruses
Leporipoxvirus	**Myxoma**, Shope fibroma, hare fibroma and squirrel fibroma viruses
Molluscipoxvirus	**Molluscum contagiosum** virus
Yatapoxvirus	**Tanapox** and yaba viruses
Suipoxvirus	**Swinepox** virus
Capripoxvirus	**Sheeppox**, goatpox and lumpy skin disease viruses
Avipoxvirus	**Fowlpox**, canarypox, pigeonpox, sparrowpox, turkeypox, quailpox and juncopox viruses
Parapoxvirus	**Orf**, pseudocowpox, stomatitis papulosa and chamois contagious ecthyma viruses

[†]Prototypic members are denoted in bold case

Table I-2. Examples of Poxvirus Immune Evasion Strategies†

VIROKINES

Type	*ORF (Virus)*
Complement Binding Proteins	VCP (vaccinia virus)
Serine Proteinase Inhibitors (Serpins)	SERP-1 (myxoma)
EGF/TGFα-like Growth Factors	MGF (myxoma virus)
VEGF-like Growth Factors	A2R (orf virus)
CC Chemokines	MC148R (molluscum contagiosum)

VIROCEPTORS

Type	*ORF (Virus)*
Soluble IL-1β Receptor	B15R (vaccinia virus)
Soluble TNF Receptor	M-T2 (myxoma virus)
Soluble IFN-α/β Receptor	B18R (vaccinia virus)
Soluble IFN-γ Receptor	M-T7 (myxoma virus)
Soluble Chemokine Binding Protein	M-T1 (myxoma virus M-T7 (myxoma virus)
7-Transmembrane Chemokine Receptor	K2R (swinepox virus) Q2/3L (capripox virus)
Soluble IL-18 Binding Protein	ORF54L (molluscum contagiosum)
Soluble IL-2/IFN-γ/IL-5 Binding Protein	ORF not identified (tanapox virus)
Soluble GM-CSF Binding Protein	ORF not identified (orf virus)

VIROMITIGATORS OF APOPTOSIS

Features or Known Function	*ORF (Virus)*
Caspase inhibitor	CrmA (cowpox virus)
Localizes to mitochondrial membranes	M11L (myxoma virus)
Host range virulence factor – ankyrin motifs	M-T5 (myxoma virus)
ER Resident protein –RDEL motif	M-T4 (myxoma virus)
Blocks TNF-R/Fas signalling pathways?	M-T2 (myxoma virus)
Bcl2 homologue	A179 (African swine fever virus)
Viral FLIPs -death effector domains	MC159/160 (molluscum contagiosum)
Oxygen radical scavenger	MC066L (molluscum contagiosum)
IAP homologue – caspase inhibitor	A224L (African swine fever virus)
PKR inhibitor	E3L (vaccinia virus)

†Only prototypic examples are shown

Table I-3. Human Chemokines and their Receptors

CC chemokines	*Receptor*
RANTES, MIP-1α, MIP-1β, MCP-2, MCP-3	CCR1
MCP-1, MCP-2, MCP-3, MCP-4	CCR2
Eotaxin, Eotaxin-2, RANTES, MCP-2, MCP-3, MCP-4	CCR3
TARC	CCR4
RANTES, MIP-1α, MIP-1β	CCR5
MIP-3α	CCR6
MIP-3β	CCR7
I-309	CCR8
RANTES, MIP-1α, MIP-1β, MCP-1	CCR9

CXC chemokines	*Receptor*
IL-8, GCP-2	CXCR1
IL-8, GROα,β,γ, NAP-2, ENA78, GCP-2	CXCR2
IP-10, MIG, I-TAC	CXCR3
SDF-1α,β	CXCR4
BCA-1	CXCR5

C chemokines	*Receptor*
Lymphotactin	XCR1

CX_3C chemokines	*Receptor*
Fractalkline	CX_3CR1

Abbreviations: RANTES, regulated upon activation normal T-cell expressed and secreted; MIP, monocyte inflammatory protein; MCP, monocyte chemoattractant peptide; TARC, thymus and activation-regulated chemokine; IL-8, interleukin-8; GRO, growth related oncogene; NAP-2, neutrophil activating peptide-2; ENA78, epithelial neutrophil activating peptide; GCP-2, granulocyte chemotactic protein-2; IP-10, interferon-γ inducible protein; MIG, monokine induced by interferon-γ; SDF-1, stromal cell-derived factor-1; BCA-1, B cell attracting chemokine-1

Table I-4. Virus-Encoded Modulators of Chemokines and Chemokine Receptors

Viral Chemokine Ligand Homologues (vCk)

ORF	VIRUS	HOMOLOGUE	FEATURES
MC148R	molluscum contagiosum	MCP-1	broad spectrum CC & CXC chemokine antagonist
K4/vMIP-II	human herpesvirus-8	MIP-1α	broad spectrum CC, CXC, & CX_3C chemokine antagonist
K6/vMIP-I	human herpesvirus-8	MIP-1α	broad spectrum chemokine antagonist
BCK/vMIP-III	human herpesvirus-8	MIP-1β	n/d
U83	human herpesvirus-6	MIP-1α	n/d
M131/MCK-1	murine cytomegalovirus	MIP-1β	n/d
Not determined	stealth virus	Groα/MGSA	n/d
Not determined	Marek's disease virus	IL-8	n/d

Viral Chemokine Receptor Homologues (vCkR)

ORF	VIRUS	HOMOLOGUE	FEATURES
ORF74	human herpesvirus-8	CXCR2	constitutively signalling and agonist-independent receptor
U12	Human herpesvirus-7	CCR	n/d
U12	Human herpesvirus-6	CCR3	CC chemokine signalling receptor
ECRF3	herpesvirus saimiri	CXCR2	CXC chemokine signalling receptor
US28	human cytomegalovirus	CCR1	CC chemokine signalling receptor; HIV entry coreceptor
UL33	human cytomegalovirus	CCR1	localizes to virus envelope particles
M33	murine cytomegalovirus	CCR1	important for viral dissemination to salivary glands
ORF74	murine γherpesvirus-68	CXCR2	n/d
ORF74, E1,E6	equine herpesvirus-2	CXCR2/CCR1	n/d
K2R	swinepox virus	CXCR2	n/d
Q2/3L	capripox virus	CCR4	n/d

Viral Chemokine Binding Proteins (vCkBP)

ORF	VIRUS	HOMOLOGUE	FEATURES
M-T1	myxoma virus	?	broad spectrum CC chemokine inhibitor
S-T1	Shope fibroma virus	?	broad spectrum CC chemokine inhibitor
35kDa	rabbitpox virus	?	broad spectrum CC chemokine inhibitor
C23L/B29R	vaccinia virus (strain Lister)	?	broad spectrum CC chemokine inhibitor
G3R	variola (smallpox) virus	?	broad spectrum CC chemokine inhibitor
D1L/H5R	cowpox virus	?	broad spectrum CC chemokine inhibitor
M-T7	myxoma virus	IFN-γ receptor	binds C, CC, CXC chemokines via heparin binding domains

Figure I-1. *Bam*H1 map of the myxoma virus genome.

Shown is an illustration of the *Bam*H1 map of the myxoma virus genome with vertical lines indicating *Bam*H1 restriction enzyme sites. The locations of several open reading frames encoding known virulence factors (black boxes), M-T1, M-T2, M-T4, M-T5, M-T7, SERP-1, MGF and M11L, and the length of the terminal inverted repeats (TIR) are also shown.

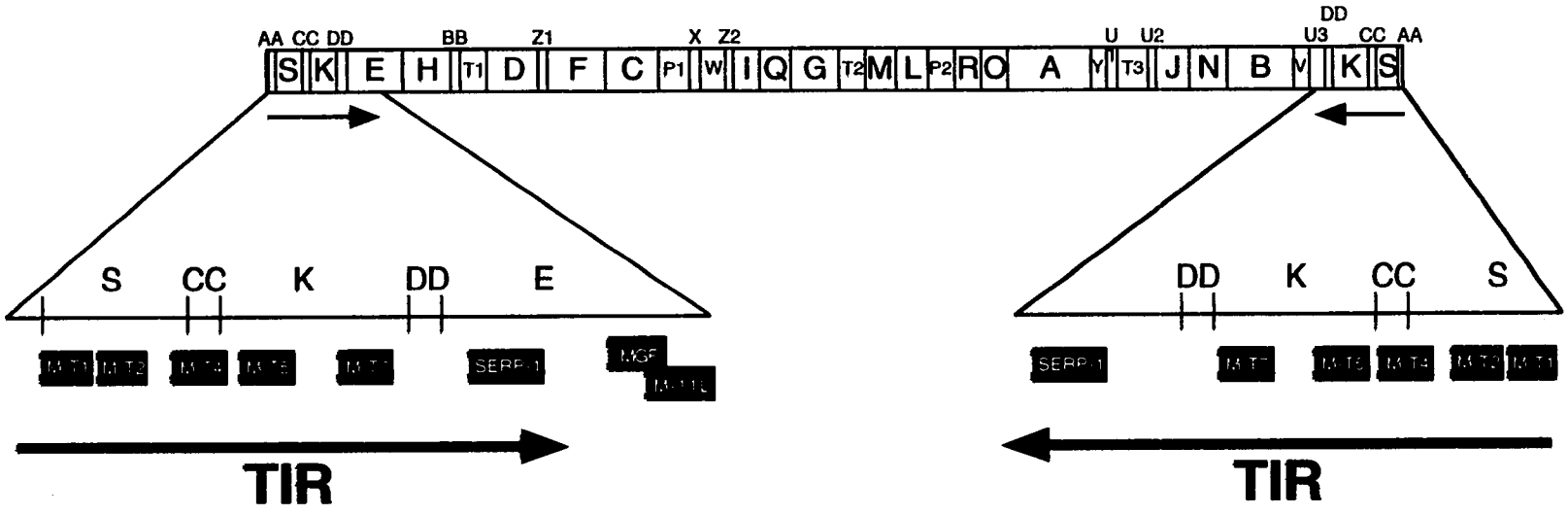
AA CC DD
BB
Z1
X Z2
U U2
DD
U3 CC AA
S K E H T1 D F C P1 W I Q G T2 M L P2 R O A Y T3 J N B V K S
S CC K DD E
DD K CC S
MGF
SERP-1
SERP-1
TIR
TIR

Figure I-2. Viral strategies for modulating chemokines and chemokine receptors.*

To thwart host defenses, viruses express three classes of proteins which alter the function of chemokines or chemokine receptors during infection: (1) virus-encoded chemokine receptor homologues which may sequester chemokines or promote viral dissemination; (2) virus-encoded chemokine ligand homologues which function as competitive antagonists; and (3) virus-encoded secreted chemokine binding proteins which sequester and neutralize soluble or surface-bound chemokine gradients in infected tissues.

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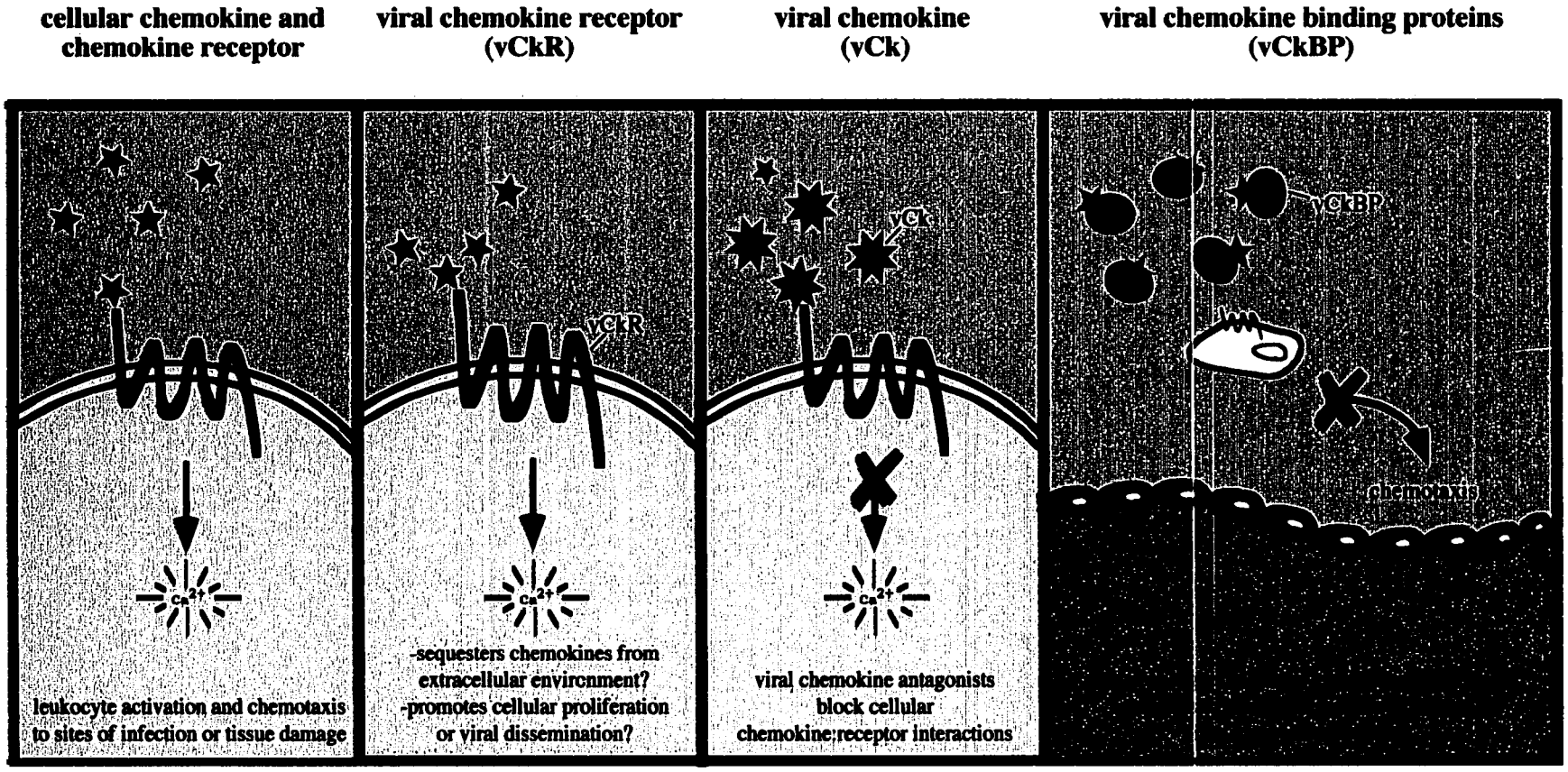
cellular chemokine and chemokine receptor
viral chemokine receptor (vCkR)
viral chemokine (vCk)
viral chemokine binding proteins (vCkBP)
vCkR
vCk
vCkBP
chemotaxis
Ca2+
Ca2+
Ca2+
leukocyte activation and chemotaxis to sites of infection or tissue damage
-sequesters chemokines from extracellular environment?
-promotes cellular proliferation or viral dissemination?
viral chemokine antagonists block cellular chemokine:receptor interactions

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CHAPTER II

THE PURIFIED MYXOMA VIRUS IFN-γ RECEPTOR HOMOLOGUE, M-T7, INTERACTS WITH THE HEPARIN-BINDING DOMAINS OF CHEMOKINES*

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INTRODUCTION

It is becoming increasingly evident that poxviruses have evolved a multitude of strategies to subvert the development of the host inflammatory response, thereby fostering virus survival in the wild (22, 41, 42). In particular, poxviruses encode specific gene products that have significant homology to cellular cytokine receptors and may function by sequestering the host cytokine before their interaction with their cognate receptors (3, 23). The myxoma virus T7 protein, M-T7, is a functional soluble IFN-γreceptor homologue that has previously been demonstrated to bind IFN-γ with high affinity, and inhibit its anti-viral activities in a species specific manner (25, 44).

We have recently shown that M-T7 is a crucial virulence factor in progression of myxomatosis in European rabbits (24). Rabbits infected with a myxoma virus in which both copies of the M-T7 open reading frame had been disrupted recovered fully and displayed a vigorous and effective cellular immune response to infection. In particular, the absence of M-T7 expression resulted in an elevation of reactive-leukocyte levels in secondary lymphoid organs, contributing to efficient virus clearance. Furthermore, dermal tissue surrounding sites of wild-type virus infection showed a striking disruption of inflammatory cell migration while tissues from a rabbit with an M-T7 deletion virus infection showed this apparent blockade of leukocyte infiltration to be relieved (24). Since it has yet to be demonstrated that IFN-

γ blockading alone influences inflammatory cell trafficking, we speculated that M-T7 may perform additional functions apart from binding and inhibiting rabbit IFN-γ.

The activation and recruitment of specialized leukocyte populations to sites of viral infection and inflammation is largely orchestrated by a growing superfamily of chemoattractant cytokines called chemokines (4, 19, 37). To investigate a possible relationship between M-T7 and other pro-inflammatory cytokines such as the chemokine family, we purified M-T7 to homogeneity and characterized the protein *in vitro*. In this report we show that in addition to binding rabbit IFN-γ, purified M-T7 interacts with multiple members of the chemokine superfamily. Experiments using various NH_2- and COOH-terminal chemokine mutants suggest that this novel activity may be facilitated through interactions between M-T7 and the conserved heparin-binding domains found in a wide spectrum of chemokines.

RESULTS

Purification and Characterization of M-T7

To determine whether M-T7 may have additional activities independent of rabbit IFN-γ binding, we purified M-T7 to homogeneity to further characterize its biochemical properties *in vitro*. M-T7 protein was purified from proteins secreted from myxoma virus-infected cells by a two-step procedure using FPLC. As shown in Fig. II-1A, concentrated myxoma supernatants were fractionated by anion-exchange and gel-filtration chromatography using Mono-Q and Superdex-200 columns, and protein fractions containing a prominent single species of approximately 37 kDa

under denaturing conditions were detected. To verify that the purified 37 kDa protein species was indeed the product of the myxoma virus T7 gene, the purified M-T7 sample was subjected to immunoblotting using an anti-M-T7 antibody that had been affinity purified using a bacterially produced GST-T7 fusion protein. Immunoblotting analysis revealed a major protein species of approximately 37 kDa and a smaller minor variant of approximately 35 kDa from crude myxoma virus-infected cellular supernatants (Fig. II-1B, lane 1). Identical M-T7 isoforms were also detected from the pooled fractions collected after both steps of the purification procedure (Fig. II-1B, compare lanes 1-3). Similar SDS-PAGE profiles have been detected with a previously described anti-M-T7 antibody prepared exclusively from a bacterial T7-GST produced protein (24). To determine if these isoforms were a result of alternate glycosylation states, purified M-T7 was subjected to treatment with *N*-glycosidase F. Treatment of M-T7 with this enzyme resulted in the deglycosylation of the major 37-kDa M-T7 species to approximately 32 kDa under denaturing conditions, whereas the minor 35 kDa form of M-T7 appeared resistant to this treatment (Fig. II-1B, lane 6). These data indicate that although the major M-T7 species was *N*-glycosylated, the minor M-T7 isoform may have been post-translationally modified by a different mechanism. Since M-T7 from crude myxoma supernatants has previously been shown to bind and inhibit rabbit IFN-γ (25), we tested whether the purified protein also retained this biological activity. Purified M-T7, when incubated with rabbit IFN-γ and cross-linked, produced a shifted complex of approximately 53 kDa (Fig. II-1B, lane 5) as previously observed (24). Thus, the purified protein was indeed M-T7 and

retained the biological property of rabbit IFN-γ binding previously ascribed to this mxyoma virus IFN-γ receptor homologue.

During the purification of M-T7 protein, it was noticed that the native M-T7 species migrated as a single species on gel filtration columns of an approximate molecular mass of 175 kDa, suggesting that M-T7 is an oligomeric species. To clarify the discrepancy of the protein's apparent molecular mass observed under denaturing conditions and non-denaturing gel filtration studies, the mass and oligomeric status of the purified protein was assessed by mass spectrometry and ultracentrifugation studies. According to mass spectrometry, the calculated mass of M-T7 was 33.0 kDa. As the theoretical molecular mass of M-T7 is only 29.9 kDa, the difference was most likely due to the extent of *N*-linked glycosylation. Sedimentation equilibration analysis (see Materials and Methods) revealed that the purified native M-T7 protein behaved as a single uniform species with a calculated molecular mass of approximately 113 kDa (data not shown). Based on these data, we predict that M-T7 likely exists as a stable trimer in solution. This also suggests that the apparent larger mass of M-T7 based on its behavior by Superdex-200 gel filtration chromatography was due to anomalous behavior of the protein likely due to glycosylation and/or structural asymmetry of the homotrimer.

Purified M-T7 Protein Binds to Chemokines

Given the pathogenic profiles of rabbits infected with a M-T7 knockout virus, we speculated that M-T7 may be capable of performing additional biological functions apart from the binding and inhibition of rabbit IFN-γ (24). Using a gel

mobility shift chemical cross-linking assay, we investigated whether purified M-T7 could bind to other cytokines that may help explain the phenotype observed from the pathology of the deletion virus. Although M-T7 exhibited species specificity for rabbit IFN-γ, we were limited to screening with human and murine cytokines in our assays since many rabbit cytokines are unavailable. As shown in the immunoblot in Fig. II-2, M-T7 protein did not bind murine IL-1β, human IL-2, human IL-3, murine IL-4, murine IL-6, murine IL-7 (lanes 1-7) or murine IFN-α (lane 9). However, we readily observed a shifted complex between M-T7 and the human chemokine IL-8, when these two proteins were incubated together (lane 8). The retarded complex of approximately 45 kDa as monitored by SDS-PAGE, corresponded to the predicted size of M-T7 monomer (37 kDa) binding to IL-8 (8 kDa) in a 1:1 stoichiometric ratio. Thus, this observation indicated that purified M-T7 has the ability to bind to at least one other cytokine in addition to rabbit IFN-γ, and prompted us to investigate the specificity of this novel interaction.

To examine the specificity of M-T7 and chemokine interactions, we investigated whether M-T7 could bind to members of other chemokine subfamilies or whether this interaction was restricted to the CXC subfamily, which includes IL-8. We therefore tested the ability of M-T7 to bind to a variety of chemokines using a similar gel-shift assay as described above. As shown in Fig. II-3A, M-T7 was found to interact with all representative members of the CC subfamily (human RANTES, MCP-1, MCP-3) (lanes 2-4), the CXC subfamily (human IL-8, PF4, IP-10, NAP-2, MGSA) (lanes 5-10), and the C subfamily (murine Ltn) (lane 11) of chemokines. It appears, therefore, that M-T7 binds multiple chemokines from the chemokine

subfamilies tested. Neither purified M-T2 (39) nor SERP-1 (29), both of which are soluble myxoma virus glycoproteins, were able to bind ^{125}I-labeled RANTES (data not shown) under similar conditions, suggesting that chemokine interactions are not a general property exhibited by glycosylated poxvirus proteins. It has previously been demonstrated that M-T7 displays species ligand selectivity by binding to rabbit IFN-γ, but not to murine or human IFN-γ (25). The M-T7 interaction with chemokines, however, did not display this selectivity, because M-T7 was able to bind to rabbit, murine or human chemotactic ligands (Fig. II-3A). To examine if the shifted M-T7-containing complexes shown in Fig. II-3A reflected an interaction of the viral protein with chemokines, we produced a parallel immunoblot probed with an anti-human RANTES antibody. The RANTES antibody did not cross-react with M-T7 alone (Fig. II-3B, lane 1). RANTES was detected as a shifted complex of approximately 50 kDa when incubated with a stochiometric equivalent amount of M-T7, but not when incubated alone (Fig. II-3B, compare lanes 2 and 3). This interaction of M-T7 with RANTES could be effectively inhibited by the addition of molar excess amounts of rabbit IFN-γ (Fig. II-3B, lanes 4-6) but not by murine or human IFN-γ (data not shown), demonstrating that chemokine and rabbit IFN-γ binding sites may overlap or may be shared.

To demonstrate that the binding of M-T7 with chemokines was independent of cross-linking, we also performed solid-phase binding assays in which the ability of radiolabeled chemokines to bind to immobilized M-T7 was tested under native conditions. Specific binding of radiolabeled RANTES was observed when incubated in the presence of M-T7 (Fig. II-4A), confirming the interactions between M-T7 and

chemokines demonstrated earlier by cross-linking assays. This suggests that M-T7 interacted with the chemokine RANTES in the absence of a cross-linker and under physiological conditions. To gain an approximate value for the affinity at which chemokines bind M-T7, we next determined the IC_{50} for displacement of ^{125}I-labeled RANTES binding in M-T7 cross-linking shift assays. Under conditions of saturable binding, we observed that the IC_{50} for displacement of labeled RANTES was approximately 900 nM for unlabeled RANTES (Fig. II-4B).

Purified M-T7 Interacts with the Heparin-Binding Domain of Chemokines

Chemokines are basic polypeptides which have been proposed to interact with at least the NH_2-terminal regions of chemokine receptors that are rich in acidic residues (17, 18). It has been previously demonstrated that an NH_2-terminal domain preceding the first cysteine residue of IL-8, the ELR motif, is essential for binding to IL-8 type receptors (7, 9, 16). ELR deletion mutants of IL-8 are cannot bind their cognate receptors or elicit a biological response in target neutrophils. To determine whether the ELR sequence of IL-8 may be a determinant for binding M-T7, we tested the ability of M-T7 to bind to a variety of IL-8 analogs containing successive deletions in the NH_2-terminus or an IL-8 analog that contained a partial deletion in its COOH-terminus (Fig. II-5A). All NH_2-terminal deletion IL-8 mutants (Fig. II-5A, lanes 2-6) were able to bind to M-T7 including the IL-8 analog in which the entire ELR motif had been deleted (Fig. II-5A, lane 6). Variation in binding intensities observed may be due to the level of cross-linking efficiency in this experimental assay. These data indicate that, unlike binding to CXCR1 and CXCR2 cellular

chemokine receptors, the NH_2-terminal region of IL-8 is not necessary for M-T7 binding.

In addition to binding specifically to their receptors, chemokines interact with glycosaminoglycans such as heparin and heparan sulfate (47) through an α-helix at the COOH-terminus. This interaction is thought to facilitate chemokine localization to the endothelial wall, generating a solid-phase gradient for leukocyte trafficking along the vasculature (36). A human IL-8 partial COOH-truncation mutant analog, hIL-8{1-66}, which displays a markedly lower heparin affinity (47) than does its full-length counterpart, was unable to bind M-T7 (Fig. II-5A, compare lanes 7 and 8). We have also observed that M-T7 is not able to bind to the hIL-8{4-52} analog (data not shown) which lacks the complete COOH-terminal α-helix and displays no heparin binding (47). These results indicated that M-T7 bound to IL-8, and possibly other chemokines, via the COOH-terminal domain suggesting that this soluble viral protein may be interacting with these chemotactic ligands in a heparin-like fashion. To confirm this, heparin competition studies were performed. Increasing mass ratios of heparin effectively prevented the binding of M-T7 with RANTES (Fig. II-5B, lanes 3-8), whereas the addition of heparin had no effect on the interaction of M-T7 with rabbit IFN-γ (Fig. II-5B, lanes 9 and 10). Addition of heparin itself did not appear to alter M-T7 mobility (Fig. II-5B, lanes 1 and 2). Collectively, these data suggested that M-T7 may interact with the heparin-binding region found in a variety of chemokines of the CXC, CC, and C classes.

DISCUSSION

The proper recruitment and activation of specialized leukocyte subsets to sites of viral infection constitute pivotal components of the host defense for efficient clearance of a pathogen. Viruses, therefore, have coevolved multiple anti-inflammatory mechanisms in an attempt to circumvent the arsenal of the host's primary defense systems (23, 28, 42). Poxviruses encode a variety of gene products that target the cytokine machinery that regulates the earliest stages of the inflammatory response. These include inhibitors of cytokine synthesis, release, and signal transduction, as well as a variety of cytokine receptors homologues (35). Viral mimics of cytokine receptors have been demonstrated to function by binding to their respective ligands with high affinity and preventing normal cytokine-cellular-receptor interactions. There is also increasing evidence of poxviral virulence factors that may have multiple biological properties that cannot be predicted from conventional sequence analysis. For example, the myxoma virus TNF receptor homologue, M-T2, also blocks apoptosis in myxoma virus-infected rabbit lymphocytes by a mechanism that appears to be independent of its extracellular TNFα binding property (20, 40). Also, a glycoprotein secreted by tanapox virus has previously been shown capable of binding three distinct cytokines, namely IFN-γ, IL-2, and IL-5 (12). In the case of M-T7, sequence analysis was used to predict its demonstrated function as a soluble IFN-γ receptor (26, 44). Although, IFN-γ does indeed appear to be a major target for the biological activity of M-T7 *in vitro*, the pathogenic profile of rabbits infected with M-T7 deletion viruses indicate that M-T7 may be a multifunctional virulence factor (24).

Here we report a new activity for M-T7 apart from binding and inhibiting rabbit IFN-γ, namely, promiscuous interaction with members of the chemokine superfamily.

Chemokines appear to be of paramount importance in the early host response to viruses (10, 30, 34). Viral proteins dedicated to subverting these proinflammatory mediators would therefore be expected. Several virus chemokine receptor homologues, sharing sequence similarity to seven-transmembrane-type cellular receptors, have been identified (1, 27, 38). These include the gene products encoded by cytomegalovirus US28 (6, 31, 38); herpesvirus samiri ECRF3 (2, 32); human herpesvirus-6 UL78, U12, and U51 (14); equine herpesvirus-2 ORF74 and E1 (43); and two poxviruses, swinepox K2R (21) and capripox Q2/3L (5). Although both US28 and ECRF3 have been demonstrated to be functional signalling receptors by binding multiple CC and CXC chemokines, respectively, their roles in viral pathogenesis remain unclear. Sequence analysis of M-T7, however, indicates no homology to any of the extracellular regions of any chemokine receptors identified to date. Considering that M-T7 is a soluble protein with significant amino-acid similarity to the extracellular ligand-binding region of cellular IFN-γ receptors, we hypothesized that its interaction with chemokines would thus be distinct from the interaction of chemokines with their cognate cellular receptors. Given that rabbit IFN-γ specifically inhibited RANTES binding to M-T7 (Fig. II-3B), it is possible that the two ligands compete for binding to identical or overlapping M-T7 binding sites. Alternatively, a conformational change in M-T7-IFN-γ complex could occlude the chemokine-binding domain of M-T7, although this remains to be determined by further studies which map the domains of chemokine and rabbit IFN-γ binding. Note

that the ability to bind to chemokines does not appear to be a biochemical property shared by all poxviral IFN-γ homologues. The IFN-γ receptor homologue from vaccinia virus (strain WR) was unable to form cross-linked complexes with radiolabeled chemokines in similar binding assays (15). We have also recently identified another family of poxviral secreted chemokine binding proteins that are distinct from M-T7. Like M-T7, the M-T1 family of 35-kDa poxvirus proteins binds both CXC and CC chemokines, although the biochemical mechanism of this interaction remains to be demonstrated (see Chapter III) (15).

Native M-T7 is shown to be a trimer that is *N*-glycosylated, but further investigations are needed to determine whether the carbohydrate moieties of this glycoprotein are important for the interaction. Several lines of evidence suggest the binding of M-T7 with the chemokines described here may be analogous to the interactions between chemokines and heparin. Firstly, unlike most chemokine receptors that have been identified, M-T7 binds promiscuously to members of all three chemokine subfamilies tested, suggesting that a common conserved structural motif is involved. Secondly, a COOH-terminal IL-8 mutant, which displayed decreased affinity for binding heparin in previous work, were also unable to interact with M-T7 in our studies. In contrast, NH_2-terminal determinants that dictate CXC chemokine binding, such as the ELR motif, were not essential for M-T7 binding. Thirdly, heparin specifically displaced the binding of chemokines to M-T7. We propose, therefore, that this novel M-T7 activity may be facilitated through the α-helix heparin-binding region commonly found within multiple chemokines.

The biochemical characteristics of M-T7-chemokine interactions suggested that M-T7 protein alone might not effectively occlude chemokine receptor triggering. However, it is still plausible that M-T7 could perturb chemokine localization in the extracellular matrix and/or affect the correct presentation of chemokines to target leukocytes, neither of which can be readily monitored with current *in vitro* chemotaxis assays. Consistent with this hypothesis is the data demonstrating a sub-μM affinity between RANTES and M-T7, a value reported similarly for chemokine-glycosaminoglycans interactions (48). Given the prodigious quantity of M-T7 secreted from an infected cell, in excess of 10^7 molecules/cell/hour (26), we propose that M-T7 is an ideal candidate for interaction with multiple classes of chemokines in virus-infected tissues. A similar role of a chemokine sink has also been proposed for a non-signalling chemokine receptor found on the surfaces of erythrocytes (11). Nevertheless, the dramatic effect of M-T7 loss on leukocyte migration in myxoma virus-infected tissues suggests that further studies to define the *in vivo* relationship between extracellular M-T7 protein and the activities of chemokines that mediate leukocyte influx into virus-infected tissues are warranted.

