INTERACTION OF COMPLEMENT WITH HUMAN CYTOMEGALOVIRUS

by

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ABSTRACT.

In the absence of specific anti-human cytomegalovirus (HCMV) antibodies, complement has a negligible neutralizing effect on the virions. Under these conditions, the presence of activated C3 fragments, but not C9, were found on the HCMV virions. There are no known viral complement inhibitors encoded in the HCMV genome, but the presence of host-encoded complement inhibitors, CD55, CD46, and CD59, on the HCMV virions may explain the virion's ability to regulate complement. In the presence of anti-HCMV antibodies, complement enhanced the neutralizing ability of the immunoglobulins by 2-3 fold. Complement activation in the presence of antibodies occurred primarily by the classical complement activation pathway and was complete, as assessed by the presence of C9.

CD55 (decay-accelerating factor) and CD46 (membrane co-factor protein) regulate complement at the level of C3 and belong the regulators of complement activation (RCA) gene cluster. CD59 regulates complement at the level of the terminal lytic pathway and does not belong to the RCA gene cluster. HCMV infection of fibroblasts and glioblastoma cells resulted in a 3-8 fold increase in the expression of CD55 and CD46 by 72 h p.i., but infection with herpes simplex virus or adenovirus had no effect. An increase in C3 convertase regulation was also found on the HCMV-infected cells using a purified complement component assay. By contrast, CD59 expression was decreased on HCMV-infected cells by 50% by 72 h p.i., similar to the decrease observed for HLA class I.

CD55 expression was increased in HCMV-infected cells at the level of protein, mRNA, and transcription of the gene as assessed by using a variety of techniques. Similarly, the levels of CD59 mRNA decreased in the HCMV-infected cells, paralleling the observations for protein expression, but radiolabel

pulse-chase analysis identified a decreased CD59 protein survival. Indirect evidence suggested that the immediate early or early genes from HCMV were responsible for the altered expression of host complement inhibitors, but CD55 promoter activity and protein expression were unaffected by the presence of isolated HCMV immediate early genes.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Abs	absorbance
AD169	common lab strain of HCMV
Ad5	human adenovirus type 5
Ag	antigen
ANOVA	analysis of variance
Ast	astrocyte(s)
β1H	factor H (soluble complement regulator)
bp	basepair(s) of nucleic acid(s)
BSA	bovine serum albumin
C'	complement
C3, etc	third complement component, etc
C3b	large activation fragment of C3
C3dg	inactive, covalently-bound C3 fragment, CR2 ligand
C4b	large activation fragment of C4
C4d	inactive, covalently-bound C4 fragment
C4bp	C4-binding protein
C8bp	C8-binding protein
CAT	chloramphenicol acetyl-transferase
CD	cluster designation (international protein classification)
CD4	cell-surface marker for T-cell (helper subtype) and monocytes
CD8	cell-surface marker for T-cell (cytotoxic/suppressor subtype)
CD34	hematologic stem cell marker
CD35	complement receptor 1 (CR1)
CD46	Membrane co-factor protein (MCP)
CD55	decay-accelerating factor (DAF)
CD59	Membrane inhibitor of reactive lysis (MIRL)

cDNA	complementary DNA from mRNA
CML	chronic myelogenous leukemia
CMV	cytomegalovirus
CPE	cytopathic effect
CPM	counts per minute
CR1	complement receptor 1 (CD35)
CR2	complement receptor 2 (CD22)
CR3	complement receptor 3 (CD11b/CD18)
CSF	cerebrospinal fluid
CTP	cytosine triphosphate
D	day(s)
DH5α	competent E. coli for transformation
DHFR	dihydrofolate reductase
DMEM	Dulbecco's minimum essential medium
DNA	deoxyribonucleic acid
E	early HCMV gene(s)
E2F	eukaryotic transcription factor 2
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-(beta-aminoethylester) tetraacetic acid
ELAM	endothelial-leukocyte adhesion molecule
ELISA	enzyme-linked immunosorbent assay
Fab	variable, antigen-binding region of an immunoglobulin
Fc	constant region of an immunoglobulin
FC	flow cytometry solution
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate

fos	a human oncogene
gB	glycoprotein B (major envelope protein for CMV or HSV)
gC	glycoprotein C (major envelope protein for HSV)
gH	glycoprotein H (major envelope protein for CMV or HSV)
gp41	part of the major HIV virion envelope protein
gp115	part of the CMV gB virion envelope protein
gp350	EBV complement regulating protein
GPI	glycophosphoinositol
gpIII	synonym for CMV gB virion envelope protein
GVB	gelatin containing veronal-buffered saline solution
h	hour(s)
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
h p.i.	hours post infection
HSV-1	herpes simplex virus type 1
ICAM	member of the integrin family of proteins
IE	immediate early HCMV gene(s)
IE1	immediate early HCMV gene #1
Ig	immunoglobulin
IgG	gamma class of immunoglobulin
IgM	mu class of immunoglobulin
IL	interleukin
Inab	specific CD55-deficient phenotype
Int	international
INF	interferon
IRS	internal repeat sequence
kb	kilobase pairs

kDa	kilodaltons
L	late HCMV gene(s)
Lac Z	β-galactosidase gene
LPS	bacterial cell wall lipopolysaccaride
M	molar
MAC	membrane-attack complex
Man	mannose
mCi	millicurries (radioactivity)
MEM	minimal essential medium
MHC	major histocompatibility antigen (mouse HLA)
min	minute(s)
MIRL	synonym for CD59
ml	milliliter(s)
mm	millimeter(s)
mM	millimolar
MOI	multiplicity of infection
Mono	monocyte
Mr	relative molecular mobility
mRNA	messenger ribonucleic acid
mtr	group of immortalizing genes found in the HCMV genome
myc	a human oncogene
Na	sodium salt
ng	nanogram(s)
NK	natural killer cells
nm	nanometer(s)
NHS	normal human serum
p15E	complement activating protein from C-type retroviruses

p55	part of the CMV gB virion envelope protein
p86	the CMV gH virion envelope protein
p130	part of the CMV gB virion envelope protein
PAA	phosphoacetic acid
PAGE	polyacrylamide gel electrophoresis
PBM	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PHA	phytohemaglutanin
p.i.	post-infection
PIPLC	phosphoinositol-specific phospholipase C
PFU	plaque forming unit(s)
Plt	platelet(s)
PMN	polymorphonuclear cells
PNH	paroxysmal nocturnal hemoglobinuria
Rab C'	rabbit serum containing active rabbit complement
Rc/CMV	eukaryotic expression vector
RCA	regulators of complement activation (gene cluster)
RNA	ribonucleic acid
rpm	revolutions per minute
RSB	reticulocyte standard buffer
SCR	short consensus repeat
SDS	sodium dodecylsulfate
SIV	simian immunodeficiency virus
SW30Ti/SW50	swinging bucket rotors rated for 30 K and 50 K respectively
TBS	tris-buffered saline
TBST	tris-buffered saline containing 0.05% Tween 20 detergent
THP-1	monocytic leukemia cell line

TRS	terminal repeat sequences
U	units
U373-MG	glioblastoma cell line
U_L	unique long region of the cytomegalovirus genome
U_S	unique short region of the cytomegalovirus genome
ug	microgram(s)
ul	microliter(s)
UV	ultra violet light
VBS	veronal-buffered saline
vol	volume
w/v	weight/volume
w/w	weight/weight
X-gal	chromogenic substrate for β-galactosidase

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Dedication;

This thesis is dedicated to Dana Devine, who always believed; to my wife Linda who always faced my difficulties with me; and to my parents, Bob and Lorna, without whose constant support and encouragement this would not have been possible.

1. BACKGROUND:

This dissertation focuses on a proposed mechanism by which human cytomegalovirus (HCMV)-infected cells evade clearance by specific antibodies and complement, and briefly addresses the potential role of complement in the *in vivo* clearance of extracellular HCMV virions. HCMV, an enveloped, double-stranded DNA virus, is a member of the herpesvirus family, and as such replicates in the nucleus and is capable of latent infection. The means by which the virus evades the host immune response during latent infection is not well understood. HCMV is a ubiquitous viral agent. Depending on the population studied, up to 100% of individuals may have been infected with the HCMV by middle-age, most without any obvious signs or symptoms (Borysiewicz et al 1983, 1986, Schrier et al 1986, Riddell et al 1991). HCMV antibody prevalence rates in North America range from 30% to 80%, while some areas in Africa and the Far East reach 100% seropositivity (Tegtmeier 1989, Ho 1991). Asymptomatic infections in normal individuals are often accompanied by virus shedding in urine, saliva, semen, breast milk, and virus can occasionally be isolated from blood. HCMV also accounts for about 15% of all cases of mononucleosis, a non-life threatening and self-resolving illness (Rinaldo et al 1980, Kaariainen et al 1966, Klemola et al 1965).

The most severe pathologies are associated with congenital HCMV infection or infection during immunosuppression. Approximately 1-2% of infants are infected in utero and another 6-60% (depending on geographic location) become infected during the first 6 months of life as the result of birth canal or breast milk transmissions (Ho 1991, Reynolds et al 1973). HCMV transmission to the fetus during gestation can occur during primary maternal infection or during re-infection/reactivation of latent HCMV infection. However, the pathological consequences seem to be more associated with a maternal primary infection (Ho 1991). Although HCMV is a ubiquitous viral agent and evidence of infection can approach 100% of certain populations, there are some rare diseases in which HCMV has been speculated to play

a pathological role. These include various cancers, atherosclerosis, Guillain-Barré syndrome, Charcot-Marie-Tooth disease, post-perfusion syndrome, diabetes mellitus, hepatitis, hemolytic anemia/ thrombocytopenia, and gastrointestinal disease (reviewed in Huang and Kowalik 1994).

Other than congenital HCMV infection, the other large patient group at risk for complications arising from HCMV infection of immunosuppressed individuals. Patients can become immunosuppressed by HIV infection or iatrogenically, through organ or bone-marrow transplantation procedures. The transplantation group is of major concern, since surgical procedures and post-transplant care can require the transfusion of large numbers of blood products. The ability of HCMV to be carried along with these blood products appears to be quite efficient (Bowden 1991). Besides the concerns associated with patient morbidity and mortality, HCMV infection has been reported to be associated with a higher rate of graft rejection or the development of graft-versus-host disease (Lopez et al 1974, Lonnqvist et al 1984, Rinaldo et al 1980). Furthermore, many strategies are being developed to decrease the risk of HCMV infection from blood transfusion including transfusing only blood products that do not contain anti-HCMV antibodies or filtering out the leukocytes, which seem to harbor infectious HCMV (Bowden 1991). All of these modalities are costly: mass screening of blood products, filters, infusion of intravenous immunoglobulins, post-rejection complications and re-transplantation. Further understanding of the HCMV biology is necessary before one can develop more effective strategies to reduce morbidity and mortality associated with HCMV infection.

1.1 HCMV biology and life cycle.

HCMV is a member of the herpesvirus family. Inclusion into the family Herpesviridae is based on the architecture of the virion. A typical herpesvirion consists of (1) a core containing a linear, double-stranded DNA, (2) an icosadeltahedral capsid, containing 162 capsomeres with a hole running down the

long axis, (3) an amorphous, sometimes asymmetric material that surrounds the capsid, designated the tegument, and (4) an envelope containing viral glycoprotein spikes on its surface (Roizman 1990; Figure 1A).

HCMV is the largest of the herpesvirus family; an HCMV virion is between 150-200 nm and has a genome of approximately 230 kilobase pairs. Analysis of the genome of the AD169 laboratory strain of HCMV has identified 208 open-reading frames and potential protein products (if open reading frames are limited to <300 bp; Chee et al 1990). The HCMV genome has a complex arrangement; sequences from both termini are repeated in an inverted orientation and juxtaposed internally, dividing the genome into two components, each of which consists of unique sequences flanked by inverted repeats. Therefore, since both components can invert relative to each other, extracted DNA from virions exists as four equimolar isomers. Since not all herpesviruses have the ability to isomerize, the HCMV genome is referred to as a type E genome which indicates this ability (Roizman 1990). HCMV is further classified into the Betaherpesvirinae subfamily, which indicates a restricted host range (non-exclusive to this subfamily), a long reproductive cycle, ability to establish a latent infection, hematogenous spread and possible site of latency, and that infected cell frequently become enlarged (cytomegalia).

While the cellular receptor for HCMV has not yet been elucidated, the viral envelope glycoproteins gH (or p86; see section 1.3.2) and gB (or p130/55; see section 1.3.2) have been reported to be responsible for HCMV ligand-cellular receptor interactions (Rasmussen 1991). Upon absorption, uncoating, and entry of viral DNA into the nucleus, expression of the HCMV genome is sequentially regulated (Figure 1B). According to the kinetics of gene expression, the genes encoded by HCMV can be categorized into three kinetic classes: immediate-early (IE), early (E), and late (L) genes (Stinski 1990; Figure 2). IE genes of HCMV encode a group of regulatory proteins with strong transactivating activities. IE expression is not only needed to activate subsequent E gene expression, but the IE gene products also transactivate the

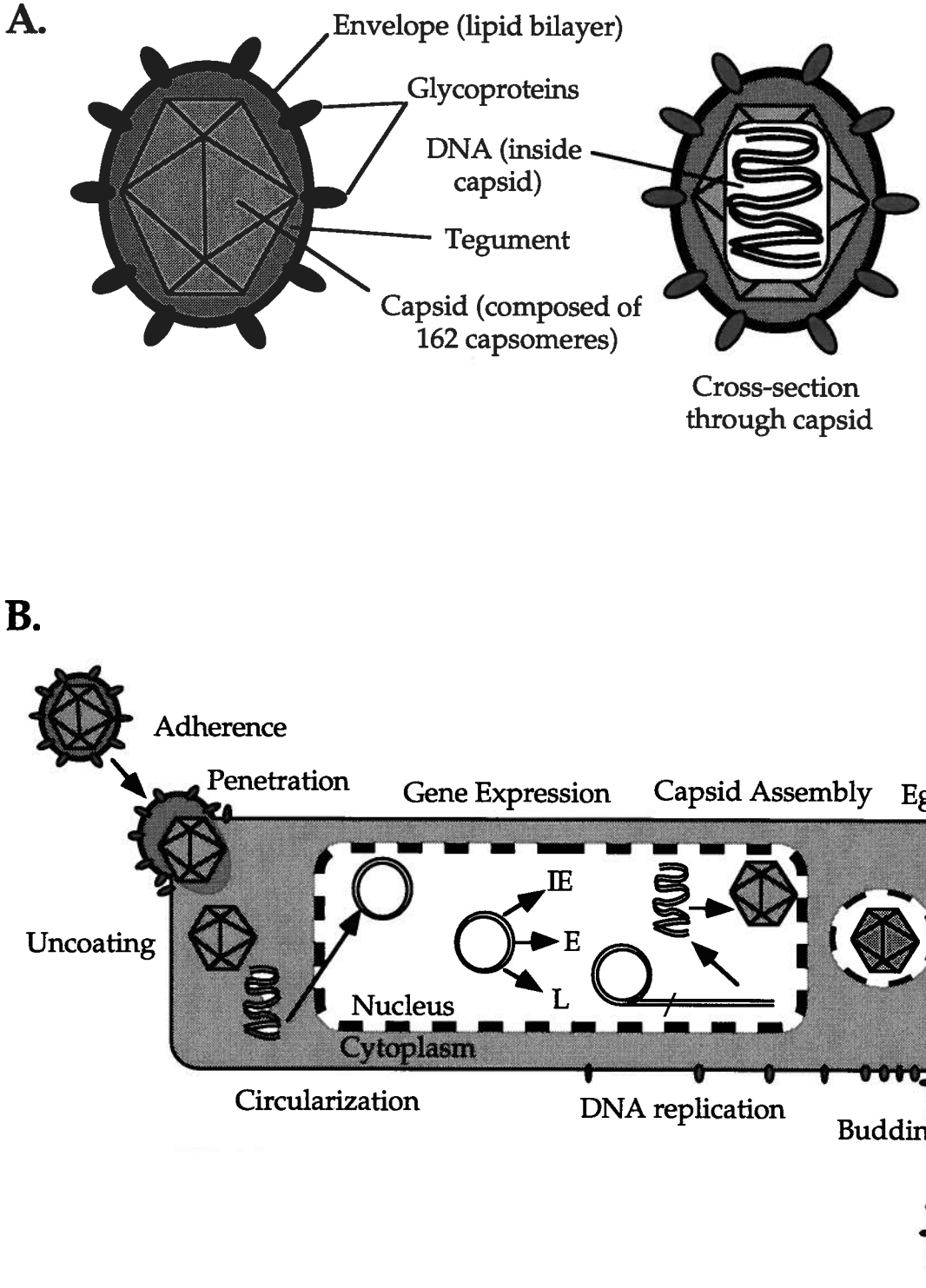


Figure 1. (A) Schematic representation of the herpesvirion, seen through a cross section of the envelope with spikes (glycoproteins) protruding from its surface. (B) Diagrammatic representation of the HCMV replication cycle. The abbreviations denote the immediate early (IE), early (E), and late (L) gene expression.

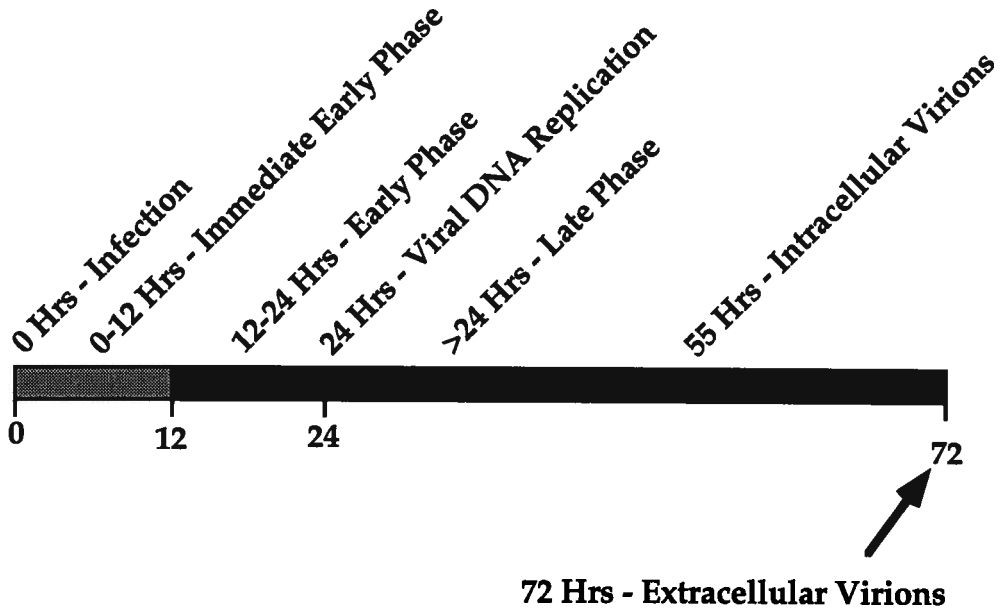


Figure 2. Diagrammatic representation of the HCMV replication cycle in human fibroblasts.

promoters of certain cellular genes (see section 1.6). HCMV E genes encode enzymes and factors involved in viral DNA replication and aid in the regulation of late gene expression. HCMV L genes are expressed following the initiation of HCMV DNA replication and these genes encode the structural components of the virion. Capsid assembly occurs in the nucleus and infectious intracellular HCMV can be detected at 55 h post-infection. The release of extracellular HCMV can be detected after 72 h post-infection *in vitro* and continues until cell death.

1.2 Cell types infected by HCMV.

The CMV family of viruses are species specific. With few exceptions CMV from one species will not infect cells from another (Plummer 1973). Furthermore, unlike HSV, another member of the herpesvirus family, HCMV will not infect all cell types isolated from a permissive host. Some of the most compelling evidence for *in*

vivo cell specificity comes indirectly from investigation of HCMV-associated pathology of immunosuppressed patients.

1.2.1 Post-transplantation/transfusion HCMV infection.

The largest body of evidence for the site of HCMV persistence or latency was identified serendipitously via transmission of HCMV through blood products to transplant recipients. HCMV-seronegative transplant recipients can acquire primary HCMV infection from either contaminated blood products or from the transplanted organ (or bone marrow) itself, if these are obtained from HCMV seropositive individuals. Individuals who have serological evidence of previous exposure to HCMV, on the other hand, can reactivate latent HCMV or be re-infected. Eighteen to eighty-three percent of HCMV-seronegative transplant recipients who receive blood products or allografts from HCMV-seropositive donors will become infected with HCMV depending on the transplantation setting (data summarized in Bowden 1991). Furthermore, 70% of seropositive patients, for whom no attempt at HCMV screening of products is made, have evidence of active HCMV infection post-transplantation, but the origin of the HCMV was not elucidated (Bowden et al 1986). This contrasts sharply to the 0-6% incidence of HCMV infection of HCMV-seronegative recipients receiving transplants and blood products from HCMV-seronegative donors (data summarized in Bowden 1991). However, even these HCMV infections in seronegative recipients receiving products from seronegative donors may reflect the limitations in the screening procedure; HCMV DNA has been reported occasionally in monocytes in the absence of detectable specific antibody (Taylor-Wiedeman et al 1991). It is the relatively high rate of transmission of HCMV via blood products which lends support to the hypothesis that a major latency reservoir for HCMV is contained within the blood. The decrease in HCMV transmission following leukofiltration of blood products as well as reports that HCMV can be found in buffy coat preparations from blood donors, congenitally infected children, and transplant recipients strongly

suggests the mononuclear peripheral blood leukocyte fraction is the site of latency for HCMV (Diosi et al 1969, Kaariainen et al 1966, Lang and Noren 1968, Fiala et al 1975, Smith et al 1993). One multi-center controlled study reported that 21% of newborn infants transfused with unfiltered blood products developed HCMV infection, whereas a group of infants transfused with leukodepleted blood products did not develop HCMV infection (Gilbert et al 1989). The rare transmission of HCMV when measures are taken to reduce the transmission via blood products may suggest that there are additional cell types involved in HCMV latency/persistence.

1.2.2 *In vitro* HCMV infection of hematologic cells.

Several *in vitro* studies have attempted to elucidate the particular cell which is infected in the leukocyte fraction. At the least differentiated level, there is some evidence that clonogenic bone marrow progenitors (CD34+ cells) are permissive for HCMV infection (Maciejewski et al 1992). However, even though HCMV virions appear to enter a large number of CD34+ cells and some viral progeny is produced, the infected cells quickly lose their expression of the CD34 antigen, indicating induction of cellular differentiation, prior to expression of HCMV late genes. Of infected CD34+ cells which express HCMV late genes following loss of the CD34 antigen, all of the productively infected cells were found to belong to the myelomonocytic lineage (Maciejewski et al 1992). These findings provide additional evidence that the cells related to the leukocyte fraction are responsible for harboring HCMV. Additionally, the use of polymerase-chain reaction techniques recently identified the presence of HCMV DNA, which does not necessarily indicate a productive infection, in the monocyte fraction of healthy donors (Taylor-Wiedeman et al 1991).

Infectious HCMV has occasionally been found in buffy-coat or mononuclear cell preparations (Rinaldo et al 1977, Fiala et al 1975, Jordan 1983, Stanier et al 1989) obtained from patients with clinical HCMV infection; but only rarely has the virus

been isolated from healthy donors (Diosi et al 1969). A low number of purified T-cells (3 per million) isolated from transplant patients produced plaques when co-cultured with fibroblasts; purified B-cells did not (Garnett 1982). However, a minimal contamination of T-cells with monocytes could not be ruled out. Early studies using in situ hybridization found that 0.03 - 2% of the peripheral blood mononuclear (PBM) cells from 8 asymptomatic individuals hybridized strongly with a probe for the HCMV immediate early gene-1 (IE-1; Schrier et al 1985). Further analysis of one individual, who had 2% HCMV IE-1 (+) PBM, found that 2.4 % of the CD4(+) cells and 0.8% of the CD8 (+) cells hybridized with an IE1 probe. However, it is important to note that the authors alluded to similar findings with monocytes, and that all monocytes also express CD4 which was used to identify the T-helper cells. Other studies relying on indirect immunofluorescence with monoclonal antibodies identified only IE HCMV genes, but not late genes nor the presence of any intracellular virions by electron microscopy, in peripheral blood cells (Rice et al 1984). Determination of cell types found IE expression in monocytes>NK cells>>B lymphocytes >CD8(+) T cells>>CD4(+) T cells. Interestingly, two separate groups found that low passage HCMV isolated from patients was far more capable of infecting PBMs than fibroblast-conditioned laboratory strains of HCMV (Rice et al 1984, Einhorn and Ost 1984). However, lack of detection of late HCMV gene expression maybe indicative of an abortive infection since IE1 genes can be expressed in non-permissive cell lines (De Marchi 1983, La Femina and Hayward 1983).

Rice et al (1984) indicated that the most receptive cell type for HCMV IE gene expression was the monocyte and examinations of biopsies of transplanted organs from patients with HCMV disease indicated that mononuclear inflammatory cells are the predominantly infected cell type (Gnann et al 1988, Wiley et al 1986). Directed by these findings other investigators stimulated primary monocytes or monocytic cell lines to become fully permissive to HCMV infection (Weinshenker et al 1988, Lathey and Spector 1991, Ibanez et al 1991). A monocytic leukemia cell line, THP-1, was

found to allow the expression of late HCMV proteins and release infectious virus only after differentiation by phorbol esters (Weinshenker et al 1988). However, a similar treatment of promyelocytic or T cell lines (HL60, HUT 102, and Molt-4) did not result in late gene expression or virus production (Weinshenker et al 1988). Primary monocytes could be induced to express late HCMV genes and produce significant amounts of HCMV if they were first co-cultured with phytohemagglutinin P-stimulated T-cells then treated with hydrocortisone (Lathey and Spector 1991) or differentiated with Con A (Ibanez et al 1991). In all cases however, permissive HCMV infection was only induced after the monocytic cells were stimulated to form multinucleated giant cells.

1.2.3 Infection of non-hematologic cell types.

The most common *in vitro* model of HCMV infection uses human fibroblasts with the laboratory strain of HCMV, AD169. Classically, investigation of *in vivo* distribution of HCMV utilized histological evidence of specific nuclear and cytoplasmic inclusions. Examination of tissue from individuals acutely infected with HCMV, for the presence of cytomegalic cells, has identified the virus in a wide variety of organs (Myerson et al 1984, Wiley et al 1986, Ho 1982). Generally, infection has been restricted to cells of epithelial and endothelial origin. However, evidence was put forward at the 1994 International Herpesvirus Workshop to suggest the fibroblast may play a central role as an *in vivo* host cell. Sinzer et al (1994) studied samples from lung and gastrointestinal tract of patients with symptomatic HCMV infections and found that the most common HCMV-infected cell type identified was the fibroblast. Furthermore, these investigators noted expression of late genes in endothelial cells, monocytes/macrophages, fibroblasts, smooth muscle cells and epithelial cells. These data suggest that while endothelial cells, monocytes/macrophages and polymorphonuclear cells may play a crucial role in the hematogenous spread of the virus, fibroblasts, smooth muscle cells and epithelial cells are probably as important to

the multiplication and spread of HCMV via cell-cell contact in infected tissues. *In vitro* studies also confirm that cells of epithelial (Smith 1986, Heieren et al 1988, Numazaki et al 1989a,b), endothelial (Ho et al 1984, Smiley et al 1988, Lathey et al 1990), smooth muscle (Tumilowicz et al 1985), and fibroblast (Compton 1993, Smith 1986) origins are permissive for HCMV infection.

1.3. *In vivo* control of HCMV infection.

Regardless of the cell types infected *in vivo* , pathological consequence of HCMV infection is associated with immunosuppression, for example post-transplant, infectious mononucleosis, HCMV meningitis/encephalitis. HCMV infection may also be acquired congenitally. However, in the immunocompetent host a significant immune response to HCMV can be seen.

1.3.1. Cellular immune response to HCMV infection

Cellular immunity plays a central role in controlling HCMV. Post-transplantation studies have demonstrated that recovery from HCMV infection correlates closely with the levels of HCMV-specific cytotoxic responses (Quinnan et al 1982, Rook et al 1984, Reusser et al 1991). More recently, provocative studies where CD8+ and CD4+ cells from transplant recipients were collected before transplantation, pre-stimulated against HCMV, and re-introduced after transplantation, observed a protection from subsequent HCMV disease (Cheng-Rong et al 1994). Even though the cytotoxic effects of T-cells are more commonly associated with CD8+ cells, the recovery of CD4+ HCMV-specific lymphoproliferative responses was found to be obligatory for the endogenous reconstitution of CD8+ cytotoxic effects (Quinnan et al 1982, Reusser et al 1991). However, upon immunosuppression many of these patients experience a reactivation of latent HCMV suggesting that cellular immunity may control HCMV infections, but it does not clear the virus from the host.

1.3.2. Humoral immune response to HCMV infection.

Antibody-mediated complement cytolysis is one of the primary mechanisms by which the host immune system eliminates virus-infected cells (Sissons and Oldstone 1980). HCMV infection of immunocompetent individuals results in a normal humoral response and the subsequent generation of stable levels of anti-HCMV IgG antibodies. Acute HCMV infection leads to the rapid generation of anti-HCMV antibodies of the IgM class (Langenhuysen 1972). A positive anti-HCMV IgM response correlated with active infection in mononucleosis patients and the titer was highest during viremia (Rasmussen et al 1982), and subsequently decreased over a period of a few months, while the IgG class of anti-HCMV antibodies increased and remained stable (Langenhuysen 1972, Rasmussen et al 1982). The levels of IgM anti-HCMV antibodies were also found to increase after reactivation (or re-infection) of HCMV in patients and the amount of anti-HCMV IgM measured was speculated to be related to the severity of both primary and recurrent HCMV infections (Rasmussen et al 1982).

Anti-HCMV antibodies, which arose following HCMV mononucleosis, were found to react with HCMV proteins of 66, 50, 135 and 42 kDa within 2 weeks after symptoms (Hayes et al 1987). The longest response to develop was antibody against a 92 kDa protein, which rose slowly for a month or more. The kinetics of anti-HCMV antibodies against the other HCMV proteins were intermediate between these two patterns. The antibody response to HCMV antigens located in the nuclear and extracellular virions continued to increase for more than three months after symptoms, whereas response to the cytoplasmic HCMV antigens peaked within one to two months (Hayes et al 1987).

Several groups have also investigated which epitopes on the HCMV virion are most important for neutralizing anti-HCMV antibodies. The most common epitopes appear to be envelope glycoproteins of approximately 130 kDa, 86 kDa, and 55 kDa (Britt 1984, Nowak et al 1984, Pereira et al 1984, Rasmussen et al 1985a). The 55 kDa protein (p55) was reported to be a post-transcriptionally processed form of the 130

kDa protein (p130) (Pereira et al 1984, Rasmussen et al 1985a). The gene of the p130/55 glycoprotein has been identified (Mach et al 1986) and shown to be homologous to the HSV glycoprotein B (gB) protein (Cranage et al 1986). The second protein (p86) was identified as glycoprotein H (gH) which also shares homology with HSV gH (Cranage et al 1988, Gompels and Minson 1986). A major difference in complement requirement was observed when these proteins were inoculated into mice or guinea pigs: the anti-gB antibodies generated require the additional presence of complement to neutralize HCMV virions, while anti-gH antibodies were neutralizing independent of complement (Pereira et al 1982, 1984, Nowak et al 1984, Britt 1984, Rasmussen et al 1984,1985a,b). Interestingly, if the antibodies were raised against gB expressed in E. coli, and therefore unglycosylated, the resultant neutralizing anti-gB antibodies raised in mice were complement independent (Britt 1988).

1.3.3 Complement and antibody-mediated HCMV neutralization.

The role of complement-mediated neutralization enhancement has been well documented. Addition of guinea pig complement to commercially available human IgG preparations has been found to increase the ability of those preparations to neutralize HCMV, as determined by plaque assay, from between 2- to 16 fold (Lewis et al 1986, Eizuru et al 1988). Other groups have found that the enhanced neutralization observed with complement addition is roughly the same as that observed when 0.24 mg/ml of rabbit anti-human IgG was added (Rundell and Betts 1982). However, the mechanism by which complement enhances antibody-mediated neutralization, for example by steric hindrance or virolysis, has not been elucidated.

1.3.4 The complement cascade.

The complement system consists of at least 20 immunologically non-cross-reactive and distinct plasma proteins. Together they represent a significant

proportion of the plasma proteins as their cumulative concentration exceeds 3 mg/ml. Upon interaction with activators, these proteins interact with membranes and with one another in an orderly sequential manner (Figure 1.1). The complement components can be grouped into two well characterized activation pathways, the classical and the alternative pathways. Activation of the classical pathway is most often initiated by the binding of the first complement component (C1) to antigen-antibody complex; however, other molecules, including polyanions, certain viral proteins, and lipid A of lipopolysaccharides, may also mediate classical pathway activation in the absence of antibody. C1 exists in the serum as a calcium-dependent complex of three subunits C1q, C1r, and C1s. C1q binds to the Fc region of immunoglobulin complexes, triggering the autoactivation of C1r, which then cleaves and activates C1s. Activated C1s then cleaves C4 into C4a and C4b. C4b has a reactive internal thioester bond, which is exposed upon cleavage and mediates the covalent attachment of C4b to the cell surface through an ester or amide bond to protein, carbohydrate, or lipid moieties. C4b then binds C2 in the presence of magnesium, The nearby activated C1s cleaves C2 into C2a, which remains bound to C4b and forms the classical C3 convertase, and C2b, which diffuses away. C4b2a is capable of cleaving C3 into C3a and C3b, which also has an internal thioester bond and covalently attaches to the surface of the cell. The cleavage of C3 signals the beginning of the common terminal lytic pathway (discussed below), and the end of the classical activation pathway.

