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**Preservation of natural endothelial cytopathogenicity of
cytomegalovirus by propagation in endothelial cells**

Waldman, W. James, Ph.D.

The Ohio State University, 1990

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Ann Arbor, MI 48106

**PRESERVATION OF NATURAL ENDOTHELIAL CYTOPATHOGENICITY
OF CYTOMEGALOVIRUS BY PROPAGATION IN
ENDOTHELIAL CELLS**

DISSERTATION

**Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of the Ohio State University**

By

W. James Waldman, B.S., M.S.

*** * * * ***

The Ohio State University

1990

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This work is dedicated to my family: to my wife Kay, and my daughter Brenn, for their understanding, support, and unconditional love; to my parents Arthur and Sunny Waldman, for their confidence and assistance; to my mother- and father-in-law Eloise and Arlie White, for their faith in me and their constant encouragement; to my sister-in-law Elise Evans and her family, for their friendship and support; and especially to the memory of my brother Paul, whose confidence in me and whose pride in my accomplishments shall remain with me forever.

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FIELDS OF STUDY

Major Field: Pathology

Studies in human viral pathogenesis, specifically, cytomegalovirus/endothelial cell interactions

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CHAPTER I

INTRODUCTION

I. 1. Rationale

Human cytomegalovirus (CMV) infection is widespread among the world population. Although prevalence varies with age, socioeconomic status, and geographic location,^{1 2 3} it has been estimated that overall, 50-80% of the population shows evidence of CMV exposure by adulthood.⁴ Most infections are apparently asymptomatic;⁵ however, the virus is capable of causing a mononucleosis-like syndrome⁶ and certain types of liver dysfunction.⁷ Although severe disease is rarely a consequence of CMV infection in otherwise healthy individuals, two populations are at risk of developing serious complications, one being the unborn. CMV remains the most common congenital infection,⁸ with a prevalence of 0.4-2.3% of all live births.⁹ Infection of the fetus can be particularly devastating, resulting in a variety of congenital malformations, especially of the central nervous system,^{10 11 12 13} liver disease,^{10 11} and even pre- or neo-natal death.⁴ Additionally, infected infants who are asymptomatic at birth often experience delayed CNS sequelae such as sensorineural hearing loss,^{14 15} moderate

to severe mental retardation,¹⁶ IQ deficit,^{14 17} as well as varying degrees of psychomotor or behavioral complications.^{16 18}

The other major risk-prone group consists of those individuals with compromised immune function, in whom CMV infection is a leading cause of death.¹⁹ Although this is not a new observation,²⁰ it gains new consequence with the growth in numbers of immunosuppressed individuals that has occurred in the last decade.^{21 22 23} Individuals with impaired cellular immunity, attributable to either iatrogenic manipulation or disease process, are at extreme risk of disseminated and frequently fatal infection with this virus. Since the 1960's CMV has overwhelmed recipients of organ transplants and, recently, patients with the acquired immune deficiency syndrome (AIDS). Although pulmonary infection is most common,²⁴ the virus manifests itself in nearly all organs.

Despite its significant health impact, the pathogenesis of CMV disease is poorly understood. The modes of transmission of the virus, as well as its routes of dissemination, are not entirely clear. Its potential to establish latency is strongly implicated,²⁵ however the latent state remains undefined.¹⁹ Specific sites of latency, as well as mechanisms of reactivation also remain elusive.¹⁹ In response to these uncertainties, evidence continues to accumulate implicating significant endothelial involvement in these

processes. Careful studies have indicated that the endothelial cell is a common site of infection regardless of the organ involved. Evidence for CMV infection has been found in endothelial cells within the lung,²⁶ skin,²⁷ retina,²⁸ and gastrointestinal tract,^{29 30 31 32 33} as well as spleen and pancreas,²⁴ and peritoneum.³⁴