MATERIALS AND METHODS

Cells and Viruses. v-Myxlac, a derivative of myxoma virus (strain Lausanne) containing a β-galactosidase marker gene, has been described elsewhere (33). v-Myxlac was routinely propagated in baby green monkey kidney (BGMK) cells (a gift from S. Dales, University of Western Ontario). BGMK cells were cultured in

Dulbecco's minimal essential medium (DMEM) supplemented with 10% newborn calf serum (Life Technologies, Gaithersburg, MD).

Cytokines and Chemokines. Recombinant murine (m)IL-1β, human (h)IL-2, hIL-3, mIL-4, mIL-6, mIL-7, mIFN-α were kindly donated by Dr. Hsaing-fu Kung (Biological Response Modifiers Program, NCI) and recombinant rabbit (r)IFN-γ was a gift from Genentech Inc. (San Francisco, CA). Synthetic chemokines--hRANTES, full length hIL-8 {1-72}, monocyte chemoattractant protein (hMCP)-1, hMCP-3, platelet factor-4 (hPF4), neutrophil activating peptide-2 (hNAP-2), melanoma growth stimulatory activity (hMGSA), interferon-γ-inducible protein (hIP-10), mLtn, full length rIL-8 {1-79}, and chemokine truncation mutant analogs--hIL-8 {1-66}, rIL-8 {6-79}, rIL-8 {9-79}, rIL-8 {11-79} and rIL-8 {12-79}--were synthesized with *tert*-butoxycarbonyl chemistry on an Applied Biosystems 430A peptide synthesizer as described elsewhere (8).

Purification of M-T7. v-Myxlac was adsorbed onto roller bottles containing $3X10^8$ BGMK cells at a m.o.i. of 2.5 PFU for 3 h, washed with phosphate-buffered saline (PBS), and incubated with serum-free DMEM at 37°C. After 18 h, the medium was harvested, centrifuged at 10,000 X *g* for 1 h to remove virus and cellular debris, concentrated 10-fold with a stirred ultrafiltration cell (Amicon, Beverly, MA), and dialyzed against 20 mM bis-Tris (Sigma Chemical Co., Mississauga, ON) buffer, pH 6.0. M-T7 was purified from concentrated myxoma virus-infected cellular supernatants using fast performance liquid chromatography (FPLC). Approximately 5 mg of concentrated supernatant was loaded onto a Mono-Q HR5/5 (Pharmacia, Piscataway, NJ) column that had been pre-equilibrated with low-ionic-strength 20

mM bis-Tris buffer, pH 6.0. Proteins were eluted from the anion-exchange column by increasing the salt concentration of the elution buffer to 500 mM sodium chloride in a step gradient. Protein fractions eluting between 120-175 mM sodium chloride were subjected to sodium dodecyl sulphate-12% polyacrylamide gel electrophoresis (SDS-12% PAGE) and visualized by silver staining (13). Mono-Q fractions containing M-T7 were pooled, dialyzed against PBS overnight, and loaded onto a PBS-equilibrated Superdex-200 (Pharmacia, Piscataway, NJ) gel filtration column that had been precalibrated with known molecular weight size standards (Pharmacia, Piscataway, NJ). Eluted proteins were analyzed by SDS-PAGE as above. Fractions containing purified M-T7 were pooled, concentrated five-fold with a Centriprep (Amicon, Beverly, MA) concentrator, and stored at 4°C. Approximately 80 μg of M-T7 per mg of crude myxoma virus-infected cellular supernatants was routinely obtained.

Deglycosylation of M-T7. One microgram of M-T7 was resuspended in 100 mM sodium phosphate buffer, pH 7.0, and boiled for 10 min in the presence of 1% SDS. The denatured protein was then diluted by the addition of 100 mM sodium phosphate buffer, (pH 7.0), 20 mM EDTA, 5% Triton X-100, and 1.1 mM dithiothreitol and incubated with 0.2 U of *N*-glycosidase F (Boehringer Mannheim, Laval, PQ) at 37°C. After overnight incubation, the reaction products were dialyzed against sodium phosphate buffer, pH 7.0, concentrated to the original volume with an Ultrafree microconcentrator (Millipore, Bedford, MA) and analyzed by immunoblotting with an M-T7 antibody.

Sedimentation Equilibrium Studies. Analytical ultracentrifugation studies were performed at 8000 rpm in PBS at 20°C on a Beckman Spinco Model E

analytical ultracentrifuge using absorbance optics. The molecular weight (M) of M-T7 was calculated as $(2RT/(1-\upsilon\rho))(d(\ln y)/\omega^2 d(r^2))$ where R is the universal gas constant; T is the temperature in Kelvin; ν is the partial specific volume of the protein; ρ is the solvent density; ω is the angular velocity; and $d(\ln y)/d(r^2)$ is the slope calculated from a plot of ln$y$ versus r^2, where y is the concentration in absorbance units at 280 nm and r is the distance from the axis of rotation (cm). A linear slope from the plot of ln$y$ versus r^2 (data not shown) indicated that M-T7 behaved as a single uniform species in solution. On the basis of amino acid composition alone, ν was calculated to be 0.72. However, as M-T7 is glycosylated, ν for the secreted glycoprotein was calculated to be 0.70 using the formula $(M_1\nu_1+M_2\nu_2)/(M_1+M_2)$, where $M_1\nu_1$ and $M_2\nu_2$ are the weights and partial specific volume of the protein and sugar component, respectively.

Gel Mobility Shift Cross-linking Assay. The interaction of M-T7 with various cytokines was assessed using a chemical cross-linking assay as described previously (44). Briefly, purified M-T7 was incubated with the appropriate cytokine or chemokine ligand for 2 h at room temperature. For competition binding studies, increasing amounts of rabbit IFN-γ, unlabeled RANTES or intestinal porcine heparin (Sigma Chemical Co.) competitors were incubated simultaneously with the ligands used in the reaction. After incubation, the protein complexes were cross-linked by the addition of 1-ethyl-3-(3-dimethyaminopropyl)carbodiimide (EDC) (Sigma Chemical Co.) to a final concentration of 40 mM for 30 min at room temperature and the reaction was quenched by the addition of 1/10 volume of 1.0 M Tris, pH 7.5. SDS-loading buffer was added to the mixtures, the samples were boiled for 3 min,

subjected to SDS-12% PAGE, and the resulting protein complexes were analyzed by immunoblotting. For analysis of ^{125}I-labeled RANTES competition assays, cross-linked complexes were analyzed by autoradiography and quantified on a Fujix BAS 1000 phosphorimager with the MacBAS program. The relative affinity of M-T7 for RANTES was determined by measuring the 50% inhibitory concentration (IC_{50}) of unlabeled RANTES required for displacement of labeled RANTES in M-T7 cross-linking assays by a modification of a technique previously described (45, 46).

Immunoblotting Analysis. M-T7 antiserum was prepared as described previously (24) except that rabbits were injected with FPLC column-purified M-T7 protein in Freund's complete adjuvant. Polyclonal antiserum was further subjected to affinity purification against an immobilized bacterial-produced M-T7-glutathione *S* transferase (GST) fusion protein, as outlined elsewhere (24). Detection of M-T7 was performed by immunoblotting analysis as described previously (39) except that the blots were incubated with a 1:5,000 dilution of affinity-purified anti-M-T7 antibody for 1 h. Detection of RANTES-M-T7 complexes by immunoblotting was performed in a similar manner with a 1:1,000 dilution of anti-human RANTES antibody (R&D Systems, Minneapolis, MN) and a 1:5,000 dilution of horseradish peroxidase-conjugated donkey anti-goat immunoglobulin G antibody (Jackson ImmunoResearch Laboratories, West Grove, PA).

Solid-Phase M-T7 Binding Assay. hRANTES was radiolabeled with ^{125}I (Dupont NEN, Mississauga, ON) by using Iodobeads (Pierce, Rockford, IL) according to the manufacturer's recommendations. Falcon 96-well immunoplates were coated with 100 ng of purified M-T7 in 50 μl of PBS overnight at 4°C. Wells

were blocked with 5% skim milk powder in Tris-buffered saline (TBS) containing 0.2% Tween-20 for 4 h at room temperature and then incubated with ^{125}I-labeled RANTES in 100 µl blocking buffer for 2 h. Wells were washed three times with TBS-0.2% Tween and removed, and radiobound counts were measured on a Packard 5780 gamma counter. To determine the specific binding of M-T7 to RANTES, nonspecific binding in the presence of 50-fold excess cold RANTES was subtracted from total binding.

Figure II-1. Analysis of purified M-T7 glycoprotein.

M-T7 was purified from crude supernatants of vMyxlac-infected cells by column chromatography and analyzed by silver staining (A) and immunoblotting (B). (A) Crude supernatants (lane 1) or pooled fractions containing 500 ng of M-T7 following Mono-Q (lane 2) and Mono-Q-Superdex-200 (lane 3) column chromatography were subjected to SDS-12% PAGE and visualized by silver staining. (B) M-T7, as detected by immunoblotting analysis with an affinity anti-M-T7 antibody, from crude myxoma virus supernatants (lane 1) or pooled fractions following Mono-Q (lane 2) and Mono-Q-Superdex-200 (lane 3) column chromatography. One microgram of purified M-T7 was incubated in the absence (lane 4) or presence (lane 5) of 1 μg of rabbit IFN-γ and cross-linked as outlined in Materials and Methods, and complexes were detected by immunoblotting following SDS-12% PAGE. M-T7 was deglycosylated by treatment with *N*-glycosidase F (lane 6) as outlined in Materials and Methods. SDS-PAGE markers are shown on the right of each panel.

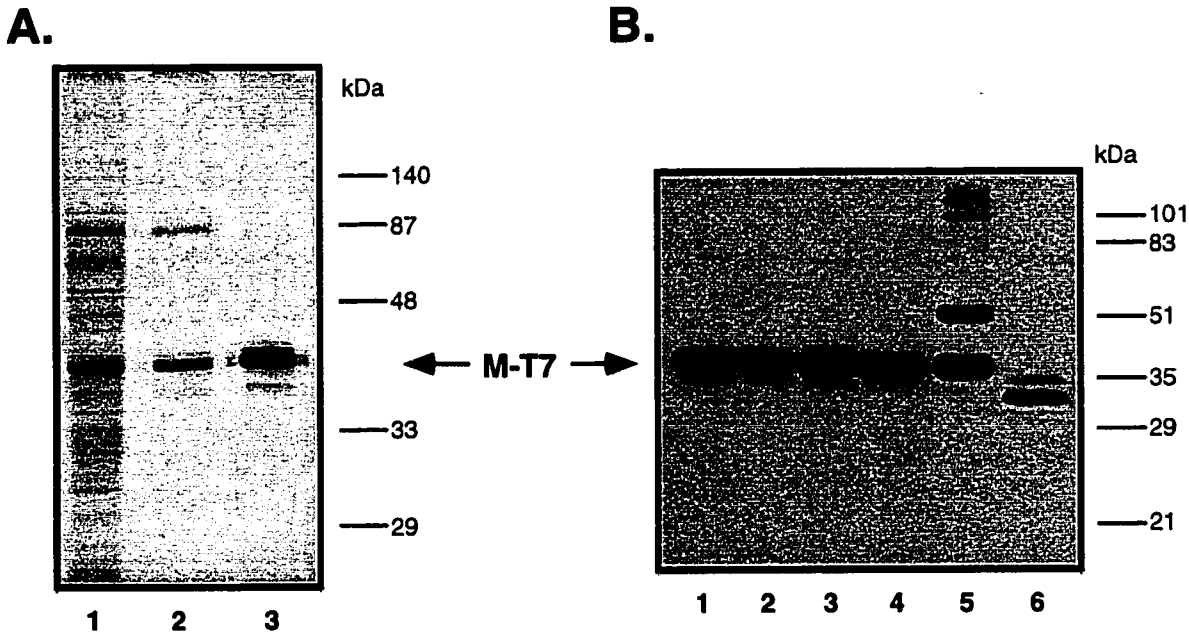

A.
B.
kDa
140
87
48
33
29
M-T7
1
2
3
kDa
101
83
51
35
29
21
1
2
3
4
5
6

Figure II-2. Purified M-T7 binds to rIFN-γ and hIL-8 but not other cytokines tested.

One microgram of purified M-T7 was incubated alone (lane 1) or with 1 μg of the indicated cytokine (lanes 2 through 10), cross-linked with M-T7 as described in Materials and Methods and analyzed by immunoblotting with an affinity-purified anti-M-T7 antibody. SDS-PAGE markers are shown on the right, and arrows indicating novel shifted M-T7-cytokine complexes are on the left.

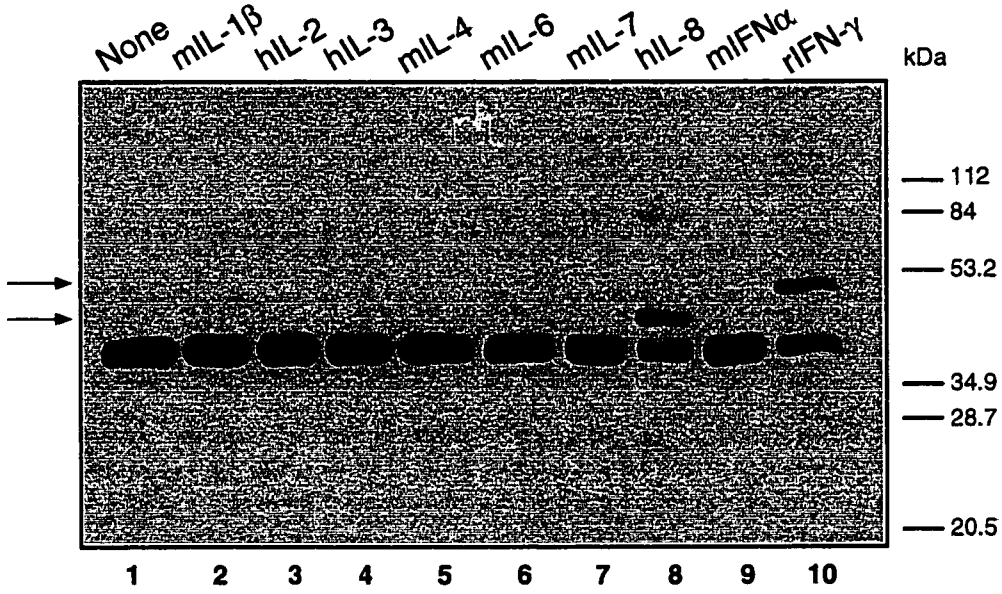
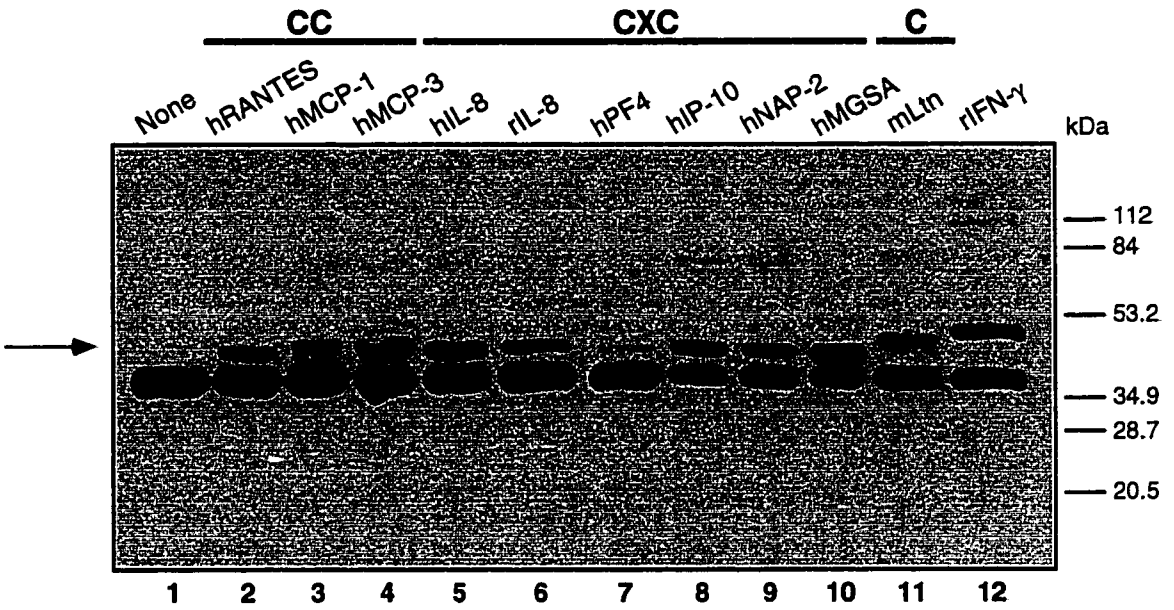

None
mIL-1β
hIL-2
hIL-3
mIL-4
mIL-6
mIL-7
hIL-8
mIFNα
rIFN-γ
kDa
112
84
53.2
34.9
28.7
20.5
1
2
3
4
5
6
7
8
9
10

Figure II-3. M-T7 binds members of the CC, CXC, and C subfamilies of chemokines.

(A) One microgram of purified M-T7 was incubated alone (lane 1) or with 1 μg of the indicated chemokine (lanes 2 through 12) cross-linked and analyzed by immunoblotting as described in the legend to Fig. II-2. The arrow indicates shifted M-T7-chemokine complexes. (B) Binding of RANTES to M-T7, as detected by anti-human RANTES antibody, can be inhibited by molar-excess amounts of rIFN-γ. Stoichiometrically equal amounts of M-T7 (lane 1) and hRANTES (lane 2) were incubated together in the absence (lane 3) or presence of 0.01X (lane 4), 0.1X (lane 5), or 1.0X (lane 6) molar-excess amounts of rIFN-γ, cross-linked as described in the text and analyzed by immunoblotting with an anti-human RANTES antibody. SDS-PAGE markers are shown on the right of each panel.

A.


CC
CXC
C
None
hRANTES
hMCP-1
hMCP-3
hIL-8
rIL-8
hPF4
hIP-10
hNAP-2
hMGSA
mLtn
rIFN-γ
kDa
112
84
53.2
34.9
28.7
20.5
1 2 3 4 5 6 7 8 9 10 11 12

B.

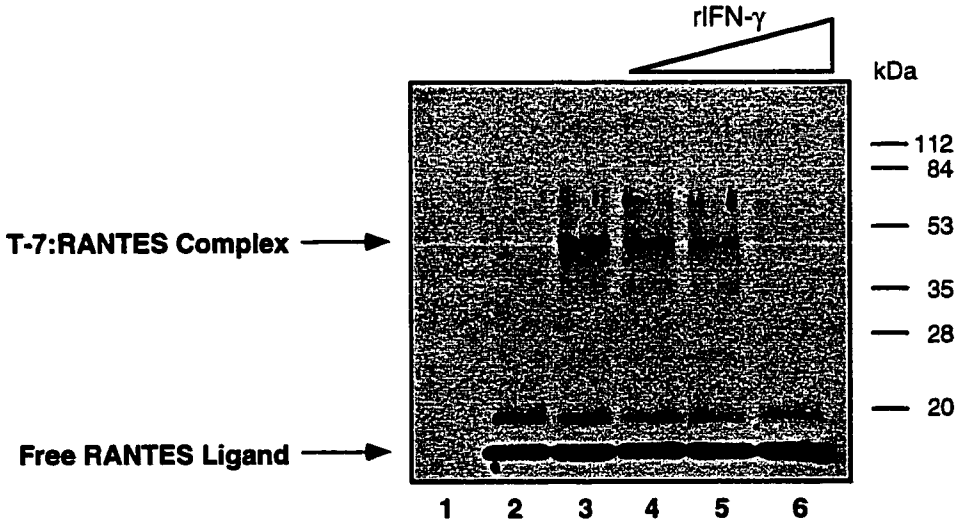
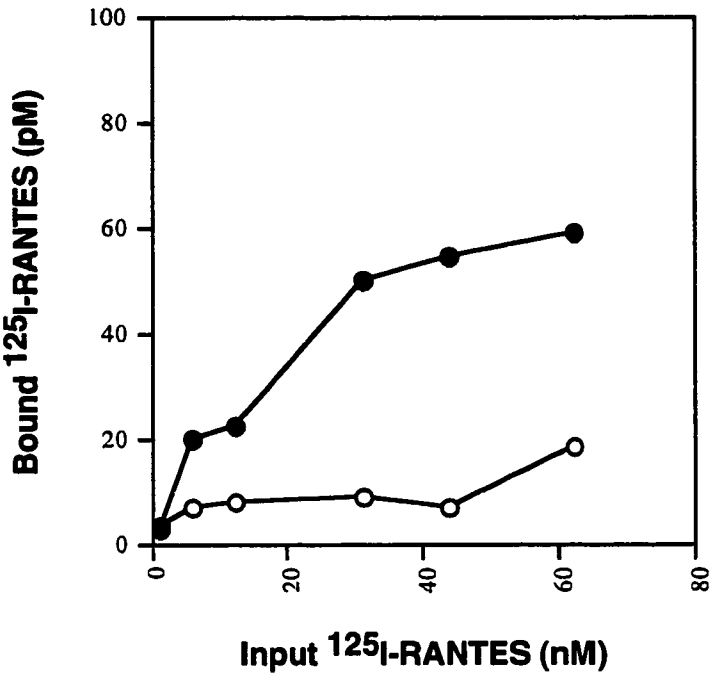

rIFN-γ
kDa
T-7:RANTES Complex
Free RANTES Ligand
112
84
53
35
28
20
1 2 3 4 5 6

Figure II-4. Solid-phase and self-competition cross-linking binding assays of M-T7 to ^{125}I-labeled RANTES.

(A) Results of a representative experiment performed in triplicate of solid-phase binding of ^{125}I-labeled RANTES to M-T7 (solid circles) or bovine serum albumin (control open circles) as described in Materials and Methods. (B) Determination of the RANTES concentration required to displace 50% binding of ^{125}I-labeled RANTES to M-T7. The amounts of ^{125}I-labeled RANTES bound to M-T7 were quantified by phosphorimaging after cross-linking assays and plotted against the amounts of increasing cold RANTES competitor. From the graph, the IC_{50} of RANTES for M-T7 was determined to be approximately 0.9 μM.

A.


Bound 125I-RANTES (pM)
100
80
60
40
20
0
0
20
40
60
80
Input 125I-RANTES (nM)

B.

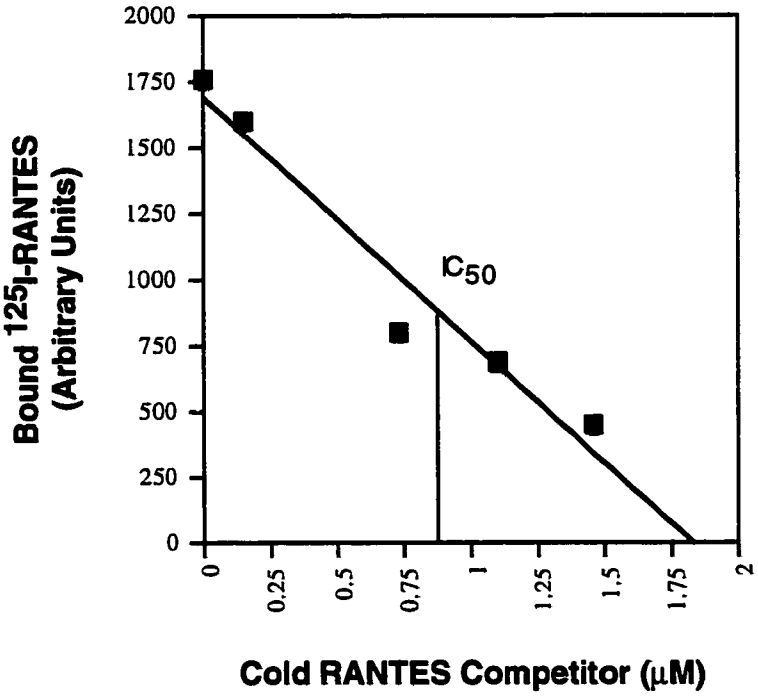
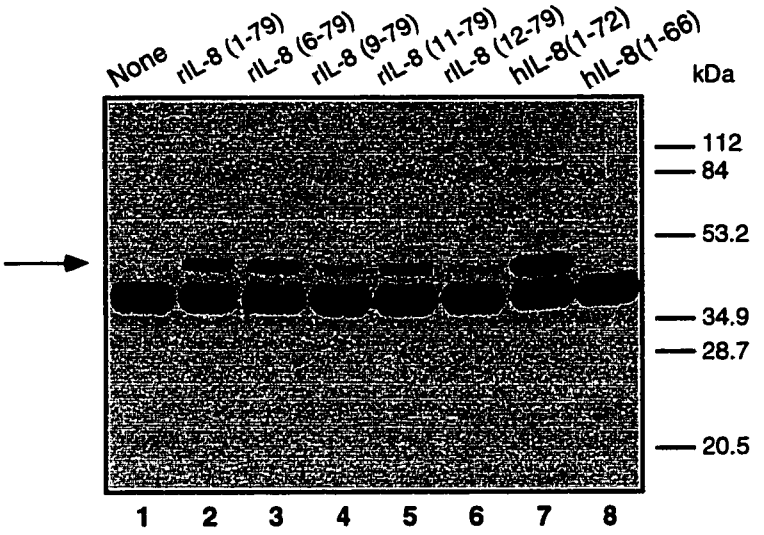

Bound 125I-RANTES (Arbitrary Units)
2000
1750
1500
1250
1000
750
500
250
0
IC50
0
0.25
0.5
0.75
1
1.25
1.5
1.75
2
Cold RANTES Competitor (μM)

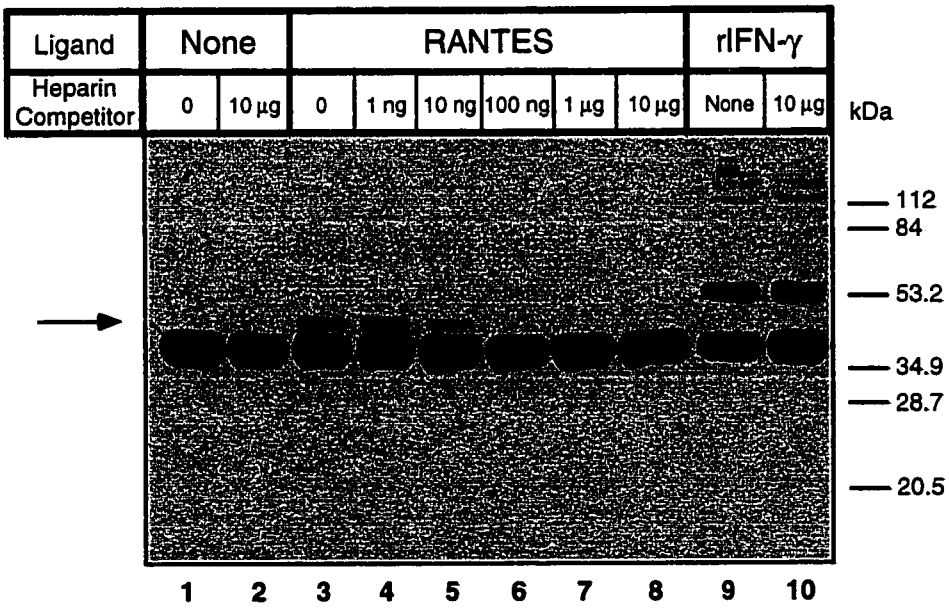
Figure II-5. M-T7 binds to rIL-8 containing NH_2-terminal deletions but is unable to bind to an hIL-8 COOH-terminal-deletion mutant.

(A) One microgram of purified M-T7 was incubated alone (lane 1) or with 1 μg of the indicated mutant chemokine (lanes 2 through 8), cross-linked as described in the text; binding complexes were analyzed by immunoblotting with an anti-M-T7 antibody. (B) Heparin competes with M-T7 for binding to RANTES but not to rIFN-γ. One microgram of M-T7 was incubated alone (lane 1) or with 1 μg of either hRANTES (lanes 3 through 8) or rIFN-γ (lanes 9 and 10) in the absence (lanes 1, 3, and 9) or presence (lanes 2, 4 through 8, and 10) of increasing amounts of heparin, cross-linked as described in the text and analyzed by immunoblotting with an anti-M-T7 antibody. Arrows to the left of each panel show M-T7-chemokine complexes, and SDS-PAGE markers are indicated on the right.

A.


None
rIL-8 (1-79)
rIL-8 (6-79)
rIL-8 (9-79)
rIL-8 (11-79)
rIL-8 (12-79)
hIL-8(1-72)
hIL-8(1-66)
kDa
112
84
53.2
34.9
28.7
20.5
1 2 3 4 5 6 7 8

B.


Ligand
None
RANTES
rIFN-γ
Heparin Competitor
0
10 µg
0
1 ng
10 ng
100 ng
1 µg
10 µg
None
10 µg
kDa
112
84
53.2
34.9
28.7
20.5
1 2 3 4 5 6 7 8 9 10

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CHAPTER III

THE T1/35kDa FAMILY OF POXVIRUS-SECRETED PROTEINS BIND CHEMOKINES AND MODULATE LEUKOCYTE INFLUX INTO VIRUS-INFECTED TISSUES*,†

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†This study was performed in collaboration with Dr. Kathryn A. Graham (University of Alberta, Edmonton, Canada)

INTRODUCTION

One of the key features of the early inflammatory response to an initial virus challenge is the influx and activation of leukocytes which help initiate the earliest phases of antiviral immune activation (27, 41). Neutrophils, monocytes/macrophages, and NK cells in particular participate in the first wave of cellular infiltration but require directional signals in order to migrate to the injured tissues bearing infected cells (5, 35). The activities of multiple chemotactic cytokines, collectively known as chemokines, are believed to be critical in this process (5, 10, 30). Here we report a novel class of secreted poxvirus proteins that interact with a broad spectrum of chemokines *in vitro* and retard the extent of leukocyte influx into virus infected lesions *in vivo*.

Poxviruses are large DNA viruses that replicate and direct viral gene expression from the cytoplasm of infected cells (23). Myxoma virus, a member of the *Leporipoxvirus* genus, is a well characterized rabbit pathogen that induces a benign disease in its natural host, the South American rabbit (*Sylvilagus brasiliensis*) (9, 18). However, in the European rabbit (*Oryctolagus cuniculus*) myxoma virus infection results in myxomatosis, a condition that is usually lethal. In contrast, rabbitpox virus is a member of the *Orthopoxvirus* genus and induces a disease syndrome in European rabbits that is also generally lethal but has a distinct pathogenicity spectrum (15). Both the rabbitpox virus and myxoma virus systems provide unique insights into the fundamental mechanisms by which poxviruses cause immune dysfunction (17, 18).

Like many viruses, poxviruses have evolved a number of strategies to counteract the host's immune system. In particular, several secreted poxvirus proteins have been shown to interfere with the host immune response to viral infection by blocking the function of important extracellular immunoregulatory molecules. Myxoma virus as well as orthopoxviruses such as vaccinia virus and rabbitpox virus have been shown to encode a variety of secreted proteins which mimic host cytokines or cytokine receptors (21, 32).

As part of our on-going search for novel secreted poxvirus inhibitors of the cytokine network, supernatants from poxvirus infected tissue culture cells were screened for viral proteins which interact with proinflammatory chemokines that induce immune cell chemotaxis into sites of injury or infection (22). In this report we demonstrate that many, but not all, poxviruses encode a member of a family of secreted proteins, collectively termed T1/35kDa, that bind a variety of CC and CXC chemokines *in vitro*. Furthermore, *in vivo* infection with a rabbitpox virus mutant that does not express the secreted 35kDa chemokine binding protein revealed pronounced alterations in the influx of extravasating leukocytes into virus-infected rabbit tissues.

