MECHANISMS OF HUMAN CYTOMEGALOVIRUS PERSISTENCE IN MONOCYTE-DERIVED MACROPHAGES

by

Kenneth N. Fish

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This is certify that the Ph.D. thesis of

Kenneth N. Fish

Has been approved

[redacted]

Professor in charge of thesis

Member [redacted]

Member [redacted]

Member [redacted]

[redacted]

Associate Dean for Graduate Studies

Abstract

MECHANISMS OF HUMAN CYTOMEGALOVIRUS PERSISTENCE IN MONOCYTE-DERIVED MACROPHAGES

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Monocytes/macrophages are key cells in the pathogenesis of human cytomegalovirus (HCMV). Although HCMV replication in monocytes is restricted to early events of gene expression, productive infection occurs in differentiated macrophages *in vitro*. The best characterized *in vitro* primary monocyte/macrophage system that allows HCMV replication is based on cocultivation of monocytes with Concanavalin A (Con A) stimulated autologous non-adherent cells. This thesis further characterised the Con A-macrophage system by studying the kinetics of viral replication, viral protein expression, and cellular changes in macrophages that occur as a result of HCMV infection. In addition, the cytokines INF-γ and TNF-α were identified as being essential for HCMV-replication in Con A-stimulated monocyte-derived macrophages (Con A-MDM). Although HCMV infected Con A-MDM produce similar viral titers as infected human fibroblasts (HF), the viral kinetics are delayed in Con A-MDM. In addition, virus remains cell associated and is non-lytic in Con A-MDM. In contrast, HCMV infection of HF cells results in the accumulation of both intracellular and extracellular virus, and eventually lyses the cells. Interestingly, the majority of Con A-MDM intracellular infectious virus is sequestered into vacuoles that contain the major HCMV glycoprotein B (gB) and the resident medial Golgi enzyme mannosidase II, which don't enter the cellular degradation system. Finally, although gB is found on the cell surface of HF cells, almost no gB is detected at the PM in Con A-MDM by confocal microscopy. In Con A-MDM, gB steady-state expression is

tightly regulated, keeping the majority of the protein in or near the Golgi. This specific gB intracellular routing is regulated by the phsophorylation of an acidic cluster sorting signal in the gB cytoplasmic tail. Therefore, HCMV uses intracellular trafficking machinery to target gB to specific cellular assembly compartments. The final virion envelope is acquired when tegumented-capsids bud into gB vacuoles. Once inside the vacuole, the mature virions are proposed to be protected from the host immune system and cellular degradation pathway. These findings emphasize the cell-specific differences that occur during HCMV infection and therefore stress the importance of examining viral replication in biologically relevant cell types.

This thesis is based on the following papers:

1. Growth kinetics of HCMV are altered in monocyte derived macrophages.
2. A novel mechanism for persistence of human cytomegalovirus in macrophages.
3. Steady-state plasma membrane expression of HCMV gB is determined by the phosphorylation state of ser_{900}.
4. Clathrin-dependent endocytosis is not required for envelopement of HCMV.
5. Interferon-γ and tumor necrosis factor-α specifically induce formation of cytomegalovirus-permissive monocyte-derived macrophages that are refractory to the antiviral activity of these cytokines.

We are all Worms,

but I am a Glow worm.

(Winston Churchill)

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GLOSSARY

Mononuclear phagocytes: includes monoblasts, promonocytes, monocytes, and macrophages.

Resident macrophages: macrophages occuring in specific sites in normal, non-inflamed tissues. However, they may also be observed in small numbers in an inflammatory exudate. They are sometimes called 'normal macrophages'.

Activated macrophages: exhibiting an increase in one or more functional activities, or the appearance of a new functional activity. Both resident and exudate macrophages can be activted.

Exudate macrophages: macrophages occurring in an exudate and identifiable on the basis of peroxidase activity, immunophenotype, and cell kinetics. They derive from monocytes and have many of the characteristics of the latter. Exudate macrophages are felt to be the precursors of resident macrophages.

Elicited macrophages: macrophages attracted to a given site because of a particular stimulus. An elicited population of macrophages in heterogeneous both developmentally and functionally.

ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
CMV	cytomegalovirus
Con A	concanavalin A
CSF	colony stimulating factor
DNA	deoxyribonucleic acid
DAPI	4'-6'diamidino-2-phenylindole
dpi	days post infection
E	early
EBV	Epstein-Barr virus
ER	endoplasmic reticulum
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FACS	fluorescence activated cell sorter
HCMV	human cytomegalovirus
HSV	herpes simplex virus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HBSS	Hank's balanced salt solution
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonicacid]
HFF (HF)	human foreskin fibroblasts
ICAM-1	intercellular adhesion molecule-1
IE	immediate-early
IFN	interferon
IL-1	interleukin-1
IL-2	interleukin-2
IgG	immunoglobulin G
kDa	kilodaltons
LA	late
LPS	lipopolysaccharide
MCMV	mouse cytomegalovirus
M-CSF	macrophage colony-stimulating factor
MDM	monocyte derived macrophage
MEM	minimal essential medium
MHC	major histocompatibility complex
M/M	monocyte/macrophage
MNGC	multinucleated giant cell
MOI	multiplicity of infection
mRNA	messenger RNA
MT	mirotubule
MW	molecular weight

PBL	peripheral blood leukocytes
PBML	peripheral blood mononuclear leukocytes
PBS	phosphate-buffered saline
PE	phycoerythrin
PFU	plaque forming unit
PM	plasma membrane
RT-PCR	reverse transcriptase-polymerase chain reaction
SD	standard deviation
TGF	transforming growth factor
TNF	tumor necrosis factor

Chapter 1

INTRODUCTION

1.0 HUMAN CYTOMEGALOVIRUS

1.1 Historical background

H. Ribbert was first to describe cytopathic effects characteristic of cytomegalovirus infection in a 1904 report. His report was of an observation in the kidney of a stillborn child where he observed enlarged "protozoan-like" cells containing inclusion bodies (199). Subsequently, in 1907 Lowenstein described intranuclear and cytoplasmic inclusion bodies in the salivary glands, lungs, kidneys and liver of infants and fetuses who died from a variety of causes (145). In 1921 Goodpasture and Talbot introduced the term "cytomegalia" to describe the cytopathic effects that resulted from this unknown disease (88). After Von Glahn and Pappenheimer demonstrated that inclusion bodies occurred in cells infected by herpesviruses in 1924, they concluded that these cytomegalia cytopathic effects were caused by a virus related to the herpesviridae (277). In 1953, Minder used an electron microscope to provide evidence, which supported Von Glahn's and Pappenheimer's hypothesis, that a herpesvirus was the causative agent of cytomegalia cytopathic effects (164). The initial name for the new virus was the "salivary gland virus", since virus-associated cytopathic effects were frequently observed in

salivary glands. Because cytomegaloviruses are highly species-specific, the virus wasn't isolated until 1956, when the development of new tissue culture techniques made it possible to culture human cells (288). The first human cytomegalovirus (HCMV) laboratory strains to be isolated were AD 169 (207), Davis (289), and Smith (235). Although the Smith strain was isolated from the salivary glands, the virus was renamed cytomegalovirus (from the Greek 'kytos': cell and 'megalos': large) to describe the wide-spread pathogenicity of the virus on several of the bodies organs.

1.2 Classification

The virus family Herpesviridae is made up of more than 80 distinct herpesviruses that have been isolated from a wide variety of animal species. All members of this family share four structural features: (1) a core containing a linear, double-stranded DNA; (2) an icosadeltahedral capsid, approximately 100-110 nm in diameter, containing 12 pentameric and 150 hexameric capsomeres; (3) globular material that is asymetrically arranged around the capsid and designated as the tegument; and (4) an envelope surrounding the entire structure, which contains glycoproteins (294). As a result of these structural similarities, the members of the Herpesviridae can not be readily differentiated by electron microscopy. However, evolutionary pressures have resulted in broad diversification of

the herpesviruses. Viruses are differentiatable from each other by size and arrangement of their genome, base composition, and a number of biological properties.

Many herpesviruses get their name from the clinical conditions or the disease they cause (e.g., pseudorabies virus), after their hosts (e.g., herpesvirus hominis), the cell pathology they cause (e.g., CMV), or their discoverers [e.g., Epstein-Barr virus (EBV)]. Herpesviruses are subdivided into three groups according to biological properties: alphaherpesvirinae; betaherpesvirinae, and gammaherpesvirinae. The alphaherpesviruses [e.g., herpes simplex virus type 1 (HSV-1) and varicella zoster virus (VZV) (204)] have a variable host range both *in vivo* and *in vitro*, a short reproductive cycle, and rapidly spread throughout susceptible cells in culture, which results in mass destruction of the cells. In humans, HSV infection may result in oropharyngeal and genital blisters, while VZV causes chickenpox (varicella) and shingles (zoster).

Beteherpesvirus (e.g., HCMV) classification is based on host specificity, length of replication cycle compared to other herpesviruses, and cytopathic effects (105). As a group they show a high degree of morphological similarity with other members of the Herpesviridae (298). CMVs are highly species-specific in both replication and pathogenesis. For example, mouse CMV does not infect human cells and human CMV

does not infect monkey cells. In addition, within their natural host they show cell-type specificity for replication and pathogenesis (8). CMVs are highly disseminated in nature and have been found in most animal species including mice, horses, guinea pigs, rats, bovines, cats, pigs, and monkeys (287). Although the viral genomes differ between the species, CMV targets similar organs. Therefore, CMV infection results in a comparable pathogenesis within these hosts.

The gammaherpesviruses [e.g., EBV and human herpesvirus 8 (HHV-8)] usually establish latency in lymphoid tissue, are able to infect either B or T cells, have a variable reproductive cycle duration and cytopthology, and are usually limited to the host they naturally infect. The clinical syndromes associated with these viruses are infectious mononucleosis and nasopharynx carcinoma, which are caused by EBV, and more recently, Kaposi's Sarcoma, which is associated with HHV-8 (211).

The classification between alphaherpesviruses and gammaherpesviruses is based on host range and characteristics of latent infection; while the classification between betaherpesviruses and the others is based primarily on the viral life cycle length and the slow development of pathology in cell culture. In addition, more recently classification has been determined on DNA sequence information. For

example, HHV-8 was classified as a gammaherpesvirus once the viral genome was sequenced. Furthermore, although all herpesviruses are capable of establishing latency after a primary infection, the cell type used as a host is usually different between the subfamilies. For example, the alphaherpesviruses establish latency in sensory ganglia, while HCMV has recently been shown to establish latency in monocytes/macrophages (240).

1.3 Characteristics of HCMV

1.3.1 Core and DNA

The core of a mature HCMV virion contains the linear viral double-stranded DNA genome in the form of a "toras"—a bulging, knobby strand (79, 172). The HCMV genome is the largest and most complex of all known herpesviruses with a molecular weight of 150-155 x 10^6 daltons (approximately 240 kbp) with a density of 1.716 g/cm^3 (corresponding to a G + C content of 57%) (56, 81, 129). The viral genome consists of a unique short (U_S) and a unique long (U_L) segment of DNA. Each segment is tethered by terminal repeats (TR) and inverted internal repeats (IR) (129). Open reading frames are, therefore, designated with either a U_S, U_L, or IR prefix depending on genome location. During CMV DNA replication the U_S and U_L components are capable of inversion with regard

to one another (129), so that four populations of DNA molecules exist in infected cells. These populations consist of a prototype; an inversion of the U_S component relative to the prototype orientation; an inversion of the U_L component; and inversions of both the U_S and U_L components (100, 223, 295). In addition, the HCMV genome possesses pac sequences, which are used for cleavage and packaging of the virion DNA and are highly conserved among herpesviruses (55, 127, 166).

The HCMV genome has over 200 open reading frames (ORFs), and only a small fraction of these have been thoroughly characterized (40). Only 33 of the identified ORFs have substantial amino acid similarity to other known herpesvirus proteins (40). HCMV proteins can be divided into two categories: the non-structural proteins and the structural proteins. In general, the non-structural proteins are transcribed from genes prior to DNA synthesis and are responsible for establishing infection and preparing the cell for virus replication. The majority of the proteins encoded by these genes are enzymes involved in transcriptional regulation or synthesis of the viral DNA template. However, recently some of these non-structural proteins have also been found to interfere with MHC class I pathway antigen processing events (118, 293). Structural proteins, which form the virion, are usually transcribed from late genes

only after viral DNA synthesis and include the capsid, tegument and envelope proteins.

1.3.2 Physical properties

Even though HCMV is a heat-labile virus (137), the virus is more stabile at 36°C than at 4°C (189) and has a half-life of 60 minutes at 37°C. In addition, direct treatment of HCMV with phytohemagglutinin inactivates the virus (114) and similar to other viruses, HCMV can be inactivated by exposure to ultraviolet light.

1.3.3 Capsid, tegument, and envelope

1.3.3.1 Capsid

The viral DNA is encapsulated within an icosahedrally symmetric capsid structure (20-sided body) of 90-110 nm, consisting of 162 capsomeres (pentameres or hexameres) (105). The HCMV capsid is minimally comprised of the major capsid protein (MCP); the minor capsid protein (mCP); the minor capsid binding protein, which is a 34 kDa protein that is homologous to an HSV protein that interacts with viral DNA and assists in the insertion of viral DNA into the capsid; the smallest capsid protein; the assembly protein; and the proteinase (40, 83). The genes that correspond to these capsid-associated proteins are listed in Table 1.1. Three different

forms of the nuclear capsid have been identified in infected cells: A capsids are clear because they lack the assembly protein (empty—contains no viral DNA); B capsids appear translucent because they contain the assembly protein, but not DNA (an empty translucent core—contains no viral DNA); and C capsids (mature—contains viral DNA) lack the assembly protein, since this protein is transported out of the capsid at the time of DNA packaging (113, 267). While only naked capsids (without tegument or viral envelope) are detected in the nucleus of infected cells, the cytoplasm contains tegumented capsids as well as fully mature enveloped particles (267). Whether the cytoplasmic non-enveloped particles are degraded or eventually enveloped is not known.

Table 1.1 HCMV genes and their products that are involved in capsid assembly.

Gene	Gene Product
UL86	major capsid protein
UL85	minor capsid protein
UL46	minor capsid binding protein
UL48/49	smallest capsid protein
UL80.5	assembly protein
UL80A	proteinase

1.3.3.2 Tegument

Roizman and Furlong (205) introduced the term tegument to describe the dense material found between the capsid and envelope. The tegument thickness of HCMV particles can vary; therefore, mature virions vary in size from 150-200 nm. In addition to mature virions, an aberrant form of the virion forms, referred to as dense bodies, which contain mainly

tegument proteins and glycoproteins, but no viral DNA or nucleocapsid. Another infectious particle contains the central core, capsid, tegument, and envelope but lacks viral DNA; therefore these particles are unable to produce progeny.

The HCMV tegument region is composed of more than 20 proteins. The majority of these proteins are phosphoproteins (115, 243). The products of several genes that encode tegument proteins have been well characterized. The UL32 gene encodes the basic phosphoprotein pp150, which is a major component of the virion and has been suggested to be more associated with the capsid structure than with the envelope because the viral protein is absent from dense bodies that lack capsid but present in virions or enveloped particles that contain a capsid but not viral DNA. In addition, pp150 posses highly antigenic properties (106, 203). The pp28 tegument protein is encoded by the UL99 gene and is an extremely hydrophilic and highly immunogenic protein (158, 196). The lower matrix phosphoprotein pp65 is encoded by UL83 and is found in both virions and dense bodies (203, 208). This tegument protein has recently been shown to be dispensible for HCMV replication *in vitro* (215). The UL82 gene encodes the upper matrix protein pp71, which has been recently shown to be a transactivator of gene expression (143). The pp71 tegument protein

is produced in small amounts compared to pp65 and is almost exclusively found in virions (203, 208).

1.3.3.3 Envelope

The HCMV envelope surrounds the tegumented capsid and is comprised of a lipid bilayer membrane that is host cell derived and contains viral glycoproteins and some host proteins. Only a small fraction of the 55 ORFs predicted to encode viral glycoproteins have been characterized by investigators. Within these ORFs, there are clusters of genes encoding glycoproteins with similar structure and perhaps similar function. The virion envelope is known to carry at least eight glycoproteins in as many as four glycoprotein complexes in which glycoproteins interact with one another to achieve a specific subcellular location and/or function (40, 115, 185, 195, 243). One might predict that several minor envelope glycoproteins exist in the virion envelope given the large number of ORFs that are predicted to encode for integral membrane proteins. In support of this hypothesis, HSV, which is a much simpler herpesvirus, has 14 viral membrane proteins in its envelope. Therefore, there are likely more viral proteins associated with the HCMV envelope.

HCMV gB (encoded by the UL55 gene) is the most abundant glycoprotein detected in the HCMV virion envelope (27). HCMV gB is

synthesized as a 105 kDa polypeptide precursor; this precursor is processed into a highly glycosylated 130 kDa precursor glycoprotein, which is cleaved by furin to produce a heterodimer protein (gp55 and gp116) (276). gB55 and gB116 form the gcI complex. gB is a type I glycoprotein containing a signal sequence, an extracellular/lumenal domain, a transmembrane (TM) domain, and a 135 aa cytoplasmic tail (26, 90, 185). Although the gB cytoplasmic tail contains a consensus casein kinase II (CKII) site that is phosphorylated both *in vitro* and *in vivo* (18, 175), nothing is known about the function of gB phosphorylation.

Two other well characterized glycoproteins are gH and gL, which are the products of the UL75 and UL115 genes, respectively. gH, gL, and an unidentified 145 kDa protein complex together to form the gcIII complex. HCMV gB, gH, and gL are homologous to the HSV-1 glycoproteins gB, gH, and gL, which have been shown to play a role in virus entry into cells (243). In addition to the viral proteins found associated with the virion envelope, host-encoded proteins may either be incorporated into or tightly associated with the virion envelope (85, 92, 162). These proteins may also play a role in HCMV biology.

1.4 HCMV entry, replication, and egress

1.4.1 Entry

Even though biochemical, immunological, and ultrastructural analyses have been used to study some of the major steps of the HCMV life cycle, the HCMV infectious cycle is relatively poorly characterized. The HCMV viral infectious cycle occurs in a sequential pattern of events. Entry of HCMV into host cells begins when a virion attaches to a cell through interaction of the glycoprotein complexes gcI-III with their cognate cellular receptors. The virus penetrates the plasma membrane (PM) by a pH-independent fusion mechanism that occurs between the viral envelope and the PM, which results in release of the viral tegumented capsid, which contains the viral double-stranded DNA, into the cytosol (46, 234). Subsequently, the capsid is thought to be transported to the centrosome by the microtubule (MT) motor protein dynein (236). Once at the centrosome, the capsid detaches from the microtubule by an unknown mechanism, and docks with a nuclear pore, at which time the viral genome is delivered into the nuclear compartment. Once the viral DNA has entered the nucleoplasm, a cascade of gene expression occurs that results in the transcription of viral genes, which is followed by protein synthesis and DNA replication. Subsequently, the assembly of viral progeny, and release of infectious virus into the extracellular milieu

occurs. During these replication events virus infection results in extensive modification of host cell function and proteins. These modifications are essential because they ensure that the viral infectious cycle occurs in an efficient manner.

Neither the cellular receptors nor the receptor-ligand interactions that mediate HCMV binding to the cell surface and fusion events are known. However, the initial, relatively nonspecific, viral attachment is proposed to occur between the HCMV gB envelope protein and cell surface heparan sulfate proteoglycans (47, 123, 124). HCMV attachment to surface heparan sulphate proteoglycans has been proposed to facilitate binding of virions to secondary, high affinity receptors, which must occur prior to fusion of the virion with the cellular PM (47). Taylor and Cooper reported the first evidence for a specific HCMV receptor in 1989. In this study the binding of radiolabelled virus to cells was inhibited by treatment with protease (257). In addition, these authors demonstrated that fibroblasts bound saturable levels of radiolabelled HCMV. Furthermore, two independent studies identified what appeared to be the same membrane protein present in both fibroblasts and lymphoid cells with a molecular weight of 30-34 kDa that binds HCMV (5, 258). The 30-34 kDa protein was later identified as annexin II (299). The annexin II molecule-gB interaction was recently shown not to be important for viral entry into

fibroblast cells (188). Other putative HCMV receptors have also been identified, such as a 92.5 kDa cell membrane protein that binds gH-derived anti-idiotypic antibodies (125, 126). Another cell surface protein, aminopeptidase N (CD13) has also been implicated as a receptor in HCMV entry (84, 237). Giugni et al. demonstrated that exposure of HF cells to CD13-specific monoclonal antibodies prior to HCMV infection, blocked HCMV binding and entry (84, 85). The viral protein that interacts with cell surface CD13 and the role this receptor plays in HCMV entry remain elusive.

1.4.2 Replication

The replication cycle of HCMV is slow in comparison to HSV, requiring 48-72 hours to yield detectable levels of progeny virus in HCMV-infected fibroblasts (167). The comparatively slow kinetics is related to relatively slow rates of transcription and DNA replication in these cells. Studies have defined three kinetic classes of genes that are transcribed: immediately early (IE), early (E), and late (L) genes (57, 282).

The first HCMV genes expressed after viral entry are the IE genes, which are dependent on cellular transcription factors for activation, and do not require *de novo* viral protein synthesis (283). Therefore, transcription of IE genes proceeds in the presence of protein synthesis inhibitors such

as cycloheximide or anisomycin. IE genes are expressed from three distinct segments of the genome (57, 282, 283). One of these genomic segments contains two continuous regions expressed at high levels to produce two transcripts designated IE-1 and IE-2 (251, 253). Differential splicing of mRNAs in these two regions as well as the presence of alternative polyadenylation sites produces transcripts that encode the predominant IE proteins with molecular weights of 72kDa (IE-1, UL123) and 86kDa (IE-2, UL122). Generally, the IE proteins are non-structural nuclear proteins, which have been shown to transcriptionally regulate both homologous and heterologous promoters (167).

In HF cells the early phase of HCMV infection begins approximately 4-8 hours post infection and proceeds until the onset of viral DNA synthesis, which is approximately 24 hours after infection (283). This time course of expression is delayed by hours or days in several primary cell culture systems (e.g. macrophages and endothelial cells). Generally, the HCMV E genes are defined as those genes that are transcribed even when viral DNA replication is blocked with HCMV DNA polymerase inhibitors such as phosphonoacetic acid, gancyclovir or foscarnet. Approximately 75% of the HCMV genome is transcribed during the early phase of infection, which results in the production of non-structural

proteins involved in viral DNA replication such as the HCMV DNA polymerase (167), as well as certain structural proteins (e.g. gB).

HCMV L genes encode structural viral proteins such as capsid proteins, matrix proteins, tegument, and envelope glycoproteins (167). Some of these genes are transcribed at low levels prior to viral DNA replication, but efficient transcription of late genes requires viral DNA synthesis. During late gene expression, which in HF cells starts at 24-48 hours post infection and lasts until cell lysis, infectious virus particles are produced in the cell.

1.4.3 Egress

Although biochemical, immunological, and ultrastructural analyses have been used to study the egress pathway of HCMV, the mechanisms involved in virion egress (the exit of the capsid from the nucleus and its envelopment) are controversial. HCMV is considered to use similar mechanisms as other herpesviruses for its viral infectious cycle. Early experiments performed to examine mechanisms of viral assembly and egress in herpesviruses were performed using HSV-1. The majority of results from experiments performed to identify the egress pathway fall into one of two proposed models. The first model suggests that HSV-1 capsids acquire their envelope by budding through the inner nuclear

membrane, which contains immature glycoproteins. Once in the inner nuclear space, the enveloped capsids are transported to the Golgi, via the lumen of the endoplasmic reticulum and the cisternal space, where their glycoproteins are modified (116). Finally, mature virions are transported to the cell surface in cytoplasmic vacuoles, which fuse with the PM, releasing virus into the extracellular space. To support this hypothesis, Johnson and Spear found that in HSV-infected cells treated with monensin, the virus did not reach the cell surface but accumulated in intracytoplasmic vacuoles (116). Since monensin blocks vesicular transport from the Golgi to the PM, the authors suggested that egress of HSV occurs via the Golgi apparatus.

Experiments supporting the second egress model (35, 78, 194, 246, 291), known as the de-envelopment–re-envelopment model, suggest that the nucleocapsid buds through the inner nuclear membrane, acquiring an envelope, followed by de-envelopment as the enveloped capsid fuses with the outer nuclear membrane, releasing the naked capsid into the cytosol. These experiments also propose that final envelopement occurs in the trans-Golgi network (TGN) and endosomes, where terminally modified glycoproteins have accumulated, since this step is sensitive to treatment of cells with brefeldin A (67). In addition, a recent study by Browne et al., which targeted HSV gH to the ER or to the medial-Golgi by

adding specific trafficking signals to the glycoprotein's cytoplasmic tail, supports this hypothesis; whereas gH with ER localization motifs resulted in the secretion of noninfectious gH-less virions, the Golgi localization signal resulted in the production of infectious virus with normal gH content (30). The results of Browne et al. suggest that the HSV-1 envelope is acquired in the TGN.

In both egress models, transport across the nuclear membrane is hypothesized to be mediated by gB and gH localization to the nuclear membrane. Although this hypothesis is supported by the presence of glycoproteins associated with perinuclear-enveloped virions in HSV, evidence in other herpesvirus infections is lacking. In addition to other similarities between the egress models, neither model takes into account that different herpesvirus family members may utilize alternative pathways for assembly and egress. If cell- and herpesvirus-specific envelopment pathways exist, then a multitude of pathways, which may have small or large differences, would exist.

1.5 HCMV clinical importance

Human cytomegalovirus is an opportunistic virus that has evolved to co-exist with immunologically healthy individuals. Between 60 and 100% of the population experience HCMV infection during their lifetime (25, 76).

Generally, primary HCMV infection occurs during childhood and results in life-long persistence of the virus in a latent state. All bodily fluids are possible sources of HCMV transmission and most children become infected via breast milk (163) or via transmission from child to child in day care centers and schools (91, 178). While HCMV infection is usually asymptomatic or manifested by mononucleosis-like symptoms, recurrent infections are seen in immunocompromised patients who lack the ability to immunologically contain virus, which places them at a high risk of HCMV disease and may even result in death (Golden, 1994; Levinson 1992; Griffiths 1993). Therefore, due to the escalation in the number of patients that are immunosuppressed, the interest in HCMV pathology has increased. Despite recent advances in prophylaxis drugs, HCMV is the leading viral cause of birth defects and is a major cause of morbidity and mortality in transplant patients and AIDS patients (Griffiths, 1993; Prentice, 1994; McKenzie, 1991; Saltzman, 1992; Crumpacker, 1988; Macher, 1983; Britt, 1996).

1.6 Pathogenesis

HCMV disease manifestation may result from a number of mechanisms: i) the virus can cause direct lysis of target cells, ii) cytotoxic T cells might be recruited by the host's immune system to lyse infected cells, or iii)

synergism between HCMV and another virus may cause pathology. Moreover, HCMV might activate host oncogenes or trigger autoimmunity through priming of cytotoxic T cells that react with host antigens. In addition, HCMV can establish latent infections and pathogenesis can result from reactivation. Latent infection is defined as the persistence of viral genome without extensive gene expression and without the presence of infectious virus. Normally only a small minority of viral genes are expressed in cells that harbor latent virus. Cells could be affected by infection with HCMV in a latent form in one or both of two potential manners: either reactivation of virus could occur so that an acute infection resulted, or there might be subtle perturbation of the normal function of the latently infected cell.

1.7 Tissues targeted by HCMV during acute disease

Symptoms of patients with acute HCMV disease predict the involvement of a variety of organ systems. Histological analyses of autopsy tissues obtained from patients with HCMV disease have demonstrated infected cells in virtually all organs. A number of different cell types (including monocytes, fibroblasts, endothelial cells, epithelial cells, stromal cells and neuronal cells) are infected in patients with HCMV disease (23, 52, 61, 64, 68, 77, 86, 108, 122, 132, 148, 149, 165, 200, 201, 219, 224, 228-231,

238, 259, 269). The most frequently infected cell types found during HCMV disease are epithelial cells, endothelial cells, and macrophages. Although the vast majority of studies on HCMV replication have been performed in virally-infected HF cells, evidence that these cells are infected *in vivo* has only recently been reported (228). In this report several organs with HCMV infected fibroblasts were observed including the lungs, intestine, and placenta; however, there is no evidence that HF cells are very important in HCMV pathogenesis. Examination of viral replication in other cell types such as monocyte-derived macrophages (MDM) has revealed significant differences in the kinetics of viral replication, viral cytopathic effect, and release of virus from the cell (112). In addition, epidemiological studies have demonstrated that peripheral blood mononuclear cells (PBMC) are an important source of HCMV. Furthermore, assessment of separated cell populations from PBMC in *in vivo* HCMV infections have identified monocytes as the predominant infected cell type (4, 52, 61, 68, 108, 119, 122, 148, 200, 238, 259). Therefore, monocytes/macrophages are a more relevant cell type than HF cells to study HCMV replication *in vitro*.

1.8 Monocytes/macrophages

Before what is known about HCMV infection of macrophages is presented, the origin of these cells will be discussed.

1.8.1 Origin

Hematopoietic stem cells are the precursors from which all the cellular elements of the blood arise. These pluripotent cells divide giving rise to two specialized types of stem cells: a common lymphoid progenitor and a myeloid progenitor. The lymphoid stem cells give rise to T and B cells while the myeloid stem cells give rise to leukocytes, erythrocytes and megakaryocytes. Leukocytes as a whole are collectively termed the polymorphonuclear leukocytes because of the shape of their nuclei, or granulocytes because of their characteristic staining in blood smears. The polymorphonuclear leukocyte family contains the monocytes, basophils, eosinophils and neutrophils.

Colony-forming unit, granulocyte-macrophages (CFU-GM) are the specific progenitors of monocytes and neutrophils (156). Under the regulation of colony stimulating factors (CSFs), the CFU-GM differentiate into monoblasts and then either promonocytes or promyelocytes. CFU-GM are thought to become committed to differentiate into either monocytic or granulocytic cells prior to entering the promonocyte and promyelocyte stage, respectively (12).

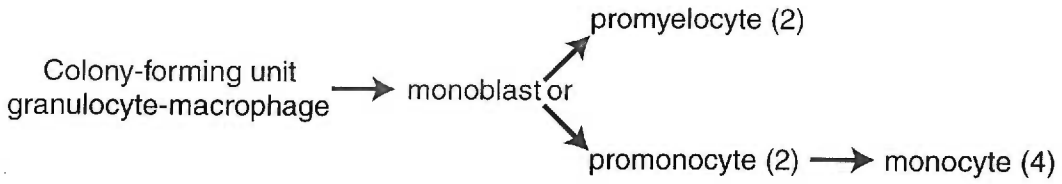

Figure 1.1 The origin of macrophages in the bone marrow.

The monoblast is considered to be the least mature mononuclear phagocytic cell (12). A monoblast divides into two promonocytes, which each are believed to give rise to two monocytes. Monocytes remain in the marrow for less than 24 hours before entering the peripheral blood, where they either circulate throughout the body or associate with marginating pools (157, 274). In the average adult there are approximately 500 monocytes per microlitre of blood. Once in the blood, the monocyte circulating half-time in humans is approximately 60-70 hours (292). Monocytes enter extravascular tissue from the peripheral blood by first adhering to the endothelium through high molecular weight glycoproteins. The decision to migrate into a particular organ in the absence of an inflammatory response is usually random and usually corresponds to the size of the organ (12). Once in the tissue, monocytes do not re-enter the circulatory system (273).

Once monocytes enter an organ, they differentiate into their tissue counterpart, the macrophage (271). The final fate of tissue macrophages is unknown; however, the overall turnover rate of tissue macrophages

must be considerable because of the number of monocytes produced. In addition, this turnover rate can increase during an acute inflammatory reaction, which leads to an increase in the production of monocytes and a decrease in the overall time spent prior to migrating to their host organ (272).

	Monoblast →	Promonocyte →	Monocyte →	Macrophage
DNA synthesis	+++	++	+	+/-
Phagocytosis	-	+	++	++++
Lysosomes	-	+	++	++++
IgG & C3 receptors	+/-	+	++	++++
Peroxidase activity	+	++++	++	+/-

Table 1.2 Characteristics of cells of the mononuclear phagocyte system.

Although the *in vivo* control elements that regulate monocytopoiesis have been elusive, two haematopoietic growth factors, which are synthesized and secreted by macrophages, are well understood: (1) macrophage colony-stimulating factor (M-CSF) and (2) granulocyte macrophage colony-stimulating factor (GM-CSF). Both M-CSF and GM-CSF stimulate the production and function of mononuclear phagocytes. In addition, macrophages secrete cytokines (IL-1 and TNF) that stimulate the

local production of M-CSF and GM-CSF by fibroblasts and endothelial cells. Furthermore, M-CSF and GM-CSF not only stimulate the production of mononuclear phagocytes (13, 29, 171), but GM-CSF also stimulates these newly produced cells to secrete TNF (34).

1.8.2 Morphology of mononuclear phagocytes

Monocytes are relatively small cells measuring 12-15 μm in diameter. The PM of monocytes appear ruffled compared to that of T and B cells, and their nuclei occupy approximately 50% of the cell. The nucleus is generally kidney-shaped with the Golgi usually located at the nuclear indentation.

Macrophages are extremely dynamic secretory and phagocytic cells and are fundamental to the initiation and orchestration of the immune response. Depending on the method used to stimulate monocytes to macrophages, the monocyte-derived macrophage (MDM) has variable characteristics. In general MDM are large cells that are irregularly shaped and have overly large cytoplasms. Fully differentiated MDM measure 25-100 μm in diameter and can have anywhere from a single nucleus to more than 100 nuclei, which vary in shape and size. The cytoplasm is extremely vacuolarized with variable numbers of fluid vacuoles, pinocytic vesicles, endosomes and lysosomes. The surfaces of macrophages are

known to develop large areas of ruffling membrane during fluid uptake. The entire PM turns over in as little as 30 minutes (250). Microtubules and microfiliments are abundant and well organized in macrophages, and the actin network is extremely dynamic, altering in response to changes in the microenvironment of the macrophage.

Through receptor-ligand binding, macrophage receptors sense changes in the local environment. Monocytes and macrophages have numerous receptors, which are responsible for regulating cellular growth, differentiation, activation, recognition, secretion, endocytosis and phagocytosis in response to their constantly changing environment. Fc and complement receptors were the first identified (16, 139). Fc receptor-ligand interaction results in the reorganization of the cytoskeleton, which facilitates endocytosis of the receptor-ligand complex. Similarly, complement receptors signal the macrophage to engulf their ligands, opsonized bacterial particles or viruses containing complement. Another molecule proposed to be involved in the regulation of phagocytosis is CD14, which is found on all cells of the monocytic lineage (101). During allogeneic stimulation of the immune system, CD14 molecules on monocytes and macrophages are suggested to deliver stimulatory signals to alloreactive T-cells and up-regulate monocyte/macrophage phagocytosis (216).

1.8.3 Cytokines that affect monocyte/macrophage function

Cytokines are proteins produced by many cells within the body and they function as messengers between cells. Cytokines are multifunctional proteins that signal other cells to become activated, call cells into an area of infection (chemokines), hold cells in the area, signal cells to prepare for an invading organism, and induce damage or death in cells. Some cytokines facilitate their activity independently, others require or enhance their effects through the presence of other cytokines. A complete description of cytokines and their functions is far beyond the scope of this introduction; however, some of the important cytokines in macrophage activation are discussed below.

A wide variety of biological effects are generated by receptor-cytokine interaction. Tumor necrosis factor (TNF)-α (256), interleukin (IL)-2 (150), IL-4 (182), M-CSF (155), and GM-CSF (155) up-regulate macrophage-activating factor (MAF) (87). MAF was later called INF-γ and therefore, for the remainder of this thesis INF-γ will only be used. IFN-γ is secreted by T-cells and is required to induce the 'primed' state of macrophage activation. Primed macrophages are not cytolytic, but easily become so when exposed to bacterial lipopolysaccharide (LPS). Once a primed macrophage has been exposed to LPS they become 'fully

activated' and are able to phagocytose and destroy a variety of pathogens.

In addition to depending on cytokines produced by other cells, macrophages produce several cytokines that either directly or indirectly affect their activities. One of the most important cytokines secreted by macrophages is tumor necrosis factor (TNF)-α (60). In the early 1900's, researchers discovered that bacterial infections occasionally caused certain tumors to become necrotic and regress. The LPS component of the gram-negative cell wall of bacteria was found to be responsible for the tumor regression. LPS was later found to act as a triggering signal; macrophages exposed to LPS can secrete lytic mediators when bound to tumor cells. Macrophages use these lytic mediators to kill tumor cells, this process is called macrophage-mediated tumor cytoxicity and is regulated by TNF-α.

The role of TNF-α extends beyond its ability to kill certain tumors. TNF-α is also capable of killing various bacteria and other microorganisms. In addition, TNF-α can act on a variety of other cells like B-cells, T-cells, endothelial cells, and neutrophils, inducing them to secrete pro-inflammatory factors capable of enhancing the inflammatory response. If the inflammation-inducing organism persists for too long,

however, a great deal of damage to the body's own cells will result. Therefore, the production and secretion of TNF-α is tightly regulated.

IL-1 is secreted by macrophages as well as several other cells and has numerous functions. Listed among its functions are co-stimulation of T-cells, promotion of B-cell maturation and division, chemoattractant for neutrophils and macrophages, and increased expression of adhesion molecules on the walls of blood vessels. In addition, IL-1 is also a stimulus for macrophage activation and is responsible for the fever associated with many infections (its original name was "endogenous pyrogen").

Three forms of IFN: IFN-α, IFN-β, and IFN-γ (see above for information on INF-γ) have been identified. Macrophages secrete both IFN-α and IFN-β in response to viral infections. Both IFN-α and IFN-β have potent anti-viral effects (186). Secretion of these cytokines stimulates neighboring cells to establish antiviral defenses; cells up-regulate antiviral and antimitotic effects, and increase MHC Class II expression. In addition, INF-α stimulation of natural killer cells results in an increase in cell activity.

1.8.4 Antigen processing and presentation in macrophages

In addition to cell and matrix receptors, macrophages have special

molecules on their cell surfaces that interact with molecules on other immune cells. Specifically, macrophages use MHC Class I and II on their membranes to communicate with other cells of the immune system. MHC Class I molecules are expressed on virtually all cells and present peptides generated in the cytosol to CD8 T cells. If a cell presents a foreign peptide, usually from a cytosolic pathogen, on a Class I molecule at the PM, the presenting cell will be targeted for cell death by CD8 T cells. The ability of the immune system to survey cellular contents through proteins expressed on the cell surface is an important means of eliminating sources of new viral particles and cytosolic bacteria to free the host of infection.

The uptake, degradation, and presentation of antigen by MHC class II are not well understood. It is not known whether uptake of antigen is receptor-mediated or is due to general, non-specific mechanisms of cell invagination. Pathogens targeted to the acidified vesicular compartments of macrophages, dendritic cells and B cells are degraded. In addition to having a low pH, degradation vacuoles contain proteolytic enzymes. Once an antigen is digested, questions arise as to the mechanisms by which peptides emerging from the degradation vacuoles are escorted to the DM compartment, where they are loaded onto MHC class II molecules. After MHC Class II/peptide complexes form, they traffic to

the PM where they bind and activate CD4 T Cells. Activated CD4 T cells can stimulate B lymphocytes to produce antibodies or stimulate macrophages to destroy pathogens in their vesicles.