These findings suggest the need for an in vitro model of CMV/endothelial cell interaction; however, several recent in vitro investigations of endothelial cell infection by CMV have yielded conflicting results. Friedman et al.³⁵ reported a failure to infect endothelial cells with the Davis strain of CMV, while Ho et al.³⁶ described infection of human endothelial cells by CMV AD169 and two clinical isolates. Additionally, Ho et al.³⁶ noted enhanced cytopathic effect (CPE) in cells inoculated with the clinical isolates as compared with that observed in AD169-infected cultures. In a recent investigation, Smiley et al.³⁷ reported productive infection of human umbilical vein endothelial cells with the Towne strain of CMV without accompanying morphologic cytopathology. Thus the specific nature of the CMV/endothelial cell interaction appears to be both qualitatively and quantitatively dependent upon virus strain and/or the in vitro propagation history of the virus.

Traditionally, CMV has been propagated in fibroblasts, as these cells seem to be the most permissive for virus

reproduction. Although this has proved an effective means for generating large quantities of virus for study, extensive propagation of the virus in this cell type may result in a selection process, restricting the host range of the resulting progeny, while limiting its spectrum of cytopathic potential, as alluded to by Weller.³⁸ In light of the recent emergence of the role of the endothelial cell in human CMV infection, an alternative approach might be in order.

I. 2. Objectives of the Investigation

It is proposed that a strain of CMV raised in endothelial cells may accurately model the CMV/endothelial cell interactions observed in vivo. In pursuing this line of reasoning, a recent clinical isolate of CMV (CMV VHL) has been propagated through multiple passages in human endothelial cells, and separately, in fibroblasts. The infectious properties of the resulting progeny have been investigated and compared with those of the fibroblast-raised laboratory strain, CMV AD169.

This study is defined by the following specific hypotheses:

I. Endothelial cells are natural hosts for cytomegalovirus infection. This issue has been explored by assay of CMV-inoculated endothelial cell cultures for viral DNA, viral antigens, viral enzyme activity, viral repro-

duction, development of virus-specific cytopathology, as well as by direct visualization of intracellular viral particles.

II. Viral properties, including expression of cytopathic potential, viral reproduction, and cell tropism are influenced by the host system selected for in vitro propagation. This hypothesis has been investigated by comparing the characteristics of two sub-strains of CMV which were derived from a single clinical isolate, but propagated separately in fibroblasts and endothelial cells.

I. 3. Overview

The rationale for this investigation has been summarized in this introductory chapter. Objectives of the study, as defined by specific hypotheses have also been listed. In Chapter II, previously published results of experiments bearing particular relevance to this work are detailed. General experimental methods are described in Chapters III, IV, and V, followed by accounts of the specific experiments performed in the course of this investigation (Chapters VI, VII, and VIII). Concluding with Chapter IX, the results of these studies are summarized and the overall significance of this work is discussed.

CHAPTER II

LITERATURE REVIEW

II. 1. Overview

This chapter provides a review of published information relevant to the investigation. The common characteristics of the Herpes group of viruses are listed, followed by a description of the structure and life cycle of cytomegalovirus, justifying its classification as a Herpesvirus. The pathogenic properties of the virus are discussed, beginning with early medical observations of "cytomegalia", and following through with a description of the moderate to severe complications now known to be associated with CMV infection. Clinical studies implicating endothelial involvement are summarized, followed by detailed descriptions of the several published in vitro experiments relating to CMV/endothelial cell interactions. Finally, a discussion of the traditional method of CMV propagation and its potential impact upon previous experimental results is presented.

II. 2. Herpesviruses: Definition and General Characteristics

The Herpesviruses are widely disseminated in nature. Of over 80 identified to date, six have been isolated from humans. These include herpes simplex 1 and 2 (HSV 1 & 2), varicella-zoster (VZV), Epstein-Barr virus (EBV), cytomegalovirus (CMV),³⁹ and most recently, human Herpesvirus 6 (HHV 6).⁴⁰ Structurally, all members of the Herpes group are characterized by a large, double-stranded, linear DNA genome contained within an icosadeltahedral capsid, surrounded by a host-cell derived envelope.⁴¹