RESULTS

Several Members of the Poxvirus Family Express Secreted Chemokine Binding Proteins

Since poxviruses are known to encode multiple secreted immunomodulatory proteins, we screened supernatants prepared from poxvirus-infected tissue culture

cells for soluble viral proteins that could bind to representative members of the chemokine subfamilies. Iodinated RANTES, a CC chemokine, and IL-8, a CXC chemokine were incubated with tissue culture supernatants, exposed to a chemical cross-linker (EDC), and the presence of shifted complexes representing novel protein interactions was assessed. The addition of RANTES (Fig. III-1A) or IL-8 (Fig. III-1B) to supernatants from poxvirus-infected cells resulted in novel shifted cross-linked complexes of 53kDa for the leporipoxviruses, myxoma virus (lane 2) and Shope fibroma virus (lane 3), and 49kDa for the orthopoxviruses, raccoonpox virus (lane 4), cowpox virus (lane 6), rabbitpox virus (lane 7), and vaccinia virus (strain Lister) (lane 8). However, supernatants harvested from swinepox virus (lane 5), vaccinia virus (strain WR) (lane 9), and uninfected cells (lane 1) did not contain any detectable shifted protein species. The cross-linked products exhibited comparable mobility when either RANTES or IL-8 were used in the experiment, suggesting that either the same viral protein was responsible for binding both chemokines or that the virus encodes two or more chemokine binding proteins of similar size. If the molecular mass of RANTES or IL-8 is subtracted from the observed mobility of the shifted complexes, the putative viral chemokine binding protein would be predicted to be ~45kDa for the leporipoxviruses, myxoma virus and Shope fibroma virus, and ~41kDa for the orthopoxviruses, vaccinia virus, rabbitpox virus, cowpox virus, and raccoonpox virus. In addition, the CC chemokines MIP-1α, MIP-1β, and MCP-1 gave similar results in cross-linking assays (data not shown). These data indicated that several poxviruses encode and secrete chemokine binding protein(s) that interact with both CC and CXC chemokines.

If both RANTES and IL-8 bind to the same site on the same protein then they should be able to effectively compete with each other, depending on their relative affinities. Therefore, self- and cross-competition studies were performed (Fig. III-2) in which unlabeled ligands were used to compete with ^{125}I-labeled chemokine for binding to a protein in supernatants from myxoma virus-infected cells. RANTES binding was self-competed at a concentration of 10-fold molar excess unlabeled chemokine (Fig. III-2A). In a similar experiment unlabeled IL-8 self-competed with ^{125}I-labeled IL-8 for binding to the viral protein (data not shown). Cross-competition studies between RANTES and IL-8 showed that unlabeled RANTES effectively inhibited binding with labeled IL-8 (Fig. III-2B) to the myxoma virus protein. However, a parallel analysis showed that concentrations of IL-8 of up to 200-fold excess were not able to inhibit the binding of ^{125}I-labeled RANTES (Fig. III-2C). Control cytokines were also tested for the ability to inhibit RANTES and IL-8 binding. At concentrations of 200-fold excess, IL-3, -4, -6, -7, -9, -10, and -11 were unable to block the binding of RANTES (Fig. III-2D) or IL-8 (data not shown) to the secreted myxoma virus chemokine binding protein. These binding analyses suggest that both RANTES and IL-8 specifically interact with the same site on the poxviral protein, but that RANTES binds with a higher affinity.

Sequence of Candidate Chemokine Binding Proteins

In order to identify the poxvirus ORFs which encode the chemokine binding proteins we took advantage of the fact that although no detectable chemokine binding activity was observed from cells infected with vaccinia virus (WR), a complex could

be readily detected in supernatants from cells infected with vaccinia virus (Lister) (Fig. III-1, lanes 8 and 9). These two vaccinia strains are very similar, but exhibit a few notable differences. One such difference is the expression of a 35kDa secreted protein from vaccinia virus (Lister), designated C23L and B19R, which is truncated in vaccinia virus (WR) to a 7.5kDa species (11, 29). Homologous ORFS have also been sequenced in the genomes of variola virus and cowpox virus, all of which are closely related to the T1 gene of Shope fibroma virus (15, 36). Therefore, members of the orthopoxvirus (rabbitpox virus 35kDa) and leporipoxvirus (myxoma virus M-T1) gene families were chosen for assessment as possible chemokine binding proteins. The myxoma virus M-T1 ORF, which is a homologue of the Shope fibroma virus T1 gene, is the first ORF from the termini of the virus genome (Fig. III-3A). It is located within the *Bam*HI S fragment and is present in two copies within the terminal inverted repeats. Since previously reported myxoma virus DNA sequences (37) extend only into the 5' end of the T1 coding sequence, we have completed the nucleotide sequence extending from the initiating M-T1 codon to the end of the myxoma virus *Bam*HI S fragment (Fig. III-3B). The 783-bp myxoma virus M-T1 gene encodes a 260-amino-acid protein with a putative signal sequence and two predicted *N*-glycosylation sites. The related rabbitpox virus secreted 35kDa protein, which was previously identified using amino terminal sequencing (15), is located within the terminal inverted repeats mapping within the *Hind*III B and C fragments of the rabbitpox virus genome, and is therefore also present in two copies. The 777-bp rabbitpox virus 35kDa protein gene encodes a 258-amino-acid protein that contains a signal sequence but only one predicted *N*-glycosylation site (Fig. III-3C). Based on

deduced amino acid sequence the M-T1 and the 35kDa proteins are predicted to be 28.3 and 27.7kDa, which is less than the calculated 41- to 45-kDa mobilities (Fig. III-1), but the predicted *N*-glycosylation of both proteins suggests that the sizes of the fully processed secreted proteins will be in the range of the observed chemokine binding species.

The derived amino acid sequences of M-T1 and rabbitpox virus 35kDa were compared to the sequences of other known members of the T1/35kDa family (Fig. III-4). Within the orthopoxvirus 35kDa family there is 81-99% amino acid identity, whereas the leporipoxvirus T1 proteins are about 70% identical; however, between the leporipoxvirus and orthopoxvirus members, there is only about 40% amino acid identity (Table III-1). In comparison to the orthopoxvirus 35kDa family members, the leporipoxvirus T1 proteins have insertions of 19 (myxoma virus) and 16 (Shope fibroma virus) amino acids and both share a common 9-amino-acid deletion in the amino terminal region. However, all 8 cysteines in the predicted mature proteins are strictly conserved among all of the homologues, suggesting that the overall protein folding domains are likely maintained. Database searches have revealed no significant homology between the T1/35kDa poxvirus family and any of the reported chemokine serpentine receptors or indeed to any other proteins in the database. Thus, the closely related family of T1/35kDa proteins are candidate chemokine binding proteins based on their expression pattern in different vaccinia virus isolates, but the sequence information does not provide predictive insights into the basis for any interaction, other than to exclude receptor mimicry.

Identification of Myxoma Virus M-T1 and Rabbitpox Virus 35kDa Proteins as Chemokine Binding Proteins

Although it is demonstrated elsewhere that the purified myxoma virus IFN-γ receptor homologue, M-T7, binds to chemokines *in vitro* (see Chapter II) (13), unfractionated secreted proteins obtained from cells infected with a myxoma virus construct in which the M-T7 gene has been disrupted (24) still exhibited a chemokine binding species (Fig. III-5A, lane 1). This indicated an independent chemokine binding activity in crude supernatants from virus-infected cells that is distinct from M-T7. Using recombinant poxviruses, the ability of the myxoma virus M-T1 and rabbitpox virus 35kDa secreted proteins to interact with chemokines was assessed. The M-T1 ORF was inserted, under the control of a synthetic late promoter, into the *tk* locus of a vaccinia virus (WR) background, that itself does not express any chemokine binding protein activity, to create VV-T1. To assess the potential ability of the homologous orthopoxvirus 35kDa protein to bind chemokines, a rabbitpox virus mutant in which the gene encoding the 35kDa secreted protein has been deleted, RPVΔ35, was utilized (15). Supernatants from cells infected with the two sets of recombinant viruses were reacted with ^{125}I-labeled RANTES to test for CC chemokine binding (Figs. III-5A and III-5B). Supernatant from cells infected with VV-T1 (Fig. III-5A, lane 4), but not the parent vaccinia virus (WR) (Fig. III-5A, lane 3), formed 53kDa cross-linked complexes with RANTES. Furthermore, the size of the VV-M-T1/RANTES complex (Fig. III-5A, lane 4) was comparable to the complex generated from myxoma virus-infected supernatants (Fig. III-5A, lane 2),

indicating that the myxoma virus M-T1 gene does in fact express a secreted CC chemokine binding protein of the correct size.

Supernatants from cells infected with RPVΔ35 were compared with the parental rabbitpox virus for RANTES binding activity, to confirm that the 35kDa secreted protein of rabbitpox virus was also responsible for CC chemokine binding. As shown in Figure III-5B, rabbitpox virus supernatants contained a protein which could be cross-linked to RANTES, forming a 49kDa complex (Fig. III-5B, lane 1) but supernatants harvested from cells infected with RPVΔ35 did not exhibit any detectable chemokine binding protein (Fig. III-5B, lane 2), verifying that rabbitpox virus 35kDa protein, like M-T1, binds RANTES.

To prove that the same viral proteins could bind to CXC in addition to CC chemokines, the rabbitpox virus 35kDa protein was assessed for its ability to bind IL-8 (Fig. III-6A). Similar to the RANTES binding profile, rabbitpox virus (Fig. III-6A, lane 1) supernatants exhibited IL-8 binding activity, detectable as a 49kDa cross-linked protein complex, whereas the RPVΔ35 (Fig. III-6A, lane 2) or uninfected (Fig. III-6A, lane 3) supernatants did not form any comparable cross-linked species with IL-8. M-T1 expressed from VV-T1 but not vaccinia virus (WR) also exhibited IL-8 binding activity comparable to that observed in Figure III-5A for RANTES (data not shown). A Coomassie-stained SDS-PAGE gel (Fig. III-6B) of untreated supernatants illustrates the 35kDa secreted protein of rabbitpox virus which was absent in RPVΔ35 supernatants; note that in the gel system used here this viral protein migrated at ~41kDa. To show that IL-8 and a specific viral protein were both detectable within the shifted complexes, immunoblots of IL-8/35kDa cross-linking assays were probed

with either an anti-IL-8 antibody or the anti-35kDa antiserum, which was previously generated against the 35kDa, C23L, protein of vaccinia virus (Lister) (29). The anti-IL-8 antibody detected the IL-8/35kDa complex derived from rabbitpox virus-infected cell supernatants (Fig. III-6C). In addition to the shifted complex containing IL-8 (Fig. III-6C, lane 1), monomers and cross-linked dimers of IL-8 were observed in all cases. The anti-35kDa antiserum detected the 35kDa protein of vaccinia virus (Lister) in supernatants from infected cells (Fig. III-6D, lane 1) and also detected the shifted species cross-linked with IL-8 (Fig. III-6D, lane 2). Thus, these experiments indicated that the T1/35kDa family of secreted poxvirus proteins are soluble chemokine binding proteins that bind to members of both the CC and CXC classes of chemokines.

Solid-Phase Binding Analysis of Purified M-T1

The results obtained thus far depend upon chemical cross-linking to demonstrate the chemokine interactions with the T1/35kDa members. To show that this interaction occurs in the absence of cross-linking with a physiologically relevant affinity, solid-phase equilibrium binding studies was performed. Saturable binding of radiolabeled RANTES to immobilized M-T1 was demonstrated at nanomolar concentrations of RANTES (Fig. III-7A), demonstrating that the interactions observed earlier between the soluble viral proteins and chemokines occur under physiological conditions and are independent of cross-linking. To quantify the affinity of M-T1 and RANTES interaction, Scatchard analysis of the solid-phase

binding data was subsequently performed and yielded a dissociation constant (K_d) of approximately 73 nM (Fig. III-7B).

The Rabbitpox Virus 35kDa Protein Influences Leukocyte Migration in Infected Tissues

To determine if members of the T1/35kDa family of chemokine binding proteins influence leukocyte migration *in vivo*, their role in the early inflammatory process during poxvirus infection was assessed using the rabbitpox virus system. Rabbits were infected with rabbitpox virus or RPVΔ35 at a low (50 PFU) or high ($5X10^4$ PFU) dose of virus and tissue samples were harvested at various times in order to measure leukocyte infiltration into primary sites of infection. Although histological analysis of the tissue sections resulting from the higher virus dose on Days 1 and 2 post-infection revealed only minimal differences in cellular infiltration between the lesions induced by the two viruses, by Day 3 there were distinctive differences. The lower virus inoculation dose exhibited the same trend, but the differences were less evident, possibly because of reduced tissue dosages of secreted viral proteins in general. While the deep dermal layer of lesions infected with wild-type rabbitpox virus (Fig. III-8A) still exhibited only a few scattered infiltrating cells at 3 days post-infection, the lesions infected with RPVΔ35 (Fig. III-8B) were characterized by a significant leukocyte influx and an accompanying edema typical of an acute inflammatory reaction. To characterize the infiltrating cells, tissue sections were immunostained with an anti-rabbit CD43 antibody which specifically stains rabbit lymphocytes and monocytes/macrophages. In the RPVΔ35 tissue sections

about 30% of the infiltrating cells can be immunostained for rabbit CD43, while the remaining 70%, based on nuclear morphology, are presumed to be CD43-negative granulocytes, which are predominantly neutrophils. In the rabbit, granulocyte subclasses have not been as extensively characterized by surface marker expression studies as in the mouse and human systems and the term heterophil is sometimes used to describe such rabbit polymorphonuclear leukocytes. In the lesions caused by the parental rabbitpox virus, very few (<3%) of the cells in the dermal layer can be stained at all for CD43. Thus, in the absence of the 35kDa chemokine binding protein in rabbitpox virus infection there was an increased infiltration of multiple classes of immune cells, predominantly granulocytes, macrophages and NK cells, into the infected lesion. This suggests that *in vivo* the expression of the 35kDa secreted chemokine binding protein functions during the early stages of rabbitpox virus infection to reduce the initial influx of extravasating leukocytes into the site of infection.

DISCUSSION

Poxviruses encode an impressive array of proteins which assist in virus evasion of the collective host defense systems (3, 19, 33). The relationship between the leporipoxvirus T1 and orthopoxvirus 35kDa genes was previously noted by sequence homology analysis (15); however, no function has been previously attributed to this family of proteins. We now report that the T1/35kDa family of secreted proteins bind both CC and CXC chemokines *in vitro* and, in the case of

rabbitpox virus infection, modulate early stage leukocyte migration into virus-infected lesions *in vivo*.

Chemokine binding proteins are secreted from cells infected by several different poxviruses, including myxoma virus, Shope fibroma virus, rabbitpox virus, raccoonpox virus, cowpox virus, and vaccinia virus (Lister), but not vaccinia virus (WR) or swinepox virus. It was unexpected that the cowpox virus-derived supernatants exhibited chemokine binding, as previous analysis of [^{35}S]Met-labeled supernatants from cowpox-infected cells did not reveal a comparable 35kDa protein species (15). However, in this study immunoblot analysis using antiserum prepared against the 35kDa homologue of vaccinia virus (Lister) (29) in fact revealed a smaller related protein in the cowpox virus supernatant. This protein, which is approximately 4 kDa smaller in size than the rabbitpox virus protein, likely corresponds to a smaller secreted protein that was observed in the previous study, but not at that time attributed to the 35kDa protein homologue. The resolution of the gel shifts presented here (Fig. III-1) did not illustrate this size difference between the orthopoxvirus 35kDa homologues but these can be observed under different gel conditions.

Although the secreted T1/35kDa proteins are not related to any known cytokine receptor species, numerous cell surface virus-encoded chemokine receptors with classic seven-transmembrane domains have been previously identified. The viral proteins US28, of human cytomegalovirus, and ECRF, of herpesvirus saimiri, are functional serpentine chemokine receptors (1, 26); however, their role in viral pathogenesis remains unclear. Several other viruses encode similar transmembrane proteins with homology to chemokine receptors, including two herpesviruses (HHV-

6, HHV-8) and two poxviruses (capripox and swinepox viruses) but these proteins have not yet been shown to be functional for chemokine binding or signal transduction (6, 16, 26).

The observation that the complexes induced by either RANTES or IL-8 are identical in size for each virus suggests that the same polypeptide is responsible for binding both CC and CXC classes of chemokines. This notion is supported by the following data: (1) unlabeled RANTES inhibited binding of radiolabeled IL-8 to the viral protein; (2) the expression of one ORF, M-T1, from a recombinant vaccinia virus (WR) construct confered upon the virus the ability to secrete a protein which bound both RANTES and IL-8; and (3) the deletion of the single ORF encoding the 35kDa secreted protein from rabbitpox virus resulted in the loss of all chemokine binding activity from the supernatants of cells infected by this virus. Although the previously characterized viral chemokine receptors are fairly class restricted, binding either CC or CXC chemokines, the T1/35kDa proteins are far less restricted in terms of binding specificity. However, M-T1 clearly binds RANTES more avidly than IL-8 because RANTES effectively displaced IL-8 binding but IL-8 fails to displace RANTES binding. In addition, we were unable to detect saturable binding of purified M-T1 to IL-8 under solid-phase binding conditions (data not shown). Collectively, these results indicate that both IL-8 and RANTES bind to the same site on the viral protein, but that RANTES binds with a higher affinity.

To determine whether chemokine binding to the M-T1/35kDa proteins solid-phase occurs under physiological conditions, binding analysis was performed in the absence of cross-linking. As the binding inhibition study using unfractionated

supernatants suggested that RANTES has apparent greater affinity for the viral protein than IL-8, RANTES was selected for this experiment. The calculated 73 nM K_d of RANTES binding with purified M-T1 was comparable with CC chemokine binding to cell-surface receptors (12) but whether these soluble proteins competitively displace receptor/ligand binding or triggering remains to be determined.

The function of the M-T1/35kDa proteins can also be assessed by infecting susceptible animals with poxvirus mutants in which the M-T1/35kDa gene has been deleted. Unlike myxoma virus, rabbitpox virus expresses only a single chemokine binding protein; therefore rabbitpox virus was chosen for this study. Previous analysis of rabbitpox virus has shown that virus infection of European rabbits induces an almost uniformly lethal disease even when the secreted 35kDa gene is deleted, and no differences in the pathology were observed between the parental and mutant virus strains. However, when injected intranasally into mice, the RPVΔ35 virus did induce an inflammatory reaction that developed more rapidly during the initial stages of infection (15). There are several important differences between that study and the experiments reported here. Previously, both the parental and mutant viruses were administered to the same rabbit, on opposite sides, at a dose of 500 PFU per site. In this study each rabbit received only one virus at a dose of 50 PFU or $5x10^4$ PFU. The results we observed with the lower 50 PFU virus dose was consistent with the previously published experiment. However, when we injected a significantly higher dose of $5x10^4$ PFU, the effect of the deletion in the RPVΔ35 virus was evident. Here we show in the RPVΔ35 virus-infected lesions that the influx of at least two classes of infiltrating leukocytes, comprised of CD43-positive lymphocytes (possibly NK

cells) and monocytes/macrophages as well as CD43-negative granulocytes, occurred at significantly greater levels by Day three following infection, compared to the parental virus. Although this increase in leukocyte infiltration did not cause overall attenuation in terms of eventual mortality levels, it should be noted that the relationship between virulence and immunomodulatory proteins can be complex. For example, deletion of the IL-1β receptor in vaccinia virus causes an increase in virulence in mice following an intranasal route of inoculation but a decrease in virulence following intracranial inoculation (2, 34).

Distinct domains of chemokines have been identified that are necessary for binding to their cognate serpentine receptors and to glycosaminoglycans on both cell surfaces and on the extracellular matrix (39, 40). The poxviral soluble chemokine binding proteins, alone or in concert with other proteins, could in theory act by either blocking signaling through the chemokine receptors or alternatively by interfering with chemokine gradients mediated by interaction with glycosaminoglycans. Inhibition of either function could have a profound effect on leukocyte chemotaxis in complex tissues (20). Although the biochemical basis for the T1/35kDa protein activities *in vivo* is not understood, it is known that most chemokines bind tightly to glycosaminoglycans such as heparan sulfate proteoglycans (39). As the T1/35kDa proteins are all very acidic, with predicted p*I* values of 4.3-4.7, it is plausible that they interact with chemokines via the conserved heparin-binding domains; further studies, including mutagenesis analysis of the viral proteins, will be required to address this issue.

There is increasing evidence that chemokines play an important role in the early inflammatory responses to viruses (7). Moreover, the recent claim that chemokines can act as the major HIV suppressor factors and the fact that chemokine receptors act as cofactors for HIV entry suggests that chemokines may significantly contribute to the outcome of viral disease progression and viral pathogenesis (4). Thus, it would be expected that at least some viruses have evolved counteractive extracellular proteins to alter the ability of chemokines to activate and direct inflammatory cells to the site of virus infection. The mechanism by which the T1/35kDa family of secreted proteins modulate chemokine activities remains to be demonstrated, but this new superfamily may very well provide useful protein probes with which to investigate the complex biological roles of chemokines as regulators of leukocyte trafficking during inflammatory syndromes in general.

MATERIALS AND METHODS

Cells and Viruses. The poxviruses myxoma virus (strain Lausanne), Shope fibroma virus (strain Kasza), rabbitpox virus (strain Utrecht), raccoonpox virus, swinepox virus, vaccinia virus (strains Western Reserve and Lister) were all obtained from the American Type Culture Collection (ATCC). Cowpox virus (strain Brighton Red) was a gift from D. Pickup. The myxoma virus used throughout this study is a recombinant, vMyxlac, which has a β-galactosidase cassette inserted in an intragenic location (28). Myxoma virus in which the M-T7 ORF is disrupted, vMyxlac-T7gpt, was previously described (24). Rabbitpox virus in which the 35kDa ORF is deleted,

RPVΔ35, was also described previously (15). Myxoma virus, Shope fibroma virus, and the vaccinia viruses were routinely passaged in a baby green monkey kidney (BGMK, a gift from S. Dales) cell line and rabbitpox virus, raccoonpox virus, and cowpox virus were routinely passaged in a rabbit kidney cell line (RK13, from the ATCC) in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum (Life Technologies, Gaithersburg, MD). Swinepox virus was routinely passaged in a swine kidney (ESK, from the ATCC) cell line in Ham's F12 medium (Life Technologies, Gaithersburg, MD) with 10% newborn calf serum (Life Technologies, Gaithersburg, MD).

Vaccinia virus expressing the myxoma virus M-T1 gene, VV-T1, was generated by subcloning a 1.2 kb *Kpn*I-*Bam*HI fragment (containing the M-T1 gene) from the myxoma virus *Bam*HI S fragment into the *Kpn*I-*Bam*HI sites of (8),creating pMJ-T1 in which M-T1 is targeted for insertion in the vaccinia thymidine kinase gene. The plasmid pMJ-T1 was then used to generate VV-T1 by homologous recombination into vaccinia virus (WR).

***In vitro* Chemokine Binding Assays and Immunoblots.** Serum-free, concentrated supernatants were prepared from poxvirus infected cells as previously described (38). Briefly, 2 x 10^7 cells were infected at a m.o.i. of 10, washed extensively with phosphate buffered saline (PBS) after the adsorption period, and incubated in serum free medium (10 ml) for 12-16 h at 37°C. The medium, or supernatant, was then collected and concentrated 15-fold using Amicon centriprep 10 concentrators (Beverly, MA). The human chemokines RANTES and IL-8 were labeled with ^{125}I using Iodobeads (Pierce, Rockford IL). Five mg of chemokine was

reacted in 175 µl PBS with one iodobead, and 0.2 mCi $Na^{125}I$ (Dupont NEN, Missasauga, ON) for 7 min. The mixture was then passed over a KwikSep column (Pierce) and eluted with PBS to separate the labeled protein from unbound $Na^{125}I$.

Chemokine binding assays were performed by both cross-linking and direct solid-phase binding protocols. Cross-linking of chemokines with viral proteins was measured by mixing concentrated secreted viral proteins from virus-infected cells (5 µl) with ^{125}I-labeled chemokine (25 ng) and 10 mM sodium phosphate buffer (pH 7.0) in a total volume of 15 µl, and the mixture was then incubated at room temperature for 2 h. The protein complexes were cross-linked by the addition of 2 µl of 200 mM 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC) (SIGMA Chemical Co, Missasauga, ON) in 0.1 M potassium phosphate buffer, (pH 7.5) for 15 min. Another 2 µl aliquot of 200 mM EDC was added and incubated for 15 min, followed by the addition of 2 µl 1M Tris Cl, pH 7.5, (SIGMA Chemical Co) to quench the reaction (Upton *et al.*, 1992). The cross-linked products were then subjected to 12.5% denaturing SDS-PAGE and autoradiography. Competition assays using unlabeled ligand were performed as previously described (25). In addition to chemokines, the competitors used were human IL-3, murine IL-4, murine IL-6, murine IL-7, murine IL-9, human IL-10, and human IL-11, and were kindly supplied by Dr. Hsaing-fu Kung (NIH-Biological Response Modifiers Program).

To confirm that the higher order cross-linked complexes contain both IL-8 and viral protein, cross-linked complexes were prepared as described for the gel shift assay. Cross-linked complexes were separated on denaturing SDS-PAGE and immunoblotted as previously described (31). Immunoblots were probed with anti-

human-IL-8 (1/1,000 dilution, R&D Systems, Minneapolis, MN) and detected using horse radish peroxidase-conjugated donkey anti-human IgG (1/2,000 dilution, Jackson ImmunoReasearch Laboratories, West Grove, PA) and a chemiluminescence detection system (Amersham, Oakville, ON) according to the manufacturer's recommendations.

The antibody A18691 (29) was also used to detect the 35kDa protein on immunoblots. This polyclonal antiserum, a generous gift of Arvind Patel (MRC Virology Unit Glasgow, Scotland), was previously prepared using the purified 35kDa protein from vaccinia virus (Lister), encoded by the C23L ORF, as the immunogen. The antiserum was used at a dilution of 1/10,000, detected with horse radish peroxidase-conjugated goat anti rabbit IgG at 1/5,000 dilution (Jackson ImmunoReasearch Laboratories), and visualized using chemiluminescence.

Solid-Phase Binding of M-T1 to RANTES. M-T1 was fractionated from tissue culture supernatants of myxoma virus-infected cells by column chromatography (14). Purified M-T1 protein (50 ng) was immobilized on Falcon 96-well immunoplates in 50 µl of PBS and incubated overnight at 4°C. Wells were blocked with 5% skim milk powder in Tris-buffered saline (TBS) containing 0.2% Tween-20 for 4 h at room temperature, and then incubated with ^{125}I-labeled RANTES in 100 µl blocking buffer for an additional 4 h. Wells were then washed extensively with TBS-0.2% Tween buffer, removed, and radiobound-counts were measured on a Packard 5780 gammacounter. To determine the specific binding of M-T1 to RANTES, non-specific binding in the presence of 100-fold excess cold RANTES was

routinely subtracted from total binding. All assays were performed in triplicate and the affinity of M-T1 for RANTES was determined by the method of Scatchard.

DNA Sequence Analysis. To facilitate sequence analysis, DNA was subcloned, or amplified by PCR, from the myxoma or rabbitpox virus genomes. A 1.2 kbp *Kpn*I-*Bam*HI fragment containing the M-T1 gene was subcloned from the myxoma virus *Bam*HI S fragment into pBluescript KS+, generating the plasmid pBS-M-T1. The gene that encodes the rabbitpox virus 35kDa protein was amplified from rabbitpox virus DNA by PCR using VENT polymerase (Life Technologies, Gaithersburg, MD) and the primers 5'-GCGCTCGAGATGAAACAATATA-TCGTCCTG and 5'-GCGAAGCTTTCAGACACACGCTTTGAG. The PCR product was digested with *Xho*I and *Hind*III, and cloned into *Xho*I/*Hind*III sites in pBluescript KS+ (Stratagene, La Jolla, CA), yielding pBS-RPV-35k.

With pBS-M-T1 and pBS-RPV-35k as templates, both strands of the myxoma virus M-T1 and rabbitpox virus 35kDa genes were sequenced, using nested oligonucleotides, on an ABI 373 DNA sequencer with *Taq* cycle sequencing to a redundancy of greater than fivefold. The sequences were analyzed using Genetics Computer Group (Wisconsin, MI) programs.

Infection of Rabbits with Rabbitpox Viruses. Female New Zealand White rabbits were given intradermal infections on both flanks with 50 PFU or $5X10^4$ PFU of wild-type rabbitpox virus or RPVΔ35. A total of 12 rabbits were infected and tissue samples of the primary skin lesions were collected at 1, 2, and 3 days post-infection from 2 rabbits per dose for both viruses. Two lesions from each rabbit were assessed. Fixed tissues were paraffin-embedded, sectioned, and immunostained with

anti-rabbit-CD43 antibody (Spring Valley Laboratories, Woodbine, MD) to stain for infiltrating rabbit leukocytes, particularly T-cells and monocytes/macrophages, as previously described (24) except that hematoxylin was used as a counterstain.

Table III-1. Percentage Similarity and Identity Between the Amino Acid Sequences of M-T1 and Several Family Members.*

Virus	Myx T1	SFV T1	RPV 35kDa	VV (lis) 35kDA	VV (cop) 35kDa	VAR 35kDa	CPV 35kDa
Myx T1		84.4	56.0	56.0	55.3	57.3	57.0
SFV T1	70.4		58.1	58.5	59.2	60.3	62.3
RPV 35kDa	37.8	40.2		99.6	99.6	96.0	92.3
VV (lis) 35kDA	38.2	41.1	99.2		100.0	96.4	92.7
VV (cop) 35kDa	38.3	41.6	98.8	99.6		96.2	92.3
VAR 35kDa	37.7	42.6	94.1	94.9	94.1		88.9
CPV 35kDa	39.6	41.6	85.0	85.8	85.0	85.1	

*Similarity is shown at the top right; identity is shown at the bottom left. Accession numbers are given in the legend to Fig. III-4

Figure III-1. Secretion of chemokine binding proteins from poxvirus-infected cells.*

Supernatants from cells which were uninfected (mock, lane 1), or infected with myxoma virus (MYX, lane 2), Shope fibroma virus (SFV, lane 3), raccoonpox virus (RcPV, lane 4), swinepox virus (SPV, lane 5), cowpox virus (CPV, lane 6), rabbitpox virus (RPV, lane 7), vaccinia virus (strain Lister) (VV(lis), lane 8), or vaccinia virus (strain Western Reserve) (VV(wr), lane 9) were cross-linked with ^{125}I-labeled RANTES (A) or ^{125}I-labeled IL-8 (B). The arrow points to a 53-kDa cross-linked species in MYX (lane 1) and SFV (lane 2); a smaller complex of 49 kDa can be observed in RcPV (lane 4), CPV (lane 5), RPV (lane 7) and VV(lis) (lane 8). Molecular weight standards from top to bottom are 101, 83, 50.6, 35.5, 29.1, and 20.9 kDa.

*Experiments represented in Figure III-1 were performed in collaboration with Dr. Kathryn A. Graham (University of Alberta, Edmonton, Canada)

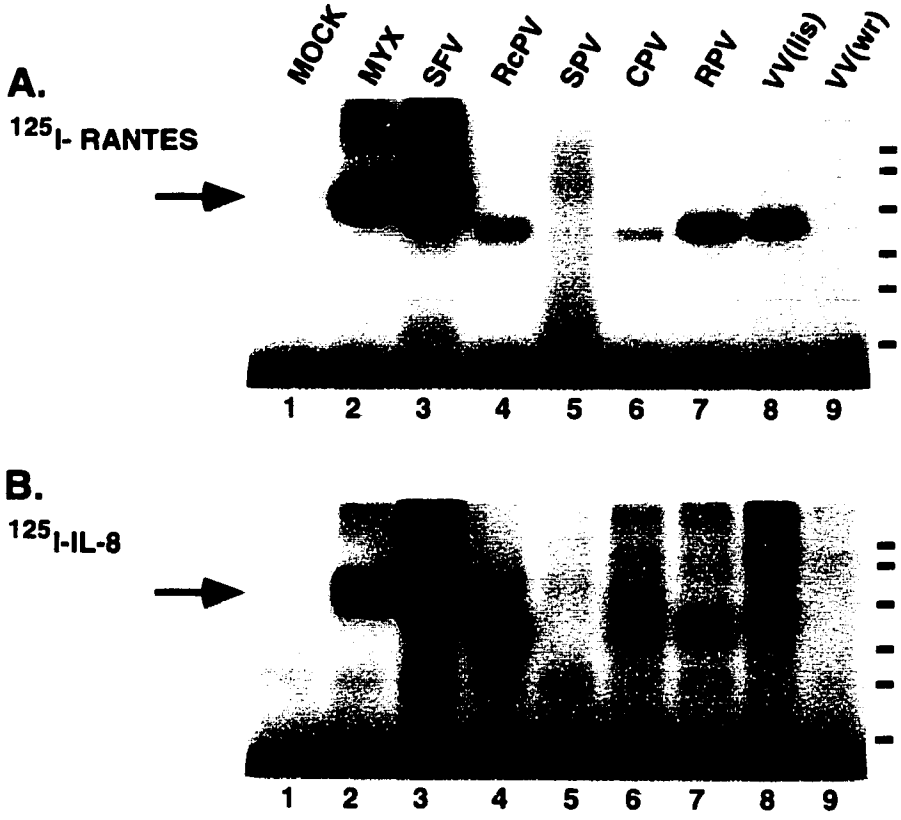

A.
^{125}I- RANTES
MOCK
MYX
SFV
RcPV
SPV
CPV
RPV
VV(lis)
VV(wr)
1 2 3 4 5 6 7 8 9
B.
^{125}I-IL-8
1 2 3 4 5 6 7 8 9

Figure III-2. Binding inhibition studies with the myxoma virus chemokine binding protein.