The alternative complement activation pathway does not require the presence of immunoglobulins. Activation of the alternative pathway is more commonly associated with bacterial cells, soluble immune complexes, or tumor cells. An essential feature of the alternative pathway is the amplification of C3 convertase. C3b, which is generated by low-grade fluid-phase C3 spontaneous activation (or the classical pathway), binds to the cell surface and factor B binds to C3b in the presence of magnesium to form the precursor of the alternative C3 convertase, C3bB. Factor D

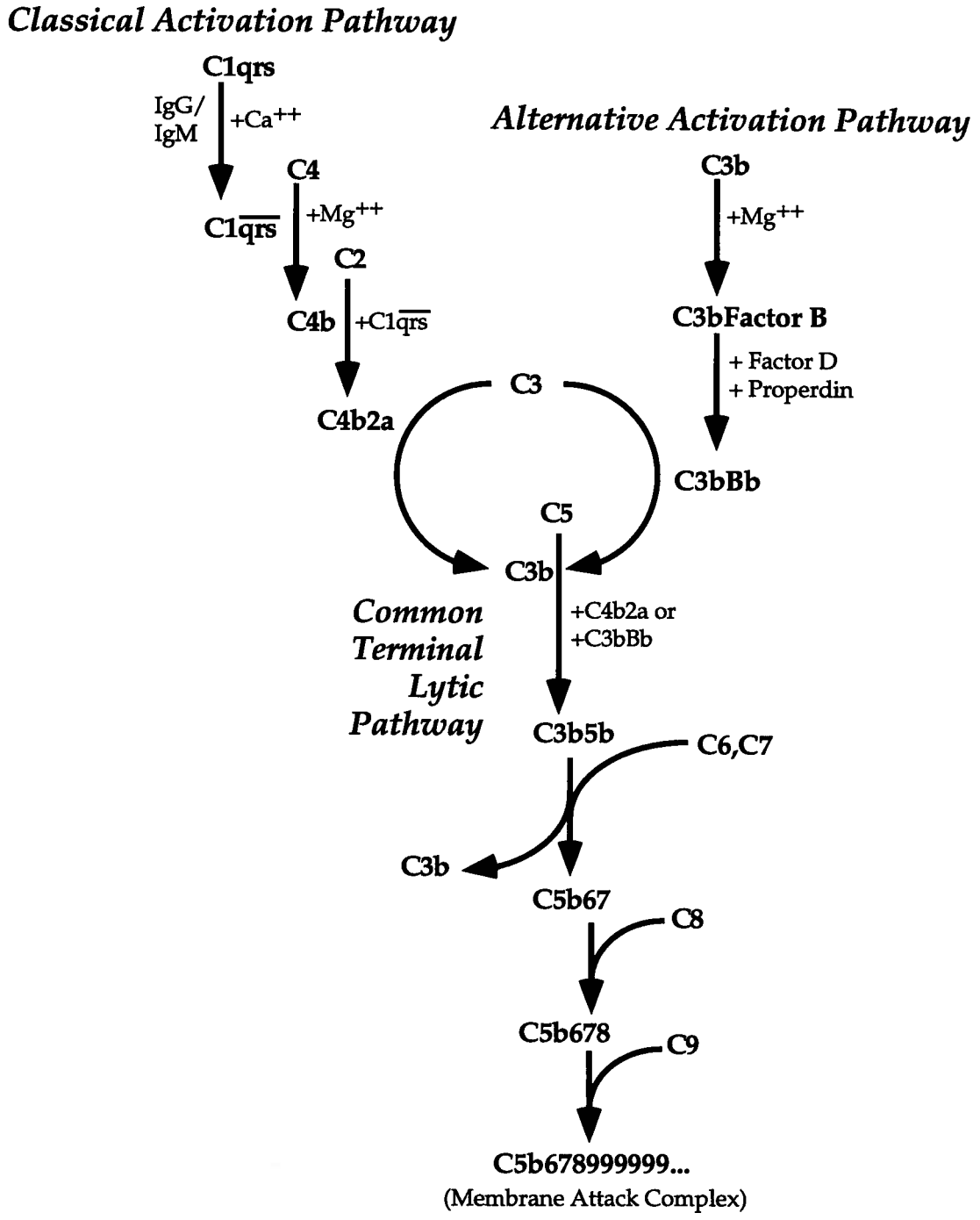


Figure 3. Diagrammatic representation of the complement cascade. Lines drawn over complement components indicate an activated state.

cleaves factor B to form the activated convertase, C3bBb, and this process is accelerated by the binding of properdin which stabilizes the interaction of C3b and factor B. The alternative pathway C3 convertase also generates C3b which will form the C5 convertase and the classical and alternative pathways join into the common terminal pathway at this point.

The common terminal lytic pathway starts with the cleavage and activation of C5. C4b2a3b and C3bBb both have the ability to cleave C5 into C5b and C5a. C6 then binds to and stabilizes C5b (otherwise, dissociation of C5b from the C5 convertase complex occurs). The C5b6 complex then reacts with C7 to form a trimolecular complex, C5b67. This complex then weakly associates with the membrane and the association of C8 makes the complex more hydrophobic which in turn causes a stronger binding of C5b678 to the membrane. The association of C9 initiates the final insertion of this complex across the membrane, and additional binding of multiple C9 subunits causes the formation of the lytic pore which can be visualized by electron microscopy (Berry and Almeida 1968). This terminal complex of complement is called C5b-9 or the membrane attack complex (MAC). The mechanism by which the MAC causes cell death has been reviewed elsewhere (Morgan 1989), and some investigators suggest that complete formation of the lytic pore is not required to induce cell death (Pramoonjago et al 1992, Dankert and Esser 1985, Morgan et al 1987).

1.4. Host-encoded complement inhibitors.

The complement system provides a potent means of recognizing and eliminating foreign elements. It is critical, however, to focus these actions on foreign particles and to prevent inadvertent attack against host tissue. Integral to this function are regulatory proteins of the complement system. Such components provide a means of separating "self" from "non-self" during complement attack. Prior to 1980, several plasma proteins were known to fulfill this role. However, in the early 1980's two additional regulatory proteins of the complement system, decay-

accelerating factor and membrane cofactor protein, were identified; closely followed by the discovery of CD59 in the mid 80's (discussed below). This section will discuss several aspects of host complement inhibition and some of the viral immune evasion mechanisms which have evolved and mimic the actions of these host proteins.

1.4.1. The regulators of complement activation (RCA) gene cluster.

The human RCA gene cluster is a 900 kb region located at the q32 region on the long arm of chromosome 1 (Weis 1987; Lublin 1987,1988) which encodes a family of proteins that are structurally and functionally related. Pulsed-field gel electrophoresis and Southern blot analysis has revealed that the following genes are located there (in order): CD46 (membrane co-factor protein) - CD35 (complement receptor 1) - CD21 (complement receptor 2) - CD55 (decay-accelerating factor) - C4 binding protein (C4bp) α subunit - C4bp β subunit. Another structurally related complement regulator is located around 500 kb from this group and can also be considered part of the RCA gene cluster (Rey-Campos et al 1988; Carroll et al 1988; Bora et al 1989; Pardo-Manuel et al 1990). Additionally, there appears to be some partial duplication of the CD35, C4bpα, and CD46 genes which are also located in this gene cluster, but their roles in complement regulation, if any, remain to elucidated (Hourcade et al 1992, 1990b,1988; Wong et al 1989; Pardo-Manuel et al 1990). All of the characterized proteins share the ability to bind C3 or C4 fragments, act as a co-factor for factor I-mediated inactivation of C3 or C4 and/or dissociate C3 convertases. All of these proteins also show a particular structural organization based on the presence of an internal repeat of ~60 amino acids that share a framework of highly conserved residues called a short consensus repeat (SCR; Reid et al 1986; Ahearn and Fearon 1989, Coyne 1992). These similarities and the juxtaposition of the genes may support the hypothesis of a common ancestral gene which diversified by partial gene duplication. Discussed below are the relevant complement regulating proteins: decay-accelerating factor (CD55), membrane cofactor protein (CD46), complement

receptor 1 (CD35), complement receptor 2 (CD21), C4-binding protein, factor H, membrane inhibitor of reactive lysis (CD59), and C8-binding protein.

1.4.1.1 CD55 or decay-accelerating factor (DAF).

CD55 is constitutively expressed on most cells with the exception of natural killer cells (Nicholson-Weller et al 1986). CD55 is not a typical membrane bound protein since it has no transmembrane region, but is linked to the cell surface by a glycophospholipid (GPI) anchor (Davitz et al 1986, Mahoney et al 1992). This anchor is added in the endoplasmic reticulum and on most cells is susceptible to cleavage with phosphoinositol-specific phospholipase C (PIPLC; Davitz et al 1986, Thomas et al 1990, Caras et al 1987). CD55 also exists as two isoforms; erythrocyte CD55 is a 70 kDa protein which has a slightly altered GPI composition (Davitz et al 1986, Roberts et al 1988, Kinoshita et al 1985) while and the rest of the cell types which express CD55 have a 78 kDa protein (Kinoshita et al 1985). This relative mass difference has been speculated to be due to differences in the glycosylation of the core protein (Lublin et al 1986). Deficiencies of CD55 have been reported to be in part responsible for the increased hemolysis associated with paroxysmal nocturnal hemoglobinuria (PNH); an acquired myelodysplastic disease in which all GPI-anchored proteins are deficient or missing entirely (Selvaraj et al 1988, Fujioka and Yamada 1992, Norris et al 1994, Ohashi et al 1994). However, some individuals who are selectively deficient in CD55 mRNA levels and protein expression with normal levels of the other GPI-linked proteins have been described (Inab phenotype; Tate et al 1989, Telen et al 1988, Merry et al 1989).

CD55 regulates complement by inactivating the classical and alternative C3 convertases through acceleration of the dissociation of the bimolecular enzymes (C4b2a and C3bBb) into subunits; this occurs only on the surface of cells which bear CD55 (Holguin et al 1992, Fujita et al 1987, Medof et al 1984). CD55 binds to the intact convertases with a higher affinity than to the separate components (Pangburn 1986),

and CD55 rapidly dissociates C2 and factor B only after being cleaved to their active fragments (Fujita et al 1987). CD55 also binds to C3b alone and may competitively inhibit convertase formation (Pangburn 1986). The overall ability of CD55 to regulate C3 deposition is greater than CD46, however, together they are more effective than singly (Iwata et al 1994). Although CD55 is composed of four short consensus repeats (SCR) of ~60 amino acids each, the entire C3 convertase dissociating activity was mapped (by molecular manipulation of cDNA constructs and blocking by monoclonal antibodies) to the third SCR (Coyne et al 1992). However, the spacing of SCR3 seems to play an important role. It is interesting to note that the GPI anchor played no role in the efficiency of CD55's regulatory activity since transmembrane versions, created by manipulating cDNA constructs, resulted in molecules with identical complement regulatory activity (Lublin and Coyne 1991).

1.4.1.2 CD46 or membrane cofactor protein (MCP).

CD46 is expressed constitutively on most cells with the exception of red blood cells (Cho et al 1991, Cole 1985, Yu 1986, Seya 1988, McNearey 1989). Unlike CD55, CD46 is bound to the cell surface through a transmembrane region. A distinguishing structural characteristic of CD46 on SDS-PAGE is the presence of two broad protein species with relative masses of 59-68 kDa and 50-58 kDa (Johnstone et al 1993). These two forms differ by the presence or absence of a heavily O-glycosylated region encoded by exon 8, but still consist of 4 SCR's. However, the tissue distribution of the different isoforms is less well defined than the distributions for CD55; some tissues express both forms of protein and this further varies between individuals (Johnstone et al 1993). A detailed study reports that of the 14 introns found in the CD46 gene, a large inter-organ and inter-individual variation exists in the combination of introns included in mRNA species (Johnstone et al 1993). This is in contrast to CD55 which has a major and minor mRNA species in all cell types but produces only one protein (Thomas and Lublin 1993).

CD46 regulates complement activation by interacting with cell-bound C3b, and to a lesser extent C4b, and acting as a co-factor for factor I, which cleaves C3b and C4b to inactive fragments (Seya and Atkinson 1989, Adams et al 1991). Interestingly, even though factor I can cleave C3b to the smaller C3c and C3dg fragments in the presence of factor H (see below), there seems to be some controversy as to whether factor I can cleave C3b to C3dg or only to iC3b in the presence of CD46 (Seya and Atkinson 1989, Adams et al 1991). CD46 has minimal activity when it is soluble, and therefore, CD46 is considered an intrinsic cofactor for the cleavage of C3b bound to the same cell surface (Seya and Atkinson 1989). SCR deletion mutants were constructed to determine which of the four SCR of CD46 contribute to ligand binding and cofactor activity. The third and fourth SCR were important for both ligand binding and cofactor activity of C3b and C4b, but deletion of SCR1 only decreased the binding of C4b (Adams et al 1991). Further, deletion of SCR2 did not decrease binding of C3b, but deletion of SCR2 abolished the cofactor activity. This suggests that binding of CD46 to complement fragments is not always sufficient for cofactor activity. It has also been reported that CD46 preferentially associates with and more efficiently inactivates the C3b dimers in the alternative C5 convertase than other membrane bound forms of C3b (Seya et al 1991), and may have minimal regulatory activity towards classical pathway activation (Kojima et al 1993).

1.4.1.3 CD35 or complement receptor 1 (CR1).

The tissue distribution of CD35 is more restricted than CD55 or CD46. Erythrocytes, B lymphocytes, a subset of T lymphocytes, monocytes, neutrophils, eosinophils, glomerular podocytes, and follicular dendritic cells are the only cell types documented to express CD35 (Fearon 1980, Wilson et al 1983, Reynes et al 1985, Gelfand et al 1975, Kazatchkine et al 1982). A soluble form of CD35 also has been found in plasma in picomolar concentrations that sedimented as a broad peak in ultracentrifugation (Yoon and Fearon 1985). Like CD46, CD35 is attached to the cell

surface by a transmembrane region. However, unlike CD46 and CD55, CD35 is made up of 30 SCR's and has a relative molecular mass of 190 kDa. Since every eighth SCR is a highly homologous repeat, this very large protein can be further organized into 3 long homologous repeats, a structural organization which is unique to CD35 (Ahearn 1989). However, this organization only holds true for the most common allotype. A total of four allotypic forms of CD35 have been identified. The size and frequency of occurrence (within brackets) is as follows: 190 kDa (0.82) > 220 kDa (0.18) >.160 kDa (<0.01) = 250 kDa (<0.01). These forms seem to differ by increments of 30 kDa which is comparable to the Mr of one long homologous repeat (LHR) and other evidence which suggests unequal translocation as being the mechanism by which they arose is reviewed in Fearon and Ahearn 1989.

Molecular cloning and deletion experiments have identified separate C3b and C4b recognition sites within CD35 (Klickstein et al 1988). The C3 recognition sites were found to exist in both the LHR-B and -C regions, while LHR-A was found to contain the primary C4 recognition site. CD35 can regulate the alternative pathway activation by three mechanisms: impairing uptake of factor B by C3b, displacing Bb from the C3bBb convertase, and acting as a cofactor for the cleavage of C3b to iC3b, C3c and C3dg by factor I (Fearon 1979, Seya and Atkinson 1989, Seya et al 1991, Pangburn 1986). Similarly, CD35 inhibits the classical pathway by impairing uptake of C2 by C4b, displacing C2a from the C4b2a convertase, and promoting the cleavage of C4b to C4c and C4d by factor I (Iida and Nussenzweig 1981, Pangburn 1986). Thus CD35 has similar complement activation regulating mechanisms as both CD55 and CD46, with one important difference: the tissue distribution of CD35 is limited and it mainly acts in an extrinsic fashion, i.e. on C3b/C4b deposited on bystander cell surfaces (Ross and Medof 1985, Medof et al 1982, 1983). This implies that CD35 plays a minimal role in intrinsic cellular protection from complement mediated lysis. Finally, CD35 has recently found a prominent place in protection of *in vivo* models

from complement mediated tissue damage, following intravenously administered soluble, recombinant CD35 (Piddlesden et al 1994, Homeister et al 1993).

1.4.1.4 CD21 or complement receptor 2 (CR2).

The tissue distribution of CD21 is even more restricted than CD35; it is expressed in mature B lymphocytes, human thymocytes, rare T lymphoblastoid cell lines, pharyngeal and cervical epithelium, and in follicular dendritic cells (Ross et al 1973, Iida et al 1983, Tsoukas and Lambris 1988, Menezes et al 1977, Tatsumi et al 1985, Fingeroth et al 1988, Young et al 1986, Sixbey et al 1987, Reynes et al 1985). CD21 is attached to the cell surface by a transmembrane region, and the extracellular domain is composed of 15 SCR's (Weis and Fearon 1985). There are no LHR's within CD21 as there are in CD35; however, there is a less conserved repeating pattern of homology involving every fifth SCR (Ahearn and Fearon 1989). The mature, cell-surface expressed CD21 has an Mr of 145 kDa and the N-linked oligosaccharides have been linked to extended biological half-life (Weis and Fearon 1985).

CD21 binds to the C3d fragment of C3, which is available for binding in iC3b, C3dg, and C3d (Weis et al 1984). Of the 15 SCR's contained within CD21, both the N-terminus SCR1 and SCR2 were required for the binding of C3dg and the Epstein-Barr virus (Lowell 1989). However, the complement regulatory role of CD21 is not clear. Some investigators document that CD21 is responsible for the activation of the alternative pathway (Theofilopoulos et al 1974, Baker et al 1977, Schreiber 1980, Ramos et al 1985), while others reported CD21 factor I-cofactor activity for the cleavage of iC3b to C3dg and C3c (Mitomo et al 1987). The role of CD21 is more prominent in B-cell activation and as the Epstein-Barr virus receptor (reviewed in Ahearn and Fearon 1989).

1.4.1.5 Remaining members of the RCA gene cluster.

Factor H is a soluble serum protein which is present at a high concentration in serum (350 μg/ml). It is an elongated molecule of approximately 160 kDa, and is composed of 20 SCRs (Reid et al 1986, DiScipio 1992, Ripoche et al 1988). Electron microscopic analysis found that factor H was an asymmetric, elongated molecule, with one end slightly larger and more rounded than the other (Smith et al 1983, DiScipio 1992). Five allelic variants of factor H have been identified using isoelectric focusing and neuraminidase treatment (Rodriguez de Cordoba and Rubenstein 1984, 1987). Factor H not only acts a cofactor for factor I cleavage of C3b to C3c and C3d, but also accelerates the decay and inhibits the reformation of alternative C3 and C5 convertases (Whaley and Ruddy 1976a, Fisher and Kazatchkine 1983). The nature of the cell surface to which C3b is attached effects the activity of factor H; the presence of sialic acid greatly enhances the factor I cofactor activity (Fearon 1978, Pangburn and Muller-Eberhard 1978). Factor H binds to the C3d portion of C3 and can compete for binding with CD35 (Pangburn 1986). Both the C3b-binding and cofactor activities have been reported to reside in the 38 kDa N-terminus (Alsenz et al 1984, 1985). Factor H also binds properdin, but at a site distinct from the C3b-binding site (DiScipio 1981), and very weak C4b-binding ability for factor H has also been reported (Pangburn 1986, Whaley and Ruddy 1976b). Finally, there is one report of a mutant Raji human B-cell line which did not secrete factor H into the medium, but expressed functional factor H on it's cell surface (Demares 1989). This could implicate factor H as a cell surface complement regulator; however, it remains to be determined whether this phenomenon is replicated *in vivo* , or whether it is related to the recently reported factor H receptor (Avery and Gordon 1993).

C4bp is the only other functionally characterized complement regulator in the RCA. Like factor H, C4bp is soluble and is found in serum at a concentration of 150 μg/ml; unlike factor H, C4bp is composed of a combination of 2 separate subunits which are both encoded in the RCA gene cluster. C4bp is about 570 kDa and has a

unique spider-like molecular structure with seven extended tentacles as observed by high resolution electron microscopy (Dahlback et al 1983). This "spider" is composed of 7 α-chains, which each has a C4b binding site and one β-chain (Dahlback et al 1983, Nagasawa et al 1983). Even though each C4bp molecule has the potential to bind 7 molecules of C4b, four C4b were shown to be bound per C4bp at physiological strength (Ziccardi et al 1984). The β-chain does not bind C4b, but instead binds and regulates the coagulation factor protein S (reviewed in Dahlback 1991). Despite these differences, each α-chain of C4bp still contains 8 SCRs, which are distinctive to the RCA family. The binding of C4bp to C4b is important to the regulation of the classical pathway C3 convertase (C4b2a). The binding of C4bp to C4b accelerates the natural decay of the C2a subunit from the C4b2a complex and functions as a cofactor for the cleavage of C4b into iC4b and C4d (Adams et al 1991).

1.4.2 CD59 or membrane inhibitor of reactive lysis (MIRL).

CD59 is not a member of the RCA gene cluster; it is located at the p13 region of the short arm of chromosome 11 (Heckl-Ostreicher et al 1993). Furthermore, CD59 does not share the SCR structural organization or functional similarities with the RCA gene cluster. It does have a GPI anchor like CD55, CD59's absence has also been implicated as a contributor to the pathology of PNH (Ohashi et al 1994, Fujioka and Yamada 1992, Yamashina et al 1990). CD59 has a wide tissue distribution (Meri et al 1991). It is a small 18 kDa, highly glycosylated protein (Holguin et al 1989, Davies et al 1989), and acts to inhibit complement activation. This occurs by inhibition of the incorporation of C8 and multiple C9 molecules into the terminal complement lytic complex, which results in abolition of the formation of the lytic pore (Rollins et al 1991). However, there is some controversy surrounding the initial findings of species restriction for the effectiveness of CD59's activity (Rollins et al 1991, Morgan et al 1994).

1.4.3 C8 binding protein (C8bp) or homologous restriction factor.

This is the least well characterized complement regulator, and its association with the other complement inhibitors by genomic location is unknown. C8bp was first isolated from red blood cells and found to have an Mr = 65 kDa (Schonermark et al 1986, Zalman et al 1986). C8bp was also found on monocytes and lymphocytes, and C8bp's sensitivity to PIPLC suggests that, like CD55 and CD59, it is attached to the cell surface by a GPI anchor (Hansch et al 1988). Furthermore, C8bp has also been reported to be absent on PNH red blood cells (Zalman et al 1987, Hansch et al 1988). More recently another group has identified a related protein they call membrane attack complex inhibitory protein (MIP), but whether this is the same as C8bp remains to be elucidated (Watts et al 1990).

Functionally, C8bp resembles CD59. C8bp blocks the formation of the membrane attack complex (MAC) by binding to C8, C9, or both (Schonermark et al 1986, Zalman et al 1986), and incorporation of C8bp into PNH cells reduced their susceptibility to reactive lysis (Zalman et al 1987, Hansch et al 1988). A urinary form of C8bp was also reported. The soluble C8bp appeared to lack the GPI anchor, but also appeared to inhibit the assembly of the MAC at a different site that the erythrocyte-bound form (Zalman et al 1989).

1.5. Complement-mediated cytolysis and virus-infected cells

Antibody-mediated destruction of virus-infected cells is an effective method for virus clearance by the immune system (reviewed in Sissons and Oldstone 1980). However, certain viruses have evolved evasion strategies which allow virus-infected cells to inhibit complement attack, or at least to decrease complement activation long enough to produce viral progeny. So far these viruses include vaccinia and a few members of the herpesviridae as discussed below.

1.5.1 Soluble vaccinia complement inhibitor.

Vaccinia produces a 35 kDa major secretory protein, which is structurally related to C4 binding protein and is so abundant it can be visualized in cell supernatants by staining of polyacrylamide gels with Coomassie blue. This protein was not found in attenuated strains of vaccinia, and was only found to be effective inhibiting activation of the classical pathway (Kotwal and Moss 1988, Kotwal et al 1990).

1.5.2 Herpesviridae complement inhibitors

The strategy evolved by members of the herpesvirus family is somewhat different; the complement inhibiting activity is found within virion envelope proteins. These are expressed on the surface of virus-infected cells and have additional functions important to infectivity. The Epstein-Barr virus (EBV) encodes a 350 kDa glycoprotein (gp350) which mediates the binding to the EBV receptor, complement receptor 2 (CD21, see below), accelerates the decay of the alternative pathway C3 convertase (but not the classical C3 convertase), and has cofactor activity for factor I (a serum complement regulating protein) which in turn mediates the cleavage of C3b to the inactive fragment C3dg and C4b to inactive C4c (Nemerow et al 1987, Mold et al 1988). It is interesting to note that EBV only infects B-cells (which express CD21), and that gp350 enhances the production of C3dg on the surface of EBV virions and EBV-infected cells. Thus the surface of EBV infected cells and EBV virions have two separate ligands, gp350 and C3dg, for the receptor, CD21. Similarly, herpes simplex type 1 and 2 (HSV-1 and HSV-2) have complement inhibiting functions which co-localize with the glycoprotein C (gC) a heparin-binding protein important to HSV virion attachment to permissive cells (Herold et al 1991). The complement inhibiting function of gC was identified as an ability to bind C3b and accelerate the decay of the alternative pathway C3 convertase (Harris et al 1990, McNearney et al 1987, Hatano et al 1988, Smiley et al 1985, Fries et al 1986). However, gC was found to lack both factor

I co-factor activity or classical C3 convertase decay accelerating activity (Fries et al 1986). Additionally, two other members of the herpesviridae which have proteins of high homology to HSV gC, porcine pseudorabies virus and bovine herpesvirus-1, were both found to have C3b binding ability and may have complement regulatory functions (Huemer et al 1993, Huemer et al 1992). However, two separate investigators have reported that HCMV does not have a C3b-binding protein (Smiley et al 1985) or activity like EBV's gp350 (Nemerow et al 1987).

1.5.3 Complement regulation on HCMV-infected cells

Even though an HCMV-encoded complement inhibiting protein has not been identified, HCMV infected cells still seem to have an enhanced ability to regulate complement. The serum levels of complement-fixing anti-HCMV antibodies increase following infection and are detectable for a prolonged period following HCMV infection (Spencer and Anderson 1972). Other studies have identified antigenic HCMV proteins which are expressed on the surface of infected cells early in infection (Amadei et al 1983). However, two separate groups have reported that serum from latently infected patients, which contain anti-HCMV antibodies and complement, are incapable of lysing HCMV-infected cells *in vitro* , except under special circumstances (Betts and Schmidt 1981, Middeldorp et al 1986). The special circumstances are met by the use of serum isolated from patients within the acute phase of HCMV infection, and this activity disappeared within 6-8 weeks after onset of infection. This activity was suggested by Betts and Schmidt (1981) to be caused by the presence of IgM class anti-HCMV antibodies. In contrast, Middeldorp et al (1986) found that cytolytic antibodies were distributed through both IgG and IgM class antibodies. This author attributed the cytolytic activity to the presence of antibodies binding to late membrane expressed HCMV proteins. However, under all conditions the cells could only be lysed 72 h post-infection (p.i.), by which time fibroblasts are producing infectious extracellular virus (McAllister et al 1963; see Figure 1.2). Furthermore, 20% of latently

infected patients were reported to have antibodies directed against membrane associated HCMV antigens (Middeldorp et al 1985), and in the samples which had cytolytic activity, binding of antibodies was observed transiently at 6 h p.i. and increased from 18 h p.i., long before the ability of the HCMV-infected cells to be lysed at 72 h p.i. (The and Langenhuysen 1972, Middeldorp et al 1985, 1986). This early binding of potentially cytolytic anti-HCMV without complement-mediated lysis until 72 h p.i., suggests the presence of a complement inhibitor.

1.6 Alterations in host protein expression induced by HCMV infection.

The most intensely studied alteration of protein expression induced by HCMV infection is the decrease in HLA class I; due to the reduced efficiency of cytotoxic T cell-mediated cytolysis of HCMV-infected cells, presumably caused by reduced cell recognition (Laubscher et al 1988, Warren et al 1994). Initially, HCMV infection of fibroblasts was reported to increase the expression of HLA class I (Grundy et al 1988), and β-2-microglobulin was reported to bind to HCMV virions (Grundy et al 1987a, McKeating et al 1987) and enhance HCMV infection of fibroblasts (Grundy et al 1987b). These initial findings resulted in the speculation that HLA class I was the cellular receptor for HCMV. However, shortly thereafter, numerous groups reported a decrease of HLA class I on the surface of HCMV infected fibroblasts (Yamashita et al 1993, Barnes and Grundy 1992, Gilbert et al 1992, Beersma et al 1993). The level of HCMV interference with HLA class I expression is thought to be after the transcriptional level since no alteration in the mRNA levels have been observed (Browne et al 1990). Early reports supported the hypothesis that a viral protein (H301, U_L18) bound and sequestered beta-2-microglobulin, thus preventing the normal association and expression of HLA class I chains (Stannard 1989, Tysoe-Calnon et al 1991, Browne et al 1990). However, deletion of the H301 (U_L18) gene from the HCMV genome did not abolish the HLA class I down-regulation (Browne et al 1992).

More recent reports identified an accelerated HLA class I heavy chain half-life, coinciding with infection by HCMV (Beersma et al 1993, Warren et al 1994). The steady state levels of HLA class I heavy chains (both free chains and those complexed with beta-2-microglobulin) were reduced, even though pulse-chase analysis indicated that initial synthesis was not affected (Beersma et al 1993). HCMV-infection was also accompanied by the loss of post-translational modifications associated with the cis/medial Golgi compartment, and the possible presence of a 'virus-assisted' HLA heavy chain-specific protease was therefore suggested. However, no alterations in the cellular levels of β-2-microglobulin were detected nor were novel β-2-microglobulin complexes with viral proteins seen when immunoprecipitating with antibodies specific for β-2-microglobulin.

Conversely, HCMV infection of cells also causes increase of total cellular mRNA as well as an increase of host gene products involved with DNA replication, including human thymidine kinase and hamster dihydrofolate reductase (Estes and Huang 1977, Benson and Huang 1990, Wade et al 1992). Other intracellular proteins found to increase with human HCMV infection include heat shock protein-70 and certain proto-oncogenes (Santomenna and Colberg-Poley 1990, Boldogh et al 1991, Boldogh et al 1990). HCMV infection of endothelial cells has also been found to rapidly increase the secretion of interleukin-6 (IL-6; Almeida et al 1994). HCMV infection of the monocytic leukemia cell line, THP-1, resulted in the increase in IL-1β mRNA levels (Iwamoto 1990), but not in secreted protein (Kline et al 1994); however, it did result in an increased secretion of an IL-1 receptor agonist. Lastly, HCMV-infection of endothelial cells also resulted in an increased polymorphonuclear cells and indirect evidence suggested that the increased adherence was mediated by an increase in ELAM-1 (Span et al 1991). However, the mechanism of adherence appears to be indirect, since the presence of anti-IL-1 antibodies partly inhibited this phenomenon.

This thesis investigates the influence of HCMV infection on the expression of the host-encoded complement inhibitors. The influence of membrane-bound inhibitors is emphasized, because under the conditions of the *in vitro* experiments mentioned above (Middeldorp et al 1986, Betts and Schmidt 1981), soluble factors would be removed and could not mediate the inhibition of complement. This does not exclude the possibility that HCMV infection influences the expression of soluble complement inhibitors and/or that the increased expression of soluble complement regulators would not be as effective at protecting HCMV-infected cells.

The overall goal of this work is to understand how HCMV-infected cells evade antibody-mediated C'-cytolysis. The elucidation of the interaction of HCMV with it's host cell may lead to the development of techniques to manipulate the patient's immune response to clear endogenous and exogenous HCMV and decrease the morbidity, mortality of HCMV-associated disease, and graft rejection. The specific aims of this thesis were directed to test the hypothesis that HCMV infection of host cells leads to an altered complement regulation on the cell surface. We set about testing this hypothesis by determining:

1) The degree of complement binding and neutralization of free HCMV virions.
2) The effect of viral infection on the surface expression of host-encoded complement inhibitors.
3) The effect of viral infection on the transcription of host-encoded complement inhibitors.

For these studies I utilized fibroblasts since they are the most commonly used cell type for *in vitro* experiments and relate directly to HCMV complement-resistance (Middeldorp et al 1986, Betts and Schmidt 1981). A second adherent permissive glioblastoma cell line, U373-MG, was also used to address cell specificity issues; and

finally, I used the THP-1 monocytic leukemia cell line because they are reported to be permissive for HCMV infection and represent the cell type most probably involved as the HCMV persistence reservoir (Weinshenker 1981).

2. MATERIALS AND METHODS:

2.1. Materials.

Bovine serum albumin (BSA, fraction five), EDTA (ethylene-diamine tetraacetate) and EGTA(ethyleneglycol-bis-(beta-aminoethylester) tetraacetic acid) were obtained from Sigma (St. Louis, MO). Scintillation cocktails (Ready Safe and Ready Prot.), and all ultracentrifuge rotors were from Beckman (Palo Alto, California). The scintillation cocktail used for the kinetic partitioning CAT assay, Econofluor, and all radioactive reagents were purchased from Dupont-NEN (Mississauga, Ontario) except [^{32}P]-labeled dCTP which was purchased from ICN (St. Laurent, Quebec). All tissue culture products were purchased from Gibco/BRL (Burlington, Ontario). Purified complement components: C3, factor B, and factor D were provided by Dr. Devine, and cobra venom factor was purchased from Jackson Immunoresearch (Avondale, PA).

2.2. Cells and viruses.

Primary explant fibroblasts were derived from human neonatal foreskin (HuFF), using the method described by Korn et al (1983), and cultured in DMEM (Gibco/BRL; Burlington, Ontario) supplemented with 100 U penicillin/ml , 100 mg streptomycin/ml , and 5% FCS. Primary fibroblasts were used between the second and ninth passage. The glioblastoma cell line, U373-GB, was obtained from American Type Culture Collection (ATCC: catalogue # HTB-17). These cells were cultured in MEM with non-essential amino acids supplemented with 1 mM sodium pyruvate, Earle's basic salt solution, 100 U penicillin/ml , 100 mg streptomycin/ml , and 5% FCS. The human epithelial cell line, HeLa, and the monocytic leukemia cell line, THP-1, were also purchased from the ATCC, and propagated in Eagle's DMEM supplemented with 100 U penicillin/ml , 100 mg streptomycin/ml , and 5% FCS.

The laboratory strain of human cytomegalovirus used for these studies was AD169, obtained from the ATCC. Patient isolates of HCMV were a gift of Dr. Eva Thomas, Children's Hospital, Vancouver, B.C. The identification of patient isolates as HCMV was made using both plaque assays and immunocytochemical assays at Children's Hospital, Vancouver, B.C. The KOS strain of Herpes Simplex-1 (HSV-1) was a gift from Dr. Frank Tufaro (University of British Columbia, Vancouver, B.C.). The Ad5 strain of Human Adenovirus was a gift of Dr. Wilf Jefferies (University of British Columbia, Vancouver, B.C.).

2.3. Antibodies.

The description and sources for murine monoclonal antibodies used in this study are listed in Table 1. Peroxidase-conjugated goat anti-rabbit IgG (used for Western blot analysis) or fluorochrome-conjugated (either phycoerythrin or FITC) goat anti-mouse IgG or anti-rabbit antisera (used for flow cytometry and indirect immunofluorescence analysis, respectively) were purchased from Jackson Immunoresearch (Avondale, PA). Western blot analysis of complement inhibitors on cells or virions utilized: the polyclonal rabbit anti-CD55 was raised using DAF isolated from RBC by the method of Nicholson-Weller et al (1982); the polyclonal rabbit anti-CD59, a gift of Dr. C.J. Parker (University of Utah); the polyclonal anti-CR1, a gift of Dr. B. Paul Morgan (University of Wales); and the monoclonal anti-CD46 (M75 - see Table 1).

Western blot analysis of complement components C3 and C9 utilized polyclonal antibodies raised in goats and purchased from Quidel (San Diego, CA) and detected with a peroxidase-conjugated anti-goat IgG antibody purchased from Jackson Immunoresearch (Avondale, PA). Western blot analysis of human IgG utilized a peroxidase conjugated primary antibody raised against human IgG heavy and light chains (Jackson Immunoresearch). Normal rabbit sera was used as a control in Western blot analysis and was obtained from Cedarlane Labs (Hornby, Ontario).

The control antibodies used in flow cytometry consisted of an isotype-matched monoclonal anti-MHC class II (Isotype IgG_{2a}; gift of Dr. W. Jefferies) or a mouse monoclonal antibody raised against Aspergillus niger glucose oxidase (isotype IgG_1, DAKO laboratories, Santa Barbara, CA), which is neither expressed nor inducible in mammalian tissues.

Table 1. Monoclonal antibody description and sources.

Antibodies	Ag recognized	Source[1]
1H4, 12G6, 11D7	CD55	Dr. W.F. Rosse
BRIC229	CD59	Dr. B.P. Morgan
MIRL1	CD59	Dr. C.J. Parker
3D9	CR1	Dr. C.J. Parker
M75	CD46	Dr. D.M. Lublin
J4-48	CD46	Dako Laboratories
W6/32	HLA class I	Dr. W. Jefferies
9.3F10	HLA class II	Dr. W. Jefferies
Cat #9466SA	C3d	BRL (Burlington, ON)

1. Affiliations of sources: Dr. W.F. Rosse - Duke University; Dr. B.P. Morgan - University of Wales; Dr. C.J. Parker - University of Utah; Dr. D.M. Lublin - Washington University; Dr. W. Jefferies - University of British Columbia. DAKO laboratories products are distributed from Santa Barbara, CA.