Although these viruses vary greatly in their host range and replication kinetics, all share the capacity to establish a latent state in an appropriate host.³⁹

II. 3. Structure of Cytomegalovirus (CMV)

II. 3. 1. The CMV Genome

As with all members of the Herpes group, the genome of CMV consists of a large, linear, double-stranded DNA molecule. The genome of CMV is, in fact, the largest among the human Herpesviruses, extending approximately 240,000 base pairs (240 Kb), with a molecular weight (mw) of approximately 145×10^6 daltons.⁴² CMV DNA has a density of $1.716\text{--}1.717 \text{ g/cm}^3$ (G + C content = 58%) facilitating its separation from human DNA (in infected cells) by density gradient centrifugation.^{43 44}

The gross structure and organization of the CMV genome bears strong resemblance to that of herpes simplex.⁴⁵ CMV DNA is arranged in two segments, the longer of the two (L) representing approximately 82% of the entire length of the molecule, and the shorter (S) representing approximately 18% (Figure 1). Each segment consists of an internal unique sequence (U_L and U_S) flanked by repeats. The junction between the L and S segments is composed of a series of internal repeat sequences (IR_L and IR_S) which, in an inverted form, also flank the ends of the molecule as terminal repeats (TR_L and TR_S).^{46 47}

Another genetic characteristic shared with herpes simplex is the tendency of the CMV genome to isomerize.⁴⁵ As demonstrated by electron microscopy of partially denatured DNA⁴⁸ and restriction pattern analysis,^{42 47 49} a given clone of CMV contains four isomeric forms of DNA in which the U_L and U_S segments are found to be inverted with respect to one another (Figure 1). This effect is thought to result from recombination events mediated through interaction between the terminal and internal repeat regions during rolling circle replication of the genome.^{45 50}

Regarding the fine structure of the CMV genome, although restriction fragment profiles vary considerably among isolates,^{51 52 53 54 55} all strains seem to share approximately 80% homology.⁵⁶

A. CMV GENOME

B. ISOMERIC FORMS

Figure 1: Diagrams representing A) the basic structure of the double stranded DNA genome of CMV, and B) the four isomeric forms generated during CMV DNA replication. a, b, c, d, e, and a', b', c', d', e' represent complementary sequences comprising the terminal (TR) and internal (IR) repeats flanking the unique (U) regions of the long (L) and short (S) segments. x, y, and z, representing unique genetic sequences, have been added to demonstrate the rearrangements resulting from isomerization. mw = molecular weight (adapted from Somogyi et al.)

II. 3. 2. The CMV Virion

The structure of the complete CMV particle is again typical of the Herpes group of viruses.⁴⁵ The genome is wrapped in an apparent torus about a fibrillar spool within the viral core. Encasing the core is a 100-110 nm capsid, an icosadeltahedron composed of 150 hexameric and 12 pentameric capsomeres. Surrounding the capsid is an assymetrically distributed, amorphous globular matrix termed the tegument. The complete virion is enclosed in a bilayered, host-cell derived envelope, although the precise sub-cellular origin of the viral envelope is not completely understood.⁵⁸

II. 4. The Life Cycle of CMV

The major steps in the life cycle of CMV are described below and summarized in Figure 2. As is apparent in the following discussion, many details regarding cellular infection and viral reproduction remain to be elucidated.

II. 4. 1. Entry into the Host Cell

As an obligate intracellular parasite, the first step in the life cycle of CMV must necessarily be the process of entry into a suitable host cell. Although the biochemical basis for CMV adsorption to the host cell surface remains elusive, recent studies suggest that β_2 microglobulin-coated virus may bind to class I HLA molecules expressed on

FIGURE 2: Schematic diagram of the life cycle of cytomegalovirus.

the cell membrane.⁵⁹ Alternatively, Taylor and Cooper⁶⁰ reported the expression by human fibroblast cells, of a CMV-specific, saturable surface receptor, possibly of glycoprotein composition.