Self-competitions for chemokine binding were done using secreted proteins from cells which were infected with myxoma virus and cross-linked with (A) ^{125}I-labeled RANTES in the presence of increasing concentrations of unlabeled RANTES or with (B) ^{125}I-labeled IL-8 in the presence of increasing concentrations of unlabeled IL-8. Alternatively, (C) a cross-competition was done in which myxoma virus-derived supernatants were reacted with iodinated RANTES in the presence of increasing concentrations of unlabeled IL-8. In panels A, B, and C, ligand was added in the absence of competitor (lane 1) or in the presence of competitor at concentrations of 0.5X (lane 2), 1.0X (lane 3), 2.5X (lane 4), 5X (lane 5), 10X (lane 6), 50X (lane 7), 100X (lane 8), and 200X (lane 9) fold molar excess. Also, (D) labeled RANTES was reacted with myxoma virus supernatants in absence of competitor (lane 1), in the presence of 200 fold molar excess of unlabeled RANTES (lane 2), or 200-fold molar excess of a variety of non-chemotactic cytokines including huIL-3 (lane 3), muIL-4 (lane 4), muIL-6 (lane 5), muIL-7 (lane 6), muIL-9 (lane 7), huIL-10 (lane 8), and huIL-11 (lane 9). Molecular weight standards are as described in the legend to Fig. III-1.

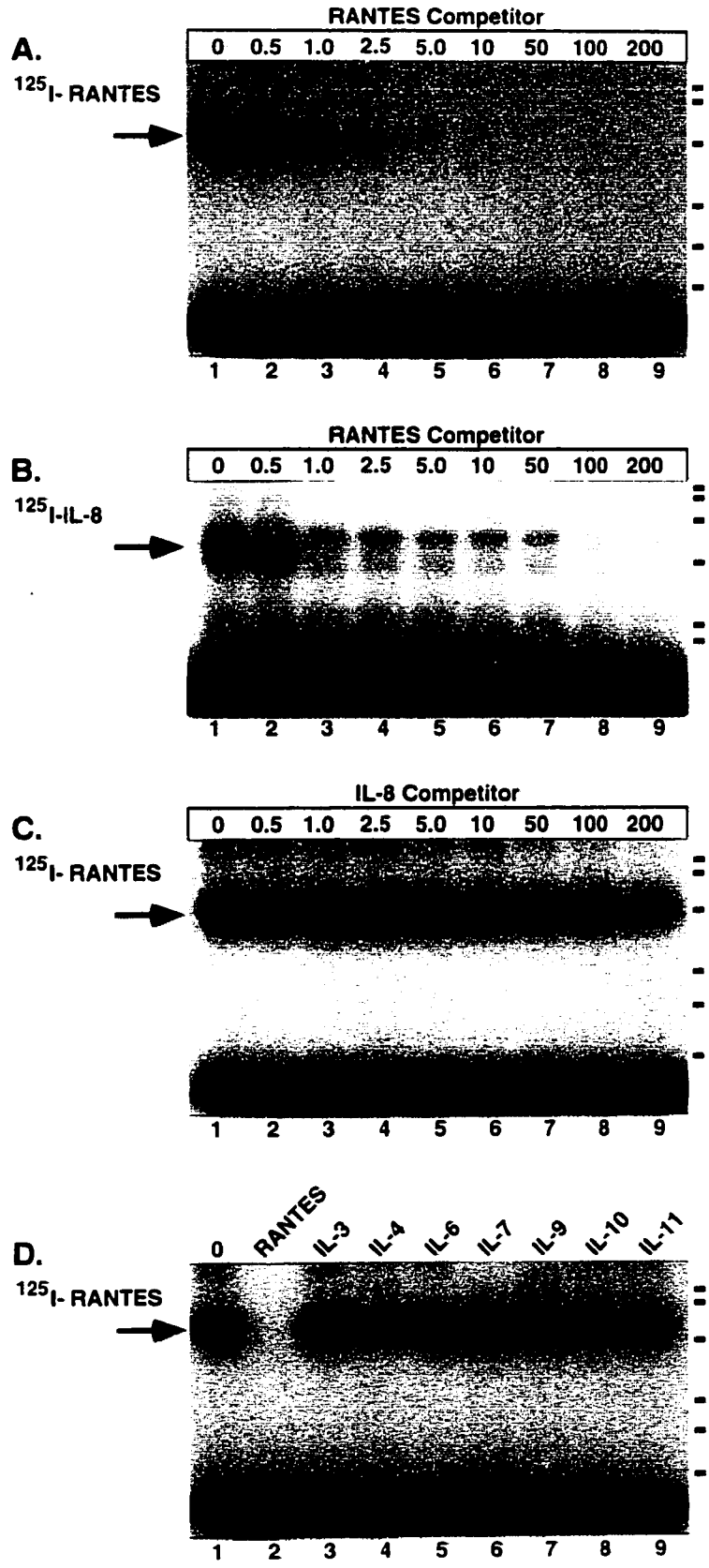
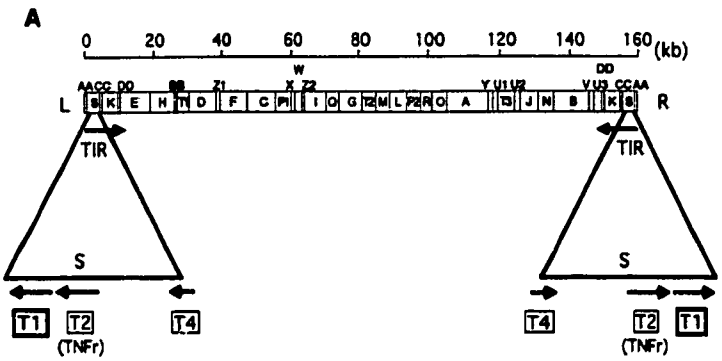

A.
^{125}I- RANTES
RANTES Competitor
0 0.5 1.0 2.5 5.0 10 50 100 200
1 2 3 4 5 6 7 8 9
B.
^{125}I-IL-8
RANTES Competitor
0 0.5 1.0 2.5 5.0 10 50 100 200
1 2 3 4 5 6 7 8 9
C.
^{125}I- RANTES
IL-8 Competitor
0 0.5 1.0 2.5 5.0 10 50 100 200
1 2 3 4 5 6 7 8 9
D.
^{125}I- RANTES
0 RANTES IL-3 IL-4 IL-6 IL-7 IL-9 IL-10 IL-11
1 2 3 4 5 6 7 8 9

Figure III-3. Location of the M-T1 gene in the myxoma virus genome and the nucleotide and deduced amino acid sequences of the myxoma virus M-T1 and rabbitpox virus 35kDa open reading frames.*

(A) A *Bam*HI restriction map of the myxoma genome shows the location of characterized genes in the *Bam*HI S fragment. These genes are present in two copies as they are fully within the terminal inverted repeats (TIR). Sequences of the myxoma virus M-T1 (B) and rabbitpox virus 35kDa (C) open reading frames are shown. The derived amino acid sequences are shown with the predicted signal sequence cleavage sites (arrow) and putative N-glycosylation sites (underline).

*Experiments represented in Figure III-3 were performed in collaboration with Dr. Kathryn A. Graham (University of Alberta, Edmonton, Canada)



B

```
1 ATGAAACGCCTGTGTGTATTATTCGCGTGCCTGGCCGCGACCCTCGCGACGAAGGCATC
  M K R L C V L F A C L A A T L A T K G I

61 TGCAGACAAGCGCGAAGATGTCCGATACATGGGAATAGACGCCGTGGCCAAAATTACAAAG
   C R Q G E D V R Y M G I D A V A K I T K

121 AGGACTACCGGAAGCGACACGCCGTGTCAGGGTCTGCGTACGACTATTGAATCCGCGTAT
    R T T G S D T F C Q G L R T T I E S A Y

181 ACAGAAGACGAAAACGAAGACGATGGCGCGACGGGTACGGAGCAGCCCGACGATCTTAGC
    T E D E N E D D G A T G T E Q P D D L S

241 GAGGAATACGAGTACGACGAAAACGACGAATCGTTTCTAACCGGTTTCGTGATCGGAAGT
    E E Y E Y D E N D E S F L T G F V I G S

301 ACTTACCACACGATCGTCGGAGGAGGACTCTCCGTCACGTTCGGATTTACGGGATGTCCT
    T Y H T I V G G G L S V T F G F T G C P

361 ACCGTTAAGGCGATATCCGAACACGTCAAAGGCGCCACGTCTACGTCCGACTGTCCAGC
    T V K A I S E E V K G R E V Y V R L S S

421 GACGCTCCTTGGAGAGATACGAATCCCGTGTCTATGAACCGTACAGAGGCGCTCGCCCTA
    D A P W R D T N P V S M N R T E A L A L

481 CTCGACACGTGTGAAGTGTCCGTAGATATCAAATGCAGTCGCGTCAACGTAACCGAAACG
    L D T C E V S V D I K C S R V N V T E T

541 ACGTACGGAACCGCCGCCGCTTGTCCCGCGTATAACTCAAGCGACGAGACGCAGTCATATT
    T Y G T A A L V P R I T Q A T R R S E I

601 ATCGGATCTACCCTGGTCGACACGGAATGTGTGAAGAGTCTAGACATAACCGTCCAAGTG
    I G S T L V D T E C V K S L D I T V Q V

661 GGTGAAATGTGTAAGAGAACGTCTGATCTCTCGGCGAGAGACAGTCTTAAGGTAAAGAAC
    G E M C K R T S D L S A R D S L K V K N

721 GGCAAACTACTCGAGGACGATATCCTTGTCCTTCGTACGCCTACCCTCAAGGCGTGTAAC
    G K L L E D D I L V L R T P T L K A C N

781 TAATCCTATCTACGATCGATGTCGTATTTTTCTGACCGTTACGCGTCACGTTTTTATACC
    *

841 TATATAAATAGTTAAAACGCATATAGGGAATACCGCTCGCTTTTTTTCCTTCGTAGTTG

901 TTTACCCGCTCGATAGATCGCGTCGAGGAAGTACCAACCGTGACCACTCCTCCGGCGGGG

961 ATCC
```

C

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1 ATGAAACAATATATCGTCCTGGCATGCATGTGCCTGGCGGCAGCTGCTATGCCTGCCAGT
  M K Q Y I V L A C M C L A A A A A M P A S