1.9 HCMV infection of monocytes

Functional defects occur in the peripheral blood leucocytes of patients with HCMV mononucleosis. Specifically, the adherent mononuclear cells of the monocyte/macrophage lineage suppress the Con A response of autologous lymphocytes (202). This initial report was confirmed by Carney and Hirsch (36) who observed a decreased Con A responsiveness of unseparated PBMC from mononucleosis patients by incubating monocytes from these patients with normal lymphocytes. Rice et al. (200) also found that *in vitro* HCMV infection of peripheral blood leukocytes resulted in decreased mitogen and antigen responsiveness. They predicted that the decrease in response was due to HCMV infection of monocytes (200, 281). When the monocytes were analyzed independently, HCMV infection resulted in a decreased ability to support the proliferative responses of normal T cells (36, 220).

Several groups have extensively studied which aspects of monocyte function may be responsible for mediating the suppressive effect that occurs in HCMV infected monocytes. In 1981 Garnett

demonstrated that when adhered monocytes were infected with HCMV, cells lost microextensions (80). As a result, the cells rounded up and changes occurred in surface topography. HCMV infection was also demonstrated to reduce monocyte mobility, adherence, and ability to phagocytose yeast particles (63, 64). These results suggest that HCMV infection of monocytes may disrupt cytoskeletal elements.

1.10 The involvement of macrophages in HCMV pathogenesis

A prominent source of HCMV during acute disease is cells in the peripheral blood. (52). HCMV is known to be transmitted to patients through transfusion of the leukocyte fraction of the peripheral blood (4, 15, 22, 42, 65, 103, 210, 262, 263, 297). Although HCMV can be isolated from both the mononuclear and polymorphonuclear cell fractions (36, 201), a low percentage of blood cells from asymptomatic individuals are infected with virus. These observations contrast with higher frequencies of HCMV positive leukocytes found in liver and kidney biopsies from transplant patients that are immunosuppressed (10). Examination of separated cell populations from the PBMC obtained from HCMV seropositive individuals has led to the conclusion that monocytes are the predominant infected cell type (259). These observations are consistent

with the conclusion that macrophages are the predominant HCMV infected infiltrating cell in tissues (228).

Several animal models have been established to understand mechanisms involved in latency and reactivation of CMV (31, 96, 197, 198, 300). In murine organ transplant models, reactivation of murine cytomegalovirus (MCMV) has been shown to be influenced by the state of immunosuppression and histoincompatibility between the donor and the recipient (31, 96, 197, 198, 300). In MCMV latently infected mice, the spleen, kidneys, and bone marrow are important sources of virus (120, 154, 176). Reactivation of MCMV in latently infected mice can occur through either intraperitoneal injection of thioglycollate, a reagent that stimulates monocytes to differentiate (190), or allogeneic stimulation (176, 213). The peripheral blood of latently infected animals has also been demonstrated to be a reservoir of virus since allogeneic stimulation of PBMC resulted in activation of MCMV replication (176, 213). Although the identification of the cell type in the blood of MCMV-infected mice remains controversial, their studies suggest that T cells, B cells or monocytes may be potential sites for viral latency (120, 154, 176).

Several primary monocyte/macrophage systems have been established to examine mechanisms of HCMV activation in myeloid cells (33, 36, 112, 122, 202, 217). These studies have demonstrated that the

ability of the virus to replicate in macrophages is dependent on the state of cellular differentiation of the macrophage. Infection of unstimulated monocytes resulted in either the lack of viral gene expression or replication restricted to immediate early gene products (112). The block in HCMV replication in unstimulated monocytes was not at the level of virus entry and fusion with the cell, but rather at the level of early and late gene expression (112).

Prior to the initiation of this thesis project, the best characterized *in vitro* primary monocyte/macrophage system that allowed HCMV replication was based on cocultivation of monocytes with Con A stimulated autologous non-adherent cells (112, 217). In this system, stimulated monocytes differentiate into several morphologically distinct macrophage phenotypes (Con A-MDM), which include multinucleated giant cells (MNGC). These MDM can be maintained for long periods of time without the addition of exogenous cytokines and a fraction of the cells can be productively infected by HCMV.

1.11 Myeloid lineage cells harbor latent HCMV

Similar to other herpesviruses, it has been proposed that HCMV establishes life-long latency in the host after a primary infection, which is characterized by persistence of the viral genome without the production of

infectious virus. Transmission of HCMV infection has been shown to occur through transfusion of blood products, bone marrow grafts and solid organs (25, 43, 159, 262), but the latent reservoir of HCMV has not been identified. Even though *in vitro* infection of Con A-MDM with HCMV results in a productive infection without cell lysis, monocyte/macrophages from seropositive donors that have been Con A-stimulated do not reactivate HCMV.

Myeloid cells provide an ideal site of latency for a virus that is closely linked to the immune system for activation. HCMV has previously been reported to infect $CD34^+$ pluripotent stem cells both *in vitro* (148, 165, 170, 226) and *in vivo* (153, 278). However, these findings do not explain why virus is not consistently found in all mature peripheral cell lineages (32, 219, 238, 279). A recently established culture system demonstrating HCMV infection of $CD33^+$ myeloid progenitor cells *in vitro* suggests that myeloid cells may be infected early during hematopoiesis (132, 133). In this model, *in vitro* infection of myeloid progenitor cells results in production of infectious HCMV after the cells are caused to differentiate into $CD14^+$ macrophages. While $CD33^+$ myeloid progenitor cells would be an obvious site for HCMV latency, conclusive proof that these cells are a site of HCMV latency *in vivo* would require that virus be reactivated from these $CD33^+$ cells taken from healthy individuals.

Aims of the present study

This thesis focuses on analyzing HCMV replication and persistence in MDM, which are key cells in the biology of the virus. Macrophages are a heterogeneus population of terminally differentiated myeloid lineage cells. Since myeloid progenitor cells in the bone marrow are susceptible to HCMV infection *in vitro*, they are thought to be latent reservoirs of virus (148, 165) and, therefore, a biologically relevant cell type in which to study HCMV replication. Although previous studies demonstrate that HCMV replication is restricted to early events of gene expression in monocytes, little is known about HCMV replication in myeloid lineage cells. Therefore, the aims of the present study are:

Overall aim

- To define the role peripheral blood monocytes play as a HCMV reservoir and vector for HCMV dissemination to target tissues.

Specific aims

- To study the delayed growth kinetics and compartmentalization of HCMV in monocyte/macrophages.
- To study the cellular components necessary for the differentiation of monocytes to HCMV permissive macrophages.
- To study HCMV envelopment in macrophages.

Chapter 2

Materials and Methods.

Isolation and culture of MDM. PBMC were isolated from the blood of HCMV-seronegative donors selected from a pool of donors at Oregon Health Sciences University. Heparinized whole blood was underlayed with Histopaque (Sigma Chemical Co., St. Louis, Mo.) and centrifuged at 1,500 rpm in a Sorvall RT6000B centrifuge for 25 min. at room temperature. The PBMC band was collected, washed twice with sterile saline and once with serum-free media, and resuspended at 4 $x10^7$ cells per ml in Iscove's medium (GIBCO Laboratories, Grand Island, NY) with 1.0% penicillin-streptomycin solution (GIBCO) and 10% pooled human serum (prepared from the Oregon Health Sciences University donor pool). Approximately 1.5 $x10^7$ PBMC were plated per chamber onto Lab-Tek 2 chamber slides (Nunc, Inc., Naperville, Ill.) or approximately 5 $x10^7$ PBMC were plated onto 60-mm-diameter Primaria culture dishes (Becton Dickinson, Lincoln Park, NJ) and incubated at 37° C with 7% CO_2 for infections. Adherent cells were induced to differentiate by cocultivation with Con A (5.0 µg/ml; Sigma)-treated nonadherent cells for 24 h. Subsequently, all nonadherent

cells were removed and adherent MDM were cultured in complete 60/30 medium (60% AIM-V medium and 30% Iscove's medium [GIBCO] supplemented with 10% human serum and 1% penicillin-streptomycin). The adherent cells were greater than 99% esterase positive at 72h (112). MDM cultures were fed every 3 days with 50% fresh medium and 50% spent medium clarified by centrifugation. Replacement of this conditioned medium at each feeding was necessary for optimal MDM differentiation. Day 1 of differentiation is defined as the day after the initial PBMC isolation and stimulation with concanavalin A.

HCMV infection of HF cells, U373 cells and MDM. Several recent isolates of HCMV or the laboratory strain AD169 was used to infect HF cells, microglial cells (U373 MG; ATCC), and primary cultures of MDM. These isolates (I-G, PH, PO, and LY) were isolated from transplant patients with HCMV disease and passaged through HF cells and frozen below passage 12 in liquid nitrogen (112, 200). Frozen samples from this stock were thawed and passaged for three additional rounds through HF cells prior to culture infections. Cell-free supernatants from HCMV-infected HF cells were used as the source of inoculum for HF and U373 cell cultures as previuously described (112). MDM cultures containing approximately 5 x 10^6 cells per 60-mm-diameter culture dish or 3 x 10^6 cells per slide chamber were infected at 8-10 days post differentiation with

infected HF supernatants containing approximately 10^8 PFU per dish. Virus inoculum was incubated with the MDM for 2 h, removed with thorough washing, and replaced by conditioned MDM medium.

All plaque assays were performed by plating sonicated samples or culture supernatants onto monolayers of HF cells at 75% confluence. After an initial 24 hours of viral adherence at 37°C, cells were washed twice in medium and overlaid with DMEM medium containing 10% FBS, 2 mM L-glutamine, 100 IU/ml of penicillin, 100 µg/ml of streptomycin, and 0.5% autoclaved SeaKem agarose (Sigma). The cultures were incubated for 14 days, with feeding every fourth day. The cells were fixed with 25 % formaldehyde in PBS for 15 minutes, stained with a 0.05% solution of methylene blue, and plaques were counted (290).

Immunocytochemistry. Uninfected and HCMV-infected cultures were fixed in methanol or were fixed 20 minutes at room temperature in buffered picric acid-paraformaldehyde (PAPF; 2% paraformaldehyde and 15% picric acid) and permeablized with 0.3% Triton X-100 in phosphate-buffered saline (PBS). Monolayers were blocked with 20% normal goat serum or 10% normal goat serum plus 10% human AB serum in PBS and incubated for 1 hour at 37°C with a 1:100 dilution of one or more of the primary antibodies listed in table 2.1. Binding of primary antibody was detected with fluorescein isothiocyanate (FITC)- or tetramethyl rhodamine

isocyanate (TRITC)- or Cyanine-5- (Cy-5; Biological Detection Systems, Inc., Pittsburgh, Pa.) conjugated secondary antibodies raised in the appropriate species and visualized on an upright Leitz fluorescent microscope or a Leica Confocal Laser Scanning Microscope equipped with a Leitz Fluorovert-FU Microscope and argon/krypton laser. Cultures were exposed to 1 µg/ml of the DNA stain DAPI to visualize nuclei. The Slowfade Antifade Kit (Molecular Probes, Inc.) was utilized to ensure minimal fluorescence fading and where indicated the Quantimet 500 fluorescence analysis program (Leica) was used to quantitate fluorescence.

Table 2.1 HCMV and cellular specific antibodies.

Rabbit anti-	Mouse anti-
*IE72	pp65
**pp71	gB N-terminus (27)
***gB C-terminus	LAMP-1 (H4A3; (11, 37)
mannosidase II (169)	LAMP-2 (H4B4; (11, 37)
transferrin receptor (296)	alpha-tubulin (Amersham Life Sciences)
	beta-tubulin (E7; (45)
HLA-DR (L243; Becton Dickinson)	
	Rab5 (Santa Cruz Biotechnology, Inc.; (39)

*Generated using IE 72 recombinant protein.

**Generated using recombinant protein from an E. coli expression system (121).

***Generated to a GST gB C-terminal tail chimeric protein, where the entire cytoplasmic tail of gB was fused to GST.

Drug-Mediated Modification of MDM Organelles. Prior to fixation MDM were pretreated with 10 µM taxol (Molecular Probes, Inc., Eugene,

OR.) for 30 minutes at 37° (242) to ensure that depolymerization of microtubules was not due to experimental procedures (54). In the nocodazole experiments, macrophages were treated for various intervals up to six days with 2.5 µg/ml nocodazole (Methyl-(5-[2-Thienylcarbonyl]-1H-Benzimidazol-2-YL)-Carbamate; Sigma) which allows for the total depolymerization of the microtubule network without resulting in cell death or lifting of MDM off the culture substrate.

BODIPY C_6-ceramide (3,3'-dihexyloxacarbocyanione iodide $DiOC_6$ (9); Molecular Probes, Inc.) was used to examine the integrity of the Golgi in MDM. Mock and HCMV infected MDM cultures were exposed to $DiOC_6$ (10 µg/ml) for 10 minutes at different dpi, rinsed and maintained in normal culture medium for 1 hour before visualization by fluorescence microscopy.

HCMV vacuole isolation from MDM. Isolation of HCMV-infected vacuoles was performed as follows. At 13 dpi a serum coated coverslip was placed over the MDM culture and incubated for 24 hours. Removal of the coverslip displaced the PM from the cell exposing the intracellular organelles. Subsequently, the cells were rinsed lightly to recover the HCMV containing vacuoles which were separated from free virus by centrifugation in a Sorvall RT6000B at 500 x g for 15 mins. Plaque assays were performed with pelleted cellular organelles and supernatant on HF cells as described above.

Polymerase Chain Reaction of HCMV immediate early and late antigen. For RNA isolation, HCMV-infected MDM grown in 60mm culture dishes were lysed directly by adding RNA STAT-60™ (1.5 ml/dish) (TEL-TEST "B", INC. Friendswood, TX). The RNA was then chloroform extracted and precipitated using isopropanol. After a 75% ethanol rinse the RNA was suspended in DEPC-treated water, and used for PCR analysis. Reverse transcriptase PCR and cDNA amplification was carried out using the Perkin Elmer Cetus (Norwalk, CT) GeneAmp Thermostable rTth Reverse Transcriptase RNA PCR Kit. For the reverse transcription reaction a 3' oligonucleotide primer along with 250 ng of total RNA were used along with 17 µL master mix (see Perkin Elmer Cetus Protocol) at 70°C for 10 minutes. The resulting product was then transferred to another tube containing the 5' primer and 79 µL of master mix (see Perkin Elmer Cetus Protocol) for 2 minutes at 95°C for 1 cycle, then 1 minute at 95°C and 1 minute at 60°C for 35 cycles, and finally, 7 minutes at 60°C for 1 cycle. All 5' oligos used for PCR were end-labeled with ^{32}P ATP utilizing polynucleotide kinase and FPLC purified. The RT-PCR products were seperated on a 5% acrylamide gel in 1X TBE. Immediate early (IE) primers amplified a 435–base-pair sequence of the MIE gene within exon 4 (IE-1/UL123) of this gene (40). The primers were Primer MIE-4, 5'-CCAAGCGGCCTCTGATAACCAAGCC-3', and Primer MIE-5, 5'-

CAGCACCATCCTCCTCTTCCTCTGG-3' (58). We also used IE primers spanning the exon 6/7 junction (IE-2/UL122), which generate a 659 bp product from exon 5 and a 198 bp product from exon 6/7 (40). The primers were 55/86-5', 5'-GCACACCCAACGTGCAGACTCGGC-3', and 55/86-3', 5'-TGGCTGCCTCGATGGCCAGGCTC-3'. Late primers amplified a 401–base-pair sequence that corresponds to sequences in the 3' end of pp65 RNA that also codes for pp71 (therefore also amplifying pp71 mRNA) (208). The primers were pp65–3205, 5'-CCCGCTACCCGATCGTGTGCGAATCACCC-3', and pp65–3579, 5'-CCGGGGACAGTCCGGCTTGGGTGTCC-3'. Primers specific for gB, a late glycoprotein, were used to amplify a 360 bp fragment from the center of the RNA (50). The primers were gB5', 5'-AGCTGCGTGACCATCAACCAAACC-3', and gB3', 5'-CTGCGAGTAAAGTTCCAGTACCC-3'. Finally, the primers specific for the β actin control, used to determine the integrity of the RNA, were β actin-L, 5'-CCTTCCTGGGCATGGAGTCCTG-3', and β actin-R, 5'-GGAGCAATGATCTTGATCTTC-3'.

Expression of gB by vaccinia virus infection in HF and U373 cells. Vaccinia virus (VV) strain WR was used in these studies. Recombinant VV (RVV) were constructed using a modification of a previously described method (24, 275). The point mutants gB_{ala} and gB_{asp}

were constructed using PCR with the amino terminal primer gB_{wt} N-term (5'-TCGTCTGATGCATCCACGGCG-'3) and the carboxy terminal primer gB_{ala} C-term (5'-CTAGCTGAGCGGCCGCTCAGACGTTCTCTTCTTCGTCGGC GTCTTTC-'3) or gB_{asp} C-term (5'-CTAGCTGAGCGGCCGCTCAGACGTT CTCTTCTTCGTCGTCGTCTTTC-'3)]. The PCR fragments from the PCR mutagenesis of AD169 gB were digested with NsiI and NotI and cloned into an EcoRI site in $Rep4DegB_{wt}$, resulting in $Rep4DegB_{ala}$ and $Rep4DegB_{asp}$. The genotype of the new clones was confirmed by sequence analysis and restriction enzyme digestion with EcoRI. gB was excised from the Rep4De clones with Xho I and the resulting 2.7 Kb fragments were cloned into the VV insertion selection plasmid pZVneo (99) digested with Xho I. Orientation was confirmed using StuI and BglII restriction digest analysis and cycle sequencing. Homologous recombination, selection and partial purification of recombinant viruses was performed as described by VanSlyke et al. (275). RVV 1-12-11 was chosen for gB_{wt}, 13-24-13 was chosen for gB_{asp}, and 25-36-33-38 was chosen for gB_{ala} expression in vaccinia virus. Expression of gB_{wt} and the gB point mutation substitutions gB_{ala} and gB_{asp} were performed essentially as described previously (24). In addition, a vaccinia virus was constructed

that expresses a dynamin dominant-negative mutant (RVV dyn_{K44A}) (51) as described above.

***In vitro* phosphorylation of GST-gB constructs.** Fusions of native and mutated gB cytoplasmic tails with GST were produced by PCR amplification of the appropriate full-length gB construct in pZVneo (see RVV construction) and cloned into the *Bam*HI site of pGEX 3X (Pharmacia). GST chimeras expressed in bacteria were used for *in vitro* phosphorylation assays. GST-gB (1 µg) was incubated at 30° C for 20 min in the presence of 0.1 mM [γ-^{32}P] ATP (4000 c.p.m./pmol) in a final volume of 30 µl. CKII (10 U; ICOS) was assayed in 50 mM Tris (pH 7.2), 150 mM KCl and 10 mM $MgCl_2$. CKI (10 U; ICOS) was assayed in 50 mM Tris (pH 7.5), 150 mM NaCl and 60 mM $MgCl_2$.

***In vivo* phosphorylation of gB.** Confluent HF and U373 cells ($5x10^6$) cultured in 75 mm flasks were infected with RVV at a multiplicity of infection of 5 and incubated at 37° C. At 2 h post-infection, the medium was replaced with phosphate-free MEM (Gibco) supplemented with 5% dialyzed FBS. At 3 h post-infection, [^{32}P] sodium orthophosphate (3 mCi/ 5 x 10^6 cells) was then added to the medium and incubated for an additional 4 h. Following labeling, the cells were harvested in 1 ml cold RIPA buffer + protease inhibitors. The lysates were clarified by centrifugation at 16,000 g for 10 minutes at 4° C in an Eppendorf

microcentrifuge. The supernatant was transferred to a new tube containing 5 µl mouse IgG and incubate on ice ten minutes with continuous mixing. 20 µl Protein A-Sepharose was added followed by incubation on ice for ten minutes with continuous mixing. The samples were centrifuged and supernatant was transferred to a new tube. Samples were exposed to 20 µl Protein A-Sepharose again to clear. The samples were then transferred to a new tube containing 10 µl gB 7-17 and incubated overnight at 4° C with continuous mixing. This step was followed by addition of 20 µl Protein A-Sepharose; the total mixture was incubated on ice on ice 2 hours with continuous mixing.

Radiolabelling and surface biotinylation of gB. We used radiolabelling and surface biotinylation to measure relative amounts of gB at the PM of U373 cells infected with RVV gBwt, gB_{ala}, or gB_{asp}. U373 cells infected with RVV gB_{wt}, gB_{ala} or gB_{asp} were pulsed labeled for 12 hours with ^{35}S-methionine and ^{35}S -cysteine at 2 dpi. After removal of the label, cells were incubated with NHS-SS-biotin (Pierce, Rockford, Illinois 61105; stock of 200 mg/ml DMSO) at 4°C. After a one hour labeling period the cells were rinsed with HBSS and prepared for SDS-PAGE analysis. The cells were harvested in 1 ml cold RIPA buffer + protease inhibitors. The lysates were clarified by centrifugation at 16,000 g for 10 minutes at 4° C in an Eppendorf microcentrifuge. Biotinylated protein was recovered from

sample supernatants by precipitation with ImmunoPure Immobilized Avidin (35 µl, 50% slurry; Pierce) after which the beads were washed. Biotinylated gB was eluted from the avidin beads by boiling in 50 µl 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% SDS buffer for 5 min. Samples were then centrifuged and the supernatants were transferred to new tubes containing 5 µl mouse IgG and incubate on ice ten minutes with continuous mixing. 20 µl Protein A-Sepharose was added followed by incubation on ice for ten minutes with continuous mixing. The samples were centrifuged and supernatants were transferred to a new tube. Samples were exposed to 20 µl Protein A-Sepharose again to clear. The samples were then transferred to a new tube containing 10 µl gB 7-17 and incubated overnight at 4° C with continuous mixing. This step was followed by addition of 20 µl Protein A-Sepharose; the total mixture was incubated on ice on ice 2 hours with continuous mixing. The immunoprecipitated protein was then analyzed on a SDS-PAGE gel.

Internalization Experiment. gB antibody uptake experiments were performed in RVV gB_{wt} or RVV gB_{asp} infected U373 cells. At 6 hours post infection, mouse anti-gB N-terminus antibody was applied to cells for 30 minutes. Cells were then rinsed and incubated for a 30 minute chase period followed by fixation. Non-permeabilized cells were stained with a cyanine-5 anti mouse secondary conjugate, rinsed, permeabilized and

stained with a TRITC anti mouse secondary conjugate. Cells were rinsed again, exposed to rabbit anti gB C-terminus and then a FITC anti-rabbit secondary conjugate.

HCMV vacuole isolation from MDM. Isolation of gB coated cellular vacuoles from HCMV-infected U373 cells was performed using Mini MACS columns (Miltenyi Biotec, Bergish Gladbach, Germany). At 7 dpi a serum-coated coverslip was placed over the U373 culture and incubated for 24 hours. The cultures were washed twice in cold HBSS prior to removal of the coverslip, which displaced the PM from the cells exposing their intracellular organelles. Subsequently, the plates were rinsed lightly in Mini MACS buffer (PBS containing 5 mM EDTA and 0.5 % BSA) to recover the cellular organelles, which were then incubated with a gB monoclonal antibody (1:100) and 160 µl Mini MACS beads conjugated with rat-anti-mouse antibodies for 20 minutes at 4°C. Each Mini MACS column was washed with 15 ml of Mini MACS buffer before the addition of the respective sample. Vacuoles coupled to Mini MACS beads were separated from the samples by flow through the column in a magnetic field under flow resistance. Each column was subsequently washed with 4 ml Mini MACS buffer, followed by the removal of the magnetic field. The collected vacuoles were removed from the column using complete DMEM. Small aliquots of each sample were analyzed by confocal microscopy

using a lipid dye (as described above) and a gB rabbit antibody conjugated to TRITC to ensure the purity of isolated vacuoles. Samples were sonicated and analyzed by a plaque assay as described above.

Generation and use of adenovirus vectors. All recombinant DNA steps used standard techniques. Three recombinant adenoviruses (Adeno) were constructed that expressed either dynamin-1 (dyn) wild-type (wt), dyn_{K44A} (a dyn dominant negative point mutant), or dyn_{G273D} (a temperature sensitive dyn dominant negative mutant). The general outline of the cloning strategy is depicted in Figure 1. Methods for production and use of virus are previously described in detail elsewhere (97). The levels of accumulated protein were regulated by the concentration of doxycycline, amount of virus used to infect cells, and length of time following removal of doxycycline. For these experiments, to prevent toxic effects, U373 cells were infected for 2 hours with 50-200 pfu/cell in PBS lacking $CaCl_2$, containing 20ng/ml doxycycline. Subsequently monolayers were washed twice with doxycycline free media. Cells were further incubated for 16-18 hours to express the indicated recombinant proteins. These conditions did not produce toxic effects to the cells, and were chosen to provide an adequate signal for our localization and functional studies. In all experiments, controls included cells that were i) uninfected, ii) infected with dynamin-I dominant negative mutants in which expression

was fully repressed by 20ng/ml doxycycline, or iii) infected with a control virus encoding dynamin-I wt. The addition of 20 ng/ml doxycycline to the culture medium caused a complete loss of dynamin-I specific signal in immunofluorescence and biochemical studies (not shown). Adenovirus vectors encoded dynamin-I wt or dominant negative mutants (tag at the N-terminus) containing an HA epitope tag. Previous studies have shown that the epitope tagged versions dynamin I demonstrate the same localization and function as the respective non-tagged versions (51).

Internalization experiment. To measure gB internalization from the PM, tetracycline responsive U373 cells (U373 tTA) were infected with an adenovirus expressing a temperature sensitive dynamin dominant-negative mutant (Adeno dyn_{G273D}) as described above. At 2 hours post infection the virus innoculum was removed and the culture temperature was switched from room temperature to 30°C (wt endocytosis kinetics). After 18 hours, the cultures were coninfected with RVV gB_{ala} or gB_{asp}. At 9 hours post RVV infection, the cultures were pulsed labeled with ^{35}S-methionine and ^{35}S-cysteine. After a one hour labeling period the cells were rinsed with DMEM and fresh DMEM was added, following the media change the temperature was shifted from 30°C to the non-permissive temperature of 38°C, which results in inhibition of endocytosis within 15 minutes. After a one hour incubation period at 38°C, metabolically labeled

10 cm cell cultures (1 x 10^6 cells) were rinsed three times with 4°C PBS containing 0.1 mM $CaCl_2$ and 1.0 mM $MgCl_2$ (=PBS+) and incubated on ice for 10 min in the same buffer. After rinsing the cultures twice with PBS+, cell surface biotinylation was initiated by addition of 4 ml of 0.5 mg/ml EZ-Link™ NHS-SS-Biotin (Pierce 21331) (a reagent that is cleaved in the presence of a reducing agent) in PBS+ to each dish. EZ-Link™ NHS-SS-Biotin is added from a freshly prepared 200 mg/ml stock in PBS+ right before use. After addition of the biotinylation reagent, the cultures were incubated on ice for 30 min with gentle agitation. The reaction was quenched by rinsing the cultures five times at 4° C with DMEM/15 mM Tris, pH 7.8, with a 10 min incubation in DMEM/Tris/50 mM ethanolamine between the third and fourth washes. The ethanolamine was added from a 1M stock (1.22 ml/20 ml H_2O, pH 7.8). For the 0 time point, the biotinylated monolayers were then immediately lysed with 500 ml of lysis buffer supplemented with 0.6% SDS, 15 mM Tris, and protease inhibitors and boiled for 3 min. gB was immunoprecipitated using a gB monoclonal antibody and Protein A-Sepharose beads. The immunoprecipitated protein was then eluted from the Protein A-Sepharose beads by boiling for 4 min in 40 µl of immunoprecipitation buffer containing 10% SDS and 2 mM PMSF. A fraction of the eluted protein was added to SDS-PAGE sample buffer to quantitate the total amount of gB in the cells. The

remainder was diluted with immunoprecipitation buffer supplemented with 0.5% bovine serum albumin, 10 mM N-ethylmaleimide, 2 mM PMSF, and enough Triton X-100 to bring the final detergent ration to 1.0% Triton and 0.2% SDS. Biotinylated protein was recovered from this sample by a second round of precipitation with ImmunoPure Immobilized Avidin (35 ml, 50% slurry; Pierce 20219) after which the beads were washed. gB was then eluted from the avidin-agarose by boiling in SDS-PAGE sample buffer for 5 min and analyzed (along with the total protein sample) by SDS-PAGE. As a control, cells were treated with BFA prior to metabolic labeling. For the 15 and 30 minute time points of internalization, the cultures were rinsed and then the culture temperature was shifted back to 30°C. Directly or after the internalization interval, the cell cultures were placed at 4°C to inhibit further internalization and treated with the reducing compound 2-mercaptoethanesulfonic acid (MESNA; Sigma Chemical Co., St. Louis, USA) to reduce surface NHS-SS-biotin. Surface labeled cultures were incubated at 4° C three times for 20 min each with 50 mM MESNA in 50 mM Tris-HCl, pH 8.7, 100 mM NaCl, 2.5 mM $CaCl_2$ prior to thorough rinsing with PBS+ containing 20 mM Hepes, to measure internalized gB (194).

gB antibody uptake experiment. To determine where in the cell endocytosed gB_{wt} trafficks, U373 cells were infected with HCMV. At two

days post HCMV-infection (MOI of 3), infected cultures were co-infected with $Adeno_{G273D}$, as described above. Cultures were maintained at 30°C for 18 hours prior to the return of the culture temperature to 38°C, which inhibits endocytosis. After 6 hours at 38°C the cells were placed on ice and incubated with a monoclonal gB-specific antibody for one hour. After rinsing, the culture temperature was shifted to 30°C to allow for wild-type endocytosis kinetics. At 45 minutes post-internalization, the cell cultures were rinsed, followed by preparation for immunofluorescence analysis by confocal microscopy. In addition to internalized gB, cells were stained for total HCMV gB using a polyclonal antibody specific for gB antigen.

Tissue preparation for structural EM analysis. To examine virus-infected U373 cells by electron microscopy (EM), uninfected and infected cells were harvested at 10 days post HCMV infection and fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at +4°C for 16-20h. Fixed cells were collected by scraping and washed in 0.15 M sodium cacodylate (pH 7.4). Specimens were post-fixed in 1% osmium tetroxide in the same buffer for 1 h at 4°C, dehydrated in ethanol followed by acetone, and embedded in LX-112. Sections of uninfected and infected cells on grids were washed and contrasted with uranyl acetate-oxalate for 5 min, embedded in 2% methyl cellulose containing 0.2%

uranyl acetate, and examined with a calibrated Philips 420 electron microscope at 80 kV.

Chapter 3

Growth kinetics of human cytomegalovirus are altered in monocyte-derived macrophages.

by Kenneth N. Fish, Alison S. Depto, Ashlee V. Moses, William Britt, and Jay A. Nelson

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pages 55-75 of this Thesis

All experiments presented in this chapter were performed by Kenneth N. Fish.

ABSTRACT

Stimulation of monocyte/macrophages (M/M) with activated nonadherent cells allows productive nonlytic growth of human cytomegalovirus (HCMV), but the viral replication cycle is delayed relative to replication of HCMV in human fibroblasts (HF). Analysis of infected monocyte-derived macrophages (MDM) mRNA for major immediate early (MIE 86, 72, 55) and late (pp65, gB) gene expression by reverse transcription (RT) polymerase chain reaction (PCR) indicates that transcription peaks at 3 and 7 days post-infection (dpi) respectively. In contrast, in HF controls, mRNA for MIE and late gene expression peaked at 5 and 48 hours post infection (hpi) respectively. Consistent with RT-PCR experiments, double-label antibody experiments first detected MIE antigen expression at 12 hpi peaking at 3 dpi, and late (pp65 or gB) antigen expression at 5 dpi peaking at 7 dpi. MIE antigen was not detected between 3-7 dpi but reappeared and was co-expressed with pp65 in enlarged MDM nuclei at 7 dpi. After 7 dpi macrophages with numerous vacuoles containing large amounts of pp65 and gB were observed in culture. These vacuoles were frequently seen at cellular contact points, suggesting that cell-to-cell transfer of virus was the major mode of viral transmission. Consistent with this observation, infectious virus was recovered from MDM cellular lysates but not culture

supernatant. The delayed growth and compartmentalization of HCMV in macrophages may allow the cell to accommodate the viral replication cycle without cell lysis. In addition, the macrophage may function as a vehicle for cell-to-cell transmission of HCMV.

INTRODUCTION

Epidemiological studies have demonstrated that peripheral blood mononuclear cells (PBMC) are an important source of human cytomegalovirus (HCMV). Although HCMV is occasionally isolated from peripheral blood of individuals during acute disease (36, 201), virus is rarely detected in PBMC from HCMV seropositive asymptomatic patients. Viral expression in PBMC from the latter individuals is infrequent and limited to early events (173, 202, 264, 287). Interestingly, examination of biopsy tissue from organ transplant patients with HCMV disease reveals a high proportion of inflammatory cells that exhibit extensive viral expression in comparison to the frequency observed in peripheral blood (86). The above observations suggest that the state of cellular activation plays a significant role in induction of viral replication.

Assessment of separated cell populations from PBMC in natural or *in vitro* HCMV infections have identified monocytes as the predominant infected cell type (4, 52, 61, 68, 108, 119, 122, 148, 200, 238, 259).

These cells are derived from myeloid progenitor cells in the bone marrow, which are susceptible to HCMV infection *in vitro* (148). The myeloid progenitor cells are thought to be latent reservoirs of virus and induce HCMV replication upon cellular differentiation (148, 165).

A variety of different culture methods have been developed to study the growth of HCMV in primary MDM (33, 36, 122, 202). In these systems the ability of HCMV to replicate is dependent on the stage of cellular activation or differentiation. We have described a primary M/M culture system designed to facilitate monocyte differentiation into macrophages (112, 218). In this system, monocytes are co-cultured with antigenically or mitogenically activated autologous non-adherent cells for a controlled period to allow for monocyte stimulation. Stimulated monocytes subsequently differentiate into several morphologically distinct macrophage phenotypes including multinucleated giant cells (MNGCs). Macrophages derived by this method can be maintained in long term culture without the addition of exogenous cytokines and are fully permissive for HCMV infection (112).

The vast majority of studies on HCMV gene expression have been performed in virally infected human fibroblasts (HF) cells, which do not represent a naturally infected cell in the human host. During productive HCMV infection of these cells, the virus life cycle is regulated by

sequential expression of the viral genome. Immediate early (IE) genes are rapidly transcribed after infection followed by expression of early and late gene products. In HF cells IE gene expression peaks at 5 hours post infection (hpi), while early and late gene products peak at 24 hpi and 48 hpi respectively (57, 283). We have observed that the kinetics of viral production in MDM are considerably slower than in human fibroblasts. To determine the basis of these delayed kinetics, we examined expression of representative IE and L viral genes in MDM. We found the expression of IE and L genes were dramatically delayed during productive infection and that virus appeared to be compartmentalized in vacuoles in the cytoplasm. These observations imply a unique adaptation of the virus to the cell, which may facilitate a mechanism of trafficking and cellular survival.

RESULTS

HCMV replication is delayed in MDM. MDM were cultured until the appearance of multinucleated giant cells (MNGC), which occurred between 6 and 10 days post-stimulation depending on the donor. Following MNGC formation, MDM cultures were infected with recent isolates of HCMV (I-G and PH). HCMV-infected MDM culture lysates or supernatants were assayed for the production of cell-associated or extracellular virus by plaque assay on susceptible fibroblast monolayers

(Figure 1A and 1B). In MDM lysates HCMV production for both I-G and PH was first detected at 5 dpi peaking at 7 dpi with continued high levels of virus production occurring over the next 12 days (Figure 1A). Detection of HCMV after 5 dpi culture suggested a delayed virus replication cycle relative to the infectious cycle in HF cells. In control infections of HF cells with I-G and PH, HCMV production was detected as early as 2 dpi with a peak at 3 dpi (Figure 1B). The delayed appearance of virus in MDM was also observed with another recent HCMV isolate (PO) indicating that this observation was not strain dependent (data not shown). The kinetic growth differences observed between MDM and HF cells was not due to differential attachment, absorption and nuclear localization of the viral genome in these cells [(112) and unpublished results]. Unlike infected HF cells in which virus is readily recovered from supernatants, HCMV was not detected in MDM culture supernatants (Figure 1A and 1B). This observation suggests that progeny virus remains primarily cell-associated in MDM.

The Kinetics of HCMV mRNA Expression is Altered in MDM. To determine if the delay in the appearance of HCMV in MDM is a result of altered kinetics of HCMV gene expression, the presence of mRNA's encoding proteins from the MIE region (UL122 and UL123), pp65/pp71 (UL82 and UL83), and gB (UL55) were analyzed by RT-PCR. Figure 2

represents the kinetics of mRNA expression for the HCMV isolate I-G in MDM. Similar results were also obtained for the HCMV isolate PH (data not shown). For this experiment whole cell RNA was extracted from HCMV-infected MDM at 1, 3, 5, 7 dpi and from infected HF cells at 2, 6, 12, 18, 24, 37, 48, and 72 hpi. Since the MIE gene generates multiply spliced transcripts with common and unique exons, oligonucleotide MIE primers were designed to detect expression of mRNA's encoding MIE exon 4, exon 5, and the exon 6/7 splice junction generating RT-PCR products of 435 bp, 659 bp, and 198 bp, respectively (Figure 2A and 2B). The exon 5 primers would also detect the presence of a late mRNA encoding a late 40 KDa protein (252). To detect expression from the genes encoding pp65/pp71 and gB, primers were utilized to generate products of 401 bp and 360 bp, respectively (Figure 2C and 2D). Primers to βactin RNA, which span the exon 4 and 5 junction, were used to demonstrate the integrity of the macrophage RNA as well as determine the presence of contaminating DNA.

Examination of RNA extracted from MDM by RT-PCR revealed a peak of MIE exon 4 and exon 6/7 splice junction products at 3 dpi (Figure 2A and 2B, respectively). Interestingly, the 6/7 splice junction product, which produces an mRNA encoding the IE55 kDa protein, is only observed when HF cells are treated with protein synthesis inhibitors [(254)

and data not shown]. Exon 5 signal increased through day 7 probably representing expression not only of IE 86 but also the L40 mRNA (Figure 2B). RT-PCR analysis of pp65/pp71 and gB mRNA first detected expression of these genes at 5 dpi with a peak at 7 dpi. In contrast to viral mRNA production in MDM, MIE mRNA peaked in HF cells at 5 hpi while pp65/pp71 and gB peaked at 48 hpi [(208, 254, 283), data not shown].

Expression of HCMV Proteins Is Delayed in MDM. To detect expression of viral proteins, HCMV-infected MDM were fixed at various intervals pi for a period of 7 days and stained with virus-specific antibodies by double-label immunofluorescence. Figure 3 represents the immunostaining pattern of expression for the IE 72 and pp65 proteins using the HCMV isolate I-G. Similar IE and pp65 expression patterns were also observed with HCMV isolates PH, PO and LY (data not shown) Consistent with mRNA expression patterns described above, IE 72 antigen was first detected at 12 hpi with a maximum amount of cells expressing antigen (15%) at 3 dpi (Figure 3A—red nuclei). Between 3 and 5 dpi, co-expression of both IE 72 and pp65 antigens were detected in HCMV infected MDM (Figure 3B—yellow nuclei). At 5 dpi, only pp65 antigen was observed in MDM cultures (Figure 3C—green nuclei). Interestingly, if these latter infected MDM cultures were screened only for the presence of IE 72 antigen, virus would not have been detected in

these cultures. After 7 dpi, IE 72 antigens reappeared in cultures co-expressed with pp65 antigen in large nuclei (Figure 3D and 3E—yellow nuclei). During this interval, MDM either expressing IE 72 or pp65 were also observed in foci adjacent to these co-expressing cells (Figure 3D and 3E). Cells expressing single viral antigens probably represent secondary rounds of infection. At 7 dpi when infectious virus peaks in MDM, co-expression of IE 72 and pp65 was detected in macrophages with enlarged nuclei representing about 5% of infected cells (Figure 3F). In these cells, distinct structures staining for the presence of pp65 antigen were observed closely associated with the cell nucleus. The temporal pattern of gB and gH expression was also examined by immunofluorescence and follows the same kinetics displayed by pp65 (data not shown).