Following binding to the host cell membrane, the virus must be internalized. This process is likewise poorly defined at present, but seems to proceed rapidly via some form of endocytosis and/or fusion with the plasma membrane. The de-enveloped capsid is then transported to a nuclear pore where the viral DNA is released into the nucleus.³⁹

II. 4. 2. Biochemical Events of Virus Replication

The replication-related biochemical events that ensue following entry of the viral genetic material into the host cell nucleus seem exceedingly complex. By analogy with the cascade model of protein synthesis regulation observed in herpes simplex-infected cells,⁶¹ CMV gene products have been classified as immediate-early (IE), early (E), or late (L), with the IE and E proteins appearing prior to viral DNA synthesis, and the L proteins appearing afterward.^{62 63 64} Several recent studies, however, have shown that the cascade model does not rigorously apply to CMV (some IE gene products persist throughout the reproductive cycle, and some L proteins appear prior to DNA synthesis^{65 66}). Nevertheless, it is clear that viral gene expression in CMV infected cells proceeds under temporal

and quantitative control.⁶⁷ Thus, for the sake of clarity, the traditional classification system will be used for this description.

Immediately upon nuclear entry of the viral DNA, transcription of a few restricted regions begins, presumably via cellular RNA polymerase II (as is the case for herpes simplex.⁶⁸) These IE genes are characterized by their ability to be transcribed in the absence of de novo protein synthesis, as demonstrated by the appearance of their corresponding mRNAs in the presence of cycloheximide block.^{63 82} Translation of the viral message proceeds in the host cytoplasm by the usual cellular mechanisms. These IE proteins are assumed to be primarily regulatory for subsequent viral replication-related events.^{69 70}

Transcription and translation of a series of early genes rapidly follows the appearance of IE proteins (within 1-2 hours post infection). Synthesis of E polypeptides precedes, and is requisite for viral DNA replication. Accordingly, most of the early proteins characterized to date seem to possess functional properties related to DNA synthesis. These include single-stranded DNA binding proteins,^{71 72} as well as at least two protein kinases.^{73 74} Presently, the best characterized of the early proteins is the virally encoded DNA polymerase.⁷⁵ This enzyme is an α -type polymerase, but differs from its cellular counterpart in its sensitivity to inhibition to phosphonoacetic

acid (PAA), its stimulation by ammonium sulfate, and its ability to catalyze polymerization in the presence of high salt concentrations.^{75 76}

Additionally, by analogy with the herpes simplex β -polypeptides, CMV early proteins may function in down-regulation of IE gene expression, and in the stimulation of late protein synthesis.^{61 77}

The last group of CMV proteins to appear represent products of the late genes. Their expression follows, and is dependent upon viral DNA replication.⁷⁸ These genes code primarily for structural proteins of the virion [reviewed by Landini & Michelson⁷⁹]. Many of the identified late proteins show extensive post-translational modification such as glycosylation^{80 81} or phosphorylation.⁸²

The process of early polypeptide synthesis might be construed as a means of preparation for viral DNA replication. The presence of the virally encoded DNA polymerase and the various accessory enzymes expressed by both the viral and cellular genomes, as well as the availability of nucleotides (provided through cellular metabolic pathways), permit viral DNA synthesis to commence, generally at approximately 12 hours post-infection.⁶³ As with herpes simplex, replication likely proceeds by a rolling circle mechanism, generating extended concatamers which are subsequently excised into unit-length molecules.⁴⁵ The genomic isomerization described above has been postulated

to result from recombination events mediated through interaction between the terminal and internal repeat regions during the excision process.⁵⁰

II. 4. 3. Virion Assembly

Assembly of the virion, as described in detail by Smith and deHarven,⁸³ begins in the host cell nucleus, where empty capsids can be observed approximately 72 hours post-infection. These capsids rapidly fill with viral DNA, migrate peripherally, and bud through the inner nuclear membrane into the perinuclear cisterna, retaining a portion of the nuclear membrane as the viral envelope. From the cisterna, enveloped particles migrate through the cytoplasm within vacuoles produced by evagination of the outer nuclear lamella. Subsequent fusion of these vacuoles with the cellular plasma membrane facilitates extrusion of the complete virions.