61 CTTCAGCAATCATCCTCATCCTCATCCTCGTGTACGGAAGAAGAAAACAAACATCATATG
   L Q Q S S S S S S S C T E E E N K E E M

121 GGAATCGATGTTATTATCAAAGTCACAAAGCAAGACCAAACACCGACCAATGATAAGATT
    G I D V I I K V T K Q D Q T P T N D K I

181 TGCCAATCCGTAACTGAAATTACAGAGTCCGAGTCAGATCCAGATCCCGAGTGGAATCA
    C Q S V T E I T E S E S D P D P E V E S

241 GAAGATGATTCCACATCAGTCGAGGATGTAGATCCTCCTACCACTTATTACTCCATCATC
    E D D S T S V E D V D P P T T Y Y S I I

301 GGTGGAGGTCTGAGAATGAACTTTGGATTCACCAAATGTCCTCAGATTAAATCCAGTCTA
    G G G L R M N F G F T K C P Q I K S I S

361 GAATCCGCTGATGGAAACACAGTGAATGCTAGATTGTCCAGCGTGTCCCCAGGACAAGGT
    E S A D G N T V N A R L S S V S P G Q G

421 AAGGACTCTCCCCGCGATCACTCATGAAGAAGCTCTTGCTATGATCAAAGACTGTGAGGTG
    K D S P A I T H E E A L A M I K D C E V

481 TCTATCGACATCAGATGTAGCGAAGAAGAAGAAAGACAGCGACATCAAGACCCATCCAGTA
    S I D I R C S E E E E K D S D I K T H P V

541 CTCGGGTCTAACATCTCTCATAAGAAAGTGAGTTACGAAGATATCATCGGTTCAACGATC
    L G S N I S H K R V S Y E D I I G S T I

601 GTCGATACAAAATGTGTCAAGAATCTAGAGTTTAGCGTTCGTATCGGAGACATGTGCAAG
    V D T K C V K N L E F S V R I G D M C K

661 GAATCATCTGAACTTAAGGTCAAGGATGGATTCAAGTATGTCGACGGATCGGCATCTAAA
    E S S E L E V K D G F K Y V D G S A S K

721 GGTGCAACGGATGATACTTCACTCATCGATTCAACAAACTCAAAGCGTGTGTCTGA
    G A T D D T S L I D S T K L K A C V *
```

Figure III-4. Alignments of the T1/35kDa family members.*

The PIR (release 48.0) Swiss-Prot (release 33.0), and GenBank (release 94) databases were searched with the myxoma virus M-T1 and rabbitpox virus 35kDa amino acid sequences and an alignment of homologues was constructed: from top to bottom, myxoma virus M-T1 (U62677), Shope fibroma virus S-T1 (A43692), rabbitpox virus 35kDa (U64724), C23L 35kDa of vaccinia virus (Lister) (P19063), C23L 35kDa of vaccinia virus (Copenhagen) (A42529), G3R 35kDa of variola virus (Somalia) (U18341), and ORF B of cowpox virus (L08906). Amino acids are boxed if they are conserved in both leporipoxviruses and at least four orthopoxviruses. Conserved cysteines are indicated (*).

*Data represented in Figure III-4 were obtained in collaboration with Dr. Kathryn A. Graham (University of Alberta, Edmonton, Canada)

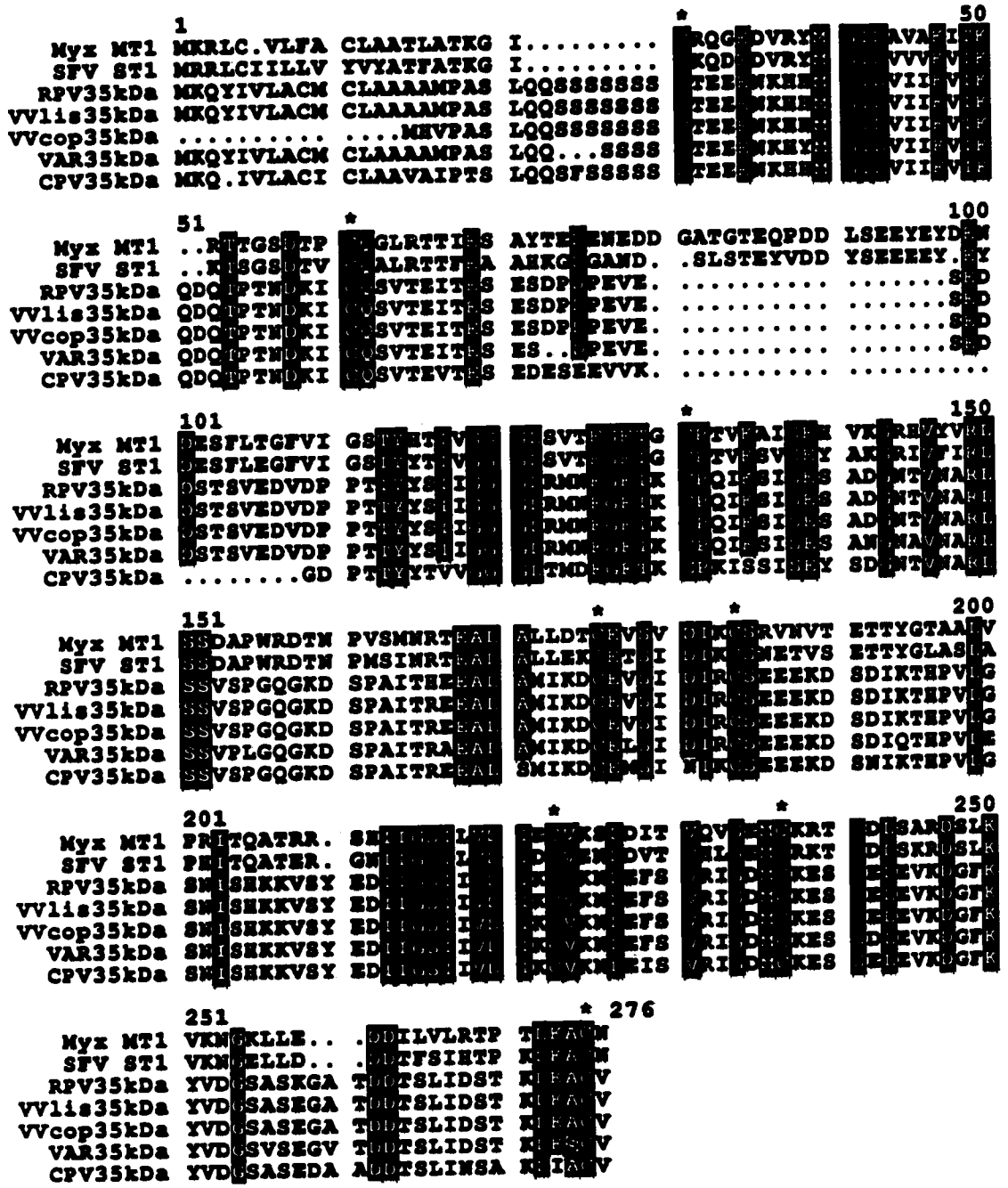
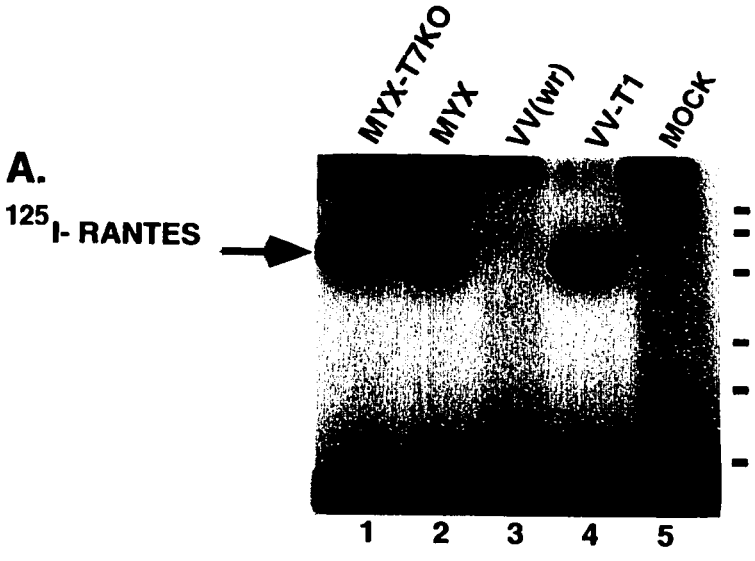

Myx MT1
SFV ST1
RPV35kDa
VV1is35kDa
VVcop35kDa
VAR35kDa
CPV35kDa

Figure III-5. Identification of myxoma virus M-T1 and rabbitpox virus 35kDa as soluble poxviral CC chemokine binding proteins using chemical cross-linking.*

(A) ^{125}I-labeled RANTES was cross-linked with supernatants from cells infected with a myxoma virus construct in which the M-T7 ORF was disrupted (Mossman *et al.*, 1996) (MYX-T7KO, lane 1), myxoma virus (MYX, lane 2), vaccinia virus (WR) (VV(wr), lane 3), vaccinia virus (WR) recombinant expressing M-T1 (VV-T1, lane 4), or from uninfected cells (MOCK, lane 5). The arrow indicates a chemokine/viral protein complex of identical size in MYX-T7KO (lane 1), MYX (lane 2), and VV-T1 (lane 4) supernatants. (B) ^{125}I-labeled RANTES was cross-linked with supernatants from cells infected with rabbitpox virus (RPV, lane 1), rabbitpox virus in which the 35kDa gene has been disrupted (Martinez-Pomares *et al.*, 1995), (RPVΔ35, lane 2) or from uninfected cells (MOCK, lane 3). The arrow indicates the 49-kDa cross-linked complex in supernatants harvested from rabbitpox virus-infected cells that is absent in RPVΔ35 and mock samples. Molecular weight standards are as described in the legend to Fig. III-1.

*Experiments represented in Figure III-5 were performed in collaboration with Dr. Kathryn A. Graham (University of Alberta, Edmonton, Canada)


A.
^{125}I- RANTES
MYX-T7KO
MYX
VV(wr)
VV-T1
MOCK
1
2
3
4
5

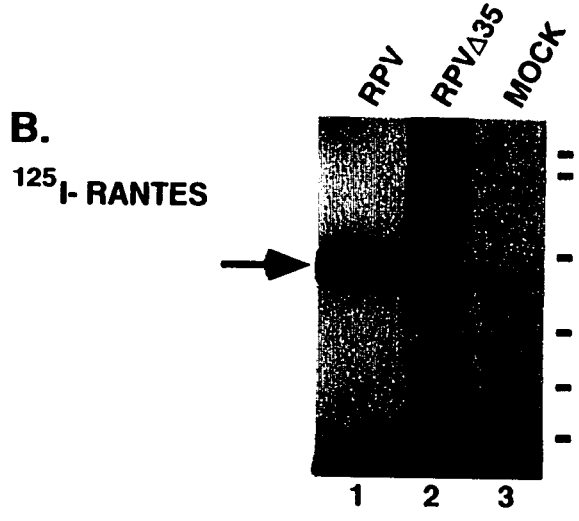

B.
^{125}I- RANTES
RPV
RPVΔ35
MOCK
1
2
3

Figure III-6. Identification of rabbitpox virus 35kDa as soluble poxviral CXC chemokine binding protein.*

(A) ^{125}I-labeled IL-8 was cross-linked with supernatants from cells infected with rabbitpox virus (RPV, lane 1), rabbitpox virus in which the 35kDa gene has been disrupted (Martinez-Pomares *et al.*, 1995), (RPVΔ35, lane 2) or from uninfected cells (MOCK, lane 3). The arrow indicates the 49-kDa cross-linked complex in supernatants harvested from rabbitpox virus-infected cells that is absent in RPVΔ35 and mock samples. In a Coomassie-stained gel (B) of the rabbitpox virus (RPV, lane 1) and RPVΔ35 (lane 2) supernatants, the arrow indicates the 35kDa rabbitpox virus secreted protein, which migrates at ~41kDa in this gel system, and is absent from the RPVΔ35 supernatants. (C) An anti-IL-8 immunoblot was performed to demonstrate that IL-8 is present in the shifted complexes. Unlabeled IL-8 (1 μg) was cross-linked alone in the absence of supernatant (IL-8, lane 4), or with supernatants from uninfected (MOCK, lane 3), or from cells infected with rabbitpox virus (RPV, lane 1), or RPVΔ35 (lane 2). The arrows indicate the monomer (M), and dimer (D) of IL-8, and the 49-kDa shifted complex (C) which contains IL-8. To show that the viral 35kDa protein is also present in the shifted complexes, an anti-35kDa immunoblot (D) was performed using the A18691 antiserum which detects the vaccinia virus (strain Lister) 35kDa protein (Patel *et al.*, 1990). Supernatant harvested from vaccinia virus-infected cells was cross-linked in the absence (lane 1) or presence of 1 μg IL-8 (lane 2); the arrow indicated the shifted species. Molecular weight standards are as described in the legend to Fig. III-1.

*Experiments represented in Figure III-6 were performed in collaboration with Dr. Kathryn A. Graham (University of Alberta, Edmonton, Canada)

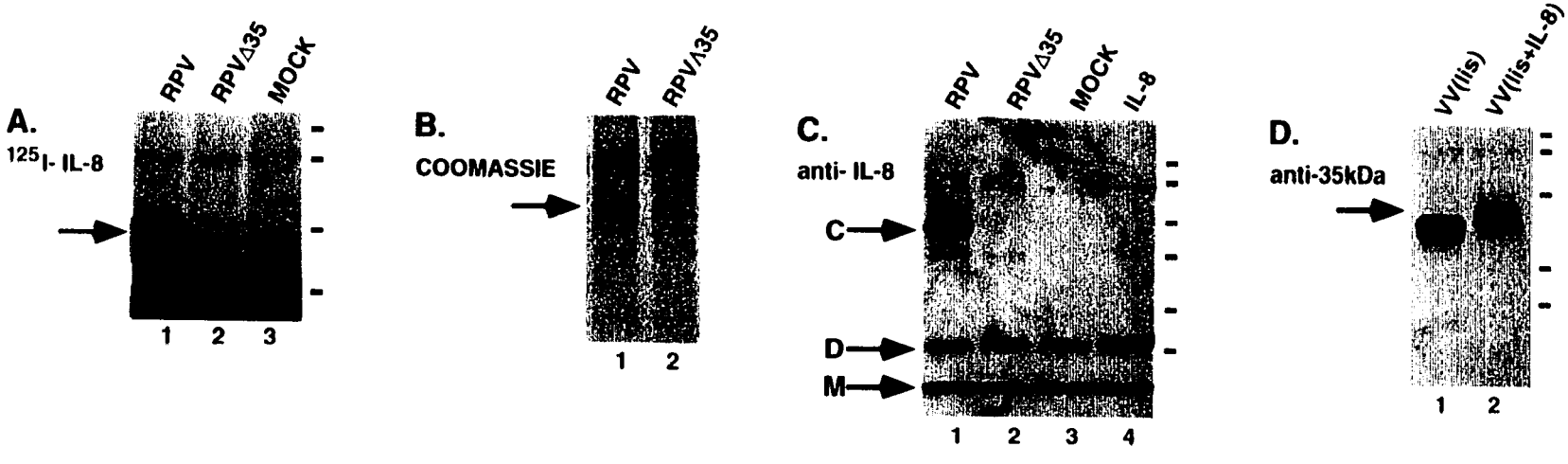
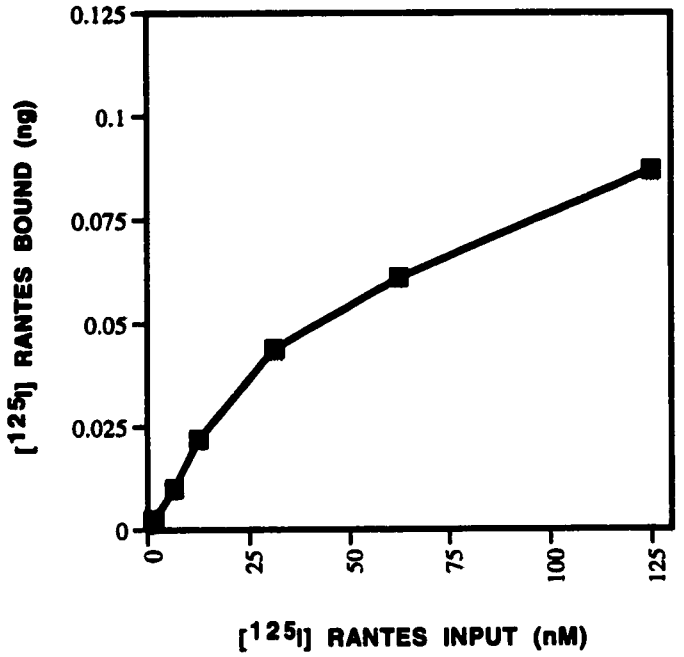

A.
^{125}I- IL-8
RPV
RPVΔ35
MOCK
1 2 3
B.
COOMASSIE
RPV
RPVΔ35
1 2
C.
anti- IL-8
RPV
RPVΔ35
MOCK
IL-8
C
D
M
1 2 3 4
D.
anti-35kDa
VV(lis)
VV(lis+IL-8)
1 2

Figure III-7. Solid-phase equilibrium binding analysis of ^{125}I-labeled human RANTES to M-T1.

(A) Solid-phase binding analysis of immobilized myxoma M-T1 protein with ^{125}I-labeled RANTES as outlined under Materials and Methods. (B) Scatchard plot analysis of the binding curve of ^{125}I-labeled RANTES with M-T1.

A.


0.125
0.1
0.075
0.05
0.025
0
[125I] RANTES BOUND (ng)
0
25
50
75
100
125
[125I] RANTES INPUT (nM)

B.

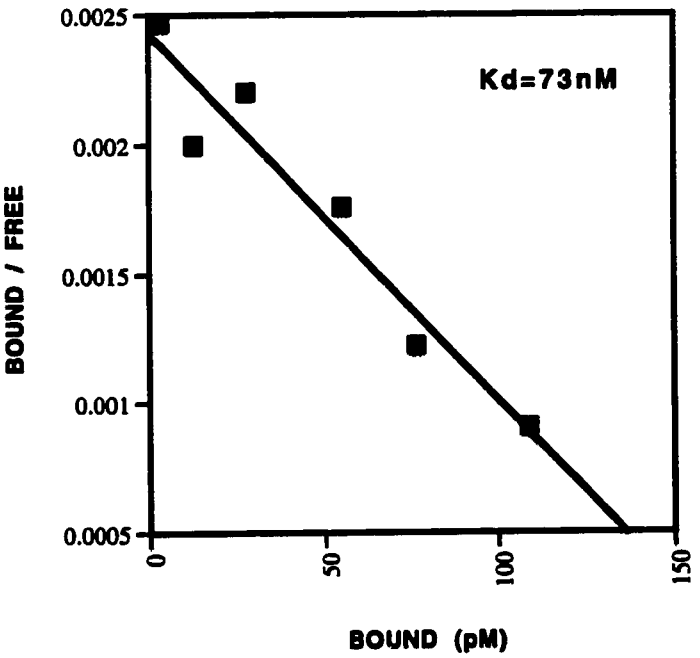
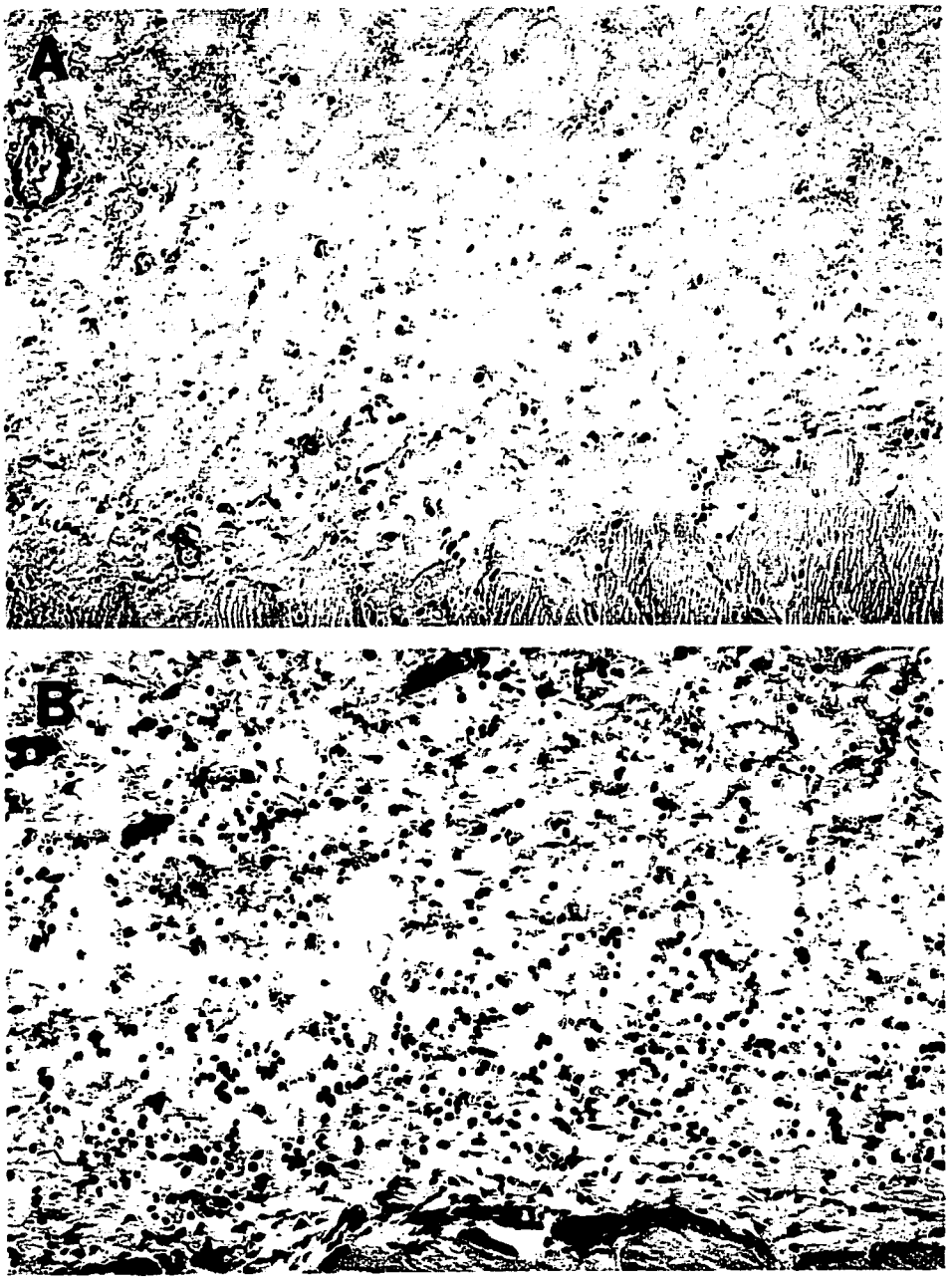

Kd=73nM
0.0025
0.002
0.0015
0.001
0.0005
BOUND / FREE
0
50
100
150
BOUND (pM)

Figure III-8. Leukocyte infiltration into dermal lesions of European rabbits infected for 3 days with $5x10^4$PFU rabbitpox virus or RPVΔ35 viruses.*

In the deep dermal layer at the primary lesion on Day 3, very little cellular infiltration was induced by rabbitpox virus (A). However, in (B) RPVΔ35-infected tissues there was extensive infiltration of CD43-positive lymphocytes and macrophages (stained brown) and CD43-negative granulocytes (stained blue). Refer to Materials and Methods for experimental details.

*Experiments represented in Figure III-8 were performed in collaboration with Dr. Kathryn A. Graham (University of Alberta, Edmonton, Canada) and Dr. Joanne Macen and Traci L. Ness (University of Florida, Gainesville, USA)


A
B

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CHAPTER IV

FUNCTIONAL COMPARISONS AMONG MEMBERS OF THE POXVIRUS T1/35KDA FAMILY OF SOLUBLE CC CHEMOKINE INHIBITOR GLYCOPROTEINS*

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INTRODUCTION

The inhibition of proinflammatory cytokines appears to be a widely adopted strategy used by poxviruses to foster virus survival in the presence of an aggressive innate host immune response (2, 15, 25). Given the paramount role of chemokines in early inflammatory responses to pathogens, it is not surprising that viruses have adopted specific countermeasures to block early signals that promote leukocyte trafficking into virus-infected tissues (11, 18). We have recently identified a new family of poxvirus-encoded 35-40-kDa secreted proteins that bind members of both the CC and CXC chemokine subfamilies *in vitro* as deduced by cross-linking analyses (see Chapter III) (7). The prototypic members of these soluble chemokine binding proteins include the major secreted 35kDa protein expressed by rabbitpox virus (RPV), a member of the *Orthopoxvirus* genus, and the M-T1 gene product of myxoma virus, a specific pathogen of rabbits belonging to the *Leporipoxvirus* genus.

In vitro, unfractionated M-T1 from crude myxoma virus-infected cellular supernatants was shown to be capable of forming cross-linked complexes with both CC (RANTES) and CXC (IL-8) chemokines, but solid-phase and displacement binding analyses suggested that M-T1 only interacts with CC chemokines such as RANTES with high avidity. Recently, other investigators have demonstrated that the major secreted 35kDa proteins expressed by a variety of orthopoxviruses (vaccinia strain Lister, variola and cowpox viruses) bind human CC chemokines with subnanomolar affinities, which effectively prevents their engagement with cell surface chemokine receptors, thereby indirectly inhibiting intracellular calcium

release and chemotaxis (1, 24). *In vivo*, we have shown that the expression of the related 35kDa protein from RPV appears to dramatically affect the infiltration of leukocytes into RPV virus-infected tissues (7).

Sequence analysis showed no apparent homology between the T1/35kDa family members and any known cellular genes, including chemokine receptors, suggesting a novel mechanism for the basis of their chemokine interaction. In fact, the leporipoxvirus T1 proteins and the orthopoxvirus 35kDa proteins share only 40% amino acid identity with each other. Given the divergent sources of T1/35kDa family members in terms of host species restriction, it was of particular interest to determine whether the interactions of these proteins with chemokines exhibited any of the species specificities noted for certain other poxvirus viroceptors, such as those specific for TNF or IFN (17, 23). In this report, we purified M-T1 protein from myxoma virus and the "major secreted 35kDa" proteins from RPV and vaccinia virus (VV) (strain Lister) to comparatively analyze their CC chemokine inhibitory properties *in vitro*. Here we show that RPV-35kDa, VV-35kDa, and M-T1 all potently inhibited the biological activities of CC chemokines with comparable efficacies in a species-nonspecific fashion. Despite the significant sequence divergence between the leporipoxvirus T1 and orthopoxvirus 35kDa proteins, these data suggest that their CC chemokine binding and inhibitory properties are likely to reside within the limited regions of conservation.

RESULTS

Purification of M-T1, VV-35kDa and RPV-35kDa Glycoproteins

Sequence analysis of the leporipoxvirus T1 and orthopoxvirus 35kDa proteins predicts no homology to any known cellular proteins in the database including known chemokine receptors; therefore, the molecular mechanisms governing the interaction between chemokines and the poxvirus proteins remains enigmatic. Given the limited (~40%) conservation of amino acid identity between the leporipoxvirus and orthopoxvirus T1/35kDa family members, we purifed M-T1 from myxoma virus and two orthopoxvirus 35kDa family members, RPV-35kDa and VV-35kDa, to apparent homogeneity to determine whether the functional specificities between these proteins differ and for comparative analyses of their inhibitory properties *in vitro*. Previous studies have shown that unlike many orthopoxviruses which express only one type of secreted chemokine binding protein (1), myxoma virus expresses a second distinct soluble glycoprotein, M-T7, which also demonstrates the biochemical property of binding to a broad spectrum of chemokines (see Chapter II) (10). Thus, to study M-T1 in the absence of M-T7, we fractionated M-T1 from proteins secreted from an M-T7 knockout recombinant myxoma virus- (vMyxlac-T7gpt) defective for M-T7 expression. VV-35kDa and RPV-35kDa were obtained from secreted proteins from VV- (strain Lister) and RPV-infected cells. Given the highly acidic nature of the T1/35kDa family of proteins (7), we exploited the use of anion-exchange chromatography to fractionate M-T1, VV-35kDa, and RPV-35kDa. As shown in Figure IV-1, concentrated proteins harvested from virus-infected cells (Figs. IV-1A, 1C and 1E, lane 1), were fractionated by a dual-step procedure using a Mono-Q column. A primary protein species of the predicted molecular mass of 35-40kDa (Figs. IV-1A, 1C and 1E, lane 2) was detected in fractions eluting at a predicted high

molar salt concentration of the elution buffer. To further purify these proteins from minor copurifying contaminants, pooled fractions containing the putative proteins were subjected to a second round of anion-exchange or gel filtration chromatography, and the corresponding fractions revealed a single prominent species of an apparent molecular mass of 43kDa for M-T1 (Fig. IV-1A, lane 3) and 35kDa for VV-35kDa (Fig. IV-1C, lane 3) and RPV-35kDa (Fig. IV-1E, lane 3) under denaturing conditions.

To verify that the proteins purified were indeed the products of the myxoma virus-T1, VV-C23L/B19R and RPV-35kDa ORFs, unfractionated (Figs. IV-1B, 1D and 1F, lane 1) and purified putative M-T1 (Fig. IV-1B), VV-35kDa (Fig. IV-1D) or RPV-35kDa (Fig. IV-1F) species were subjected to immunoblotting with antiserum designed specifically against their respective proteins. Immunoblotting analyses of protein fractions collected after both purification steps (Figs. IV-1B, 1D and 1F, lanes 2 and 3) identified a single protein species of the predicted molecular mass confirming that the purified proteins were indeed M-T1, VV-35kDa, and RPV-35kDa, respectively. Moreover, microsequencing and peptide analysis demonstrated that fractionated M-T1 was >95% pure and confirmed the N-terminal amino acid sequence, TKGICRQGE-, of M-T1 following the predicted signal cleavage sequence. Analysis of the predicted M-T1 peptide sequence reveals two putative *N*-linked glycosylation sites and a predicted molecular mass of ~30kDa (7). Given the discrepancy of the predicted (30kDa) and apparent (43 kDa) molecular masses of M-T1 observed under SDS-PAGE conditions, we tested whether these differences could be due to *N*-linked glycosylation. Treatment of purified M-T1 with the enzyme *N*-

glycosidase F resulted in the deglycosylation of the protein to ~37-40 kDa (Fig. IV-1B, lane 4), suggesting that at least one posttranslation modification of M-T1 includes the linkage of *N*-linked oligosaccharides to the viral peptide.

Purified M-T1, VV-35kDa and RPV-35kDa Bind CC chemokines

We previously demonstrated that M-T1, VV-35kDa, and RPV-35kDa from unfractionated virally infected cellular supernatants can form cross-linked complexes with chemokines *in vitro* (see Chapter III) (7). To verify that this interaction is independent of other viral proteins or virally induced cellular proteins, purified M-T1, VV-35kDa, and RPV-35kDa were tested for their ability to form protein complexes with two radiolabeled human CC chemokines, MIP-1α and MCP-1. As shown in Figures IV-2A and IV-2B, column fractions containing partially purified M-T1 or RPV-35kDa (lanes 3 and 6, respectively), or purified M-T1, RPV-35kDa, or VV-35kDa (lanes 4, 7 and 9, respectively) all demonstrated the capability to form a 1:1 shifted cross-linked complex with hMIP-1α (Fig. IV-2A) or hMCP-1 (Fig. IV-2B) in an identical manner to their unfractionated viral counterparts (lanes 2, 5 and 8). However, no complexes could be detected between the radiolabeled chemokines and secreted proteins from mock-infected cells (Figs. IV-2A and 2B, lane 1). Thus, the purified M-T1, VV-35kDa, and RPV-35kDa proteins described herein retained the previously characterized property of interacting with representative members of the CC chemokine subfamily as demonstrated in Chapter III.

M-T1 and 35kDa Proteins Block CC Chemokines from Triggering Cell Surface Receptors and Inducing Calcium Fluxes

The increase of intracellular calcium from cytosolic stores is one of the earliest events occurring in response to the binding of chemokines to cell surface seven-transmembrane G protein-coupled receptors. To determine if the M-T1 and 35kDa proteins can effectively bind chemokines and abrogate this biological activity, we measured the inhibition of chemokine-mediated calcium mobilization by the viral proteins in THP-1 monocytes and differentiated HL-60 cells induced by CC and CXC chemokines, respectively. Increasing amounts of RPV-35kDa (1-10 nM) progressively suppressed MIP-1α-stimulated intracellular calcium release in THP-1 cells as shown in the representative traces in Figure IV-3A. M-T1, RPV-35kDa, and VV-35kDa all prevented calcium mobilization in THP-1 monocytes stimulated by MIP-1α (Fig. IV-4A) or MCP-1 (Fig. IV-4B) in a dose-dependent manner. The representative insets shown in Figures IV-4A and IV-4B depict graphs as described in the Materials and Methods that were used to calculate the inhibition constant, K_i, for M-T1 inhibition against MIP-1α or MCP-1. All three viral glycoproteins possessed varying but comparable K_i values and showed potent inhibitory activity towards both human CC chemokines (Table IV-1). We also tested the purified poxvirus proteins for their ability to counter CXC chemokine-induced signal transduction in differentiated HL-60 cells expressing CXC chemokine receptors. An increased calcium response in differentiated HL-60 cells due to stimulation with IL-8 (Figs. IV-3B and 4C) was unaffected by coincubation with the M-T1 or 35kDa proteins, suggesting that the T1/35kDa family members are specific biological inhibitors of CC

but not CXC chemokines, which is consistent with previous reports for other orthopoxvirus 35kDa family members (1, 24).

Members of the T1/35kDa Family Inhibit CC Chemokine-Dependent Migration of Monocytes

Perhaps the most extensively studied activity of chemokines is their ability to stimulate gradient-dependent chemotaxis of various immune cells using modified Boyden chambers. To determine whether all members of the T1/35kDa family comparably inhibit this biological function of chemokines, we tested the ability of M-T1, VV-35kDa, and RPV-35kDa to block chemokine-mediated migration of human monocytes or neutrophils. As shown in Figure IV-5, unidirectional migration of human primary monocytes in response to human MIP-1α could be inhibited by purified M-T1 (Fig. IV-5A), VV-35kDa (Fig. IV-5B), or RPV-35kDa (Fig. IV-5C) in a dose-dependent manner. To quantify the inhibitory effects of M-T1 and 35kDa proteins in blocking MIP-1α activity, we determined the percentage of migration inhibition of monocytes in response to increasing amounts of the viral proteins (see Materials and Methods). The 50% inhibitory concentration (IC_{50}) of the T1/35kDa family for abrogating human MIP-1α activity was determined to be commensurable (6-11 nM) among the three different viral proteins (Table IV-1). To demonstrate that the observed inhibition was dependent on members of the T1/35kDa family, incubation of MIP-1α with a molar equivalent amount of SERP-1 (13), a distinct soluble myxoma virus glycoprotein with no known chemokine binding properties, did not significantly affect MIP-1α-stimulated monocyte migration (Fig. IV-5B, bottom

lane). Moreover, monocyte migration in response to fMLP and C5a, two distinct potent non-chemokine chemoattractants, was not blocked by the M-T1 or 35kDa proteins, suggesting that the inherent migratory capacity of the monocytes was not altered by the viral glycoproteins and that the inhibition of migration observed was independent of fMLP and C5a receptors (Fig. IV-5F and data not shown). To demonstrate that the inhibitory properties of the T1/35kDa family are CC chemokine specific, we also tested the ability of the poxvirus proteins to inhibit the chemoattractant activities of IL-8. Incubation of a 0.1-10-fold molar excess amount of M-T1 (Fig. IV-5D) or VV-35kDa (Fig. IV-5E) with IL-8 did not hinder the migration of human primary neutrophils in response to the CXC chemokine, which is consistent with our observations and previous reports that the 35kDa viral proteins from variola, vaccinia, and cowpox are specific inhibitors of receptor binding for CC but not CXC, chemokines (1, 24). Overall, our data showed that all members of the T1/35kDa family of proteins tested indeed display comparable activity for potently inhibiting human CC chemokines *in vitro* in a species-nonspecific fashion.

DISCUSSION

The selective activation and transmigration of reactive leukocytes mediated by chemokines are pivotal components of the early host immune response required for efficient clearance of an invading pathogen (3, 12). It is becoming increasingly evident that large double-stranded DNA viruses, such as poxviruses and herpesviruses, have evolved to encode proteins which modulate the activities of

chemokines or their receptors and thereby circumvent proinflammatory signals which promote leukocyte infiltration into virus-infected tissues (9, 11, 15, 21). Such virus-encoded chemokine modulators include mimics for both seven-transmembrane chemokine receptors and chemokine ligands, however the role of any of these genes in viral pathogenesis currently remains unclear. The most recent examples of viral chemokine regulators include soluble poxvirus chemokine binding proteins such as the myxoma virus-T7 (M-T7) glycoprotein and the T1/35kDa family of secreted proteins which are conserved among many, but not all, poxviruses (1, 7, 10, 24). Here, we purified to apparent homogeneity representative members of the T1/35kDa family from the *Leporipoxvirus* and *Orthopoxvirus* genera, namely M-T1, RPV-35kDa and VV-35kDa, and have shown that these poxviral proteins are comparably potent functional inhibitors of human CC chemokines but not CXC chemokines.

In vitro, crude preparations of M-T1 or 35kDa proteins were each capable of forming 1:1 protein complexes with members of both the CC and CXC chemokine subfamilies by chemical cross-linking, but binding inhibition and solid-phase binding analyses suggested that M-T1 interacts with CC chemokines with significantly higher affinity (Chapter III) (7). This specificity for CC chemokines has also been demonstrated by all of the other orthopoxvirus 35kDa family members tested so far (1, 24). Using calcium mobilization assays, we showed that purified M-T1, RPV-35kDa, and VV-35kDa bind and prevent human MIP-1α and MCP-1 from engaging CC chemokine receptors and inducing intracellular signalling in monocytes with comparably high affinity (K_i = 72 pM-1.02 nM). In addition, the poxvirus proteins effectively neutralize the CC chemokine activity of stimulating monocyte migration

in vitro. In contrast, neither M-T1, RPV-35kDa, nor VV-35kDa was able to prevent CXC receptor activation by IL-8 or inhibit IL-8-mediated neutrophil migration, suggesting that the biological specificity of the T1/35kDa family is targeted inhibition of the CC chemokines.

At present, the mechanism defining the specificity of CC chemokine inhibition by the soluble poxvirus chemokine binding proteins remains to be determined. Given that the T1/35kDa family of proteins are a unique class of immunomodulatory proteins with no apparent homology to any known chemokine receptors to date, they may have adopted a novel strategy for antagonizing chemokine-induced activities. Although the domains responsible for CC chemokine interactions with the M-T1 or 35kDa proteins remain to be defined, comparative functional analysis between divergent T1/35kDa family members may yield clues to their mechanism of action. Here we report that despite the significant heterogeneity in sequence identity between the leporipoxvirus T1 and orthopoxvirus 35kDa proteins, their functional activities remain essentially equivalent. This would suggest that their CC chemokine binding or inhibitory properties may occur through conserved motifs or moieties within these viral peptides. Alternatively, it is plausible that members of the T1/35kDa family adopt a uniform conformation that allows their functional inhibitory activities; however, structural studies need to be conducted to formally address this hypothesis. Importantly, the T1/35kDa family members do not exhibit the extent of species specificities observed by viroceptors from leporipoxviruses for two other cytokines, namely TNF and IFN-γ (17, 23). Thus, we predict that the inhibitory domains of CC chemokines targeted by the T1/35kDa family members to

be more conserved than the comparable domains of these latter cytokines. This conservation implies that other inhibitor molecules to this domain might exhibit a broader specificity than the CC chemokines exhibit for their cognate receptors.

CC chemokines play a primary role in the inflammatory response by inducing a wide range of biological effects on mononuclear cells, T-lymphocytes, B-lymphocytes, NK cells, dendritic cells, eosinophils, and basophils (12). Genetic evidence using homozygous MIP-1α mutant mice provides direct evidence of the antiviral role of a CC chemokine in the host immune response (5). Early during virus infection, monocytes/macrophages represent the predominant infiltrating leukocyte population into infected lesions and are major effectors for viral clearance (19). The expression of members of the T1/35kDa family during a poxvirus infection has a pronounced effect in the trafficking of reactive leukocytes into virus-infected tissues suggestive of an alteration in chemokine function *in vivo* (see Chapter III) (7, 14). Histological analysis of tissues from a rabbitpox 35kDa deletion mutant virus 3 days after infection showed a marked increase in the number of $CD43^+$ monocytes/macrophages and lymphocytes infiltrating into sites surrounding virus replication compared to a wild-type RPV infection that displayed only a sparse number of equivalent leukocytes (see Chapter III) (7). Although it is likely that the expression of M-T1 or 35kDa proteins may completely abolish all pleiotropic activities of the CC chemokines during virus infection, our evidence would suggest that at least one major biological function of the T1/35kDa protein family is to retard the activation and migration of monocytes during the early inflammatory response of the infected host. Given the fact, however, that the RPV-35kDa knockout virus is still

fully virulent for rabbits, it is possible that the increased level of infiltrating phagocytes remain poorly activated in terms of their antiviral functions. This supposition is currently being tested by knockout analysis of the M-T1 gene in myxoma virus.

MATERIALS AND METHODS