In contrast to IE expression in MDM, by 6 hpi 100% of HF cells demonstrated the prescence of IE 72, which remained throughout the course of infection (data not shown). Similarly, accelerated expression of pp65 and gB gene products in HF cells was first detected at 24 hpi, with all cells expressing by 48 hpi. Therefore, the reduced kinetics of HCMV gene expression in MDM is not an inherent property of the virus isolate but is due to cell-specific differences. Expression of viral antigen thus correlates with the previous RNA data demonstrating the delayed kinetics of HCMV infection in MDM as compared to HF cells. In addition, the

temporal appearance and distribution of viral antigen in macrophages suggests a unique adaptation of HCMV to the macrophage.

Compartmentalization and transmission of HCMV in MDM. We next examined the distribution of HCMV antigens in MDM in the later stages of viral infection. Infected cells were analyzed for MIE, pp65 or gB antigen localization by double-label immunofluorescence. At this interval HCMV infected cells with large nuclei and numerous vacuoles containing pp65 (Figure 4A) and gB (Figure 4B) were clearly evident in infected MDM cultures. The appearance of pp65 and gB containing vacuoles in MDM coincides with the recovery of infectious virus from these cultures. At late stages of infection (14 dpi) a higher frequency (20%) of cells with pp65 and gB containing vacuoles were observed in these cultures.

The lack of virus in the supernatant as well as the development of distinct HCMV foci in MDM suggested that the major mode of transmission in these cultures is by cell-cell contact and fusion events. Consistent with these observations, cells at various stages of fusion were observed with viral antigens coating intracellular surfaces at junctions of contact (Figure 4C).

Discussion

In this report we have examined characteristics of HCMV replication in MDM. We have found that relative to HF cells, HCMV infection of MDM displays a significant delay in the production of virus that is independent of viral strain. Consistent with these observations, the kinetics of HCMV gene expression was dramatically retarded in representative IE and L gene classes at the steady-state RNA and protein levels. The delay in viral expression may be a unique adaptation of HCMV for persistance in MDM and may explain the lack of cytopathic effect observed in virally infected cells (112).

The mechanism responsible for the delayed kinetics of viral gene expression is unknown. Since IE gene products are important regulators of subsequent gene expression (59, 245, 248, 251, 253, 254), the altered kinetics of IE protein production in MDM may also influence the delayed appearance of early and late viral proteins. Interestingly, IE antigen peaked at 3 dpi, disappeared at 5 dpi, and reappeared at 7 dpi in cells coexpressing pp65 or gB. These observations contrast IE expression in HF cells, which demonstrate the continuous presence of IE gene products throughout the viral replication cycle. The differences in HCMV expression patterns in the two cell types may reflect the stability of IE gene products and the rapid rate of replication in HF cells compared to MDM. The apparent reappearance of MIE gene expression at 7 dpi in

MDM may represent the detection of the L40 gene product, which contains amino acid sequences colinear with the MIE gene (252).

Another explanation for the delayed growth of HCMV in MDM is that altered gene expression occurs through differential splicing events resulting in RNAs encoding proteins with different functions. The fact that the MIE 6/7 splice junction that generates the IE 55 gene product is naturally utilized in MDM supports this possibility. Previously, utilization of this splice junction was only observed in HCMV infected HF cells treated with protein synthesis inhibitors (254). We have also observed a newly characterized spliced MIE gene product in MDM (data not shown), which is also only observed in HF cells in the presence of cyclohexamide (128). Since MIE isoformic proteins possess different transcriptional activation functions dependent on the presence of specific functional domains (251), generation of novel MIE products may accelerate or retard the growth of virus within the cell. In addition to the above possibilities, the presence of cellular inhibitors and/or absence or modification of cellular cofactors that interact with viral proteins may also play a role in HCMV growth kinetics.

HCMV infection of HF cells causes cellular fusion and lysis resulting in the release of virus in the supernatant. In contrast, virus is exclusively cell associated in MDM and persists in these cells without lytic events. Examination of MDM during late stage infection when infectious

HCMV is recovered from cell lysates revealed the presence of numerous vacuoles filled with virus. Similar observations in MDM were made with human immunodeficiency virus (HIV) (82). Ultrastructural analysis of HIV infected MDM indicated that virus was exclusively associated with intracellular vacuoles, which were not associated with the PM (180). They suggest that such HIV sequestering vacuoles are derived from the Golgi complex and are thus not endosomes. In addition, we have recently utilized double-label immunofluorescence with cellular and viral markers to demonstrate that HCMV infected vacuoles are derived from the Golgi Complex (data not shown). Therefore, it appears that both viruses utilize a common pathway for intracellular trafficking and storage in MDM. This adaptation may serve two purposes. First, storage of the virus in discrete cellular compartments may not only protect virus from cellular degradation but may also protect the cell from damage produced by viral proteins. Secondly, storage of virus in intracellular compartments that never reach the PM would allow the virus to escape detection and clearance by the immune system. The latter ominous situation would allow persistence and trafficking of virus to target tissues via a cellular vehicle while evading immune detection.

Elucidating the mechanisms of HCMV replication in MDM will be crucial in understanding viral persistence and trafficking in the human

host. The current studies indicate that HCMV replication in MDM differs greatly from HF cells. These observations underline the importance of examining viral replication in biologically relevant cell types.

Figure Legends

Figure 1: Growth of HCMV in differentiated macrophages. An HCMV one-step growth curve in infected MDM (A) and HF cells (B). MDM and HF cells were infected with HCMV recent isolates (I-G and PH) as described in Materials and Methods. Macrophages I-G (O) or PH (▪▪▪□▪▪▪) infected cells and I-G (▪▪▪◇▪▪▪) or PH (▪▪▪●▪▪▪) infected supernatants were harvested at the indicated times and viral titers were determined by plaque assay on HF cells (A). HF I-G (—○—) or PH (▪▪▪□▪▪▪) infected cells and I-G (▪▪▪◇▪▪▪) or PH (▪▪▪●▪▪▪) infected supernatants were harvested at the indicated times and viral titers were determined by plaque assay on HF cells (B).

Figure 2: PCR-RNA analysis of HCMV major immediate early (MIE) and Late (pp65/pp71; gB) gene expression in infected macrophages. Whole cell RNA was extracted from HCMV-infected (I-G) MDM at 1, 3, 5, 7 dpi. Oligonucleotide MIE primers were designed to detect the presence of exon 4 (A) generating a 435 bp product, exon 5 (B) generating a 659 bp product, and exon 6/7 splice junction (B) generating a 198 bp product. The pp65/pp71 primers (C) were designed to generate a 401 bp product. The gB primers (D) were designed to generate a 360 bp product. Oligonucleotide primers generated to produce a 202 bp product from β

actin RNA were utilized to demonstrate the integrity of the macrophage RNA.

Figure 3: Double-label immunofluorescence for the presence of MIE and pp65 gene products in HCMV-infected MDM. HCMV-infected (I-G) MDM were fixed at various intervals post infection. Double-label immuno fluorescence was performed with antibodies directed against IE 72 antigen (red) and pp65 antigen (green). IE 72 was first detected at 12 hpi peaking at 3 dpi (125x) (panel A—red nuclei). Between 3 and 5 dpi HCMV infected macrophages with both IE 72 and pp65 antigens (125x) (panel B—yellow nuclei) were detected in cells. By 5 dpi only pp65 antigen (panel C—green nuclei) was observed (62.5x). Between 5 and 7 dpi cells with large nuclei containing both IE 72 and pp65 antigens were observed (62.5x) (D) and (125x) (E). By 7 dpi cells with large nuclei and numerous vacuoles containing pp65 were observed (312.5x) in infected macrophage cultures (F).

Figure 4: Compartmentalization and spread of HCMV in MDM. HCMV-infected (I-G) macrophages were examined at 12 dpi for the presence of IE 72 (green) and pp65 (red) antigens by double-label immunofluorescence (630x) (panel A) or the prescence of gB (red) by

single-label immunofluorescence (400x) (panel B). To illustrate cell to cell transmission of HCMV in MDM cultures, HCMV-infected (I-G) MDM were examined by double-label immunofluorescence with antibody directed against gB (red) and medial Golgi (anti-mannosidase II) (22) (green) (630x) (panel C). The confocal microscopy image was focused at the level of cell contact with the tissue culture slide.

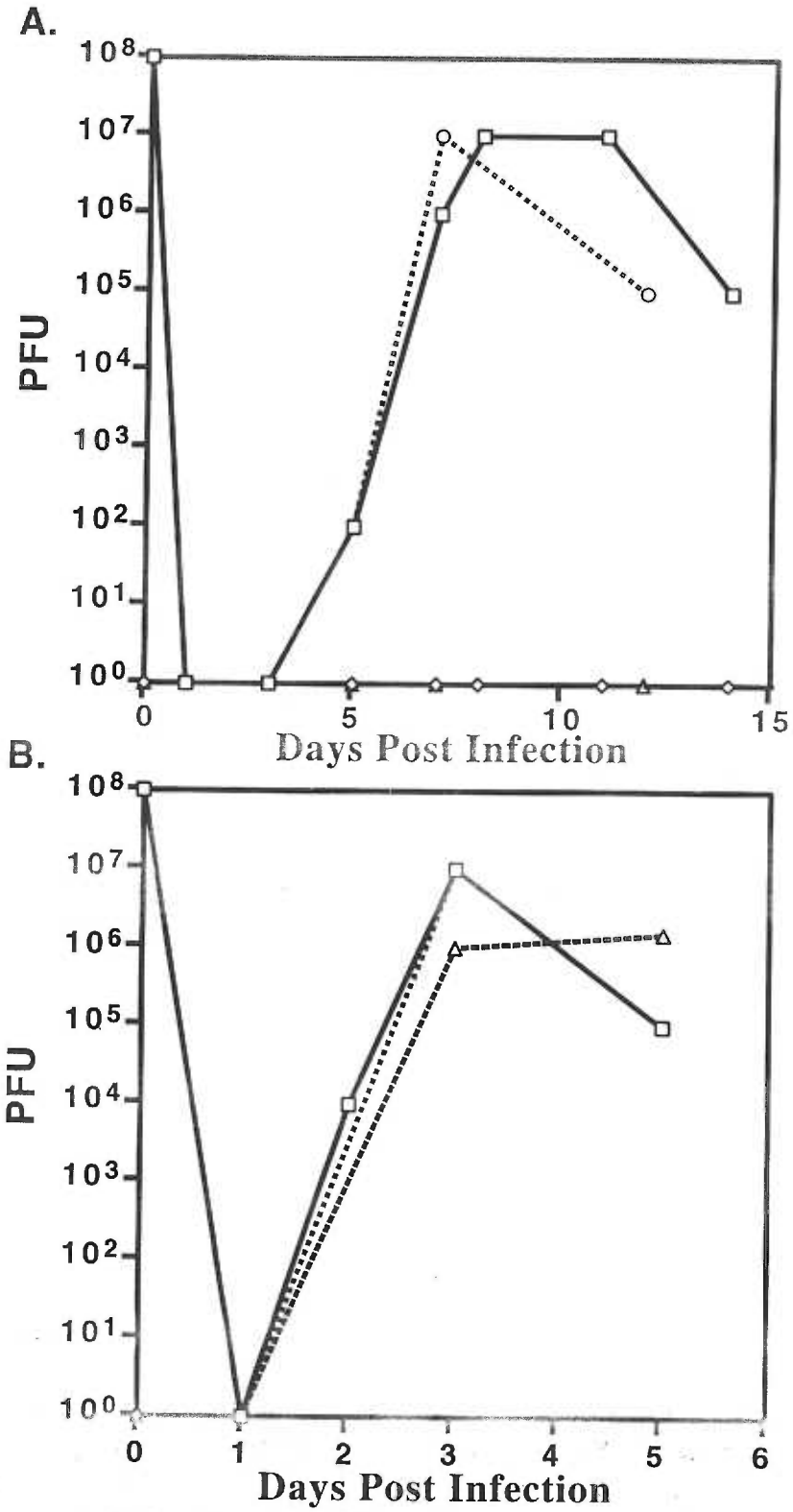

Figure 1. Growth of HCMV in differentiated macrophages.

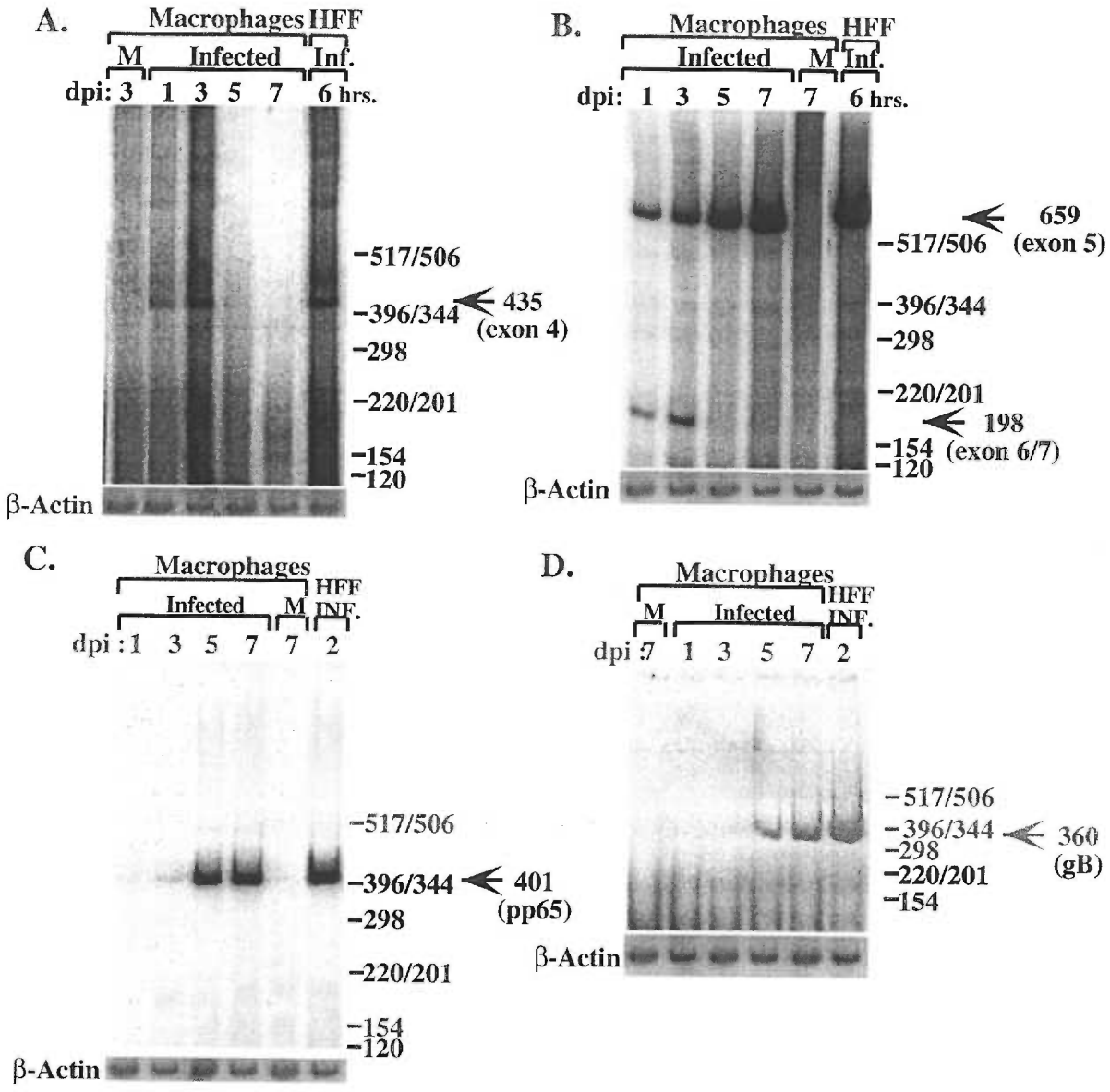

Figure 2. PCR-RNA analysis of HCMV MIE and late (pp65/pp71; gB) gene expression in infected macrophages.

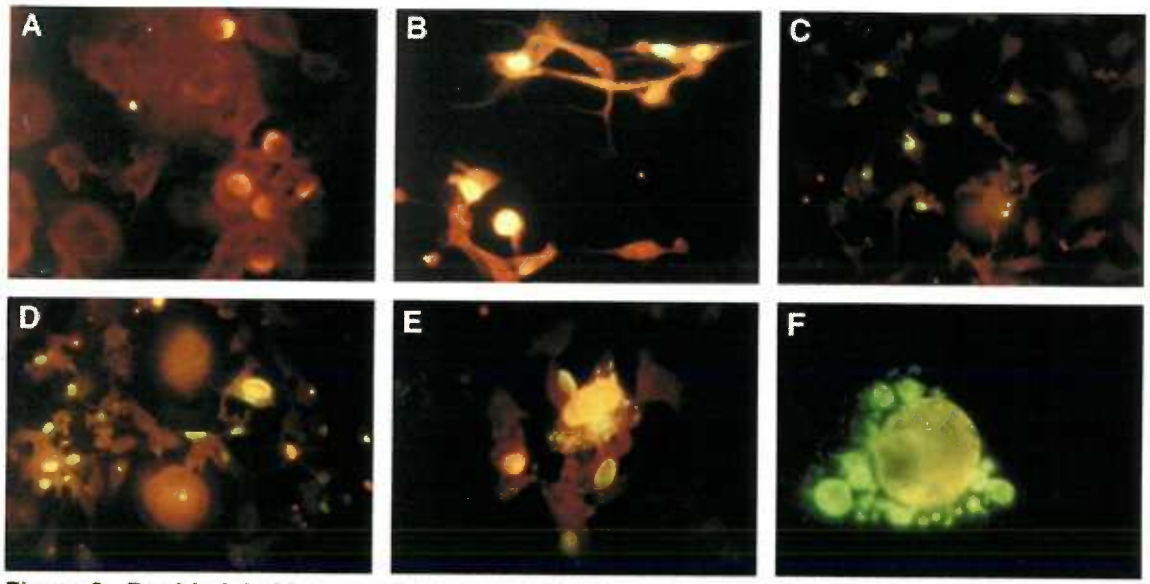

Figure 3. Double-label immunofluorescence for the presence of MIE and pp65 gene products in HCMV-infected MDM.

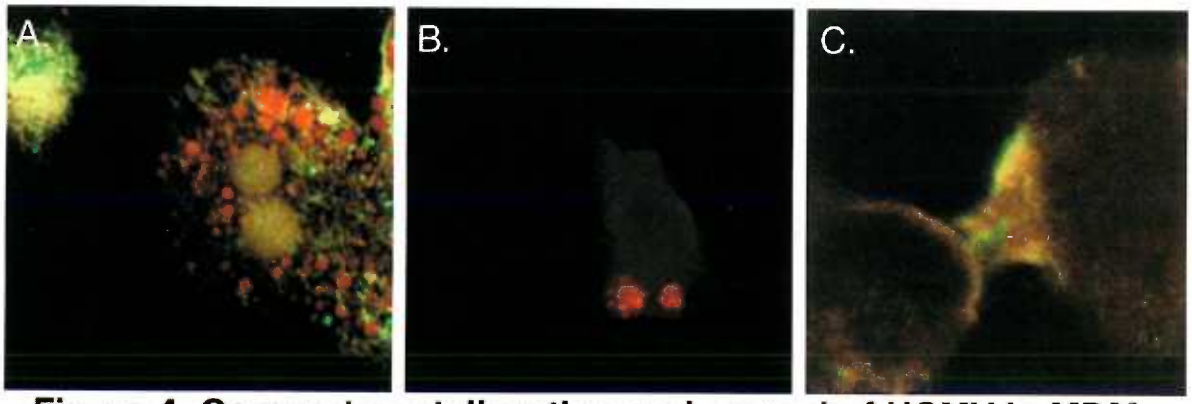

Figure 4. Compartmentalizeation and spread of HCMV in MDM.

Chapter 4

A novel mechanism for persistence of human cytomegalovirus in macrophages.

by Kenneth N. Fish, William Britt, and Jay A. Nelson

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pages 76-96 of this Thesis

All experiments presented in this chapter were performed by Kenneth N. Fish.

Abstract

Human cytomegalovirus (HCMV) infection of monocyte-derived macrophages (MDM) results in delayed and nonlytic productive viral growth. During late stages of replication, infectious virus remains cell associated in cytoplasmic vacuoles. In order to understand HCMV survival and persistence in MDM, we examined mechanisms involved in HCMV vacuole formation and trafficking in these cells. Utilizing double-label immunofluorescence with antibodies to viral and cellular proteins, HCMV vacuoles were associated with the Golgi marker mannosidase II but not with markers to early endosomes (transferrin receptor and rab5), or late endosomes and early lysosomes (Lamp 1 and 2). In addition, as late stage viral infection progressed in MDM, the cells displayed increasing abnormalities in the Golgi apparatus. Analysis of structural features of infected cells revealed the disruption of the tubulin network. These observations suggest a novel mechanism by which HCMV is vacuolized in MDM, avoiding degradation and release from the cell.

Introduction

Examination of cell types naturally infected by HCMV *in vivo* has identified monocyte/macrophages (M/M) as important cells in the biology of the virus (4, 52, 61, 119). In order for HCMV to persist in the host, the virus must avoid immune surveillance. A common mechanism that viruses use to evade immune detection is to restrict viral replication in cells until activation signals trigger the production of infectious virus. In the case of HCMV, virus infection of progenitor myeloid cells (132, 148, 165, 224, 227) as well as monocytes (68, 122, 200, 259, 260) is restricted to early events. However, differentiation of these cells into macrophages results in the production of infectious virus (112, 138). Therefore, one strategy that HCMV utilizes to evade immune detection is to remain quiescent in undifferentiated monocytes until the cells have differentiated into macrophages.

During HCMV productive infection of macrophages, the optimal situation for virus-host cell interactions is to maintain cellular survival during accumulation of virus. Recently, the viral replication cycle of HCMV in MDM was shown to be significantly delayed relative to replication in HF (72). In MDM, viral production peaked at 13-15 days post infection (dpi) in contrast to 4-5 dpi in HF cells (72). HCMV, similar to other herpesviruses, exhibits a temporal order of gene expression during productive infection of

cells. Immediate early (IE) genes are activated first, followed by early (E) and late (L) genes (245, 283). The altered growth of HCMV in MDM correlated with the delayed kinetics of viral IE and L gene expression in these cells. The retarded production of HCMV gene products may prevent the rapid increase of toxic viral factors in the cell. The delay in viral gene expression may therefore be a unique adaptation of HCMV to persist in MDM.

HCMV infection of MDM was also shown to be nonlytic and exclusively cell associated (72, 112). Infectious virus accumulated in discrete cytoplasmic vacuoles, which were not associated with PM. An important aspect of HCMV intracellular survival in the macrophage is evasion of host cell-mediated degradation induced by fusion of vacuoles with lysosomes. Pathogens utilize multiple strategies to ensure their intracellular survival within the endosomal-lysosomal system of MDM. One mechanism of microbial survival in macrophages is exemplified by *Mycobacterium tuberculosis* and *M. microti* (89, 98). These macrophage-tropic pathogens avoid destruction by producing an amine that raises the pH of the vacuoles in which they reside, preventing the vacuoles from becoming acidic, active lysosomes (89, 98). Alternatively, organisms such as *Leishmania mexicana* and *L. amazonensis* have adapted to resist degradation by lysosomal contents (7, 38). *Listeria monocytogenes* is an

intracellular pathogen that survives in the macrophage by lysing the bacterial phagosome prior to phagosome-lysosome fusion (107, 174). Finally, Salmonella enters a vacuole that is not acidified and does not appear to fuse with lysosomes (9). Therefore, intracellular pathogens have evolved a number of mechanisms to survive and persist in macrophages.

Although a number of viruses in addition to HCMV survive and persist in macrophages, the mechanisms involved in intracellular survival are unknown (184, 192). To address this issue, we examined HCMV vacuole formation in MDM. Our results suggest that HCMV uses a novel strategy for MDM intracellular survival through disruption of the microtubule network.

Results

MDM Vacuoles Contain Infectious HCMV. In previous studies of HCMV-infected MDM, numerous intracellular virus-containing vacuoles were observed at later stages of infection (Figure 1A and 1B). Since infected MDM cultures contain cells at different stages of differentiation and virus replication, identification of HCMV in these vacuoles by electron microscopy is exceedingly difficult. Therefore, to demonstrate that vacuoles staining for late viral antigens contain infectious virus, we

isolated HCMV vacuoles from infected MDM (104). In these experiments, the PM of MDM was removed by placing a serum coated coverslip over infected cultures at 13 days post infection (dpi). The coverslip with PM was removed the following day releasing free cellular organelles from MDM by a gentle rinse with media. HCMV-infected vacuoles were separated from other cytoplasmic contents by low speed centrifugation and both fractions assessed for the presence of infectious virus by plaque assay (Figure 2). The vast majority of infectious HCMV was observed in the MDM fraction that contains cellular organelles. These results indicate that the detection of HCMV late antigens in MDM vacuoles correlates with the presence of infectious virus and that very small amounts of infectious virus resides outside of these vacuoles.

Origin of HCMV Vacuoles in MDM. To determine how HCMV is compartmentalized in MDM, the origin of the vacuoles that contained virus was examined by double-label immunofluorescence utilizing antibodies directed against HCMV antigens and various cellular organelles. Utilizing antibodies to the medial Golgi-specific marker mannosidase II and either HCMV gB or pp65, the membranes surrounding HCMV-containing vacuoles stained with the Golgi marker (Figure 1B and 1C). These results indicate the Golgi origin of these HCMV vacuoles. Since Golgi-derived vacuoles enter the endosomal-lysosomal pathway, HCMV-vacuoles were

examined with specific markers for early endosomes (transferrin receptor and rab5; Figure 1D and 1E) and late endosomes/early lysosomes (LAMP 1; Figure 1F). None of these markers were found to associate with the membranes of the HCMV vacuoles in the cytoplasm of MDM. In addition, progression of HCMV infection also correlated with a reduction in lysosomal marker (data not shown). The above results indicate that HCMV vacuoles derived from the Golgi evade entry into the endosomal-lysosomal pathway.

HCMV Disruption of the Golgi Apparatus. Examination of HCMV-infected MDM with mannosidase II revealed abnormal Golgi structures associated with HCMV vacuoles. To assess the integrity of the Golgi apparatus in infected cells, MDM were exposed to BODIPY C_6-ceramide, which can specifically stain the Golgi in live cells (181). Staining of uninfected MDM revealed the normal structure of the Golgi apparatus interwoven between the nuclei of multinucleated cells (Figure 3A and 3B). HCMV-infected MDM at 8 dpi also exhibited structurally normal Golgi (data not shown). In contrast, late stage (14 dpi) HCMV-infected MDM displayed a diffuse distribution of BODIPY C_6-ceramide (Figure 3C and 3D). This observation indicates that HCMV disrupts the Golgi apparatus at late stages of infection.

Since the movement and structure of many cytoplasmic organelles is dependent on the integrity of microtubule network (136, 270), we examined the organization of this structure in infected MDM. The microtubule network in uninfected MDM demonstrated normal structural features associated with multinucleated cells (Figure 1G). However, as HCMV infection progresses in MDM cultures, cells showed increasing loss of the microtubule network (Figure 1E and 1H). Figure 1E demonstrates an HCMV-infected MDM at a late stage and 1H shows two cells in the same field at different stages of viral infection. In the latter figure, the late stage-infected cell at the top demonstrates the complete disruption of the microtubule network. While the beginning of the microtubule network disruption is observed in the earlier stage HCMV-infected MDM at the bottom. Since the integrity of the Golgi is dependent on the microtubule network, the ability of HCMV to disrupt microtubules correlates with the disorganization of the Golgi apparatus that was observed at late stages of infection.

Since MDM are non-dividing terminally differentiated cells, the absence of microtubule structure in viable infected cells was an unexpected result. To confirm that MDM are able to survive without normal microtubule structure, cells were treated with nocodazole. Nocodazole treatment results in the depolymerization of microtubules,

thus mimicking the disruption of this structure observed in HCMV-infected MDM. The viability of nocodazole-treated MDM was assessed by exposing uninfected MDM to drug for varying intervals up to six days. After removal of nocodazole, the disruption and reformation of the microtubule network was determined by immunofluorescence staining with antibody directed against tubulin (Figure 4). MDM treated with nocodazole for up to six days retained the ability to reform a normal microtubule network, indicating that MDM retain viability in the absence of a cellular microtubule structure. Therefore, HCMV-mediated disruption of the microtubule network does not result in the imminent death of the cell.

HCMV Disruption of the Microtubule Network Alters MHC Class II Expression on the Plasma Membrane. HCMV disruption of the microtubule network would result not only in an alteration in protein processing events, but also in intracellular membrane trafficking. MHC class II is assembled in the endoplasmic reticulum and transported from the Golgi to the cell surface through the endosome secretory pathway. HCMV induced disruption of the microtubule network would likely interfere with these processes. Therefore, to address this issue, the presence of MHC class II in the PM was examined throughout the course of infection. For these experiments double-label immunofluorescence was performed using antibodies directed against HCMV gB and MHC class II HLA-DR.

Quantitation of MHC class II cell surface fluorescence was determined in HCMV-infected MDM over time utilizing a Quantimet 500 fluorescence analysis program. As seen in Figure 5, reduction of MHC class II cell surface expression correlated with the progression of HCMV infection. The greatest effect was detected at 12 dpi, which correlates with the HCMV-mediated disassembly of the microtubule network. These results indicate that HCMV not only interferes with viral protein trafficking to the cell surface but also the trafficking of important cellular proteins that are involved in the antigen presentation pathways.

Discussion

In this study, we examined mechanisms of HCMV survival in MDM. Analysis of HCMV storage vacuoles with known early endosome and lysosome markers indicated that the virus evades entry into the endosomal-lysosomal system. However, the presence of mannosidase II indicates that the viral containing vacuoles are derived from the Golgi apparatus. How the viral containing vacuoles evade lysosomal fusion is unknown, but disaggregation of the microtubule network may play a role in these processes.

Macrophages represent a key cell involved in the defense of the host against pathogens. The success of a pathogen that infects

macrophages depends on the ability of the organism to evade lysosomal fusion or withstand lysosomal degradation. Macrophage pathogens have developed unique strategies to avoid cellular destruction including the release of substances that modify acidity, resistance to a harsh environment, and disruption of vacuoles (7, 9, 38, 89, 98, 107, 174). HCMV uses a novel macrophage survival approach, which involves evasion of viral containing vacuole fusion with lysosomes. The mechanisms mediating this process may be the lack of vacuolar membrane proteins, which promote lysosome fusion, or the presence of viral proteins that prevent fusion. Alternatively, lysosomal fusion may be inhibited by disruption of the microtubule network, which mediates intracellular vacuole transport through the motor proteins kinesin and dynein (270). One or all of the above mechanisms may contribute to the inability of viral containing vacuoles to fuse with lysosomes in MDM.

The ultrastructure of the cell is maintained in part by the microtubule network. This network functions to maintain placement and transport of intracellular organelles (136, 270). The major site of microtubule nucleation is the centrosome—the microtubule organization center. Polymerization and depolymerization of microtubules is a dynamic process in which the half life of a microtubule is 10 minutes. HCMV may utilize multiple mechanisms to disrupt the microtubule network. First,

HCMV may encode proteins that bind to the α and β monomers preventing polymerization similar to the drug colchicine. Second, the virus may block GTP and or GDP binding to the tubulin monomers preventing tubulin assembly into microtubules. Lastly, HCMV may disrupt the microtubule organization center inhibiting the nucleation event. Ultimately, destruction of the microtubule network would prevent transport of viral containing vacuoles to the PM.

Similar to other persistent pathogens, HCMV has developed a variety of strategies to evade detection by the immune system. The most efficient mechanism is to remain quiescent in cells until activation by some external stimuli. We (112) and others (200, 259, 260) have found that HCMV expression is restricted in monocytes but not in differentiated MDM. The quiescent state of the virus may represent the primary mechanism that HCMV utilizes to evade immune detection in these cells. As monocytes differentiate into permissive macrophages, the virus is sequestered in vacuoles that are unable to traffic to the PM. Therefore, infectious virus is retained in an environment protected from immune response. Lastly, HCMV disruption of the microtubule network indirectly alters the trafficking of proteins involved in antigen presenting pathways to the surface of the cell. The inability of the cells to present viral antigen would prevent detection by the immune system.

HCMV has developed a unique interaction with MDM, which allows both viral and cellular survival as well as evasion from immune detection. Identification of viral gene products that mediate these events will be important in elucidating mechanisms involved in MDM persistence.

Figure Legends

Figure 1. Co-localization of HCMV and cellular organelle-specific proteins in MDM. MDM were infected with HCMV and examined 10-14 dpi by confocal microscopy using the following antibodies: (A) IE72 (green) and pp65 (red); (B) pp65 antigen (red) and membrane-associated Mannosidase II antigen (green); (C) gB antigen (red) and membrane-associated mannosidase II antigen (green); (D) gB (red) and transferrin receptor (green); (E) gB (red), rab5 (green), and tubulin (blue); (F) gB (red) and LAMP-1 (green). HCMV uninfected (G) and infected (H) MDM were examined for the presence of pp71 (red) and tubulin (green) antigen by double-label immunofluoresence. Letters represent the following: N = nucleus; V = vacuole; M = mannosidase II; T = transferrin receptor; R = rab5; Tu = tubulin; L = LAMP1. The scale bar in each panel represents 10 μm.

Figure 2. Association of infectious HCMV with cytoplasmic organelles. MDM were infected with HCMV as described in Materials and Methods. The PM was removed at 14 dpi and the cells were rinsed lightly to recover free organelles from the MDM. Following centrifugation, the cytoplasmic organelle pellet and supernatant were tested by plaque assay on HF cells.

Figure 3. HCMV disrupts the Golgi apparatus. The integrity of the Golgi apparatus was assessed utilizing BODIPY C_6-ceramide, which specifically stains the Golgi in live cells. HCMV uninfected (A & B) and infected (C & D) MDM were exposed to the dye for 10 mins., rinsed, and incubated for one hr. Cells were fixed and examined by confocal microscopy. The magnification for A, C, and D is X400 and for B X630.

Figure 4. Viability of nocodazole-treated MDM. To mimic the microtubular disruption observed in HCMV-infected MDM and assess cell viability, cell cultures were treated for varying intervals with nocodazole. The integrity of the microtubule network was assessed by confocal microscopy using anti-tubulin antibodies at the following periods of treatment: (A) 6 hours, (B) 12 hours, (C) 18 hours, (D) 24 hours, (E) 4 days and (F) 6 days of nocadozole treatment. To determine MDM viability reformation of microtubule network was analyzed in cells treated with nocodazole for (G) 4 days and (H) 6 days followed by removal of drug. Cells were fixed at 24 hrs. after removal of nocodazole for examination by confocal microscopy. The magnification for A-H is X400.

Figure 5. Downregulation of MHC Class II antigen in HCMV infected MDM. The surface expression of MHC class II on macrophages was measured by recording the fluorescence intensity of macrophages labeled with a fluorescent anti HLA-DR antibody. A Quantimet 500 fluorescence analysis program was used to quantitate fluorescence. MHC class II expression was measured on days 8, 10 and 12 post infection. Fluorescence intensity of uninfected macrophages was adjusted to 100% for each time point and fluorescence intensity of HCMV-infected macrophages was expressed as a percentage of that observed in uninfected cells. Each time point represents the mean ± SD measurements for nine cells.

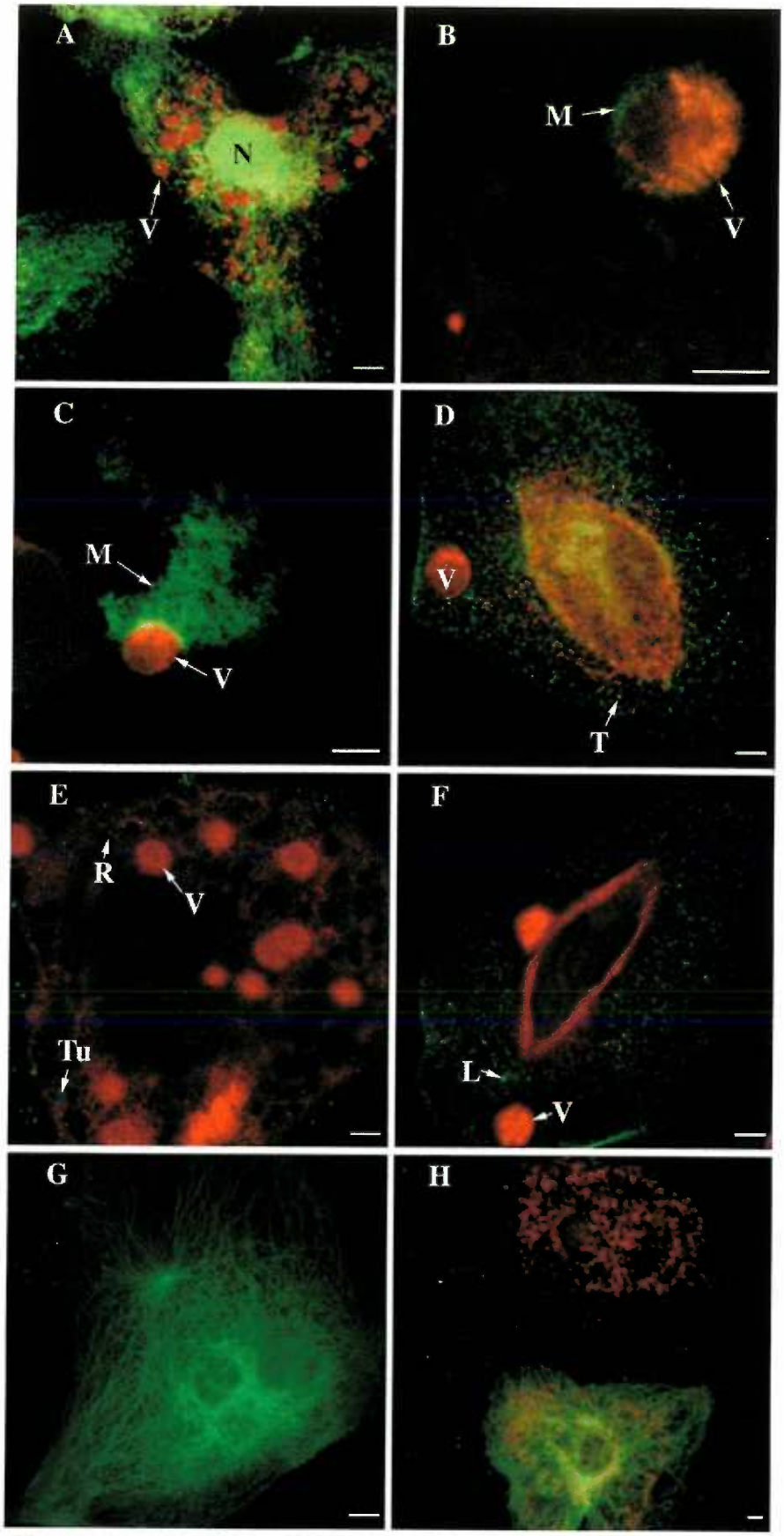

Figure 1. Colocalization of HCMV and cellular organelle-specific proteins in MDM.