It should be noted, however, that the precise sub-cellular source of the viral envelope remains an unsettled issue. Alternative models of capsid envelopment have been proposed involving a variety of host cell membrane components, such as endoplasmic reticulum, golgi, and plasma membrane.⁵⁸

II. 4. 4. Viral Latency

The events described in the previous sections represent the postulated mechanisms of active CMV reproduction in the permissive host. However, as mentioned earlier, an alternative outcome of infection (by any member of the Herpes group) is the establishment of a state of latency. Under these circumstances, viral components are present within the infected cell, but remain essentially inactive until some stimulus triggers the resumption of active replication.

Unfortunately, this very important aspect of the CMV life cycle is still largely a mystery.²⁵ Although specific latency-associated antisense transcripts have been detected in spinal and trigeminal ganglion neurons of herpes simplex-infected individuals,^{84 85 86} no analogy has been discovered for CMV infection. However, results of a recent study involving healthy, CMV-seropositive individuals suggest that cells harboring latent virus might be identified by their expression of an IE antigen.⁸⁷ The investigators found IE-positive cells of many lineages to be morphologically normal and widely distributed among various organs. These results imply that CMV can establish latency in a variety of cell types within most any organ.

Regardless of the progress that has been made, hard data regarding the mechanisms of latency are still lacking. The specific signals that determine which "life style" the

virus chooses have yet to be identified. The physical form that the virus assumes during latency is unknown, as is the qualitative and quantitative nature of the viral biochemical activity. Although immunosuppression seems to permit reactivation of latent CMV, the specific signals that trigger this process, and the mechanisms by which it occurs are still poorly understood.

II. 5. CMV as a Human Pathogen

II. 5. 1. "Cytomegalia": Early Observations

The earliest published observations of what can now be recognized as human CMV disease date back to the beginning of this century [reviewed by Ho⁸⁸]. Between 1904 and 1920, many reports appeared describing large "protozoan-like" cells containing a "central nuclear body" surrounded by an outer clear zone. Such cells were found in kidneys, lungs, liver, and thyroid gland of stillborn infants or gravely ill neonates, and in salivary glands of apparently healthy infants. The dominant theory at that time was that these inclusions represented protozoa.

Goodpasture and Talbot,⁸⁹ after observing similar pathology in the lungs, kidneys, and liver of a 6-week old child, were the first to challenge the protozoal origin of the lesions. Von Glahn and Pappenheimer⁹⁰ described the first adult case of cytomegalia, and concluded, based on previous observations of herpes virus lesions, that the

inclusions were a result of infection by a virus identical or closely related to the Herpes group. These findings were confirmed by Goodpasture⁹¹ and later, by Cowdry,⁹² both of whom demonstrated definitively the relationship between cytomegalic inclusion disease (CID) and viral infection.

The isolation of the etiologic agent of CID did not occur until the 1950's, when tissue culture techniques had advanced sufficiently to permit routine in vitro growth of human cells. In the last half of that decade, three strains of CMV were independently isolated and propagated in vitro,^{93 94 95} as described in section II. 8. below. Subsequent refinements in isolation and culture techniques, in addition to the development of immunologic staining and nucleic acid hybridization methods, have greatly improved the detection of viral infection, ushering the molecular age of clinical diagnosis.

II. 5. 2. CMV Infection in the Immunocompetent Host

Human cytomegalovirus infection is widespread among the world population. Although prevalence varies with age, socioeconomic status, and geographic location,^{1 2 3} it has been estimated that overall, 50-80% of the population shows evidence of CMV exposure by adulthood.⁴ The competent immune system is apparently capable of coping effectively with the virus without accompanying morbidity; therefore most infections remain sub-clinical.⁵

However, CMV is capable of causing disease in the apparently immunocompetent host, most notably a mononucleosis-like syndrome.⁶ % This condition resembles EBV mononucleosis, being characterized by fever, malaise, lymphocytosis, the presence of atypical lymphocytes, and occasionally, mild liver dysfunction. However, unlike the EBV-induced counterpart, no exudative pharyngitis or lymph node enlargement has been observed.