Viruses and cells. vMyxlac-T7gpt, a recombinant myxoma virus (strain Lausanne) containing a disruption in both copies of the M-T7 gene has been described elsewhere (16). Vaccinia virus (VV) (strain Lister) and rabbitpox virus (RPV) (strain Utrecht) were obtained from the American Type Culture Collection (ATCC). Myxoma and VV viruses were routinely propagated in a baby green monkey kidney (BGMK) cell line while RPV was grown in a rabbit kidney cell line, RK13 (ATCC). BGMK and RK13 cells were passaged in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% newborn calf serum (Gibco BRL), 100 units/ml penicillin (BioWhittaker Inc. and Mediatech), 100 μg/ml streptomycin (BioWhittaker Inc. and Mediatech) and 2 mM glutamine (BioWhittaker Inc. and Mediatech). Enriched populations of primary human monocytes and neutrophils were isolated from the blood of healthy donors as described elsewhere (27). Briefly, human peripheral blood was fractionated by Histopaque-1077 (Sigma Chemical Co.) density centrifugation and the resulting PBMC interface was further subjected to an iso-osmotic Percoll (Sigma Chemical Co.) gradient to yield a pure population of monocytes (>90%) as assessed by morphological criteria. Neutrophils

were isolated from Histopaque fractionated blood by 3% dextran (Sigma Chemical Co.) sedimentation followed by hypotonic lysis to eliminate red blood cells. The human monocyte leukemic cell line, THP-1 (ATCC), was routinely cultured in RPMI-1640 (Gibco BRL) supplemented with 10% fetal bovine serum, penicillin, streptomycin, 1 mM sodium pyruvate (Mediatech), 10 mM HEPES, 0.05 mM 2-mercaptoethanol, and L-glutamine at 37°C with 5% CO_2. HL-60 cells, a human monocyte leukemic cell line (ATCC), were maintained as a suspension culture in Iscove's modified Dulbecco's Medium (Gibco BRL) supplemented with 10% fetal bovine serum, penicillin, streptomycin, sodium pyruvate and L-glutamine. Expression of the IL-8 chemokine receptor type B (IL-8RB) was induced in undifferentiated HL-60 cells by culturing them in the presence of 100 nM all-*trans* retinoic acid (Sigma Chemical Co.) for 7 days as previously described (29).

Chemokines and Radiolabeling. Recombinant human chemokines, MCP-1, MIP-1α, IL-8 and GROα, and formyl-methionyl-leucyl-phenylalanine (fMLP) were purchased from commercial vendors (R&D Systems, Sigma Chemical Co.) or were generously donated by Dr. Ian Clark-Lewis (University of British Columbia, Canada). Human MIP-1α and MCP-1 were radiolabeled with ^{125}I (NEN Life Science Products) using Iodobeads (Pierce) as described in Chapter III (7).

Antibodies & Immunoblotting Analysis. To generate antisera specific for M-T1 (anti-H9MT1), a decapeptide (-LRTPTLKACN) corresponding to the C-terminal amino-acid residues 267-276 of M-T1 was synthesized by *t*-boc chemistry (Alberta Peptide Institute, University of Alberta, Canada) and used to immunize rabbits as described (4). The VV-35kDa antiserum, 18691, was a generous gift of Dr.

Arvind Patel (MRC Virology Unit, Scotland) and has been described elsewhere (20). Lyophilized gel fragments containing fractionated denatured RPV-35kDa were used to immunize rabbits and produce RPV-35kDa antisera. To detect M-T1, RPV-35kDa, and VV-35kDa proteins, an aliquot of concentrated crude supernatants from virus-infected cells or column-fractionated viral proteins containing ~250 ng of M-T1 or 35kDa were subjected to sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (SDS-12% PAGE) and transferred to HyBond-C supported nitrocellulose (Amersham Inc.) using a semi-dry transfer apparatus (Tyler Corp.) for 1 h at 50 mA. Membranes were blocked overnight in Tris-buffered saline (TBS) containing 5% skim-milk powder and 0.2% Tween-20 (ICN Biomedicals Inc.), probed with (1:10,000) anti-M-T1, (1:10,000) anti-VV-35kDa or (1:5,000) anti-RPV-35kDa antiserum for 1 h followed by a 1 h incubation with (1:5,000) horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Lab.) and visualized by enhanced chemiluminescence (Amersham Inc.). To demonstrate the specificity of the antisera against the purified proteins, parallel blots were probed with respective preimmune sera and no cross-reacting species were detected.

Purification of M-T1, VV-35kDa and RPV-35kDa. M-T1 and the 35kDa proteins were fractionated from crude supernatants from vMyxlac-T7gpt-, vaccinia virus- (strain Lister), or RPV-infected cells using fast performance liquid chromatography (FPLC). Briefly, VV and vMyxlac-T7gpt were adsorbed onto roller bottles containing 3 X 10^8 BGMK cells at a m.o.i of 2.5 PFU/cell for 3 h. The cells were washed with PBS three times and incubated with serum-free DMEM at 37°C for

12-18 h, after which the medium was harvested, centrifuged at 10,000 X *g* for 1 h to remove virus and cellular debris, concentrated 10-fold with a stirred ultrafiltration cell (AMICON) and dialyzed against low-ionic-strength buffer A (25 mM Tris buffer, pH 7.5). Then, ~5 mg of concentrated viral supernatants were loaded onto a preequilibrated Mono-Q HR5/5 (Pharmacia) anion-exchange column and washed with 20 column volumes of buffer A to remove unbound cationic proteins. Proteins were eluted from the column by increasing the counter-ion concentration from 0-600 mM NaCl in a dual-step gradient at a flow rate of 0.5 ml/min on an AKTA Purifier (Pharmacia). Aliquots of fractionated proteins were subjected to SDS-12% PAGE and visualized by immunoblotting or silverstaining (BIORAD). VV-35kDa was routinely detected in fractions containing 400-450 mM NaCl while M-T1 was consistently eluted at 500-550 mM NaCl under these conditions. To further fractionate M-T1 and VV-35kDa from slight copurifying impurities, enriched M-T1- or VV-35kDa-containing fractions were pooled and subjected to another round of Mono-Q purification. Fractions containing purified M-T1 or VV-35kDa were pooled, dialyzed in PBS overnight, concentrated 5-fold with a Centriprep (Amicon) concentrator and stored at 4°C. Then, 500 pmol of the purified myxoma virus protein was subjected to 9 cycles of automated Edman's degradation (Alberta Peptide Institute, University of Alberta, Canada) and peptide sequence analysis confirmed the N-terminal nine amino acid residues of processed M-T1.

Conditions for obtaining and purifying the RPV-35kDa protein were similar with a few differences. RPV was adsorbed onto roller bottles containing 2 x 10^8 RK13 cells at an m.o.i of 5 PFU/cell for 2 h and the cells were washed 3 times with

PBS. Serum-free virus supernatants were harvested 16 h later, clarified and concentrated as described above, and dialyzed overnight against 25 mM bis-Tris buffer, pH 6.0. Approximately 5 mg of concentrated proteins was loaded onto an equilibrated Mono-Q HR5/5 column and the bound proteins were eluted with a continuous 150-600 mM NaCl gradient at a flow rate of 1.0 ml/min. Fractions were analyzed as stated previously. RPV-35kDa eluted from the column at concentrations of 280-350 mM NaCl. Fractions containing the Mono Q-purified RPV-35kDa were pooled together, dialyzed against PBS overnight, concentrated <250 µl, and loaded onto an equilibrated Superdex 75 gel filtration column (Pharmacia) at a rate of 0.5 ml/min to remove any remaining minor contaminants. Fractions containing purified RPV-35kDa were pooled and concentrated.

Deglycosylation of M-T1. Approximately 250 ng M-T1 was deglycosylated with 0.5 units of *N*-glycosidase F (Boehringer Mannheim) essentially as described elsewhere (22), except the final reaction products were precipitated using a methanol-chloroform procedure (28) before immunoblotting.

Gel mobility shift cross-linking assay. The interactions of the M-T1 or 35kDa proteins with CC chemokines were detected using a chemical cross-linking assay as outlined in Chapter III and elsewhere (7, 26). Briefly, unfractionated or purified M-T1, RPV-35kDa, or VV-35kDa was incubated with radiolabeled chemokine for 2 h at room temperature, after which time the protein complexes were cross-linked by the addition of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Sigma Chemical Co.) to a final concentration of 40 mM for 30 min and quenched by the addition of 1/10 volume of Tris, pH 7.5. SDS loading buffer was

added to the mixtures, which were then boiled for 3 min and subjected to SDS-12% PAGE, and the resulting protein complexes were detected by autoradiography.

Calcium Flux Assays. Intracellular calcium mobilization in response to chemokine stimulation was monitored in THP-1 (for CC chemokines) and differentiated HL-60 cells (for CXC chemokines) using the fluorescent-sensitive calcium chelator, Fura-2, as previously described (8). Briefly, cells were suspended in 2 ml serum-free medium, treated with 5 μM Fura-2 AM (Molecular Probes) for 30 min in the dark at 37°C, washed 3 times with Hank's Buffered Saline Solution (HBSS) (Gibco BRL) plus 1 mg/ml BSA (Sigma) and finally resuspended at a concentration of 3 x 10^6 cells/ml. A total of 6 x 10^6 cells were transferred to a continuously stirred quartz cuvette in a RatioMaster Fluorescence Spectrometer (Photon Technology International). The emission of Fura-2 fluorescence was monitored once every second at 510 nm as a result of excitation at 340 nm (Fura-2 bound to Ca^{2+}) and 380 nm (unbound Fura-2) using Felix Version 1.2 software for Windows. Ratios of the emissions from these two excitations were automatically calculated. The peak change in the ratio following the addition of a 10 nM dose of human chemokine in the presence or absence of purified viral protein was measured and quantified as a percentage of the response obtained from subsequent stimulation with 10 μM ATP to control for variations between different cell preparations. Each assay was performed in triplicate. The overall calcium mobilization activity was calculated as (peak change in ratio due to chemokine stimulation in presence of viral protein)/(peak change in ratio due to chemokine stimulation alone). The inhibition constant, K_i, defined as the concentration of M-T1 or 35kDa protein necessary to

block 50% chemokine receptor engagement, was calculated as the *y*-intercept from the graphs plotting the relationship of $D_t(1-i)/i$ which is defined as the (concentration of M-T1 or 35kDa)(remaining activity)/inhibition against the fraction of remaining activity.

Chemotaxis Assays. Cell migration of monocytes and neutrophils were evaluated using a 48-well microchemotaxis chamber technique (6). Prior to migration assays, monocytes and neutrophils were washed and resuspended in RPMI-1640 medium containing 1 mg/ml bovine serum albumin (BSA). A 30 µl aliquot of chemoattractant (with or without indicated molar excess quantities of M-T1 or 35kDa chemokine binding proteins) diluted in RPMI-1640 medium containing 1 mg/ml BSA was placed in the lower wells and 50 µl of cell suspension (1.5×10^6 monocytes/ml or 1.0×10^6 neutrophils/ml) were placed in the upper wells of a chemotaxis chamber (Neuroprobe). The two compartments of the chamber were separated by a 5-µm pore size polycarbonate filter (Neuroprobe). After incubation for 90 min (for monocytes) or 60 min (for neutrophils) at 37°C, the filter was removed, fixed and stained with LeukoStat (Fisher Scientific) and the number of migrating cells in three high-power-fields was enumerated. Migration assays were performed in a minimum of three independent experiments and the results are expressed as the mean value (±SD) of cellular migration of triplicate samples. The percent inhibition of migration was calculated as follows: {1 - (chemokine stimulated migration in presence of viral protein - spontaneous migration/chemokine stimulated migration in absence of viral protein - spontaneous migration)} X 100. The IC_{50} of the M-T1 or 35kDa proteins

required to block CC chemokine biological activity was determined from the plots of percentage of migration inhibition versus concentration of input viral proteins.

Table IV-1. T1/35kDa Inhibition Constants for CC chemokines

	M-T1	RPV-35kDa	VV-35kDa
Inhibition constant (K_i)[a]			
MIP-1α	0.456 nM	1.021 nM	1.027 nM
MCP-1	0.188 nM	0.072 nM	0.825 nM
50% Inhibitory concentration (IC_{50})[b]			
MIP-1α	10.50 nM	6.30 nM	10.53 nM

[a] K_i values calculated from the *y*-intercept of graphs plotting percentage inhibition of calcium flux by T1/35kDa (Fig. IV-4) as defined under Materials and Methods.

[b] IC_{50} of T1/35kDa determined from the graphs of percentage inhibition chemotaxis vs. concentration of T1/35kDa (Fig. IV-5) as defined under Materials and Methods.

Figure IV-1. Purification of myxoma virus-T1, vaccinia virus-35kDa, and rabbitpox virus-35kDa proteins.*

M-T1 (A and B), VV-35kDa (C and D) and RPV-35kDa proteins (E and F) were fractionated from supernatants of virus-infected cells and analyzed by silverstaining (A, C, and E) and immunoblotting (B, D and F). Crude supernatants (lanes 1) or pooled fractions containing purified M-T1 or 35kDa proteins following Mono-Q or gel-filtration chromatography (lanes 2 and 3) were subjected to SDS-12% PAGE and visualized by silverstaining or immunoblotting using anti-M-T1 peptide antiserum (B), anti-VV-35kDa antiserum (D) or RPV-35kDa antiserum (F) as described in Materials and Methods. Purified M-T1 was deglycosylated by treatment with *N*-glycosidase F (B, lane 4) as described in Materials and Methods. SDS-molecular weight markers are indicated to the right of each panel.

*Experiments represented in Figure IV-1E were performed in collaboration with Traci L. Ness (University of Florida, Gainesville, Florida)

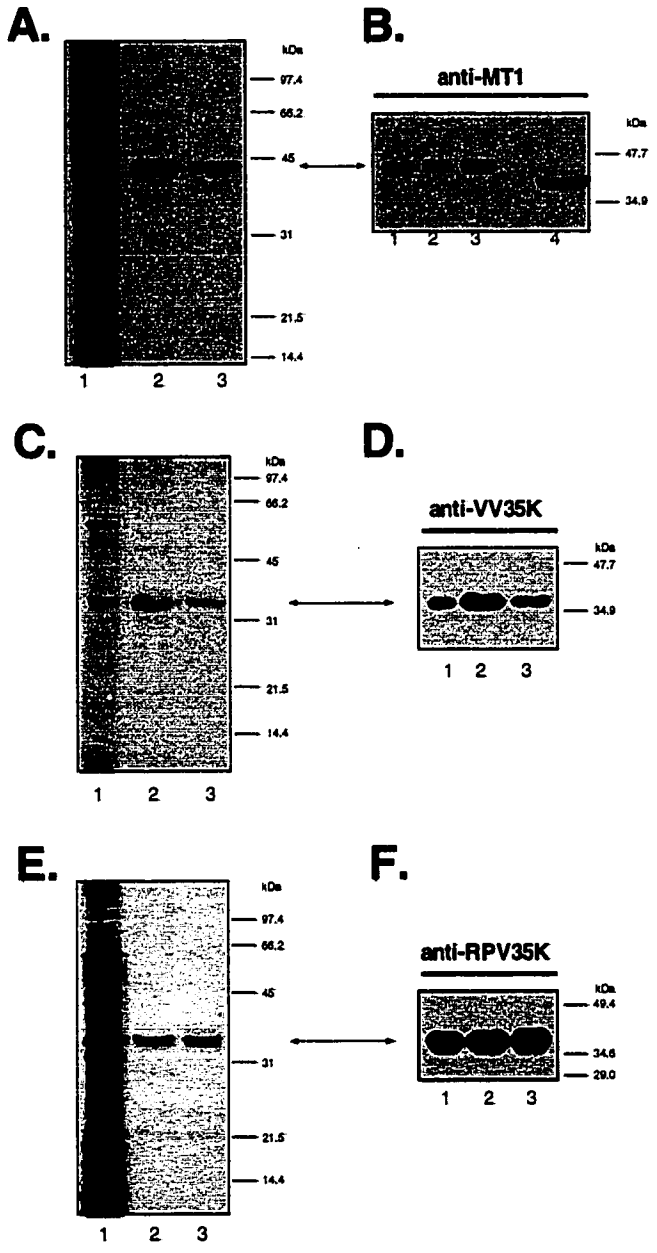
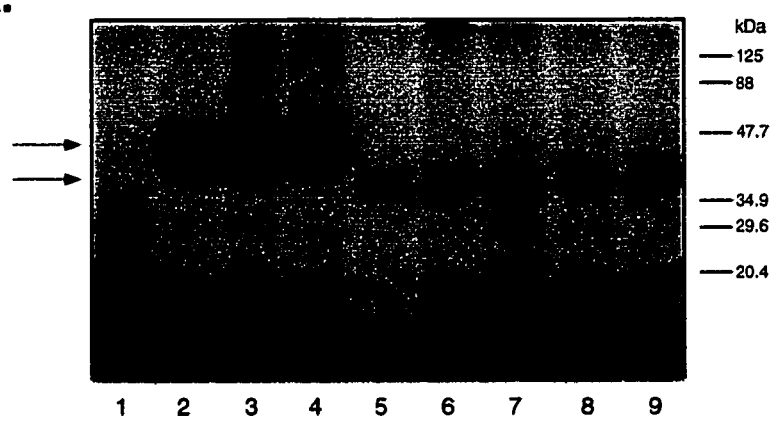

A.
B.
anti-MT1
kDa
97.4
66.2
45
31
21.5
14.4
47.7
34.9
1 2 3
1 2 3 4
C.
D.
anti-VV35K
E.
F.
anti-RPV35K
49.4
34.6
29.0

Figure IV-2. Purified M-T1, VV-35kDa, and RPV-35kDa interact with CC chemokines *in vitro*.

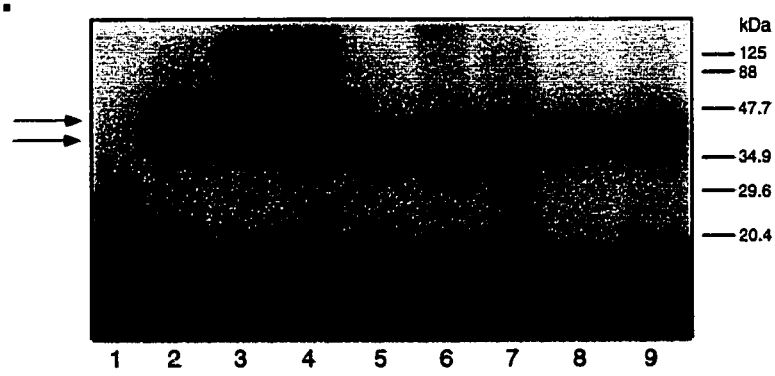
^{125}I-labeled human MIP-1α (A) or ^{125}I-labeled human MCP-1 (B) was incubated alone (lanes 1) or with myxoma virus-infected cellular supernatants (lanes 2), partially purified M-T1 (lanes 3), purified M-T1 (lanes 4), crude RPV-infected cellular supernatants (lane 5), partially purified RPV-35kDa (lane 6), purified RPV-35kDa (lane 7), crude VV (strain Lister) supernatants (lanes 8) or purified VV-35kDa (lanes 9) and cross-linked as described in Materials and Methods. The formation of M-T1- or 35kDa-chemokine-shifted complexes (arrows) was detected by autoradiography after SDS-12% PAGE. SDS-molecular weight markers are shown to the right of the panels.

A.



^{125}I MIP-1α

B.



^{125}I MCP-1

Figure IV-3. Effect of RPV-35kDa on chemokine-induced intracellular calcium mobilization.*

Chemokine was briefly preincubated (5 min) with the indicated amount of purified RPV-35kDa protein before adding it to Fura-2-loaded cells. The arrow designates the time that the chemokine with or without RPV-35kDa protein was added. Changes in calcium concentration were monitored by ratio fluorescence as described in Materials and Methods. Intracellular calcium mobilization was measured in (A) THP-1 monocytes treated with 10nM of the human CC chemokine, MIP-1α or (B) differentiated HL-60 cells treated with 10 nM of the human CXC chemokine, IL-8, in either the presence or absence of RPV-35kDa protein. The tracings shown are from a single experiment representative of three separate experiments.

*Experiments represented in Figure IV-3 were performed in collaboration with Traci L. Ness (University of Florida, Gainesville, Florida)

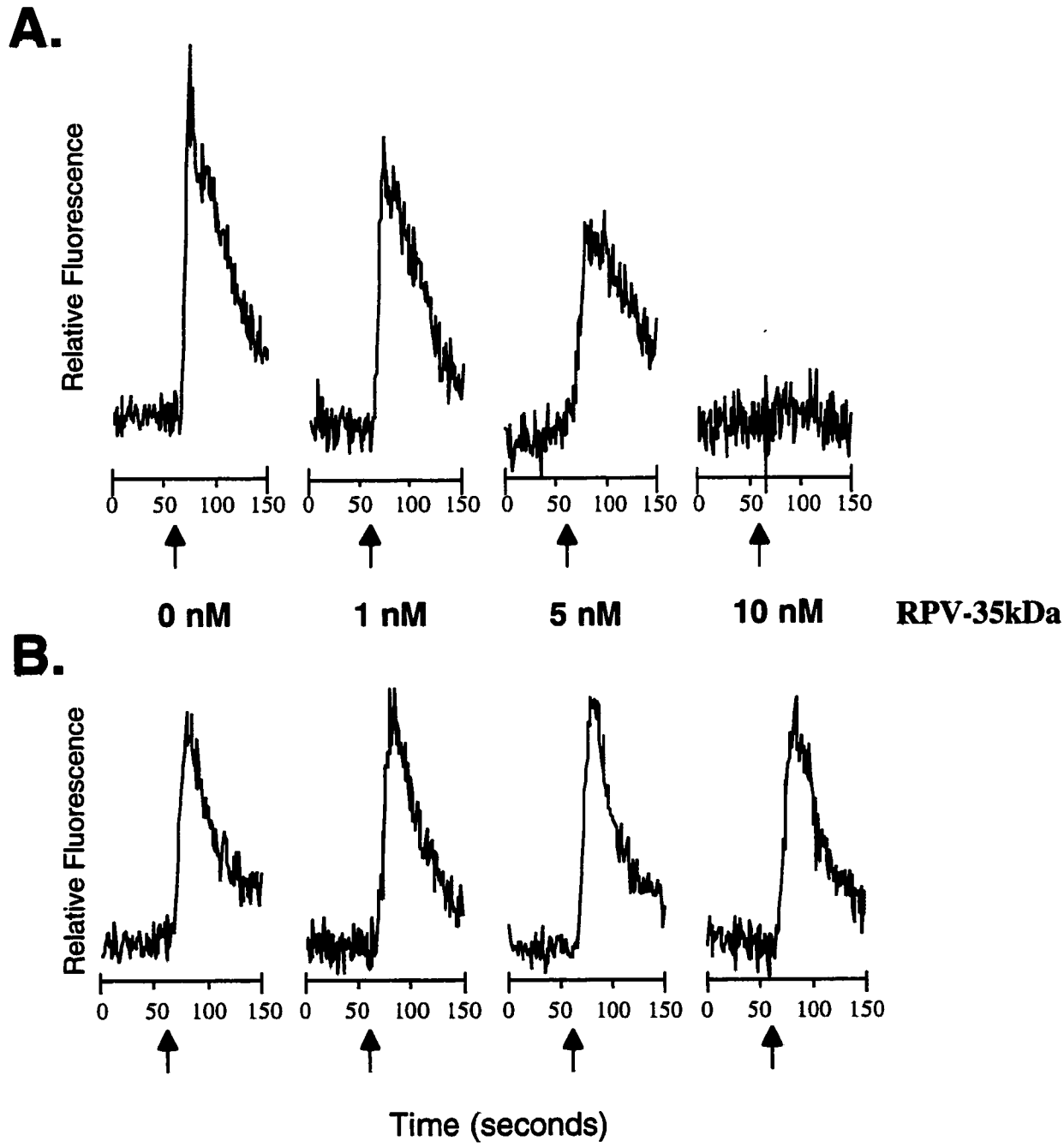
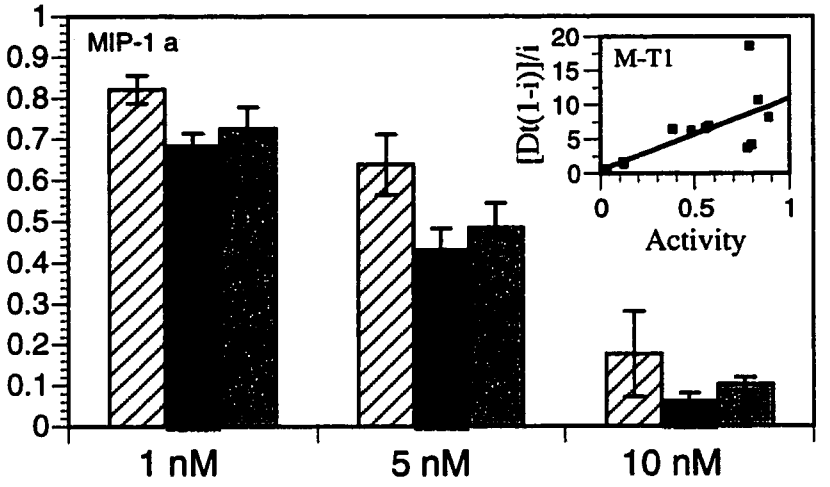

A.
Relative Fluorescence
0 50 100 150
0 50 100 150
0 50 100 150
0 50 100 150
0 nM
1 nM
5 nM
10 nM
RPV-35kDa
B.
Relative Fluorescence
0 50 100 150
0 50 100 150
0 50 100 150
0 50 100 150
Time (seconds)

Figure IV-4. Inhibition of chemokine-induced calcium mobilization by the T1/35kDa protein family.*

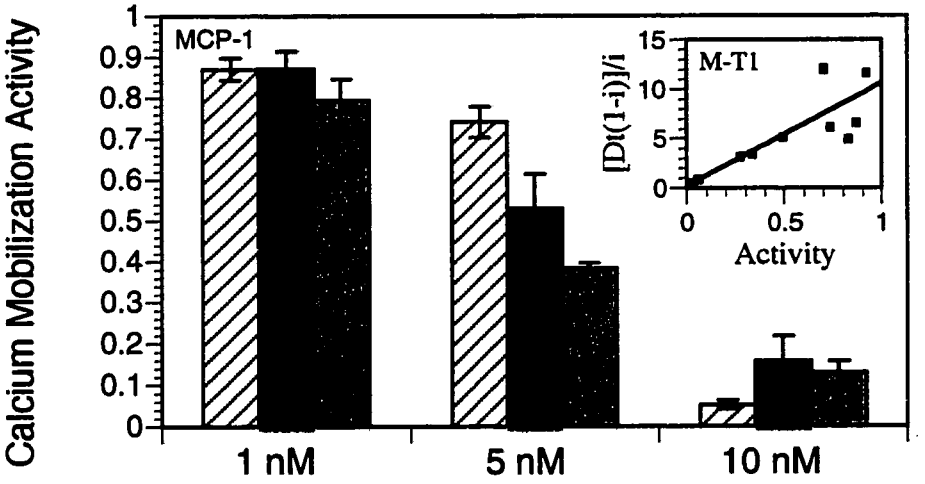
(A) Changes in the ratio of fluorescence of Fura-2-loaded THP-1 cells were monitored in response to stimulation with 10 nM MIP-1α in the presence of indicated concentrations of M-T1 or 35kDa proteins. All values were normalized to the response achieved from chemokine alone. Normalized calcium mobilization activities are shown in the presence of M-T1 (hatched bars), RPV-35kDa (black bars), or VV-35kDa (grey bars). The insets depict a representative graph used to determine the inhibition constant, K_i, for M-T1 inhibition of MIP-1α as described in Materials and Methods. (B) Calcium mobilization in THP-1 cells treated with 10 nM MCP-1 in the presence of the viral proteins. The inset shown illustrates the determination of the K_i of M-T1 against hMCP-1. (C) Differentiated HL-60 cells were loaded with Fura-2 and exposed to 10 nM IL-8 with purified M-T1 or 35kDa proteins. All of the data shown are from three separate experiments.

*Experiments represented in Figure IV-4 were performed in collaboration with Traci L. Ness (University of Florida, Gainesville, Florida)

A.


MIP-1 a
M-T1
[Dt(1-i)]/i
Activity
1 nM
5 nM
10 nM

B.


MCP-1
M-T1
[Dt(1-i)]/i
Activity
Calcium Mobilization Activity
1 nM
5 nM
10 nM

C.

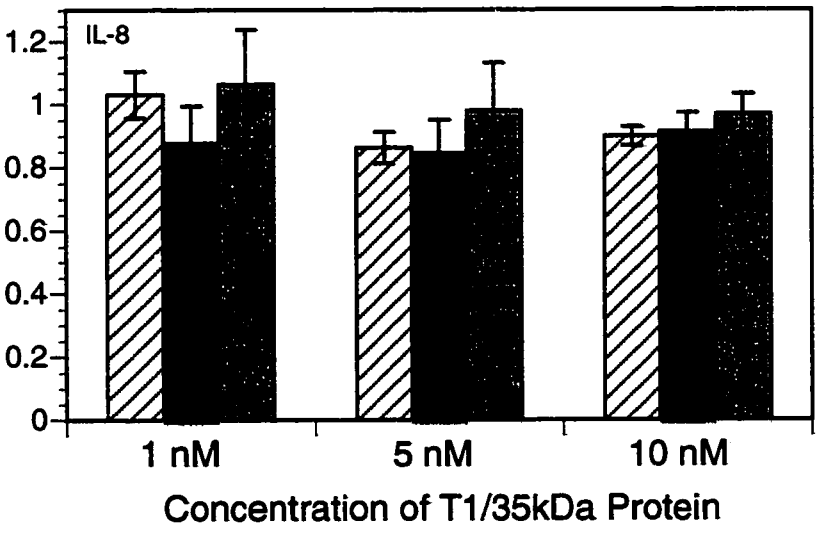
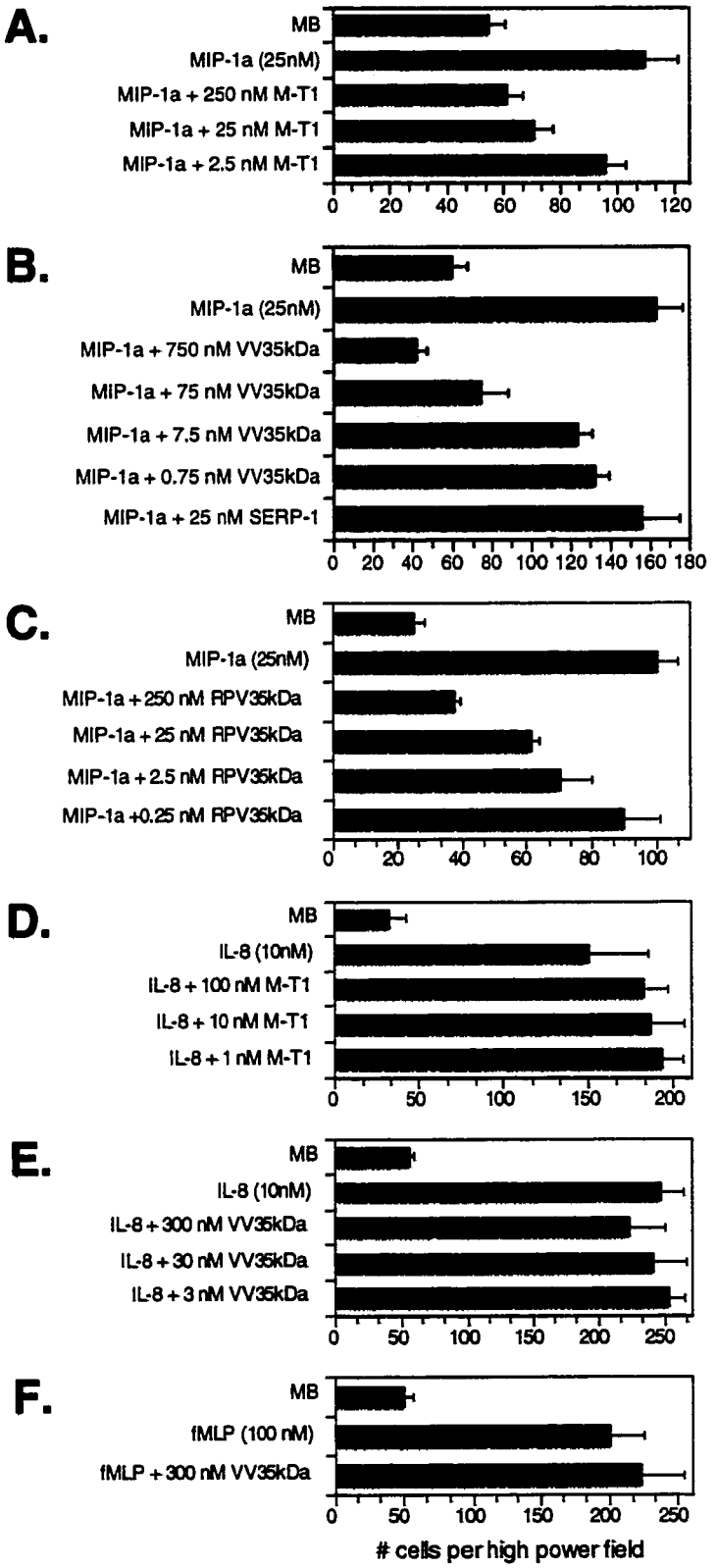

IL-8
1 nM
5 nM
10 nM
Concentration of T1/35kDa Protein

Figure IV-5. M-T1 and 35kDa proteins block monocyte migration induced by CC-chemokines.

Shown are representative migration assays performed in triplicate with primary human monocytes in response to 25 nM human MIP-1α, primary human neutrophils in response to 10 nM human IL-8, primary human monocytes in response to 100 nM fMLP, or spontaneous cell migration in response to migration buffer (MB) alone. Pre-incubation of increasing amounts of M-T1 (A), VV-35kDa (B), or RPV-35kDa (C) with MIP-1α blocked stimulated migration of primary monocytes. MIP-1α-stimulated migration was not inhibited by incubation with a control purified poxvirus secreted glycoprotein, SERP-1 (B, bottom lane). Pre-incubation of M-T1 (D) or VV-35kDa (E) with IL-8 does not block migration of primary neutrophils. VV-35kDa did not alter fMLP-mediated migration of primary monocytes (F). Migrated cells were enumerated in 3 random high-power fields by microscopy and the results expressed as the mean number of migrated cells ± SD.


A.
MB
MIP-1a (25nM)
MIP-1a + 250 nM M-T1
MIP-1a + 25 nM M-T1
MIP-1a + 2.5 nM M-T1
0 20 40 60 80 100 120
B.
MB
MIP-1a (25nM)
MIP-1a + 750 nM VV35kDa
MIP-1a + 75 nM VV35kDa
MIP-1a + 7.5 nM VV35kDa
MIP-1a + 0.75 nM VV35kDa
MIP-1a + 25 nM SERP-1
0 20 40 60 80 100 120 140 160 180
C.
MB
MIP-1a (25nM)
MIP-1a + 250 nM RPV35kDa
MIP-1a + 25 nM RPV35kDa
MIP-1a + 2.5 nM RPV35kDa
MIP-1a +0.25 nM RPV35kDa
0 20 40 60 80 100
D.
MB
IL-8 (10nM)
IL-8 + 100 nM M-T1
IL-8 + 10 nM M-T1
IL-8 + 1 nM M-T1
0 50 100 150 200
E.
MB
IL-8 (10nM)
IL-8 + 300 nM VV35kDa
IL-8 + 30 nM VV35kDa
IL-8 + 3 nM VV35kDa
0 50 100 150 200 250
F.
MB
fMLP (100 nM)
fMLP + 300 nM VV35kDa
0 50 100 150 200 250
cells per high power field

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CHAPTER V

ROLE OF THE MYXOMA VIRUS SOLUBLE CC CHEMOKINE INHIBITOR GLYCOPROTEIN, M-T1, DURING MYXOMA VIRUS PATHOGENESIS*

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INTRODUCTION

It is now evident that viruses utilize multiple mechanisms to oppose host defenses to ensure their survival within an immunocompetent host organism (15, 28, 46, 47). Poxviruses, the largest known DNA viruses, are particularly adept at subverting the host inflammatory response by expressing a collective arsenal of proteins that inhibit cytokines (5, 26, 30, 43). Many poxviruses express proteins that bear significant amino acid similarity to the extracellular ligand-binding portion of cellular cytokine receptors. These viral cytokine receptor mimics, or viroceptors, are often secreted glycoproteins that function by inhibiting competitively the cytokine ligands from binding to their cognate cellular receptors (28). To date, functional poxviral cytokine receptor homologues have been identified for receptors of tumor necrosis factors (TNF) (14, 19, 44), interferon- (IFN-)α/β (8, 49), IFN-γ (33, 52), and interleukin-1β (IL-1β) (2, 48).

Myxoma virus, a member of the *Leporipoxvirus* genus, causes a highly lethal systemic disease known as myxomatosis in the European rabbit, *Oryctolagus cuniculus* (10, 11, 27). Given the well-documented natural history and pathogenic profile of myxoma virus and its rabbit host, myxomatosis provides an excellent model for the study of virus-host interactions. The pathogenic features of myxomatosis include extensive hemorrhagic lesions at the primary site of virus inoculation that is characterized by tissue degeneration and necrosis. By 7-10 days post-infection (p.i.), the virus effectively disseminates through the host lymphoreticular system and induces numerous internal and external secondary lesions that become visibly evident

around the ears, eyelids, lips, nares and genitalia. Furthermore, severe dysfunction in cell-mediated immunity facilitates supervening Gram-negative bacterial infections of the nasal and conjunctival mucosa and respiratory tract; within 10-14 days, infected rabbits invariably experience dyspnea and succumb to death (10, 11).

Like all poxviruses, myxoma virus contains a large (~160 kbp) double-stranded DNA genome with covalently closed hairpins and terminal sequences that are inverted and repeated (29). While the central portion of most poxvirus genomes express proteins essential for virus replication and morphogenesis, genes that map toward the termini have been shown to be important for determining virus virulence and host range (32). The deletion or disruption of these virulence genes usually has no effect on virus replication *in vitro*, but can exert a demonstrable effect on influencing the pathogenic profile within an infected host. To date, the biological roles of many distinct myxoma virus genes in contributing to virus virulence in infected laboratory rabbits have been characterized (6, 13, 24, 31, 34, 35, 41, 51). For example, the M-T2 and M-T7 myxoma gene products have previously been shown to be functional soluble viral cytokine receptors that competitively inhibit the pro-inflammatory activities of TNF and IFN-γ, respectively (36, 44). Disruption of these genes alters pathogenesis and/or markedly attenuates the virus suggesting that these proteins function in disrupting the host anti-viral cytokine circuitry during infection (35, 51).

We recently showed that the first ORF from the termini of the myxoma virus genome, termed M-T1, encodes a 43 kDa secreted glycoprotein that binds to a broad range of CC chemokines with high affinity in a non-species specific fashion (see

Chapter III) (12, 18). Homologous relatives of M-T1 have also been detected in some strains of vaccinia, rabbitpox, cowpox, ectromelia, raccoonpox, Shope fibroma, camelpox and variola (smallpox) viruses (4, 12, 18, 45). This family of related secreted poxvirus 35-40 kDa glycoproteins is collectively known as the T1/35kDa family of poxvirus chemokine binding proteins. Although members of the T1/35kDa family show variable homology amongst one another, sequence analysis predict no identifiable relationship to currently known cellular proteins, including chemokine receptors (4, 12, 45). Recently, purified M-T1 protein from myxoma virus was demonstrated to block human CC chemokines such as MIP-1α and MCP-1 from binding to their cognate receptors with an inhibition constant (K_i) of ~1.0 nM (see Chapter IV) (18). Moreover, purified M-T1 potently inhibited chemokine-dependant migration of human monocytes *in vitro* (18). In contrast, neither M-T1, nor its homologous 35kDa counterparts, were capable of neutralizing CXC chemokine activity, indicating that the T1/35kDa viral proteins are specific biological inhibitors for the CC but not CXC subfamily of chemokines (4, 18, 45).

The biological role of one T1/35kDa family member has previously been examined in a rabbitpox virus (RPV) model of pathogenesis (25). RPV, a member of the *Orthopoxvirus* genus, has a broader host range and has a distinct disease phenotype to myxoma virus in infected-European rabbits. Sequence comparisons between the RPV-35kDa and myxoma virus M-T1 proteins reveal significant sequence divergence (40% amino acid identity) between the two family members (Chapter III) (12). Mice infected intranasally with an RPV 35kDa-null mutant displayed an exacerbated illness in comparison to a wild-type RPV virus infection

suggesting that this T1/35kDa family member may modulate the host inflammatory response *in vivo* (25). Detailed histological analysis of RPV-infected rabbit lesions in subsequent studies demonstrated that RPV-35kDa alters leukocyte trafficking significantly during the early phases of RPV infection (see Chapter III) (12).

Given the potent *in vitro* CC chemokine inhibitory properties of the T1/35kDa family members demonstrated in Chapter IV, we investigated the biological role of the M-T1 glycoprotein during myxoma virus pathogenesis in the European rabbit. European rabbits were infected with a recombinant myxoma M-T1-deletion mutant virus and assessed for differences in pathological profiles. Here, we demonstrate that M-T1 markedly influences the migration of inflammatory cells, particularly macrophages, into virus-infected tissues during the initial phases of infection. However, the increased number of phagocytes are relatively ineffective at clearing the M-T1 deletion mutant virus, suggesting that other myxoma viral proteins are able to neutralize the antiviral activities of the infiltrating macrophages.

RESULTS

Kinetics of M-T1 Expression

Previous transcriptional analysis of homologous M-T1 genes in other poxviruses, namely S-T1 of Shope fibroma virus and 35kDa of vaccinia virus (strain Lister), have demonstrated that at least these two members of the T1/35kDa family are transcribed at both early and late times (22, 42). To determine the kinetics of M-T1 expression during high multiplicity (m.o.i.=10) myxoma virus infection, we