2.4. Serum samples.

Serum samples were obtained from healthy volunteers in our and neighboring laboratories. Samples were drawn by venipuncture using serum-separator type vacutainer tubes. The blood was allowed to clot at room temperature for 10 min. prior to centrifugation at 3000 xg. The serum was immediately removed and divided

into 250 µl aliquots into sterile eppendorf tubes. The serum samples were frozen immediately at -70°C and used within 4 months of collection to ensure that the complement activity in the samples remained optimal. Some aliquots were heat-inactivated at 56°C for 30 min. to remove the complement activity when required. Aliquots from all serum samples were also sent the Vancouver branch of the Canadian Red Cross transfusion and blood services to be tested for the presence of HCMV antibodies using the Abbott EIA total HCMV antibody test (Hamilton, ON). All serum samples were coded and all experiments performed blind as to the status of the presence of HCMV antibodies.

2.5. IgG depletion from serum samples.

The anti-HCMV antibody status of many of the individuals was measured at intervals of 4-6 months. Serum samples which contained anti-HCMV IgG antibodies, but were negative for anti-HCMV IgM were depleted of IgG, resulting in the removal of all anti-HCMV antibodies. These depleted sera were confirmed as seronegative by Abbott EIA. For IgG depletion, 700 µl of fresh serum was loaded on a 1 ml protein G-sepharose column which had been equilibrated with 20 mM sodium phosphate (pH=7.0). The serum was allowed to enter the column, the flow was then stopped and the serum allowed to incubated in the sepharose for 30 min. at 4°C. Flow through the column was resumed and 0.5 ml fractions collected; the depleted serum was easily identified by it's yellow color and the serum containing fractions were pooled and concentrated to the original 700 µl volume. The depleted serum was concentrated using a Amicon centrifugal concentrator with a molecular weight cut-off of 10 kDa to avoid losing the smaller complement components (i.e. factor D). The complement activity of the depleted serum was checked by the clinical hematology laboratory at University Hospital and found to be at the low end of the normal range (classical pathway=114-202, alternative pathway=92-152). The IgG retained by the protein G-sepharose column was eluted using a 0.1 M glycine buffer (pH=3.0), and 0.5

ml fractions collected. The absence of IgG in the depleted serum and presence of IgG in the eluted fractions was determined by Western blotting analysis (see below) using a peroxidase-conjugated polyclonal antibody directed against human IgG (see Figure 11 in results section).

2.6. Virus Infection.

The laboratory strain of human cytomegalovirus, AD169, was used to infect cell lines at a multiplicity of infection (MOI)=10 for investigations of complement inhibitor expression changes. Mock-infected controls consisted of cells incubated with UV-inactivated HCMV using conditions previously reported; briefly, half of the viral stock to be used for infection of cells was put in the UV-stratalinker (254 nm) for 15 min. on maximum irradiation just prior to incubation with cells (Nishiyama and Rapp 1980). UV-inactivated HCMV produced no plaques when subsequently tested by standard plaque assay techniques, and data collected for cells incubated with UV-inactivated HCMV was statistically indistinguishable from data collected for uninfected cells. Infection of fibroblasts with human adenovirus type 2 was carried out at an MOI=50, whereas infection with the KOS strain of herpes simplex virus-1 (HSV-1) was performed at an MOI=10. Culture conditions for adenovirus and HSV-1 were the same as those described above for HCMV.

Stocks for HCMV were propagated by infecting 150 cm^2 T-flasks containing fibroblasts at 85% confluency at an MOI<0.01, to minimize the production of non-infectious enveloped-particles and dense bodies (virions containing no HCMV DNA; Irmiere and Gibson 1983) and defective interfering particles (incomplete and incorrect HCMV genomes; Ramierez et al 1979, Stinski et al 1979), which leads to a decrease in infectivity. These flasks were harvested when 100% cytopathic effects (cell rounding and vacuolization) was observed, usually between 10-14 days p.i., with a cell scraper. Intracellular virions were released by passing the harvested cell debris through a 25-gauge needle three times. The cell debris was then removed by centrifugation of the

samples at 600 xg, leaving the virions in the supernatant. This supernatant usually contained roughly $5x10^6$ plaque-forming units (pfu) per ml, and was sufficient for viral infections of adherent cells. However, HCMV stocks could be further concentrated by centrifugation of the precleared supernatant at 20,000 xg for 1 hr at 4°C, and the magnitude of the concentration could then be controlled by the amount of media the pellet was resuspended in. Concentration of the HCMV stocks was necessary to attain the necessary MOI for the infection of THP-1 cells and as a preparatory step for HCMV virion purification, and the concentration procedure also served to remove any soluble factors (i.e. cytokines or interferons, etc.) in the media of the original stock and reduce extraneous variables. Propagation of the HSV-1 and human adenovirus stocks was performed by the labs from which they were obtained.

2.7. Standard Plaque Assay.

One confluent 100 mm dish of human fibroblasts was disaggregated using trypsin (0.25% bovine trypsin with EDTA, Gibco/BRL, Burlington, Ontario), resuspended in 12 mls of media and split between two 6-well dishes (Gibco/BRL, Burlington, Ontario). Next morning, the cells were usually at about 85% confluency, which is optimal for viral infection. The virus sample to be assayed was then serially diluted; 50 µl added to 450 µl media, the dilution was mixed, and 50 µl removed for addition to the next tube containing 450 µl media, etc. One hundred microliters of undiluted virus and each serial dilution were added to a corresponding labeled well in the 6-well dish (in duplicate). Unconcentrated virus stocks were tested from dilutions of 10^0 to 10^{-5} (effective dilution of 10^{-1} to 10^{-6}, taking into account the fact that only 100 µl was added to each well), while concentrated stocks were tested to dilutions as high as 10^{-8}. Mock-infected controls were also run in parallel to compare uninfected cell morphology. The 6-well dishes were incubated for 1 h in the cell incubator and rocked every 10 minutes to stop the cell layer from drying out. Following the incubation, the virus was aspirated and the unbound virus removed by

washing with 1 ml of PBS. The dishes were incubated at 37°C, 5% CO_2 for 10-14 days and plaque development was checked daily by inverted microscopy. To ensure the plaques formed were the product of the initial infection and not subsequent release of virus into the supernatant by initially infected cells, the cells were overlaid with DMEM with 10% FBS containing 1% agarose or overlaid with DMEM with 5% FBS containing 0.05 mg/ml of human IgG (containing high titers of anti-HCMV antibodies as determined by functional neutralization assay). Both of these methods were equally effective at stopping the formation of extraneous plaques. Visualization of the plaques was enhanced by fixing the cells for 10 min. at room temp with 5% formaldehyde in saline solution and then staining the cell layer with 0.05% methylene blue in distilled water for 30 min. After rinsing the cell layer with distilled water and allowing them to dry, the plaques were counted under an inverted microscope and appeared as dark blue cells exhibiting CPE against a pale blue background.

2.8. HCMV neutralization assay.

Six-well dishes of fibroblasts were prepared as stated above, but the amount of virus was kept constant for each well (roughly 40-80 pfu per well, calculated and diluted from known frozen HCMV stocks which had been previously plaque assayed). For each well, 125 µl of HCMV stock was placed in a sterile eppendorf tube. The control well, which yielded between 40 and 80 plaques was mixed 1:1 with media, while the other viral aliquots were mixed 1:1 with human serum samples (either samples of undiluted serum or samples of the serum serially diluted in cell media). Each serum sample was tested as undiluted (1/2 dilution since it was mixed 1:1 with virus) and also diluted by increments of 1/2 to a dilution of 1/1024. The samples were incubated with the virus aliquot for 30 min. at 37°C then duplicate 100 µl samples were taken from each tube and incubated with the cell layers in the 6-well dishes for 1 h. Controls were incubated with serum only to identify a potential presence of HCMV in the serum sample. Following the infection of the cell layers for

1 h at 37oC, the samples were aspirated and the unbound virus removed by rinsing the cell layers with PBS. The cell layers were then allowed to develop as listed for the plaque assay. Variations of the described neutralization assay were necessary for some experiments and these are identified in the results section.

2.9. Western blot analysis.

Whenever Western blot analysis was being used to compare protein expression amongst cell types, or between HCMV-infected and uninfected cells of the same type, the numbers of cells per lane were equalized to a common cell number. If comparisons were being made between cells and HCMV virions, the amount of protein per lane was equalized. Cells were disaggregated using 15 mM EDTA in phosphate-buffered saline (PBS) containing 30 mM sodium azide, centrifuged at 4000 xg, and resuspended in 100 µl of EDTA/PBS and counted using a hemocytometer. The cell concentration of all samples was equalized by increasing the volume of the most concentrated samples. An equal amount of 2X non-reducing sample buffer (0.12 M Tris (pH 6.8), 4% SDS, 20% glycerol, and 0.1% Triton X-100) was added to each sample and the cells were lysed by repeated freeze-thaw cycles, followed by heating in a boiling water bath for 5 min. prior to loading on the SDS-polyacrylamide gel (SDS-PAGE; Laemmli, 1970). Whenever polyacrylamide gels were being used to investigate the presence of complement components on the surface of HCMV virions, each lane was loaded with an equivalent amount of protein or an equal volume if the protocol had initially equalized the amount of virions and serum. Furthermore, when complement components were being investigated, the gels were run under reducing conditions (the 2X sample buffer contained 10% β-mercaptoethanol).

Four different concentrations of polyacrylamide were used for the lower resolving portion of the gel depending on the size of the protein being investigated: 5%, 7.5%, 10%, and 15% (in order for larger proteins to smaller proteins). The lower resolving portion of the SDS-PAGE always contained the same concentrations of SDS

(0.01%), Tris (pH=8.8) (0.375 M), ammonium persulfate (0.01%), and TEMED (0.005%). Regardless of the polyacrylamide concentration of the resolving portion of the gel, the upper stacking portion of the gel was constant: 3.3% polyacrylamide with 0.26 M Tris (pH=6.8), 0.1% SDS, 0.1% ammonium persulfate, and 0.005% TEMED.

Amersham rainbow-prestained protein mass standards (Oakville, ON) and BRL prestained protein mass standards (Burlington, ON) were concomitantly run on each gel and used to estimate the relative molecular mass of the proteins being investigated. The gels were run at a constant 30 mA per side until the bromophenol blue stain, from the sample buffer, was just above the end of the gel. The gels were then removed and equalized for 30 min. in Western blot transfer buffer (25 mM Tris, 192 mM glycine, and 20% (v/v) methanol [pH=8.4]) and then electrophoretically transferred (1.0 Amp Hour) to nitrocellulose paper. After blocking overnight with 3% powdered milk and 3% BSA in Tris-buffered saline containing 0.05% Tween 20 (TBST), the membranes were incubated with polyclonal or monoclonal antibodies (in TBST) directed against the protein of interest. The primary antibody was detected by a subsequent incubation with a horse radish peroxidase-conjugated secondary antibody (directed against the heavy and light chains of the immunoglobulin subclass for the species the primary antibody was raised in) after the excess primary antibody was removed by washing 3X in TBST. Excess secondary antibody was removed by three washes in TBST, and immunoreactive bands were detected with the Amersham ECL chemiluminescence Western blot detection kit and Amersham Hyperfilm X-ray film (Oakville, ON). Background staining patterns were always determined by pre-probing the Western blot with the appropriate secondary antibody before the primary antibody and any bands observed (usually none) were noted.

2.10. Complement component co-purification with HCMV virions.

The specific association of complement components with purified HCMV virions was determined by incubating serum samples with virions under varying

conditions. HCMV virions were purified from cell fragments by discontinuous sucrose gradient centrifugation. The HCMV stocks used for the purification were generated by inoculating 5 large T-flasks at an MOI<0.01, allowing the monolayer to reach 100% CPE, and concentrating the virus down to 1 ml using the protocol listed in the virus propagation section above. The discontinuous sucrose gradient was made by layering 2 mls of each of the following in a 12 ml polyallomer tube, in order: 60%, 50%, 40%, 30%, and 20% (w/w) sucrose in Tris-buffered saline (TBS) (12.5 mM Tris (pH=7.4), 0.88% sodium chloride). The concentrated HCMV stock was resuspended in 1 ml of reticulocyte standard buffer (10 mM Tris (pH=7.4), 10 mM KCl, 1.5 mM $MgCl_2$, 0.25 M sucrose) and 0.5 ml was loaded per discontinuous gradient. The gradients were centrifuged in a Beckman SW30Ti rotor at 18,000 rpm for 2 h at 4°C, and the HCMV virions were found as an opalescent band at the interface between the 50% and 60% sucrose layers. The band was extracted using a 3 cc syringe and a 20-gauge needle, and resuspended in 3.5 mls TBS and the virions were pelleted at 32,000 rpm for 30 min. at 4°C using a Beckman SW50.1 rotor. As a control, an equal number of fibroblasts used for initial virus propagation were disrupted by passing the cells 3X through a 25-gauge needle, then treating the resultant suspension as though it were the supernatant containing HCMV from a viral propagation; even though no bands were seen on the discontinuous sucrose gradient nor any pellet in subsequent centrifugation steps, the samples were collected at the same level and treated identical to those samples containing HCMV virions (cell lysate control).

For optimal complement activation conditions, the virion pellet was resuspended in veronal-buffered saline (GVB; 5.0 mM sodium barbitol, 0.15 M NaCl, and 0.2% gelatin [pH 7.5]) containing 2.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$ (GVB^{2+}). One hundred microliters containing $5x10^6$ pfu of HCMV virions in GVB was incubated with each of the following: 100 µl of GVB^{2+}, 100 µl HCMV-seropositive serum, 100 µl seropositive serum with 15 mM EDTA or 15 mM EGTA, 100 µl seropositive serum depleted of IgG, and HCMV-seronegative serum. A control

containing only HCMV-seropositive serum and 100 µl GVB was also included to ensure the post-incubation washes removed the unbound complement components. All mixtures were incubated for 30 min. at 37°C then 3.5 ml of GVB without cations containing 15 mM EDTA (GVB-EDTA) was added and the virions pelleted by centrifugation at 32,000 rpm in a Beckman SW 50.1Ti rotor for 30 min. The pellet was resuspended in GVB-EDTA and the centrifugation procedure repeated. The pellet was then resuspended in RSB and the non-specifically associated complement components were removed from the HCMV virions by layering the mixture on top of a 30% sucrose pad (3.5 ml) and centrifuging at 32,000 rpm in a Beckman SW 50.1Ti rotor for 1 h. This last wash step was repeated three times; the percentage of sucrose required and the number of washes required to remove the unbound complement components were empirically determined in the preliminary studies by using 100 µl of seropositive serum, without HCMV virions. Following the wash steps the resulting pellet was resuspended in 100 µl of 2X reducing sample buffer and 25 µl for each sample was used for SDS-PAGE and Western blot analysis using polyclonal antibodies raised in goats against human IgG or human complement components C3 or C9.

2.11. Immunocytochemistry.

For immunofluorescence studies on the glioblastoma cell line, U373-MG, and primary fibroblasts, one hundred to 200 cells were grown overnight at 37°C on acid-etched coverslips. Polyclonal antibodies were used at a concentration of 10 µg/ml, the monoclonal anti-HLA class I was used at 5 µg/ml, and the fluorescein-conjugated anti-mouse or anti-rabbit IgG secondary antibodies were used at a concentration of 1 µg/ml. All procedures were carried out at 4°C and all solutions were filtered prior to the addition of antibodies and contained 30 mM sodium azide to prevent antibody patching and capping. No aldehyde fixative can be used for the investigation of CD55 expression since this completely abrogates antibody binding to this antigen.

Background staining was determined using normal rabbit serum or the monoclonal antibody directed against HLA class II as the primary antibodies. Cells to be examined by epifluorescence were washed twice with phosphate-buffered saline (PBS; pH=7.4) then with PBS containing 1% bovine serum albumin (BSA). Primary and secondary antibody incubations were for 30 min. and separated by three rinses with PBS/1% BSA. Following the secondary antibody incubation, the cells were rinsed once with PBS/1% BSA, once with PBS, mounted, and photographed with a Zeiss Axiophot microscope equipped with epifluorescence optics.

2.12. Flow cytometry studies.

Adherent cell lines were disaggregated by incubation with 15 mM EDTA at 4°C and suspended in cold flow cytometry (FC) solution (1% BSA, 15 mM EDTA, 15 mM sodium azide in PBS). Exposure to EDTA did not interfere with antibody recognition of CD55, CD46, CD59, CR1 or HLA class I. All procedures were carried out at 4°C and all solutions contained 30 mM sodium azide to prevent endocytosis or antibody patching and capping. Briefly, the cells were rinsed in the FC solution, centrifuged for 4 min. at 4,000 rpm in a Beckman TLA-100 centrifuge, and resuspended in FC solution at $5x10^6$ cells/ml. Fifty microliters of cells were incubated for 30 min. with an equal volume of primary monoclonal antibody. The saturating amounts of each monoclonal antibody were predetermined for each cell line. Following three washes in FC solution, cells were incubated in fluorescent-labeled secondary antibodies. After the unbound secondary antibodies were removed, cells were analyzed on an EPICS Profile flow cytometer and using Cytologic software (Coulter, Hialeah, FL). Non-specific antibody binding was determined by using monoclonal anti-MHC class II as the primary antibody (5 μg/ml) or the monoclonal control antibody directed against a non-mammalian protein (see antibody section; 1 μg/ml). Repeated measures analysis of variance (ANOVA) was used to initially determine statistically significance. One-way ANOVA and post-hoc analysis using

Fisher's least significant test and Bonferroni's correction for multiple comparisons was used to identify differences between groups.

2.13. Northern blot analysis.

Total RNA was extracted from HCMV-infected or uninfected cells at varying times post-infection using the guanidium isothiocyanate method (Turpin and Griffiths, 1986). Briefly, monolayers of fibroblasts or glioblastoma cells, which were uninfected or infected with HCMV (MOI=10), were grown in three 150 mm for each condition investigated (i.e. mock- vs. HCMV-infected) and harvested at various times. At the time of harvest, the monolayers were washed once with PBS and 7 mls of guanidium isothiocyanate solution (4.5 M guanidium isothiocyanate, 5 mM Na-citrate pH 7.0, 0.1 M 2-mercaptoethanol, 0.5% sarkosyl) was added to each of the three monolayers sequentially so that total volume did not exceed 8.5 mls; all residuals were collected by scraping. The cells were disrupted by three passages through a 25-gauge needle. The cell lysate is layered on top of 4 mls of 5.7 M cesium chloride containing 50 mM EDTA (pH=7.4), which is in an 11 ml polyallomer quickseal tube. The tubes were balanced, sealed, and centrifuged at 60,000 rpm for 6 hours at 20°C in a Beckman near vertical rotor. The RNA pellet was resuspended in 10 mM Tris-HCl (pH=7.4) containing 2 mM EDTA and 0.5% SDS, then extracted twice with 12 mls of 1:4 butanol:chloroform. The aqueous phase was then ethanol precipitated with 12 mls of 100% ethanol following the addition of 1/10 vol. of 3.0 M Na acetate. The total cellular RNA pellet was then resuspended in 100 µl of distilled water and the RNA concentration measured at 260 nm. RNA was stored at -70°C after the sample had been adjusted to a constant RNA concentration of 10 µg/µl.

Thirty micrograms (3 µl) of total RNA were separated on a formaldehyde (6%)-agarose (1%) gel after being mixed with 5 µl RNA sample buffer (55 mM MOPS, 6% formaldehyde, 5% glycerol, 0.1 mM EDTA, 0.04% bromophenol blue, 0.04% xylene cyanol) and being denatured at 65°C for 15 min. Total RNA from HeLa cells (10 µg)

was included as the positive control for CD55. The RNA was osmotically transferred to non-charged nylon membranes using 3 M NaCl/0.3 M Na-citrate, and hybridized with radiolabeled RNA anti-sense probes specific for CD46, CD55 or CD59 mRNA. Radio-labeled anti-sense probes were made using a random primer kit (Bethesda Research Laboratories, Burlington, ON) and [^{32}P]-labeled dCTP (ICN; St. Laurent, Quebec). Plasmids containing CD55 cDNA were provided by D.M. Lublin, Washington University, plasmids containing CD59 cDNA were provided by W.F. Rosse, Duke University, and plasmids containing CD46 cDNA were provided by J.P. Atkinson, Washington University.

2.14. Radio-immunoprecipitation studies.

Monolayers of fibroblasts growing in 60 mm dishes were infected with HCMV at a MOI=10, while control cells were uninfected. After 1 h the virus was removed and replaced with complete medium. At 24 h p.i. cells were washed three times with cysteine-free medium and labeled for 50 min. with 100 μCi/ml [35S]-cysteine (1117 Ci/mmol) in cysteine-free medium containing 5% dialyzed FCS. At the completion of labeling, cells were either harvested immediately or washed three times and incubated in DMEM with 10% FBS (which contained excess cold cysteine) for 2 or 4 h. Cells were harvested by washing the monolayer with cold PBS and incubated for 15 min. with 1 ml cold lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Nonidet P-40 (NP-40), 1% Na-deoxycholate).

Immunoprecipitations were performed by first adding 1/10 volume of a mixture of 10% NP-40, 10% Na-deoxycholate, and 1% SDS to aliquots of cell lysates to be precipitated. The samples were precleared by incubation with protein G-sepharose (pre-washed in 20 mM phosphate (pH=7.4), containing 2% BSA) while rocking at 4^{o}C for 2 h. The protein G-sepharose was pelleted by using a benchtop Beckman centrifuge, the pellet was saved as the preclear control, and the supernatant transferred to a new tube. Ten microliters of rabbit polyclonal anti-CD59 antibody

was added to each sample and incubated, while rocking at 4°C, for 2 h; at which time protein G-sepharose was added to capture the anti-CD59 antibody as described above for preclearing. All protein G-sepharose pellets (including the preclear controls) were washed with 0.5 ml of the following solutions; wash buffer #1 (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40), wash buffer #2 (20 mM Tris-HCl pH 8.8, 150 mM NaCl, 1% NP-40, 0.2% SDS), wash buffer #3 (20 mM Tris-HCl pH 6.8, 150 mM NaCl, 1% NP-40, 0.2% SDS). The final pellet was resuspended in 2X reducing sample buffer, heated at 100°C for 5 min. and separated by SDS-PAGE. Gels were fixed for 45 min. in 7% acetic acid containing 25% methanol, dried, and exposed to Kodak XAR-5 film for autoradiography (usually between 3 days and 2 weeks).

2.15. Alternative pathway C3 convertase activity assay.

The protocol used to measure C3 convertase activity was a modified version of one previously published for platelets (Devine et al 1987). HCMV-infected or uninfected cells were harvested and incubated with 30 mM NaN_3, 30 mM 2-deoxy-D-glucose, 10 mM gluconic acid-delta-lactone in PBS to block cell metabolism. Equal numbers of infected or uninfected cells were suspended in magnesium-free GVB (0.2% gelatin in veronal-buffered saline), 1 mg of C3, and 200 µl of preformed fluid phase C3 convertases (cobra venom factor (CoVF) factor B, and factor D). After 30 min. at 37°C, fluid phase C3 convertases and any unbound C3 were washed away, and half of the cells were removed for quantitation of baseline C3 deposition. The cells were resuspended in GVB with 5 mM $MgCl_2$; 50 µl factor B and 50 µl factor D were added and incubated for 10 min. at 37°C. One milligram of C3 was added and the incubation was stopped after 1 h at 37°C by the addition of 20 mM EDTA. After washing, the amount of cell-bound C3 on all cell preparations was determined by flow cytometry using monoclonal anti-C3d (Gibco/BRL, Burlington, ON). In the absence of factor B, anti-C3d binding was equivalent to that seen with non-immune antibody controls. Data were pooled for time points between 36 and 72 h p.i. (N=4),

as no significant trend could be established between these time points. Statistical significance was determined by one-tailed paired t-tests.

2.16. CD55 promoter constructs.

The CD55 bacteriophage genomic clones were previously identified, mapped, and partially sequenced (Post et al 1990). A 4.6-kb HindIII fragment encoding the 5'-flanking region, 5'-untranslated region/signal peptide, and part of short consensus repeat 1 was subcloned into pBluescript KS (Statagene, San Diego, CA). Subsequent restriction digests and manipulations were performed as described in Thomas and Lublin (1993), and I received the following 5'-deletion constructs of the CD55 promoter from Dr. DM Lublin who is located at Washington University (all of which contained the transcriptional start site and 84 additional base pairs of the transcribed region; see figure 34): -2800, -796, -206, -77, -54, and -36. The basal promoter activity of all of these, except the -2800 to +84 construct, were previously reported when transfected into K562, EBV-transformed B-cells, Molt 4 and HeLa cells (Thomas and Lublin, 1993). I created two additional 5'-deletions from the -796 to +84 construct to investigate the large region left between the -796 and -206 from the received constructs. The CD55 promoter had been inserted at the BssH II restriction site in the SP65 polylinker region, which meant the Pst I and Xba I (Gibco/BRL, Burlington, ON) restriction sites from the SP65 polylinker region were located between the +84 end of the CD55 promoter and the start of the 5'-end of the chloramphenicol acetyl-transferase (CAT) reporter gene. By removing the Pst I and Xba I restriction fragments from the -796 to +84 CD55 promoter in SP65-CAT plasmid, then inserting the isolated fragment into a SP65-CAT plasmid (devoid of previous CD55 promoter insertions); the -425 to +84 and -275 to +84 CD55 promoter constructs were created. The restricted fragments were isolated by separating the cut plasmid on a 1% agarose gel, staining the gel with ethidium bromide, excising the band of appropriate size under UV-light visualization, purifying the DNA using the Geneclean purification

system (Gibco/BRL, Burlington, ON), and ligating the fragment into SP65-CAT (pre-cut with the appropriate restriction enzyme) with T4 ligase (Gibco/BRL, Burlington, ON). Since two orientations were possible, the correct orientation of single colony plasmid DNA was identified by Sac I restriction enzyme (Gibco/BRL, Burlington, ON) digest after separation on a 1% agarose gel and staining with ethidium bromide. The SP65-CAT plasmid contained the ampicillin resistance gene so all plasmids were propagated in transformed DH5α E. coli (subcloning efficiency) grown in 2X YT media (1.6% tryptone, 1.0% yeast extract, 0.5% NaCl) containing 100 μg/ml ampicillin, or on plates made from 2X YT media containing 1.5% agar and 100 μg/ml ampicillin.

2.17. Other plasmids.

The β-galactosidase (β-gal) or Lac-Z gene in the Rc/CMV plasmid (Invitrogen, San Diego, CA) was a gift from Dr. X. Wu (University of British Columbia) and was utilized to measure the efficiency of transfection. The isolated HCMV immediate early (IE) genes were obtained from various sources: Plasmids containing the UL 36, UL37, UL38 and UL36-38 IE genes were a generous gift of Dr. AM Colberg-Poley (George Washington University, Washington D.C.) and are described in Colberg-Poley et al (1992). Plasmids containing the IE1, IE2, and US3 IE genes were a generous gift of Dr. R Ruger (Boehringer Mannheim, Penzberg, Germany) and are described in Colberg-Poley et al (1992).

2.18. Plasmid transfection into cells.

Plasmid DNA to be used for transfection was purified from RNA, proteins, and bacterial DNA by banding the plasmid DNA in 1.2 g/ml cesium chloride by centrifugation at 100,000 rpm at 25°C in a Beckman near-vertical rotor in a Beckman benchtop ultracentrifuge. The bands were visualized by pre-addition of ethidium bromide; after extracting the band with a 20-gauge needle and 3 cc syringe, the ethidium bromide was removed by 3 consecutive n-butanol extractions followed by

ethanol precipitation. The DNA concentration was measured at 260 nm and the DNA concentration standardized to 1 μg/μl prior to storage at -70°C. The glioblastoma, fibroblast, and HeLa cells grown in 6-well dishes at 70% confluency were transfected using the calcium chloride method: 5 μg plasmid DNA was co-precipitated with 20 μg of salmon sperm DNA in HEPES-buffered saline (pH=7.0) with 2 M $CaCl_2$ (per well). The cells were shocked with 20% glycerol in HEPES-buffered saline (pH=7.0) 12 h later, and some of the glioblastoma cells and fibroblasts were infected at 24 h post-transfection for 1 h. For transient co-transfection assays investigating, 5 μg of each plasmid was added to the transfection mixture (including a plasmid containing the β-gal gene), no super-infection by HCMV was performed, and cells were harvested at 48 h post-transfection for measurement of CAT activity or flow cytometry. The cells were then harvested 48 h after being glycerol shocked and the intracellular CAT activity and mock controls were cells treated identically, but the 5 μg of plasmid DNA was replaced with sterile water.

2.19. Measurement of CAT activity.

The intracellular CAT activity, which could only have originated from the plasmid, since mammalian cells do not naturally contain this gene, was assayed using a kinetic partitioning assay (Neumann et al, 1987). The cells were harvested by trypsinization and each well was resuspended in 1 ml PBS, put in a 1.7 ml eppendorf tube, and pelleted by centrifugation in a benchtop Beckman centrifuge. Each cell pellet was resuspended in 100 μl of PBS and the intracellular CAT was released by three cycles of freeze-thaw using a 100% ethanol/dry ice bath and a 37°C water bath. The cell debris was cleared by centrifugation (15,000 rpm in a Biofuge 17 centrifuge) and 30 μl of each sample was put in a scintillation vial. After mixing each sample with 210 μl of the CAT reaction mixture (100 mM Tris-HCl (pH=7.8), 1.0 mM [^{14}C]-acetyl CoA (0.1 μCi), and 1.0 mM chloramphenicol) and 5 ml of the hydrophobic scintillation fluid, Econofluor (Dupont NEN research products, Boston, MA), was

carefully layered on top of the reaction mixture. Since the covalent attachment of the chloramphenicol to the [^{14}C]-acetyl-CoA makes the latter more hydrophobic, the basic principle of this assay utilizes the fact that only the covalently linked [^{14}C] will enter the hydrophobic scintillation fluid and be detected by the scintillation counter. Therefore, by counting the vials at regular intervals and graphing the cpm as a function of the accumulated time for that count, the amount of CAT present in the sample is directly proportional to the slope of the graph. This technique also allowed the investigators to measure the activity in the linearly increasing portion of the reaction and avoid artifactually low readings from points at which the concentration of the substrates became limiting. CAT activity measured for each sample was performed in triplicate, and each finding was replicated at least three times.

2.20. β-gal activity assay.

For all transient co-transfection assays and transfected cells used for flow cytometry, parallel cell cultures were also created to investigate the transformation efficiency. The monolayers were grown in 6-well dishes and the cells were washed twice with PBS prior to fixation with 4% formaldehyde and β-gal activity investigated. Cells containing the β-gal gene turn blue when incubated overnight with PBS containing 2 mM MgCl, 10 mM $FeCN_2$, 10 mM $FeCN_3$, and the chromogenic substrate for β-gal, 1 μg/μl (0.1%) X-gal (Gibco/BRL, Burlington, ON). The transfected cell monolayer was observed under the microscope: the total number of cells and number of blue cells were counted for 10 fields, and the transfection efficiency estimated from the ratio of these two numbers.

3. RESULTS:

3.1 CHAPTER 1. Complement effects on HCMV virions.

The presence of complement has been reported to enhance the ability of specific anti-HCMV antibodies to neutralize HCMV virions, as detected by plaque assay (Lewis et al 1986, Eizuru et al 1988). However, the role of complement alone in HCMV neutralization has not been addressed, nor has the pathway responsible for the enhanced antibody neutralization. These questions will be addressed in this chapter.

3.1.1 The role of complement in neutralization of HCMV virions.

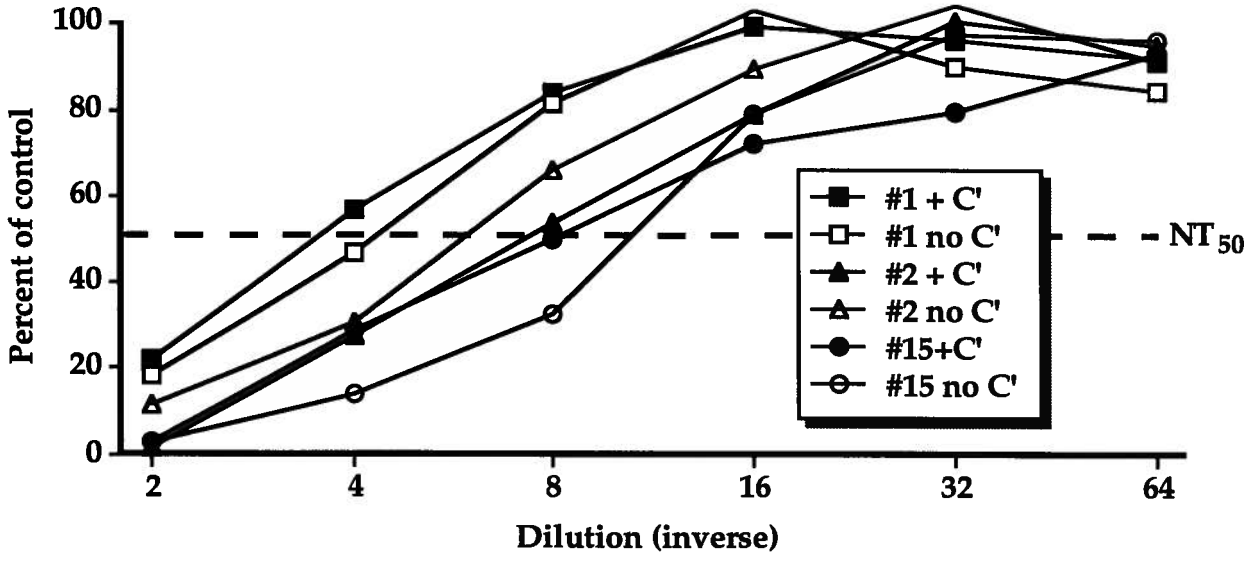
The first set of experiments were designed to determine the role of complement in the neutralization of HCMV. Most of the experiments in this section utilize a variation of the standard plaque assay, called the neutralization assay. In this assay the amount of virus was kept constant (roughly 60-80 plaques per 9.6 cm^2) but the human serum mixed and incubated with the virus was serially diluted. Therefore, the more neutralization activity present in the serum the greater the serum dilution required before plaques were observed. The dilution of serum which yielded half the plaques compared to virus mixed with media only is referred to as the NT_{50}, and was reported as the inverse of the dilution (i.e. a dilution of 1/64 is reported as 64). The presence of anti-HCMV antibodies was also measured on the same samples by the Abbott EIA method at the Vancouver branch of the Canadian Red Cross Blood Transfusion Services. Interestingly, incubation of seronegative serum with virus resulted in NT_{50} ranging from <2 to 8 (3 representative samples of more than 10 experiments shown in Figure 4). Heat-inactivation of the complement in seronegative serum samples from donors #1, 2, 7, and 15 did not effect the results, nor did the addition of rabbit complement to sample #7 (Figure 4 and 5), which suggests these observations cannot be attributed to the effect of complement. The NT_{50} values for the seropositive individuals #9, 20, 26, and 27 ranged from 64 to >256 (Figure 6 and 7),

but unlike the seronegative samples, heat inactivation of the serum sample #9 decreased the neutralization activity and adding rabbit complement to sample #9 increased the activity greatly (Figure 7). The difference between the effects of the intrinsic complement and the added rabbit complement likely represents the relative dilution of the intrinsic complement, coincident with the dilution of the antibodies.