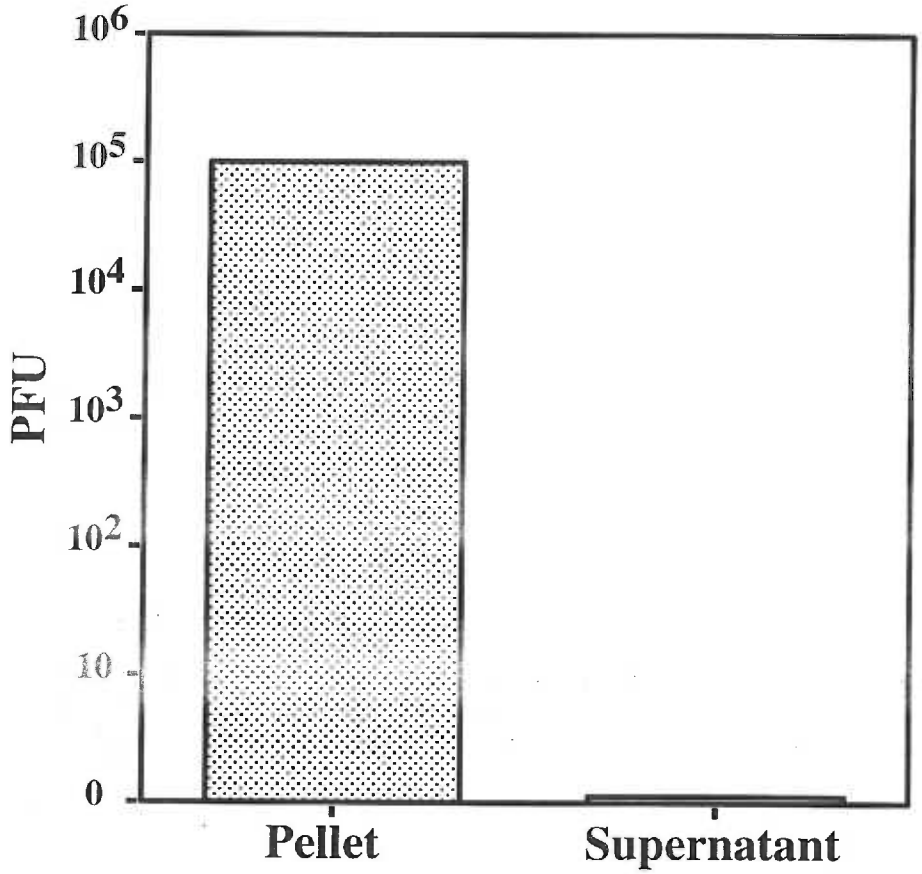

Figure 2. Association of infectious HCMV with cytoplasmic organelles.

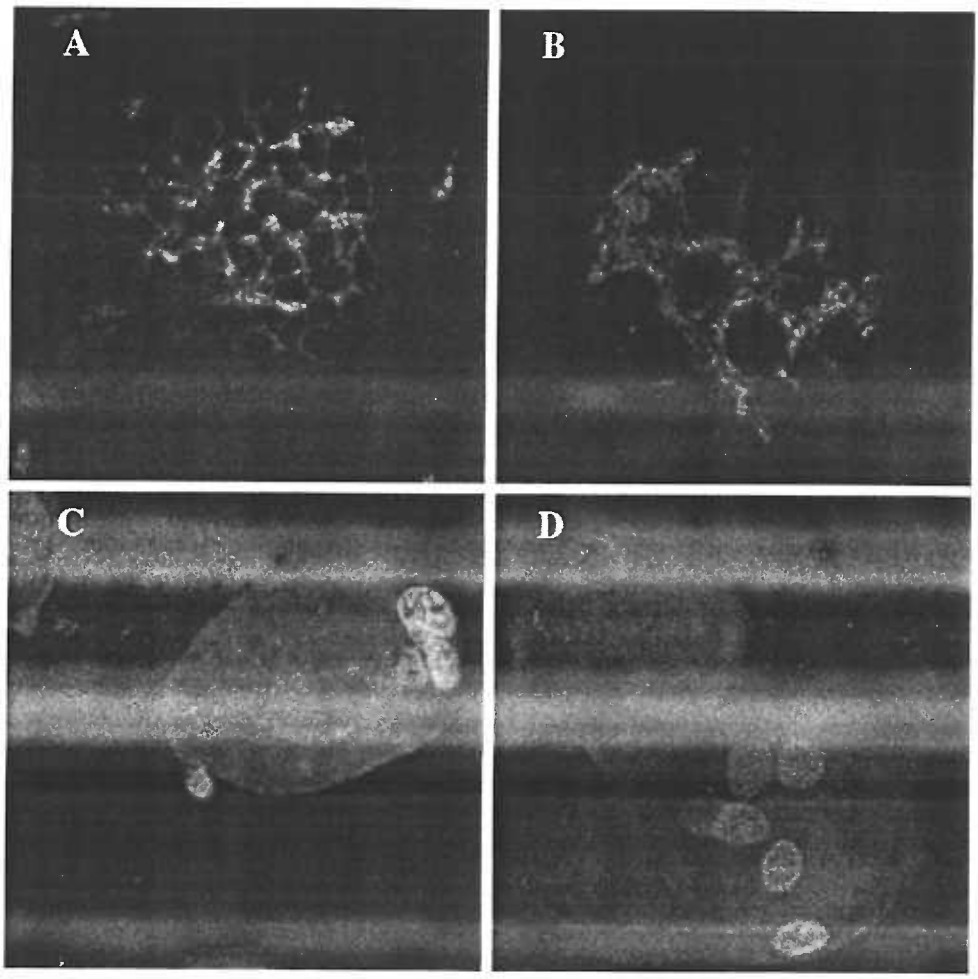

Figure 3. HCMV disrupts the Golgi apparatus.

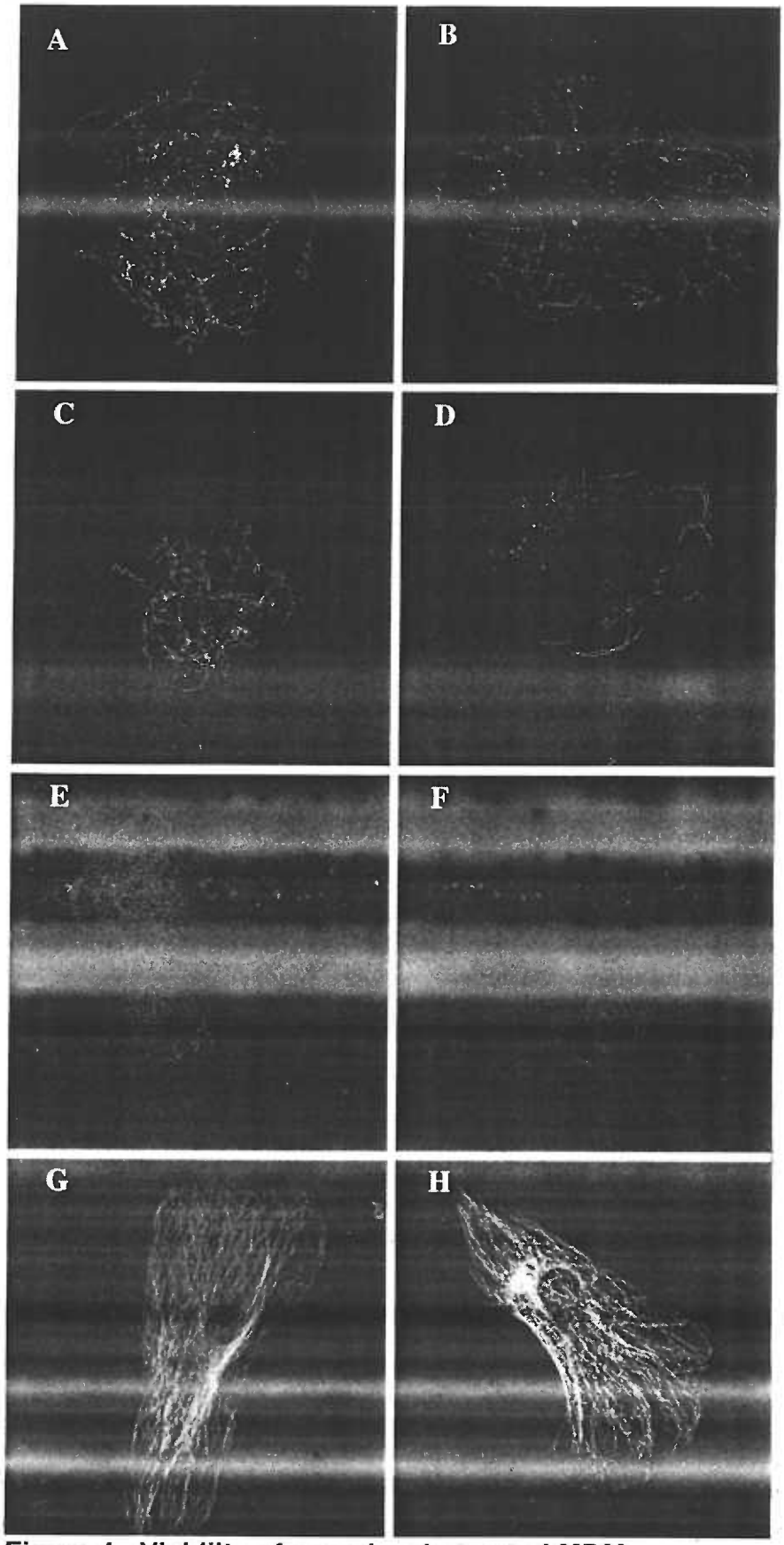

Figure 4. Viability of nocodazole-treated MDM.

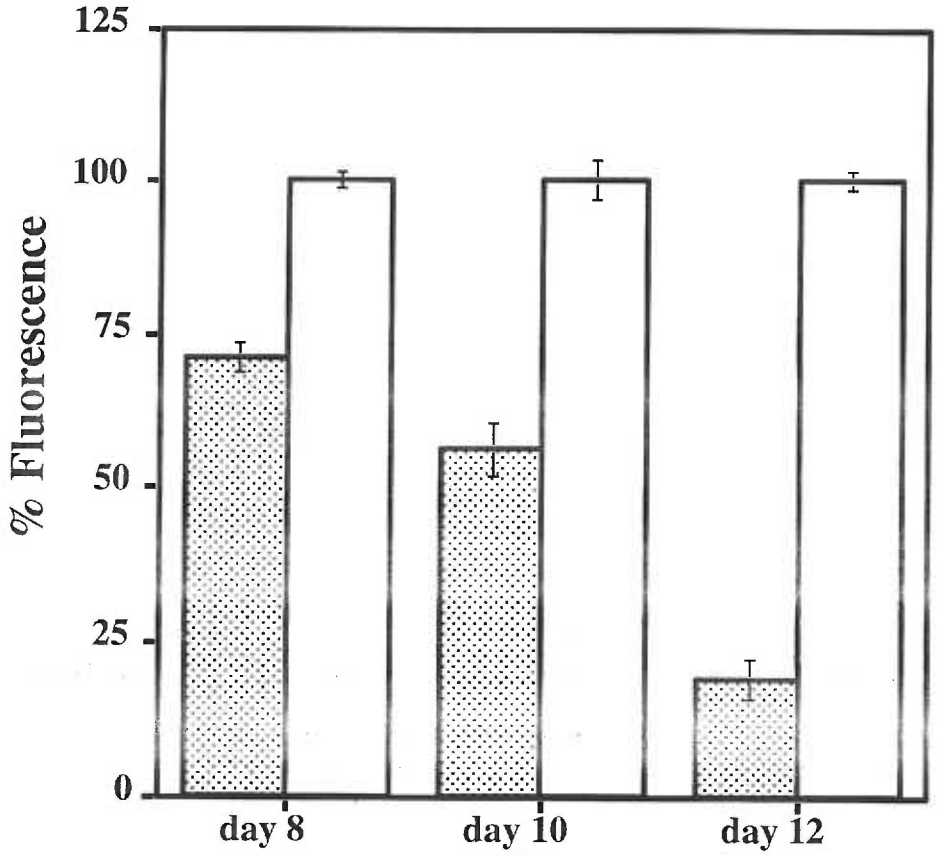

Figure 5. Downregulation of MHC class II antigen in HCMV-infected MDM.

Chapter 5

Steady-state plasma membrane expression of HCMV gB is determined by the phosphorylation state of ser_{900}.

by Kenneth N. Fish, Cecilia Soderberg-Naucler, and Jay A. Nelson

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pages 97-124 of this Thesis

All experiments presented in this chapter were performed by Kenneth N. Fish.

Abstract

Human cytomegalovirus (HCMV) infection of an astrocytoma cell line (U373) or human fibroblast (HF) cells results in a differential cell distribution of the major envelope glycoprotein gB (UL55). This 906 amino acid type I glycoprotein contains an extracellular domain with a signal sequence, a transmembrane domain, and a 135 amino acid cytoplasmic tail with a consensus CKII site located at a serine residue at position 900 (Ser_{900}). Since phosphorylation of proteins in the secretory pathway is an important determinant for intracellular trafficking, the state of gB phosphorylation was examined in U373 and HF cells. Analysis of cells expressing wild-type (WT) and site-specific gB mutants indicated that the glycoprotein was equally phosphorylated at a single site, Ser_{900}, in both U373 and HF cells. To assess the effect of charge on gB surface expression in U373 cells, Ser_{900} was replaced with an aspartate (Asp) or an alanine (Ala) residue to mimic the phosphorylated and nonphosphorylated state, respectively. Expression of the Asp but not the Ala gB mutation resulted in an increase in the steady-state expression of gB at the PM in U373 cells. In addition, treatment of U373 cells with the phosphatase inhibitor tautomycin resulted in the accumulation of gB at the PM. Interestingly, the addition of a charge at Ser_{900} trapped gB in a low level cycling pathway at the PM, preventing trafficking of the protein to the

trans-Golgi network or other intracellular compartments. Therefore, these results suggest that a tautomycin-sensitive phosphatase regulates cell-specific PM retrieval of gB to intracellular compartments .

Introduction

Viral glycoproteins mediate a number of essential functions in the virus life cycle, including entry into the host cell, assembly of viral progeny, and release of infectious virus (110). Glycoprotein maturation occurs through post-translational modification during sequential transport through the cellular secretory pathway. Viral proteins use cellular trafficking pathways to concentrate at a subcellular location at which capsid envelopment is thought to occur. The mechanisms involved in the localization of glycoproteins to sites of viral assembly are poorly understood.

Human cytomegalovirus (HCMV), a member of the herpesvirus family, demonstrates cell-specificity for virus assembly and release (105). The mechanisms for virus assembly and egress are still unclear, although attachment of membrane-bound viral glycoproteins to tegumented capsid is believed to play an important role in this process. The most abundant glycoprotein detected in the HCMV virion envelope is gB (UL55) (27). HCMV gB is synthesized as a 105 kDa polypeptide and processed into a highly glycosylated 130 kDa precursor glycoprotein. After glycosylation, the gB precursor is cleaved by furin to produce a heterodimer protein (gp55 and gp116) (244). gB is a type I glycoprotein containing a signal sequence, an extracellular/lumenal domain, a transmembrane (TM) domain, and a 135 aa cytoplasmic tail (26, 90, 185). The cytoplasmic tail

contains a consensus CKII site, which is phosphorylated both *in vitro* and *in vivo* (18, 175, 268).

HCMV infects several different cell types in patients with disease including monocytes, fibroblasts, endothelial cells, epithelial cells, and stromal cells (52, 61, 64, 68, 77, 86, 108, 132, 148, 149, 165, 200, 201, 219, 228-231, 238, 259, 269). However, the vast majority of studies on HCMV replication *in vitro* have used HF. Examination of viral replication in other cell types such as MDM and endothelial cells revealed significant differences in the kinetics of viral replication, viral cytopathic effect, and release of virus from the cell (73, 75, 112). Interestingly, unlike infected HF cells in which virus is readily recovered from supernatants, HCMV infection of MDM resulted exclusively in the accumulation of intracellular but not extracellular infectious virus, which was sequestered in numerous intracellular vacuoles whose membranes contained gB. In addition, comparison of HF cells and MDM by confocal microscopy revealed the presence of gB at the PM of HF cells, but not MDM. These observations suggest cell-specific pathways for gB intracellular trafficking.

The surface expression of viral glycoproteins is affected by their steady-state expression, transport to the PM, and rates of internalization from the cell surface. Deletion and point mutational analysis of the C-terminal domain of cell surface receptors and viral glycoproteins has

revealed sequence motifs, which are used by adapter molecules to sort the proteins to coated pits, where they become internalized. Internalization signals have been identified for several cellular proteins including furin, low-density lipoprotein receptor, transferrin receptor, polymeric Ig receptor, and epidermal growth factor receptor. Comparison of the sequences of these proteins indicates that a common structure, rather than sequence, is necessary for internalization. Recent studies on viral glycoproteins have uncovered how viruses have evolved to take advantage of this regulated endocytosis pathway (209, 302).

Recent studies with furin have demonstrated that the state of glycoprotein phosphorylation can affect the steady-state expression of a protein at the PM. Since HCMV gB displayed cell-specific PM expression, we examined the effect gB phosphorylation had on gB trafficking in different cell types. Our results indicate that gB displays a cell-specific steady-state expression of protein at the cell surface, which is regulated by a tautomycin-sensitive phosphatase. In addition, the presence of a charged residue at the phosphorylation site, which mimics the phosphorylation state, results in gB vacuoles that remain near the PM. These data suggest that the cell-specific differences in surface expression of gB are due to altered states of gB phosphorylation, which appears to be mediated by a tautomycin-sensitive phosphatase.

Results

Steady-state HCMV gB exhibits cell-specific differences in intracellular trafficking. Previous studies of HCMV permissive cells indicated that production of virus remained exclusively cell associated in U373 cells and macrophages in contrast to HF cells, which generated significant amounts of supernatant virus [(71, 72) and unpublished observations]. Since gB is an essential part of the viral envelope, we used confocal microscopy to examine gB accumulation and compartmentalization in these cell types. In HCMV infected HF cells gB was observed in cytoplasmic vacuoles as well as at the PM (Figure 1A). However, in macrophages as well as U373 cells gB was not detected at the PM and was restricted to intracellular vacuoles (Figure 1B and 1C). Similar gB expression patterns were obtained in HF and U373 cells infected with a recombinant vaccinia virus that expressed wild type gB ($RVVgB_{wt}$) (Figure 4A and 4D). The cellular differences in gB localization suggested alteration in the trafficking patterns of this protein in these cells.

Phosphorylation of HCMV gB occurs only at Ser_{900}. Previous studies have demonstrated that phosphorylation within acidic cluster motifs is an important determinant for protein sorting to intracellular compartments. Recently, the CKII consensus sequence sequence D-S^{p}_{900}-D-E-E-E-N in the HCMV gB carboxy terminal tail was shown to be

phosphorylated *in vitro* and *in vivo* (175, 268). However, other potential phosphorylation sites within gB were not examined in this study. To address this issue, a gB/GST fusion protein was constructed in which the entire 135 aa gB tail was fused to GST (GST-gB_{wt}) in order to determine the ability of CKII to phosphorylate Ser_{900} in the context of the entire gB cytoplasmic tail. In addition, the point mutation Ser_{900} to Ala_{900} (GST-gB_{ala}) was constructed as a fusion protein and used to determine if CKII phosphorylation of Ser_{900} was specific for this site in an *in vitro* phosphorylation experiment. As demonstrated in Figure 2A, GST gB_{wt} was an efficient substrate for CKII while replacement of Ser_{900} with Ala abolished phosphorylation. To assure stable expression of both GST-gB_{wt} and GST-gB_{ala}, expression was analyzed on denaturing SDS-PAGE gels using Coomassie briliant blue staining. Figure 2B shows that both GST-gB chimeras are stably expressed in similar quantities. In addition, neither phosphorous alone nor CKI and ^{32}P were able to phosphorylate the gB tail; therefore, Ser_{900} phosphorylation was specific. Thus, the above point mutant experiments demonstrate that Ser_{900} is the only amino acid in the gB tail that is phosphorylated by CKII *in vitro*.

To determine whether gB is phosphorylated *in vivo*, ^{32}P-labeled HF and U373 cells were infected with HCMV. Immunoprecipitation of gB from HCMV-infected HF and U373 cell lysates indicated that the protein is

naturally phosphorylated *in vivo* in both cell types (Figure 3A). These results suggest that the cell-specific differences in the presence of gB at the PM of HF and U373 cells is not due to the inability of CKII to phosphorylate the protein in either cell type. To determine whether gB Ser_{900} is the only residue phosphorylated *in vivo*, U373 and HF cells were infected by recombinant vaccinia viruses expressing either WT (RVV gB_{WT}) or gB containing Ser_{900} replaced with an Ala residue (RVV gB_{Ala}). While infection of U373 and HF cells with RVV gB_{WT} resulted in phosphorylation of gB in both cell types, mutation of the Ser_{900} residue abrogated phosphorylation of the glycoprotein (Figure 3B). Therefore, Ser_{900} is the only amino acid in gB that is phosphorylated in both U373 and HF cells.

A charge at Ser_{900} results in cell surface expression of gB in U373 cells. The recombinant vaccinia viruses RVV gB_{wt}, RVV gB_{ala} and a virus that expresses gB with a point mutation which replaces Ser_{900} with an aspartate residue (RVV gB_{asp}) were utilized to determine if the state of gB phosphorylation affects intracellular routing. The point mutants gB_{ala} and gB_{asp} were generated to mimic the non-phosphorylated and phosphorylated states of gB Ser_{900}. Western blot analysis of RVV gB_{wt}-, gB_{ala}-, or gB_{asp}-infected HF cells revealed similar levels of gB production (data not shown). In addition, the localization of gB was evaluated by

confocal microscopy in HF and U373 cells infected with RVV gB_{wt}, gB_{ala}, or gB_{asp}. gB was detected at the cell surface of HF cells infected with RVV gB_{wt} (Figure 4A), but not at the surface of U373 cells (Figure 4D). Figure 4A and 4D show that gB expressed by RVV gB_{wt} retains the differential expression pattern observed with gB expression in both HCMV-infected HF and U373 cells, respectively. Infection of HF cells or U373 cells with RVV gB_{ala} resulted in a similar cellular distribution of gB as infection with RVV gB_{wt} (compare Figure 4A with 4B, and 4D with 4E, respectively). In contrast, when U373 cells were infected with RVV gB_{asp}, a substantial amount of gB was detected at the cell surface of U373 cells (Figure 4F). In addition, RVV gB_{asp} infection of HF cells resulted in increased expression of gB at the PM compared to gB_{wt} (compare Figure 4A with 4C).

To analyze the overall expression of gB at the cell surface of U373 cell cultures, surface biotinylation of RVV gB_{wt}, gB_{ala}, or gB_{asp} infected cell monolayers was performed. Specifically, U373 cells infected with RVV gB_{wt}, gB_{ala}, or gB_{asp} were pulsed labeled for 12 hours with ^{35}S-methionine and ^{35}S -cysteine at 2 dpi. After removal of the label, cells were incubated with NHS-SS-biotin at 4°C for 30 minutes. Surface biotinylated proteins were immunoprecipitated from culture extracts using immobilized avidin. After biotin-avidin complexes were disrupted by boiling, immobilized avidin

was cleared by centrifugation. GB was then immunoprecipitated from the sample supernatants using a monoclonal antibody and analyzed by SDS-PAGE. Figure 5 demonstrates that substantially more gB_{asp} than gB_{ala} is detected at the surface of U373 cells.

These results suggest that phosphorylation of Ser_{900} plays a key role in the trafficking of gB, since replacing Ser_{900} with a charged amino acid (Asp) allows surface expression in both U373 and HF cells. One possible explanation for these observations is that the delivery of gB to the PM depends on the phosphorylation state. Alternatively, the state of gB phosphorylation may regulate internalization or recycling at the PM.

Tautomycin treatment of U373 cells results in gB_{wt} cell-surface expression. The altered trafficking of gB in U373 cells infected with gB_{asp} suggests that the phosphorylation state of Ser_{900} may play an important role in gB trafficking. However, experiments above indicate that gB is equally phosphorylated in both HF and U373 cells. The phosphorylation of gB in these cells may also be influenced by the presence of differential phosphatase activities that regulate the phosphorylation state of gB. To determine whether phosphatases regulate gB cell surface expression, U373 cells infected with RVV gB_{wt} or RVV gB_{ala} were treated with either the phosphatase inhibitor okadaic acid (100 nM; inhibitor of the protein phosphatase 2A) or tautomycin (100 nM; inhibitor of the protein

phosphatases 1/2A). While addition of okadaic acid to RVV gB_{wt}- and RVV gB_{ala}-infected U373 cells had no effect on gB localization (data not shown), addition of tautomycin to RVV gB_{wt}-infected U373 cells resulted in gB accumulation at the cell surface (Figure 4G), whereas an accumulation of gB was not detected in RVV gB_{ala}-infected U373 cells (Figure 4H). These observations suggest that the lack of cell-surface expression of gB in U373 cells compared to HF cells is due to a specific phosphatase activity.

Trafficking of gB to the PM is not dependent on the state of gB phosphorylation. To determine if gB without a charged residue at position 900 trafficks to the PM, U373 cells were coinfected with the recombinant vaccinia viruses described above in addition to a vaccinia construct that expresses a dynamin dominant-negative mutant (RVV dyn_{K44A}). The dynamin mutant prevents dynamin-mediated transport of surface molecules back to the cytoplasm by blocking clathrin-dependent endocytosis (51). Coinfection of U373 cells with RVV dyn_{K44A} and RVV gB_{wt} (Figure 4I), gB_{asp} (Figure 4J), or gB_{ala} (Figure 4K) resulted in the accumulation of gB at the PM in U373 cells. Surface gB expression was not observed in RVV gB_{wt}-infected U373 cells coinfected with vaccinia virus expressing wild-type dynamin (data not shown); therefore, expression of gB at the cell PM is not the result of vaccinia virus infection.

These data indicate that both phosphorylated and non-phosphorylated gB can traffic to the cell surface. Furthermore, these observations indicate that the internalization of gB from the PM occurs via a clathrin-dependent pathway. These experiments support the hypothesis that gB trafficks to the PM in a charge-independent manner and is then internalized from the cell surface to an intracellular compartment at a cell-specific rate.

gB_{asp} remains near the PM upon internalization. Antibody uptake studies were performed to determine if gB that is internalized at the PM accumulates into cytoplasmic vacuoles. U373 cells were infected with RVV gB_{wt} for 6 hours followed by a 30 minute exposure of live cells to a monoclonal antibody specific for the N-terminus of gB. After rinsing, the cells were incubated for 30 minutes at 37° C, fixed, prepared for immunofluorescence analysis and examined for antibody internalization by confocal microscopy. Expression of WT gB resulted in the accumulation of the glycoprotein in cytoplasmic vacuoles (Figure 6A). As a control antibody to the HCMV tegument protein pp65 was added in parallel experiments and did not stain cells (data not shown). Interestingly, when cells were infected with RVV gB_{asp}, gB was internalized but remained near the PM (Figure 6B). Thus, in U373 cells, gB_{wt} is rapidly endocytosed upon reaching the cell surface, sorted upon endocytosis, and concentrated on

the surface of vacuoles. In contrast, gB_{asp} internalizes but remains in small vacuoles near the PM.

Discussion

Here we demonstrate that phosphorylation of HCMV gB in HF and U373 cells occurs *in vivo* only at the CKII site in the cytoplasmic domain, and that the phosphorylation state of the gB carboxy terminal tail is one of the important determinants for intracellular trafficking. We also show that gB PM expression occurs in a cell-specific manner. Specifically, the steady-state expression of gB at the cell surface in U373 cells is dependent on the phosphorylation state of Ser_{900} in the gB cytoplasmic tail. Cell-specific differences were shown to be associated with a tautomycin sensitive phosphatase, not by CKII activity. In addition, our results suggest that gB trafficks to the PM in a phosphorylation-independent manner. Therefore, the cell-specific difference in the steady-state expression of gB at the cell surface is the result of the state of phosphorylation of Ser_{900}, which effects either the internalization rate of gB from the PM or recycling to the PM. Finally, gB accumulates in cytoplasmic vacuoles upon leaving the PM. These observations suggest that formation of these vacuoles may be required for HCMV capsid envelopment.

The intitial finding that gB was not on the PM of HCMV infected U373 cells contrasts with previous findings of gB expression in constitutively expressing stable U373 cell lines (268, 301). The difference may be explained either by the overexpression of the glycoprotein in the cell line or the use of FACS analysis rather than immunofluorescence to detect gB. In any event our data is in agreement that gB is present on the U373 PM but the steady-state amount varies greatly depending on the cell type due to the presence of a charged residue at Ser_{900}.

The processes involved in HCMV assembly and egress are controversial and are considered to use mechanisms similar to other herpes viruses (167). The herpesvirus model suggests that nucleocapsids assembled in the nucleus acquire a temporary envelope by budding through the nuclear membrane, followed by de-envelopment at the outer nuclear membrane (19, 193). Transport across the nuclear membrane is hypothesized to be mediated by gB and gH localization to the nuclear membrane. While the latter event may be correct, experiments have not been reported that differentiate gB localization at the nuclear membrane from the rough endoplasmic reticulum (RER), which are in close proximity. This issue may be resolved using double-labeling experiments with viral envelope antibodies in combination with antibodies to RER or nuclear membrane markers. The final HCMV envelope is proposed to be acquired

in the TGN since this step is sensitive to brefeldin A treatment (67). This latter step is logical since several groups of viruses acquire their envelope glycoproteins in the secretory pathway during assembly (214, 255).

The cytoplasmic tails of a number of viral glycoproteins that enter the secretory pathway have been shown to contain selective trafficking signals, which direct proteins to different cellular compartments (62, 140, 147, 209, 222, 302). Surface expression of viral glycoproteins is determined by the cellular steady-state expression of the protein, transport to the PM, and rates of internalization of protein from the cell surface. Internalization occurs through both clatherin-dependent and -independent pathways. The C-terminal domains of several membrane proteins contain amino acid motifs, which constitute internalization signals. These proteins include furin, low-density lipoprotein receptor, transferrin receptor, polymeric Ig receptor, epidermal growth factor receptor, Varicella-Zoster virus (VZV) Fc receptor gE and the SIV transmembrane protein gp41 (21, 62, 117, 140, 147, 168, 209, 222, 280, 302). Comparison of the sequences of these proteins indicates that a common structure rather than sequence is necessary for internalization. The VZV gE envelope glycoprotein contains two TGN targeting sequences in the cytoplasmic domain, which include an AYRV motif and acidic amino acid cluster (302). The presence of either of these sequences is sufficient to cause

internalization of protein from the PM and targeting to the TGN. The Tyr-dependent motif in the cytoplasmic tail of the simian immunodeficiency virus gp41 transmembrane protein is another example of an internalization signal that regulates glycoprotein expression at the cell surface (209). The signals for internalization of HCMV gB are unknown.

Protein localization to subcellular compartments may also be influenced by secondary modifications that occur in a cell-type specific manner. For example, the glycoproteins produced by Sindbis virus are modified in the secretory pathway of both vertebrate and insect cells, but have cell-specific trafficking patterns, which affect the subcellular location of virus assembly (212). Thus, in vertebrate cells, viral assembly and budding occurs at the PM. In contrast, in insect cells, virus buds into intracellular vacuoles, which fuse with the PM and release virus into the extracellular fluid. The Sindbis virus glycoproteins are transiently phosphorylated; inhibitors of phosphorylation prevent production of infectious virus (144). These observations suggest that the phosphorylation state of Sindbis glycoproteins may determine either glycoprotein trafficking or viral assembly. Similarly, HCMV also demonstrates cell-specificity for virus release, and the phosporylation of gB may determine this event.

In summary, understanding the mechanisms involved in gB trafficking may be important in determining mechanisms of viral envelopment and intracellular sequestration. Future work will determine the importance of gB expression on the PM in these processes.

Figure Legends

Figure 1. Confocal images of gB staining in HCMV-infected cells. HF cells (Panel A), U373 cells (Panel B) and monocyte derived macrophages (Panel C) were infected with HCMV as described in Materials and Methods. HF cells were fixed at 3 dpi, U373 cells were fixed at 7 dpi, and MDM were fixed at 14 dpi. Cells were permeabilized and stained with a monoclonal mouse anti-gB antibody. Magnifications = 303X.

Figure 2. *In vitro* CKII phosphorylation of a GST-gB C-terminal tail chimeric protein. A GST-gB_{wt} or gB_{ala} C-terminal tail chimera was attached to microbeads and treated with either CK II or CK I and ^{32}P orthophosphate followed by magnetic bead purification and PAGE analysis. The gB_{wt} but not gB_{Ala} was phosphorylated by CKII (Panel A). However, neither of the chimeric proteins were phosphorylated by CKI. Both GST-gB_{wt} and GST-gB_{ala}, were stably expressed as determined by analysis on denaturing SDS-PAGE gels (Panel B).

Figure 3. gB is phosphorylated in HF and U373 cells *in vivo*. HCMV (Panel A), RVV gB_{wt} (Panel B), or RVV gB_{Ala} (Panel B) infected HF and U373 cells were labeled with inorganic ^{32}P followed by immunoprecipitation with gB specific rabbit antisera and a preimmune

control sera. *In vivo* phosphorylated gB is detected in HCMV and RVV gB_{WT}, but not RVV gB_{Ala} infected HF and U373 cells.

Figure 4. Subcellular localization of gB in HF and U373 cells. Confocal images of gB staining were obtained in cells infected with RVV gB_{wt}, RVV gB_{Ala}, or RVV gB_{asp}. Panels A, B, and C represent HF infected cells and panels D, E, and F represent U373 infected cells. Cells were stained before permeabilization or at 4°C (representing surface gB) with mouse anti-gB (green) and post-permeabilization (representing total gB) with mouse anti-gB (red). Panels A and D represent RVV gB_{wt} infections, panels B and E represent RVV gB_{ala} mutant infections, and panels C and F represent RVV gB_{asp} mutant infections. Surface gB staining will be a combination of pre-permeabilization or 4°C and post-permeabilization (yellow), which is observed in panels A, B, C, and F. Panels G and H are confocal microscopy images demonstrating the presence of gB in vaccinia virus infected cells. U373 cells were infected with either RVV gB_{wt} (panel G) or RVV gB_{ala} (panel H) and subsequently treated with the phosphatase inhibitor tautomycin. Cells were stained with mouse antibody to gB pre-permeabilization or at 4°C(green) (surface gB) and mouse antibody to gB post-permeabilization (red) (total gB). The accumulation of gB trafficking to the surface was only observed in the

RVV gB_{wt} infection (yellow panel G). To demonstrate that transport of gB to the cell surface is not affected by the state of phosphorylation, we coinfected U373 cells with RVV gB_{wt} (panel I), gB_{asp} (panel J), or gB_{ala} (panel K) with RVV dyn_{K44A}. Magnifications for panels A-F and I-K = 303X; for panels G-H = 473X.

Figure 5. Steady-state cell surface expression of gB_{asp} is greater than gB_{wt} and gB_{ala} on the plasma membrane of U373 cells. U373 cells were infected with RVV gB_{wt}, gB_{ala}, or gB_{asp} followed by surface biotinylation to analyze differences in gB PM expression. Specifically, ^{35}S-labeled U373 cells infected with RVV gB_{wt}, gB_{ala} or gB_{asp} were pulsed with NHS-SS-biotin, followed by analysis of immunoprecipitated surface gB by SDS-PAGE. This figure demonstrates that substantially more gB_{asp} than gB_{ala} is detected at the surface of U373 cells. These results support the hypothesis that a charge at position 900 in the gB cytoplasmic tail increases surface expression in U373 cells.

Figure 6. Endocytosis and intracellular targeting of gB in U373 cells. gB antibody uptake experiments were performed in RVV gB_{wt} (panel A) or RVV gB_{asp} (panel B) infected U373 cells. At 6 hours post infection, mouse anti-gB N-terminus antibody was applied to cells for 30 minutes.

Cells were then rinsed and incubated for a 30 minute chase period followed by fixation. Non-permeabilized cells were stained with a cyanine-5 anti mouse secondary conjugate (blue; stable surface gB), followed by rinsing, permeabilization and staining with a TRITC anti mouse secondary conjugate (red; internalized gB). Cells were rinsed again and exposed to rabbit anti gB C-terminus followed by a FITC anti-rabbit secondary conjugate (green; total gB). Therefore, internalized gB is both green and red (yellow vacuoles), PM gB is blue, red, and green (white PM staining) and gB that was absent from the PM during the 30 minute mouse anti-gB N-terminus antibody exposure is green. Magnifications = 473X.

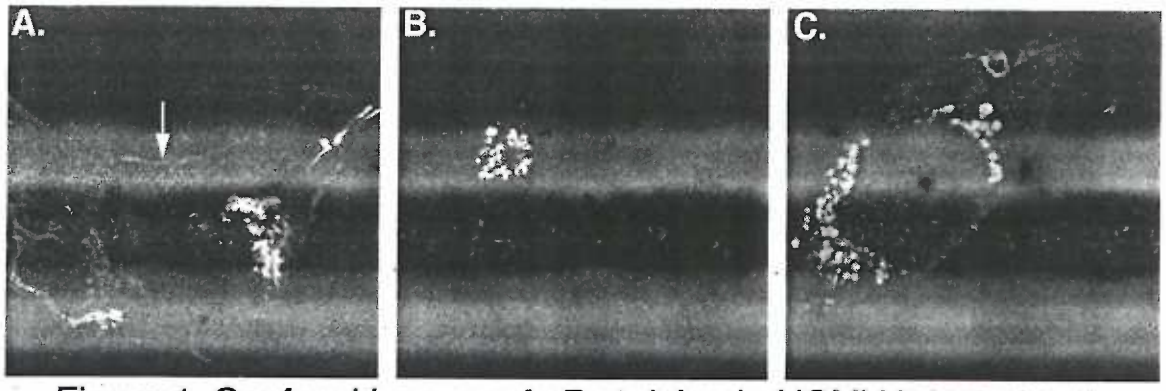


Figure 1. Confocal images of gB staining in HCMV-infected cells.