immunoblotted myxoma virus-infected cellular supernatants that were harvested at various times p.i.. As shown in Figure V-1A, M-T1 protein was detected in the medium of infected cells as early as 2-4 h p.i. and accumulated increasingly during the late times of virus replication. By 12 h, greater than 1 x 10^7 molecules/cell of M-T1 were routinely observed in the medium of infected cultures (Table V-1). Analysis of the levels of accumulated proteins during infection demonstrated no significant reduction in the amounts of soluble M-T1 even after 24 h p.i., indicating that the secreted M-T1 polypeptide accumulates as a highly stable ~40-45 kDa species (Fig. V-1A and Table V-1). To determine if the mature M-T1 glycoprotein was secreted continuously during the course of infection, infected cell cultures were washed thoroughly at various times, replaced with fresh medium at 4 h intervals and analyzed for M-T1 expression throughout the virus replication cycle. Processed M-T1 was observed to be continuously secreted from infected cells at comparable rates (~1 x 10^6 molecules/cell/h) both at early and at late times of infection with maximal expression (6.67 x 10^6 molecules/cell) occurring between 4-8 h p.i. (Fig. V-1B and Table V-1). In addition, soluble M-T1 detected both at early and late times were equally efficacious in binding to CC chemokines (data not shown). Thus, biologically active and significant amounts of soluble M-T1 appear to be continuously secreted through the course of myxoma virus infection, and accumulate in the extracellular environment as a highly stable 40-45 kDa species.

Construction and Analysis of a Recombinant M-T1-Deletion Mutant Virus

To ascertain the biological role of M-T1 in viral virulence, we constructed a recombinant M-T1-deletion mutant myxoma virus, vMyxlacT1^{-}, that failed to express M-T1. Since the genomic location of the M-T1 ORF is within the terminal inverted repeats, it is present as duplicate copies in the myxoma virus genome. Both copies of the M-T1 ORF were deleted and replaced with a dominant selectable marker gene, *E. coli* guanosine phosphoribosyl transferase (Ecogpt), using a similar homologous recombination procedure between the parental virus (vMyxlac) and an engineered transfer vector as outlined elsewhere (6, 24, 35). A construct bearing flanking sequences of M-T1 separated by Ecogpt, pKS-T1KO (Fig. V-2 and Materials and Methods), was transfected into vMyxlac-infected cells and individual foci surviving three rounds of passage in mycophenolic acid selection were harvested. Plaque-purified viral isolates were propagated further and analyzed by PCR and Western blotting. As a control, a recombinant M-T1 revertant virus, vMyxT1R, in which the intact M-T1 ORF was restored fully into its original loci, was constructed using a similar genetic approach.

To verify the deletion of M-T1, viral DNA harvested from plaque-purified isolates was subjected to PCR analysis using 5' and 3' primers that hybridize to specific M-T1 flanking sequences. Deletion/disruption of M-T1 by Ecogpt insertion can be easily visualized by size discrimination of the PCR products under agarose gel electrophoresis and the presence of any contaminating isolates bearing wild-type M-T1 sequences can be readily identified. As shown in Figure V-3A, whereas the wild-type M-T1 sequence produced an amplified product of ~890 bp (lanes 1 and 3), the integration of Ecogpt within the disrupted M-T1 sequence yielded a PCR product of

~1000 bp (lane 2). In addition to an isolate containing a mixed pool of both wild-type and vMyxlacT1$^-$ recombinants (Fig. V-3A, lane 4), one isolate comprising a pure population of vMyxlacT1$^-$ recombinants (Fig. V-3A, lane 5) was identified and chosen for further characterization. PCR analysis from a vMyxT1R viral recombinant demonstrated only the reverted wild-type M-T1 sequence (Fig. V-3A, lane 6).

To verify whether the vMyxlacT1$^-$ recombinant virus was defective in M-T1 expression, secreted proteins from virus-infected cells were examined by Western blotting using specific anti-M-T1 antisera. Whereas the 43kDa secreted M-T1 glycoprotein was readily detected in the supernatants of cells infected with the parental virus (Fig. V-3B, lane 2) or vMyxT1R recombinant (Fig. V-3B, lane 4), M-T1 was completely absent from the medium of vMyxlacT1$^-$-infected (Fig. V-3B, lane 3) or mock-infected (Fig. V-3B, lane 1) cultures. Similarly, lysates from vMyxlacT1$^-$-infected cells were devoid of intracellular M-T1. Furthermore, several other distinct myxoma soluble glycoproteins, namely SERP-1 and M-T7, were expressed from vMyxlacT1$^-$ and vMyxlac in comparable amounts indicating that the deletion of M-T1 did not alter the expression of additional myxoma soluble proteins (data not shown). Taken together, these data confirm that vMyxlacT1$^-$ is a pure recombinant myxoma M-T1 deletion mutant virus that is incapable of M-T1 expression.

M-T1 is Non-Essential for Replication *In Vitro*

Previous studies demonstrated that virulence factors from poxviruses are typically non-essential for growth in tissue culture. We characterized the growth

characteristics of vMyxlacT1$^-$ *in vitro* in several susceptible rabbit and primate cell lines. Single-step growth curve analyses demonstrated no defects in the ability of vMyxlacT1$^-$ to replicate in cultured primate BGMK (Fig. V-4A) or rabbit RK-13 (Fig. V-4B) fibroblast cells compared with the parental vMyxlac virus. The productive infection of rabbit lymphocytes is thought to be crucial for efficient dissemination of the virus via the host afferent lymphatic channels *in vivo*. vMyxlacT1$^-$ was observed to productively infect a rabbit T-lymphocyte cell line, RL-5, with no observable defects in replication or in causing premature cell death of infected cells (data not shown). Thus, vMyxlacT1$^-$ appears to be dispensable for virus replication in cultured cells *in vitro*.

Pathogenesis of vMyxlacT1$^-$-Infected European Rabbits

To examine the biological role of M-T1 in virus virulence, European rabbits were infected with vMyxlacT1$^-$ and monitored for any changes in the clinical manifestations of myxomatosis. As summarized in Table V-2, rabbits infected with either the parental vMyxlac or recombinant vMyxT1R viruses were indistinguishable and developed the classical symptoms of myxomatosis, including the development of fulminating lesions at the primary site of inoculation, multiple secondary lesions, blepharoconjunctivitis and supervening bacterial infections. By Day 10-14, all vMyxlac- and vMyxT1R-infected rabbits were sacrificed due to the increased severity of the disease. Surprisingly, vMyxlacT1$^-$-infected rabbits also experienced a similar pathogenic profile including the development of primary and secondary lesions, opportunistic bacterial infections, and five out of six (>80%) rabbits became

moribund and required euthanasia (Table V-2). However, one striking phenotypic difference was an augmented inflammation of the primary lesions from the vMyxlacT1^{-}-infected rabbits during the early stages of the disease. By 2-3 days p.i., the primary lesions at the initial site of vMyxlac inoculation were barely discernible (Table V-2). In contrast, the primary lesions of rabbits infected with the deletion-mutant virus were significantly larger, and displayed heightened inflammation (reddening and swelling) and a notable surrounding edematous zone (Table V-2). Although the elevated size and inflammation of the vMyxlacT1^{-}-lesions were morphologically distinct from the vMyxlac-lesions between 1 and 3 days p.i., the primary nodules became indistinguishable by 7 days after infection. In general, the vMyxlacT1^{-}-infected rabbits also experienced more edema, conjunctivitis, and a slightly accelerated development of bacterial infections; however, given the variability of symptomology between rabbits, we were not able to assess these differences as significant. Thus, the absence of the M-T1 viral chemokine binding protein resulted in an increased localized inflammatory response at the primary inoculation site during the early phases of the infection, but had no major significant effects in attenuating the progression of disease or in the mortality of infected European rabbits.

Histological Analysis of vMyxlacT1^{-}-Infected Tissues

Gross pathological examinations showed that the primary vMyxlacT1^{-}-tissue lesions exhibited greater inflammation and were considerably larger than their wild-type virus-infected counterparts during the first 3 days of infection. Given these

notable differences in the early inflammatory response at the primary site, tissue samples were resected at 2, 3, and 7 days p.i. for complete histological analysis. Microscopically, the epidermis of the vMyxlacT1$^-$ primary sites showed more reactivity and hyperplasia than vMyxlac tissues indicative of an intense inflammatory response occurring at 2-3 days p.i. (data not shown). Stained tissue sections revealed striking differences in cellular infiltrates within the deep dermis of the primary lesions between the two sets of rabbits (Fig. V-5). By 2 and 3 days p.i., a mild focal inflammatory response with a sparse number of infiltrating leukocytes were observed in the dermal tissues of vMyxlac primary sites (Figs. V-5A and V-5C). In contrast, a intense widespread inflammatory reaction with a marked increase in heterophil and mononuclear rabbit leukocytes was observed in the deep dermal layer of vMyxlacT1$^-$ primary tissues (Figs. V-5B and V-5D). To determine if M-T1 was modulating the influx of leukocytes within infected tissues, rabbit leukocytes permeating the dermal tissues were enumerated. In the absence of M-T1 expression, we observed an approximate three- to four-fold increase in the number of leukocytes migrating deeply into the primary tissues (Fig. V-6A), suggesting that M-T1 alters the trafficking of inflammatory cells *in vivo* during the early phases of myxoma virus infection.

M-T1 Blocks Monocyte/Macrophage Influx *in vivo*

We previously demonstrated that M-T1 potently inhibits CC chemokines from inducing the chemotactic migration of primary monocytes *in vitro* (18). We predicted, therefore, that M-T1 may also abrogate CC chemokine-mediated migration of inflammatory effectors belonging to the monocyte/macrophage cell lineage during

virus infection *in vivo*. The discrimination of rabbit leukocyte sub-classes by surface marker expression is poorly defined and few reagents currently exist for optimal immunohistochemical analysis of rabbit tissues. To characterize the effects of M-T1 on monocytic influx during myxoma virus infection, tissue sections were immunostained with RAM11, a murine monoclonal antibody that is specific to rabbit monocytes/macrophages (20, 50). As shown in Figure V-6B, there was a significant elevation in the percentage of infiltrating rabbit monocytes/macrophages into the dermal tissues early (2-3 days) during vMyxlacT1$^-$ infection. We were unable to accurately assess the differences in RAM11-positive leukocytes in the Day 7-tissues due to widespread tissue necrosis, or determine which additional leukocyte subpopulations may have altered migration patterns by vMyxlacT1$^-$. Nevertheless, our data suggest that the expression of the M-T1 glycoprotein has profound effects on influencing the effective migration of rabbit inflammatory leukocytes, such as monocytes/macrophages, during viral infection *in vivo*, consistent with the biochemical property of M-T1 as a potent CC chemokine inhibitor *in vitro*.

Viral Burden in Infected Lesions

Macrophages play a central role in the early response to virus infection by facilitating intrinsic resistance mechanisms and generating secreted antiviral cytokines (38). Since myxoma virus expresses a variety of proteins that target host inflammatory cells, we examined tissues from primary lesions to determine if the increased level of macrophages observed during vMyxlacT1$^-$ infection had any effects on virus clearance. Since the parental vMyxlac and mutant vMyxlacT1$^-$

viruses both express an *E. coli* lacZ transgene, localization of either virus within infected-tissues can be readily identified by staining sections with an antibody against β-galactosidase. No significant qualitative differences in the level of β-galactosidase staining, as a marker for viral gene expression, were observed in the primary or secondary dermal tissues from vMyxlac- or vMyxlacT1$^-$-infected rabbits at 3 days p.i. (Fig. V-7). Thus, although an increased number of infiltrating phagocytes were present in vMyxlacT1$^-$-infected primary tissues at 3 days p.i., the viral burden of both the mutant and the wild-type myxoma viruses remained at comparable levels. We conclude that despite the increased levels of infiltrating macrophages in lesions infected with vMyxlacT1$^-$, the effective antiviral activities of these responding phagocytes were likely compromised by the summated activities of other anti-inflammatory myxoma viral proteins.

DISCUSSION

A major strategy used by poxviruses in thwarting the host-inflammatory response to virus infection involves the sequestration of crucial host biological response modifiers such as key cytokines critical for orchestrating the anti-viral responses (5, 7, 47). A growing number of secreted poxvirus glycoproteins including vIFNα/β-receptors, vIFNγ-receptors, vIL-1β-receptors, and vTNF-receptors, have been identified with activities aimed at disrupting host cytokine networks (29). In addition to being capable of effectively neutralizing the anti-viral activities of their respective cytokines *in vitro*, many of these viroceptors have been shown to function

as anti-inflammatory virulence factors since their deletion alters the pathogenesis of their infected hosts *in vivo* (30).

The coordinated recruitment of immune cells to sites of virus infection is a key feature of the early host inflammatory response to pathogenic insult (38). Chemokines are believed to play a critical role in this process by selectively activating and mobilizing monocytes, T-lymphocytes, dendritic cells, natural killer cells and granulocytes to sites of infection and damage (21). As a consequence of the important roles that chemokines play in orchestrating inflammatory processes, certain viruses have devised a number of countermeasures for neutralizing the activities of chemokines or their receptors (17, 37, 39, 53). There are currently three strategies of chemokine subversion known to be used by viruses: 1) virus-encoded chemokine ligand homologues that function as antagonists 2) virus-encoded cell-surface chemokine receptor homologues and 3) virus-encoded secreted chemokine binding proteins such as the myxoma virus M-T1 glycoprotein. However, the significance of many of these viral proteins in contributing to viral pathogenesis or in abrogating the activities of chemokines *in vivo* has not been fully addressed.

We previously showed that the myxoma virus M-T1 glycoprotein binds to a broad-spectrum of CC chemokines with high affinity and effectively sequesters soluble chemokine ligands from engaging their cell-surface receptors (see Chapters III and IV) (12, 18). In addition, using *in vitro* chemotaxis assays, M-T1 was shown to be capable of blocking MIP-1α and MCP-1 from stimulating the unidirectional migration of primary human monocytes, suggesting that M-T1 may play a fundamental biological role in myxoma virus pathogenesis by functionally retarding

the proper trafficking of rabbit phagocytes *in vivo* (18). Here, we demonstrate that the expression of M-T1 during myxoma virus infection *in vivo* results in marked perturbations of the extent and amount of leukocyte infiltration into virus-infected tissues although these additional phagocytes exert only minimal effects on virus-induced mortality.

Our present study demonstrates that the myxoma M-T1 glycoprotein is secreted continuously during early and late times of virus infection and is non-essential for virus replication *in vitro*. Given the potent *in vitro* CC chemokine inhibitory properties of M-T1 demonstrated in Chapter IV, we were surprised to discover that the deletion of M-T1 from myxoma virus does not significantly reduce the lethality of the myxomatosis disease progression in infected European rabbits. Rather, M-T1 was shown to have demonstrable alterations in localized inflammation surrounding the primary sites of infection but the increased numbers of infiltrating phagocytes were still relatively ineffective at virus clearance. Rabbits infected with an M-T1-null mutant experienced a notable elevation of inflammation at the primary lesions in comparison to an infection with the parental virus during the early phases of infection. The heightened inflammatory response observed during gross examinations of vMyxlacT1^{-}-infected rabbits was also confirmed by histological analysis. Deletion of M-T1 resulted in an increase in the extravasation of rabbit monocytes/macrophages, and perhaps additional leukocytes, into infected dermal tissues. Thus, M-T1 appears to play a significant role in altering the directional migratory signals that are required for influx of inflammatory cells during the acute-

phase response to myxoma virus infection, as predicted by the *in vitro* activities of all the T1/35kDa family members as potent CC chemokine inhibitors (4, 18, 45).

Our results are in agreement with prior investigations of a distinct M-T1 family member, namely RPV-35kDa, in a RPV model of pathogenesis (25). The deletion of 35kDa ORF from RPV resulted in a modest acceleration of illness in infected Balb/c mice and no overall difference in the pathogenesis of infected European rabbits (12, 25). However, histological analysis in subsequent studies revealed dramatic alterations of lymphocyte influx in primary rabbit tissues during early phases of infection (12, 25). Another example of the difficulty of using morbidity as an end-point measurement for poxvirus virulence is the case of deleting the vIL-1β receptor of vaccinia virus that resulted in an accelerated pathogenicity and morbidity following intranasal inoculation, but attentuation, following intracerebral injection (3, 48). Thus, it has been proposed that certain viroceptors may play a role in protecting the host from the detrimental effects caused by the excessive production of certain cytokines in response to infection (1). In this respect, it is entirely plausible that M-T1 may function in moderating the level of the host acute-phase response in order to facilitate viral replication and spread during the early phases of myxoma virus infection.

The elevated levels of infiltrating phagocytes observed in vMyxlacT1^{-}-tissues did not appear sufficient to resolve virus infection since the detectable viral burden was essentially identical between vMyxlac- and vMyxlacT1^{-}-infected tissues. It is possible that the infiltrating phagocytes during vMyxlacT1^{-} infection remain poorly activated and unable to clear virus infection, and at least one of the other known

secreted myxoma virulence factors, SERP-1, is known to function by directly inhibiting inflammatory cells (20, 24). Thus, although M-T1 may effectively suppress the chemoattraction of inflammatory effector cells early during the course of infection, the consolidated activities of additional myxoma virus factors likely contribute to preventing these increased numbers of phagocytes from blocking the development of full-scale myxomatosis pathogenesis.

In addition to M-T1, myxoma virus also expresses a second distinct soluble glycoprotein, M-T7, which has been demonstrated to bind chemokines *in vitro* and influence leukocyte influx during myxoma virus infection (see Chapter II) (16, 35). However, unlike M-T1 which prevents chemokine-receptor interactions, M-T7 has been postulated to function in a distinct manner by disrupting the establishment of solid-phase chemokine gradients along the extracellular matrix (16, 17). In addition, M-T1 modulates cellular influx significantly earlier than the inhibition of cellular infiltration observed previously with M-T7 (35), suggesting that these chemokine binding proteins are functionally non-redundant during myxoma virus infection

MATERIALS AND METHODS

Cells and Viruses. vMyxlac, a myxoma virus (strain Lausanne) derivative bearing a β-galactosidase marker cassette in an intergenic location, was used as the parental wild-type strain in this study and has been described previously (40). The construction of both recombinant myxoma M-T1-deletion mutant (vMyxlacT1^{-}) and revertant (vMyxT1R) viruses are described below. All viruses were propagated in

African baby green monkey kidney cells (BGMK) (ATCC). BGMK cells were passaged in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% newborn calf serum (Gibco BRL), 100 units/ml penicillin (BioWhittaker Inc.), 100 µg/ml streptomycin (BioWhittaker Inc.) and 2 mM glutamine (BioWhittaker Inc.). RK-13 cells, a rabbit kidney cell line (ATCC), were maintained under similar conditions.

Construction of Recombinant vMyxlacT1$^-$ and vMyxT1R Viruses. To create the myxoma M-T1-deletion mutant virus, vMyxlacT1$^-$, both copies of the M-T1 ORF were deleted/disrupted by insertion with a dominant selectable marker as shown by the schematic in Fig. V-2. A pBluescript vector containing the entire M-T1 gene, pBS-M-T1 (12), was restriction digested with *Pst*I and *Bam*HI to remove a majority of the M-T1 coding sequence creating the vector pKS-5'T1. A fragment containing a small 3' flanking portion of M-T1 was amplified from pBS-M-T1 by PCR using a 3' M-T1 primer containing an engineered *Pst*I site, T1-3'Pst (CTACCCTGCAGGCGTGTAAC), and a forward primer corresponding to a downstream vector sequence. The amplified products were cloned into a pT7Blue T-vector (Novagen) and a 192 bp *Pst1-Bam*HI fragment was excised and ligated into pKS-5'T1, creating the plasmid pKS-T1del. In addition to flanking sequences, the construct contained the 5' 64 bp and 3' 20 bp coding sequences of M-T1 separated by an internal *Pst*I site. A *Pst*I cassette containing the *E. coli* guanosine phosphoribosyl transferase (Ecogpt) gene whose expression is driven by the vaccinia 7.5K promoter (9) was inserted into pKS-T1del, creating pKS-T1KO.

vMyxlacT1$^-$virus was generated by homologous recombination with the pKS-T1KO plasmid using a vMyxlac infection/transfection procedure utilizing mycophenolic acid selection (9) as previously described (34, 35). To verify the disruption of M-T1 and insertion of the Ecogpt cassette, primers flanking the 5' (5'T1-ATAGAAGGATCCTATCATGAAAC) and 3' (3'T1-CGACATGGATCCTAGATAGGATTA) sequences of M-T1 were generated and PCR analysis was performed. vMyxlacT1$^-$ was subsequently used along with the plasmid pBS-M-T1 to create a myxoma M-T1 revertant virus, vMyxT1R, in which the intact M-T1 gene was restored fully into its original loci by a similar procedure described elsewhere (23, 34).

Immunoblotting Analysis. Soluble M-T1 was detected from virus-infected cellular supernatants using Western blotting analysis as described in Chapter IV (18). Briefly, BGMK cells were infected with vMyxlac, vMyxlacT1$^-$, or vMyxT1R at a m.o.i. of 10. After 1 h, cells were washed thoroughly with phosphate-buffered saline (PBS) to remove unabsorbed virus and cultured overnight (or for various times as indicated) with fresh serum-free media. Supernatants were then harvested, clarified of virus and cellular debris, and concentrated 10-fold using spin micro-concentrators (Millipore). Concentrated supernatants were resolved by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (SDS-12% PAGE) and transferred to HyBond-C supported nitrocellulose (Amersham Inc.) using a semi-dry transfer apparatus (Tyler Corp.) for 1 h at 50 mA. Membranes were blocked overnight in Tris-buffered saline (TBS) containing 5% skim-milk powder and 0.2% Tween-20 (ICN Biomedicals Inc.), and then probed with (1:5,000 dilution) polyclonal anti-M-T1 peptide antisera

(Chapter IV) for 1 h. Then, blots were washed with TBS-0.2%Tween-20, re-probed with (1:5,000 dilution) horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Jackson Laboratories), and subsequently visualized by enhanced chemiluminescence (NEN Life Sciences).

Quantitation of Secreted M-T1 from Infected Cells. Supernatants containing secreted myxoma virus proteins were harvested at various times p.i. and immunoblotted by a procedure similar to that described above. To calculate the amount of soluble M-T1 produced during myxoma virus infection, chemiluminescent M-T1 bands were detected using a FX Molecular Imaging System (Bio-Rad) and quantified using purified M-T1 protein standards.

Single-step Growth Curve Analysis. Six-well multidishes containing 5×10^5 BGMK or RK-13 cells were infected with vMyxlac or vMyxlacT1$^-$ at an m.o.i. of five for 1 h at 37°C. After viral absorption, the cells were washed three times with PBS and incubated with DMEM-containing serum. Infected cultures were harvested at 0, 4, 8, 24, and 48 h p.i., and viral titers were determined on BGMK cells as previously outlined (34). All infections were performed in triplicate and results expressed as the average viral titers (log plaque forming units (PFU)/10^5 cells) ± SD.

Pathogenesis of Virus-Infected Rabbits. Adult female, New Zealand White rabbits (*Oryctolagus cuniculus*) were purchased from a local supplier and housed within a Biohazard Level 3 containment facility in compliance with guidelines of the Canadian Council on Animal Care. Rabbits were injected intradermally with 1000 PFU of virus below each thigh. Virus titers were verified prior to experimentation to ensure the accuracy of the dosage inoculum. For gross pathology studies, six rabbits

were inoculated with vMyxlacT1$^-$(M-T1-deletion mutant virus); three rabbits with vMyxlac (parental wild-type strain); and three with vMyxT1R (recombinant M-T1 revertant virus). The infected rabbits were observed daily for clinical symptoms of myxomatosis and any rabbits that appeared moribund were promptly euthanized using an intravenous overdose of pentobarbital. For histological studies, six rabbits were each inoculated with vMyxlac or vMyxlacT1$^-$ as described above. Two rabbits from each group were sacrificed at 48 h, 72 h, and 7 days p.i., and subjected to a complete post-mortem examination. Tissue sections were harvested following necropsy and stored in neutral-buffered 10% formalin prior to histological analysis (discussed below).

Immunohistological Analysis. Infected rabbit tissue samples were paraffin-embedded, and 5-μm sections were stained with hematoxylin and eosin (H&E) for microscopic analysis. To ascertain specific infiltrating leukocyte sub-populations within infected lesions, sections were further analyzed by immunohistochemical staining as described elsewhere (35). To detect rabbit monocyte/macrophages infiltrates, samples were stained with a rabbit macrophage-specific monoclonal antibody, RAM11 (a kind gift from Dr. E. Raines, University of Washington), at a dilution of 1:500 (50). Positive-stained (RAM11) rabbit monocytes/macrophages were enumerated from two independent sets of blinded rabbit sections per time point by microscopy using three random high-power-fields and the results expressed as the percentage of infiltrating RAM11+ macrophages per total number of leukocytes per field in the deep dermal tissue. To evaluate the presence of the virus within infected tissues, samples were immunostained with