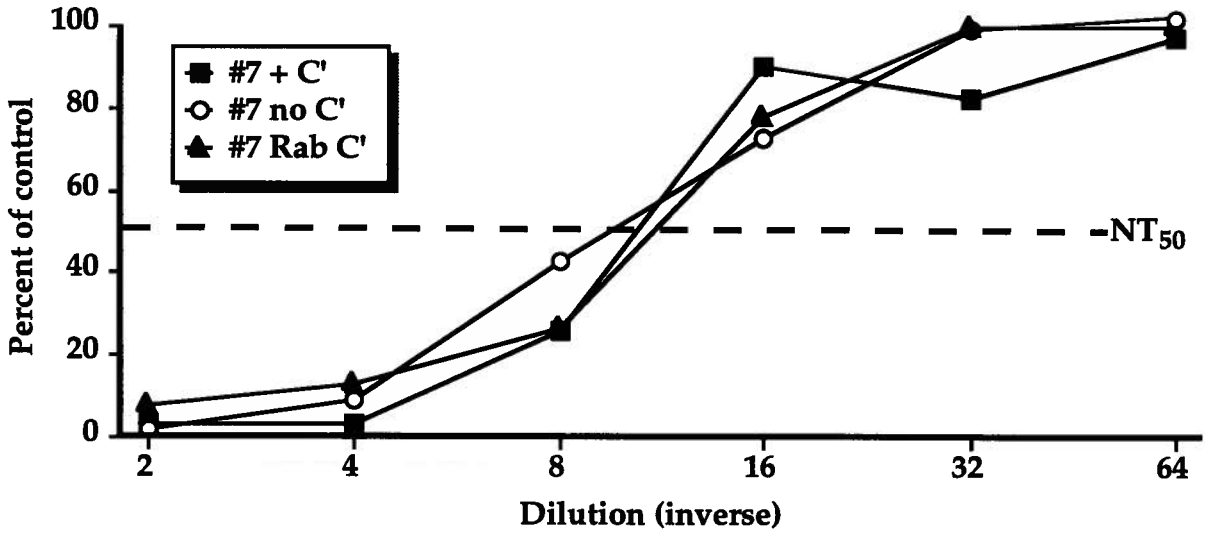
By adding an equal amount of undiluted serum to an aliquot of a viral stock, the difference in neutralizing activity between seronegative and seropositive serum samples is better illustrated by standard plaque assay (Figure 8). A twenty percent decrease was observed when seronegative serum was mixed with the HCMV stock compared to a 3800-fold decrease when mixed with seropositive serum. Again heat-inactivation of complement did not vary these effects in the seronegative sample; however, heat-inactivation of the seropositive serum decreased the neutralization by half. In a separate experiment using a different set of donors, depleting the IgG from the seropositive sample using protein G-sepharose reduced that serum sample's ability to neutralize HCMV to the level of a seronegative serum sample.

It is important to note that the HCMV serological status for all individuals was gauged by their Abbott EIA test results. There may be some limitations for this test since it is a first generation ELISA. Repeat testing of all individuals before and after assured that the individuals were not in the process of seroconverting, since the Abbott EIA is designed to detect the IgG class of immunoglobulins. More sensitive, specific peptide ELISA tests are available now, but these tests have limitations as well, and none of the assays available are very good at determining the presence of weakly cross-reacting antibodies directed against other herpesviruses. However, the similarity between seronegative serum and the IgG-depleted serum in the pages that follow provide the best argument for the accuracy of the Abbott EIA determinations.

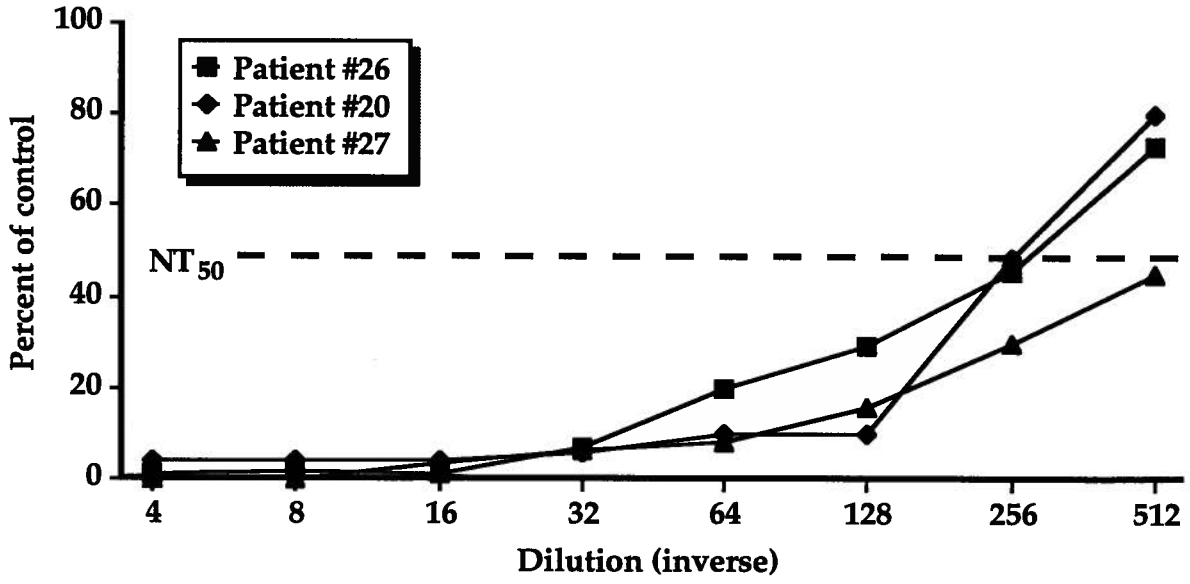
↓ **Figure 4. Serum samples obtained from three individuals (coded as #1, #2, and #15),which were determined to be negative for the presence of anti-HCMV antibodies by the Abbott EIA method, were subsequently used in a functional HCMV neutralization assay. The number of plaque-forming units present, after a 30 min. incubation of a virus aliquot with serial dilution for each serum, was expressed as a percentage of the number of plaques formed when only tissue culture media was added to the HCMV aliquot. The percentage of plaques formed is compared to the final dilution of serum used in the graph below. Open symbols represent the same samples tested after the intrinsic complement components were heat inactivated (56°C, 30 min.). Each point represents the average determined on two aliquots of the same sample run simultaneously.**



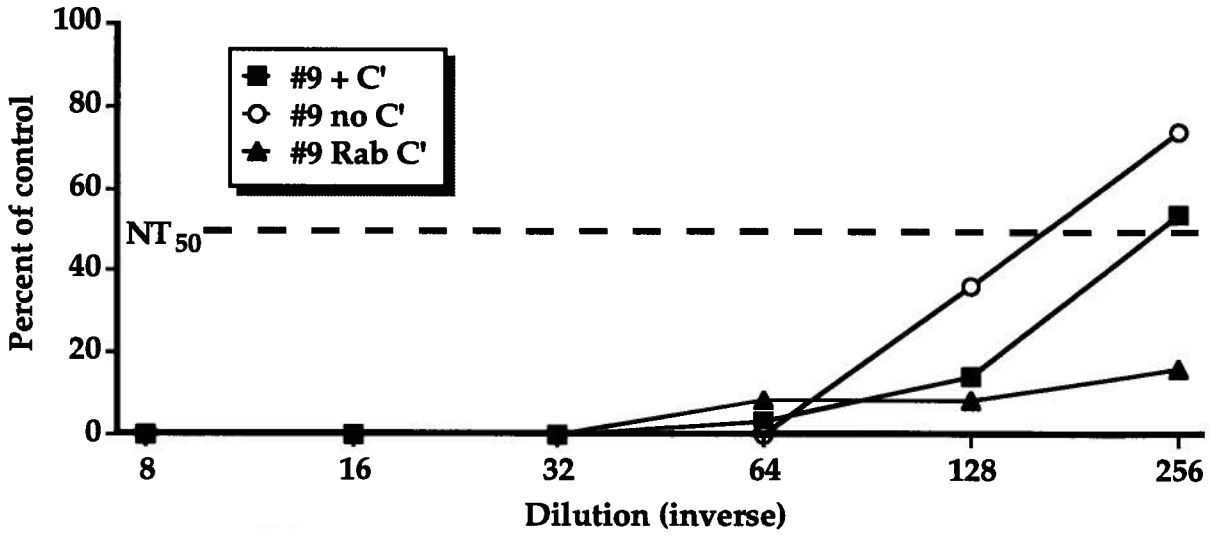
↓ Figure 5. Using the same neutralization assay in figure 4, HCMV aliquots were incubated with serial dilutions of another serum sample (found to be seronegative by the Abbott EIA method). The variables tested include neutralization activity with the intrinsic complement activity left intact (closed square), neutralization activity with the intrinsic complement activity removed via heat inactivation (open circle), or neutralization activity in the presence of additional rabbit complement (to a final concentration which is two-fold higher than the intrinsic complement concentration; closed triangle). Each point represents the average determined on two aliquots of the same sample run simultaneously. Identical results were obtained when seronegative serum samples #42 and #15 were used.



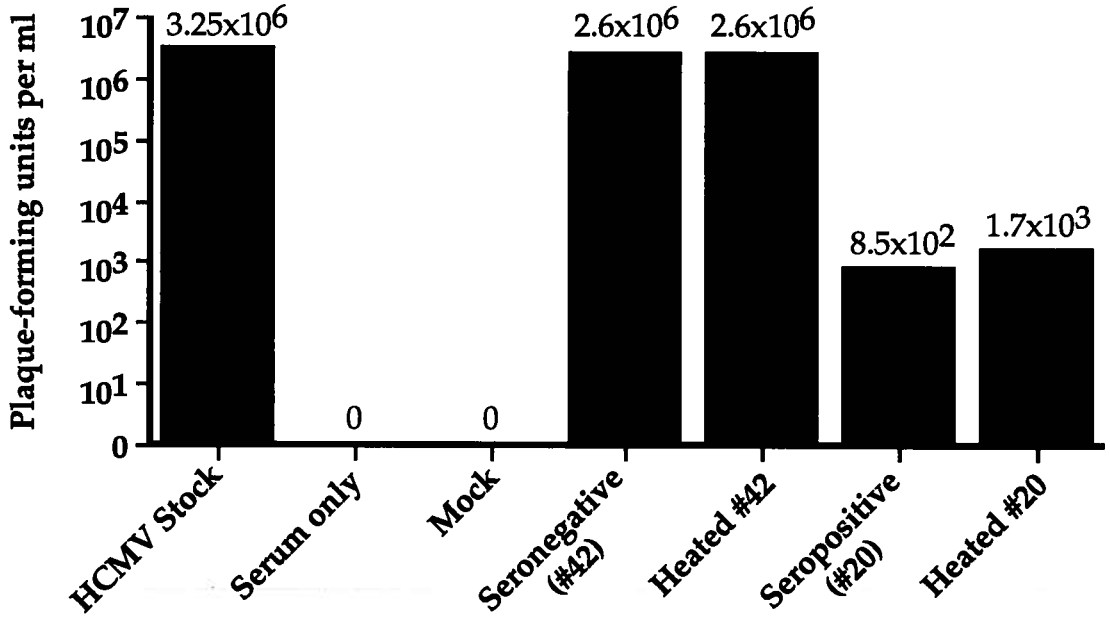
↓ **Figure 6. Serum samples obtained from three individuals (coded as #20, #26, and #27) were determined to have anti-HCMV antibodies by the Abbott EIA method and were subsequently used in the HCMV neutralization assay outlined in Figure 4. The graph below demonstrates the percentage of plaques formed as a function of the dilution of serum. Heat inactivation of intrinsic complement components was not used as a variable in this experiment because of the high dilution factors involved. Each point represents the average of two aliquots of the same sample run simultaneously.**



↓ Figure 7. Using the same neutralization assay in figure 4, HCMV aliquots were incubated with serial dilutions of another serum sample (found to be seropositive by the Abbott EIA method). The variables tested include neutralization activity with the intrinsic complement activity left intact (closed square), neutralization activity with the intrinsic complement activity removed via heat inactivation (open circle), or neutralization activity in the presence of additional rabbit complement (to a final concentration which is two-fold higher than the intrinsic complement concentration; closed triangle). Each point was determined in duplicate and identical results were obtained when seropositive serum sample #26 was used.



↓ **Figure 8. A standard plaque assay was performed on an HCMV stock after aliquots of HCMV had been incubated with an equal volume of medium (viral stock), seronegative serum (#42), or seropositive serum(#20). The effect of the intrinsic complement was determined by heat-inactivating (56°C, 30 min.) both serum samples in a parallel incubation (labeled as ɔheated). The presence of HCMV in the serum samples was also determined and found to be the same as the mock infection. Each value was determined in duplicate and the actual values are given at the top of each bar.**



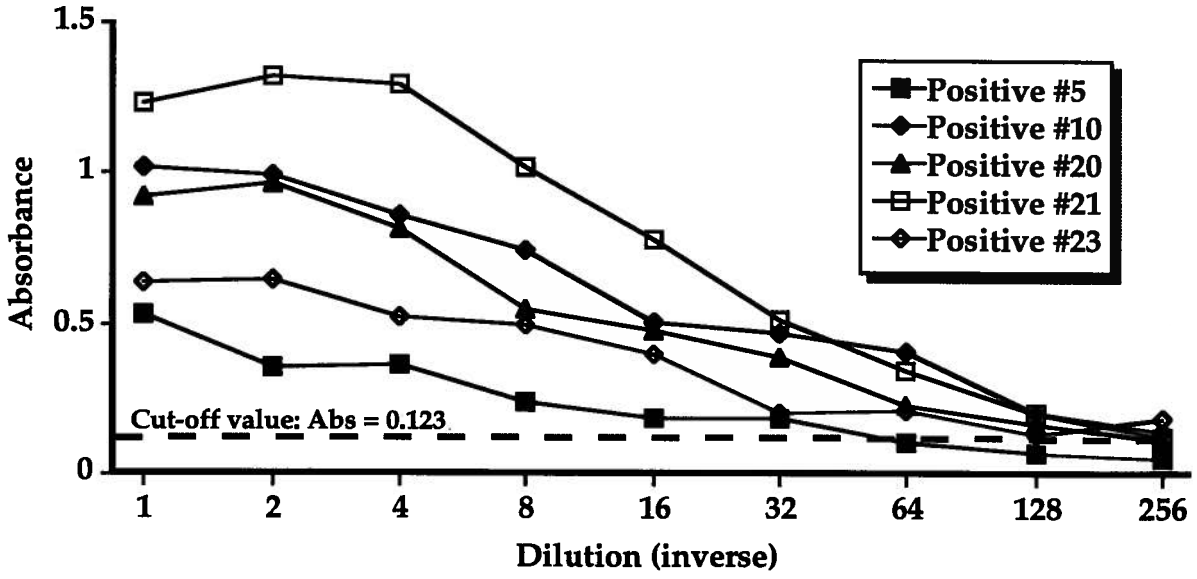
3.1.2 Neutralizing titer correlation with amount of specific anti-HCMV antibody.

An Abbott EIA was used to assess the level of anti-HCMV antibody. Although the supporting documentation provided with this test kit suggested that the amount of colorimetric product produced (measured by absorbance) was directly proportional to the amount of anti-HCMV antibody present in the sample, we failed to find a close correlation in all samples. In general, dilution of the serum samples did decrease the absorbance, but many of the samples did not decrease linearly, some values even increased slightly for serial points of sample dilution (Figure 9). Comparisons were then made between the anti-HCMV antibody titer and the functional neutralizing ability of the serum sample as determined by the NT_{50} assay discussed in the previous section. If the NT_{50} values are graphed as a function of the antibody titer, there is still no correlation observed (Figure 10). This may reflect the epitopes identified by the various antibodies produced during affinity maturation.

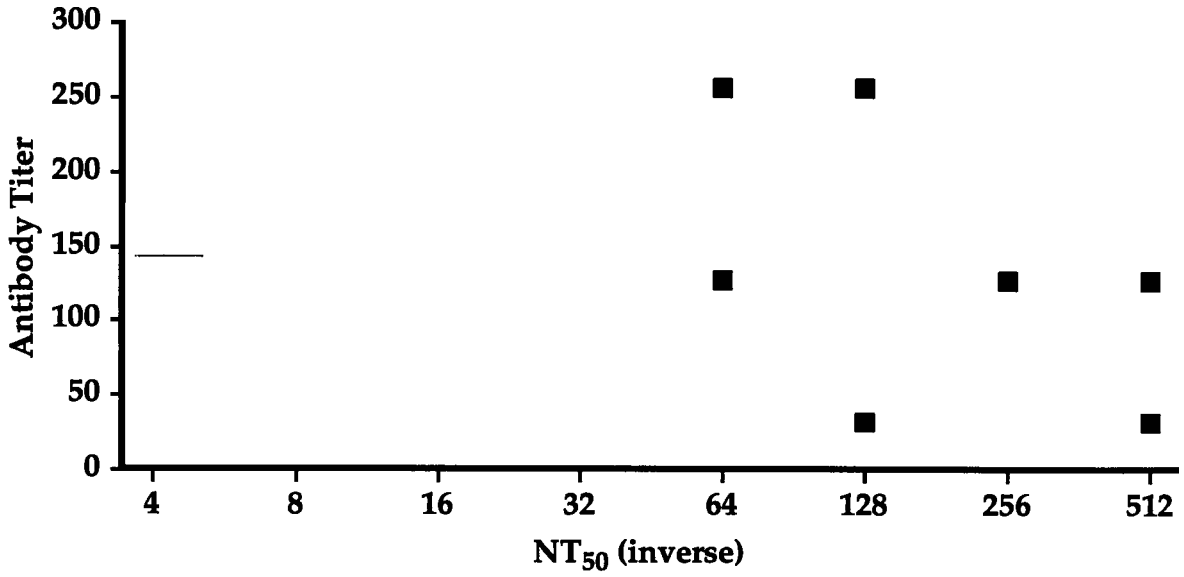
3.1.3 Identification of complement activation pathway with purified virions.

These experiments were designed to identify the complement activation pathway responsible for the enhancement of antibody-mediated neutralization. The role of antibodies was addressed by two different methods: First, sucrose purified virions were incubated with both seropositive and seronegative serum samples, and second, with seropositive serum samples in which the IgG was removed by a protein G-Sepharose. The Western blots shown in Figure 11 demonstrate the fractions collected after IgG-depletion using a protein G-Sepharose column (A), and the subsequent elution profile of the bound IgG with a 0.1 M glycine buffer (B). The activity of both the alternative and classical activation complement pathways of the depleted serum were within the normal range as assessed by functional hemolytic assays performed in the routine clinical laboratory at University Hospital (Vancouver, B.C.). The classical pathway function was unaffected by IgG depletion because the antibody used in the assay is an exogenous anti-rabbit erythrocyte antibody.

↓ **Figure 9. Serum samples from 6 individuals tested positive for anti-HCMV antibodies by the Abbott EIA method. These samples were then serially diluted in PBS, and the absorbance of the dilutions determined by this method. The cut-off absorbance (dotted line) was the value=0.123; absorbance readings below this absorbance were considered seronegative. The absorbance readings do not always decline linearly, and higher initial values do not always correspond to a higher extinction dilution (dilution which falls below the cut-off value).**



↓ **Figure 10. Seven seropositive samples, for which the antibody titer was determined (by extinction dilution as demonstrated in Figure 9) were subsequently tested in the functional HCMV neutralization assay. This graph depicts the NT_{50} values graphed as a function of the extinction dilution: again the correlation was fairly low (R^2=0.315)**



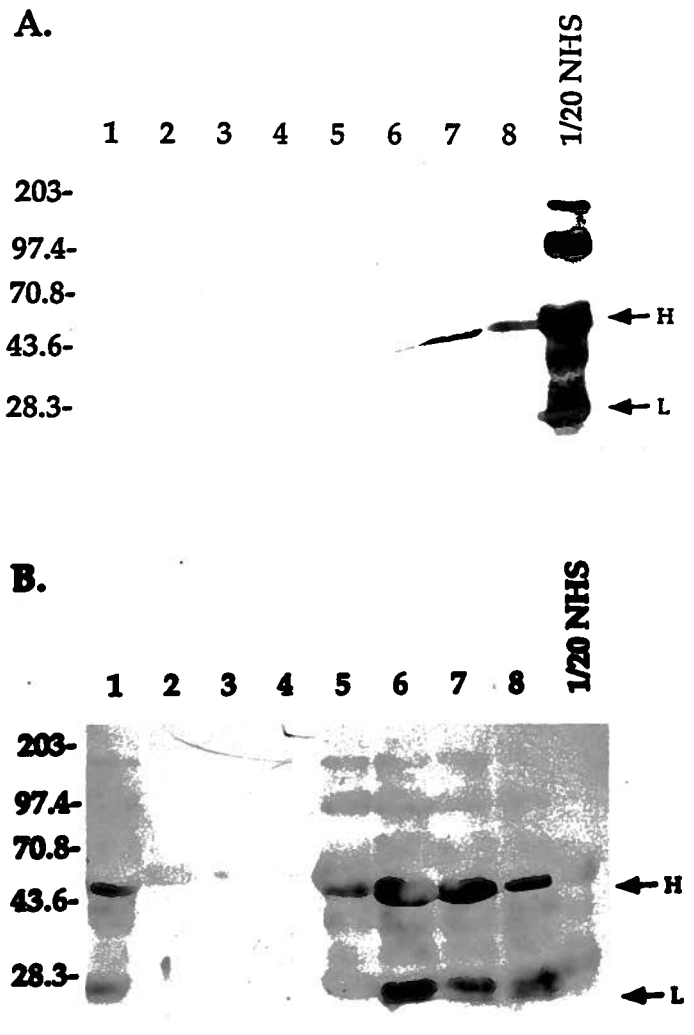
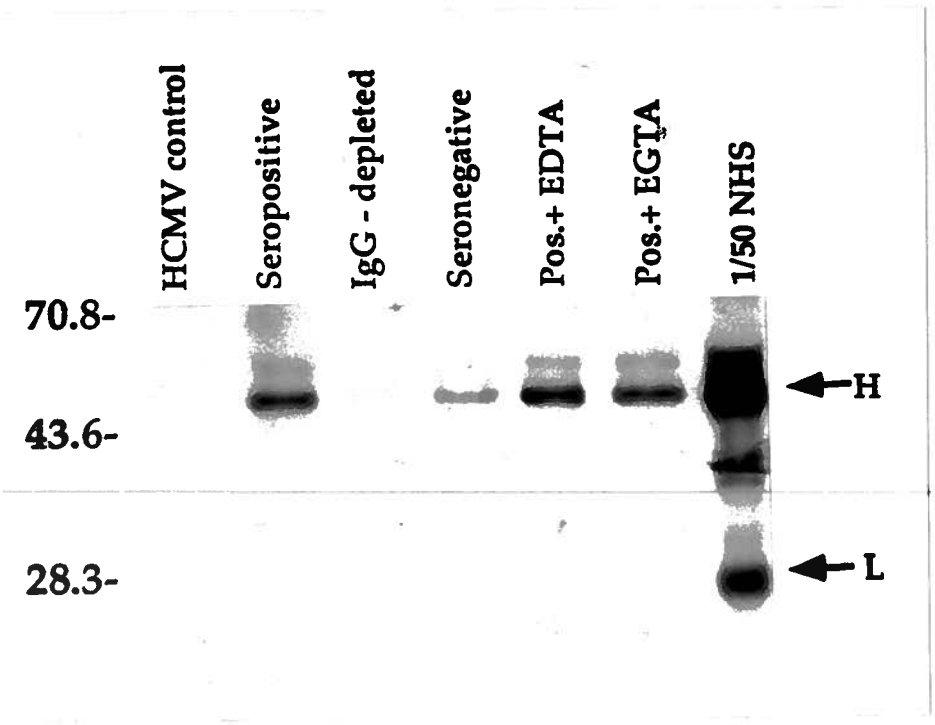


Figure 11. (A). Western blot analysis for IgG present in fractions collected after using a protein-G sepharose column to deplete IgG from an HCMV-seropositive serum sample. Samples from the first 8 fractions were separated under reducing conditions by SDS-PAGE. After transfer to nitrocellulose the Western blot was probed with a polyclonal anti-human IgG antibody. The first four fractions contained a majority of the total protein and were pooled and concentrated to their original volume (see materials and methods). As seen in (A) these fractions contained negligible amounts of the heavy chain of IgG (53 kDa), while this band is readily apparent in the 1/20 dilution of a sample of HCMV-seropositive serum (NHS) run as a control. (B). The same column was subsequently rinsed with a 0.1 M glycine buffer and samples of the eluted fractions treated the same as for (A). The 53 kDa heavy-chain of IgG is readily apparent in fractions 5-8, and are much stronger than that observed for the 1/20 NHS control. Pre-probing both (A) and (B) with the secondary antibody yielded no signal in either case.

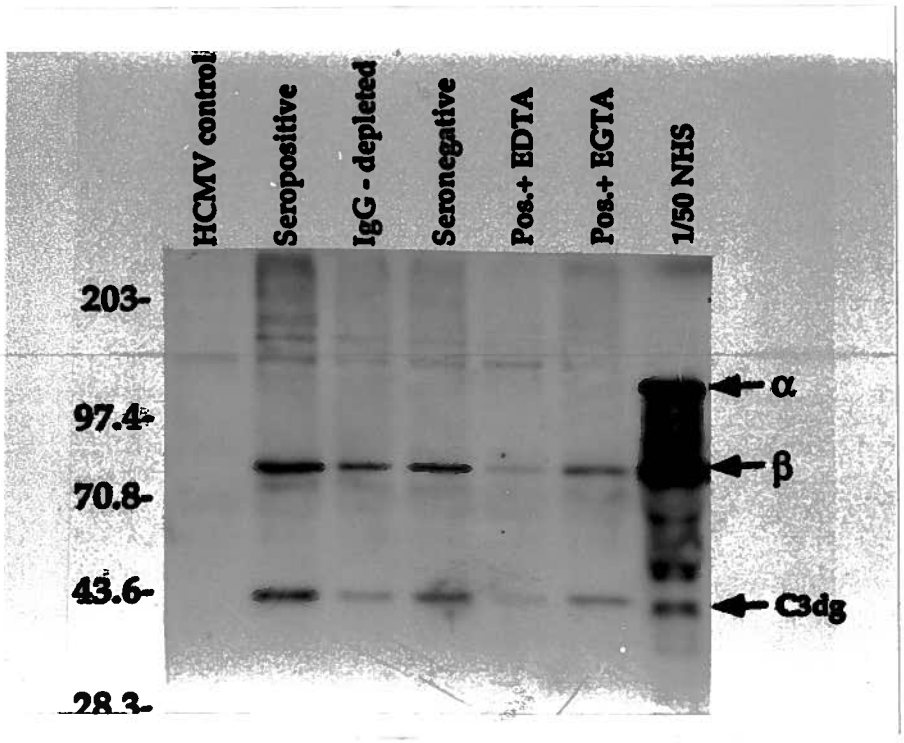
Sucrose-density gradient purified HCMV virions were incubated with serum samples under various conditions for 30 minutes at 37 °C, and the unbound soluble proteins removed by a series of sucrose-density gradient purifications. In addition to unaltered seropositive serum, HCMV virions were also incubated with seropositive serum in the presence of EGTA or EDTA. EGTA is a specific calcium chelator which inhibits the classical complement activation pathway, but does not affect the alternative pathway since it only requires magnesium. However, EDTA chelates both magnesium and calcium and serves to identify non-specific association of complement components with HCMV virions since activation cannot occur without these divalent cations. Under these various conditions, a large amount of IgG was bound to the virions incubated with seropositive serum, regardless of the presence of EDTA or EGTA (Figure 12). No IgG co-purified with virions incubated with IgG-depleted seropositive serum, and a very weak signal was observed for virions incubated with seronegative serum. The antibodies associated with virions after incubation with seronegative serum may represent cross-reacting antibodies directed against other herpesviruses or false-negative results generated by the ELISA method used to test the serum. However, these possibilities are unlikely: equivalent amounts of C3 deposition are seen between virions incubated with seronegative serum and serum depleted of all IgG (see below), and these results were consistent amongst four separate seronegative serum samples in my preliminary investigations. Only the heavy (H)-chain was observed by PAGE under reducing conditions which may indicate a higher avidity of the polyclonal anti-human IgG anti-serum for 53 kDa H-chains than 25 kDa light (L)-chains.

We next studied the complement activation of the classical and alternative pathways. The co-purification of C3 with virions incubated with seropositive serum in the presence of EDTA (where no complement activation should occur) indicated a low level of non-specific association of C3 with HCMV virions under the experimental conditions used (Figure 13). The C3 associated with the virions

↓ **Figure 12. Western blot analysis for IgG bound to the surface of virions. Virions were first incubated with serum samples then unbound proteins removed by sucrose density gradient purification. Samples of virions were separated under reducing conditions by SDS-PAGE, and a large amount of the 53 kDa heavy chain of IgG co-purified with virions incubated with seropositive serum, regardless of the presence of EDTA or EGTA. Negligible amounts of IgG were observed when seropositive serum depleted of IgG was incubated with purified virions, and the weak band observed for seronegative serum and virions indicates the non-specific association under these conditions. A 1/50 dilution of HCMV-seropositive serum (NHS) was used as a control and pre-probing of the blot with secondary antibody only yielded no signal. IgG-depleted = seropositive serum depleted of IgG with a sepharose column as in Fig. 11, pos. = seropositive serum.**



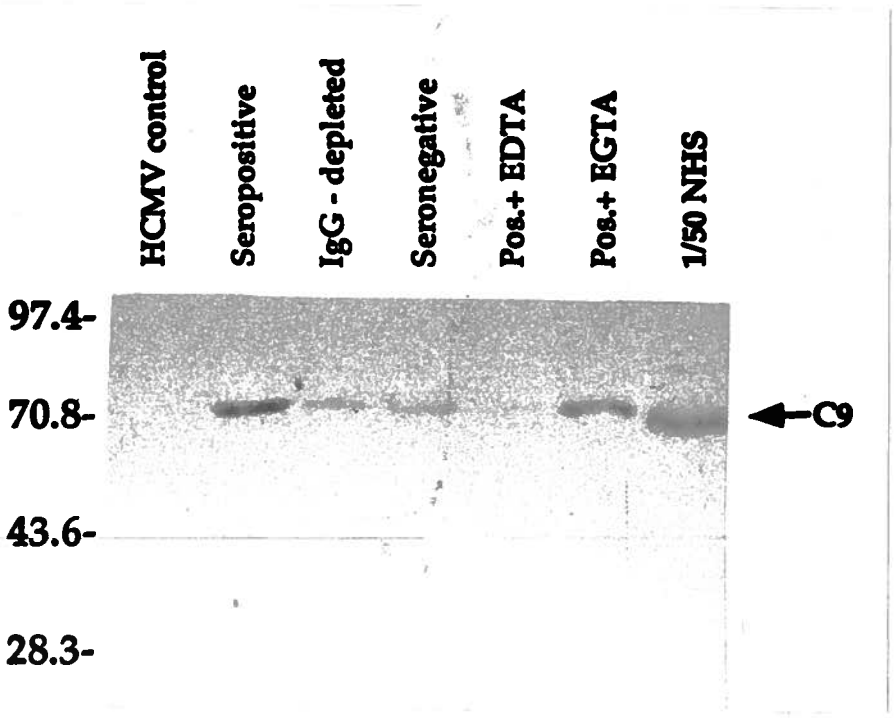
↓ **Figure 13. Western blot analysis for C3 bound to the surface of virions. Virions were first incubated with serum samples then unbound proteins removed by sucrose density gradient purification. Samples of virions were separated under reducing conditions by SDS-PAGE, and the largest amount of the 75 kDa β-chain of C3 co-purified virions incubated with seropositive serum, in the absence of EDTA or EGTA. The amount of C3 associated with the virions incubated with seropositive serum in the presence of EDTA is the lowest and represents the non-specifically associated C3. The amount of C3 associated with virions incubated with IgG depleted seropositive serum, seropositive serum with EGTA, and seronegative serum was intermediate and represents the background plus a low activation of the alternative pathway. The higher Mr smearing indicates covalently-bound C3 α-chain, and the 43 kDa band represents the proteolytically processed C3dg fragment of the α-chain. These findings were consistent regardless of whether the incubations were run at low or high ionic strength. The same controls and abbreviations as listed for Fig. 12 apply.**



incubated with seronegative serum, seropositive serum + EGTA (where only the alternative pathway is active), and IgG-depleted seropositive serum were very similar, indicating some alternative pathway activation does occur. However, much more C3 is associated with the virions incubated with seropositive serum in the absence of EGTA and EDTA. This indicates that the activation of the classical pathway is the largest contributor to complement activation products on the virions in the presence of virion-bound antibody. Furthermore, greater evidence of covalent linkage of the α-chain of C3, as visualized by higher bands and darker smearing at a high molecular weight by SDS-PAGE, was prevalent for the seropositive serum sample. Interestingly, a band was seen at 43 kDa which is the expected molecular weight for the C3dg fragment of C3. The molecular weight is slightly higher than the band seen in the NHS control lane, and it is possible that the band in the control lane represents the smallest fragment, C3d. *In vitro* , the C3g fragment, which has an Mr of 5 kDa, can be cleaved from C3dg by using trypsin (Ross and Medof 1985). Thus, C3d may be the fragment produced during the processing and repeated freezing and thawing of the control serum (the same aliquot was used as a control for multiple gels), rather than the fragment produced by C3b inactivation on the surface of virions. The levels of C3dg corresponded to the general levels of C3 co-purifying with the virions, and the possibility of a cross-reaction with the H-chain of IgG was ruled out by subsequent staining of the same blot with anti-human IgG antibody; which identified a band about 8 kDa larger. This implies that C3 bound to HCMV virions is processed by factor I to inactive fragments. These results were identical regardless of whether the experiment was performed under low ionic strength conditions (all components suspended in GVB; Figure 13) or under physiological ionic strength conditions (all components suspended in TBS).

Finally, the extent of complement activation was investigated by staining for the presence of C9 associated with the virions. No C9 was found to associate with the virions in the presence of EDTA, suggesting minimal non-specific association. The

↓ Figure 14. Western blot analysis for C9 bound to the surface of virions. Virions were first incubated with serum samples then unbound proteins removed by sucrose density gradient purification. Samples of virions were separated under reducing conditions by SDS-PAGE, and the largest amount of the 71 kDa C9 protein co-purified with virions incubated with seropositive serum, in the absence of EDTA or EGTA. Low amounts of C9 were associated with virions incubated with IgG depleted seropositive serum and seronegative serum, while the amount of C9 associated with virions incubated with seropositive serum and EGTA was intermediate. No C9 was associated with the virions in the presence of EDTA, indicating negligible background. These findings were consistent regardless of whether the incubations were run at low or high ionic strength. The same controls and abbreviations as listed for Fig. 12 apply.