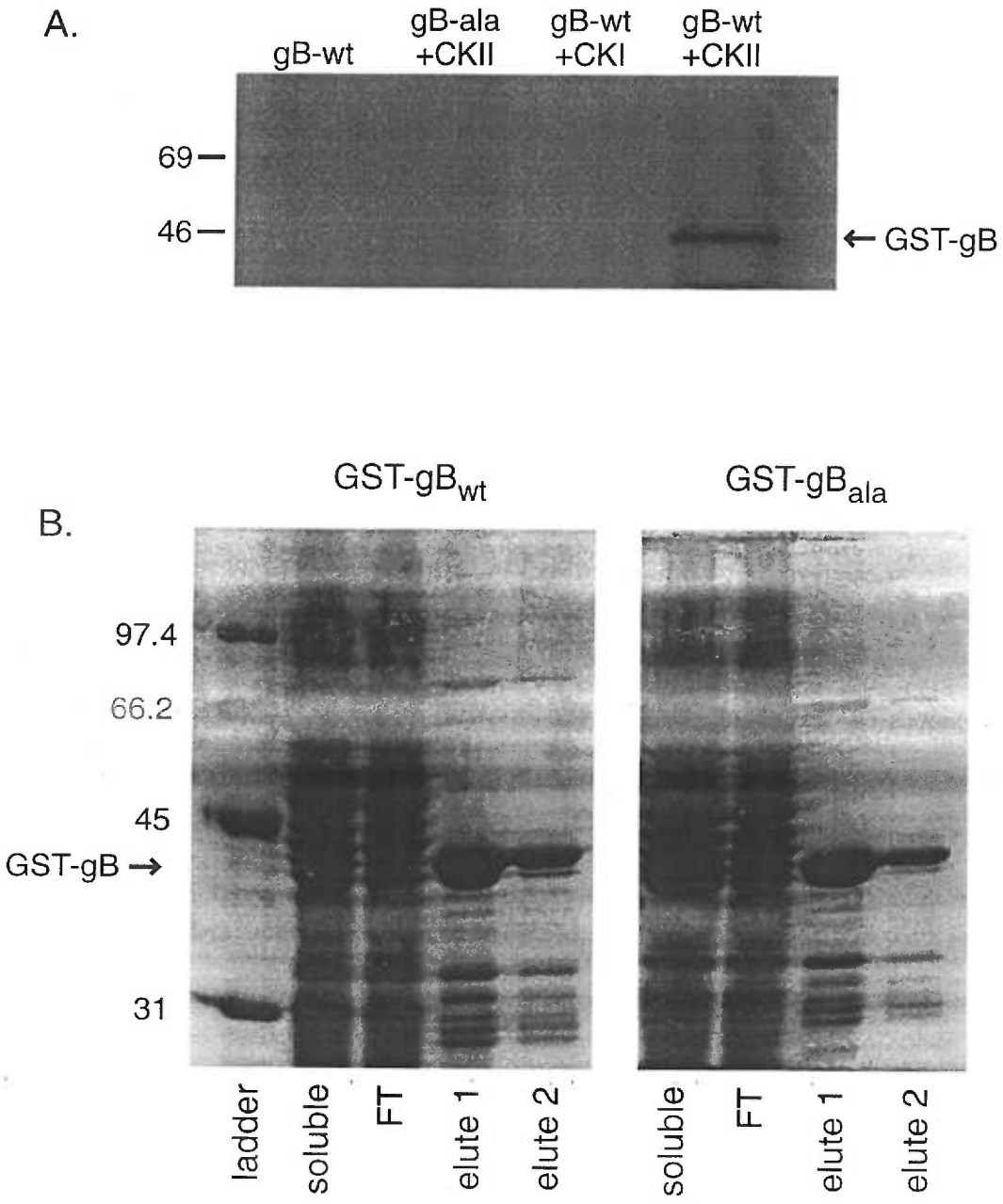


Figure 2. In vitro CKII phosphorylation of a GST-gB C-terminal tail chimeric protein.

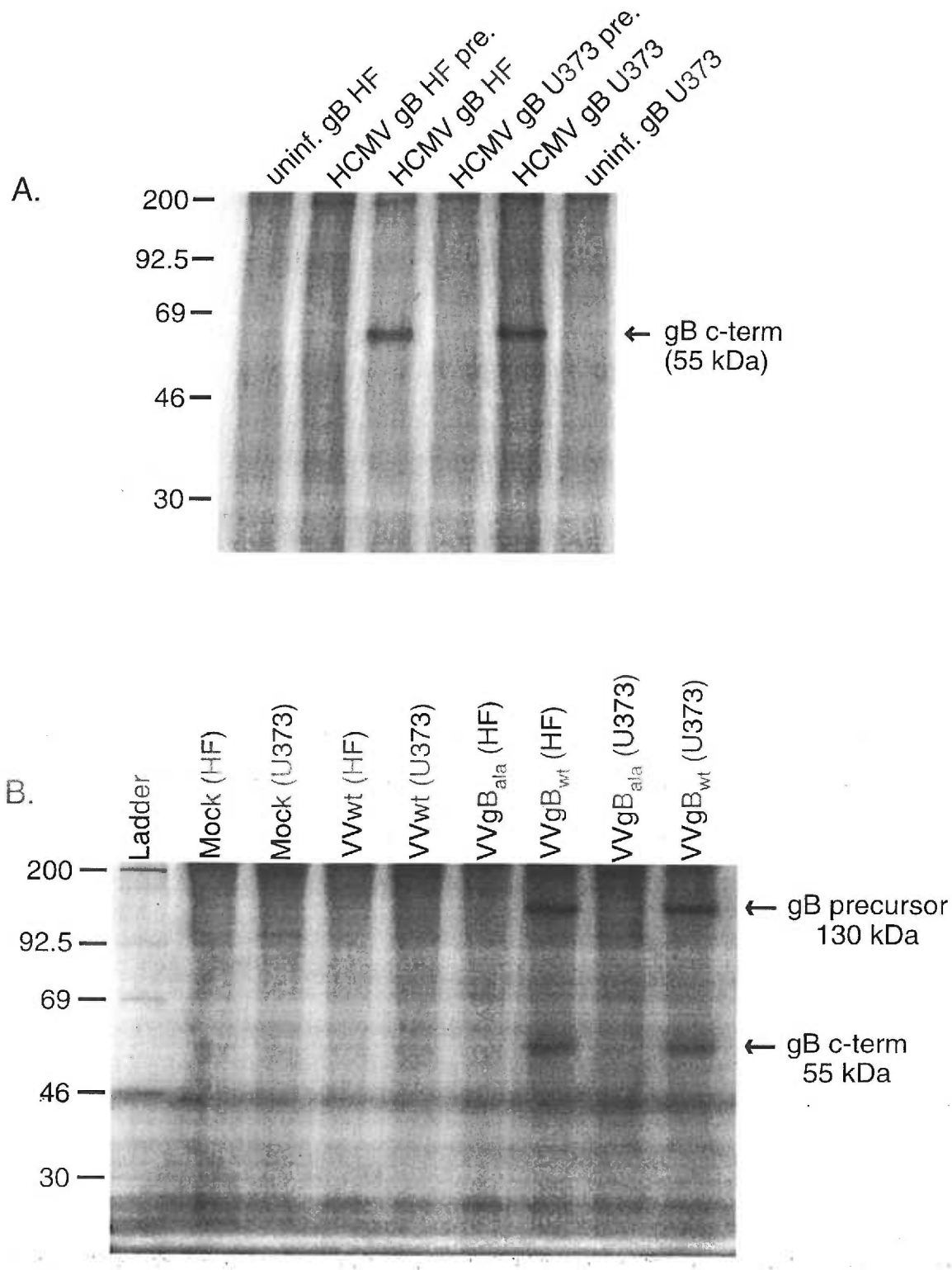


Figure 3. gB is phosphorylated in HF and U373 cells in vivo.

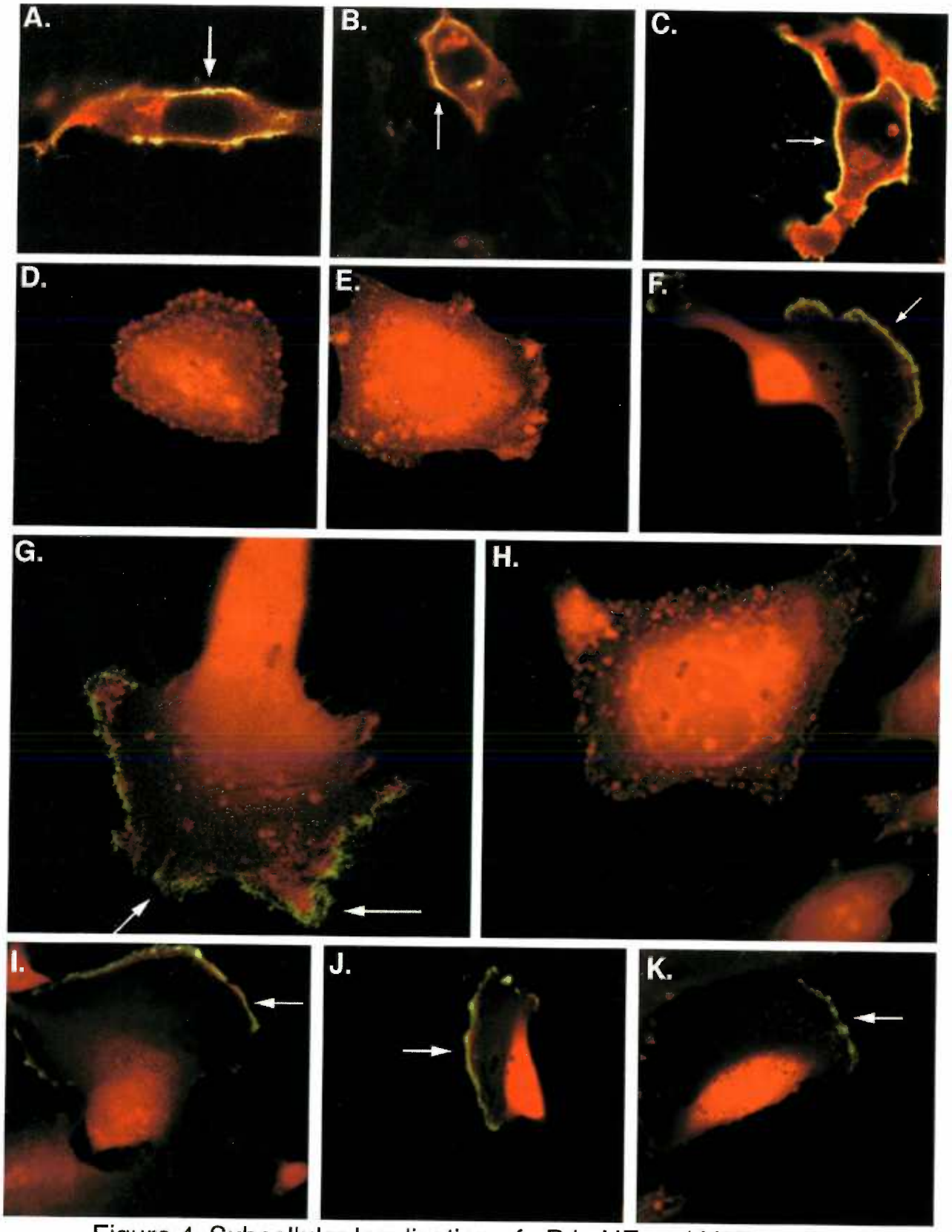


Figure 4. Subcellular localization of gB in HF and U373 cells.

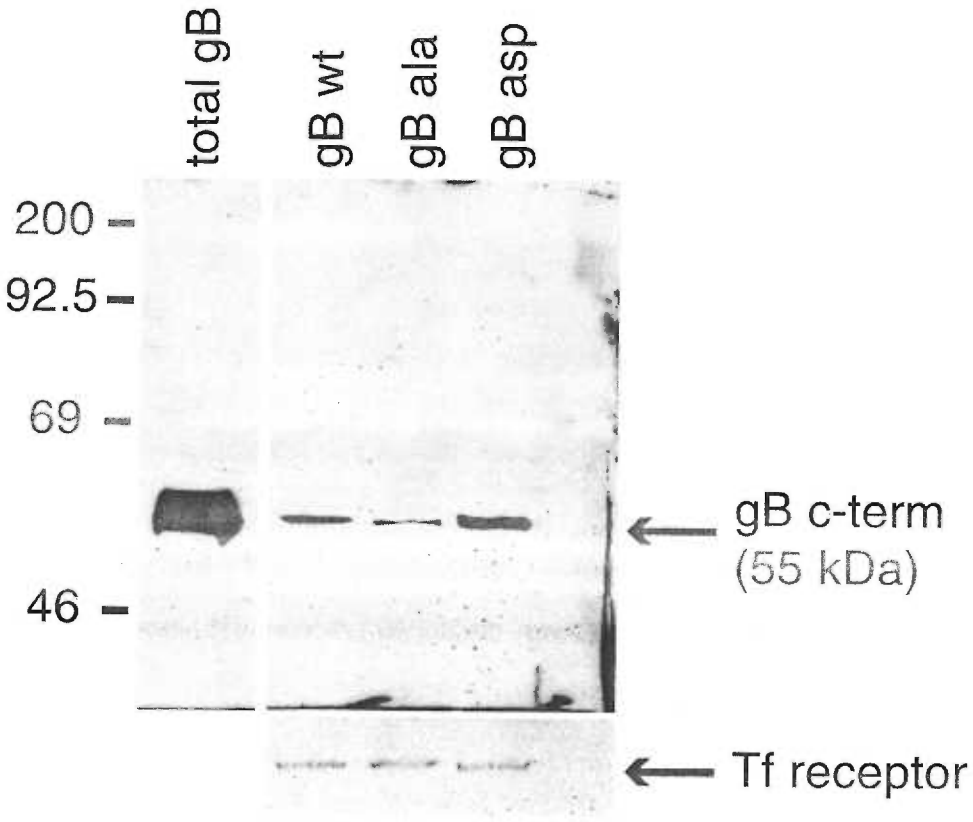


Figure 5. Steady state cell surface expression of gB_{asp} is greater than gB_{wt} and gB_{ala} at the PM of U373 cells.

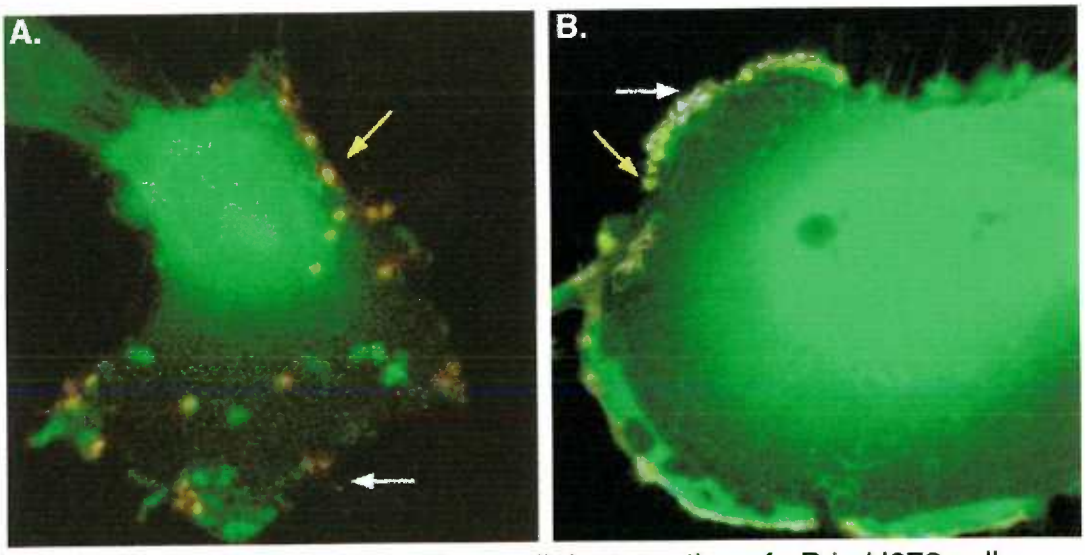


Figure 6. Endocytosis and intracellular targeting of gB in U373 cells.

Chapter 6

Clathrin-dependent endocytosis is not required for envelopment of HCMV.

by Kenneth N. Fish, Yoram Altshuler, Cecilia Soderberg-Naucler, Dan Streblow, and Jay A. Nelson

A manuscript

pages 125-152 of this Thesis

All experiments presented in this chapter were performed by Kenneth N. Fish except for figure 2C and 2D, which were performed by Cecilia Soderberg-Naucler.

Abstract

The HCMV glycoprotein gB is one of the major receptor-binding proteins of the virus and is proposed to be the most abundant glycoprotein in the virion envelope. Although the steps involved in envelopment of the HCMV nucleocapsid are not clear, concentration of viral glycoproteins in specific cellular compartments is believed to play an important role in viral envelopment. Recently, gB PM accumulation was shown to occur in a cell-specific manner. The cell-specific difference in the steady-state expression of gB at the PM was shown to be dependent on the state of phosphorylation of an acidic cluster sorting signal in the gB cytoplasmic tail. How phosphorylation regulates the expression of gB at the PM is not understood. However, since both phosphorylated and dephosphorylated gB accumulate at the PM in the presence of a clathrin-dependent endocytosis block, cell-specific differences in gB PM expression are not due to the inability of the protein to traffic to the PM. gB phosphorylation may affect the intracellular trafficking of PM gB. Therefore, we examined mechanisms of gB PM internalization and the role of PM and intracellular gB in the viral envelopment process in this study. Examination of PM and cytoplasmic fractions from infected cells indicated that the majority of infectious virus was associated with intracellular vacuoles that contained gB on their surface. In correlation with these observations, mature HCMV

virions were observed in gB coated cytoplasmic vacuoles by immuno-EM. The targeting of PM gB to these vacuoles was analyzed by antibody uptake experiments, which demonstrated that upon endocytosis, PM gB is targeted to vacuoles that had glycoproteins on their surface. Further analysis using a dominant negative dynamin mutant, which blocks clathrin-dependent endocytosis, determined that recovery of PM gB was not necessary for efficient virus envelopment. Our results suggest that HCMV uses the cellular trafficking machinery to concentrate essential virion proteins in the vicinity of the Golgi via a PM independent pathway, creating a subcellular location for virion assembly.

Introduction

Viral glycoproteins, which mediate a number of essential functions in the virus life cycle (110), have become useful tools for the scientific community to use in exploring mechanisms involved in protein trafficking through the cellular secretory pathway (66, 69, 111). For the successful formation of infectious virus, viral glycoprotein maturation must occur, which includes proper folding and post-translational modification during sequential transport through the cellular secretory pathway. Once modified, viral glycoproteins utilize the cellular trafficking machinery to concentrate collectively at a subcellular location where capsid envelopment occurs. Some enveloped viruses such as retroviruses assemble and bud at the PM, while others (e.g. coronaviruses) bud into intracytoplasmic vacuoles that fuse with the PM releasing infectious virions (179, 255). Pathways used by viruses to target their glycoproteins to viral assembly compartments have been difficult to identify.

Although biochemical, immunological, and ultrastructural analyses have been used to study the egress pathway of herpesviruses, the mechanisms involved in virion egress (the exit of the capsid from the nucleus and its envelopment) are controversial. Early experiments performed to examine mechanisms of herpesvirus assembly and egress were performed using HSV-1. The majority of results from experiments

performed to identify the egress pathway fall in to one of two proposed models. One model suggests that HSV-1 capsids acquire their envelope by budding through the inner nuclear membrane, which contains immature glycoproteins (116). Once in the space between the inner and outer nuclear envelopes, the enveloped capsids are transported to the Golgi, via the lumen of the endoplasmic reticulum and the cisternal space, where their glycoproteins are modified. Finally, mature virions are transported to the cell surface in cytoplasmic vacuoles, which fuse with the PM, releasing virus into the extracellular space. To support their hypothesis, Johnson and Spear found that in HSV-infected cells treated with monensin, the virus did not reach the cell surface but accumulated in intracytoplasmic vacuoles (116). Since monensin blocks vesicular transport from the Golgi to the PM, the authors suggested that egress of HSV occurs via the Golgi apparatus.

The other egress model, known as the de-envelopment–re-envelopment model, suggests that the nucleocapsids bud through the inner nuclear membrane, acquiring an envelope, followed by de-envelopment as the enveloped capsid fuses with the outer nuclear membrane, releasing naked capsids into the cytosol (35, 78, 194, 246, 291). These experiments also propose that final envelopement occurs in the Golgi area (medial-Golgi, TGN, and endosomes), where terminally

modified glycoproteins have accumulated, since this step is sensitive to treatment of cells with brefeldin A (67). An additional recent study by Browne et al., which targeted HSV gH to the ER or to the medial-Golgi by adding specific trafficking signals to the glycoprotein's cytoplasmic tail, supports this hypothesis; whereas gH with ER retention motifs resulted in the secretion of noninfectious gH-less virions, the Golgi localization signal resulted in the production of infectious virus with normal gH content (30). The results of Browne et al. suggest that the HSV-1 envelope is acquired in the trans-Golgi network (TGN).

Although the steps involved in capsid envelopment are disputed, there is a general consensus that attachment of membrane-bound viral glycoproteins to tegumented capsid plays an important role in virus assembly and egress. In both egress models, transport across the nuclear membrane is hypothesized to be mediated by gB and gH localization to the nuclear membrane. This hypothesis is based on the presence of glycoproteins associated with the perinuclear-enveloped HSV virions. In addition to other similarities between the egress models, neither model takes into account that different herpesvirus family members may utilize alternative pathways for assembly and egress.

The cytoplasmic tails of a number of herpesvirus proteins (302) that enter the secretory pathway contain selective sorting signals, which direct

these proteins to different subcellular compartments. The varicella-zoster virus (VZV) gE protein is a prime example of a viral glycoprotein that contains specific trafficking signals in its cytoplasmic tail. Specifically, internalization of the gE protein from the PM requires a YAGL motif located in the protein's cytoplasmic domain (177). In addition, the cytosolic domain of gE contains an AYRV sequence and an acidic amino acid patch sequence; these sequences are both able to direct the protein to the TGN (6, 302). Another example, the cytoplasmic tail of the pseudorabies virus (PRV) gE protein, was recently shown to contain trafficking signals that are required for the incorporation of gE into virion envelopes (265).

Human cytomegalovirus (HCMV), a member of the herpesvirus family (subfamily betaherpesvirinae), demonstrates cell-specificity for virus assembly and release (105). Although HCMV is considered to use similar mechanisms described for other herpes viruses for its viral infectious cycle, betaherpesvirus replication kinetics are severely delayed when compared to alphaherpesvirus replication. The most abundant glycoprotein detected in the HCMV virion envelope is gB (UL55) (27). Although HCMV gB has not been successfully deleted from the virus, probably because gB-expressing permissive cells are lacking, the correlate protein in HSV is essential for entry and fusion (206). HCMV gB

is synthesized as a 105 kDa polypeptide precursor, which upon being glycosylated in the ER, is transported to the Golgi where the protein is further modified, resulting in a 130 kDa protein. Subsequently, gB is cleaved by furin in the TGN into two products, gp55 and gp116; these products remain associated via disulfide bonds formed in the ER (28, 90, 244). gB is a type-I glycoprotein in which the gp116 exists only as a surface component and the gp55 contains a transmembrane (TM) domain, a very long cytoplasmic tail (135 aa), and a surface domain (26, 90, 185). In addition, the gB cytoplasmic tail contains a consensus CKII site, which is phosphorylated *in vitro* and *in vivo* (175, 268).

Recently, we have demonstrated phosphorylation of the gB CKII site *in vivo* and found that the state of gB phosphorylation is one of the important determinants for intracellular cell-specific trafficking (74). We compared the steady-state expression of gB in U373 cells to that of HF cells and found that the steady-state expression of gB at the cell surface in U373 cells depends on the phosphorylation state of Ser_{900} in the gB cytoplasmic tail. In addition, we demonstrated that the lack of cell surface gB expression in MDM and U373 cells correlated with the inability of these cells to release virus. In contrast, gB is easily detected at the PM in HF cells infected with HCMV, and virus is release into the culture suppernatant. Therefore, the differential trafficking of the HCMV viral

glycoproteins may regulate intracellular sites of viral assembly and release.

Previously, utilizing double-label immunofluorescence (IF) with antibodies to viral and cellular proteins, we showed that cytoplasmic vacuoles, which contained HCMV gB on their surface, were associated with the Golgi marker mannosidase II but not with markers to early endosomes (transferrin receptor and rab5) or late endosomes and early lysosomes (Lamp 1 and 2) (71). These vacuoles were found to be associated with the Golgi at early times post infection and to be dispersed throughout the cell cytosol at late times post infection. In the present study, we reinvestigated the origin of these vacuoles and the role they play in virus envelopment. Specifically, here we present a hypothesis on the mechanisms involved in the formation of these gB cytoplasmic vacuoles, and we show that these vacuoles contain the majority of the intracellular infectious viruses. Therefore, understanding the cellular pathways that HCMV exploits in the targeting of gB to intracellular viral assembly compartments may help elucidate the mechanisms by which intracellular viral sequestration occurs.

Results

Intracellular gB cytoplasmic vacuoles contain infectious HCMV. Previous studies have demonstrated that HCMV infection of U373 cells results in the production of intracellular but not extracellular virus. Examination of infected cells for the production of late viral proteins indicated that HCMV antigens were localized to vacuolar compartments. These observations suggested that infectious virus was sequestered in specific vacuoles, which may be sites of viral assembly. To examine potential sites of HCMV envelopment, virus infected U373 cells were analyzed by immunofluorescence and immuno-EM. Figure 2A shows an immunofluorescent staining of gB on the surface of intracellular vacuoles adjacent to the Golgi complex at 3 days post HCMV infection. At late times post infection and at greater magnification a punctate gB staining pattern is visible within these gB vacuoles (Figure 2B). Examination of these cells by immuno-EM using antibody to gB revealed the presence of mature viral particles within these vacuoles (Figure 2C). Viral particles were also observed budding into cytoplasmic vacuoles (Figure 2D). These results suggest that the HCMV final envelope is acquired from gB vacuoles.

To demonstrate that vacuoles staining for late viral antigens contain infectious virus, we isolated HCMV vacuoles from infected U373 cells. In

these experiments, the PM of U373 cells was disrupted by placing a serum-coated coverslip over infected cultures at 7 days post infection (dpi) followed by removal of the coverslip with bound cellular PM. Exposed free cellular organelles were collected and intracellular gB coated vacuoles were separated from other cytoplasmic contents and free intracellular virus by purification over Mini MACS columns. Subsequently, all fractions were assessed for the presence of infectious virus by plaque assay. Greater than 90% of total intracellular infectious HCMV was associated with gB coated vacuoles (Figure 3). These results in combination with the immuno-EM observations, indicate that the site of envelopment and sequestering of intracellular virus are gB encoated vacuoles.

HCMV PM gB recycles into intracellular compartments. Previous studies have suggested that HCMV gB at the PM is sorted, internalized, and transported to viral assembly compartments (194). To examine this issue and follow the trafficking of PM gB upon internalization, U373 tTA cells were coinfected with HCMV and an adenovirus that expressed a temperature-sensitive dominant negative dynamin mutant (Adeno dyn_{G273D}). The dynamin mutant prevents dynamin-mediated transport of surface molecules back to the cytoplasm by blocking clathrin-mediated endocytosis (51). Figure 4 demonstrates that PM gB internalizes and colocalizes with intracellular gB on the surface of

cytoplasmic vacuoles, which appear to be juxtaposed the Golgi (Figure 4; yellow staining). These experiments support the hypothesis that HCMV gB trafficks to the PM, where it is sorted, internalized, and targeted to viral assembly compartments.

The state of Ser_{900} phosphorylation determines recycling of gB to the PM after internalization. HCMV gB was recently shown to be phosphorylated both *in vitro* and *in vivo* at Ser_{900}, which is part of a consensus CKII site in the cytoplasmic tail (18, 74, 175). Replacement of Ser_{900} in gB with an aspartate (gB_{asp}) or an alanine (gB_{ala}) residue to mimic the phosphorylated and nonphosphorylated state, respectively, demonstrated that phosphorylation only occurred at Ser_{900} and that the steady-state surface expression of gB was dependent on the state of gB phosphorylation (74). However, gB trafficking from the TGN to the PM was shown to occur in a charge-independent manner. Therefore, to measure the internalization rate of PM gB, U373 tTA cells were coinfected with recombinant vaccinia virus (RVV) that expressed gB_{ala} or gB_{asp} (RVV gB_{ala} and RVV gB_{asp}, respectively) and Adeno dyn_{G273D}. After 18 hours post Adeno dyn_{G273D} infection at endocytosis permissive conditions (30°C), cultures were infected with RVV gB_{ala} or RVV gB_{asp}. At nine hours post RVV infection, cells were pulsed labeled for one hour with ^{35}S-methionine and ^{35}S-cysteine followed by a one hour chase at the nonpermissive

endocytosis temperature (38°C). Cell cultures were subsequently incubated with NHS-SS-biotin, a reagent that is cleaved in the presence of the reducing agent MESNA, to label cell surface proteins followed by treatment with MESNA. At time 0 post internalization, all biotinylated gB was reduced by MESNA treatment (Figure 5). By 15 minutes post internalization the majority of gB_{ala} and gB_{asp} was protected from MESNA reduction. Although gB_{ala} was still protected after 30 minutes, gB_{asp} was sensitive to MESNA. The re-expression of gB_{asp}, but not gB_{ala}, at the PM suggests that phosphorylated gB is trapped in a PM/endosomal loop that rapidly returns charged gB to the PM.

HCMV envelopment is not mediated by clathrin-dependent endocytosis. To examine if glycoprotein recovery from the PM was necessary for the production of infectious virus, we utilized an adenovirus construct that expresses a dynamin dominant-negative mutant (Adeno dyn_{K44A}). This mutant prevents dynamin-mediated transport of surface molecules back to the cytoplasm by blocking clathrin-dependent endocytosis (51). U373 cells coinfected with Adeno dyn_{K44A} and HCMV results in the presence of gB at the PM and an increase in syncitia formation (compare Figure 6A to 6B). To determine the amount of gB that escaped the endocytosis block, coinfected cultures were pulsed with a gB monoclonal antibody at 2 days post HCMV infection. Figure 6C

demonstrates that gB, which trafficks to the PM, is not endocytosed during the incubation period. Culture lysates or supernatants of coinfected U373 cells were assayed for the production of cell-associated or extracellular virus by plaque assay on susceptible fibroblast monolayers (Figure 6D and 6E). Figure 6D and 6E show that virus production remained similar to controls over a 5 day period. These results suggest that the trafficking of gB to the PM is not essential for virion envelopment.

Discussion

The HCMV major glycoprotein gB is believed to be a critical component of the virion. Here, we have demonstrated that the subcellular location of gB designates the location of infectious virus in the cell. Specifically, we showed that the gB C-terminal tail is associated with the membranes of intracellular vacuoles that contain infectious virus. In addition, we demonstrated that even though gB accumulates in cytoplasmic vacuoles upon leaving the PM, the fraction of gB that trafficks to the PM is not essential for the normal production of infectious virus. Therefore, our results suggest that HCMV uses the cellular trafficking machinery to concentrate essential virion proteins in the vicinity of the Golgi, creating a subcellular location for virion assembly.

HCMV is considered to use similar mechanisms that have been described for other herpes viruses for viral envelopment. Stackpole originated the idea that capsids exit the nucleus and subsequently enter the cytoplasm by an envelopment–de-envelopment process almost 30 years ago (246). Once in the cytoplasm, the tegumented nucleocapsid interacts with membrane-bound viral glycoproteins, which results in envelopment of the capsid. Recently, this model has been supported by several envelopment studies that used HSV and other herpesviruses (30, 35, 67, 78, 194, 291). Although the basic idea that envelopment occurs in cytoplasm is supported by these studies, the identity of the subcellular compartment in which the final virion envelope is acquired is controversial. Recent findings from two groups suggest the final envelopment is acquired in the Golgi (30, 67). However, mature virions have rarely been seen in the Golgi (206). In contrast, a recent report hypothesized that envelopment occurs in endosomes that are coated with viral glycoproteins, which are targeted to the endocytic compartment from the PM (194). In support of this hypothesis, Tooze et al. showed by EM that some HCMV was found in early endocytic compartments (266). However, EM can not determine the compartment from which these cytoplasmic vacuoles are derived because of the static nature of EM.

In addition to the controversy of the origin of the herpesvirus assembly compartment, little is known about how viral envelope proteins are targeted to these compartments. Although a regulated internalization of the VZV gE and gI proteins from the PM occurs (177), which direct the protein to the TGN (6, 302), the results presented here refute the necessity of this pathway for HCMV envelopment.

Not until now has HCMV research significantly contributed to the efforts of the herpesvirus field to define the mechanism by which virus envelopment occurs. Although our results could be construed to fit either of the two herpesvirus egress models, which are both based on experiments using HSV-1, we feel that our findings strengthen the de-envelopment–re-envelopment model. Recently, we demonstrated that phosphorylation of the CKII site in the cytoplasmic domain of gB occurs *in vivo*, and gB PM steady-state expression occurs in a cell-specific manner, which is dependent on the phosphorylation state of Ser_{900} in the gB cytoplasmic tail. Cell-specific differences were shown to be associated with a tautomycin sensitive phosphatase, not by CKII activity. Here we show that the cell-specific difference in the steady-state expression of gB at the PM is the result of the state of phosphorylation of Ser_{900}, which affects the retrieval of gB to the PM. Phosphorylated gB is selectively retrieved to the PM after endocytosis. In contrast, endocytosed

dephosphorylated gB is localized to intracellular pools of gB. These results suggest that a regulated local cycling loop exists between the PM and early endocytic compartments in which phosphorylated gB is quickly returned to the PM after being endocytosed and dephosphorylated gB moves along the endocytic pathway once endocytosed. Although studies that employed dominant negative dynamin demonstrated that the regulated PM retrieval pathway is not necessity for virion envelopment, this local cycling loop may mirror that described recently for the TGN localization of furin (168). Therefore, the state of gB phosphorylation may determine the protein localization to the TGN/endosomal system.

Although the steps involved in HCMV capsid envelopment are disputed, there is a general consensus that attachment of membrane-bound viral glycoproteins to tegumented capsid plays an important role in virus assembly and egress. Therefore, the understanding of how virion envelope proteins are sorted is crucial to identifying the subcellular HCMV envelopment compartment. However, the mechanisms involved in the subcellular targeting of glycoproteins are poorly understood. Here, we have identified a potential role for HCMV gB phosphorylation and have demonstrated that HCMV uses the host cell trafficking machinery to concentrate HCMV glycoproteins on the surface of vacuoles, which are formed from the Golgi for envelopment. However, the mechanisms of

glycoprotein targeting and HCMV envelopment shown here might be cell-specific. Previous studies of HCMV permissive cells indicated that production of virus remained exclusively cell associated in U373 cells and macrophages and was nonlytic. In contrast, HCMV infection of HF cells resulted in both intracellular and extracellular localization of progeny virus, and the lyses of the cells. In addition, HCMV accumulates in large cytoplasmic vacuoles in infected MDM and U373 cells. These observations emphasize the cell-specific differences that occur during HCMV infection and suggest that viral envelopment may vary depending on the cell type studied.

Figure Legends

Figure 1. Construction of recombinant adenoviruses. All recombinant DNA steps used standard techniques. This figure depicts the general outline of the cloning strategy. Methods for production and use of virus are previosly described in detail elsewhere (97).

Figure 2. HCMV acquires its final envelope by budding into gB cytoplasmic vacuoles. To determine how HCMV is enveloped in U373 cells, HCMV gB vacuoles were analyzed at high magnification by both IF and EM. First, the area of the Golgi was analyzed by confocal microscopy using a gB monoclonal antibody (Panel A). At 630X magnification the formation of intracellular vacuoles off the Golgi can be seen at early times post infection. At later times post infection these vacuoles appear to contain viral particles (Panel B—2000X magnification). When gB vacuoles are examined by immuno-EM using a gB monoclonal antibody mature viral particles can be seen inside containing gB antigen in their envelopes (Panel C). When we examined the Golgi area at early times after infection we never saw viral particles budding into the Golgi itself, but frequently saw viral particles budding into vacuoles adjacent to the Golgi (Panel D).

Figure 3. Isolation of intracellular gB vacuoles that contain HCMV. To demonstrate that vacuoles staining for late viral antigens contain infectious virus, we isolated HCMV vacuoles from infected U373 cells. In these experiments, the PM of MDM was removed by placing a serum-coated coverslip over infected cultures at 7 days post infection (dpi). The coverslip with PM was removed the following day releasing free cellular organelles from U373 cells by a gentle rinse with media. Intracellular vacuoles that had gB associated with their membranes were separated from other cytoplasmic contents and free intracellular virus by purification over Mini MACS columns and all fractions were assessed for the presence of infectious virus by plaque assay. These results show that the vast majority of infectious HCMV was associated within gB coated vacuoles.

Figure 4. PM HCMV gB recycles to intracellular pools of gB. To determine where PM gB is targeted to upon internalization, U373 tTA cells were infected with HCMV. At two days post HCMV infection, the culture was infected with $Adeno_{G273D}$. After 2 hours post $Adeno_{G273D}$ infection, the culture temperature was shifted to 30°C (wt endocytosis kinetics) for 18 hours, which was followed by a shift in culture temperature to the nonpermissive temperature of 38°C for six hours to accumulate gB at the PM. The cultures were subsequently incubated with a gB monoclonal

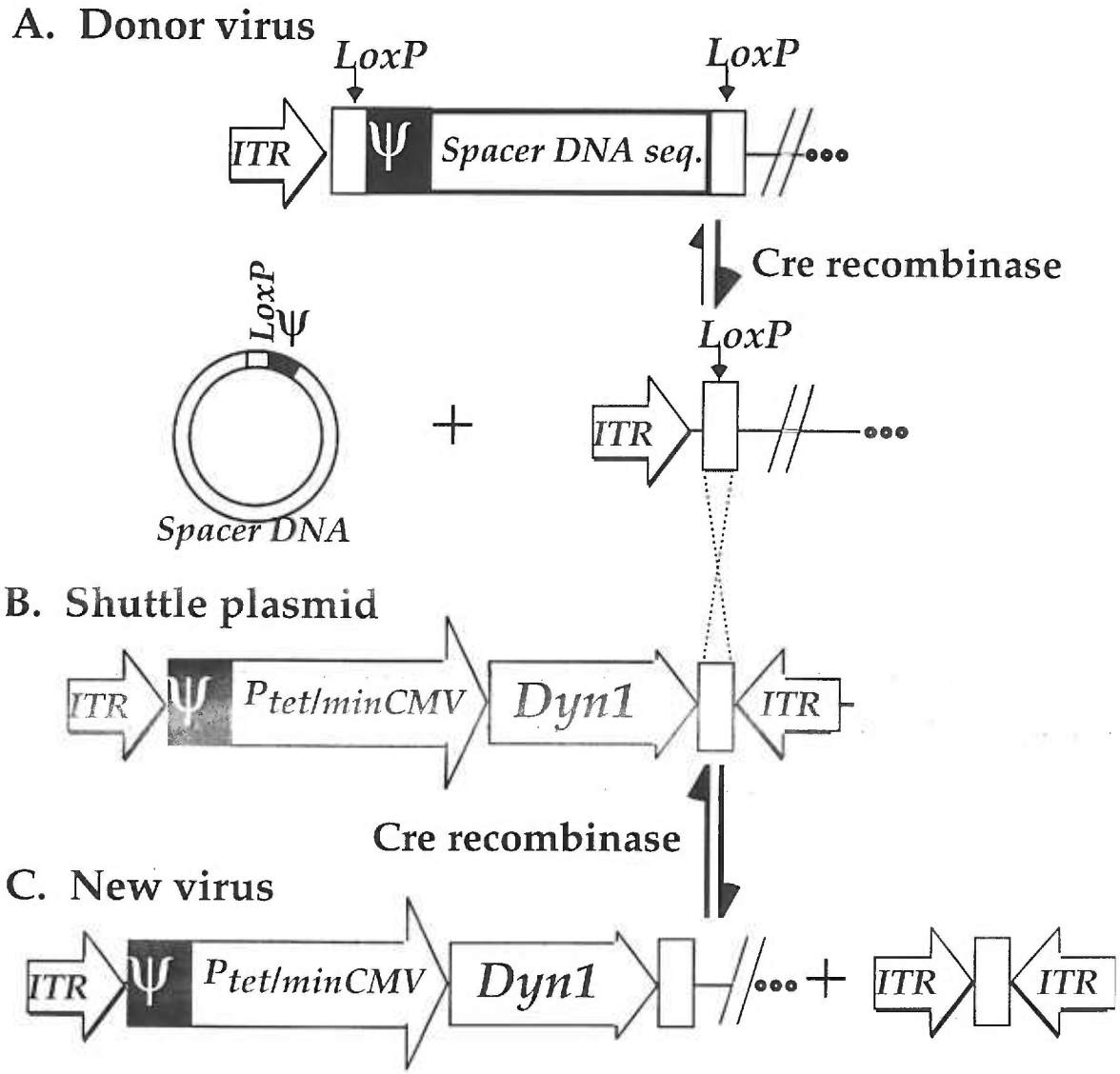
antibody at 4°C for one hour. After rinsing, the culture temperature was then shifted back to 30°C and after 45 minutes, were fixed and stained with a TRITC-secondary antibody to mouse (Red), followed by staining for the presence of total gB with a rabbit anti-gB antibody and an FITC-anti rabbit secondary antibody (Green). In addition, the nucleus was visualized using DAPI (Blue). These results demonstrates that some of the gB that reaches the cell surface is able to internalize and colocalize with intracellular gB on the surface of cytoplasmic vacuoles, which appear to be juxtaposed the Golgi. Magnification = 630X.