Less frequently, isolated liver dysfunction has been associated with CMV infection,⁷ as characterized by hepatomegaly and elevated liver function tests.

Regardless of the clinical outcome, it seems probable, based on epidemiological data, that following primary infection by the virus, a state of latency is established within the host. If so, this places most individuals in a position of life-long susceptibility to complications that may develop as a result of viral reactivation.

II. 5. 3. Congenital CMV Infection

Although severe disease is rarely a consequence of CMV infection in otherwise healthy individuals, two populations are at risk of developing serious complications, one being the unborn. In fact, prior to the recent explosion of the immunosuppressed population brought about by organ transplantation and the acquired immune deficiency syndrome (AIDS), complications resulting from congenital CMV

infection represented the sole major health impact associated with the virus. In spite of efforts to reduce its prevalence through improved hygiene and pre-natal care, CMV remains the most common congenital infection,⁸ with a prevalence of 0.4-2.3% of all live births.⁹

Infection of the fetus occurs by transplacental transmission of the virus following primary maternal infection or reactivation from latency.^{18 97} Although pre-existing maternal immunity may afford the fetus some degree of protection from overwhelming systemic infection, such immunity does not prevent reactivation or intrauterine infection. However, results of several large scale clinical studies suggest that serious fetal sequelae are much more likely to result from primary maternal CMV infection than from reactivation of latent virus.^{97 98 99}

The effects of fetal infection can be particularly devastating, and include a variety of congenital malformations, especially of the central nervous system,^{10 11 12 13} liver disease,^{10 11} and even pre- or neo-natal death.⁴ Additionally, infected infants who are asymptomatic at birth often experience delayed CNS sequelae such as sensorineural hearing loss,^{14 15} moderate to severe mental retardation,¹⁶ IQ deficit,^{14 17} as well as varying degrees of psychomotor or behavioral complications.^{16 18}

II. 5. 4. CMV Infection in Allograft Recipients

Another population at risk of developing serious CMV-associated complications consists of organ transplant recipients, in whom CMV infection is a leading cause of death.¹⁹ Although this is not a new observation,²⁰ it gains new consequence with the growth in numbers of allograft recipients that has occurred in the last decade.^{21 22} Individuals with impaired cellular immunity, attributable to the transplantation process and the associated pharmacological immune suppression, are at extreme risk of disseminated and frequently fatal infection with this virus.

Often the source of the virus is the allograft recipient himself, with drug-induced immunosuppression permitting reactivation of previously latent CMV.¹⁰⁰ Alternatively, donor strains of latent virus may accompany the graft, then reactivate within the new host.^{101 102} Finally, latent virus may be introduced into the transplant recipient via transfusion of blood products.¹⁰³

Regardless of the route of transmission, CMV has earned notoriety as one of the most troublesome sources of transplantation-associated complications, with as many as 90% of allograft recipients affected.¹⁰⁰ Although evidence of viral infection has been found in nearly all organs, pulmonary infection remains most common.²⁴ CMV pneumonia is a major cause of death among both renal allograft recipients¹⁰⁴ and

bone marrow transplant patients.¹⁰⁵ Diffuse mucosal ulceration has been associated with CMV infections of the gastro-intestinal tract in these patients.^{22 31} CMV retinitis is another common complication, often progressing to a vision-threatening stage.^{28 106} Although somewhat controversial, post-transplantation renal glomerulopathy may also be related to CMV infection.^{107 108} CMV hepatitis has been reported in liver transplant recipients,^{109 110} at times accompanied by vanishing bile-duct syndrome.¹¹¹ Recently, an etiologic role has been proposed for CMV in the accelerated development of atherosclerosis within cardiac grafts.¹¹² In addition to the above examples, evidence for CMV infection in transplant recipients has been found in nearly every organ of the body,²⁴ and has been postulated to play a role in graft rejection.¹⁰³