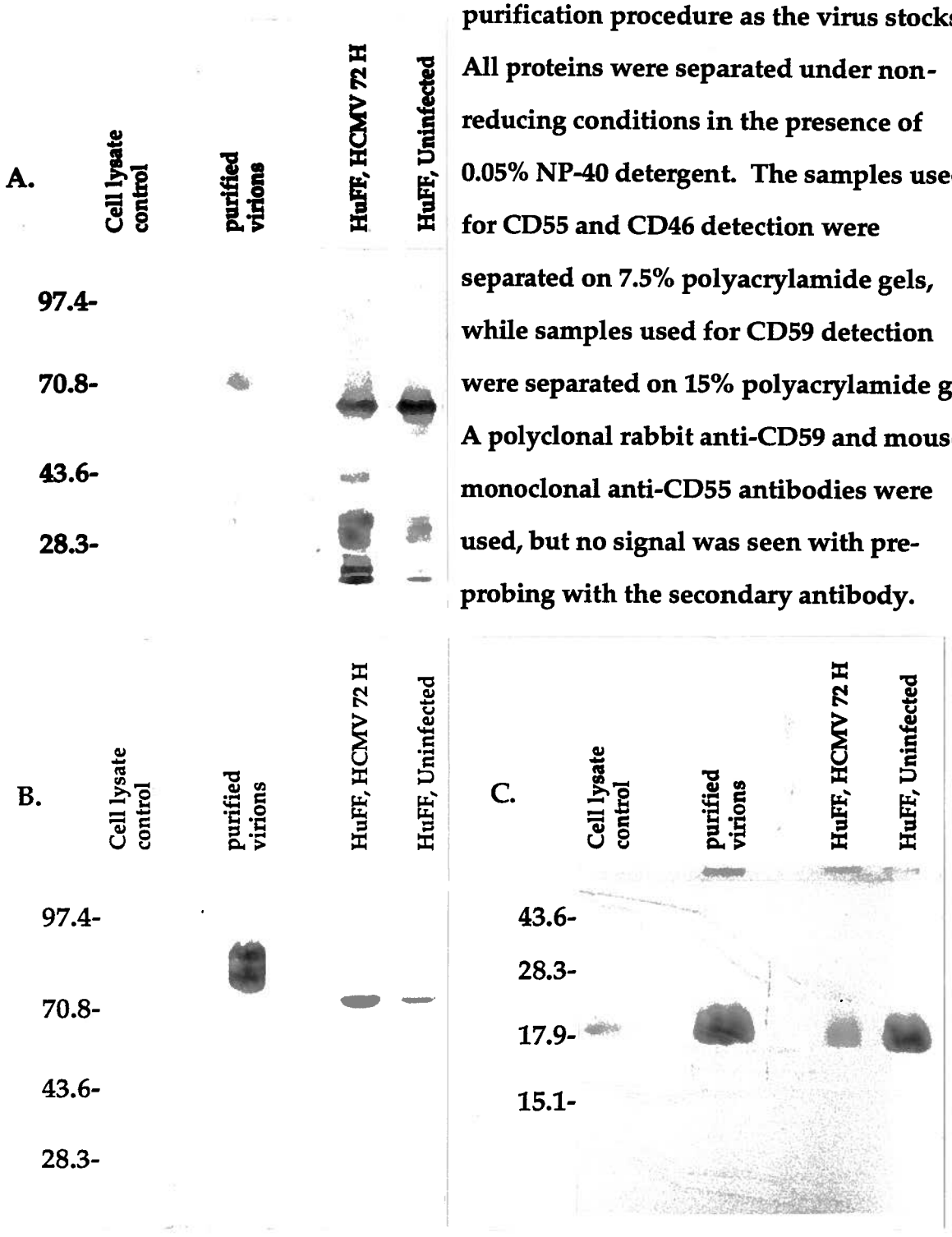
amount of C9 associated with virions incubated with seronegative serum and IgG-depleted seropositive serum was low, a little more was observed with seropositive serum + EGTA, and the most C9 was associated with virions when incubated with seropositive serum in the absence of EDTA or EGTA. Again, these results were consistent whether the experiment was conducted under low or physiological ionic strength conditions (Figure 14). This suggests that minimal activation of complement occurs in the absence of anti-HCMV antibodies; but when anti-HCMV antibodies are present, activation occurs mainly by the classical pathway and proceeds to completion with the deposition of C9 into the virions.

3.1.4 Host Complement Inhibitors associated with HCMV Virions.

The presence of what appears to be C3dg with HCMV virions suggests that some complement regulation may also occur on the virions. To address the possible presence of host encoded complement inhibitors on the virions, Western blotting techniques were utilized on sucrose-density gradient purified HCMV virions to determine whether host-encoded complement inhibitors were present. Further, Western blot results were compared for virions, HCMV-infected cells, and uninfected cells. A cell lysate control was prepared by lysing an equal number of fibroblasts (equal to the amount used to propagate the virus) using a fine needle and the cell debris was then treated identically to the virus preparation. This control addressed the possible non-specific co-migration of host cell complement inhibitors to the same density at which HCMV virions are found, thus causing artifacts. Figure 15 demonstrates the presence of CD46, CD55 and CD59 in the lanes with purified HCMV virions. The lysate control also identifies the presence of a small amount of CD59, indicating a small non-specific co-migration of CD59 at the same density as the HCMV virions. However, a much larger amount of CD59 is present in the purified virion sample; this enrichment suggests CD59 is associated with the virions. Interestingly, no CD46 or CD55 was detected in the lysate control, while vast amounts

of CD55 and a lesser amount of CD46 were associated with the HCMV virions. Since equal amounts of purified virions were used in Figure 15 A, B, and C, the relative enrichment of CD55 and CD59 associated with the HCMV virions is probably a reflection of their common glycolipid anchor, as compared to the transmembrane cellular attachment of CD46. The ease of incorporation of glycolipid anchored proteins into exogenous membrane surfaces was a phenomenon which aided in the initial determination of the complement regulating ability of CD55 and CD59 (Davitz et al 1986, Holguin et al 1989). Further, the apparent molecular mass for the CD55 associated with virions was more variable than the CD55 found on uninfected fibroblasts or fibroblasts infected with HCMV for 72 h (Fig 15C). Cross-reactivity of the anti-CD55 monoclonal antibody with a viral protein was ruled out by obtaining similar results with monoclonal anti-CD55 antibodies directed against different epitopes. There is also no homology between the region bound by the anti-CD55 monoclonal antibody and the prospective proteins determined using the published sequence of AD169 and the computer programs Geneworks and DNA Strider . The Mr of the CD46 on the purified virions is not as variable as the virion CD55, nevertheless, the virion CD46 had a higher Mr than the uninfected fibroblast or fibroblasts infected with HCMV for 72 h. These differences in CD55 and CD46 Mr between cells and virions may represent altered glycosylation occurring in cells which have been infected with HCMV for 10-12 days during HCMV propagation. Other authors have noted that the SDS-PAGE separation of HCMV-encoded virion glycoproteins results in disperse bands (Benko and Gibson 1986). There even appears to be more glycoprotein size heterogeneity in HCMV-encoded glycoproteins when compared to simian CMV. In preliminary experiments in which the HCMV-infected fibroblasts were harvested at times later than 72 Hp.i., the altered forms of CD55 were seen and became predominant compared to uninfected cell CD55 as the cells approached death.

↓ **Figure 15. Western blot analysis for the presence of CD46 (A), CD55 (B), or CD59 (C) in sucrose density gradient purified virions as compared to HCMV-infected and uninfected human foreskin fibroblasts (HuFF). The cell lysate control represents uninfected HuFFs mechanically disrupted and then exposed to the same purification procedure as the virus stocks. All proteins were separated under non-reducing conditions in the presence of 0.05% NP-40 detergent. The samples used for CD55 and CD46 detection were separated on 7.5% polyacrylamide gels, while samples used for CD59 detection were separated on 15% polyacrylamide gels. A polyclonal rabbit anti-CD59 and mouse monoclonal anti-CD55 antibodies were used, but no signal was seen with pre-probing with the secondary antibody.**



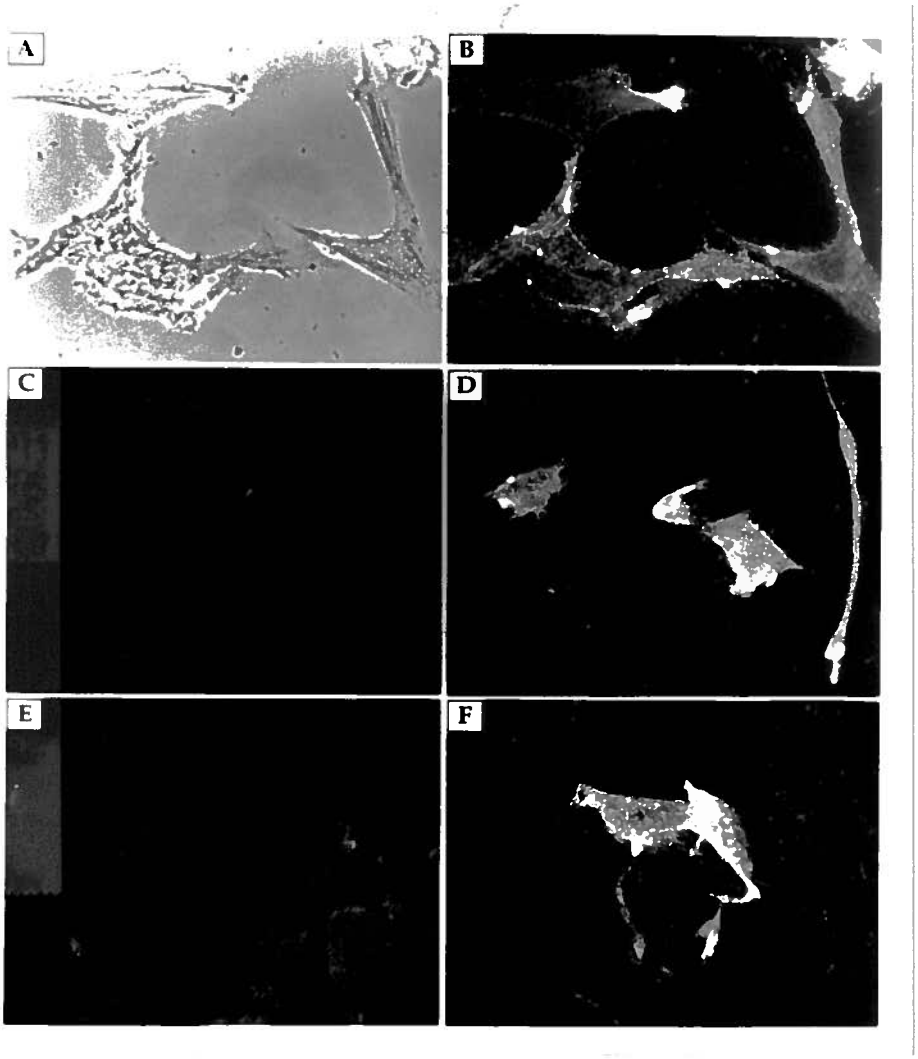
3.2 CHAPTER 2. Changes in complement inhibitor expression on adherent cells infected with HCMV.

Concurrent with my studies of purified HCMV virions, I investigated the possible alterations in host complement inhibitor expression on HCMV-infected cells.

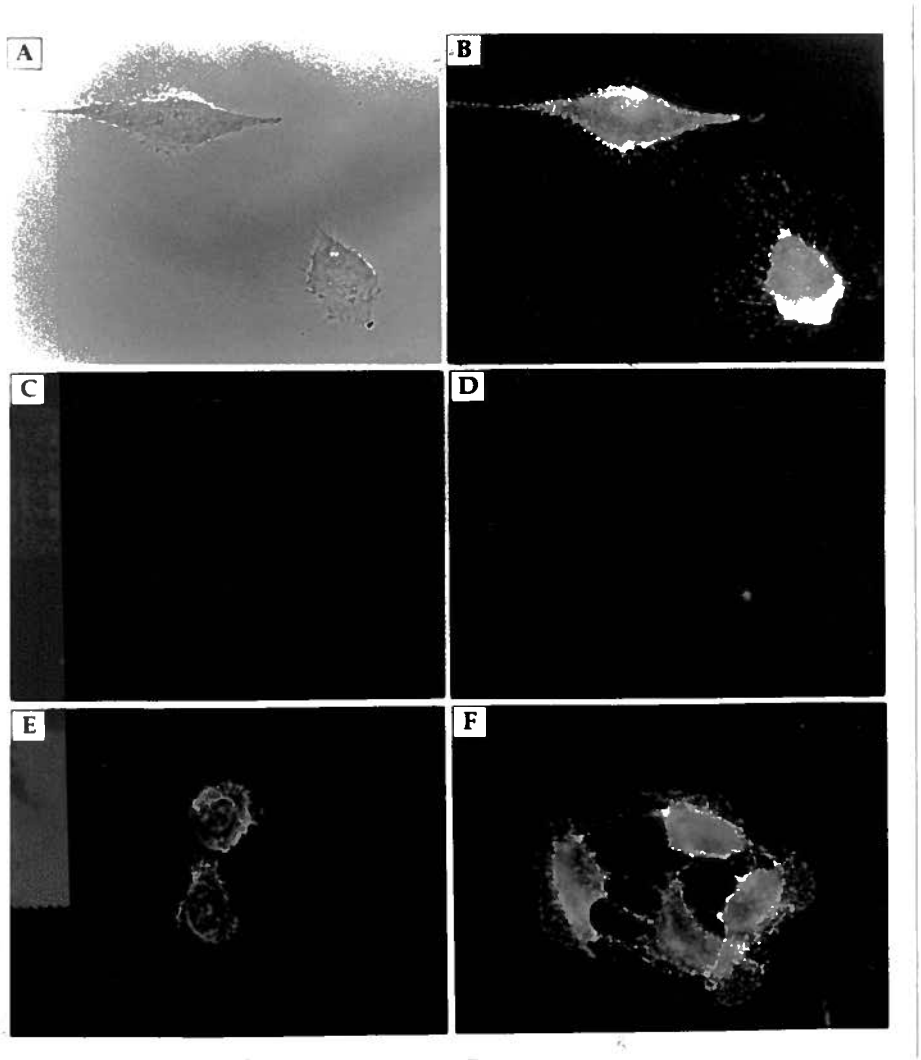
3.2.1 Complement inhibitor expression on uninfected cells.

Only certain human cell types are fully permissive for HCMV infection. I selected two different fully-permissive cell types: primary explant human foreskin fibroblasts, which are the most common model for *in vitro* HCMV infection, and the human glioblastoma cell line, U373-MG. Using indirect immunofluorescence (IIF) techniques, the expression of HLA class I, CD46, CD55, and CD59 were confirmed on uninfected fibroblasts (Figure 16) and glioblastoma cells (Figure 17). CD55 expression on glioblastoma cells was negligible by IIF techniques, but Western blot (Figure 18), flow cytometry (see section 3.2.2), and Northern blot (see section 3.4.1) techniques confirmed low but consistent constitutive expression of CD55 on this cell type. The levels of CD46 on both cell types (Fig 16E and 17E) seemed relatively weak compared to CD59 expression; however, this probably reflects the smaller amounts of a monoclonal antibody (anti-CD46) bound to the investigated antigen (single epitope recognition) as compared to a polyclonal antibody (anti-CD59; multiple epitope recognition). Western blot techniques demonstrated that the Mr of CD55 found on fibroblasts, glioblastoma cells, and primary fetal astrocytes was the same as that seen for CD55 expressed on monocytes (78 kDa), and not the lower Mr observed for erythrocyte CD55 (72 kDa) (Figure 18 A and B). However, the Mr for CD59 was identical (18-20 kDa) across all of the cell types investigated (Figure 18 C), as was the Mr for CD46 (65 kDa; Figure 18 D).

↓ **Figure 16. Indirect Immunofluorescence analysis investigating the presence of cell surface proteins on fibroblasts. Primary explant foreskin fibroblasts were grown on coverslips as seen by light microscopy in A. These cells were incubated with mouse monoclonal antibodies directed against HLA class I (B) or CD46 (E) and visualized with a FITC-conjugated anti-mouse IgG antibody, or the cells were incubated with rabbit polyclonal antibodies raised against CD55 (D) or CD59 (F) and visualized with a FITC-conjugated anti-rabbit IgG antibody. Background staining was determined by using non-immune rabbit serum and the FITC-conjugated anti-rabbit IgG secondary antibody (C). Magnifications: A, B, and C= 40X, D, E, and F= 63X.**



↓ **Figure 17. Indirect Immunofluorescence analysis investigating the presence of cell surface proteins on the glioblastoma, U373-MG, cell line. Glioblastoma cells were grown on coverslips as seen by light microscopy in A. These cells were incubated with mouse monoclonal antibodies directed against HLA class I (B) or CD46 (E) and visualized with a FITC-conjugated anti-mouse IgG antibody, or the cells were incubated with rabbit polyclonal antibodies raised against CD55 (D) or CD59 (F) and visualized with a FITC-conjugated anti-rabbit IgG antibody. Background staining was determined by using non-immune rabbit serum and the FITC-conjugated anti-rabbit IgG secondary antibody (C). Magnifications: A, B, and C= 63X, D, E, and F= 40X.**



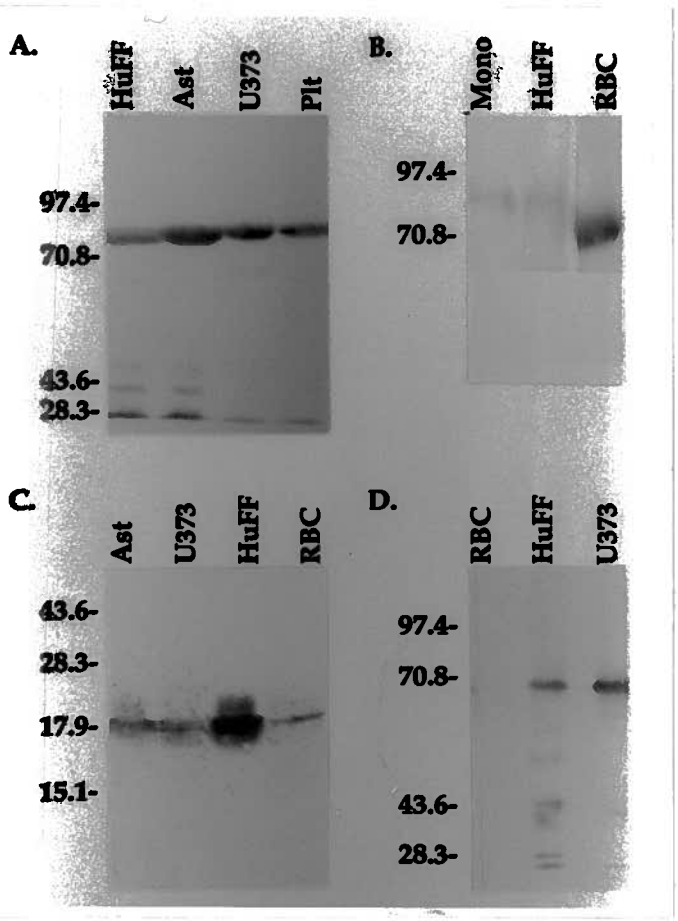


Figure 18. Western blot analysis on whole cell lysates to investigate relative molecular mass for CD55 (A and B), CD59 (C) and CD46 (D) on a number of different cell lines. Proteins in A, B, and D were separated on 7.5% polyacrylamide gels, while proteins in C were separated on a 15% polyacrylamide gel. Relative protein mobilities were compared between fibroblasts (HuFF), glioblastoma cells (U373), astrocytes (Ast), erythrocytes (RBC), platelets (Plt), and a monocytic leukemia cell line, THP-1 (Mono). Pre-probing with conjugated secondary antibody yielded no signal.

3.2.2 Complement inhibitor expression alteration with HCMV infection.

The cell surface expression of CD55, CD46, CD59, and HLA class I proteins during HCMV infection of human fibroblasts was measured using monoclonal antibodies and flow cytometry. Flow cytometry profiles for CD55 (Figure 19) demonstrated increases in cell fluorescence following infection with HCMV (MOI=10). Figure 19 also shows the binding of the isotype control antibody, which was identical for uninfected and HCMV-infected fibroblasts. Moreover, increases in cell fluorescence appeared to be uniform, and the mean fluorescence increased with

the time of infection, which suggests that the majority of cells in the population were infected. The time-course and magnitude of these results also suggest that CD55 expression was not enhanced as a result of the immediate release of internal pools of CD55, as has been described for neutrophil responses to a variety of stimuli (Berger and Medof 1987). Figure 20 displays the cumulative data for CD55, CD46, CD59, and HLA class I. CD55 expression on HCMV-infected fibroblasts increased two-fold ($p<0.01$) at 24 h post-infection (p.i.), while CD46 expression did not increase significantly until 48 h p.i. ($p<0.01$). By 72 h p.i., there was a 10-fold increase in CD55 ($p<0.001$) and a 3.4-fold increase in CD46 ($p<0.01$) as compared to mock-infected cells. Interestingly, CD59 expression decreased in response to HCMV infection, which suggests that the enhanced cell surface complement inhibitor expression mediated by HCMV is not universal (Figure 20). By 24 h p.i., the CD59 expression had decreased 30% ($p<0.05$), a trend which continued until by 72 h p.i. the expression of CD59 on HCMV-infected fibroblasts was 50% that measured on mock-infected controls ($p<0.01$). I also observed down-regulation of HLA class I expression, which has been reported previously (Gilbert et al 1993, Barnes and Grundy 1992, Yamashita et al 1993), but the HLA class I down-regulation was not significant until 48 h p.i. ($p<0.001$). However, in this thesis I am using the down-regulation of HLA class I as an indirect measure of the uniformity of HCMV infection.

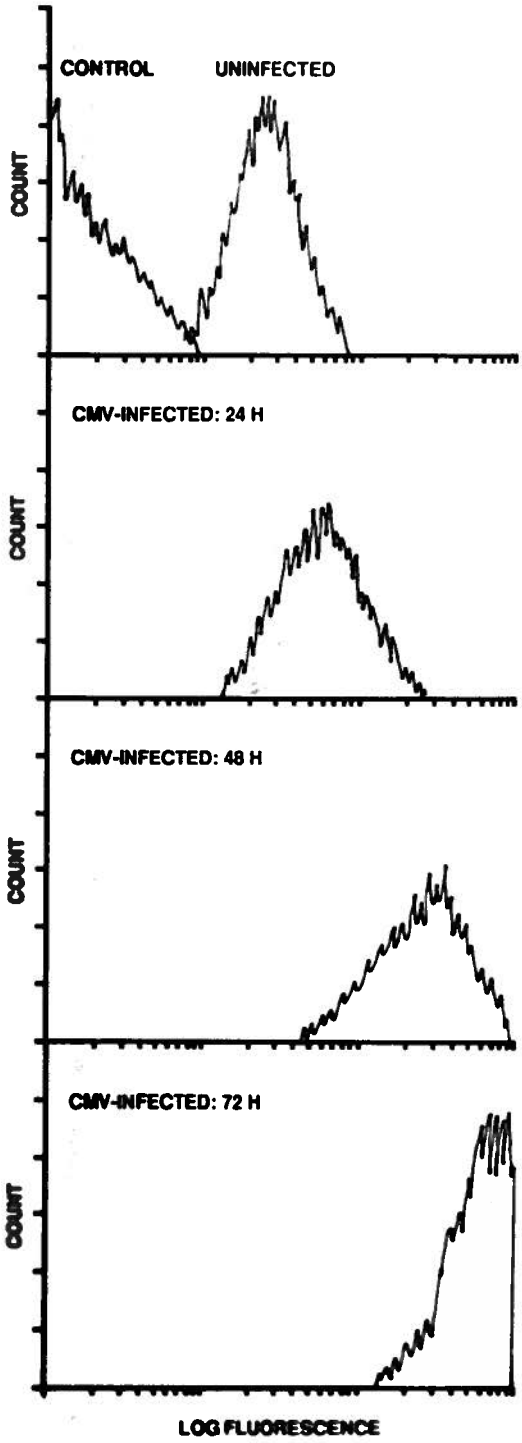
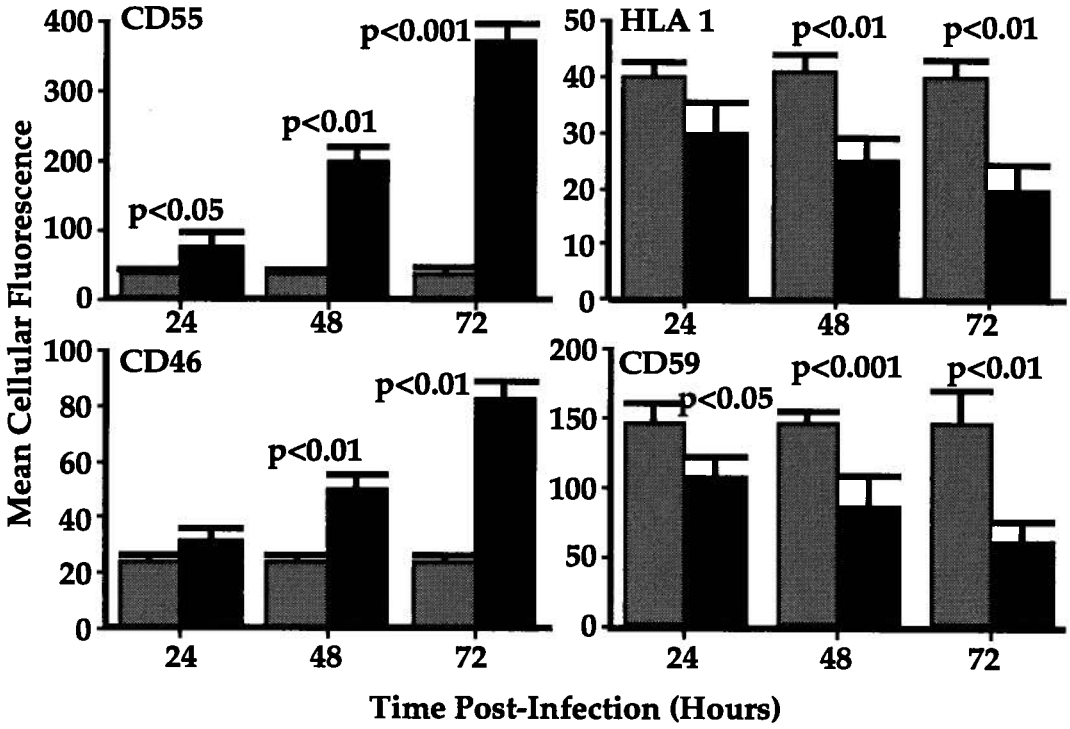


Figure 19. Composite of representative flow cytometric analysis profiles using monoclonal anti-CD55 antibody to detect surface expression on uninfected fibroblasts (top) and on HCMV-infected (strain AD169) at 24, 48, and 72 hours post-infection. Binding of isotype-matched control antibody is also shown in the top histogram (left) and was identical for HCMV-infected and uninfected cells.

↓ **Figure 20. Flow cytometric analysis of the cell surface expression of CD55, CD46, CD59, and HLA class I on mock-infected (gray bars) and HCMV-infected (black bars) human foreskin fibroblasts (HuFF) at 24 hour intervals. Predetermined saturating amounts of specific monoclonal antibodies were used for each antigen, and no change was observed when an isotype-matched control antibody was used. Error bars indicate one standard deviation, N=3.**



I investigated the possible role of an Fc-receptor in the enhanced antibody binding by using isotype-matched control antibodies as well as binding of the phycoerythrin-labeled secondary antibody only. Since, the Fc portion of the antibody removed as part of the phycoerythrin labeling process, it is uninfluenced by Fc receptors and these results are summarized in Table 2. No difference in the non-specific antibody binding was measured between uninfected cells and cells infected with HCMV (MOI=10, 72 h p.i..) indicating the increased antibody binding and mean cell fluorescence resulted from increased CD55 and CD46 antigen expression. In preliminary experiments, I blocked the Fc receptor on HCMV infected cells by pre-

incubating cells with 5% HCMV-seronegative human serum, and since the secondary antibody is pre-absorbed against human serum components, this did not contribute to the signal. No difference was measured in the mean cellular fluorescence for cells which had the Fc receptor blocked and those which had no pretreatment, indicating the Fc receptor had minimal influence on the binding of the mouse monoclonal antibodies used.

Table 2. Mean cellular fluorescence of non-specific antibody binding to uninfected and HCMV-infected cells.[1]

	HCMV (72 h p.i.)	No HCMV	Statistics[2]
Isotype control and secondary Abs[3]	0.260 ± 0.02	0.216 ± 0.02	NS
Secondary antibody only	0.207± 0.02	0.163 ± 0.02	NS
Statistics	NS	NS	

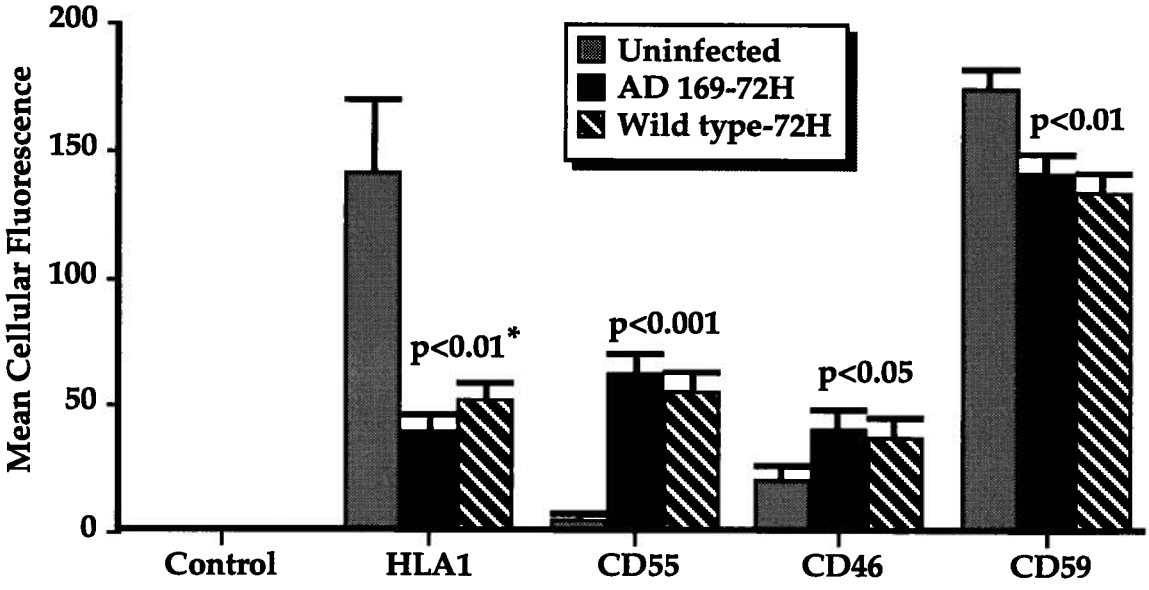
1. Mean cellular fluorescence is given as the mean +/- standard deviation, each condition performed in triplicate. Cells were infected with HCMV at an MOI=10 and harvested at 72 h p.i.

2. Comparisons were performed amongst all groups by T-tests with the additional use of Bonferroni's correction for multiple comparisons. NS=not significant.

3. The secondary antibody was a phycoerythrin-conjugated Fab fragment of a donkey anti-mouse H and L chain antibody. The isotype matched control was a mouse monoclonal anti-HLA class II antibody. Abs=antibodies

To investigate whether or not the HCMV-induced increase in CD55 and CD46 was restricted to fibroblasts, I replicated the experiments using another permissive cell line, the U373-MG glioblastoma cell line. Figure 21 represents a comparison amongst glioblastoma cells which were mock-infected, infected with the AD169 strain of HCMV, or infected with a low passage patient isolate of HCMV at a MOI=10 for 72 h. It is readily apparent that the relative increases in CD55 and CD46 are identical to those measured on HCMV-infected fibroblasts. Figure 21 also demonstrates the expression of CD55 on glioblastoma cells is significantly above background. Similar to the findings for fibroblasts, a 19% and 23% decrease in cell-surface CD59 expression was observed for glioblastoma cells infected with AD169 strain and wild type, respectively ($p>0.01$). Moreover, the relative CD55 and CD46 increases are identical for the lab and wild type strain of HCMV, indicating that this phenomenon is not a product of conditioning due to long-term tissue culture propagation of the virus. The statistics listed in Fig. 21 are comparisons of the mean cellular fluorescence of mock-infected cells to HCMV-infected cells (either AD169 or wild type). No statistical difference was found between the mean cellular fluorescence for cells infected with AD169 or wild type HCMV, and these results were identical for two separate isolates of wild type HCMV.

↓ **Figure 21. Flow cytometric analysis of the cell surface expression of CD55, CD46, and HLA class I on mock-infected (gray bars) glioblastoma cells and cells infected with the AD169 (black bars) HCMV or a low passage patient isolate of HCMV (striped bars). Two separate patient isolates were investigated and gave identical results, only one of these is shown. The error bars indicate one standard deviation and the statistics given are comparisons between HCMV-infected and mock-infected cells; *= this is the only sample in which the difference between HCMV strains used approached significance (p=0.07).**



3.2.3 Viral specificity of CD55/CD46 increased expression.

I considered the possibility that the CD55 and CD46 increased expression was a non-specific phenomenon, and that any viral stress may replicate these findings. To address this possibility I infected fibroblasts and glioblastoma cells with HSV-1 and adenovirus, both of which decrease the expression of HLA class I. The results of flow cytometry analysis using monoclonal antibodies (N=3 for each experimental group) are summarized in Table 3. Optimal viral infection of fibroblasts was gauged by the decrease of HLA class I on 95% of the cells for each virus.

Table 3. Flow cytometry analysis of fibroblasts under different viral and chemical exposure.[1]

At 20 h p.i.:	Mock	HSV	HCMV
HLA 1[2]	60.2 ± 5.2	13.9 ± 0.6 ***[3]	38.3 ± 3.1*
CD55	26.3 ± 2.9	26.1 ± 2.8	37.9 ± 3.9*
CD46	22.5 ± 1.2	19.5 ± 0.9	29.2 ± 1.5

At 72 h p.i.:	Mock	Adenovirus	HCMV
HLA 1	65.5 ± 4.9	24.1 ± 2.0 **	21.4 ± 1.9 **
CD55	25.6 ± 2.5	24.7 ± 0.9	150.1± 13.6***
CD46	23.7 ± 1.5	22.1 ± 0.9	55.2 ± 1.6 **

At 72 h :	Mock	1 μg/ml LPS [4]	15 μg/ml LPS
HLA 1	62.0 ± 3.5	94.5 ± 7.0 **	80.3 ± 6.9 *
CD55	25.9 ± 1.3	25.1 ± 0.9	26.5± 2.1
CD46	21.4 ± 0.5	ND[5]	22.2 ± 1.9

1. Mean cellular fluorescence is given as the mean ± standard deviation, each condition performed in triplicate. Cells were infected with HCMV and HSV at an MOI=10 and Adenovirus at an MOI=50. Cells were harvested at times indicated.

2. Predetermined saturating concentrations of monoclonal antibodies were used for analysis. HLA 1=HLA class I.

3. Statistics represent comparisons performed amongst all groups by ANOVA then Fisher's least significant difference tests with the additional use of Bonferroni's correction for multiple comparisons. Differences found to be statistically different from the values for mock-infected cells are given as *=$p<0.05$, **=$p<0.01$, and ***=$p<0.001$.