Figure 5. Endocytosed gB_{asp} rapidly recycles to the cell surface. To measure differences between gB mutants that internalizes from the PM, U373 tTA cells were infected with Adeno dyn_{G273D}, after which the culture temperature was changed to 30°C. At 18 hours post Adeno dyn_{G273D} infection, the cultures were infected with RVV gB_{ala} or RVV gB_{asp}. At nine hours post RVV infection the cultures were pulsed labeled for one hour with ^{35}S. After a one hour chase at 38°C, cell surface proteins were labeled by incubating the cultures with NHS-SS-biotin at 4°C. At the end of the chase, the NHS-SS-biotin was removed and time 0 was collected prior to shifting the culture temperature to 30°C for 15 or 30 minutes post internalization. Subsequently, the cell cultures were placed at 4° C and

exposed to MESNA to reduce surface NHS-SS-biotin. At time 0 post internalization, all biotinylated gB was reduced by MESNA treatment. By 15 minutes post internalization the majority of gB_{ala} and gB_{asp} was protected from MESNA reduction. After 30 minutes, gB_{ala} was still protected, but gB_{asp} became sensitive to reduction.

Figure 6. A block in clathrin-dependent endocytosis during HCMV infection does not affect virus production. To examine if glycoprotein recovery from the PM was necessary for the production of infectious virus, we coinfected U373 tTa cells with Adeno dyn_{K44A} and HCMV. Coinfection resulted in the presence of gB at the PM and an increase in syncitia formation (compare Panel A and B). To determine the amount of gB that escaped the endocytosis block, we pulsed cells with a gB monoclonal antibody at 2 days post HCMV infection and followed uptake over the remainder of the 5 day experimental time course. Panel C demonstrates that none of the gB that reached the PM endocytosed during the 3 day chase period. Culture lysates or supernatants of coinfected U373 cells were assayed for the production of cell-associated or extracellular virus by plaque assay on susceptible fibroblast monolayers (Panel D and E). Virus production remains similar to controls over a 5 day period.

Figure 1. Adenovirus delivery system



••• = *Remaining adeno genes*

Ψ = *Packaging Sequence*

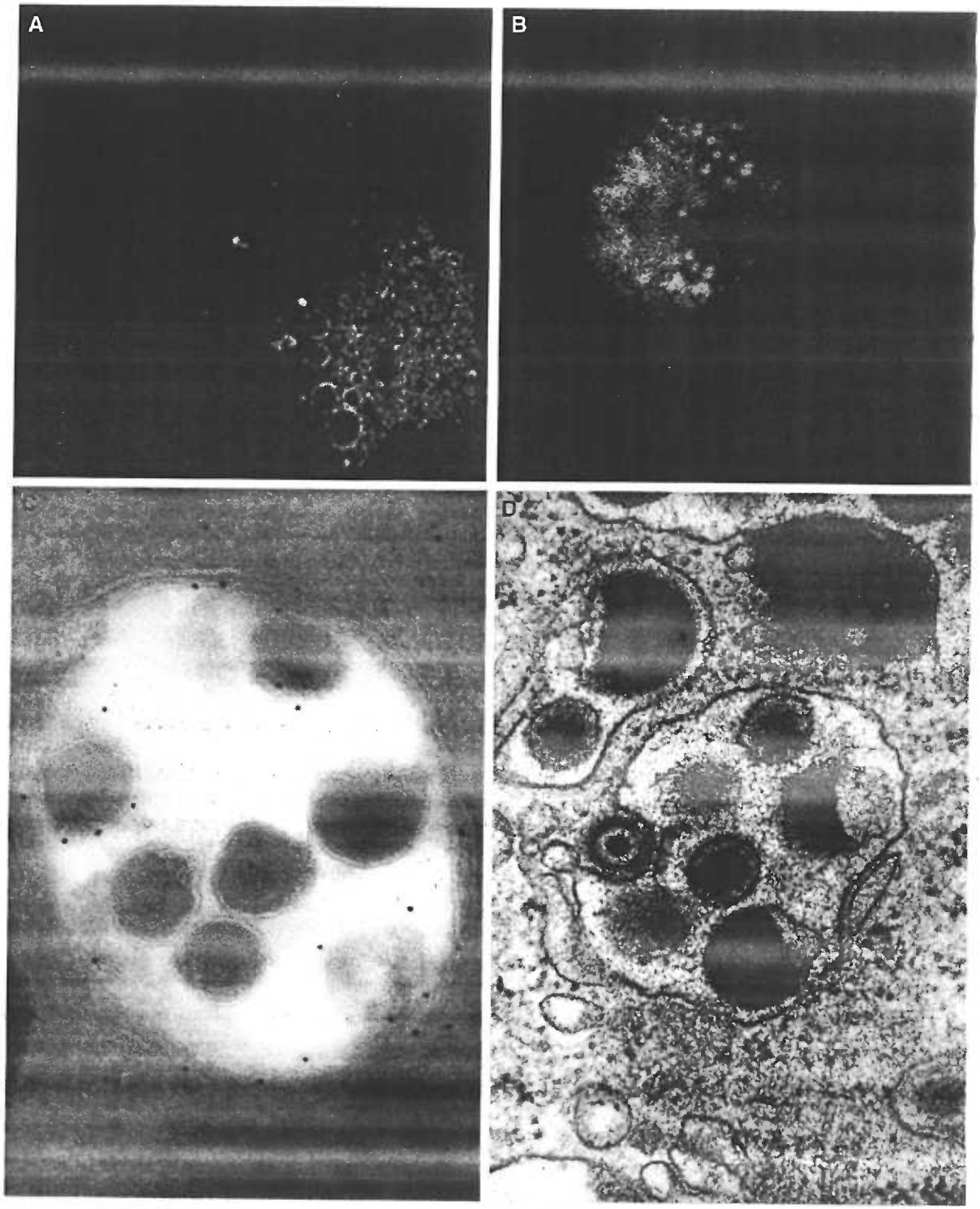


Figure 2. HCMV acquires its final envelope by budding into gB cytoplasmic vacuoles.

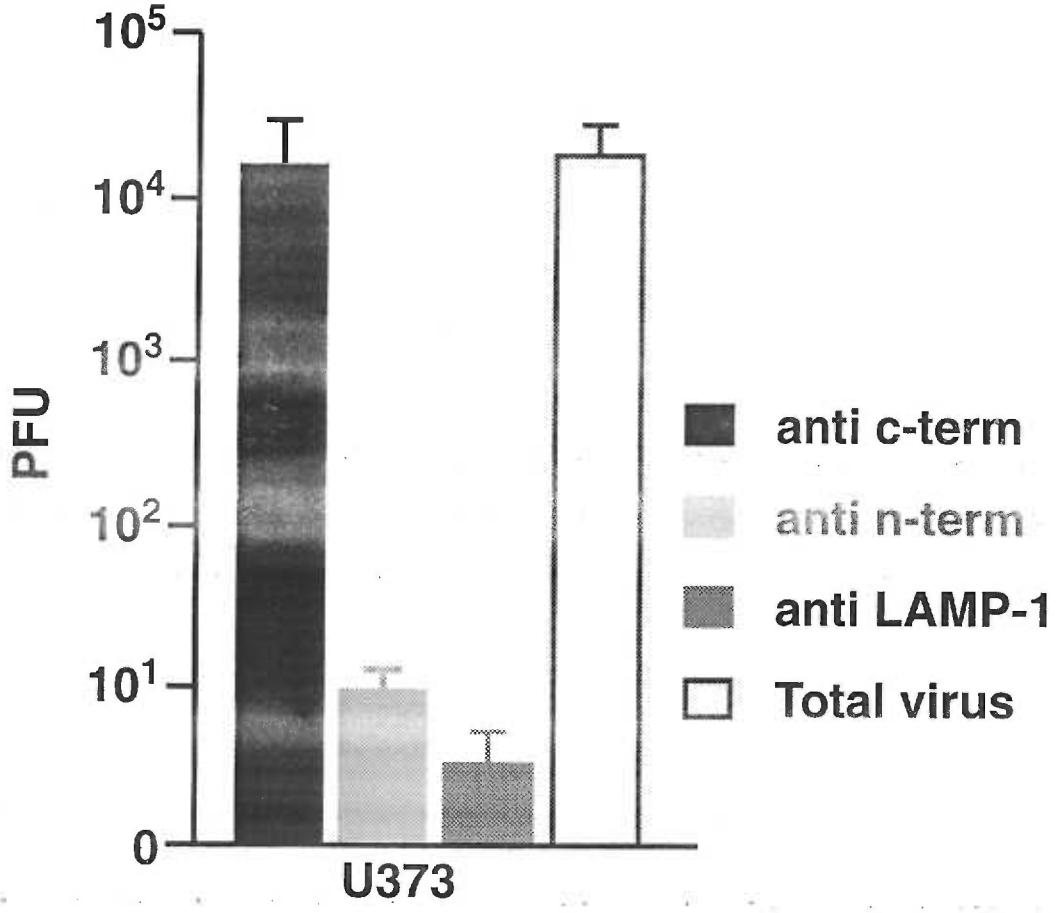


Figure 3. Isolation of intracellular gB vacuoles that contain HCMV,

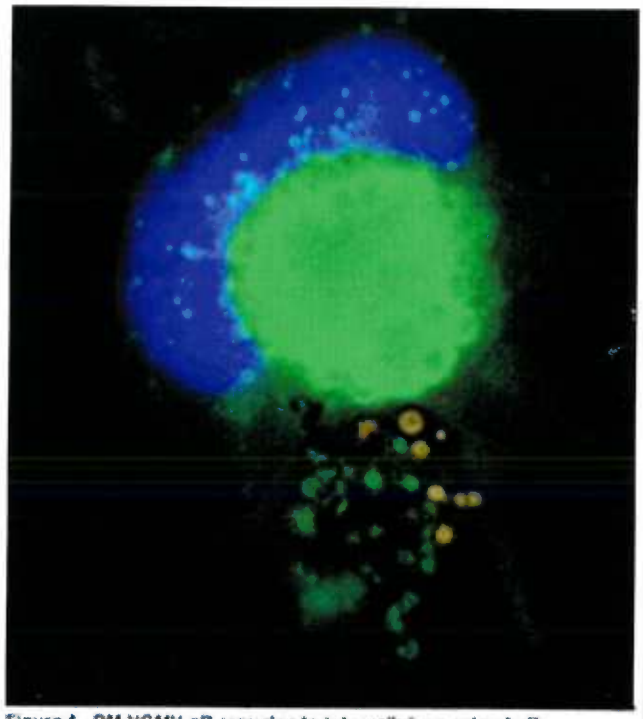


Figure 4. PM HCMV gB recycles to intracellular pools of gB.

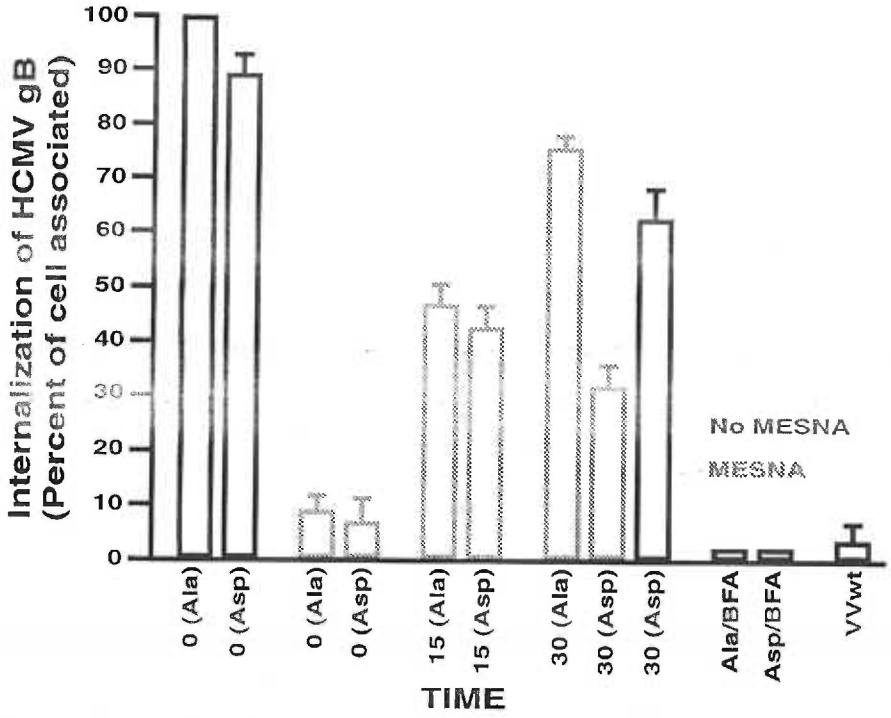


Figure 5. Endocytosed gB_{asp} rapidly recycles to the cell surface.

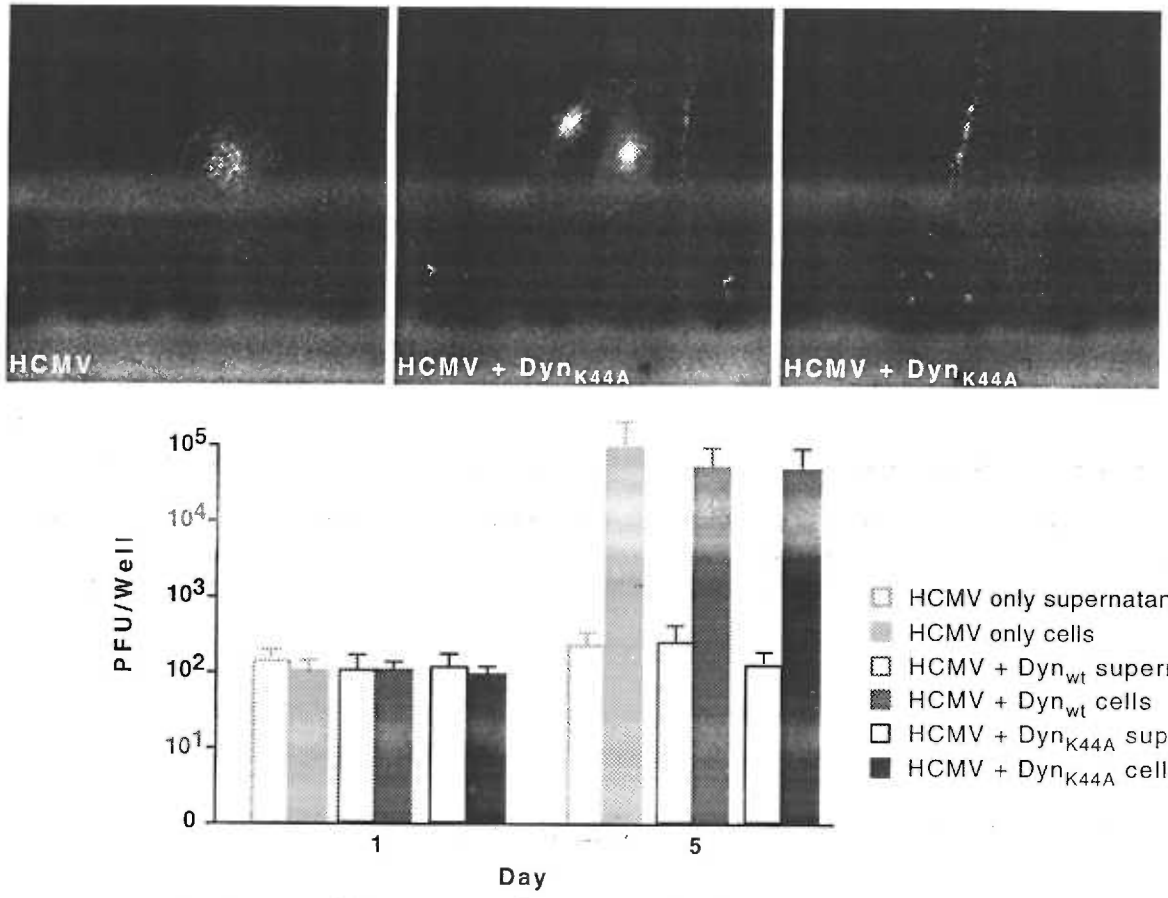


Figure 6. A block in clathrin-dependent endocytosis during HCMV infection does not affect virus production.

Chapter 7

DISCUSSION

This thesis focuses on analyzing HCMV replication and persistence in MDM, which are key cells in the biology of the virus. Macrophages are a heterogeneus population of terminally differentiated myeloid lineage cells. Since myeloid progenitor cells in the bone marrow are susceptible to HCMV infection *in vitro*, they are considered to be latent reservoirs of virus (148, 165). Although several studies have indicated that HCMV replication is restricted to early events of gene expression in monocytes, little is known about HCMV replication in myeloid lineage cells. Therefore, the goals of the present study have been (1) to investigate the correlation between monocyte differentiation and HCMV replication, (2) to determine the pathway of monocyte differentiation necessary for HCMV replication *in vitro*, and (3) to identify mechanisms of viral persistence in macrophages. These three areas of study are discussed in detail below.

Replication of HCMV in MDM.

Although a number of different cell types (including monocytes, fibroblasts, endothelial cells, epithelial cells, stromal cells and neuronal cells) are infected in patients with HCMV disease (23, 52, 61, 64, 68, 77, 86, 108,

122, 132, 148, 149, 165, 200, 201, 219, 224, 228-231, 238, 259, 269), the vast majority of studies on HCMV replication have been performed in virally-infected HF cells, evidence that these cells are infected *in vivo* has only recently been reported (228). In this report several organs with HCMV infected fibroblasts were observed including the lungs, intestine, and placenta; however, there is no evidence that HF cells are very important in HCMV pathogenesis. Examination of viral replication in other cell types such as monocyte-derived macrophages (MDM) has revealed significant differences in the kinetics of viral replication, viral cytopathic effect, and release of virus from the cell (112). In addition, epidemiological studies have demonstrated that peripheral blood mononuclear cells (PBMC) are an important source of HCMV. Furthermore, assessment of separated cell populations from PBMC in *in vivo* HCMV infections have identified monocytes as the predominant infected cell type (4, 52, 61, 68, 108, 119, 122, 148, 200, 238, 259). Therefore, monocytes/macrophages are an extremely important cell type to study HCMV replication *in vitro*.

A variety of different culture methods have been developed to study the growth of HCMV in primary MDM (33, 36, 122, 202). In these systems, the ability of HCMV to replicate depends on the stage of cellular activation or differentiation. However, since a number of functionally different macrophage phenotypes are generated depending on the

method of isolation and stimulation, varying results have been reported on HCMV replication in monocytes/macrophages (M/M). We have previously described a primary M/M culture system designed to facilitate monocyte differentiation into macrophages (72, 112, 218). In this system, monocytes are co-cultured with Con A-activated autologous non-adherent cells for 18 hours in order to allow for monocyte stimulation. Stimulated monocytes subsequently differentiate into several morphologically distinct macrophage phenotypes, including multinucleated giant cells (MNGC). Macrophages derived by this method can be maintained in long-term culture without the addition of exogenous cytokines, and these differentiated macrophages are fully permissive for HCMV infection (112).

M/M stimulated with Con A-activated autologous non-adherent cells were used to investigate the correlation between monocyte differentiation and HCMV replication. Although the ability of HCMV to productively infect these stimulated M/M varied with the time post-stimulation and, therefore, the state of monocyte differentiation, viral absorption, uncoating, and transport to the nucleus as determined by nuclear radioactivity did not vary in permissive cells (MDM 7 days post stimulation) or non-permissive cells (MDM at 1, 3, and 5 days post stimulation). These results suggest that cellular receptors for HCMV do not vary during MDM differentiation. This hypothesis is supported by the observations of Söderberg et al. (239), who

have identified CD13, which is present on both monocytes and macrophages, as one of the cellular receptors used by HCMV.

During productive HCMV infection, the virus life cycle is regulated by sequential expression of the viral genome. The MIE genes (UL122, UL123) are rapidly transcribed after infection, followed by expression of early and late gene products (57, 151, 282). The MIE region is controlled by the MIE promoter (MIEP) and encodes several isoformic regulatory proteins, which are necessary for subsequent viral gene expression. Similar to what has been observed in monocytes, HCMV expression is restricted in undifferentiated teratocarcinoma cells. Since HCMV restriction in these cells is due to inactivity of the MIEP, the activity of this regulatory region was examined in the present study in differentiating MDM cultures. The MIEP was significantly more active in the cells that were transfected at day 9 versus day 5 post-stimulation. Since the MIEP regulates the expression of regulatory proteins that may determine production of infectious virus, the major block in HCMV replication in monocytes may be at the level of MIEP inactivity. This block in HCMV expression in monocytes may be one mechanism by which virus persists in these cells without exposure to the immune system.

Further analysis of HCMV replication in differentiated MDM revealed that the expression of IE and L gene classes at the steady-state

RNA and protein levels was dramatically delayed during productive infection relative to HF cells. The mechanism responsible for the delayed kinetics of viral gene expression is unknown. However, IE gene products are important regulators of subsequent gene expression (59, 245, 248, 252-254); therefore, the altered kinetics of IE protein production in MDM may influence the delayed appearance of early and late viral proteins. The delay in viral expression may be a unique adaptation of HCMV that allows the virus to persist in MDM. The retarded accumulation of HCMV gene products may prevent the rapid increase in viral factors, which might be toxic to the cell.

These studies indicate that HCMV replication in MDM differs greatly from HF cells and, therefore, underline the importance of examining viral replication in biologically relevant cell types. In addition, they suggest the existence of a macrophage differentiation pathway that allows for HCMV replication. Therefore, the question becomes: What cellular and cytokine components are required for the differentiation of monocytes into HCMV permissive MDM?

Cytokines and cellular components.

At the onset of this thesis the identities of the cellular and cytokine components in the PBMC population that are critical for differentiating

monocytes into HCMV-permissive macrophages were unknown. Since macrophage activation is extremely complex and diverse and only 10% of Con A-MDM are productively infected by HCMV, a specific differentiation/activation pathway may be necessary for HCMV permissiveness. Therefore, the cytokines stimulated by Con A and cell-cell interactions that are required for monocyte differentiation into HCMV permissive macrophages, were examined.

Since MNGC formation correlated with the ability of virus to infect Con A-MDM cultures and MNGC are found *in vivo* in pathologic conditions such as rheumatoid arthritis, sarcoidosis, infection, foreign body reactions, and cancer (for review see 3, 20), the identity of the mechanism by which MNGC formation occurs was examined. A number of cytokines including IFN-α, IFN-β, IFN-γ, TNF-α, IL-1, IL-2, IL-4, IL-6, GM-CSF, and TGF-β play important roles in the formation of specific macrophage activation pathways, some of which lead to the formation of MNGC (for review see 2). Supernatants from Con A-stimulated PBMC were found to be sufficient to differentiate monocytes to HCMV-permissive MDM; therefore, a soluble component in HCMV-permissive MDM cultures is able to differentiate monocytes to MNGC. Therefore, several cytokines were tested to identify the soluble factor(s) responsible for the differentiation of M/M to MNGC. IL-1, IL-2, and TGF-β alone were unable to differentiate

monocytes to HCMV-permissive macrophages. In addition, even though GM-CSF or IL-4 have been found to induce MNGC formation (1, 93, 152, 191, 284, 285), these cytokines didn't induce HCMV-permissive MDM. Furthermore, the addition of IL-4 and GM-CSF to M/M induces the formation of CD83+/CD14- dendritic cells, which are not present in the Con A-stimulated cultures. In contrast, IFN-γ and TNF-α were found to be critical components for the production of HCMV-permissive macrophages. Specifically, while the effect of TNF-α and INF-γ on MNGC formation and the number of infected cells varied between the different donors, adding these cytokines to monocyte cultures consistently resulted in the formation of MDM cultures that were permissive to HCMV infection. These observations indicate the importance of TNF-α and INF-γ for the formation of HCMV-permissive macrophages.

The secretion of IFN-γ by Con A-stimulated CD8+ T lymphocytes is mediated by the binding of Con A to at least three cell surface molecules (T200, LFA-1, and Lyt-2) even in the absence of antigen (233). Con A receptors are believed to be intimately involved in antigen recognition and effector functions of CD8+ T lymphocytes (131), since these cells appear to produce IFN-γ in response to specific interactions with their target cells (93, 130, 131, 232, 233). To test if direct interactions between macrophages and CD8+ T lymphocytes are required for the formation of

permissive MDM, anti-HLA class I antibodies were added to Con A stimulated monocytes. The addition of HLA class I antibodies blocked the formation of HCMV-permissive MDM. Therefore, these observations suggest that direct contact between monocytes and T lymphocytes is essential for production of IFN-γand TNF-α in the Con A-mediated production of HCMV-permissive MDM. These results also suggest that HCMV takes advantage of two of the major antiviral cytokines to obtain a specific state of activation in macrophages that allows unrestricted replication of HCMV. Furthermore, identifying IFN-γ and TNF-α as factors that are necessary for production of HCMV-permissive macrophages has important clinical implications: Con A stimulation of T lymphocytes may mimic the activation of antigen-specific T lymphocytes during an inflammatory response. During this immune-mediated process, HCMV latently infected monocytes, which have been recruited to tissue sites and exposed to T lymphocytes, may differentiate into macrophages fully permissive for HCMV replication. The specific differentiation pathway that occurs may reactivate the latent HCMV, resulting in large quantities of virus production, which is then spread throughout the organ.

In addition to understanding how macrophages and the immune system interact with HCMV, elucidating the mechanisms HCMV uses to

replicate in MDM are essential to further understand viral persistence and trafficking in the human host.

HCMV accumulates in cytoplasmic vacuoles that are inaccessible to the MDM degradation machinery.

Although a number of viruses in addition to HCMV survive and persist in macrophages, the mechanisms involved in intracellular survival are unknown (184, 192). The optimal situation for both virus and host cell survival would be for viral accumulation to occur without killing the host cell. Therefore, after HCMV infects the permissive MDM, HCMV persistence would require a strategy for interacting with the macrophage that did not expose progeny to the immune system or completely debilitate the macrophage. The question becomes, can HCMV use macrophages as a Trojan Horse similar to lentiviruses (184, 192)?

Classically, HCMV infection of HF cells results in the production of both intracellular and extracellular virus. At late time points post infection, cells fuse and lyse, which results in the release of intracellular virus in the supernatant. In contrast, virus is exclusively cell associated in Con A-MDM and persists in these cells without lytic events. In addition, immunofluorescence analysis of HCMV infected Con A-MDM cultures after 7 dpi revealed the accumulation of viral proteins in discrete

cytoplasmic vacuoles that were not associated with the PM. These intracytoplasmic vacuoles have been proposed to contain sequestered HCMV. In support of this hypothesis, HIV also appears to accumulate in MDM vacuoles (180). Ultrastructural studies of HIV-infected MDM indicated that these vacuoles are derived from the Golgi complex (180).

In this study, the Golgi marker mannosidase II was found to be associated with HCMV infected MDM vacuoles that contain gB, which suggests that these vacuoles are derived from the Golgi apparatus. Therefore, the integrity of the Golgi apparatus was assessed in HCMV infected MDM. The Golgi apparatus in HCMV-infected macrophages became abnormal diffuse as infection progressed. Interestingly, over the same time period a similar diffuse gB staining pattern was observed. When double-label IF experiments were performed at 7 days post infection, the majority of gB colocalized with the TGN marker BODIPY FL C_5-ceramide. These observations suggest that HCMV disruption of the Golgi results in the diffuse gB staining patern late in infection.

Further analysis of vacuoles that contained HCMV gB by double- and triple-label immunofluorescence with early endosome (rab5 and transferrin receptor), late endosomes and lysosomes (lamp 1 and 2), and viral markers showed that these viral vacuoles do not contain these endocytic pathway markers. In addition, analysis of these vacuoles by IF

at high magnification, immuno-EM (using a gB monoclonal antibody) and EM revealed the presence of HCMV in gB coated vacuoles. Furthermore, vacuoles containing gB were isolated from HCMV infected cells on Mini MACS columns and their content was analyzed for virus using plaque assays. The vast majority of infectious virus was found to be associated with these vacuoles. These results suggest that the infectious HCMV particles acquire their final envelope by budding into intracellular vacuoles that have HCMV late antigens associated with their surface and that the virus then remains associated with these vacuoles. The containment of infectious HCMV in discrete cellular compartments may not only protect virus from cellular degradation but may also protect the cell from damage produced by viral proteins. The inability of the MDM to degrade newly made HCMV may allow persistence and trafficking of virus to target tissues via a cellular vehicle while evading immune detection.

Macrophages represent a key cell involved in the defense of the host against pathogens. The success of a pathogen that infects macrophages depends on the ability of the organism to evade lysosomal fusion or withstand lysosomal degradation. Macrophage pathogens have developed unique strategies to avoid cellular destruction including the release of substances that modify acidity, resistance to a harsh environment, and disruption of vacuoles (7, 9, 38, 89, 98, 107, 174). The

finding that endocytic pathway markers and HCMV gB antigen did not colocalize, suggests that virus containing vacuoles are unable to fuse with host degradation vacuoles. This avoidance of the endocytic pathway may be one mechanism that HCMV uses to survive in macrophages. The mechanisms by which HCMV is able to avoid degradation within macrophages are unknown. One potential mechanism may be that HCMV either blocks or carriers a protease that degrades vacuolar membrane proteins that are required for lysosome fusion. Alternatively, the fusion of early endosomal vacuoles with lysosomes may be inhibited by disruption of the microtubule network (270). One or all of these mechanisms may contribute to the inability of viral containing vacuoles to fuse with lysosomes in MDM.

HCMV disruption of the MT network.

To investigate how HCMV vacuoles avoid entering the host degradation system, the integrity of MT network was analyzed. The ultrastructure of the cell is maintained in part by the MT network. The major site of microtubule nucleation is the centrosome, the microtubule organization center. Polymerization and depolymerization of microtubules is a dynamic process in which the half-life of a microtubule is approximately 10 minutes. This network functions to maintain placement and transport of intracellular

organelles (136, 270); therefore, the MT network is invoved in the targeting of intracellular pathogens to the host degradation system.

During the latter stages of HCMV replication in MDM (13-15 dpi) the normal structure of the Golgi is disrupted without obvious cellular cytopathic effect. Examination of infected MDM structural elements revealed the progressive loss of an ordered MT network, which correlated with disruption of the Golgi apparatus. Since the MT network maintains placement of the Golgi and regulates intracellular vesicle movement, one would expect a cause and effect relationship between the disaggregation of the microtubules and disruption of the Golgi apparatus. However, when the MT network is stabilized with taxol disruption of the Golgi network still occurs in HCMV infected MDM.

To investigate HCMV related disruption of the MT network, the subcellular location and production of α, β, and γ tubulin subunits were analyzed by IF and western analysis. These experiments demonstrated that HCMV infection did not abrogate the production of α, β, and γ tubulin subunits. In addition, the subcellular staining pattern of γ tubulin remained perinuclear in HCMV infected cells, while α and β subunit staining became extremely diffuse throughout the cytosol. Therefore, these results suggest that HCMV disruption of the MT network is not at the level of tubulin subunit production, or through disruption of γ tubulin.

HCMV may use multiple mechanisms to disrupt the MT network, which may prevent HCMV vesicles fusing with late endosomes and lysosomes. First, HCMV may encode proteins that bind to α- and β-tubulin monomers, preventing polymerization similar to the drug colchicine. Second, the virus may block GTP and or GDP binding to the MT monomers preventing tubulin assembly into microtubules. Lastly, HCMV may disrupt the microtubule organization center by binding to γ-tubulin, which could inhibit MT nucleation. Further analysis of how HCMV disrupts the MT network may help explain why the virus has developed mechanisms to disrupt this network.

gB intracellular trafficking.

To further investigate mechanisms by which HCMV avoids cellular degradation in MDM the subcellular trafficking pattern of gB was analyzed. Interestingly, while studying viral protein expression in MDM, the subcellular localization of gB was discovered to be different in MDM compared with that in HF cells. The observation that gB trafficks differentially raises the interesting issue of how gB containing vesicles are formed and the role of gB in the coexistence of HCMV and MDM. Efforts were concentrated on gB because: 1) gB is one of the predominant glycoproteins in the HCMV envelope, 2) the gB homologues in HSV and

other herpesviruses are essential glycoproteins for entry, fusion and cell to cell spread (although this has not been demonstrated for HCMV gB), 3) the ability of gB to traffic to the cell surface correlated with the release of infectious virus, and 4) gB colocalizes with a major fraction of the infectious virus in infected cells.

The observation that infection of HF cells with HCMV resulted in the expression of gB at the PM and in cytoplasmic vacuoles, while HCMV gB could not be detected at the PM of MDM by confocal microscopy suggests that cell-specific differences exist that affect the intracellular trafficking of gB. These cell-specific differences could be the result of different trafficking machinery in MDM and HF cells, and/or the result of a secondary modification of gB. Interestingly, when the U373 microglial cell line was infected with HCMV, gB trafficking patterns similar to that of HCMV infected MDM were detected. In addition, HCMV infection of U373 cells results in a delay in virus replication kinetics similar to that described for MDM and viral infection is non-lytic and virus remains cell associated. Therefore, MDM and U373 cells display similar viral replication life cycles. Since MDM are difficult to manipulate *in vitro* and the steady-state expression of gB in the U373 cells and MDM was similar, only HF cells and U373 cells were used in further studies designed to elucidate whether cell-specific mechanisms of HCMV gB intracellular trafficking exist.

The cytoplasmic tails of a number of viral proteins (62, 140, 147, 209, 222, 302) that enter the secretory pathway have been shown to contain selective trafficking signals, which direct proteins to different cellular compartments. The surface expression of viral glycoproteins is affected not only by the cellular steady-state expression of the protein and by transport to the PM, but also by rates of internalization of proteins from the cell surface. Internalization occurs through both clathrin-dependent and -independent pathways. C-terminal domain internalization signals have been identified for several proteins including furin, low-density lipoprotein receptor, transferrin receptor, polymeric Ig receptor, epidermal growth factor receptor, VZV Fc receptor gE and the SIV transmembrane protein gp41. Comparison of the sequences of these proteins indicates that a common structure rather than a specific sequence is necessary for internalization. One example is VZV gE, which contains two TGN targeting sequences in its cytoplasmic domain (302). Both the AYRV targeting sequence and an acidic amino acid-rich region are sufficient to cause expressed protein to be internalized once it has reached the PM. Once internalized, these signals are also sufficient to target the VZV glycoprotein to the TGN. Another example is a Tyr-dependent motif in the cytoplasmic tail of simian immunodeficiency virus' transmembrane envelope protein (gp 41), which functions as an internalization signal to

regulate the expression of envelope glycoproteins at the cell surface (209).

Protein localization to subcellular compartments may also be influenced by secondary modifications that occur in a cell-type specific manner. For example, the glycoproteins produced by Sindbis virus are modified in the secretory pathway of both vertebrate and insect cells, but have cell-specific trafficking patterns, which affect the subcellular location of virus assembly (212). As a result, in vertebrate cells, viral assembly and budding occurs at the PM. In contrast, in insect cells, virus buds into intracellular vacuoles, which fuse with the PM and release virus into the extracellular fluid. The Sindbis virus glycoproteins are transiently phosphorylated; inhibitors of phosphorylation prevent production of infectious virus (144). These observations suggest that the phosphorylation state of Sindbis glycoproteins may determine either glycoprotein trafficking or viral assembly. With HCMV there is also a difference in release of virus depending on the cell type that is studied, and the virus' major glycoprotein gB contains several potential phosphorylation sites in the cytoplasmic tail.

In addition to viral proteins, the subcellular targeting of some cellular proteins has been shown to be regulated by the phosphorylation state of the protein. For example, the serine endoprotease furin, has

specific sequences in its 56 aa cytoplasmic tail that localizes furin to the TGN (222). Antibody uptake studies demonstrate that the enzyme cycles between the cell surface and the TGN (168). The trafficking signals in the furin cytoplasmic tail are mediated by two different mechanisms (117, 222). One is composed of three different primary amino acid motifs that include a dileucine-type motif, a tyrosine based motif, and an acidic cluster. The other trafficking signal is the phosphorylation state of the acidic cluster, which is mediated by CKII. The dileucine-type motif and the tyrosine based motif are internalization signals, whereas the state of phosphorylation of the acidic cluster determines targeting of the protein to the TGN.

The sequence D-S_{900}-D-E-E-E-N in the HCMV gB carboxy terminal tail fulfills the requirements for a CKII consensus sequence (X-S/T^{p}-X-X-E-E), and therefore, the role of this CKII site in gB intracellular trafficking was of interest. Point mutation analysis was used to demonstrate that gB phosphorylation occurs only at Ser_{900} in the CKII site, and that phosphorylation occurs in both U373 and HF cells *in vivo*. These results suggest that the cell-specific absence or presence of gB at the PM is not due to the inability of CKII to phosphorylate the protein in U373 cells, since gB can be phosphorylated in both cell types.

To investigate whether the state of gB phosphorylation affects intracellular routing, three recombinant vaccinia viruses (RVV) containing wild type gB (gB_{wt}) or gB that contained a point mutation at Ser_{900} were constructed. To mimic the non-phosphorylated and phosphorylated states, Ser_{900} was replaced with either an alanine (gB_{ala}) or an aspartate (gB_{asp}) residue, respectively. The subcellular location of gB was evaluated by confocal microscopy in HF and U373 cells infected with RVV gB_{wt}, gB_{ala}, or gB_{asp}. gB was detected at the cell surface of HF cells infected with RVV gB_{wt}, but not at the surface of U373 cells as previously observed. gB expressed by RVV gB_{wt} retained the differential expression pattern observed with gB expression in both HCMV-infected HF and U373 cells.

Infection of HF cells or U373 cells with RVV gB_{ala} resulted in a similar cellular distribution of gB as infection with RVV gB_{wt}, which resulted in the presence or absence of PM gB staining respectively. In contrast, when U373 cells were infected with RVV gB_{asp}, gB was easly detected at the cell surface. In addition, RVV gB_{asp} infection of HF cells resulted in increased expression of gB at the PM compared to gB_{wt}. In support of the IF data, surface biotinylation of RVV gB_{ala} or gB_{asp} infected cell monolayers demonstrated that substantially more gB_{asp} than gB_{ala} is detected at the surface of U373 cells. These results suggest that phosphorylation of Ser_{900} plays a key role in the trafficking of gB, since replacing Ser_{900} with a

charged amino acid (Asp) allows surface expression in both cell types. Therefore, the gB cytoplasmic tail acidic cluster that includes Ser_{900} serves as a sorting signal that regulates gB intracellular routing.