II. 5. 5. CMV in the Acquired Immune Deficiency Syndrome

A significant contribution to the growing immunocompromised population has come from the recent epidemic of the acquired immune deficiency syndrome (AIDS).²³ The loss of immune system integrity that occurs in AIDS leaves these patients particularly vulnerable to frequently fatal disseminated CMV infection. Nearly 100% of AIDS patients show serologic evidence of active CMV infection, and over 90% show evidence at autopsy. Although many of these infections are sub-clinical, it is estimated that life-

threatening complications result in over 7% of AIDS patients.²³

The spectrum of clinical complications arising from CMV infection in the AIDS patient closely resembles that observed in immunosuppressed allograft recipients, as described above. Common pathology includes interstitial pneumonitis,^{113 114} alimentary tract ulceration and perforation,^{32 115 116} and retinitis,¹¹⁷ as well as infection of adrenal glands and the central nervous system.^{114 118} Frequently, patients show evidence of multiple site involvement.¹¹⁴

Although the clinical picture presented here closely resembles that observed in the immunosuppressed allograft recipient, several recent studies suggest that, rather than simply representing another opportunistic agent, CMV may act synergistically with the human immunodeficiency virus (HIV). Accelerated progression toward overt disease has been reported in HIV-positive individuals showing signs of active CMV infection.¹¹⁹ On the molecular level, the CMV immediate-early gene region has been shown to trans-activate expression of genes fused to the HIV long terminal repeat (LTR) region, resulting in augmented mRNA synthesis.¹²⁰ The potential for synergistic interaction has also been suggested by the *in vitro* studies of Skolnik et al.,¹²¹ in which reproduction of HIV in lymphoblastoid cells or monocytes was enhanced by co-infection of the cells with

CMV. Indeed such co-infection has been observed in brain cells of AIDS patients.¹²² In addition to augmenting viral reproduction, CMV may act to expand the host range of HIV by upregulating cell surface Fc receptor expression, thereby permitting internalization of HIV-antibody complexes by cells not normally susceptible to HIV infection.¹²³

II. 5. 6. CMV: Additional Postulated Etiologic Associations

The oncogenic potential of certain members of the human Herpes group of viruses is well established. HSV-2 has been associated with cervical cancer.¹²⁴ EBV, an agent possessing documented transforming potential in vitro, has been assigned an etiologic role in the development of Burkitt's lymphoma and at least one form of undifferentiated nasopharyngeal carcinoma.¹²⁵ As regards CMV, a causal role in malignancy remains undefined. However the virus has been shown to stimulate host cell DNA and RNA synthesis.^{126 127 128} Significantly, cellular transformation has been induced in vitro by live virus,¹²⁹ UV-irradiated virus,¹³⁰ as well as by introduction of cloned CMV genomic fragments.^{131 132} In addition, CMV infection has recently been shown to rapidly and dramatically upregulate transcription of the proto-oncogenes c-fos, c-jun, and c-myc.¹³³ Clinically, an etiologic role for CMV has been proposed in adenocarcinoma of the prostate¹³⁴ and colon,¹³⁵

as well as in Kaposi's sarcoma.^{136 137 138 139} However, evidence has often been circumstantial, precluding the conclusive assignment of a direct causal role for CMV in the development of malignancy at this time.

Finally, it should be noted that findings of several investigations have implied a role for CMV (and other Herpesviruses) as a contributing etiologic factor in atherogenesis. This association was first suggested by results of a large scale detailed animal study, in which atherosclerosis was induced in chickens by experimental inoculation with sub-lethal titers of Marek's disease Herpesvirus.¹⁴⁰ The lesions that developed in these animals closely resembled human arteriosclerotic plaques. In a related follow-up study, Fabricant et al.¹⁴¹ demonstrated enhanced cholesterol and cholesteryl ester accumulation in cultured chicken smooth muscle cells infected with either of two avian Herpesviruses.