4. Fibroblasts were incubated for 72 h in DMEM with 5% FBS containing 1 or 15 μg/ml bacterial lipopolysaccharide (LPS).

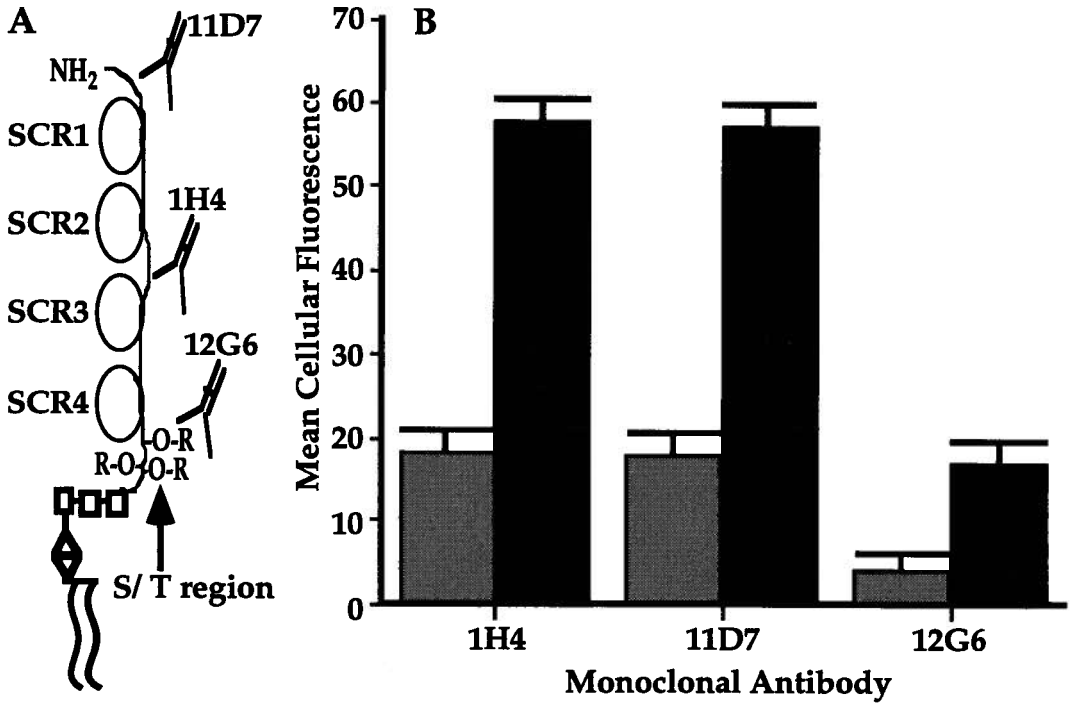
5. ND = not done.

Since HSV has a shorter lytic cycle these conditions were met by a MOI=10 at 20 h p.i.; for adenovirus these conditions were met by a MOI=50 at 72 h p.i. Table 3 demonstrates that the increase in CD55 and CD46 expression measured on fibroblasts were unique for HCMV-infection since, under these conditions, no changes in CD55 or CD46 were measured on HSV- or adenovirus-infected fibroblasts. Furthermore, incubating 1 to 15 μg/ml of bacterial lipopolysaccharide for 72 h with fibroblasts to induce activation were also unsuccessful in altering the expression of CD55 and CD46, while expression of HLA class I was significantly increased.

3.2.4 Increased CD55 is of host origin.

The largest increase in expression on HCMV-infected cells was measured for CD55. This raised the concern that the antibody against CD55 was cross reacting with a viral epitope since all experiments used the same monoclonal antibody, 1H4. Two separate methods were employed to exclude this possibility. First, flow cytometry analysis was repeated with the addition of two other CD55-specific monoclonal antibodies. As shown in Figure 22 (A), each antibody is directed against a unique epitope on CD55. The results for HCMV-infected and mock-infected fibroblasts at 72 h p.i., (Figure 22B), showed a similar relative increase in binding of anti-CD55 antibody for HCMV-infected cells. Second, Western blot analysis using an anti-CD55 polyclonal antiserum detected a 78 kDa protein in HCMV-infected fibroblasts (Figure 23), which is consistent with the reported molecular weight for CD55. Moreover, the 78 kDa band was more intense in HCMV-infected cells compared with uninfected controls, and no additional bands were detected in the HCMV-infected cells. Furthermore, a search of Genebank did not reveal any homology between CD55 and the HCMV strain AD169 genome. Taken together, these results are consistent with the hypothesis that HCMV caused the enhanced expression of host cell CD55. Further confirmatory evidence is provided by Northern blot analysis (see section 3.4.1).

↓ **Figure 22. Flow cytometric analysis of the cell surface expression of CD55 using a panel of monoclonal antibodies against different epitopes as shown in (A). Relative increases in mean cellular fluorescence were seen on HCMV-infected (black bars) compared to mock-infected (gray bars) human foreskin fibroblasts (HuFF) with all anti-CD55 monoclonal antibodies. Error bars indicate one standard deviation, and statistical analysis indicated all HCMV-infected cells had a significant increase in CD55 expression as compared to matched uninfected controls.**



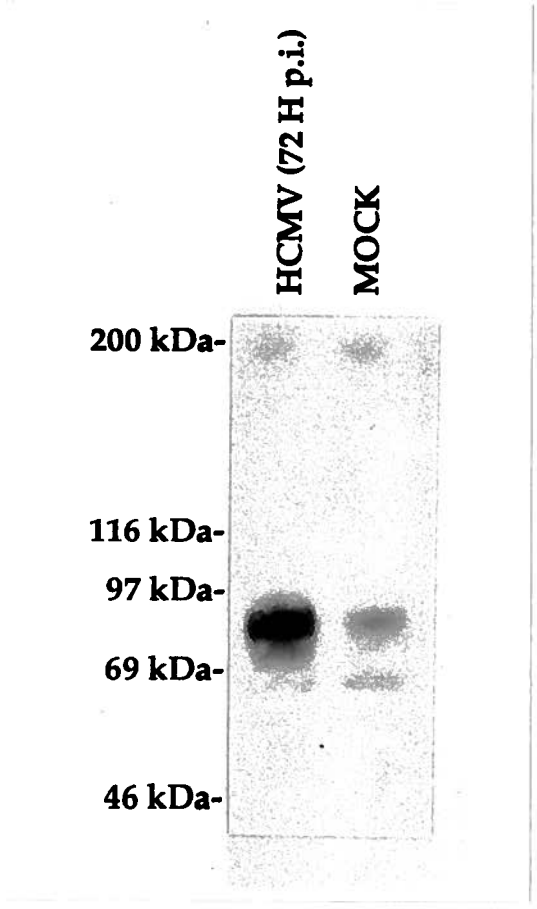


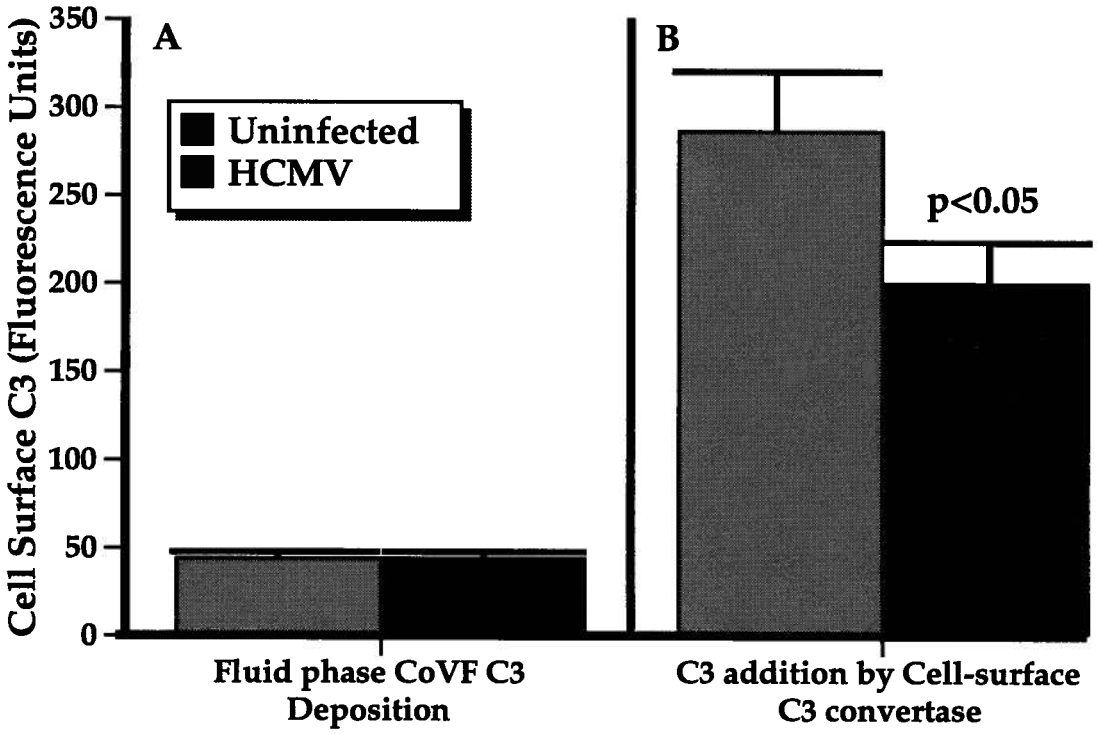
Figure 23. Western blot analysis of whole cell lysates from HCMV-infected or mock-infected fibroblasts harvested at 72 h p.i. was performed using rabbit polyclonal anti-CD55 antibodies. The intensity of the 78 kDa band detected for the infected cells was greater than that seen for the mock-infected cells and no additional bands were observed. Pre-probing of the blot with only the secondary antibody yielded no signal. Molecular weights indicated on the figure were determined using Amersham protein standards.

3.2.5 Functional properties of increased CD55 on HCMV infected cells.

Lastly, I endeavored to show that an increase in CD55 translates into a functional enhancement of complement regulation for the HCMV-infected cells. To test this hypothesis I used a purified complement protein assay to assess the cell surface activity of the alternative pathway C3 convertase, C3bBb (Devine and Rosse 1987). Figure 24 shows that similar amounts of C3b were bound to the surface of HCMV-infected or control cells from the fluid phase in the first stage of the assay. This process is known to be independent of the presence of CD55 or CD46. However, when C3bBb was formed at these sites in the second CD55-sensitive stage of the assay, the complexes on the surface of HCMV-infected cells were significantly less effective at activating C3 than C3bBb on uninfected controls (Fig 24; $p<0.05$). These results demonstrate that the increased levels of CD55 on the surface of HCMV

infected cells mediated an additional functional ability in regulating the C3-converting enzyme complexes.

↓ Figure 24. Effect of HCMV infection on C3 convertase function. HCMV-infected (black bars) or mock-infected (gray bars) fibroblasts were exposed to C3 deposition from a fluid phase C3 convertase (A) followed by cell-surface C3 convertase formation and subsequent C3 conversion (B) to assess CD55 function. Cell-bound C3 was assessed using a monoclonal anti-C3d antibody and flow cytometry. Error bars represent standard error of the mean.



3.3. CHAPTER 3. Effects of HCMV infection on THP-1 cells.

The monocyte or macrophage has been speculated to be the HCMV reservoir *in vivo* , and the THP-1 monocytic leukemia cell line is permissive to HCMV infection, but only after the cells are differentiated with phorbol esters (Weinshenker et al 1988). To confirm these reports, the amounts of virus present after HCMV infection of THP-1 cells were assayed by plaque assay on fibroblasts at regular intervals (Table 4).

Table 4. HCMV associated with THP-1 cells.

		Plaque-forming units per ml in cell supernatant[1]			
Differentiated[2]	Virus[3]	Day 0	Day 2	Day 3	Day 6
No	AD169	$3.7x10^6$	$4.3x10^5$	$1.8x10^5$	ND[4]
Yes	AD169	$9.0x10^6$	ND	$8.5x10^3$	$1.9x10^3$
Yes	Patient	$2.0x10^6$	$1.0x10^5$	$2.7x10^3$	ND
Yes & washed[5]	Patient	0	80	100	250
No & washed[5]	Patient	0	0	0	0

1. Amount of HCMV in the supernatant of infected cells was determined by removing a sample by sterile technique and performing a plaque assay using fibroblasts.

2. HCMV infection of THP-1 cells was performed while the cells were undifferentiated (non-permissive state), or after the THP-1 cells had been incubated with 300 ng/ml PMA for 72 h (permissive state).

3. The HCMV stock used was either the laboratory strain, AD169, or a low passage patient isolate of HCMV.

4. ND = not done.

5. To remove the high background of initial HCMV, the cells were extensively washed with PBS following a 1 h infection.

If the inoculating dose of HCMV was not removed from the cultures a constant decline in HCMV was seen, regardless of differentiation state, suggesting no (or an undetectable) production of HCMV above the inoculating dose. However, when the experimental conditions were altered to include extensive washing of the cells following initial infection of cells, low amounts of HCMV were produced only by the differentiated THP-1 cells. Detectable levels of HCMV were first detected at 2 days p.i. and the extracellular levels continued to increase to 6 days p.i., while no HCMV was produced by undifferentiated THP-1 cells. The amounts of HCMV produced by differentiated THP-1 cells was comparable to the amounts produced by other monocytic cell or peripheral lymphocyte cultures previously reported (St. Jeor and Weisser 1977, Lathey and Spector 1991). The low amounts of virus produced from differentiated THP-1 cells were not due to a minority of the cells being infected because greater than 80% of the cells had evidence of infection as assessed by HLA class I decrease. Although the measuring the decrease in HLA class I is not as accepted as an infectious focus assay or in situ hybridization for viral products, it is a phenomenon very specific to fully permissive infection of certain viruses. Further, a review of recent publications indicates that monocyte HLA class I expression increases or remains unchanged when exposed to cytokines, interferons α, β, ω, and γ, or bacterial cell products, indicating that this is a valid measure of HCMV infection (Chen et al 1994) However, higher viral titers may have been obtained if cell lysates had been used rather than culture supernatant, as a high degree of cell association of the virus has been speculated for monocytes (Ibanez et al 1991). In this section, I will report the effects of HCMV infection of differentiated and undifferentiated THP-1 cells on host complement inhibitor and HLA class I expression.

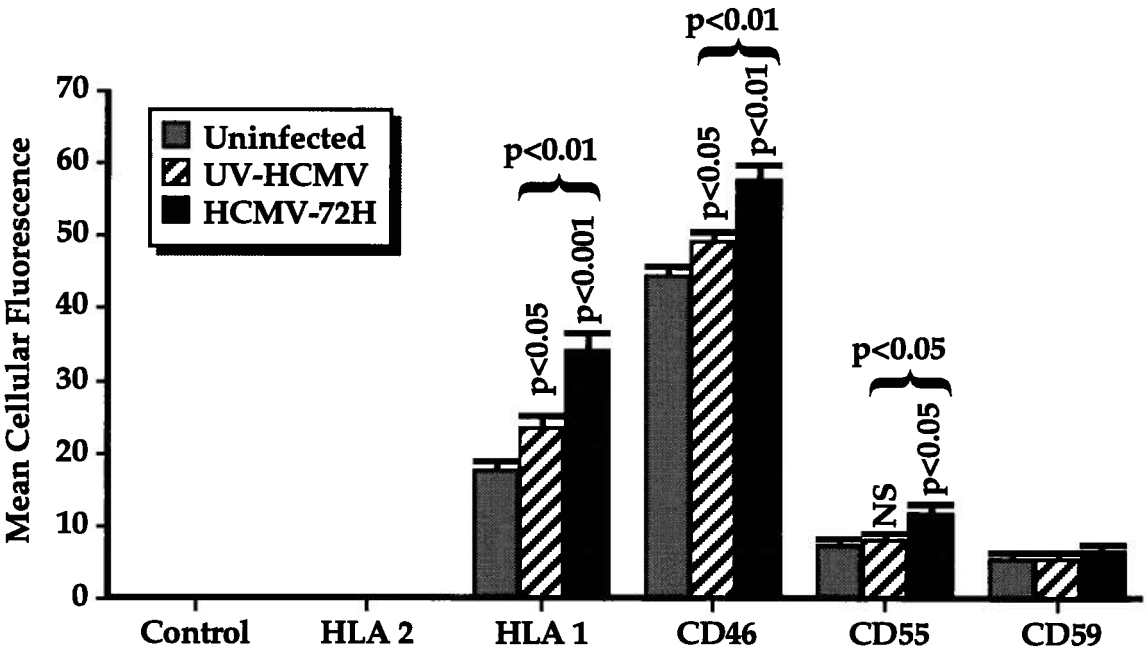
3.3.1. HCMV infection of undifferentiated THP-1 cells.

The undifferentiated THP-1 cells are non-adherent and most resemble monocytes. Routinely, $2x10^6$ cells were pelleted and resuspended in 150 μl of

concentrated virus stock (roughly $2x10^8$ pfu per ml) in a sealed tube, at 37°C for 1 h, with mixing every ten min. The cells were then suspended in 10 mls of fresh DMEM with 5% FBS and allowed to culture for a further 72H before being harvested. The infection was carried out this way to optimize infection and because the method used for infection of adherent cells was inappropriate. The virus stock was concentrated for two reasons. First, it was the only way to incubate the cells with an adequate MOI to ensure all cells would have at least one infectious virion per cell enter it. Second, concentrating the virus resulted in removing soluble chemical mediators, such as cytokines which could have been produced by the fibroblasts during HCMV propagation, and thus, reduced the confounding variables. Additionally, since monocytes are capable of engulfing proteins and this could trigger intracellular effects which alter the expression of surface proteins, a parallel incubation of a freshly UV-inactivated (254 nm) portion of the virus stock was analyzed as well. The UV-inactivated virus was always tested for the presence of plaque forming units by standard plaque assay, and none were ever found.

However, in the previous studies of adherent cell infection with HCMV, the uniform decrease in HLA class I was used as a gauge of HCMV infection. Even when non-differentiated THP-1 cells were incubated with HCMV at an MOI=100, the HLA class I expression did not decrease; in fact, as shown in Figure 25, the expression of HLA class I actually significantly increased. The increase in HLA class I was not entirely due to the effects of live HCMV, since the effects of the antigen load, as measured with UV-inactivated (254 nm) HCMV, also caused a smaller, significant increase in HLA class I expression. However, the increase in HLA class I expression measured when THP-1 cells were incubated with live HCMV was uniformly much higher than that for cells incubated with UV-inactivated HCMV in all experiments performed. Interestingly, preliminary studies in which undifferentiated THP-1 cells were infected with HSV-1 (MOI=5 for 20 h) resulted in a significant decrease in HLA class I

↓ **Figure 25. Flow cytometry analysis of cell surface expression of CD55, CD46, CD59, and HLA class I (HLA 1) on THP-1, monocytic leukemia, cells following incubation with live HCMV (MOI=50) or UV-inactivated HCMV for 72 h p.i. Vertical statistics indicate comparisons to uninfected cells, while statistics above the umbrella bracket indicate comparisons between cells incubated with live and UV-inactivated HCMV. HLA class II (HLA 2) is not expressed on this cell line and serves as a second isotype-matched antibody control.**



expression. This indicates the HLA class I increased expression is specific for HCMV under these circumstances.

The increase in CD55 and CD46 expression, while observed was not any where near the magnitude of increased expression observed for HCMV infection of adherent cells (Figure 25). The incubation of undifferentiated THP-1 cells with UV-inactivated HCMV did not significantly increase the expression of CD55, but the expression of CD55 increased 62% ($p<0.01$) at 72 h p.i. These results were the same when either the monoclonal antibody 1H4 or 11D7, directed against CD55, were used for flow

cytometry analysis. Incubation of undifferentiated THP-1 cells with UV-inactivated HCMV did increase the expression of CD46 ($p<0.05$); however, the increase in CD46 expression following incubation with live HCMV was always greater ($p<0.01$), and like the effects of HCMV infection on adherent cells, the increase in CD46 expression was always a lower magnitude than that observed for CD55. These results were consistent when either the monoclonal antibody M75 or J4-48, directed against CD46, were used for flow cytometry analysis. The lower magnitude of CD55 and CD46 expression increase, and the opposite effect on the expression of HLA class I, may reflect the limited expression of HCMV genes reported in undifferentiated THP-1 cells (Weinshenker et al 1988). The increased expression of CD46, CD55, and HLA class I was observed regardless of whether the HCMV viral stock used for infection was the AD169 strain or the patient isolates of HCMV used in the previous section.

No significant change in CD59 was observed under any of the conditions, regardless of whether the monoclonal antibody BRIC229 or MIRL1, directed against CD59, were used (Figure 25). The expression of HLA class II was previously reported to be negative for THP-1 cells (Tomoda et al 1992, Vey et al 1992), even following differentiation with phorbol esters; thus monoclonal antibodies directed against HLA class II were used as an isotype-matched negative control. Following HCMV infection, HLA class II expression did not change (Table 5). Binding of a second isotype-matched control antibody raised against Aspergillus niger glucose oxidase, a protein which is neither expressed nor inducible in mammalian cells, was also found to be unaltered by HCMV infection (Table 5). This suggests that HCMV infection does not induce HLA class II expression on undifferentiated THP-1 cells, and that the increase in expression of CD55, CD46, and HLA class I is not due to increases in non-specific binding.

Table 5. Mean cellular fluorescence of non-specific antibody binding to uninfected and HCMV-infected cells.[1]

	HCMV (72 h p.i.)	No HCMV	Statistics[2]
HLA class II[3]	2.23 ± 0.15	2.63 ± 0.36	NS
Control[3]	1.23 ± 0.10	1.25 ± 0.05	NS

1. Mean cellular fluorescence is given as the mean ± standard deviation, each condition performed in triplicate. Undifferentiated THP-1 cells were infected with HCMV at an MOI=50 and harvested at 72 h p.i.

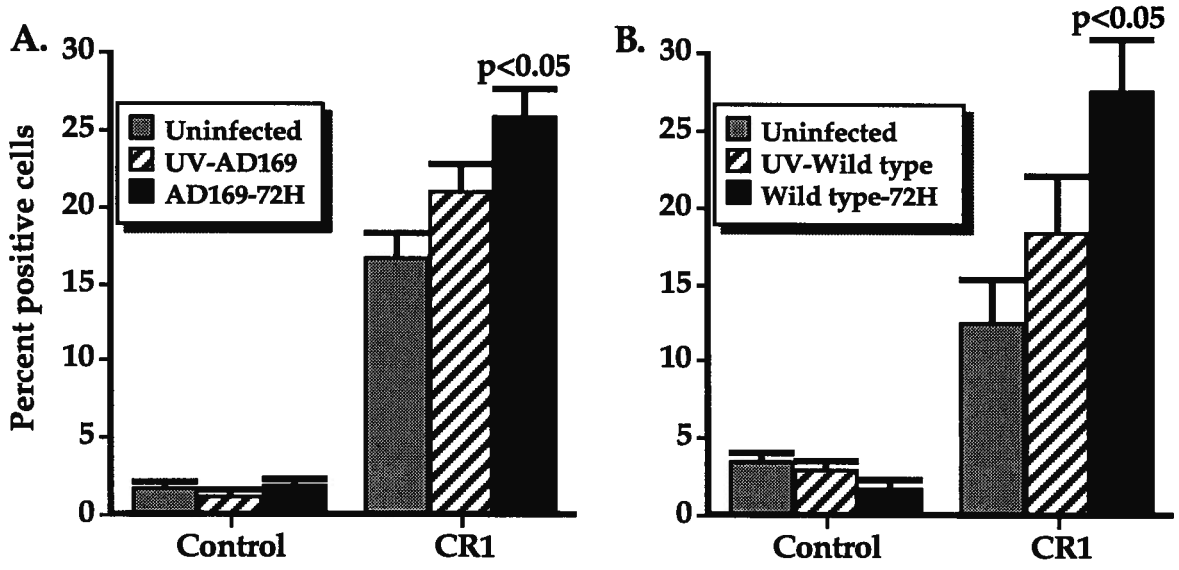
2. Comparisons were performed between HCMV-infected and uninfected cells tested with the same antibody by T-tests with the additional use of Bonferroni's correction for multiple comparisons. NS=not significant.

3. Monoclonal antibodies directed against HLA class II (isotype IgG_1) or against a yeast protein (control; isotype IgG_{2a}), which is neither expressed nor inducible in eukaryotic cells, were used as isotype-matched controls to investigate non-specific antibody binding by the cells.

The THP-1 cells also express low levels of another member of the RCA gene cluster, complement receptor 1 (CR1 or CD35; Thieblemont et al 1993), as do monocytes and macrophages (Ahearn and Fearon 1989, Thieblemont et al 1993). The effects of HCMV infection on CR1 expression were more subtle than the effect on CD55 and CD46 expression. The basal expression of CR1 is very low, but as shown in Figure 26 the percent of cells (above background) expressing CR1 increased with HCMV infection; while the expression of the control antibody did not change. No significant increase in CR1 expression was seen with undifferentiated THP-1 cells incubated with UV-inactivated HCMV, although it routinely approached significance. However, the patient isolate of HCMV appeared to have a greater ability to increase

CR1 expression than the AD169 laboratory strain of HCMV (Figure 26). These values are given as changes in percent of the population after subtraction of the background. Percent positive cells were used owing to the variability in the population and posibility that all cells may not be infected with HCMV.

↓ Figure 26. Flow cytometry analysis of CR1 expression on undifferentiated THP-1 cells after 72 h incubation with live HCMV or UV-inactivated HCMV. The only statistical significance identified was between HCMV infected and uninfected cells. Results from two representative experiments are shown using a laboratory strain, AD 169 (A), or a patient isolate of HCMV (B). Control isotype-matched antibodies directed against HLA class II or a yeast protein (see Materials and Methods) gave identical results for background.



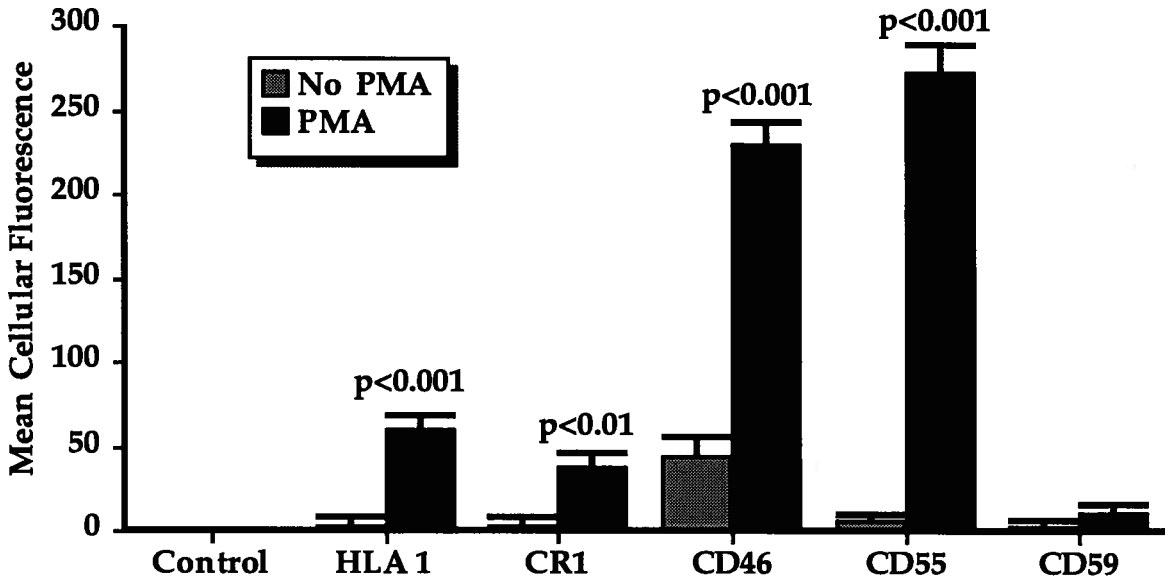
3.3.2. HCMV infection of phorbol ester-differentiated THP-1 cells.

The morphology of the THP-1 cells following differentiation into macrophage-like cells is quite different from undifferentiated THP-1 cells. In addition to increased phagocytic abilities and other functional abilities reported by others (Tsuchiya et al 1982, Mehta and Lopez-Berestein 1986), the expression of integrins and other adhesion molecules increased on THP-1 cells concomitant with their conversion to a predominantly adherent cell morphology (Prieto et al 1994). Once the THP-1 cells are adherent, they are much easier to infect with HCMV and the infection procedure is the same as for fibroblasts and glioblastoma cells. The THP-1 cells are fully permissive for HCMV infection following differentiation; extracellular HCMV is produced and can be detected by plaque assay (see Table 4 above). Unfortunately, the basal expression levels of CD46, CD55, CR1, and HLA class I also increased (Figure 27) after differentiation, which made interpreting the effects of HCMV infection post-differentiation a little difficult.

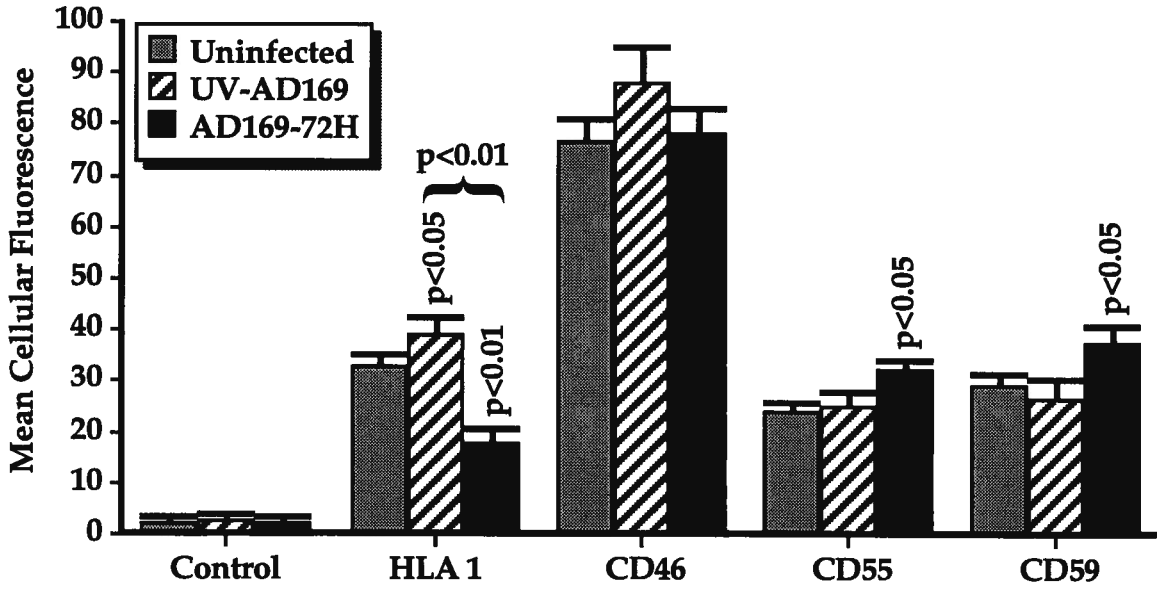
HCMV infection of differentiated THP-1 cells resulted in the significant decrease of HLA class I (Figure 28), which is opposite from the effects of HCMV infection of undifferentiated THP-1 cells. This indicated the induction of a permissive state for HCMV infection. However, incubation of differentiated THP-1 cells with UV-inactivated HCMV resulted in a small increase in the expression of HLA class I, similar to that seen when UV-inactivated HCMV was incubated with undifferentiated THP-1 cells. A 33% increase in CD55 expression ($p<0.05$) was measured on HCMV infected THP-1 cells after differentiation, while incubation of cells with UV-inactivated HCMV did not alter CD55 expression. HCMV infection did not effect CD46 expression on differentiated THP-1 cells, but CD59 expression was increased by 30% on HCMV-infected THP-1 cells (Figure 28). These last two results are markedly different than those observed for the infection of fibroblasts and glioblastoma cells, and may represent a difference in the HCMV lytic cycle in monocytic cells.

HCMV infection of the differentiated THP-1 cells also decreased the expression of CR1, which is contrary to the effect of infecting undifferentiated THP-1 cells with HCMV. Since the relative expression of CR1 on differentiated cells is still low compared to the other cell surface proteins, the percent of cells (above background) expressing CR1 is the best measure for alterations. Figure 29 shows a 3-fold decrease in the percent of cells positive for CR1 ($p<0.05$). No explanation for the contrasting effects of HCMV on differentiated versus undifferentiated cells is readily apparent.

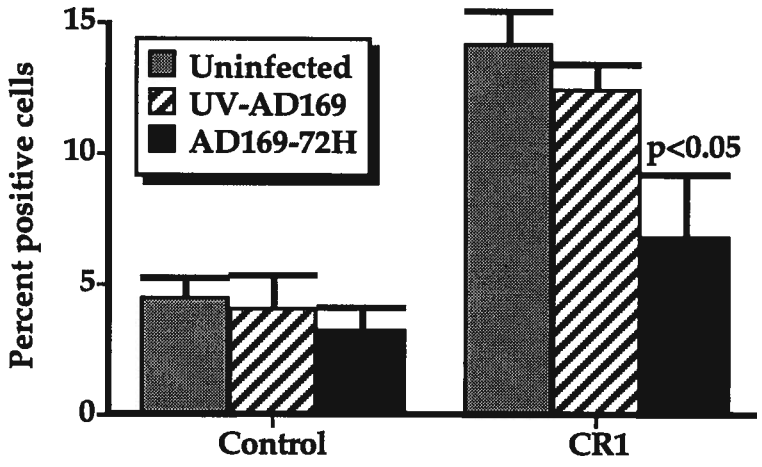
↓ Figure 27. Flow cytometry analysis of alterations in expression of CD46, CD55, CD59, HLA class I, and CR1 following differentiation with 300 ng/ml for 72 h. Isotype-matched control antibody is directed against HLA class II, and differentiation did not induce significant binding with this antibody.



↓ **Figure 28. Flow cytometry analysis of cell surface expression of CD55, CD46, CD59, and HLA class I (HLA 1) on differentiated THP-1, monocytic leukemia, cells following incubation with live HCMV (MOI=50) or UV-inactivated HCMV for 72 h p.i. The THP-1 cells were differentiated by exposure to 300 ng/ml phorbol esters for 72 h prior to infection with the laboratory strain, AD169, of HCMV. Statistics which are listed vertically indicate comparisons to uninfected cells, while statistics above the umbrella bracket indicate comparisons between cells incubated with live and UV-inactivated HCMV.**



↓ **Figure 29. Flow cytometry analysis of CR1 expression on differentiated THP-1 cells after 72 h incubation with live HCMV or UV-inactivated HCMV. The THP-1 cells were differentiated by exposure to 300 ng/ml phorbol esters for 72 h prior to infection with the laboratory strain, AD169, of HCMV. The only statistical significance identified was between HCMV infected and uninfected cells. Control isotype-matched antibodies were directed against HLA class II.**



3.4. CHAPTER 4. Mechanism of CD55 expression increase.

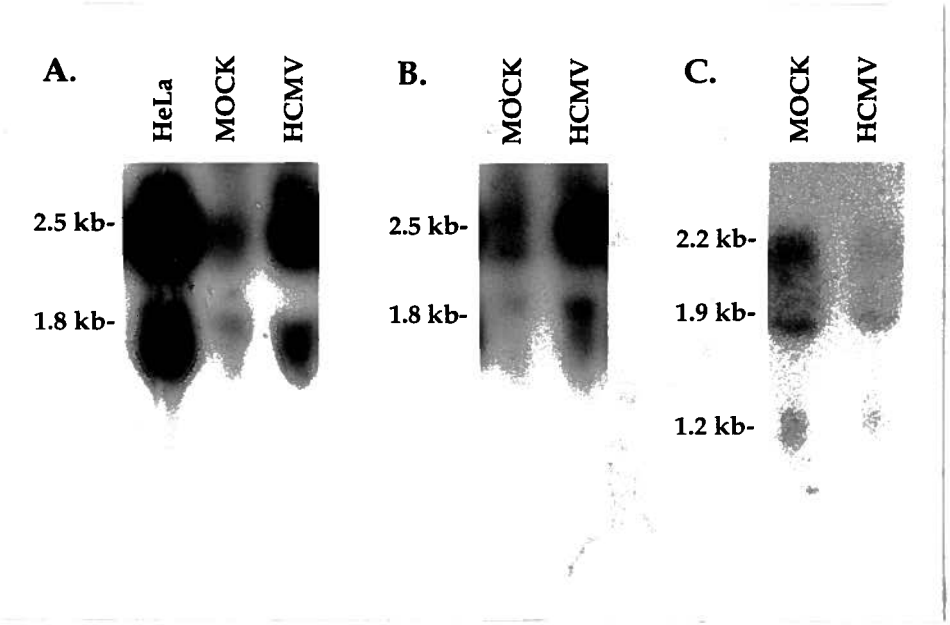
There are many mechanisms available to the cell with the common result of increased surface expression of CD55. This chapter will provide evidence suggesting that permissive HCMV infection of cells causes a increase in the transcription of the CD55 gene leading to an accumulation of CD55 mRNA species.