Although the altered trafficking of gB in U373 cells infected with gB_{asp} supports the hypothesis that the phosphorylation state of Ser_{900} plays an important role in gB trafficking, the above experiments indicate that both HF and U373 cells are able to phosphorylate gB. Since gB is phosphorylated by both HF and U373 cells, the differences in gB steady-state levels are not due to the presence of a cell-specific kinase that phosphorylates gB. The phosphorylation state of gB in these cells may be influenced by the presence of differential phosphatase activities that regulate the level of gB phosphorylation. To determine whether phosphatases regulate gB cell surface expression, U373 cells infected with RVV gB_{wt} or RVV gB_{ala} were treated with the phosphatase inhibitor okadaic acid (100 nM; inhibitor of the protein phosphatase 2A) or tautomycin (100 nM; inhibitor of the protein phosphatases 1/2A). Although addition of okadaic acid to RVV gB_{wt}- and RVV gB_{ala}-infected U373 cells did not have an effect on gB localization, addition of tautomycin to RVV gB_{wt}-infected U373 cells resulted in gB accumulation at the cell surface, whereas an accumulation of gB was not detected in RVV gB_{ala}-infected U373 cells. These observations suggest that the lack of cell-surface

expression of gB in U373 cells compared to HF cells is due to a specific protein phosphatase 1/2A activity.

The inability of dephosphorylated gB to traffic from the TGN to the PM may be another potential explanation for the observed differences in cell surface expression of gB in U373 and HF cells. Therefore, to determine if gB lacking a charged residue at position 900 trafficks to the PM, U373 cells were coinfected with the recombinant vaccinia viruses described above in addition to a vaccinia construct that expresses a dynamin dominant-negative mutant (RVV dyn_{K44A}). The dynamin mutant prevents dynamin-mediated transport of surface molecules back to the cytoplasm by blocking clathrin-dependent endocytosis (51). Surprisingly, coinfection of U373 cells with RVV dyn_{K44A} and RVV gB_{wt}, gB_{asp}, or gB_{ala} resulted in an accumulation of gB at the PM in U373 cells. Surface gB expression was not observed in RVV gB_{wt}-infected U373 cells coinfected with vaccinia virus expressing wild-type dynamin; therefore, expression of gB at the cell PM is not the result of vaccinia virus infection. These data indicate that both phosphorylated and non-phosphorylated gB traffic to the cell surface and suggest that internalization rate differences are responsible for the differential expression of gB in HF and U373 cells. Furthermore, these observations indicate that the internalization of gB from the PM occurs via a clathrin-dependent pathway. Therefore, these

experiments suggest that gB trafficks to the PM in a charge-independent manner and is then internalized from the cell surface to an intracellular compartment at a cell-specific rate.

In support of this hypothesis, Radsak et al. recently demonstrated that HCMV gB at the PM (194) is retrieved for virus envelopment. In this situation gB would traffic to the PM, where the protein would be subsequently sorted, internalized, and transported to viral assembly compartments. Therefore, the determination of which compartment gB was targeted to after internalization was of interest. To identify this compartment, antibody uptake experiments were used to track the route PM gB takes after internalization. For these experiments, tetracycline responsive U373 tTA cells (U373 tTA) were coinfected with HCMV and an adenovirus that expressed a temperature sensitive dynamin dominant negative mutant (Adeno dyn_{G273D}). These experiments demonstrated that some of the gB that reaches the cell surface is able to internalize and colocalize with intracellular gB on the surface of membranes near the Golgi. Interestingly, when similar antibody uptake experiments were performed using RVV gB_{asp} or RVV gB_{ala}, gB_{asp} was internalized but remained associated with the PM. In contrast, gB_{ala} moved into the cell and concentrated in intracellular vacuoles that appeard similar to the vacuoles formed during HCMV infection. Thus, in U373 cells, gB_{ala} is

rapidly endocytosed upon reaching the cell surface, sorted upon endocytosis, and concentrated on the surface of intracellular membranes. In contrast, gB_{asp} is internalized but remains in small vacuoles near the PM.

In order to corroborate theses IF results, cell-surface labeling experiments were performed to measure the rate at which gB internalized from the PM. Interestingly, these experiments demonstrated that gB must be dephosphorylated in order to localize with intracellular pools of gB upon internalization from the PM. If gB remains phosphorylated, the protein will recycle to the PM. These results suggest that a regulated cycling event occurs between the PM and early endocytic compartments in which phosphorylated gB is quickly returned to the PM after being endocytosed and dephosphorylated gB moves along the endocytic pathway once in the cell.

Studies in yeast (48, 49) have provided evidence for regulated cycling pathways that occur between the TGN and endosomal compartment in humans. In addition, studies with mannose 6-phosphate receptor (MPR) and the Ras-like GTPase rab 9 demonstrate that MPR cycles between the TGN and a post TGN processing compartment in a regulated fashion (241). Therefore, the cycling event at the PM in which gB_{asp} is trapped may be similar to the proposed MPR TGN/endosome

cycling loop and HCMV may use this cycle to concentrate gB in a viral assembly compartment. Further investigation will need to be done to determine if gB has evolved to use the MPR TGN/endosome cycling loop to concentrate on the surface of virus envelopment vacuoles.

The gB trafficking data presented here suggests that gB trafficking to the PM is a mechanism by which gB was targeted to intracellular gB vacuoles. The question becomes, is gB trafficking to the PM important for viral envelopment? In order to address this question, the requirement for gB to traffic from the PM to intracellular vacuoles was examined. For these experiments, U373 tTa cells were coinfected with Adeno dyn_{K44A} and HCMV. An increase in syncitia formation over HCMV infected control cultures suggested there was an increase in gB PM expression in the adenovirus infected cultures. IF experiments confirmed that there was an increase in gB PM expression in the Adeno dyn_{K44A} and HCMV coinfected cultures. Culture lysates and supernatants of coinfected U373 tTa cells were assayed for the production of cell-associated or extracellular virus by plaque assay on susceptible fibroblast monolayers. Surprisingly, virus production remained similar to production in HCMV infected controls over a 5 day period, suggesting that the trafficking of gB to the PM is not essential for production of infectious virus.

Although the steps involved in HCMV capsid envelopment are not well understood, there is a general consensus that attachment of membrane-bound viral glycoproteins to tegumented capsid plays an important role in virus assembly and egress. Therefore, the understanding of how virion envelope proteins are sorted is crucial to identifying the subcellular HCMV envelopment compartment. However, the mechanisms involved in the subcellular targeting of glycoproteins are poorly understood. Here, for the first time, a role for HCMV gB phosphorylation has been identified: HCMV uses gB phosphorylation and the host cell trafficking machinery to concentrate HCMV glycoproteins on the surface of Golgi derived vacuoles that are used for HCMV envelopment.

Further studies should be directed toward the identification of the viral genes involved in the processes introduced in this thesis and of other mechanisms required for the targeting of viral capsids to gB vacuoles. In addition, the viral protein-protein interactions that are required for the initiation of capsid envelopment need to be elucidated. Finally, prior to further analysis, the requirement for gB in HCMV replication should be determined.

SUMMARY AND GLOBAL IMPLICATIONS

The mechanisms by which HCMV persists in monocyte/macrophages were unknown at the initiation of this thesis work. This thesis defines several potential methods that HCMV and macrophages use to coexist. The most efficient mechanism is to remain quiescent in cells until activation by some external stimuli. We (112), and others (200, 259, 260) have found that HCMV early and late gene expression is restricted in monocytes but not in differentiated MDM. The quiescent state of the virus may represent the primary mechanism that HCMV utilizes to evade immune detection in these cells. As monocytes differentiate into permissive macrophages, HCMV replicates and is sequestered in vacuoles that are unable to fuse with the PM and vessicles that constitute the late endosome-lysosome degradation pathway. Therefore, infectious virus is retained in an environment protected from host-mediated degradation and the immune system. The global implications of these phenomena are two-fold. First, since HCMV replication in M/M is dependent on the state of macrophage differentiation, the virus is able to remain quiescent in monocytes until macrophage activation occurs, which results in the replication rather than destruction of intracellular HCMV. Secondly, activated M/M that harbor intracellular virus are the ideal vector for dissemination of the virus through the host.

The HCMV replication life cycle in MDM shown here is clearly different from that which occurs during HCMV infection of other cell types. For example, HCMV has recently been shown to induce a persistent noncytopathic infection in AEC (73). In this report, HCMV-infected AEC were unable to accumulate intracellular infectious virus, although a significant amount of extracellular virus was detected. This phenomenon suggests that the cells survive infection through efficient export of mature virus and toxic viral products, which may otherwise cause cellular lysis, from the cell. This survival mechanism is clearly different from that which operates in MDM. This thesis demonstrates that HCMV infection in MDM is also nonlytic, but instead of virus being rapidly exported from the cell, HCMV accumulates in large cytoplasmic vacuoles that do not fuse with the PM. These observations emphasize the cell-specific differences that occur during HCMV infection.

Although the specific cellular organs HCMV targets during acute disease have been known for almost 50 years, the site(s) for HCMV latency has been difficult to identify. The observations generated from my thesis work, suggest that monocytes or monocyte precursors would be an ideal site for latent virus to be harbored. Soderber-Naucler et al. have recently demonstrated that latent HCMV can be reactivated in a myeloid lineage cell obtained from the peripheral blood of healthy seropositive

individuals (240). Monocytes interact with multiple tissues in the body as they traffic through the blood stream and migrate into tissues. Therefore, a dynamic interaction between the cells in which virus is either activated or transmitted during extravasation may occur. These interactions may involve cell-cell contact through adhesion molecules, which subsequently trigger signal transduction events and stimulate monocytes to differentiate so that HCMV is reactivated. The virus can then be transmitted to endothelial cells lining the aorta and virus disseminated throughout the body. Therefore, a monocyte that harbors latent HCMV would function as a perfect vehicle for cell-to-cell transmission of HCMV, which would circumvent some of the immune systems efforts to detect viral pathogens.

This study clearly demonstrates the significance of macrophages in the biology of HCMV and emphasizes the importance of examining viral replication in biologically relevant cell types. The findings within this thesis may have implications for understanding reactivation of HCMV in immunocomprimised patients. Further elucidation of the mechanisms of HCMV replication and virus assembly in macrophages will be essential for future understanding of viral persistence and trafficking in the human host.

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Appendix A

Additional data.

All experiments in this section were performed by Kenneth N. Fish.

Assessment of activity of the HCMV promoter in M/M. To assess activity of the HCMV promoter, an HCMV MIEP-CAT construct containing the MIEP (-1145 to +112) that is linked to the chloramphenicol transferase gene (14) was transfected into monocytes at 1, 5, 9, 13, 16, or 20 days post-stimulation by lipofection. Cell lysates were harvested either at 2 and 3, 6 and 7, 10 and 11, 14 and 15, 17 and 18, or 21 and 22 days post-stimulation (respectively) and assayed for CAT activity. The MIEP was significantly more active in the cells that were transfected at day 9 versus day 5 post-stimulation (Figure 1A). To control for transfection differences in cells at various stages of differentiation, MIEP CAT was tagged with digoxigenin and transfected into parallel MDM cultures at 3 and 9 days post stimulation (Figure 1B and 1C). MDM that had incorporated digoxigenin were detected with anti-digoxigenin antibody coupled to alkaline phosphatase. As seen in Figure Figure 1B and 1C, a high percentage of MDM at both 3 and 9 days post-stimulation demonstrated the presence of lipofected DNA, indicating that differentiation did not affect transfection efficiency. Since the MIEP regulates the expression of regulatory proteins that may determine production of infectious virus, the major block in HCMV replication in monocytes may be at the level of MIEP inactivity. This block in HCMV

expression in monocytes may be one mechanism by which virus persists in these cells without exposure to the immune system.

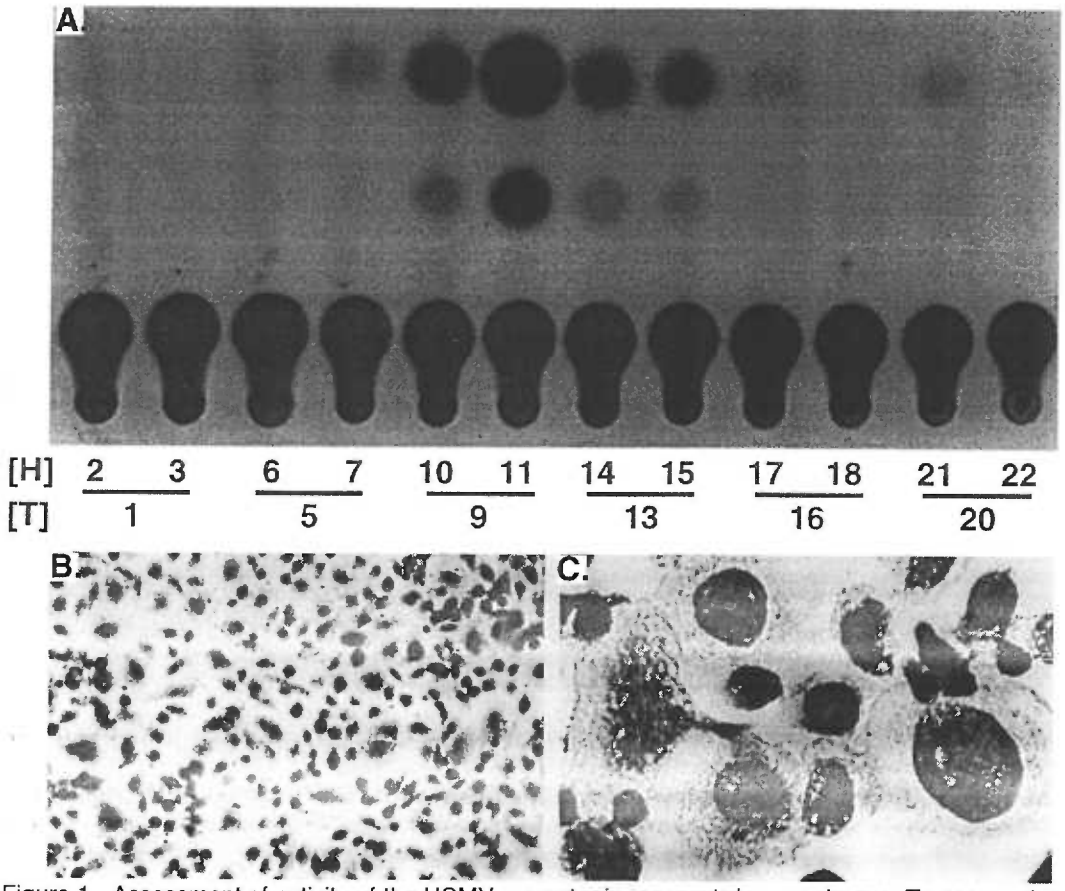

Figure 1. Assessment of activity of the HCMV promoter in monocyte/ macrophages. To assess the activity of the HCMV promoter an HCMV-CAT construct that contains the major immediate-early promoter (-1145 to +112) linked to the CAT gene was transfected into monocytes at 1, 5, 9, 13, 16, and 20 days poststimulation (dps). Cell lysates were harvested at either 2 and 3, 6 and 7, 10 and 11, 14 and 15, 17 and 18, or 21 and 22 dps and assayed for CAT activity. To control for transfection differences in cells at various stages of differentiation, major immediate-early promoter CAT was tagged with digoxigenin and transfected into parallel monocyte-derived macrophage cultures at 5 and 9 days poststimulation (B and C, respectively). Monocyte-derived macrophages that had incorporated digoxigenin were detected with anti-digoxigenin antibody coupled to alkaline phosphatase.

HCMV attachment and entry of M/M. To determine whether the restriction of HCMV expression in M/M cultures is due to the lack of viral penetration into the cell, virus with ^{3}H-labeled DNA was used to infect

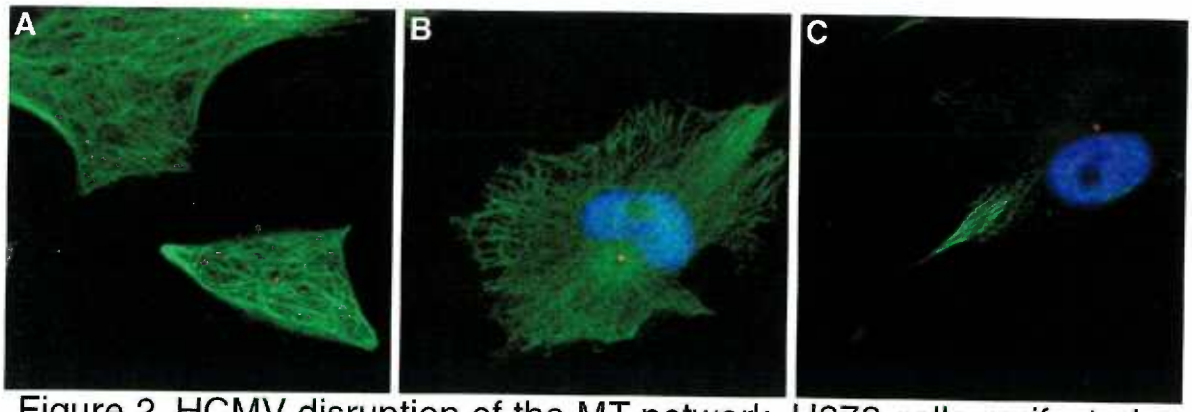

Figure 2. HCMV disruption of the MT network. U373 cells, unifected (A) or HCMV infected 3 (B) and 7 (C) dpi, were triple-labeled for the presence of IE antigen (blue), alpha-tubulin (green), and gamma-tubulin (red).

Appendix B

Interferon-γ and tumor necrosis factor-α specifically induce formation of cytomegalovirus-permissive monocyte-derived macrophages that are refractory to the antiviral activity of these cytokines.

by Cecilia Soderberg-Naucler, Kenneth N. Fish, and Jay A. Nelson

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pages 227-263 of this Thesis

Abstract

Monocytes/macrophages are key cells in the pathogenesis of human cytomegalovirus (HCMV). Although HCMV infection in monocytes is restricted to early events of gene expression, productive infection has been demonstrated in differentiated macrophages *in vitro*. We examined the cellular and cytokine components that are essential for HCMV replication in Concanavalin A–stimulated monocyte-derived macrophages (MDM). By negative selection, depletion of CD8+ T lymphocytes, but not CD4+ T lymphocytes, CD19+ B cells, or CD56+ NK cells, resulted in a 60–70% reduction in the number of HCMV-infected MDM, and a 4 log decrease in virus production. Neutralization of IFN-γ and TNF-α, but not IL-1, IL-2, or TGF-β decreased production of virus by 4 logs and 2 logs, respectively. Subsequently, addition of recombinant IFN-γ or TNF-α to purified monocyte cultures was sufficient to produce HCMV-permissive MDM. While IFN-γ and TNF-α possess antiviral properties, addition of these cytokines to permissive MDM cultures did not affect production of HCMV. Thus, rather than inhibiting replication of HCMV, IFN-γ and TNF-α specifically induce differentiation of monocytes into HCMV-permissive MDM, which are resistant to the antiviral effects of these cytokines.

Introduction

Human cytomegalovirus (HCMV) infection remains a major cause of morbidity and mortality in transplant and AIDS patients. Primary infection of HCMV results in lifelong persistence of the virus in the host, and reactivation frequently occurs in immunocompromised individuals. Several recent reports have provided strong evidence that, in addition to causing acute disease, HCMV may be involved in the development of atherosclerosis, allograft atherosclerosis (chronic rejection) in organ transplant patients, and chronic graft-versus-host disease in bone marrow transplant patients. The underlying mechanism for the role of HCMV in the development of these complications, however, is still unknown.

Although the cellular site of HCMV latency still has not been identified, epidemiological studies have implicated blood products, bone marrow grafts, and solid organs as sources of HCMV (41-44, 160, 261). Examination of transplanted organs early in the course of HCMV disease have indicated that infiltrating leukocytes are the primary source of virus (86). These infiltrating cells were identified as macrophages using double-label immunohistochemistry with antibodies directed against viral antigens and cellular markers (231). Furthermore, studies of separated peripheral blood cell populations derived from individuals with HCMV disease (142, 238, 279) or asymptomatically infected individuals (52, 259) have

identified monocytes as the predominant infected cell type. However, productive HCMV infection of monocytes is infrequent, and viral replication is restricted to early events of gene expression (260). In contrast, extensive unrestricted viral replication can be demonstrated in tissue macrophages (86, 229, 231). The increased frequency of HCMV-infected macrophages in tissues as well as the detection of late viral gene expression suggests that differentiation of monocytes into macrophages may influence the ability of the virus to replicate in these cells. In support of this hypothesis, several primary monocyte/macrophage systems that have been established to examine HCMV replication (112, 138, 149, 165, 286) have demonstrated that the ability of the virus to replicate in these cells is dependent on the state of cellular differentiation. Infection of unstimulated monocytes resulted in either the lack of viral gene expression, or a restricted replication to immediately early gene products (112, 165, 260). The block in HCMV expression in unstimulated monocytes was not at the level of virus entry and fusion with the cell, but rather at the level of transcriptional or posttranscriptional events (75, 112, 132, 133, 225).

We have previously established a primary monocyte/macrophage system in which differentiated macrophages are fully permissive for HCMV. In this system monocytes are cocultured with Con A–stimulated

autologous nonadherent cells for a defined period of time to allow for monocyte stimulation. Stimulated monocytes differentiate into different morphologically distinct phenotypes of macrophages, including MNGC. The macrophages at this stage can be maintained for long periods of time without addition of exogenous cytokines, and are permissive for HCMV infection. Since macrophage activation is extremely complex and diverse, a specific activation pathway may be necessary for HCMV permissiveness. Therefore, we examined the cell–cell interactions and cytokines involved in Con A–mediated differentiation of HCMV-permissive macrophages. Our results suggest that HCMV replication in Con A–MDM is dependent on CD8-positive T lymphocytes and IFN-γ and TNF-α production. Interestingly, neither IFN-γ nor TNF-α demonstrated HCMV antiviral effects in the infected macrophages. Since MDM were recently shown to be a site for HCMV latency in healthy individuals, knowledge of the specific activation pathway of HCMV-permissive MDM will lead us closer to understanding HCMV latency and persistence in the human host.

Materials and Methods unique to this publication.

Negative selection of blood cells prior to Concanavalin A stimulation. In order to obtain CD4+ or CD8+ T lymphocyte, B cell or NK cell depleted MDM cultures, the Mini MACS system (Miltenyi Biotec, Bergish Gladbach, Germany) was used for negative selection of the respective cell type. Freshly isolated PBMC were stained with monoclonal antibodies directed against the following cell-type specific molecules; anti-human Leu-3a (CD4, T lymphocytes), anti-human Leu-2a (CD8, T lymphocytes) both from Becton Dickinson, anti-human CD19 (DAKO-CD19, B Lymphocytes), anti-human CD56 (DAKO-CD56, NK cells), and for negative controls anti-human CD31 (DAKO-CD31, endothelial cells) all from Dakopatts, Glostrup, Denmark, or mouse IgG1 (Fc, R&D Systems, Minneapolis, MN). 1 x 10^8 cells in 500 µl serum free Iscoves medium were incubated with a titered excess of the respective antibody at 4°C for 45 minutes. The cells were washed twice in cold PBS, and resuspended in 250 µl of MACS buffer (PBS containing 5 mM EDTA and 0.5 % BSA) and incubated with 160 µl MACS beads conjugated with rat-anti-mouse IgG1 antibodies or rat-anti-mouse IgG2a and IgG2b antibodies for 20 minutes at 4°C. Each MACS column was washed with 15 ml of MACS buffer before the addition of the respective sample. PBMC coupled to MACS beads were eliminated from the samples by flow through the

column in a magnetic field under flow resistance. Each column was washed with 4 ml MACS buffer, and the collected cells were washed twice in serum free medium, and resuspended in Iscove´s complete medium with the addition of Con A (5 µg/ml) as described above. Small aliquots of each sample before and after negative selection were analyzed by flow cytometry to ensure satisfactory purity of each sample before the establishment of each MDM culture.

Blocking of HLA class I and HLA class II molecules. To block the interaction between T lymphocytes and monocytes, monoclonal antibodies directed against constant regions of HLA A B C or HLA-DR (both from Immunotech, Westbrook, ME), or isotype controls; mouse IgG2a or mouse IgG2b (both from R&D Systems) at a concentration of 35 µg/ml were preincubated with 7×10^7 cells in Iscove´s complete medium for 1 hour at 4°C before the addition of Con A. The cells were washed after 16-20 hours of incubation to remove non-adherent followed by the addition of complete 60/30 medium as described above.

Generation of condition medium from Con A stimulated PBMC. Supernatants from Con A stimulated PBMC collected at 4 and 20 h post stimulation were filtered through a 45 µm filter, and cell-free supernatants and transferred to fresh monocyte enriched cell cultures. At 7-9 days the cultures were infected with HCMV at an MOI of 10. Cells were collected

for virus titer assays by scraping at 14 days post infection as described for viral titer assays.

Neutralization of lymphokines in MDM cultures. For neutralizating experiments, polyclonal neutralizing goat antibodies against human TNF-α, IL-1α, IL-2, TGF-ß or IFN-γ(R&D Systems) were used to block the respective lymphokine produced in MDM cultures. Antibodies were added to the cultures at the same time as Con A, and were present in the cultures for 16-20 hours post stimulation. Thereafter, nonadherent cells and antibodies in the cultures were removed by three washes in serum-free medium, and the MDM cultures were cultured in complete 60/30 medium for up to 20 days.

Stimulation of monocyte enriched cultures with recombinant cytokines. Fresh PBMC at a concentration of 1.8 x 10^7cells / ml were enriched for monocytes by plastic adherence in Primaria dishes for 2h at 37°C. Non-adherent cells were removed and the adherent monocyte cultures were stimulated with recombinant IFN-γ (500 U / ml) , IL-1 (2 ng / ml) or TNF-α (10 ng / ml) (all from R&D Systems) in complete 60/30 medium. Parallel dishes were stimulated with Con A as described above. All the cultures were infected with HCMV at 9 days post stimulation at an MOI of 10. Cells were collected for virus titer assays as described

elsewhere, or fixed in 1% PFA, or methanol / acetone (1:1) for immunocytochemistry.

Flow cytometry. A fluorescence-activated cell analyzer (FACScalibur; Becton Dickinson, San Jose, CA) producing 15 mW of light at 488 nm was used for all analyses. The fluorescence signal from 10^4 cells from samples before and after negative selection was obtained. Data were handled with logarithmic amplification and fluorescence intensity was displayed on a 1024-channel, 4 decade log scale delineated in arbitrary log units. Histograms displaying the log fluorescence of FITC (FL1) of the samples before and after negative selection were generated, and the percentage of positive cells was estimated by setting the level for positive cells not to include the background staining of uninfected cells in the negative control.

Results

HCMV replication in Con A–stimulated MDM cells is dependent on CD8+ T lymphocytes. Previous studies have shown that differentiation of monocytes into HCMV-permissive macrophages is dependent on the presence of mitogen-stimulated nonadherent cells in the culture. A key characteristic of these cultures that correlates with viral permissiveness is the ability of the macrophages to form MNGC. Although the appearance of MNGC is correlated with macrophage infectibility, both MNGC as well as cells with a single nucleus stained positive for HCMV-early and -late antigens. The cellular components within the nonadherent cell population that mediate the macrophage differentiation event are unknown. To determine whether Con A–induced differentiation of monocytes to macrophages was dependent on a specific cell phenotype within the nonadherent cell fraction, depletion of different cell phenotypes from fresh PBMC was performed by negative selection techniques. Flow cytometric analysis was performed on cells before and after negative selection to ensure that the residual cell phenotype was< 3% (data not shown). Formation of MNGC was substantially inhibited when CD8+ T lymphocytes were depleted from the culture (Figure 1A and 1B). A 60–65% inhibition in the formation of MNGC was observed when compared with control cultures (Figure 1b) in all donors, whereas MNGC

formation was not changed when CD4+ T cells, CD19+ B cells, or CD56+ NK cells were eliminated from the cultures (Figure 1A and 1B). When these different cultures were challenged with HCMV, we found that the presence of the CD8+ T lymphocytes in the nonadherent cell fraction was necessary for generating HCMV-permissive MDM cells. Elimination of CD8+ cells from the cultures resulted in a 60–70% reduction of the number of HCMV-infected cells, as determined by expression of the HCMV gene products IE (Figure 2A) and gB (data not shown). In contrast, depletion of CD4+ T lymphocytes, CD19+ B cells, or CD56+ NK cells from the cultures did not affect HCMV expression in MDM (Figure 2A). Viral titer assays performed on the different cultures revealed that the production of infectious virus decreased by 4 logs with depletion of CD8+ T lymphocytes (Figure 2B). Thus, CD8+ T lymphocytes are essential for HCMV replication in Con A–differentiated MDM *in vitro*.

The formation of HCMV-permissive MDM is dependent on cell–cell contact via HLA class I molecules. To determine whether cell–cell contact between the CD8+ T lymphocytes and the monocytes was necessary for development of HCMV-permissive MDM, blocking experiments were performed using monoclonal antibodies directed against HLA class I or HLA class II molecules. Monoclonal antibodies directed against HLA class I molecules prevented the formation of MNGC by

65–70% in all experiments (Figure 3A and 3B). In contrast, an effect was not observed with the addition of HLA class II molecules, or with the addition of the respective isotype control sera (Figure 3A and 3B). Blocking of HLA class I–mediated interactions resulted in a 70–80% reduction of the number of IE-positive cells in MDM after HCMV infection (Figure 4A). However, only a 5–20% reduction in the number of HCMV-positive cells was observed using HLA class II–specific antibodies, and an effect on the number of HCMV-positive cells was not observed using isotype control sera (Figure 4A). Analysis of viral production from the respective cultures revealed that production of HCMV decreased by 4 logs in the MDM cultures that were blocked with HLA class I–specific antibodies (Figure 4B). These data suggest that cell–cell contact between CD8+ T lymphocytes and monocytes is important for differentiation of HCMV-permissive MDM, whereas only a minor effect on viral production effect was observed in the HLA class II–blocked cultures (Figure 4B). An effect on viral production was not observed in the presence of isotype control sera in these cultures (Figure 4B).

IFN-γ and TNF-α are essential for productive HCMV infection in MDM. To address the question of whether soluble components in the supernatant of Con A–stimulated PBMC were sufficient to induce differentiation of HCMV-permissive macrophages, we examined the effect

of cell-free supernatants from Con A–stimulated MDM cultures on the viral permissiveness of treated cultures. Cell-free supernatants were collected from Con A–stimulated MDM at 4 and 20 h after stimulation, and were transferred to fresh monocyte-enriched cell cultures. MDM derived from adherent monocytes supplemented with the supernatants produced at 20, but not at 4 h after Con A stimulation, underwent differentiation into HCMV-permissive MDM as determined by viral titer assays (Table I). Thus, soluble components produced by Con A stimulation of MDM 20 h after stimulation were sufficient for permissive HCMV infection in MDM.

To determine if a specific cytokine mediated HCMV-permissive MDM development, polyclonal antibodies with neutralizing activity against IFN-γ, TNF-α, IL-1, TGF-β, or IL-2 were added to Con A–stimulated cultures. The presence of neutralizing antibodies to IFN-γresulted in a 70–80% reduction in the number of MNGC formed in the cultures (Figure 5A and 5B). In addition, a 5–40% reduction of the number of MNGC was observed using TNF-α–neutralizing antibodies (Figure 5B). While the effect of TNF-α on the % of MNGC and the number of infected cells varied between the different donors used in individual experiments; each experiment resulted in a 15–25% inhibition as compared with control cultures. Development of MNGC was not affected using neutralizing antibodies against IL-1, IL-2, TGF-β or isotype control serum (Figure 5A

and 5B, and data not shown). The respective MDM cultures were challenged with HCMV 7–9 d after Con A stimulation. Neutralization of IFN-γreduced the number of HCMV IE–expressing cells by 60–90%, whereas neutralization of TNF-α reduced the number of HCMV IE–positive cells by 5–45% (Figure 6A). Furthermore, adding neutralizing antibodies to IFN-γ and TNF-α reduced HCMV production in MDM cultures by 4 logs and 2 logs, respectively (Figure 6B). Neither the number of HCMV IE–expressing MDM nor the production of virus was affected by adding neutralizing antibodies to IL-1, TGF-β, IL-2, or goat control serum to cultures (Figure 6A and 6B). Thus, the number of MNGC formed in individual cultures correlated with the % of infected cells as well as with the production of virus in each individual experiment.

To assess whether IFN-γand TNF-α were sufficient to induce differentiation of HCMV-permissive MDM, monocyte-enriched cultures were stimulated with recombinant IFN-γ, TNF-α, or IL-1. Indeed, addition of 500 U/ml of recombinant IFN-γ or 10 ng /ml of recombinant TNF-α was sufficient to induce formation of MDM (Figure 7A), which could be infected with HCMV (Figure 7B), and supported unrestricted HCMV replication (Figure 7C). The greatest amount of HCMV was observed with the IFN-γ–treated MDM that produced > 1 x 10^8 pfu/35-mm dish 14 d after infection (Figure 7C). In comparison, lesser amounts of virus were

observed in the TNF-α –treated MDM cultures (5 x 10^5–1 x 10^6 pfu/35-mm dish 14 d after infection), which was similar to the levels of HCMV produced in the Con A–stimulated MDM (Figure 7C). In contrast, recombinant IL-1 (2 ng/ml), which also stimulates monocytes/macrophages, failed to induce formation of MNGC or production of HCMV (< 500 pfu/ 35-mm dish 14 d after infection) (Figure 7C). These data clearly demonstrate a specific but independent role of IFN-γand TNF-α in the differentiation of monocytes to macrophages that are fully permissive to HCMV infection.

IFN-γand TNF-α do not affect HCMV replication in HCMV-permissive macrophages. The above results contrast the common assumption that IFN-γ and TNF-α treatment of cells negatively influences viral replication. However, while these cytokines are important for generating HCMV-permissive MDM, exposure of infected cells at later time points may inhibit viral replication. Therefore, recombinant IFN-γor TNF-α were added to IFN-γ–stimulated MDM cultures at day 1 or day 4 after HCMV infection. As demonstrated in Figure 8, neither addition of IFN-γnor TNF-α altered the production of HCMV in these cells. Although significant levels of expression of both IFN-γand TNF-α receptors were detected on differentiated macrophages (data not shown), neither IFN-γ nor TNF-α demonstrate HCMV antiviral effects in infected MDM.

Discussion

Macrophages are key cell types in the biology of HCMV, and we have developed a unique MDM system to examine the cellular mechanisms involved in viral replication. This study has identified the cellular and cytokine components in the PBMC population that are critical for differentiating monocytes into HCMV-permissive macrophages. CD8+ T lymphocytes were identified as the predominant cell type required for developing monocytes into HCMV-permissive macrophages by using negative selection of different subpopulations of Con A–stimulated PBMC. The use of monoclonal antibodies directed against HLA class I molecules also indicated the necessity of cell–cell contact between the CD8+ T lymphocyte and the monocyte in this *in vitro* system. However, cell-free supernatants from nonadherent PBMC stimulated with Con A for 20 h were sufficient to induce differentiation of HCMV-permissive MDM. Subsequent analyses performed to identify the soluble components that were responsible for macrophage differentiation revealed that IFN-γ, and to some extent TNF-α, played specific but independent roles in differentiating HCMV-permissive MDM. Interestingly, neither addition of recombinant

IFN-γnor TNF-α to HCMV-infected MDM cultures had an effect on HCMV production. Thus, HCMV appears to take advantage of two of the major cytokines with antiviral activity to obtain a specific state of activation in macrophages that allows unrestricted replication of HCMV. These observations have important implications not only in mechanisms of viral activation, but also for the use of these cytokines in antiviral therapy.

Because of the complexity of macrophage differentiation, dissecting the components required for the formation of HCMV-permissive macrophages is crucial for understanding HCMV pathogenesis. In the HCMV-permissive Con A macrophage system, MNGC formation correlated with the ability of virus to infect Con A–stimulated MDM cultures in each individual experiment. While the effect of TNF-α and IFN-γon MNGC formation and the number of infected cells varied between the different donors, adding these cytokines to monocyte cultures consistently resulted in significant production of viral progeny. These observations indicate the importance of TNF-α and IFN-γfor the formation of HCMV-permissive macrophages. MNGC are found *in vivo* in pathologic conditions such as rheumatoid arthritis, sarcoidosis, infection, foreign body reactions, and cancer (for review see references 3, 20). A number of cytokines including IFN-α, INF-β, INF-γ, TNF-α, IL-1, IL-2, IL-4, IL-6, GM-CSF, TGF-β, and LPS, play important roles for specific macrophage

activation pathways, some of which lead to the formation of MNGC (for review see reference 2). Although we have demonstrated that IFN-γ and TNF-α are critical components for the production of HCMV-permissive macrophages, GM-CSF or IL-4 have also been found to induce MNGC formation (1, 93, 152, 191, 284, 285). However, we have previously observed that GM-CSF does not induce HCMV-permissive MDM (unpublished results). In addition, IL-4 and GM-CSF induce the formation of CD83-positive/CD14-negative dendritic cells that are not present in the Con A–stimulated cultures (data not shown).

The macrophage activation pathway induced by IFN-γ and TNF-α was specific, since IL-1, IL-2, TGF-β, or GM-CSF were not critical components in the production of HCMV-permissive Con A–stimulated MDM. Previous studies have demonstrated that the secretion of IFN-γ by Con A–stimulated CD8+ T lymphocytes is mediated by the binding of Con A to at least three cell surface molecules (T200, LFA-1, and Lyt-2), even in the absence of antigen (233). Con A receptors are believed to be intimately involved in antigen recognition and effector functions of CD8+ T lymphocytes (131) since these cells appear to produce IFN-γ in response to specific interactions with their target cells (93, 130, 131, 232, 233). In this study, we found that direct interactions between CD8+ T lymphocytes and macrophages were crucial since antibodies to HLA class I blocked

HCMV infection in MDM. However, cell-free supernatants from Con A–stimulated PBMC or recombinant IFN-γ and TNF-α were sufficient to produce the HCMV-permissive MDM. These observations suggest that direct contact between monocytes and T lymphocytes was essential for production of IFN-γ and TNF-α in Con A–mediated production of HCMV-permissive MDM. Identifying IFN-γ and TNF-α as factors that are necessary for production of HCMV-permissive macrophages has important clinical implications, since Con A stimulation of T lymphocytes may mimic the activation of antigen-specific or allogeneic T lymphocytes during an inflammatory response or allograft rejection, respectively. During such immune-mediated processes, latently infected monocytes that have been recruited to tissue sites and exposed to T lymphocytes may differentiate into macrophages fully permissive for HCMV replication. Subsequent infection of adjacent cells, such as endothelial cells and smooth muscle cells, would have important clinical implications for HCMV disease.

The observation that IFN-γ and TNF-α did not negatively affect HCMV replication in MDM was unexpected since both antiviral cytokines interfere with viral replication at multiple stages. For example, administration of neutralizing antibodies against IFN-γ has been shown to increase mortality in mice infected with herpes simplex virus, and to

increase viral titers of lymphocytic choriomeningitis virus and vaccinia virus (109, 141, 247). Antiviral effects by both IFN-γ and TNF-α have also been demonstrated for HIV infection in macrophages (134, 135, 161, 187). In regard to CMV, treatment of peritoneal macrophages with recombinant IFN-γ significantly inhibits murine CMV replication *in vitro* (102). In addition, IFN-γ production by HCMV IE1–specific human CD4+ T lymphocytes has been shown to inhibit HCMV replication in U373 MG cells *in vitro* (53). Inhibition of CMV replication in murine microglia cells has also been demonstrated by recombinant IFN-γ (221). Furthermore, animal models have suggested a critical role for IFN-γ and TNF-α in regulating CMV replication. Previous studies of CMV infection in mice have demonstrated an important role of IFN-γ for the clearance of murine CMV in infected animals (146, 183), and prophylactic IFN-γ was shown to reduce mortality in CMV-infected mice (70). In support of this observation, increased murine CMV titers were detected in multiple organs in IFN-γ–depleted mice (102). Experiments that will further investigate the mechanism of antiviral resistance to TNF-α and IFN-γ in HCMV-infected MDM are in progress in our laboratory.