(1:500 dilution) anti-β-galactosidase antibody (Promega) to detect the presence of the *E. coli lacZ* gene product expressed from vMyxlac and vMyxlacT1$^-$ (35).

Table V-1. Quantification of Secreted Myxoma Virus M-T1 Glycoprotein†

	Time (Post Infection)	Total M-T1 secreted		
	hr	*μg / 10⁶ cells*	*molecules / cell*	*molecules / cell / hr*
Cumulative	4	0.19	2.70×10^6	0.66×10^6
	8	0.65	9.12×10^6	1.14×10^6
	12	1.00	1.39×10^7	1.16×10^6
	18	1.06	1.48×10^7	0.82×10^6
	24	1.40	1.97×10^7	0.82×10^6
Pulse-Chase	0-4	0.27	3.84×10^6	0.96×10^6
	4-8	0.48	6.67×10^6	1.67×10^6
	8-12	0.24	3.43×10^6	0.86×10^6
	12-18	0.28	3.93×10^6	0.65×10^6
	18-24	0.30	4.19×10^6	0.70×10^6

†Tabulation of M-T1 levels from myxoma virus-infected BGMK cells at various times post-infection was determined according to the procedures outlined in Materials and Methods.

Table V-2. Pathogenicity of vMyxlac-, vMyxT1R-, and vMyxlacT1$^-$-Infected European Rabbits

Day	vMyxlac (wild-type) and vMyxT1R (revertant)	vMyxlacT1$^-$ (knockout virus)
0	• Six NZW rabbits inoculated with 1000 pfu intradermally	• Six NZW rabbits inoculated with 1000 pfu intradermally
2 - 3	• Small (0.5 - 1 cm), slightly inflamed primary lesions	• Larger (1 - 2 cm) very inflamed primary lesions; edema of face and eyes; mild conjunctivitis
4 - 7	• Large (2 - 2.5 cm) protuberant demarcated primary lesions with necrotic centers; few secondary lesions; mild edema, conjunctivitis and rhinitis	• Large (2.5 cm) protuberant demarcated primary lesions with necrotic centers; multiple secondary lesions; moderate edema, conjunctivitis and rhinitis
7 - 12	• Multiple secondary lesions; severe supervening bacterial infections (mucopurulent discharge of eyes/nares); extreme conjunctivitis, edema and rhinitis; acute dyspnea	• Multiple secondary lesions; severe supervening bacterial infections (mucopurulent discharge of eyes/nares); extreme conjunctivitis, edema and rhinitis; acute dyspnea 1 rabbit with resolving infection
10 - 14	• All rabbits sacrificed due to disease severity	• Five of six rabbits sacrificed due to disease severity
14 - 21	———	• Complete recovery of remaining rabbit

Figure V-1. Immunoblotting analysis of M-T1 secretion from myxoma virus-infected cells.

(A) Accumulation of secreted M-T1 glycoprotein from supernatants of vMyxlac-infected BGMK cells harvested at various times post-infection and detected with an anti-M-T1 antibody. (B) Infected cells were washed at 4-h intervals and replaced with fresh serum-free medium. Newly synthesized soluble M-T1 in the medium of infected cells during the indicated times post-infection was detected by Western blotting analysis as outlined in Materials and Methods.

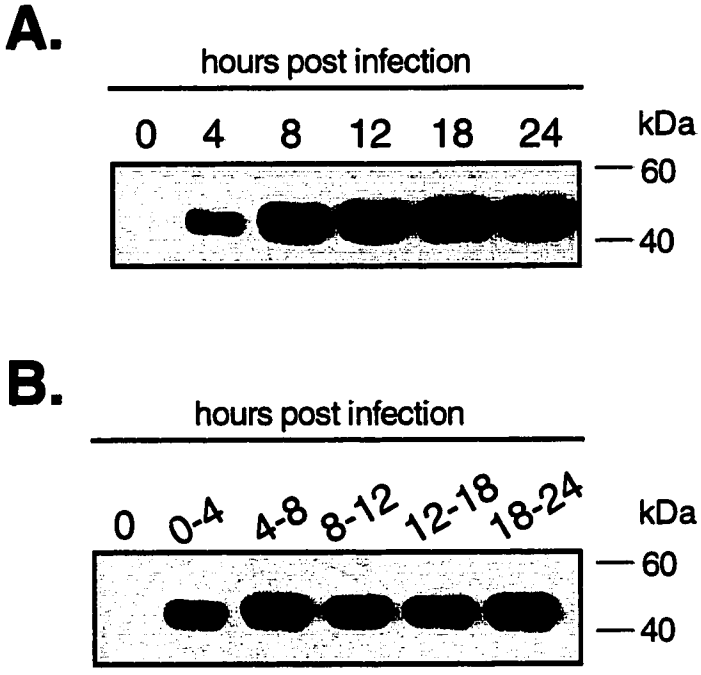

A.
hours post infection
0 4 8 12 18 24
kDa
60
40
B.
hours post infection
0 0-4 4-8 8-12 12-18 18-24
kDa
60
40

Figure V-2. Construction of a recombinant mutant myxoma virus that contains a M-T1 disruption.

Shown is a schematic representation outlining the construction of vMyxlacT1^{-}. A construct containing a 64 bp 5' coding sequence of M-T1, pKS-5'T1, was generated by restriction digestion of pBS-M-T1 with *Pst*I and *Bam*HI. A 3' terminal fragment of M-T1 was generated by PCR amplification using a primer containing an engineered *Pst*I site and cloned into a pT7Blue T-vector. The 3' M-T1 fragment was isolated after restriction digestion with *Pst*I and *Bam*HI and cloned into the complementary sites of pKS-5'T1 to generate the plasmid pKS-T1del. Digestion with *Pst*I and insertion of a *Pst*I p7.5Ecogpt cassette was used to form the M-T1 disruption plasmid, pBS-T1KO. vMyxlac-infected cells were subsequently transfected with pBS-T1KO DNA, and recombinant viruses (vMyxlacT1^{-}) resistant to Ecogpt selection were harvested and plaque-purified. Primers directed toward the 5' and 3' flanking sequences of M-T1 (indicated by asteriks) were used in PCR diagnostic analysis to verify the disruption of M-T1 and insertion of Ecogpt.

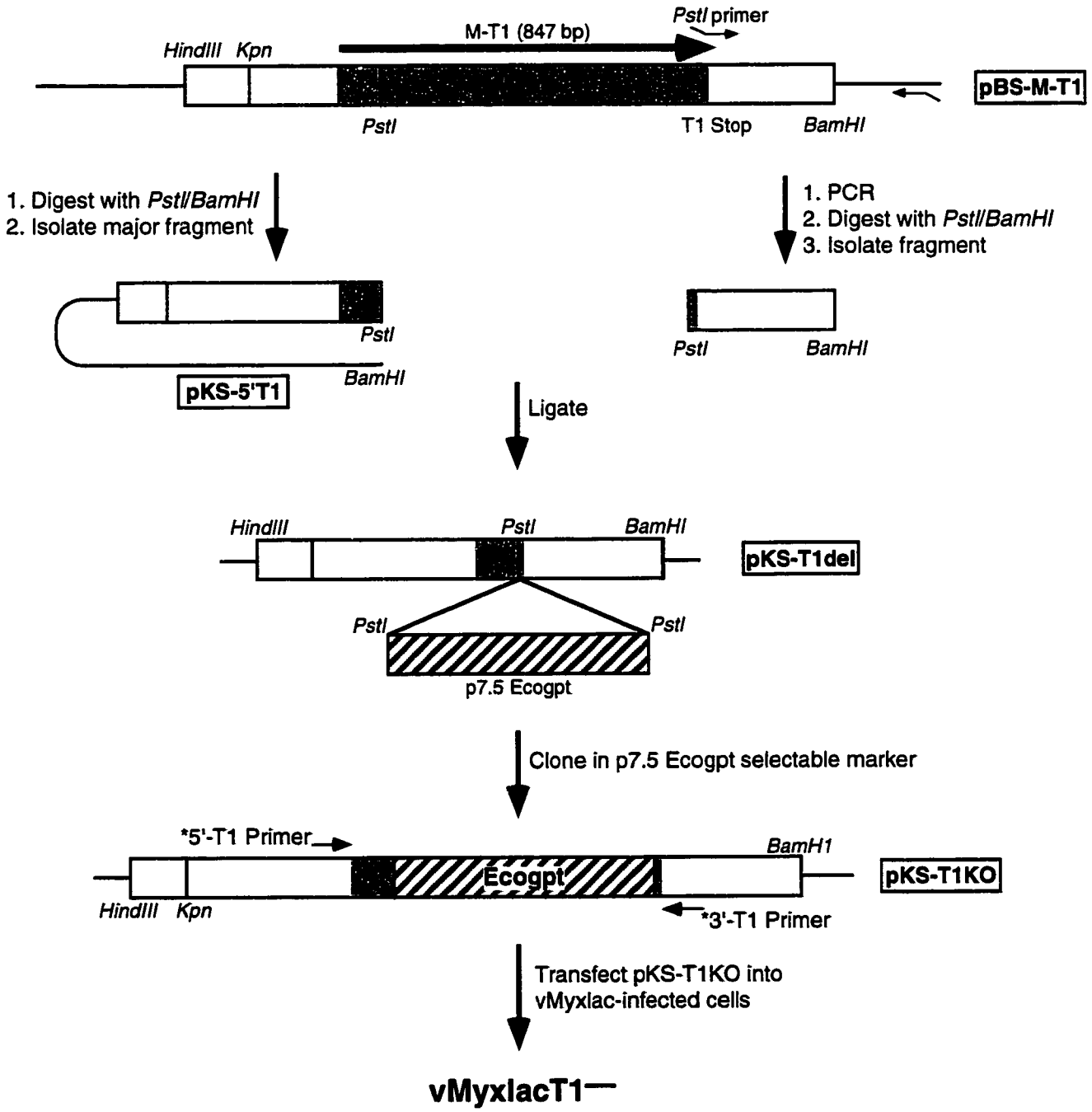

PstI primer
M-T1 (847 bp)
HindIII Kpn
pBS-M-T1
PstI
T1 Stop
BamHI
1. Digest with PstI/BamHI
2. Isolate major fragment
1. PCR
2. Digest with PstI/BamHI
3. Isolate fragment
PstI
BamHI
pKS-5'T1
PstI
BamHI
Ligate
HindIII
PstI
BamHI
pKS-T1del
PstI
PstI
p7.5 Ecogpt
Clone in p7.5 Ecogpt selectable marker
*5'-T1 Primer
BamH1
Ecogpt
pKS-T1KO
HindIII Kpn
*3'-T1 Primer
Transfect pKS-T1KO into
vMyxlac-infected cells
vMyxlacT1—

Figure V-3. Characterization of M-T1 from vMyxlac (wild-type), recombinant vMyxlacT1$^-$, and vMyxT1R viruses.

(A) Agarose gel electrophoresis of PCR products amplified by flanking M-T1 5' and 3' primers. Migration of wild-type M-T1 (lane 1) and M-T1:Ecogpt (lane 2) DNA amplified from pBS-M-T1 and pKS-T1KO plasmids, respectively. PCR analysis of viral DNA harvested from vMyxlac-infected cells (lane3). PCR analysis of viral DNA following homologous recombination and Ecogpt dominant selection identified a mixed foci (lane 4) comprised of both M-T1 and M-T1:Ecogpt, and a pure recombinant M-T1-deletion virus (lane 5) containing only M-T1:Ecogpt. Viral DNA harvested from cells infected with vMyxT1R demonstrates the presence of wild-type M-T1. Molecular size markers corresponding to *Bst*EII-cut λ standards are shown in lane 7. (B) Western blotting analysis of supernatants from mock-infected (lane 1), vMyxlac-infected (lane 2), vMyxlacT1$^-$-infected (lane 3) and vMyxT1R-infected (lane 4) cells using specific anti-M-T1 antisera. SDS-PAGE low-molecular-weight markers are indicated to the right.

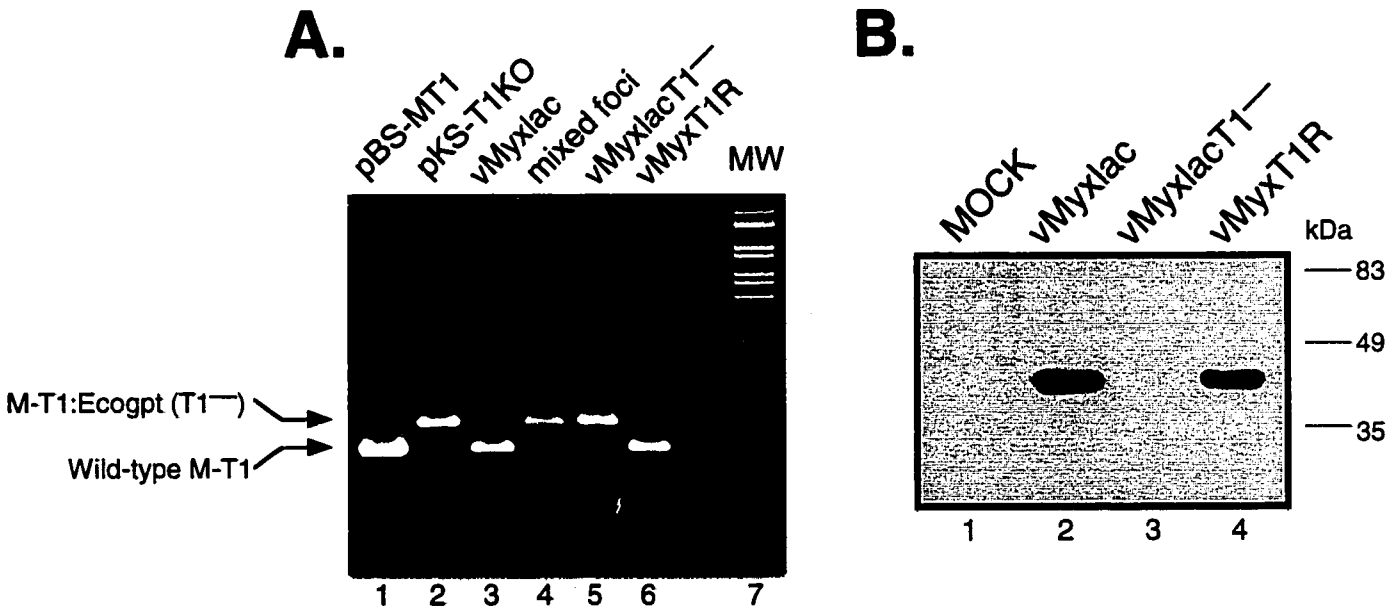
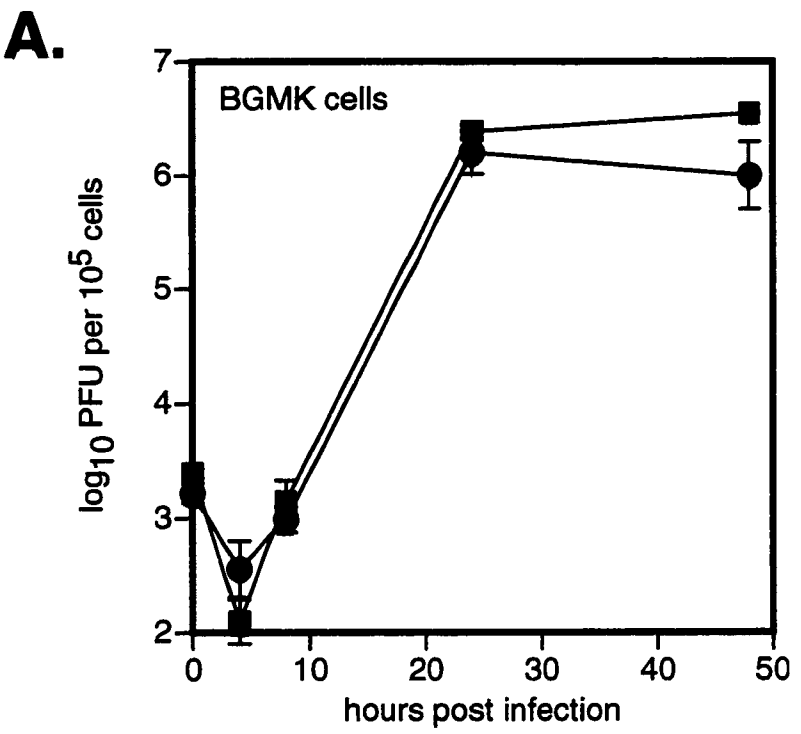

A.
pBS-MT1
pKS-T1KO
vMyxlac
mixed foci
vMyxlacT1—
vMyxT1R
MW
M-T1:Ecogpt (T1—)
Wild-type M-T1
1 2 3 4 5 6 7
B.
MOCK
vMyxlac
vMyxlacT1—
vMyxT1R
kDa
83
49
35
1 2 3 4

Figure V-4. Single-step growth curve analysis of vMyxlac and vMyxlacT1$^-$ viruses.

Baby green monkey kidney (BGMK) (A) and rabbit kidney fibroblast (RK-13) (B) cells were infected with vMyxlac (squares) or vMyxlacT1$^-$ (circles) at a multiplicity of infection of 5. The cells were harvested at 0, 4, 8, 24, and 48 h post-infection, and infectious titers were determined on BGMK cells. Virus titrations were performed in triplicate.


A.
BGMK cells
log10 PFU per 10^5 cells
hours post infection
7
6
5
4
3
2
0
10
20
30
40
50

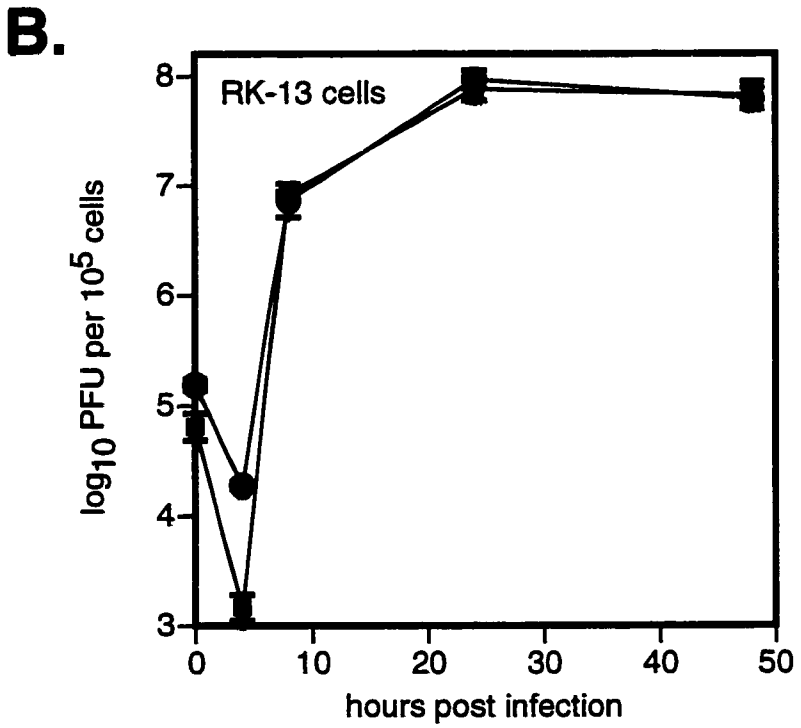

B.
RK-13 cells
log10 PFU per 10^5 cells
hours post infection
8
7
6
5
4
3
0
10
20
30
40
50

Figure V-5. Histological analysis of primary lesion tissues.

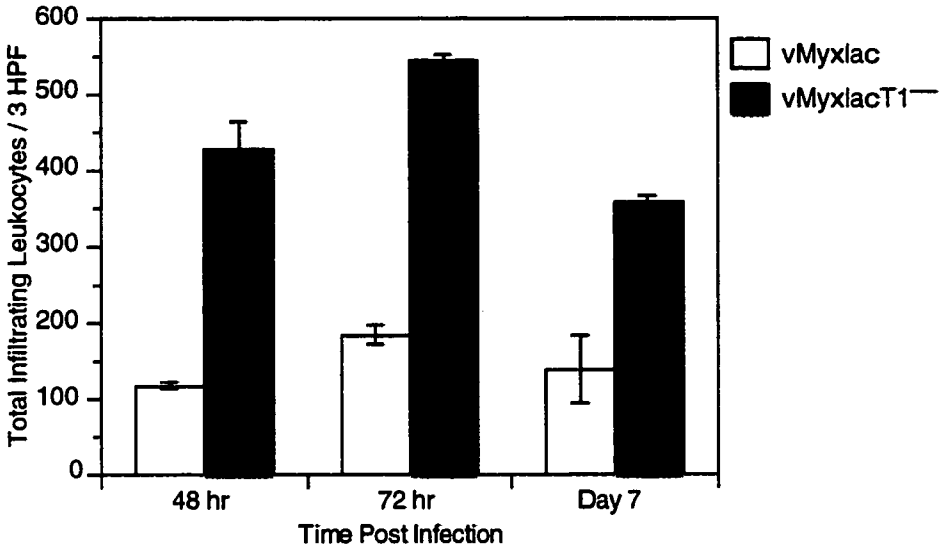
Microscopic analysis (magnification 400X) of the deep dermal layer of rabbit tissues from the primary site of inoculation with vMyxlac (A and C) or vMyxlacT1^{-} (B and D) that were harvested at Day 2 (A and B) and Day 3 (C and D) post-infection.

vMyxlacT1-
vMyxlac
B.
D.
C.

Figure V-6. M-T1 blocks leukocyte infiltration in virus infected tissues.

(A) Infiltrating rabbit leukocytes within the deep dermal layer of tissues from vMyxlac (white bars) and vMyxlacT1^{-} (solid bars) primary lesions were enumerated in three high-power-fields from H&E sectioned tissues as outlined in Materials & Methods. (B) Primary tissues from vMyxlac- (white bars) and vMyxlacT1^{-}- (solid bars) infected rabbits were harvested at 2 and 3 days p.i., and stained with RAM11 antibody. Peroxidase-stained RAM11+ monocytes in the deep dermis of infected tissues were counted in three random high-power-fields and the results expressed as the percentage of infiltrating RAM11+ monocytes/macrophages per total infiltrating rabbit leukocytes within virus-infected tissues.

A.


600
500
400
300
200
100
0
Total Infiltrating Leukocytes / 3 HPF
48 hr
72 hr
Day 7
Time Post Infection
vMyxlac
vMyxlacT1—

B.

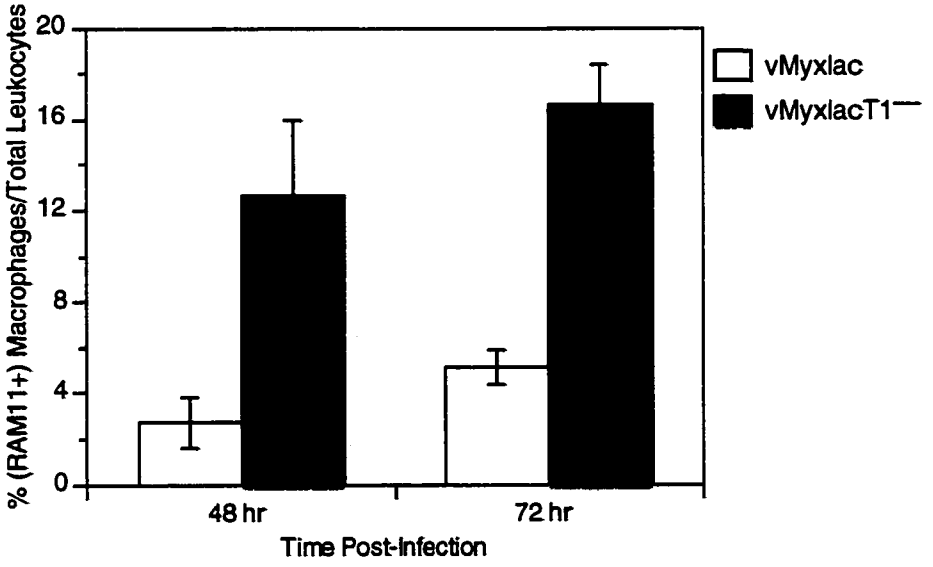

20
16
12
8
4
0
% (RAM11+) Macrophages/Total Leukocytes
48 hr
72 hr
Time Post-Infection
vMyxlac
vMyxlacT1—

Figure V-7. Viral load in infected primary dermal tissues.*

Primary tissue sections from vMyxlac- (A) and vMyxlacT1$^-$- (B) infected rabbits were harvested at 3 days p.i. and subject to immunoperoxidase staining with an anti-β-galactosidase antibody as outlined in Materials and Methods. Peroxidase-staining of β-galactosidase correlates with the presence of vMyxlac or vMyxlacT1$^-$ within infected-tissues since both viruses encode a lacZ gene marker.

*Figure V-7B was selected by Academic Press for the cover image of *Virology*, Volume 256 Issue 2, 1999.


A


B

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CHAPTER VI

GENERAL DISCUSSION AND CONCLUSIONS

For successful propagation, viruses must elude or oppose an aggressive arsenal of immune weaponry. A variety of strategies have been identified by which viruses nullify host defense mechanisms to ensure their continued survival. These tactics include the production of antigenic variants, interference with antigen presentation, inactivation of complement and antibody function, inhibition of apoptosis, and cytokine neutralization (16, 28, 33, 35). Much of our understanding of immune evasive strategies that utilize virus-encoded immunomodulatory proteins comes from studies of large DNA viruses, such as poxviruses and herpesviruses. As a result of their large genetic-encoding capacity, these viruses afford the luxury of expressing multiple proteins aimed at disabling components of the host immune system in addition to factors necessary for virus replication and assembly. A spectacular array of poxvirus-encoded proteins has now been identified that appears to subvert cellular immunity and inflammation by disrupting the function of host cytokines (2, 4, 19, 27, 32, 34). What is perhaps surprising is the extent to which poxviruses are able modulate multiple cytokine networks. To date, cytokines such as TNF, IFN-α/β, IFN-β, IL-1β, IL-2, IL-5, IL-18 and GM-CSF have been shown or may be potentially neutralized by soluble poxvirus proteins (Table I-1). With the identification of the poxvirus secreted chemokine binding proteins, M-T1/35kDa and M-T7, chemokines can now be added to the expanding repertoire of cytokines that are targeted by poxviruses.

Myxoma virus provides an attractive biological system for the study of virus-host interactions and molecular mechanisms that govern viral pathogenesis (17). The evolutionary background of the natural and susceptible rabbit hosts of myxoma virus

is well documented and the virus can be readily manipulated in the laboratory for biochemical and genetic analysis. Furthermore, myxoma virus induces an extensively characterized pathology in the susceptible European rabbit that is well suited for *in vivo* biological experimentation. A number of myxoma virus genes have been characterized that appear dispensable for virus replication *in vitro*, but whose expression contributes to the pathogenic syndrome of their infected hosts *in vivo* (24). Among these myxoma virulence factors are the soluble cytokine binding proteins, M-T2 and M-T7, which have previously been characterized as potent species-specific inhibitors of TNF and IFN-γ, respectively (22, 39). Deletion of M-T2 or M-T7 results in a severe attenuation of the virus and ameliorates the disease profile in infected laboratory rabbits (23, 36). Thus, the abrogation of cytokine activities *in vivo* appears to be an important determinant in conferring pathogenicity and allowing for increased virus survival in myxoma virus-infected European rabbits. The identification of poxvirus soluble cytokine decoy receptors such as M-T2 and M-T7 prompted us to screen for additional poxvirus soluble cytokine binding proteins that may be involved in disarming host inflammatory responses. Here, we describe the identification, biochemical and functional characterization of two distinct classes of soluble poxvirus chemokine binding proteins, M-T7 and M-T1/35kDa.

The generation of an effective acute-phase host inflammatory response is central for the clearance of an invading pathogen (25). Chemokines play a pivotal role in this process by mediating the extravasation and trafficking of immune cells into diseased and damaged tissues (3, 14, 30). It comes as no surprise, therefore, that poxviruses have evolved to encode gene products that counteract chemokine

activities. It is now generally accepted that optimal chemokine function *in vivo* depends both on high affinity interactions with their target leukocyte receptors and lower affinity interactions with glycosaminoglycans within the extracellular matrix (5, 38). Thus, the antagonism of chemokine-induced functions can be facilitated by two different but related strategies; disruption of the low or high affinity binding sites of chemokines (18). The results from this thesis indicate that myxoma virus has adopted both of these strategies for sequestering chemokines in order to effectively circumvent the host inflammatory response to virus infection. The soluble M-T1 CC-chemokine inhibitor functions by inhibiting CC chemokine-receptor interactions and the M-T7 broad-spectrum chemokine binding protein is proposed to prevent solid-phase chemokine gradients within infected tissues by inhibiting chemokine-GAG interactions (Fig. VI-1).

The chemokine binding property of M-T7 was unanticipated from its predicted sequence analysis as a soluble IFN-γ receptor homologue. Rather, pathogenic profiles of rabbits infected with an M-T7 knockout virus first revealed an unexpected linkage between the expression of M-T7 and leukocyte infiltration during infection (23). *In vitro*, M-T7 was demonstrated to bind promiscuously with multiple members of the C, CC, and CXC chemokine subfamilies in a non-species specific fashion (Chapter II), a property that is distinct from the observed class-restricted binding patterns of known cellular chemokine receptors. The interaction of M-T7 with the heparin-binding region of chemokines suggests that the virus has targeted a common domain that is structurally conserved among virtually all chemokines.

Few clues currently exist that may help to reveal the molecular basis of the novel interaction between M-T7 and chemokines. M-T7 is an *N*-linked glycoprotein and is largely acidic based on its predicted p*I* of ~4.5. It is undetermined whether the linkage of *N*-linked oligosaccharides, or whether any additional post-translational modifications such as sulfation or sialylation, to the M-T7 peptide may contribute to its novel binding activity. Interestingly, M-T7 possesses a stretch of 60 amino acids at its COOH-terminus that is unrelated to cellular IFN-γ receptors or other known cellular proteins in the database. The significance of this region in M-T7 function remains unclear. Future structure/function studies to delineate the domain(s) or amino acid determinants of M-T7 that are required for binding to chemokines will be of significant interest. Curiously, homologues of M-T7 present in other genera of poxviruses (i.e. orthopoxviruses) do not appear to bind to CC or CXC chemokines (1). It is intriguing to speculate that M-T7 possesses a unique moiety for its chemokine binding activity that may be absent in other poxvirus homologues. For example, M-T7 contains all 8 structurally critical cysteine residues that are highly conserved with mammalian IFN-γ receptors. However, in all the orthopoxvirus IFN-γ receptor homologues that have been characterized to date, the first 2 cysteines are notably missing (21). Alternatively, M-T7 may require a specific cofactor for its chemokine binding property that is present only in myxoma virus. One such candidate cofactor, a virus-encoded α2,3-sialyltransferase, has recently been isolated exclusively from myxoma virus-infected cells (7). Sialyltransferases are a family of enzymes that catalyze the transfer of sialic acid residues to *N*- and *O*-linked glycoproteins (29). Sialylation of glycoproteins is known to play important roles in

biological processes such as mediating ligand-receptor interactions in immune responses (37). Current studies are underway to determine whether modification of the M-T7 glycoprotein by the myxoma virus-encoded sialyltransferase contributes to its chemokine binding activity.

Rather than occluding chemokine receptor triggering, the biochemical characteristics of M-T7-chemokine interactions suggest that M-T7 likely perturbs chemokine localization and/or effects their correct presentation to target leukocytes in complex tissues. Unfortunately, neither of these suppositions can readily be examined by current *in vitro* chemotaxis assays. Thus, the inhibition of chemokine biological activity by M-T7 both *in vitro* and during a chemokine-mediated inflammatory response *in vivo* is an area that merits further investigation.

Members belonging to the M-T1/35kDa family of poxvirus genes were originally identified almost a decade ago but no function had been ascribed to this family until now. In vaccinia virus (strain Lister), VV-35kDa is the most abundantly secreted protein from infected cells and its genomic location within the TIRs suggested that it may play an important biological function in poxvirus virulence. Here we demonstrate that M-T1 and its homologous 35kDa proteins found in the majority of orthopoxviruses, define a new family of secreted poxviruses proteins that bind to multiple CC chemokines with high affinity, bind to IL-8 with lower affinity, and appear to potently inhibit CC chemokine proinflammatory activities.

The major biological consequence of T1/35kDa appears to be the abolishment of infiltration involving multiple inflammatory cells which are induced into sites surrounding virus infection. Although T1/35kDa was shown to interact with members

of both CC and CXC chemokine subfamilies *in vitro* as deduced by chemical cross-linking, CC chemokines appear to be the preferential targets of inhibition as demonstrated by subsequent *in vitro* biological assays (Chapter IV) (1, 31). The physiological consequence of soluble poxvirus chemokine binding proteins was also confirmed by histological analysis of poxvirus-infected tissues *in vivo*. Expression of T1/35kDa was shown to effectively attenuate leukocyte infiltration of CC chemokine-responsive leukocytes such as monocytes/macrophages and T-lymphocytes into dermal tissues of infected rabbits during the acute-phase response as predicted by its *in vitro* inhibitory properties (Chapter III and V).

Perhaps the most remarkable feature of the T1/35kDa family members is the apparent lack of sequence similarity to any known cellular proteins including seven-transmembrane spanning chemokine receptors. Unlike many of the poxvirus virulence factors that have been identified to date which are clearly derived from cellular homologues during the process of virus-host co-evolution, the origin of T1/35kDa remains ambiguous. It is intriguing to speculate that T1/35kDa is an evolutionary gene product generated by sequence shuffling which resulted in a soluble chemokine receptor mimetic. Alternatively, a more exciting possibility is that T1/35kDa represents a viral homologue of a yet unidentified novel or ancestral cellular CC chemokine receptor or regulator. The sequence of M-T1 provides modest clues to its mechanism of action: a highly acidic nature (p*I* of 4.6), two N-linked glycosylation sites, eight cysteines and multiple stretches of 3-5 amino acid residues that are absolutely conserved with all known T1/35kDa family members. Despite the considerable sequence heterogeneity (<40% amino acid identity) between M-T1 and

the orthopoxvirus 35kDa proteins, all members of the T1/35kDa tested to date possess essentially equivalent functional activities (Chapter IV) (1, 31). This suggests that conserved motifs or moieties that are shared among multiple T1/35kDa viral peptides may serve as likely targets for mutagenesis in future experiments. Alternatively, it is plausible that members of the T1/35kDa family adopt a uniform conformation that allows their chemokine binding activities and structural studies of recombinant M-T1 are also underway to formally address this hypothesis.

The biochemical characteristics of M-T1-chemokine interactions appear to be clearly distinct from those proposed for M-T7. This study, together with similar studies from Alcami, A. *et al* and Smith, C. *et al*, have demonstrated that members of the T1/35kDa family effectively block CC chemokines from binding to their cognate cell surface receptors (1, 10, 31). Thus, it is likely that the T1/35kDa viral proteins occlude a necessary receptor binding site(s) that is commonly found within the NH_2-terminus of many chemokines. Consistent with this is the demonstration that M-T1 and VV-35kDa retain binding to GAG-binding deficient chemokine mutants but have reduced affinity for NH_2-terminal RANTES mutants that have impaired receptor-binding capabilities (9). In addition, excess quantities of proteoglycans are unable to inhibit the binding of M-T1 or VV-35kDa to chemokines in binding assays (1, 9). Taken together, whereas M-T7 appears to bind to the heparin-binding site of chemokines, M-T1 interacts with high-affinity to a site that is independent of the proteoglycan-binding domain of CC chemokines. Future studies aimed at identifying the conserved CC chemokine determinants required for M-T1 binding may elucidate the basis for the broad-spectrum CC chemokine specificity of this viral protein and

may yield further insight into the mechanism of chemokine-receptor interactions in general.

There are a number of important issues that remain to be resolved concerning the relationship between M-T1 and M-T7 during myxoma virus infection. For example, it is unproven whether both of these chemokine binding proteins are synergistic with respect to each other *in vivo*. Knockout analysis reveals that M-T1 retards leukocyte influx significantly earlier (Day 2-3) than M-T7 (Day 7) during myxoma virus pathogenesis suggesting that these two proteins are not functionally redundant (Chapter V and 8, 23). In addition, M-T7 is crucial for the development of the myxomatosis whereas M-T1 has no significant effects on disease progression or in the overall mortality of infected European rabbits (8, 23). The direct contribution of M-T7's chemokine binding property to myxoma virus virulence still awaits formal confirmation and the generation of M-T7 mutants that discriminate between its IFN-γ– and chemokine-binding activities may address this important question.

A more recent development is the potential use of viral anti-inflammatory proteins for therapeutic interventions. The SERP-1 glycoprotein of myxoma virus has been recently demonstrated to have potent anti-inflammatory effects in animal models of coronary restenosis and antigen-induced arthritis (13, 15). Also, based on the strategies of viral cytokine decoy receptors (viroceptors), a new class of anti-cytokine agents has emerged in pharmaceutical drug development. Similar to the functions of the myxoma M-T2 glycoprotein, ENBREL™ (Immunex Corp.) is a soluble version of cellular TNF receptors and functions by competitively blocking the engagement of TNF to cell surface receptors (20). Based on its impressive therapeutic efficacy in

clinical trials, ENBREL™ has recently received FDA approval for the treatment of rheumatoid arthritis (26).

There is mounting evidence to suggest that chemokines contribute significantly to a manifold of disease states that are characterized by excessive or dysregulated inflammation (14). Agents that effectively neutralize chemokine activity are therefore regarded as promising reagents for anti-inflammatory therapies (6). Given the non-species-specific broad-spectrum chemokine binding properties of M-T7 and the potent CC chemokine inhibitory activities of T1/35kDa demonstrated here and elsewhere (1, 31), soluble poxvirus chemokine binding proteins may have potential as novel agents for treating certain types of acute and chronic inflammatory disorders. This application was recently demonstrated in a guinea pig skin model of allergic inflammation where VV-35kDa effectively blocked eotaxin-stimulated infiltration of eosinophils (1). In addition, both M-T1 and M-T7 have been shown to cause marked reduction of inflammatory cell migration in animal models of transplant arteriosclerosis and angioplasty restenosis (11, 12). The potential use of M-T1 and M-T7 as novel anti-chemokine agents is under active investigation by the London, Ontario, Canada based biotechnology company, Viron Therapeutics Inc.

In summary, the study and function of viral immunomodulatory proteins have not only been instructive in our understanding of virus-host interactions, but also in revealing crucial components of the immune circuitry. Myxoma virus thus continues to provide a rich biological repository of information with which to probe the disciplines of viral immunology and pathogenesis. The work presented in this thesis illustrates a novel strategy by which myxoma virus, and other poxviruses, have

evolved to circumvent the host inflammatory response to infection in order to foster their continued survival within an immune-competent host. M-T1 and M-T7 represent the first examples of secreted virus proteins that modulate chemokine function and prevent inflammatory cell migration into infected tissues. Continued investigations into poxvirus soluble chemokine binding proteins may provide further insight into the mechanisms of virus pathogenesis, the functions of chemokines in host defense, and may even generate new strategies for therapeutic interventions against a broad spectrum of syndromes associated with inappropriate inflammation.

Figure V1-1. Model of leukocyte chemotaxis inhibition by soluble poxvirus chemokine binding proteins.*

(Top) Chemokines (solid yellow circles) bound to glycosaminoglycans (GAGs) along the endothelial wall or within deep tissues provides a solid-phase gradient for leukocyte chemotaxis to sites of infection or inflammation. (Bottom) Secreted poxvirus chemokine binding proteins (red stars) alter chemokine function by preventing chemokine-receptor interactions or disrupting chemokine localization by inhibiting chemokine-GAG interactions.

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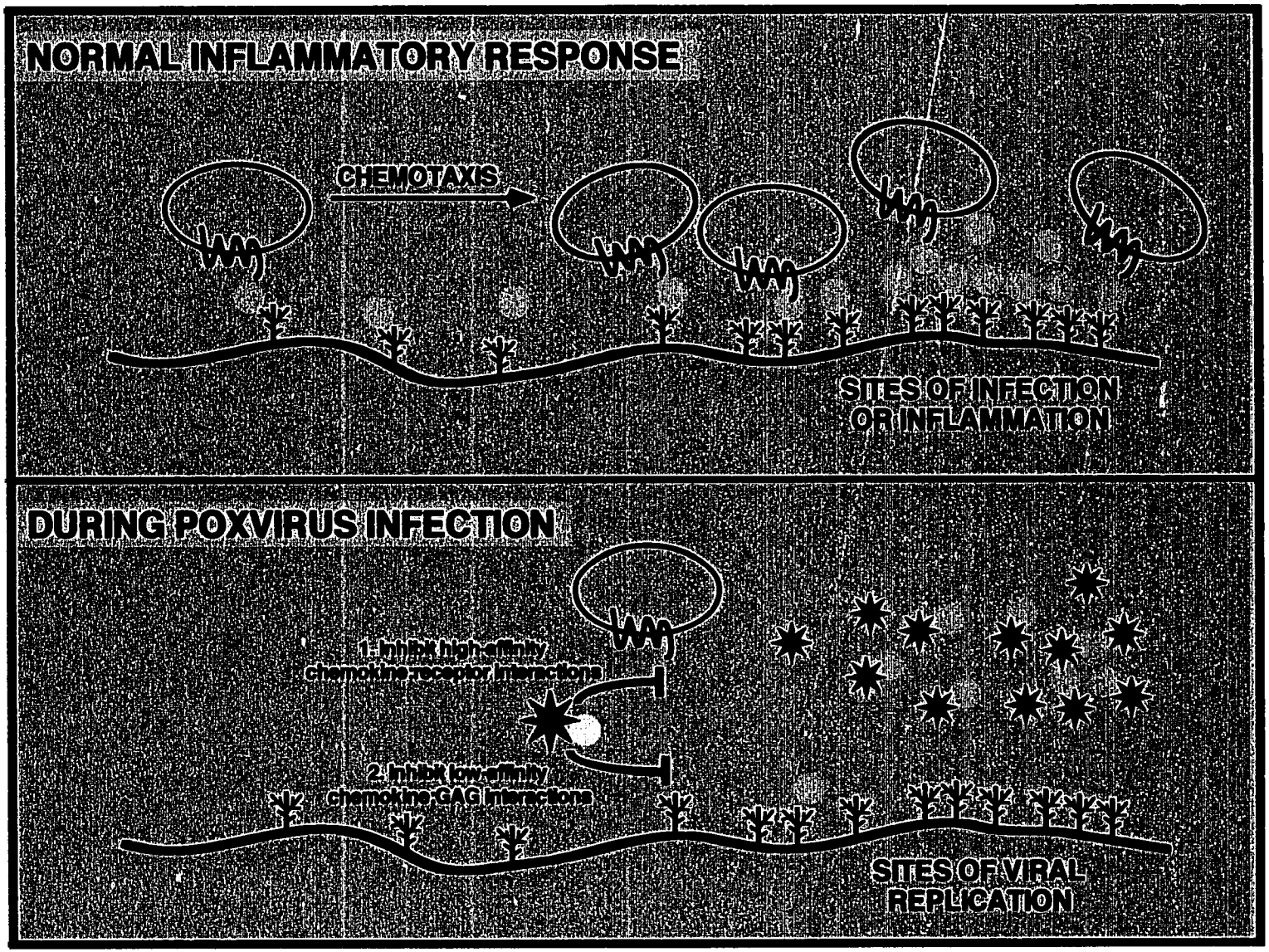
NORMAL INFLAMMATORY RESPONSE
CHEMOTAXIS
SITES OF INFECTION
OR INFLAMMATION
DURING POXVIRUS INFECTION
1. Inhibit high-affinity
chemokine-receptor interactions
2. Inhibit low-affinity
chemokine-GAG interactions
SITES OF VIRAL
REPLICATION

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