3.4.1 Northern blot analysis of HCMV-infected cells.

One possibility to account for the observed increase in CD55 expression is that HCMV infection upregulates the synthesis of CD55 mRNA. To investigate this possibility, total RNA was isolated from human fibroblasts and glioblastoma cells which were infected with the AD169 strain of HCMV for 72 h and uninfected controls. Thirty micrograms of total RNA from each was separated on a 1% formaldehyde-denaturing agarose gel and transferred to a nylon membrane. The amount of CD55 mRNA was determined by probing the nylon membrane with a radiolabeled anti-sense CD55 probe, the comparison for infected and uninfected fibroblasts and glioblastoma cells are shown in figure 30 A and B, respectively. Ten micrograms of total RNA from HeLa cells was included as the positive control for CD55 mRNA species (Thomas and Lublin 1992). Figure 30 C demonstrates the amounts of CD59 mRNA present in HCMV-infected and uninfected fibroblasts in the same samples used in figure 30 A and B, using a radiolabeled anti-sense CD59 probe. The levels of CD55 mRNA were greatly increased in HCMV infected cells, either fibroblasts or glioblastoma cells, at 72 h p.i., which parallels the flow cytometry and Western blot analysis findings. However, the amount of CD59 appeared to be diminished in HCMV infected fibroblasts compared to the uninfected cells.

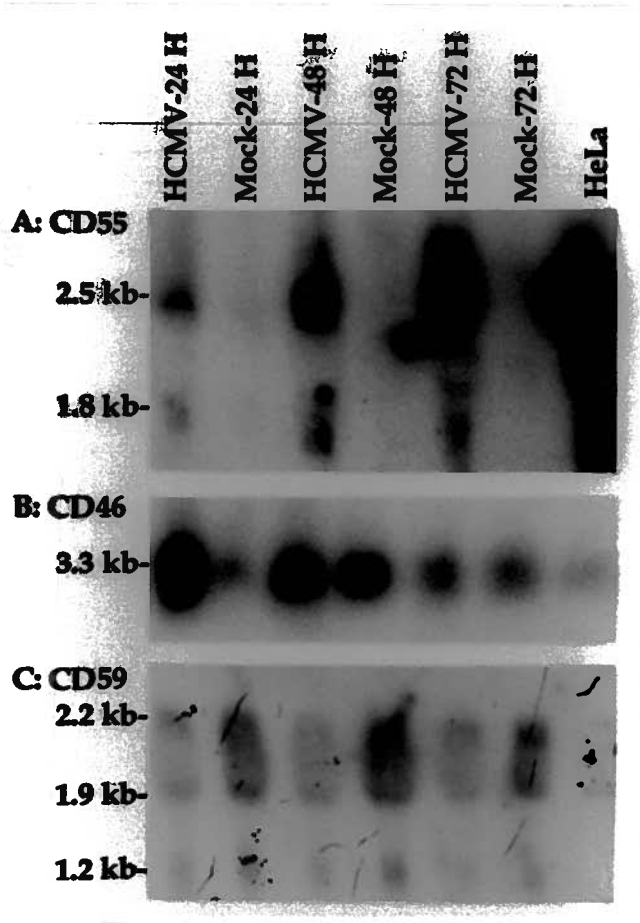
Further investigation of mRNA levels was performed by purifying total RNA from glioblastoma cells which were infected with HCMV, collecting samples from infected and uninfected cells at 24 h intervals corresponding to the time points investigated by flow cytometry (section 3.2.2). Thirty micrograms of each sample was

↓Figure 30. Northern blot analysis of HCMV-infected or mock-infected cells harvested at 72 h p.i. was performed using a radiolabeled probe made from CD55 cDNA on fibroblasts (A), or glioblastoma cells (B) or using a probe made from CD59 cDNA on glioblastoma cells (C). Thirty micrograms of total RNA was loaded for HCMV-infected cells and matched uninfected cells. Ten micrograms of total RNA from HeLa cells were included as a control for CD55 mRNA. Sizes of mRNA are listed next to each band and match those previously reported for CD55 and CD59 (Thomas and Lublin 1993, Holguin et al 1993).



separated under the same conditions described above and the nylon membranes were probed with radiolabeled anti-sense CD55, CD59, or CD46 probes (Figure 31). The amount of CD55 mRNA was increased by 24 h p.i. and continued to accumulate to the 72 h p.i., which parallels the flow cytometry findings (Figure 20). No alterations in the splicing pattern or ratio of minor and major transcripts were observed for CD55 which could account for the difference in the Mr of CD55 found on purified virions, suggesting the difference in the size of virion CD55 (Figure 15, p 67) may be due to alterations in post-transcriptional modifications.

The amount of CD46 mRNA did not demonstrate a consistent increase over the course of HCMV infection (Figure 31 B). Although the there appears to be a large increase in the CD46 mRNA at 24 H p.i., the differences between mock-infected CD46 mRNA levels through the 24 to 72 H time points makes it difficult to comment on the significance of the differences between mock-infected and HCMV-infected cells. This method may not be sensitive enough to detect alterations in CD46 mRNA, since cell surface expression only changes by 2-3 fold, as opposed to the 8-10 fold increase of CD55. Alternately, increased transcription of the CD46 gene may not be the mechanism responsible for the increased cell surface expression. The amount of CD59 mRNA appears to be decreased by 24 h p.i. and this decrease continues throughout all time points collected relative to samples collected for mock-infected



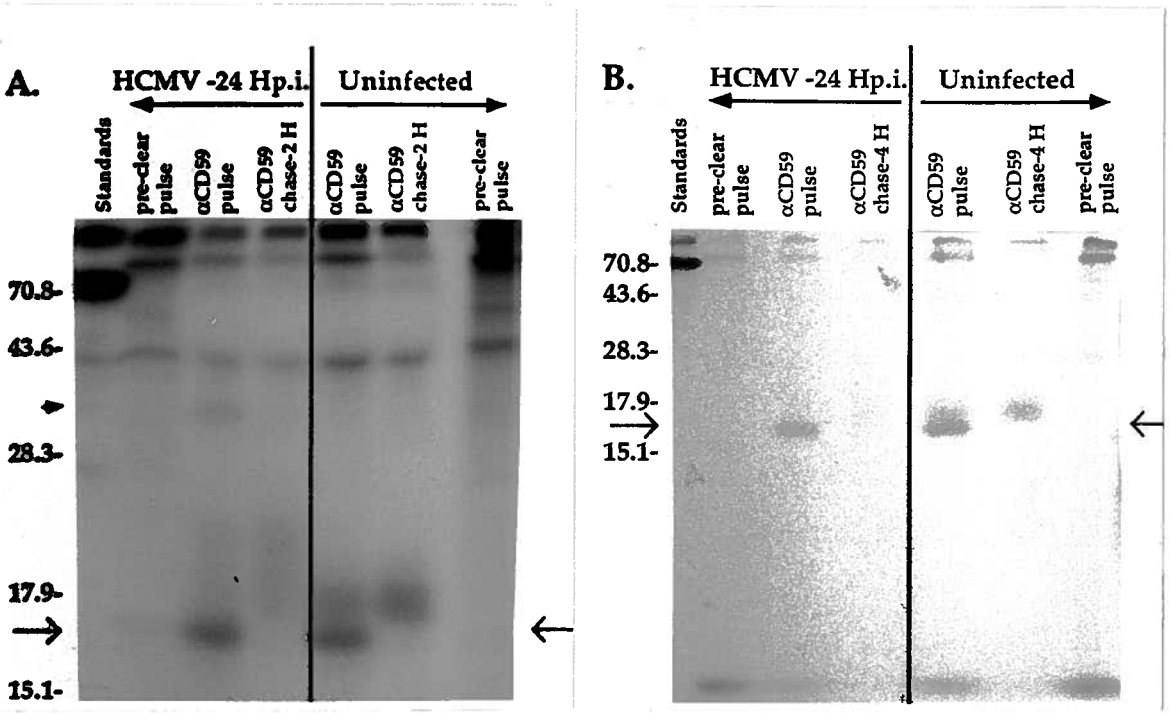
↓ Figure 31. Northern blot analysis of HCMV-infected or mock-infected glioblastoma cells harvested at 24 h intervals after infection. Thirty micrograms of total RNA from HCMV-infected or mock-infected cells for each time point were separated on a denaturing formaldehyde-agarose gel and transferred to a nylon membrane. Matching membranes were probed with radiolabeled anti-sense probes made from CD55 cDNA (A), CD46 cDNA (B), or CD59 cDNA (C).

cells (Figure 31 C); however, the magnitude of the decreaseis inconsistent with the flow cytometry findings which show a very small decrease in cell surface expression over 72 h p.i. for HCMV-infected glioblastoma cells.

3.4.2. Further investigation of decreased CD59 expression on HCMV-infected cells.

Northern blot analysis consistently reported a decrease in CD59 mRNA levels when cell were infected with HCMV. However, flow cytometry studies reported either a decrease or little change depending on the antibody used as described below. In order to understand the variability in these results, CD59 expression was further investigated using radiolabeled immunoprecipitation analysis. Equal numbers of HCMV-infected and uninfected fibroblasts were labeled with [^{35}S]-cysteine for 50 minutes at 24 h p.i. and harvested immediately or following a 2 or 4 h chase with excess cold cysteine (Figure 32). Non-specific bands were identified by performing an initial mock-immunoprecipitation by using all the reagents normally used, but omitting the addition of antibody. The polyclonal anti-CD59 antibody was then added and the samples were then subjected to a second round of immunoprecipitation. The mock immunoprecipitation was labeled as the "precleared" lane, and run adjacent to the other lanes. Figure 32 (A) shows that the amount of CD59 labeled by the initial radioactive pulse was slightly decreased at 24 h p.i.; however, processing of the precursor of 16.5 kDa to the mature 17-19 kDa form, seen after a 2 h chase with the uninfected fibroblasts was negligible following infection with HCMV. Furthermore, a second band of 34 kDa was present only in the HCMV-infected sample at the initial harvest and not in any of the other samples. Figure 32 (B), which differs only by the longer post-labeling chase, confirms these findings; again at 24 h p.i. the initial pulse of the infect cells indicates the amount of CD59 precursor was slightly decreased and no mature form can be seen. The protein labeling in (B) was not as strong as in (A), and the 34 kDa band in the HCMV-infected

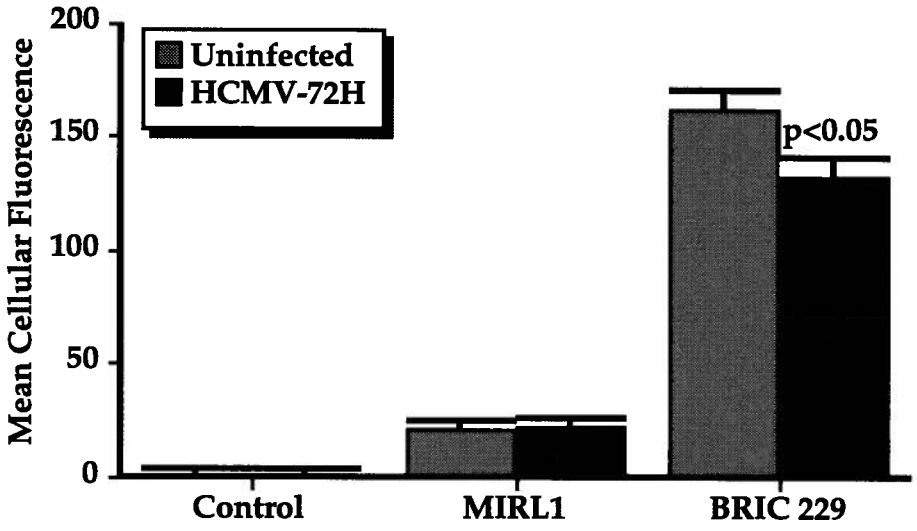
↓ **Figure 32. Autoradiograph from proteins labeled with [^{35}S]-cysteine, immunoprecipitated using a polyclonal anti-CD59 antibody and protein G-sepharose, and separated on a 15% polyacrylamide gel under reducing conditions. [^{14}C]-labeled bovine serum albumin was run in addition to unlabeled standards in both A and B. The left half of both gels represents samples from fibroblasts infected with HCMV for 24 h, while the right half represents samples harvested from uninfected cells. The lanes labeled as pre-clear pulse represent non-specific association of proteins with protein G-sepharose and indicate background banding patterns, since no immunoprecipitating antibody was added. The cells were 'pulsed' with 100 μCi [^{35}S]-cysteine for 50 min. at 37°C and lanes labeled pulse are samples collected immediately following labeling. Some samples were collected after the radiolabeled cysteine was removed and the cells incubated ("chased") with 'cold' cysteine for 2 h (A) or 4 h (B). The large arrows indicate the CD59 precursor at around 16 kDa, while the arrowhead indicates the presence of a 34 kDa band found only with the HCMV-infected cells in (A).**



is too faint to be distinguished; however, this band has consistently appeared in all pulse-chase experiments run.

The possible explanations for the different rates of CD59 decrease measured by different method include: 1) The steady-state half-life of cell surface CD59 is extremely long and the reduced production of mature CD59 does not represent a significant proportion of total, and therefore, was not observed by methods such as flow cytometry. 2) The relative dilution of the CD59 mRNA by the HCMV encoded mRNA species exaggerates the actual decrease in CD59 mRNA, and could account for the inability to observed increased CD46 mRNA levels in HCMV-infected cells. 3) The monoclonal anti-CD59 antibody used in the flow cytometry studies has a low affinity and did not accurately assess the cell surface levels of CD59. The actual reason is probably a combination of all three, but the only possibility easily assessed is the last. To address this possibility the levels of CD59 expression were investigated on HCMV-infected and uninfected glioblastoma cells at 72 h p.i., using two anti-CD59 antibodies which recognize separate epitopes (Figure 33). The basal levels of CD59-associated fluorescence on the surface of uninfected cells appear to be 8-fold higher when the monoclonal antibody BRIC229 is used. Furthermore, a significant decrease in the expression of CD59 was only detected on the HCMV infected cells, as compared to the uninfected controls, when BRIC229 was used. Therefore, the discrepancies between the decreases in CD59 expression (as measured by flow cytometric, Northern blot, and radio-immunoprecipitation methods) must be due to a long catabolic half-life for CD59 with some dilutional effects caused by the accumulation of viral mRNA.

↓ **Figure 33. Flow cytometry analysis of cell surface expression of CD59 on glioblastoma cells using two different monoclonal antibodies directed against CD59. Gray bars represent uninfected cells and black bars indicated cells infected for 72 h with HCMV. Even though the monoclonal antibodies are of the same mouse IgG class and both were used at predetermined saturating conditions, the BRIC229 monoclonal antibody is much better at detecting surface expression of CD59. The only statistical significance found for CD59 decreased expression was with the BRIC229 monoclonal antibody.**

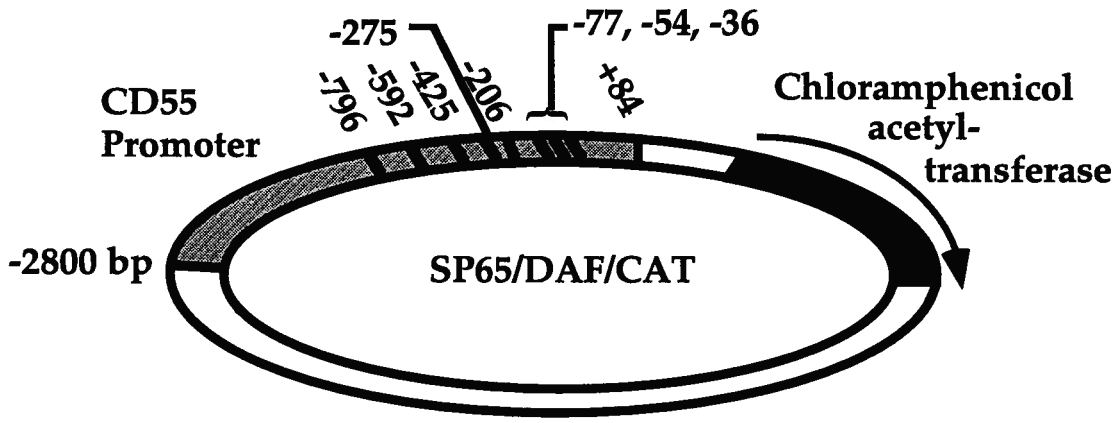


3.4.3. Requirements for CD55 promoter activity.

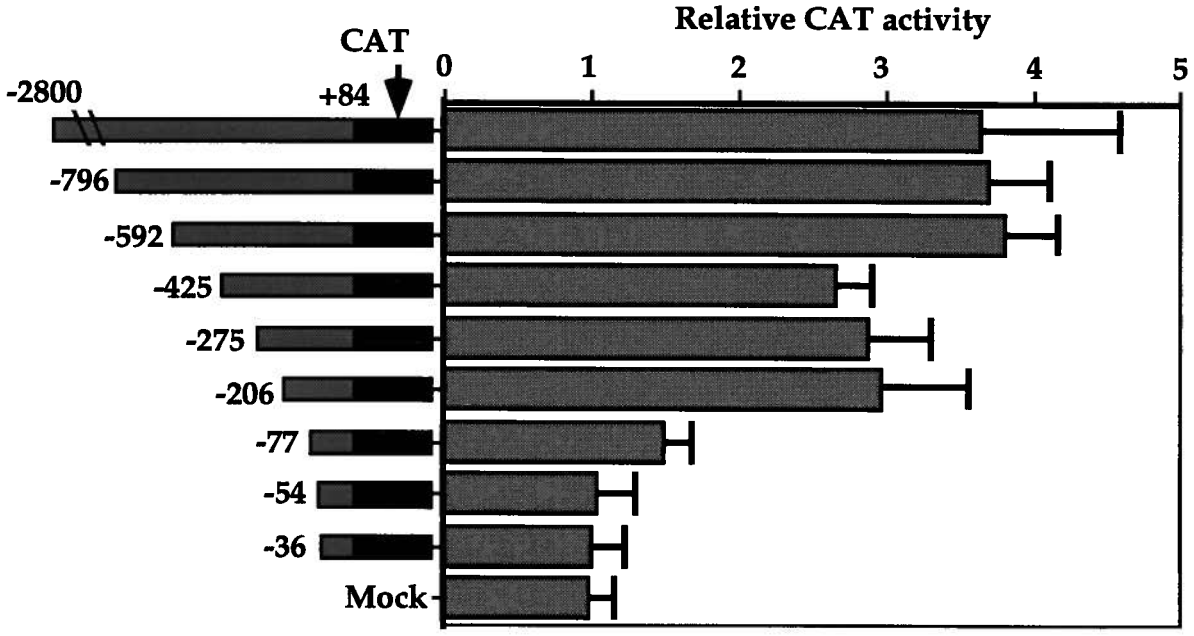
There are a number of mechanisms which could conceivably result in the accumulation of CD55 mRNA reported in section 3.4.1. To investigate the possible role of HCMV-infection on upregulation of CD55 transcription, I obtained the CD55 promoter inserted into a plasmid with a chloramphenicol-acetyl-transferase (CAT) reporter gene and several CD55 promoter 5'-deletion constructs (Figure 34). The basic requirements for the basal expression have been previously published (Thomas and Lublin 1992) for the HeLa cell line and a few other cell lines. However, I chose to use a different method of detecting CAT than the method used by Thomas and Lublin, and a few additional constructs were added including: -275, -425, -592, and -2800 to

+84. Figure 35 shows the relative CAT expression in HeLa cells under the influence of the different 5'-deletion constructs of the CD55 promoter. These findings are very similar to those reported by Thomas and Lublin: no CAT activity was seen until 77 bp of the promoter upstream of the transcriptional start site were present. However, nearly all of the full promoter activity was present with the construct containing -206 bp, and no statistically significant addition of activity was observed when up to -2800 bp were present. Figure 35 does suggest a weak pattern in which the activity of the -206, -275, and -425 constructs are slightly less than the -592, -796, and -2800 promoter constructs, but it does not stand up to statistical analysis.

↓ **Figure 34. The CD55 promoter was isolated from a bacteriophage genomic clones containing human CD55. A fragment containing 2800 bp upstream from the transcriptional start site to 84 bp downstream from the transcriptional start site (+84) was inserted into the plasmid SP65 containing the chloramphenicol-acetyl transferase (CAT) reporter gene. Deletion mutants were made by removing 5 segments of DNA with either restriction enzymes (-796 to -206 constructs) or by designing appropriate PCR primers (-77 to -36 constructs) (Thomas and Lublin 1993).**



↓ **Figure 35. Basal CD55 promoter activity was measured 48 h after transfection into HeLa cells. Intracellular CAT levels were measured using [^{14}C]-Acetyl CoA for each deletion construct measured. Almost all basal promoter activity was present when only 206 bp upstream from the start site were present. Mock activity (reporter plasmid only) CAT activity arbitrarily set=1, N=6 per point, bars=SEM.**



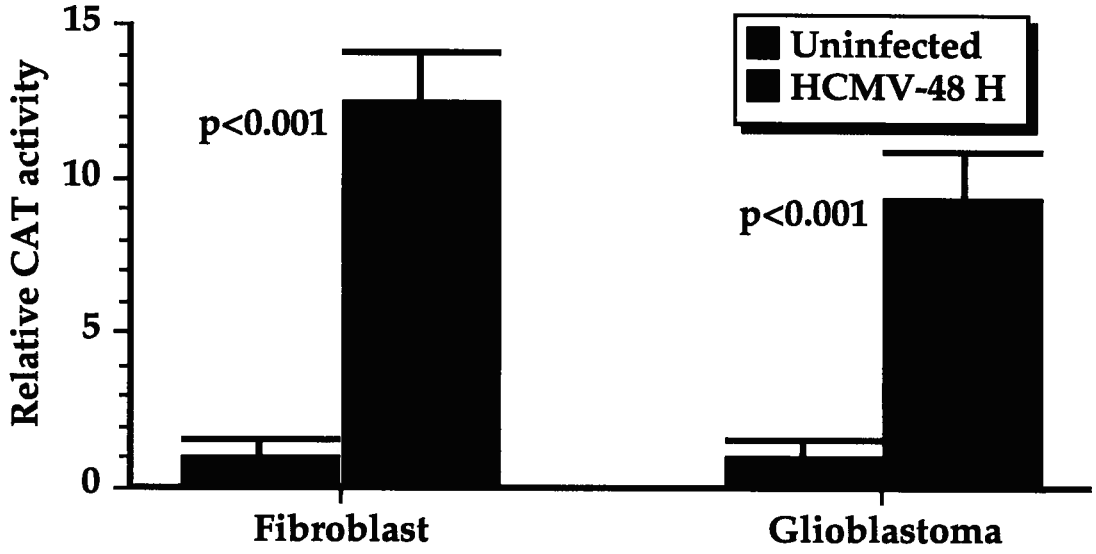
3.4.4. Effect of HCMV infection on CD55 promoter constructs.

Unfortunately, the HeLa cell line is not permissive for HCMV infection; therefore, the CD55 promoter construct containing -796 to +84 bp was transfected into glioblastoma cells and fibroblasts. Twenty-four hours after plasmid transfection of both cell types, half of the cultures were super-infected with the AD169 strain of HCMV and the other half were kept as uninfected controls. Forty-eight hours post-viral infection (i.e. 72 h post-transfection) HCMV-infected and uninfected cells were harvested and the intracellular CAT activity assessed (Figure 36). The levels of relative CAT activity of the -796 bp CD55 promoter construct were increased 10-12 fold in both cell types following HCMV infection compared to the levels in uninfected

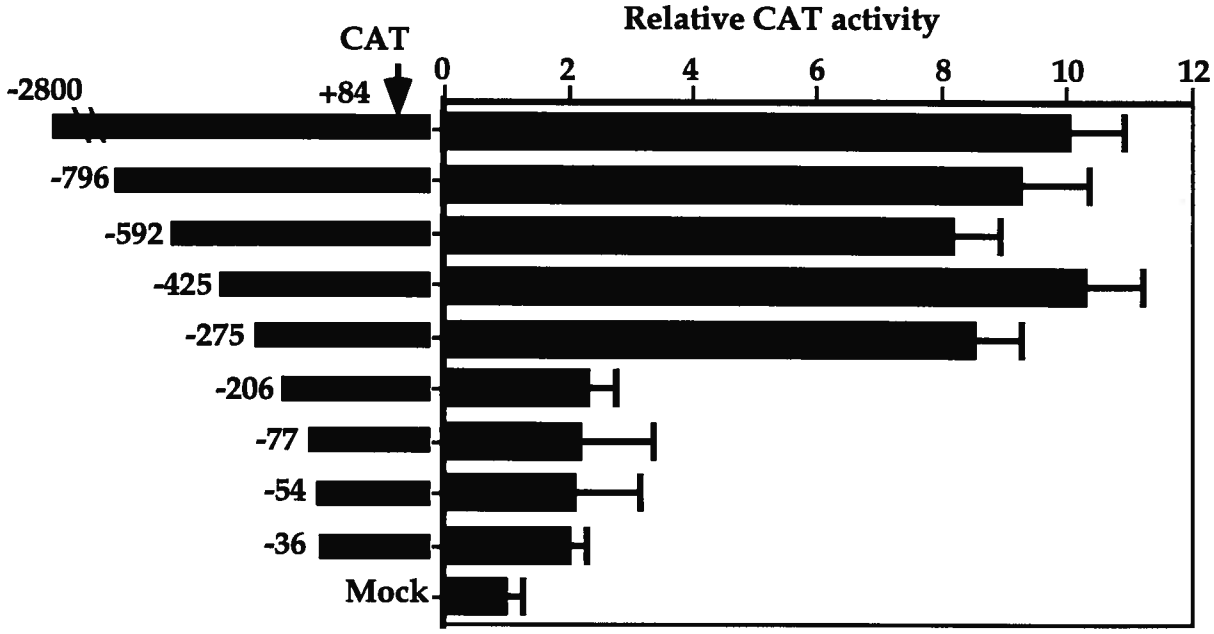
cells. This indicates that the transcription of the CD55 promoter is upregulated by HCMV infection and the accumulation of CD55 mRNA is, at least in part, caused by this increased transcription.

To address the basic requirements of the HCMV upregulation of transcription, compared to the basal transcription requirements, the 5'-deletion constructs of the CD55 promoter were used. The various constructs were transfected into the glioblastoma cell line because it survived the transfection procedure more readily than the fibroblasts. The super-infection of transfected cells was carried out as detailed above, and 48 h post-viral infection, the intra-cellular CAT activity was assessed. Figure 37 demonstrates that, unlike the basal transcription requirements, the upregulation of the CD55 promoter activity was first seen in the -275 to +85 promoter construct. No difference was seen amongst the CD55 promoter constructs larger than -275, up to and including the -2800 to +84 construct. Interestingly, no difference was seen in the basal levels of activity between the -206 and -275 construct in the HeLa cell line (Figure 35), but all of the HCMV upregulation activity seems to be between these constructs.

↓ Figure 36. Intracellular CAT activity from fibroblasts or glioblastoma cells which were transfected with the -796 to +84 CD55 promoter construct then harvested 72 h later (gray bars). The black bars represent cells treated exactly the same as those shown in gray except that they were infected with HCMV 24 h after transfection. A large increase in CD55 promoter activity was seen for both cell types when super-infected with HCMV. CAT activity is arbitrarily set at uninfected cell activity=1, N=6.



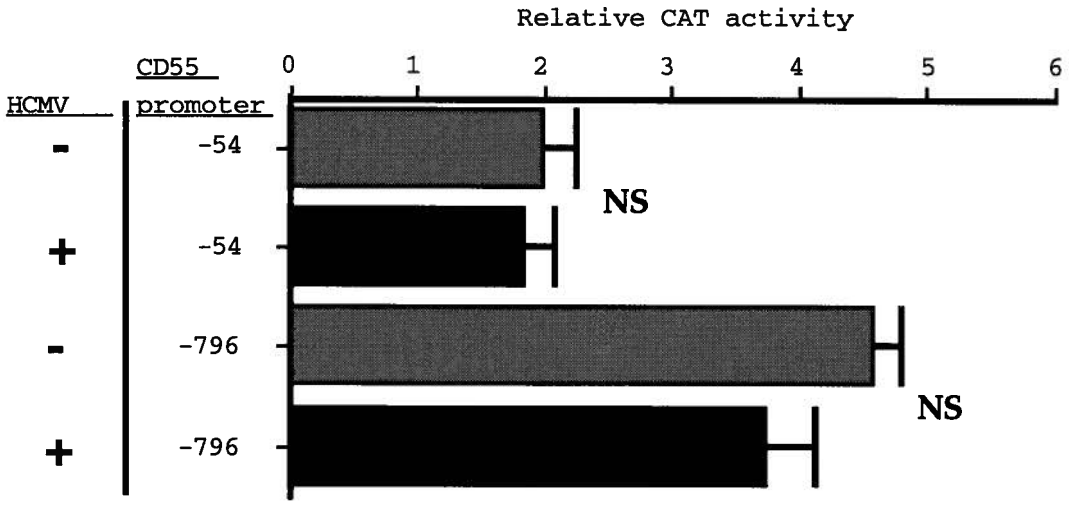
↓ **Figure 37. Intracellular CAT activity was measured for glioblastoma cells which were first transfected with the promoter constructs listed above, then super-infected with HCMV for 48 h prior to harvesting. Enhanced promoter activity required the presence of 275 bp upstream from the start site. This is different from the requirements of only 206 bp for the basal transcription. Mock activity (reporter plasmid only) CAT activity arbitrarily set=1, N=6 per point, bars=SEM.**



The basal activity of the CD55 promoter constructs was too low in the fibroblasts and glioblastoma cells to be assessed by the CAT measurement protocol I had selected. The reason it works for the HeLa cell line is readily apparent from the different intensities of the mRNA present between 10 μg of total HeLa RNA and 30 μg of total uninfected glioblastoma cell RNA seen by Northern blot analysis in Figure 30. Even though the HeLa cells are not permissive for HCMV infection, the possibility that the presence of HCMV virions was altering CD55 promoter activity without actually infecting the cells was addressed by incubating HeLa cells, transfected with the -796 CD55 promoter construct, with an amount of HCMV equivalent to a MOI=100 as determined by infection of fibroblasts (Figure 38).

No morphological changes indicative of cytopathic effect were observed by observation under the inverted microscope, while such changes were quite apparent in permissive cells infected with HCMV at the same time point. Furthermore, no alteration in the CD55 promoter activity was observed in HeLa cells incubated with HCMV. This indicates that the upregulation of the CD55 promoter activity requires permissive HCMV infection. The HCMV responsive element appears to be located in the region between -275 and -206 or may overlap the -206 restriction site, but analysis of this region did not identify any known transcription factor binding sites or known enhancer sequences.

↓ **Figure 38. Intracellular CAT activity from HeLa cells which were transfected with the -796 to +84 or -54 to +84 CD55 promoter construct. At 24 h post-transfection half of the transfected cells were incubated for 1 h with a high concentration of HCMV (black bars), then the cells were harvested 48 h later. The HeLa cells are not permissive for HCMV infection and incubation with a high amount of HCMV did not increase the CD55 promoter activity when compared to the transfected cells not incubated with HCMV (gray bars), indicating the effects of HCMV on the CD55 promoter in fibroblasts and glioblastoma cells (Figure 36) was not an artifact. CAT activity is arbitrarily set at background activity=1, N=6, error bars=SEM.**



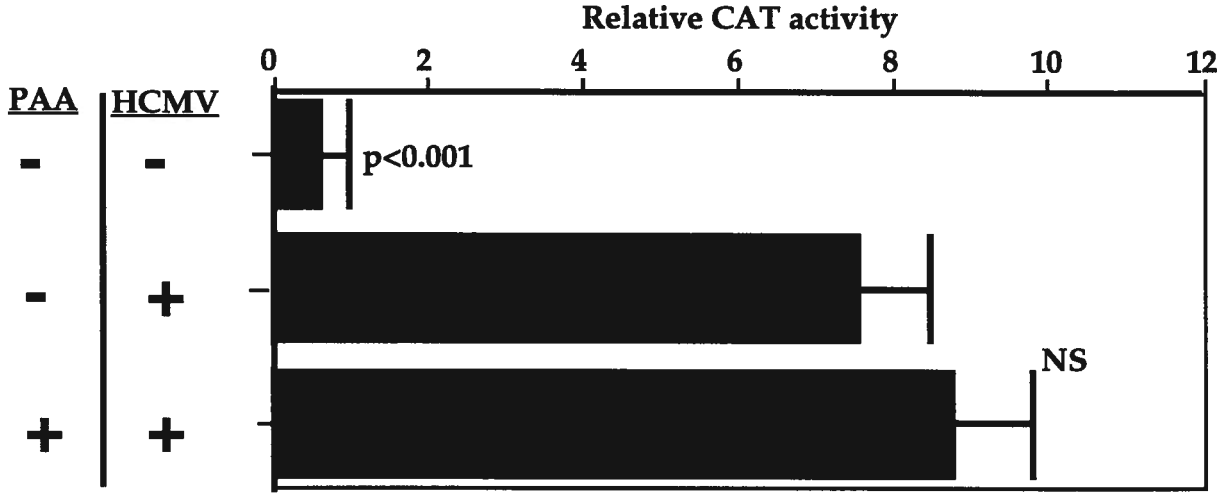
3.4.5. Effect of HCMV late gene repressor on CD55 upregulation.

A 2-fold increase in CD55 expression is measured by 24 h p.i. on HCMV infected fibroblasts, and 24 h p.i. is also the point at which the HCMV genome begins to replicate and the late HCMV genes are first expressed in infected fibroblasts (DeMarchi et al 1980). Therefore, it is probable that the HCMV immediate early or delayed early genes are responsible for the upregulation of CD55. It has been shown that infecting fibroblasts with HCMV in the presence of phosphonoacetic acid (PAA) completely inhibits the expression of the HCMV late gene expression and genome replication (Stinski 1977). Duplicate cultures infected with HCMV for 84 h p.i. in the presence of PAA did not produce infectious extracellular virus as assessed by plaque assay; while the culture infected with HCMV in the absence of PAA routinely produced between 10^3-10^4 pfu/ml of HCMV. Figure 39 demonstrates the effect of PAA on HCMV super-infection of glioblastoma cells transfected with the -796 CD55 promoter construct, as described in the previous section. The addition of PAA did not effect the CD55 promoter activity when the cells were super-infected with HCMV, since the promoter activity increased between 8-9 fold, compared to uninfected cells ($p<0.001$), regardless of whether PAA was present or not.