In contrast to the antiviral effect of IFN-γ and TNF-α, these cytokines have also been shown to positively influence viral replication. For example, IFN-γ–induced viral replication has been demonstrated in

HIV-infected monocytes and promonocytic cells (17, 135). For CMV, treatment of both immunosuppressed and immunocompetent rats with neutralizing antibodies against IFN-γ protected animals from viral infection (95). In the same study, pretreatment of cells *in vitro* with IFN-γ enhanced viral replication in both macrophages and fibroblasts (95). In regard to TNF-α, this cytokine was also shown to promote CMV replication and pathogenicity in a rat model (94). The ability of TNF-α to increase CMV production in animals may be due to the ability of this cytokine to activate the major immediate early promoter, which regulates genes that are important for viral replication (249). The positive influence of IFN-γ and TNF-α described in the above studies support our observations that these cytokines induce the formation of HCMV-permissive MDM (135). Furthermore, our results are similar to the ability of IFN-γ to induce the formation of HIV-permissive MDM. However, while the addition of IFN-γ after HIV infection reduced viral replication, we demonstrate that HCMV-permissive MDM are resistant to the antiviral effects of these cytokines. The explanation for the conflicting effects of IFN-γ and TNF-α on CMV replication in various systems may be that different cells and viruses respond differentially to cytokine treatment. In support of this hypothesis, the above studies consistently demonstrate that IFN-γ and TNF-α inhibit

HCMV replication in the mouse model, but stimulate viral replication in the rat model.

In conclusion, HCMV appears to use two of the major cytokines involved in combating a virus infection to obtain a specific state of activation in macrophages, allowing unrestricted replication of HCMV. Here we demonstrate that the production of IFN-γ and TNF-α by Con A–stimulated CD8+T lymphocytes is essential for HCMV replication in MDM cells *in vitro*. Thus, IFN-γ and TNF-α induce production of cellular factors necessary for HCMV replication in MDM at the same time as these cells become resistant to the antiviral effects of these cytokines. The identity of such factors is unknown, but experiments to reveal their identity are in progress in our laboratory. Identification of the events critical for HCMV replication is invaluable for studies of HCMV reactivation in the host, and may help to define future targets for therapeutic intervention.

Figure legends

Figure 1. The formation of Con A induced MNGC is dependent on CD8+ T lymphocytes. Negative selection of different cell phenotypes from fresh PBMC was performed to determine whether Con A-induced differentiation of monocytes to macrophages was dependent on a specific cell phenotype within the non-adherent cell fraction (a). Formation of MNGC was substantially inhibited by the lack of CD8+ T lymphocytes in the culture (C), whereas an effect was not observed when negative selection was performed without antibody (A), with anti-CD4 (CD4+ T lymphocytes, B), anti-CD19 (B cells, D), anti-CD56 (NK cells, E) or with isotype control serum (F). b represents quantification of the number of MNGC in each culture established by negative selection of different cell phenotypes, as compared to Con A-stimulated cultures (% MNGC).

Figure 2. Con A-induced differentiation of HCMV-permissive MDM is dependent on CD8+ T lymphocytes. HCMV infection is inhibited in macrophages that were established by negative selection of CD8+ T cells before Con A stimulation of fresh PBMC, but not when CD4+ T cells, CD19+ B cells, or NK cells were depleted from the cultures. A represents the % HCMV IE-expressing cells in the respective MDM culture. In addition, production of HCMV in Con A-differentiated MDM was inhibited

in MDM cultures that were depleted of CD8+ T cells, whereas an effect was not observed when CD4+ T cells, CD19+ B cells, or NK cells were depleted from the cultures (B). The figure represents the viral titer produced at day 14 after infection by the respective MDM culture in a 35-mm culture dish.

Figure 3. Formation of Con A-induced MNGC is dependent on cell-cell contact via HLA class I molecules. To determine whether cell-cell contact between the CD8+ T lymphocytes and the monocytes was important for the development of Con A-differentiated MNGC (a), blocking experiments were performed using monoclonal antibodies directed against HLA class I (B), HLA class II (C), an isotype control antibody (D), or without antibody (A). The formation of MNGC was substantially inhibited by the additing of HLA class I-specific antibodies (B). b represents quantification of the number of MNGC in the HLA-blocked cell cultures, as compared to Con A-stimulated cultures (% multinucleated macrophages).

Figure 4. Con A induced differentiation of HCMV-permissive MDM is dependent on cell-cell contact via HLA class I molecules. HCMV infection was inhibited in macrophages that were established by blocking of HLA class I molecules before Con A stimulation of fresh PBMC, but not

by blocking of HLA class II molecules (A). The figure represents the % HCMV IE-expressing cells in the respective MDM culture. In addition, production of HCMV was also significantly decreased in MDM that were established by blocking of HLA class I molecules before Con A stimulation of fresh PBMC, but not by blocking HLA class II molecules (B). The figure represents the viral titer produced at day 14 post infection by an MDM culture in a 35-mm culture dish.

Figure 5. The formation of Con A-induced MNGC is dependent on production of IFN-γ in the culture. To determine whether a specific cytokine was important for Con A-induced differentiation of monocytes into MNGC (a), neutralization of different cytokines was performed by adding neutralizing antibodies directed against IL-1 (B), TGF-β (C), TNF-α (D), IFN-γ(E), isotype goat serum (F), or without antibody (A) to fresh PBMC before Con A-induced differentiation of monocytes to macrophages. b represents quantification of the number of MNGC in each culture that was established by neutralization of different cytokines before Con A stimulation, as compared to control Con A-stimulated cultures (% MNGC). The formation of MNGC was substantially inhibited adding neutralizing antibodies directed against IFN-γ (a and b).

Figure 6. Con A induced differentiation of HCMV-permissive MDM is dependent on IFN-γand TNF-α. HCMV infection was inhibited in macrophages that were established by neutralization of IFN-γ, and to some extent by neutralization of TNF-α, whereas an effect on HCMV infection was not observed by neutralization of IL-1, IL-2, or TGF-β before esablishing MDM cultures (A). The figure represents the % HCMV IE-expressing cells in the respective MDM culture. In addition, production of HCMV was also significantly decreased in MDM cultures that were established by neutralization of IFN-γ, and to some extent by neutralization of TNF-α, whereas an effect on virus production was not observed by neutralization of IL-1, IL-2, or TGF-β before Con A stimulation of PBMC (B). The figure represents the viral titer produced at day 14 after infection by the respective MDM culture in a 35-mm culture dish.

Figure 7. IFN-γ and TNF-α induce the production of HCMV-permissive MDM. MDM cultures were established by stimulating purified monocytes with recombinant TNF-α (10 ng/ml) and IFN-γ(500 U/ml), and were tested for their ability to replicate HCMV. a represents micrographs of a Con A-stimulated MDM culture (A), a TNF-α-stimulated MDM culture (B), and a IFN-γ-stimulated MDM culture (C) 9 days after infection. HCMV infection of these cultures 9 days after stimulation resulted in a similar

number of HCMV IE-expressing cells in HCMV-infected Con A MDM cultures and TNF-α–stimulated MDM cultures (b). A significant increase in the number of HCMV IE expressing cells, however, was found in IFN-γ-stimulated MDM cultures. In contrast, IL-1 (2 ng/ml) could not induce differentiation of MDM cultures that were permissive to HCMV infection (b and c). To determine viral production in these cultures, macrophages were assessed at various intervals by plaque assay. HCMV-infected IFN-γ-stimulated MDM produced significantly more HCMV as compared to HCMV-infected Con A MDM cultures or TNF-α–stimulated MDM cultures (c), while minimal amounts of virus was detected in IL-1-stimulated MDM cultures (c).

Figure 8. IFN-γ and TNF-α do not inhibit HCMV replication in HCMV-permissive macrophages. Since IFN-γ and TNF-α possess antiviral properties, we examined the effect on HCMV replication by adding these cytokines to MDM cultures after HCMV infection. IFN-γ-stimulated monocyte enriched cultures were infected with HCMV at 9 days after stimulation at an moi of 10. Addition of IFN-γ at a concentration of 500 U / ml or 1000 U / ml, or TNF-α at a concentration of 10 ng / ml or 20 ng / ml to permissive MDM cultures at 1 and 4 dpi, did not affect HCMV production, as determined by

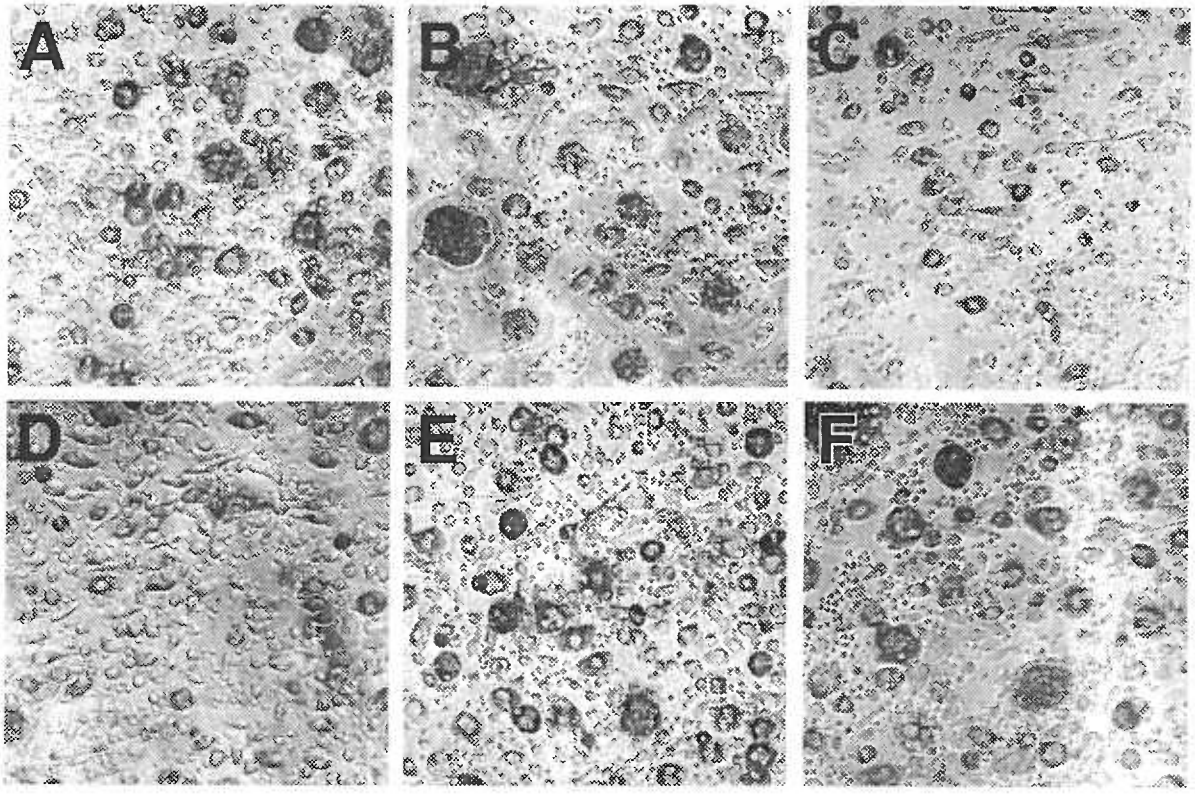
viral titer assays at day 14 after infection. Similarly, viral production was also not inhibited by simultaneous addition of both cytokines at increasing concentrations. These results indicate that rather than inhibiting the replication of virus, IFN-γ and TNF-α induce differentiation of monocytes into HCMV permissive-MDM, which are resistant to the antiviral effects of these cytokines.

Table I. Macrophage Cell-free Supernatant Induces Formation of HCMV-permissive MDM

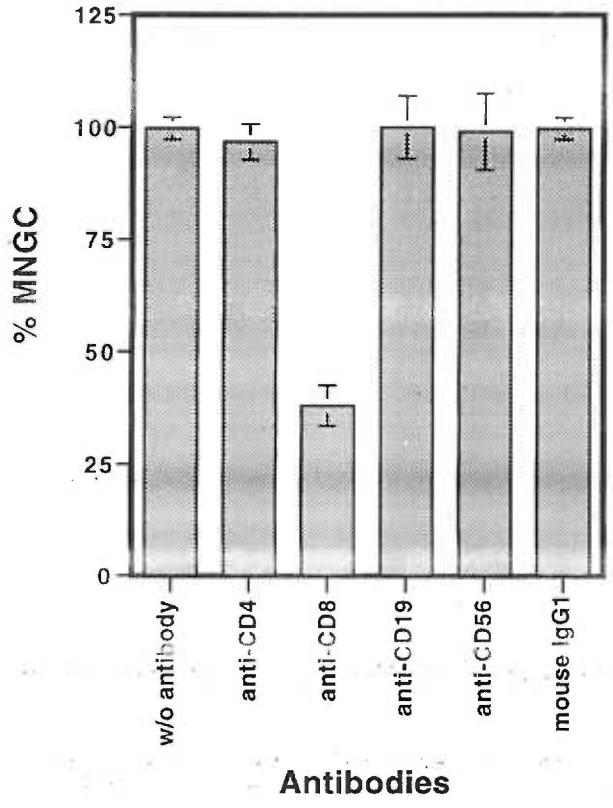
	Monocytes stimulated with cell-free supernatant from Con A—stimulated PBMC	
Con A—stimulated PBMC	4 h	20 h
2.6×10^6	—	1.7×10^6

Viral titers were determined by plaque assay on HF cells (pfu/35-mm dish at 14 d after infection). Number indicates the mean value of two experiments.

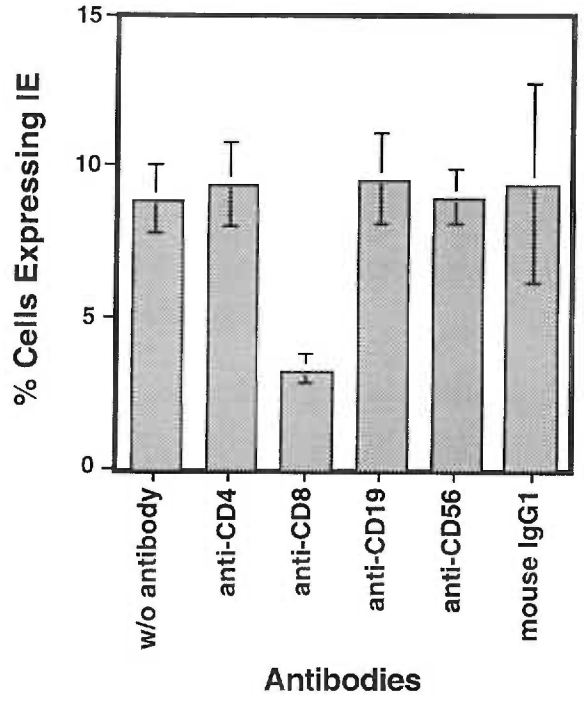
1a


A
B
C
D
E
F

1b


125
100
75
50
25
0
% MNGC
w/o antibody
anti-CD4
anti-CD8
anti-CD19
anti-CD56
mouse IgG1
Antibodies

2A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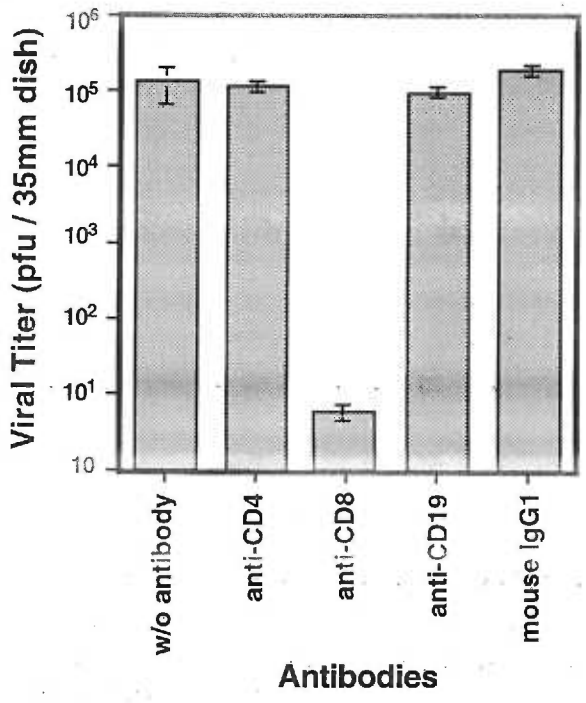
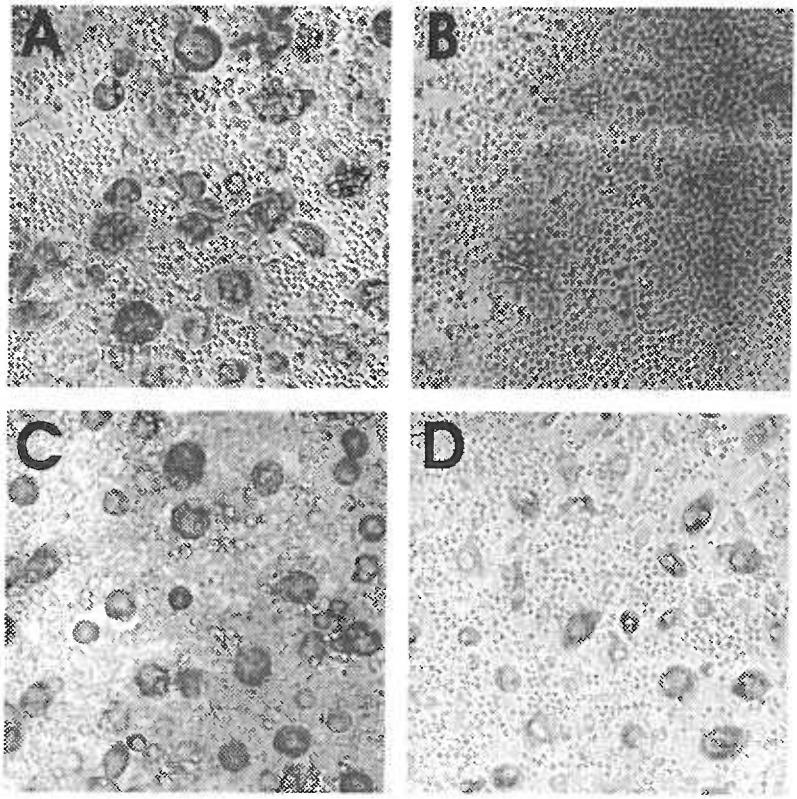
10
5
0
% Cells Expressing IE
w/o antibody
anti-CD4
anti-CD8
anti-CD19
anti-CD56
mouse IgG1
Antibodies

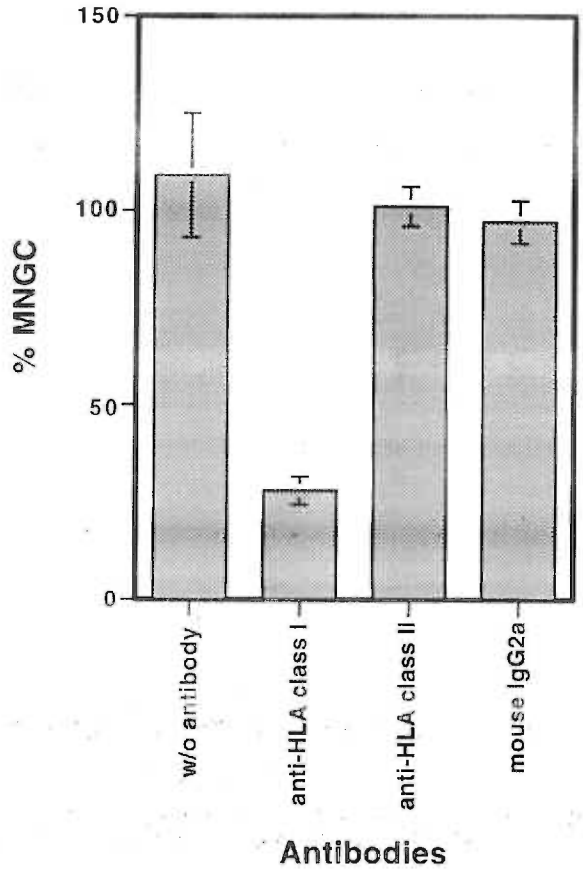
2B

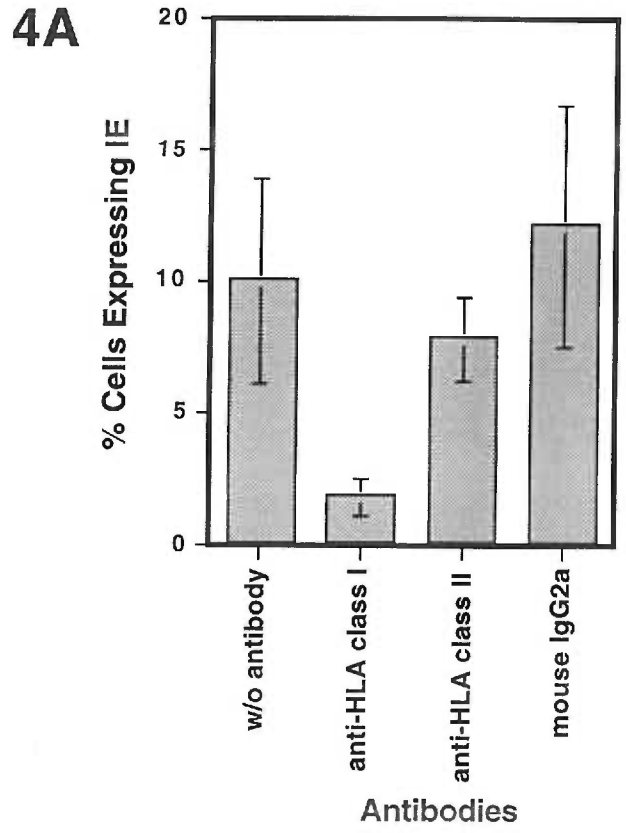

10^6
10^5
10^4
10^3
10^2
10^1
10
Viral Titer (pfu / 35mm dish)
w/o antibody
anti-CD4
anti-CD8
anti-CD19
mouse IgG1
Antibodies

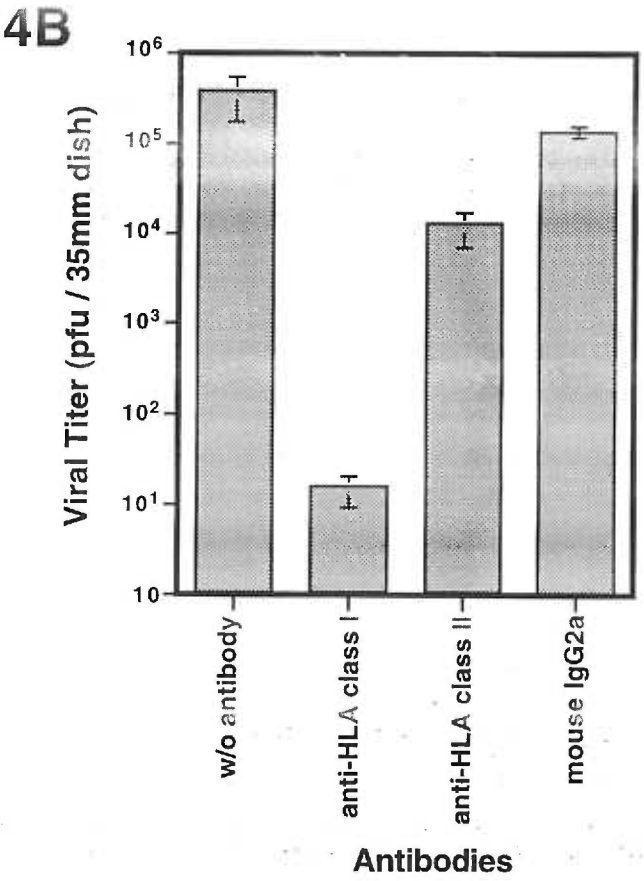
3a


A
B
C
D

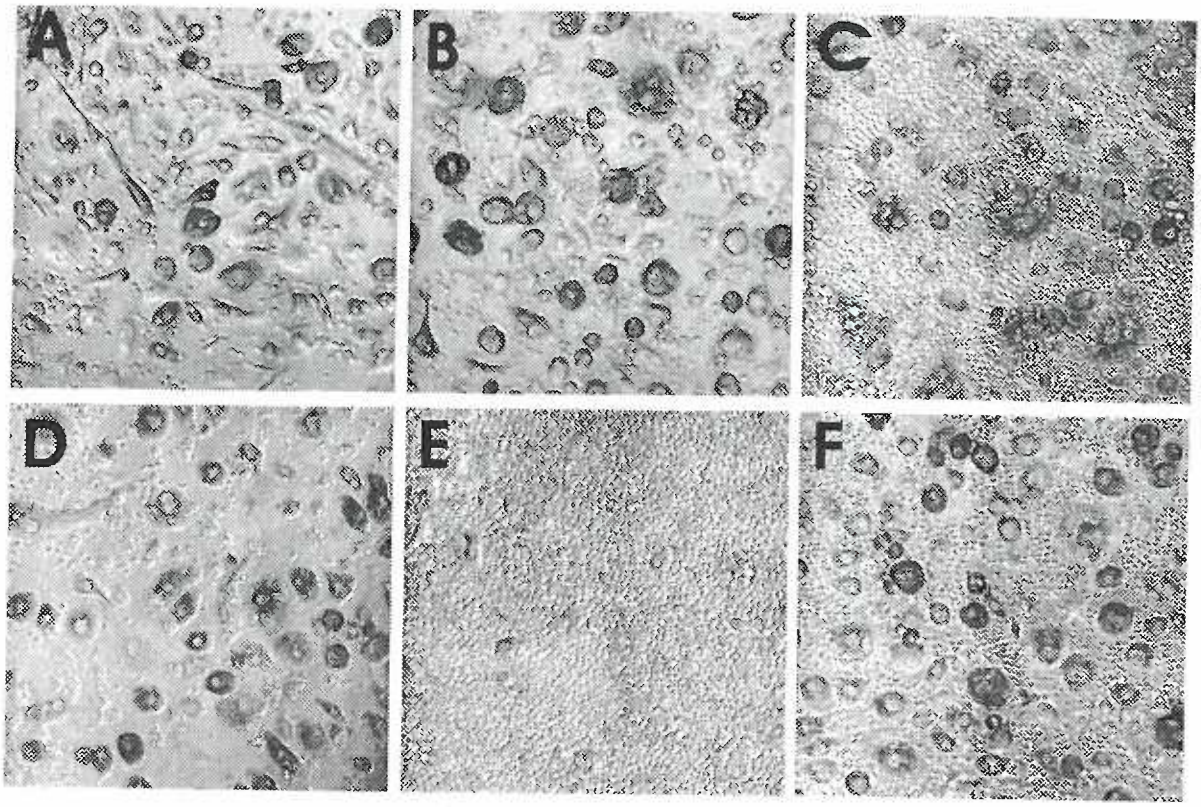
3b


150
100
50
0
% MNGC
w/o antibody
anti-HLA class I
anti-HLA class II
mouse IgG2a
Antibodies

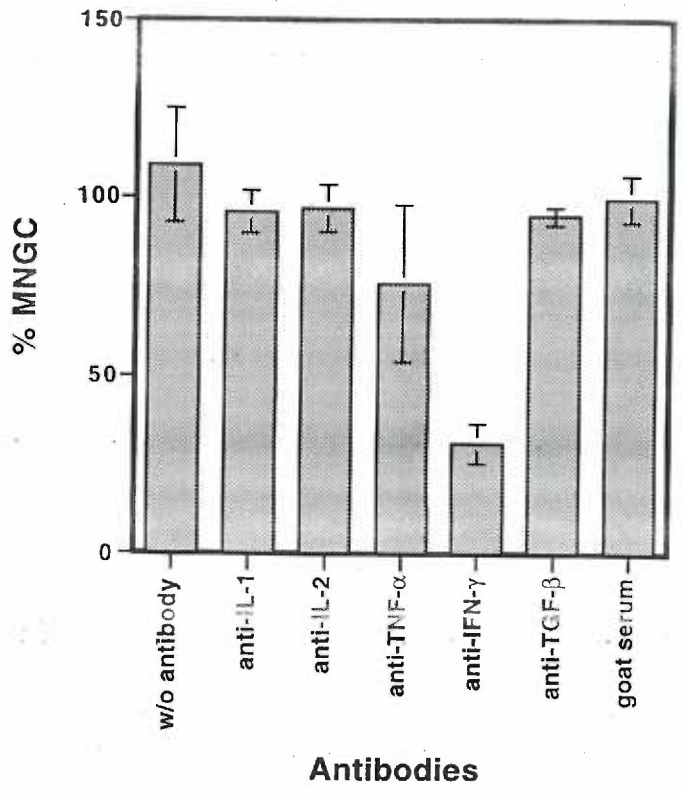

4A
% Cells Expressing IE
20
15
10
5
0
w/o antibody
anti-HLA class I
anti-HLA class II
mouse IgG2a
Antibodies


4B
Viral Titer (pfu / 35mm dish)
10^6
10^5
10^4
10^3
10^2
10^1
10
w/o antibody
anti-HLA class I
anti-HLA class II
mouse IgG2a
Antibodies

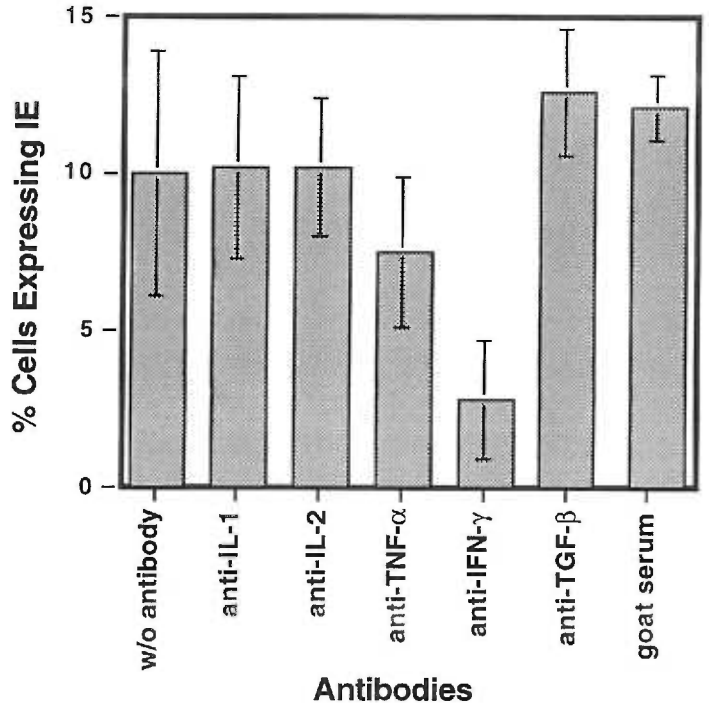
5a


A
B
C
D
E
F

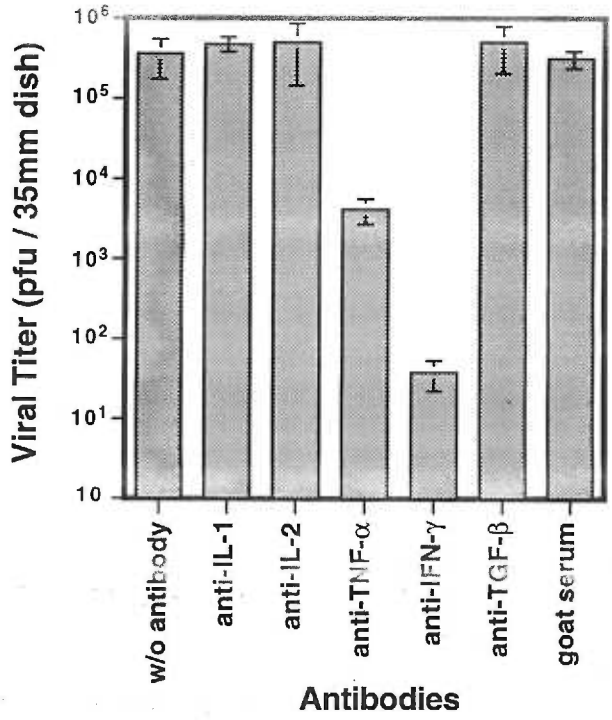
5b


150
100
50
0
% MNGC
w/o antibody
anti-IL-1
anti-IL-2
anti-TNF-α
anti-IFN-γ
anti-TGF-β
goat serum
Antibodies

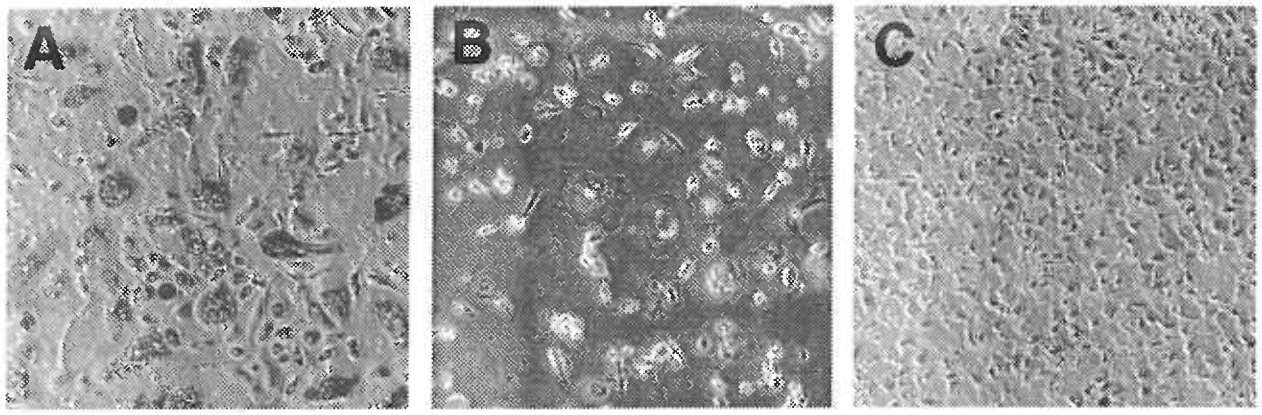
6A


% Cells Expressing IE
15
10
5
0
w/o antibody
anti-IL-1
anti-IL-2
anti-TNF-α
anti-IFN-γ
anti-TGF-β
goat serum
Antibodies

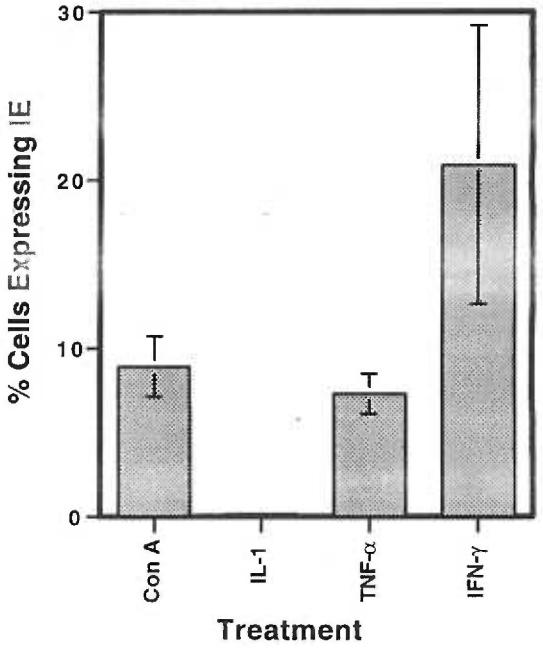
6B


Viral Titer (pfu / 35mm dish)
10⁶
10⁵
10⁴
10³
10²
10¹
10
w/o antibody
anti-IL-1
anti-IL-2
anti-TNF-α
anti-IFN-γ
anti-TGF-β
goat serum
Antibodies

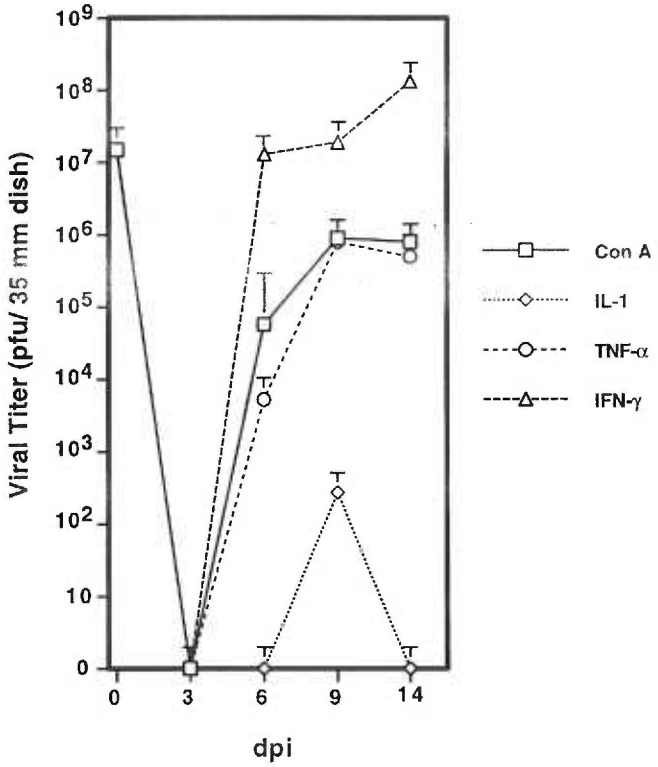
7a


A
B
C

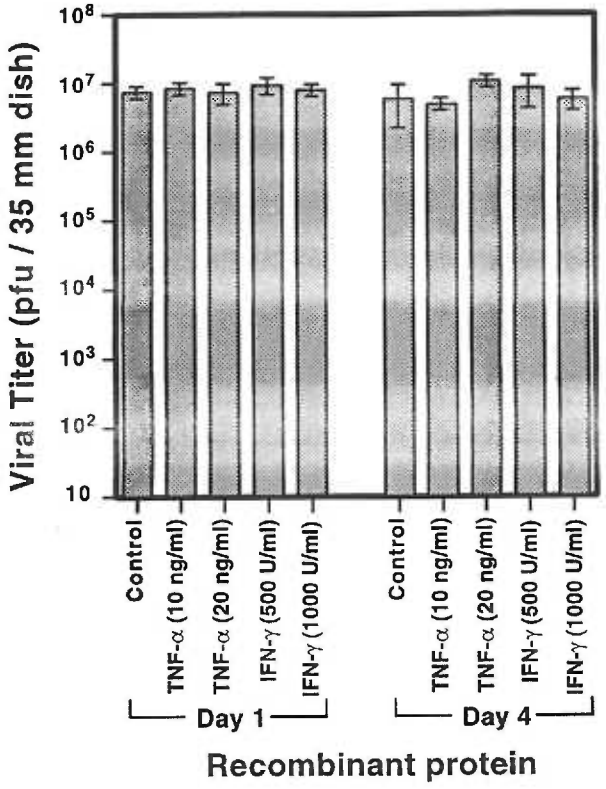
7b


% Cells Expressing IE
30
20
10
0
Con A
IL-1
TNF-α
IFN-γ
Treatment

7c


Viral Titer (pfu/ 35 mm dish)
10⁹
10⁸
10⁷
10⁶
10⁵
10⁴
10³
10²
10
0
0
3
6
9
14
dpi
Con A
IL-1
TNF-α
IFN-γ

8


Viral Titer (pfu / 35 mm dish)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10
Control
TNF-α (10 ng/ml)
TNF-α (20 ng/ml)
IFN-γ (500 U/ml)
IFN-γ (1000 U/ml)
Control
TNF-α (10 ng/ml)
TNF-α (20 ng/ml)
IFN-γ (500 U/ml)
IFN-γ (1000 U/ml)
Day 1
Day 4
Recombinant protein