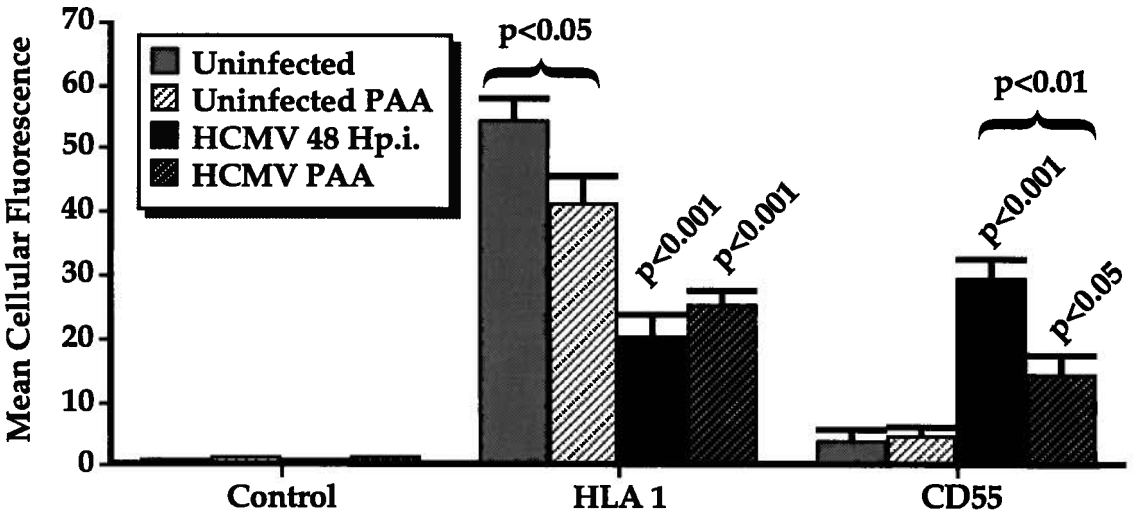
The effect of PAA was also tested using flow cytometric methods. The investigation utilized four groups of glioblastoma cells: one was mock-infected, one was mock-infected in the presence of 0.1 mg/ml PAA, one was infected with AD169 with no PAA, and the last group was infected with AD169 in the presence of 0.1 mg/ml PAA. Forty-eight hours p.i. all groups (each in triplicate) were harvested and cell surface expression of CD55 and HLA class I were assessed using monoclonal antibodies and flow cytometry (Figure 40). The decrease in HLA class I, used to assess the completeness of HCMV infection, was found to be significantly decreased by HCMV infection in the presence or absence of PAA ($p<0.001$). However, PAA alone was also observed to decrease the expression of HLA class I. Although the decrease was less than that observed for HCMV infected cells, it was observed to be

separate from the effects of HCMV ($p<0.05$). The expression of CD55 was found to be significantly increased on HCMV-infected cells in the presence and absence of PAA ($p<0.05$ and $p<0.001$, respectively). However, the increase in CD55 expression on cells infected with HCMV in the presence of PAA was found to be significantly less than in the absence of PAA ($p<0.01$).

↓ Figure 39. Intracellular CAT activity from glioblastoma cells which were transfected with the -796 to +84 CD55 promoter construct and super-infected with HCMV, in the presence or absence of 0.1 mg/ml phosphonoacetic acid (PAA), then harvested 48 h later. The large increase in CD55 promoter activity still occurred in the presence of PAA, suggesting the involvement of HCMV early or immediate early genes. CAT activity is arbitrarily set at uninfected cell activity=1, N=6, error bars=SEM.



↓ **Figure 40. Flow cytometric analysis of HCMV-infected or uninfected glioblastoma cells was performed using predetermined saturating amounts of monoclonal anti-CD55 and HLA class I antibodies. The effect of 0.1 mg/ml PAA (48 hour exposure) on the cell surface protein expression for HCMV-infected (harvested at 48 hours post-infection) and uninfected cells was determined (see legend in figure for designations). Error bars indicate one standard deviation. Statistical significance between adjacent samples is indicated by an umbrella bracket, while statistical comparison between PAA or no PAA matched pairs is listed at a 45° angle. No difference in the isotype matched monoclonal antibody was observed.**



There are two possible explanations for these results: 1) The late genes of HCMV play a partial role in the increased expression of CD55, or 2) the presence of PAA adversely affects the proper synthesis of some host proteins, as demonstrated for HLA class I. The basal expression CD55 on the glioblastoma cells did not seem to be affected by PAA, but the basal expression of CD55 on glioblastoma cells is also extremely low. Additionally, the CD55 promoter data shown in Figure 39 demonstrated no effect of PAA on HCMV upregulation of CD55, and tend to support the latter possibility.

3.4.6. No Upregulation of CD55 promoter by isolated HCMV early genes.

All of the results reported in this thesis suggest that the HCMV immediate early (IE) or early (E) genes play a role in the upregulation of CD55 expression. To address this possibility, I obtained all of the isolated HCMV immediate early genes (that are known to date) inserted in eukaryotic expression vectors from a number of sources (see materials and methods). These IE genes were transiently co-transfected with the -796 CD55 promoter and the CAT activity assessed (Table 6), and the IE genes were also transfected into cells and their effects on the endogenous CD55 expression were assessed by flow cytometry (Table 7). As shown in Tables 6 and 7, none of these genes had any effect on CD55 promoter activity or CD55 cell surface expression, either alone or in combination. To investigate the possibility that these results were negative due to low transfection efficiency, a plasmid containing the Lac Z driven by an HCMV IE promoter was added to all transfections, and the transfection efficiency was assessed on duplicate cultures by staining for Lac Z activity. In the presence of X-gal, a substrate for β-galactosidase (Lac Z) successfully transfected cells turn blue. All of the cultures of the experiments reported in Table 6 and 7 had transfection efficiencies from 8-12 % as assessed by microscopy. At these levels, the flow cytometric analysis should have been able to identify a sub-population upregulated within the untransfected cells, and the transient co-

transfection assay should be unaffected since super-infection of cells transfected by the same procedure yielded good results (see section 3.4.4). Therefore, these results suggest that these HCMV IE genes are not directly responsible for the upregulation of CD55 or CD46 on glioblastoma cells.

TABLE 6. Transient transfection assessment for co-transfection of the CD55 promoter/reporter and isolated HCMV IE genes.[1]

IE genes[2]	mg/ml[3]	Promoter construct[4]	Relative CAT activity
None	0	-796 to +84	1.00 ± 0.06
IE1, IE2	5	-796 to +84	1.06 ± 0.06
IE1, IE2, US3, UL36-38	5	-796 to +84	1.20 ± 0.20
None	0	-275 to +84	1.00 ± 0.10
IE1, IE2, US3, UL36-38	5	-36 to +84	0.95 ± 0.13
IE1, IE2, US3, UL36-38	5	-275 to +84	1.00 ± 0.13
AD169[5]	-	-796 to +84	30.1 ± 5.1

1. Promoter activity was assessed using transient co-transfection of the isolated HCMV immediate early genes and CD55 promoter construct listed for each value. N=3 for each value reported as mean ± standard deviation, relative CAT activity in cells transfected with the CD55 promoter only were arbitrarily set as equal to 1.00. Isolated HCMV immediate early genes in expression vectors were transfected into glioblastoma cells using the CaCl method listed in materials and methods.

2. The IE genes were transfected in the combinations listed, and IE genes transfected alone yielded identical results.

3. This column lists the amount of each IE construct used in that transfection.

4. This column lists the CD55 promoter construct utilized for the IE genes listed.

5. A positive control using the longest CD55 promoter and super-infected with the AD169 strain of HCMV was included to ensure the CD55 promoter used for co-transfection with the IE genes was responsive to HCMV infection.

TABLE 7. Flow cytometry analysis of CD55 expression following transient transfection with isolated HCMV IE genes.[1]

IE genes[2]	mg/ml[3]	% cells in gate 3[4]	MCF of the top 5% of cells[4]
None	0	1.53 ± 0.57	34.79 ± 0.56
IE1	5	0.96 ± 0.25	33.79 ± 0.08
IE2	5	0.63 ± 0.21	36.76 ± 4.03
None	0	1.63 ± 0.12	32.96 ± 1.17
IE1, IE2, US3, UL36-38	1	1.57 ± 0.76	32.45 ± 1.42
IE1, IE2, US3, UL36-38	2	1.87 ± 0.26	35.68 ± 2.72
IE1, IE2, US3, UL36-38	5	1.60 ± 0.14	34.03 ± 0.78
AD169[5]	-	75.4 ± 2.10	--

1. Isolated HCMV immediate early genes in expression vectors were transfected into glioblastoma cells using the CaCl method listed in materials and methods. The effect of transfection on the endogenously expressed CD55 was assessed by flow cytometry using monoclonal antibodies directed against CD55. N=3 for each value reported as mean ± standard deviation.

2. The IE genes were transfected in the combinations listed, and IE genes transfected alone yielded identical results.

3. This column lists the amount of each IE construct used in that transfection.

4. Since the transfection efficiency for this method is roughly 10%, the transfected cells with increased CD55 on the cell surface would be observed as a sub-population. Therefore, gate 3 was arbitrarily set at 1.5% for the cells which were not transfected with IE genes, so that any sub-population present with increased CD55 would be identifiable. Gate 2 was set at 5% for the cells which were not transfected with IE genes and the mean cellular fluorescence (MCF) is reported so that the increased CD55 expression would be minimally diluted by the fluorescence of the larger, untransfected population.

5. A positive control using glioblastoma cells infected with the AD169 strain of HCMV was included to ensure CD55 expression was responsive to HCMV infection in these experiments.

4. DISCUSSION

Other investigators (Lewis et al 1986, Eizuru et al 1988, Rundell and Betts 1982) have reported that complement enhances the neutralizing ability of HCMV-seropositive serum. My studies confirm this finding, and elaborate the extent of the complement binding to virions.

The accessory role for complement in assisting viral neutralization has been previously described for other viruses as well. The neutralization of equine arteritis virus requires both complement and specific antibody (Radwan and Burger 1973). Complement was also found to be essential for the neutralization for equine herpes virus-1 *in vivo* , but only during the first 2 days after infection; following this period the enhancing effect of complement decreased (Snyder et al 1981). Interestingly, a difference in the mechanism of complement enhanced neutralization was found between homotypic and heterotypic complement and antibody sources for avian infectious bronchitis virus (AIBV; Berry and Almeida 1968). Incubation of fowl sera containing indigenous antibodies with AIBV virions resulted in an increased protein halo around the virus particles when viewed by electron microscopy while incubation of AIBV virions with serum from rabbits previously inoculated with the virus resulted in the formation of 100 angstrom holes in the virion envelope. The lack of evidence for the formation of the MAC, as assessed by electron microscopy, between rabbit and fowl complement suggests that the AIBV virions contain an inhibitor for fowl complement which interferes with the complete activation of complement. The presence of a species specific inhibitor would not be surprising, since an avian virus would have evolved against the selective pressure of an avian immune system. These findings are comparable to the results presented in this thesis, except that the source of the complement inhibitors on HCMV virions appears to be host cell from which the virus was derived (an option which is also possible for the AIBV). The presence of the host complement inhibitors on the HCMV virions may also explain the lack of noticeable neutralizing ability of the seronegative sera, even though I found that

incubation of seronegative serum with HCMV virions resulted in the deposition of C3 on the virions. Reports of viral proteins which directly activate the classical complement pathway exist (Bartholomew et al 1978), but the C3 deposited on the HCMV virions probably arose from minimal activation of the alternative pathway since similar amounts of C3 were deposited on HCMV virions incubated with HCMV-seronegative serum and serum in the presence of EGTA, a classical complement pathway inhibitor.

I found the presence of antibodies greatly increased the deposition of C3 on the HCMV virions and was required to achieve a level of complement activation which resulted in significant amounts of C9 being associated with the HCMV virions. Studies reported for herpes simplex type-1 (HSV-1), using C4-, C5-, C6-deficient guinea pig serum, suggested that the presence of a functional terminal lytic pathway was not necessary for neutralization enhancement by complement (Daniels et al 1970). Furthermore, Daniels et al (1970) found that the addition of purified C4 to optimum amounts of activated C1q on HSV virions enhanced virus neutralization, but that the further addition of purified C2 and C3 only further enhanced the virus neutralization if the amount of C4 present was sub-optimal. These minimal requirements of complement for HSV neutralization are probably not reflective of the requirements for HCMV neutralization; C3 was present on HCMV virions incubated with HCMV-seronegative serum, but with a negligible effect on HCMV neutralization. However, these conclusions are tenuous since the presence of complement-activating antibodies on the virion would undoubtedly result in much greater quantities of the early complement components being deposited on the virion.

Complement assisted neutralization of some type C oncoviruses results in virolysis, as assessed by release of radiolabeled RNA and tegument enzymes (Cooper et al 1976, Spear et al 1990); indicating complement activation by these viruses goes to completion. Complement activation by HCMV virions appears to go to completion only in the presence of specific anti-HCMV antibodies as assessed by the presence of

C9 with the HCMV virions. However, whether true virolysis occurs is questionable since the virions maintain enough integrity to survive through 3x 30% sucrose purification steps post-incubation (see materials and methods).

The absolute requirement of complement for efficient neutralization was reported for the initial infection of horses with equine herpes virus-1 (Snyder et al 1981). However, the enhancing abilities of complement for antibody neutralization decreased with time, and the same effect has been reported for anti-HSV antibodies (Yoshino et al 1977). The neutralizing enhancement of complement for equine herpes virus-1 and HSV could be a combination of the switch from IgM to IgG production, since IgM is far more efficient at activating complement, or the rapid affinity maturation of the antibodies being produced, which would result in the increase in the intrinsic neutralizing abilities of the antibodies and subsequently reduce the apparency of complement's effect.

The pattern of antibody response to HCMV infection has been delineated in the literature. Patients with primary HCMV infections were found to seroconvert within the first 2-10 weeks and the mean antibody response titer reached a plateau around 10 weeks after the first symptoms were noticed, but the antibody response in patients re-infected or with a reactivated infection attained higher levels much earlier in infection (Pass et al 1983). Spencer and Andersen (1972) found that the neutralizing antibody titers rose later than antibodies detectable by indirect fluorescent assay or complement fixing assay. The enhancing effect of complement in antibody-mediated neutralization in my studies was investigated using serum samples from volunteers who had been seropositive for at least one year and, therefore, represent convalescent serum samples. It should be noted that my experiments were not carried out with pooled serum, but were performed using individual characterized sera in each experiment. To my knowledge no reports exist which indicate that the neutralizing abilities of early antibodies generated against HCMV infection are enhanced to a greater or lesser degree than antibodies generated at later stages of infection, and

samples from patients recently infected with HCMV were not readily available to me. However, one murine monoclonal antibody directed against the HCMV-envelope glycoprotein p130/55 required complement to neutralize HCMV virions while a second antibody directed against another envelope glycoprotein, p86, did not; even though they were both the same IgG subclass (IgG_{2a}). This suggests that the mechanism of complement enhancement of antibody neutralization is much more complex than simple subclass differences between early and late antibodies. Britt et al (1988) found that neutralizing monoclonal antibodies generated after inoculation of a non-glycosylated recombinant from of HCMV p130/55 did not require complement for neutralization while antibodies generated from glycosylated p130/55 did; suggesting the specificity of the antibody is more important to complement requirements.

Finally, host encoded CD55, CD46, and CD59 were found on purified HCMV virions. The greater presence of CD55 and CD59 on the virions, as compared to CD46, probably represents the difference in transfer of glycolipid-anchored proteins to budding virions compared to transmembrane proteins. However, the apparent molecular mass for the host-encoded complement inhibitors associated with virions was more variable than that found on uninfected fibroblasts or fibroblasts infected with HCMV for 72 h (Fig 15C). A similar finding has been found when comparing the molecular weights of virion glycoproteins purified from simian CMV and HCMV virions (Benko and Gibson 1986). It was indicated by these authors that the disperse appearance of the electrophoretic profile of HCMV virion glycoproteins makes it difficult to assign accurate molecular weights. Some of the variability was accounted for by heterogeneity of the glycosylation; the proteins appear to be heavily glycosylated and incompletely processed since some virion glycoproteins appear to have both high mannose and complex type carbohydrates on the same protein (Benko and Gibson 1986). It is feasible, therefore, that the glycosylation of host proteins may be affected with progressive HCMV infection. In fact, the slight alteration in host

protein processing may account for the unique ability of sera from patients acutely infected with HCMV to induce the lysis of HCMV-infected fibroblasts (Betts and Schmidt 1981, Middeldorp et al 1986). The early immune response to HCMV may include antibodies which are directed against modified epitopes on CD55, CD46, and CD59. This could decrease the ability of HCMV-infected cells, even with the upregulated expression of CD46 and CD55, to regulate complement, and would also result in the addition of more cell-bound complement activating antibodies. However, since these represent autoantibodies the affinity maturation process would likely eliminate the production of these antibodies and may explain the absence of cytolytic abilities in HCMV-seropositive serum from convalescent patients.

Complement represents one of the first lines of defense against foreign antigens, and host-encoded complement inhibitors associated with the virion may provide a reprieve from complement-mediated clearance. A second report of virions containing CD55, CD46, and CD59 was published after this body of work was completed: these host complement inhibitors being associated with HIV and SIV virions in that report (Montefiori et al 1994). Similar to Dr. Cooper's findings for HCMV (1993), Montefiori et al found that monoclonal antibodies directed against CD46 and CD59 blocked the complement regulating ability of the virions and resulted in a complement-dependent reduction in HIV and SIV infectivity. It is possible that many of the enveloped viruses passively carry along host complement regulators and that this represents a viral evolution strategy for evading the host immune system.

The presence of complement regulators on HIV and SIV virions is interesting since both viruses also activate complement, even in the absence of anti-HIV antibody (Solder et al 1989). However, the C3 fragments left on the virion surface are capable of mediating immune adherence to complement receptor-bearing cells, and complement activation has been shown to enhance the binding of HIV to CR2 by 10-fold (Montefiori et al 1992). Thus complement can alter the distribution of host cells for the virus. Similarly, the EBV glycoprotein gp350 was found to enhance the

cleavage of bound C3 to C3dg which binds to the EBV receptor, CR2, which is also the receptor for gp350 (Mold et al 1988). It is possible, therefore, that the regulated presence of C3 fragments on the surface of HCMV virions represent a mechanism which directs HCMV to bind to cells expressing C3 receptors, such as the monocyte/macrophage which may be the HCMV reservoir *in vivo* .

Concurrent with my studies of purified HCMV virions, I investigated the effect of HCMV-infection on host protein expression. The upregulation of CD55 or decay-accelerating factor on the cell surface induced by non-viral effects has been seen. Shibata et al (1991) reported that incubating mesangial cells with human complement components which were activated by unrelated immune complexes resulted in a 2-fold increase in the expression of CD55. This is relevant to HCMV-infected cells, since *in vivo* anti-HCMV antibodies would activate complement and may increase the CD55 expression further than the direct effects which I have presented in this dissertation. The increased CD55 expression that I measured for phorbol ester stimulated THP-1 cells was previously reported for phorbol ester stimulated endothelial cells (Bryant et al 1990), and was found to be associated with protein kinase C activation. The fact that HCMV infection of THP-1 cells which were pre-differentiated by phorbol esters resulted in a further increase suggests that the HCMV-induced increase maximized this stimulation pathway , or more possibly, acts via a second unrelated pathway. Interestingly, incubation of endothelial cells with tumor necrosis factor, IL-1, interferon gamma (IFNγ) and some lectins did not alter CD55 expression, but incubation of endothelial cells with the lectin wheat germ agglutinin increased CD55 expression 5-fold and incubation with lectins ConA and PHA increased CD55 expression by two-fold (Bryant et al 1991). Not surprisingly, there appears to be some difference in response amongst cell types; incubation with IL-1 and IFNγ had a negligible effect on CD55 and CD46 expression on endothelial cells (Moutabarrik et al 1993, Bryant et al 1991), but were found to increase both CD55 and CD46 expression on cultured thyroid cells (Tandon et al 1994). Exposure of

endothelial cells to histamine also resulted in a two-fold increase in CD55 expression as measured by flow cytometry, without affecting the expression of CD46 or CD59, but the amount of CD55 shed into the supernatant also greatly increased (Tsuji et al 1994), indicating that flow cytometry measurements may underestimate the magnitude of CD55 increase in my studies.

Increased expression of the other complement inhibitors have also been reported. As mentioned above, CD46 expression was increased on cultured thyroid cells exposed to IL-1 and IFNγ (Tandon et al 1994), and interestingly, elevated CD46 levels are commonly associated with leukemic tumor cell lines, except B-cell lines, and other malignancies (Seya et al 1994, Seya et al 1990, Hara et al 1992, Cho et al 1991). While I found that the expression of CD55 and CD46, which are closely related and belong to the RCA gene cluster, were both increased with HCMV infection of adherent cells, alterations in CD46 and CD55 appear to be mostly independent of one another in most other reports (Tsuji et al 1994, Moutabarrik et al 1993). Increased CD59 expression had been reported for endothelial cells incubated with IL-1β (Moutabarrik et al 1993), phorbol esters (Meri et al 1993, Holguin et al 1993), and the expression of CD59 has been reported to be elevated on colonic adenocarcinoma cells (Bjorge et al 1994). However, I only found an increased CD59 expression on the THP-1 cells when infected with HCMV or treated with phorbol esters; CD59 expression was decreased on adherent cells infected with HCMV. The only other reports of decreased CD59 expression include: endothelial cells incubated with IL-1β (Moutabarrik et al 1993), phorbol ester stimulated HL60, promyelocytic leukemia cell line (Sedlak et al 1993), and CD8(+) lymphocytes infected with the retrovirus, human immunodeficiency virus-1 (HIV-1; Weiss et al 1992).

I only observed an increase in CR1 expression on undifferentiated THP-1 cells, since the fibroblasts and glioblastoma cells did not express this complement inhibitor. Other investigators have reported similar increases in CR1 on monocytes isolated from patients with rheumatoid arthritis (McCarthy et al 1992). In my experiments, the

small increase in CR1 probably represents activation of the undifferentiated THP-1 cells because these cells are only permissive for HCMV infection after differentiation (Weinshenker et al 1988), CR1 pools have been reported to be in the secretory vesicles of neutrophils (Sengelov et al 1994), and other reports show CR1 expression increased on monocytes under conditions of activation (Leino and Lilius 1992). Conversely, the decreased expression of CR1 on differentiated THP-1 cells following HCMV infection is much more interesting. CR1 expression has been reported to be decreased on erythrocytes in patients infected with HIV (Pascual et al 1994), on erythrocytes from patients connective tissue diseases, on erythrocytes from patients with a high amount of circulating immune complexes (Corvetta et al 1991, Tausk and Gigli 1990), and on leukocytes isolated from patients with chronic myelogenous leukemia (CML; Lanza et al 1991, Lanza and Castoldi 1992). However, the relevance of decreased erythrocyte CR1 are minimal, since HIV cannot directly infect erythrocytes and immune complexes do not exist in my *in vitro* system; however, neutrophils from AIDS patients were reported to have a decreased ability to upregulate surface expression of CR1 in response to activation (Tausk and Gigli 1990). Another possible explanation may relate to the trypsin sensitivity of CR1 (Pascual et al 1994); thus, patients infected with HIV could have more circulating proteases, which could also be released from my differentiated THP-1 cells when infected with HCMV. Alternately, the expression of CR1 may indicate an alteration in cell differentiation, such as with CML, which may occur with HCMV infection.

The HCMV genes involved in upregulation of certain host proteins have been studied by other investigators. I was interested to know whether these genes could also be responsible for the changes that I observed in host-encoded complement proteins. The HCMV immediate early genes 1 and 2 (IE1 and IE2) were found to transactivate the hamster dihydrofolate reductase promoter through the transcription factor E2F (Wade et al 1992), using transient co-transfection assays. The rat brain creatine kinase promoter, which was linked to a chloramphenicol acetyl-transferase

(CAT) reporter gene, was found to respond to transient co-transfection of the isolated HCMV IE2 gene, with minimal increase with the addition of IE1 gene and a suppression of enhancement with the addition of the HCMV immediate early genes US3 or UL36-38 (Colberg-Poley et al 1992). These results are in contrast to the effects of the same genes on the human heat-shock protein-70 (hsp-70) promoter in the HeLa cell line, which is non-permissive for HCMV infection (in decreasing order of enhancement): UL36-38/US3 > UL36-38/IE1 > IE1 > IE1/US3 (Colberg-Poley et al 1992). However, some differences existed between hsp-70 promoter responsiveness in HeLa cells and in fibroblasts (which are HCMV-permissive); in fibroblasts only the IE combinations of IE1/IE2 and IE1/UL36-38 were effective at increasing hsp-70 promoter activity (Colberg-Poley et al 1992). However, I used these same constructs containing the isolated HCMV IE genes in fibroblasts and no effect was observed using the CD55 promoter. These results may indicate that increased CD55 and CD46 expression on HCMV-infected adherent cells is not mediated through direct transactivation via HCMV IE genes, rather other possibilities must be considered including alterations in cytokines, signal transduction or autocrine pathways.

More relevant to the results presented in this dissertation, isolated IE genes have been used to transactivate cellular promoters in THP-1 cells. The effects of HCMV IE1 and IE2 on IL-1β mRNA levels and IL-1β promoter activities were investigated using Northern blotting techniques and transient co-transfection of promoter-CAT constructs (Iwamoto et al 1990). Maximal effect of IE1 and IE2 genes required additional stimulation of THP-1 cells with LPS, and peaked at 3h post-addition of LPS, decreasing rapidly there after. The IE1/IE2 genes were also found to enhance the IL-1β promoter activity in LPS-stimulated THP-1 cells, in a dose dependent fashion, but the HCMV IE2 gene alone had no effect even though it synergistically increased the transactivation activity of IE1 on the IL-1β promoter (Iwamoto et al 1990). Unfortunately, subsequent investigations found that even though IL-1β mRNA levels were increased, the levels of released IL-1β protein were

found to be unaltered by transfection of LPS-stimulated THP-1 cells with IE1 and IE2 (Kline et al 1994). The transfection of these cells with IE1 and IE2 did, however, result in increased mRNA and secreted protein levels of IL-1 receptor agonist (Kline et al 1994). The increased protein IL-1 receptor agonist protein secretion required the transfection of both IE1 and IE2, and was actually suppressed when IE1 was used alone. Transient co-transfection assays investigating the effect of IE1 and IE2 on the HLA class I (A2 allele) promoter in the Jurkat (immortalized T-lymphocyte) cell line found a 4-fold increase in promoter activity when the cells were unstimulated and an 11.5-fold increase in stimulated Jurkat cells (Burns et al 1993). The effect of IE genes on the HLA class I promoter may explain the increased HLA class I expression I measured on the unstimulated THP-1 cells. However, Weinshenker et al (1988) reported that infection of THP-1 cells prior to differentiation with phorbol esters did not lead to expression of HCMV IE genes, which disputes this hypothesis.

The increases in CD55 and CD46 on unstimulated, HCMV-infected THP-1 cells was a fraction of the increases observed for HCMV-infected adherent cell lines. However, the differences between the effects in HCMV infection on HLA class I expression on differentiated and undifferentiated THP-1 cells may help to explain this. After the differentiation of THP-1 cells with phorbol esters, HCMV infection of THP-1 cells results in the significant decrease in HLA class I expression observed on HCMV-infected adherent cells, indicating a release of the block of the HCMV gene expression which mediates this effect. It is reasonable to assume that differentiation of the THP-1 cells also results in the release of the block of the HCMV-mediated increase in CD55 and CD46 expression; however, the phorbol esters used to differentiate the THP-1 cells vastly increased the expression of the complement inhibitors, confirming the findings of other investigators (Bryant et al 1990, Holguin et al 1993). Therefore, the increase in CD55 and CD46 may mask the increases induced by fully-permissive HCMV infection. The fact that an additional increase in CD55 is measured on differentiated, HCMV-infected THP-1 cells suggests that the

upregulation of CD55 is not maximized by phorbol ester stimulation, or that HCMV and phorbol esters act through separate stimulation pathways. However, there is no explanation apparent for the lack of CD59 decrease on differentiated THP-1 cells infected with HCMV. Perhaps, like the differences reported by Colberg-Poley et al (1992) for hsp-70 promoter responsiveness between HeLa cells and fibroblasts, the gene(s) responsible for CD59 down-regulation are differentially expressed amongst cell types.

I also investigated the promoter requirements for the HCMV-induced increase in CD55 promoter activity. A majority of the basal CD55 promoter activity was found in the construct containing 206 bp upstream from the transcriptional start site, but all of the enhanced CD55 promoter activity in HCMV-infected cells required 275 bp upstream from the transcriptional start site. This is in contrast to the requirements of the HLA class I (A2) promoter identified by Burns et al (1993): the basal and HCMV-induced increase in HLA-A2 promoter both required only the minimal 116 bp upstream from the transcriptional start site. However the HLA class I promoter contains 2 CCAAT boxes and a presumed TATA box in this region (Burns et al 1993), while the CD55 promoter lacks both of these elements (Thomas and Lublin 1993). Furthermore, the HLA-A2 promoter responded to transfection with the IE1 and IE2 genes, while the CD55 promoter did not; suggesting different HCMV genes are responsible. The hamster DHFR promoter was reported to require the sequence CCCGACTGCAATTTCGCGCCAAACTTGG to respond to the HCMV IE1 and IE2 genes (Wade et al 1992), which is also the sequence required for this gene to respond to adenovirus and required the transcription factor E2F. However, the region of the CD55 promoter in question (-275 to -206) does not contain any sequences with homology to the DHFR sequence, CD55 expression is not affected by adenovirus infection, and the CD55 promoter also does not respond to co-transfection with the IE1 and IE2 genes. The HCMV DNA polymerase gene is upregulated by the HCMV IE genes and Kerry et al (1994) reported the sequence of an inverted repeat found in

the DNA polymerase promoter required for a majority of the transactivating activity of HCMV infection. However, the CD55 promoter does not contain this sequence either. Given the responsiveness of CD55 expression to IL-1, IFNγ, and histamine in some cell types (Tandon et al 1994, Tsuji et al 1994), the alterations in cytokine production induced by HCMV infection (Almeida et al 1994, Iwamoto et al 1990), and the lack of responsiveness of the CD55 promoter and expression to transfection with isolated HCMV IE genes suggests that the effect of HCMV infection on CD55 and CD46 expression may be indirect and possibly linked to alterations in autocrine production. The lack of increase in CD55 and CD46 on fibroblasts incubated with bacterial lipopolysaccharide (LPS) ,adenovirus, or HSV in Table 3 address some of the possibilities. LPS was found to induce granulocyte/macrophage colony stimulating factor tumor growth factor 1β, Interleukin-1α (IL-1α), IL-1, IL-6, and IL-8, but not tumor necrosis factor-α (Xing et al 1993, Huleihel et al 1990, Schwachula et al 1994). Therefore, since LPS treatment of fibroblasts did not increase CD55 or CD46 expression, one may assume that the CD55 and CD46 expression increase is not related to these cytokines. Further, since adenovirus and HSV infection results in the expression of interferon α and β (Daly and Reich 1993, Neilsch et al 1992), but did not result in increased expression of CD55 or CD46, one may also assume that the CD55 and CD46 expression increase is not related to interferon.

Beyond the increased complement regulating activity induced by HCMV-infection, there may be other, further reaching implications to HCMV infection. CD59 and CD55 have both been associated with tyrosine kinase pathways (Shenoy-Scaria et al 1992, Morgan et al 1993, Stefanova and Horesji 1991), indirectly to signal transduction (Shibuya et al 1992) and CD55 and CD59 are found to be closely associated on the cell surface, indicating intentional organization (Stefanova and Horesji 1991). Alterations in CD55 to CD59 ratios as seen in HCMV infection of adherent cells may have major implications for the signal transduction pathways associated with them. Signal transduction through CD55 was found to be associated

with altering cytokine production and glucose consumption on monocytes (Shibuya et al 1992) and lymphocyte proliferation (Shenoy-Scaria et al 1992); while signal transduction through CD59 was found to be associated with calcium transients and cell activation in T lymphocytes. The implications of increased CD46 decreased CR1 with various carcinomas (Lanza et al 1991, Lanza and Castoldi 1992, Seya et al 1994, Seya et al 1990, Hara et al 1992, Cho et al 1991) also implies a potential role for HCMV in immortalization. Finally, increases in CD55 expression have been reported to decrease the function of natural killer cells (Finberg et al 1992), which potentially could act as a further immunological evasion strategy for the one cytotoxic cell whose effectiveness would not be reduced by the decreased HLA class I expression on HCMV-infected cells.

5. SUMMARY.

The overall objective of this project was to describe the interaction between complement and human cytomegalovirus (HCMV) virions and to determine the effect of HCMV infection on host complement inhibitor expression. There were two objectives in this thesis. **The first objective was to assess the ability of complement to neutralize purified HCMV virions.** Complement alone, in the absence of specific anti-HCMV antibody, was found to have a negligible neutralizing effect, even though small amounts of C3 were found to be deposited on the HCMV virion. Addition of specific anti-HCMV antibody resulted in the activation of both the alternative and classical pathways, deposition of large amounts of C3 on the HCMV virion, and formation of the membrane attack complex as evidenced by the deposition of C9. Although complement activation was clearly seen in seronegative specimens mixed with HCMV virions, the apparent arrest of the cascade at the C3 step suggested the presence of complement inhibitors on the HCMV virion. Further investigation identified the presence of host complement inhibitors, CD46, CD55, and CD59 on virions. These findings potentially explain the complement regulatory activity of HCMV virions in the absence of virus-encoded complement inhibitors in the HCMV genome. This is a novel observation as no reports exist in the literature of any identifiable complement regulators present on the HCMV virion, irrespective of origin.

The second objective was to determine whether HCMV infection altered the expression of host-encoded complement inhibitors. HCMV infection resulted in an increase of the C3 regulating proteins, CD55 and CD46, on adherent cells, but a decrease in the terminal complement pathway inhibitor, CD59. These effects were determined not to be cell type specific as the effect was seen on both glioblastoma cells and fibroblasts. Furthermore, virus-induced increases in CD46 and CD55 were specific to HCMV infection; they were not seen when cells were infected with either HSV-1 or human adenovirus. Preliminary studies found an enhanced complement

regulating function associated with the enhanced CD55 expression on HCMV-infected cells. This finding provides the first evidence for a new mechanism whereby virus-infected cells enhance resistance to complement-mediated cytolysis.

To further characterize the mechanism by which HCMV infection enhances expression of CD55 and decreases CD59 expression, comparisons were made in the RNA and protein synthesis pathways between HCMV-infected and uninfected cells. While the results presented here do not absolutely identify the contributions of all possible pathways, infection of permissive adherent cells clearly resulted in the accumulation of CD55 mRNA. Furthermore, evidence was provided that this mRNA increase was accompanied by a large increase in CD55 promoter activity which appeared to be mediated through a novel HCMV-responsive element located upstream from the elements required for basal transcription. Conversely, the decreased CD59 expression appeared to result from a combination of decreased CD59 mRNA levels and dysfunctional protein processing which could result in enhanced catabolism. These studies have identified CD59 as only the second host protein to decrease with HCMV infection.

Interestingly, addition of isolated, identified immediate early HCMV genes, singly or in combination, were not able to reproduce the increased CD55 promoter activity or CD55 expression, suggesting that the mechanism is much more complex than that reported for other upregulated host proteins. Further investigation of CD55 promoter activity and CD55 protein expression using larger gene segments in cosmid expression vectors as well as measurement alterations in cytokine production in HCMV infected cells may clarify the mechanism by which HCMV mediates the increased CD55 expression. Finally, since the specific deficiency of CD55 (Inab phenotype) is reported not to be associated with clinically significant episodes of hemolysis *in vivo* , future studies for HCMV infection therapy might consider including a specific CD55 inactivator to enhance the effectiveness of the normal immune response in HCMV clearance.

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