Although the evidence for an etiologic role for CMV in atherogenesis is not quite so compelling, several investigations have yielded provocative results. CMV-specific antigens and nucleic acids have been detected in arterial smooth muscle cultures derived from punch-biopsies obtained during vascular surgery for atherosclerosis.^{142 143} Indeed, human arterial smooth muscle cells have been shown to be permissive for CMV infection, and to support CMV reproduction in vitro.¹⁴⁴ In addition, several recent clinical

studies have demonstrated that CMV infection within arterial walls (as determined by in situ hybridization, immunologic staining, and polymerase chain reaction) is a common occurrence, with a higher prevalence in patients suffering from advanced atherosclerosis.^{145 146} Perhaps this association has been most strongly suggested by results of a recent investigation involving over 300 cardiac transplant recipients.¹¹² In this study, the CMV-infected group (91 patients) showed significantly higher incidence of severe graft atherosclerosis, graft rejection, and death, than did the remainder of the (CMV disease-free) patients. As with the question of the virus's oncogenic potential, insufficient evidence has accumulated to conclusively assign an etiologic role for CMV in atherogenesis. Clearly, however, enough suggestive data are available to postulate such a relationship.

II. 6. CMV Infection in Endothelial Cells: Clinical Observations

Despite its significant health impact, the pathogenesis of CMV disease is poorly understood. The modes of transmission of the virus, as well as its routes of dissemination, are not entirely clear. Its potential to establish latency is strongly implicated,²⁵ however the latent state remains undefined.¹⁹ Specific sites of latency, as well as mechanisms of reactivation also remain elusive.¹⁹ In

response to these uncertainties, evidence continues to accumulate implicating significant endothelial involvement in these processes. Studies of tissue specimens obtained from allograft recipients and AIDS patients by biopsy or at autopsy have shown that the endothelial cell is a common site of CMV infection regardless of the organ involved. Results based on morphological criteria, immunologic staining, and/or nucleic acid hybridization in situ have demonstrated the presence of the virus in endothelial cells within the lung,²⁶ skin,²⁷ retina,²⁸ and gastrointestinal tract,^{29 30 31 32 32} as well as spleen and pancreas,²⁴ and peritoneum.³⁴ In addition, the expression of CMV immediate-early antigen by endothelial cells within multiple normal organs of seropositive healthy individuals⁸⁷ suggests this cell type as a candidate for a reservoir of latent virus.

Recent advances in the understanding of the complexities of endothelial cell biology have rendered obsolete the concept of the endothelium as a simple inert barrier. In addition to functioning as an anti-thrombogenic internal vascular lining, endothelial cells, through the expression of a large array of factors, act in both agonist and antagonist capacities in the soluble coagulation system and in platelet adherence.¹⁴⁷ It has also become clear that the endothelium interacts dynamically with the immune system in leukocyte adherence, major histocompatibility complex (MHC) antigen expression, foreign antigen presen-

tation, as well as in the production and release of certain cytokines.¹⁴⁶ Furthermore, endothelial cells play an active role in angiogenesis, both under physiologic conditions (tissue regeneration, wound healing), and in a pathologic context (neoplasia, inflammatory disease).¹⁴⁷ Finally, these cells are involved in the synthesis of vascular stromal components and in vascular tone regulation.¹⁴⁷

Clearly then, CMV infection of endothelial cells may have far reaching implications. Virally induced physical damage or metabolic derangement may upset the balance of thrombogenic controls, and thus explain the role of the virus in atherogenesis. Alteration in expression and processing of surface antigens may render the CMV-infected endothelial cells highly immunogenic, leading to over-amplified, destructive inflammatory responses. Enhancement of MHC class I antigen expression has, in fact, been demonstrated in cultured endothelial cells following inoculation with CMV,¹⁴⁹ a finding which suggests a specific role for the virus in allograft rejection. Finally, it is conceivable that CMV infection might disturb the normal regulation of endothelial angiogenic activities. Herein may lie the connection between the virus and the over-proliferated, aberrant vascular patterns of Kaposi's sarcoma.

As indicated by this discussion, many important questions have arisen (and continue to arise) regarding the

response of the endothelial cell to CMV infection. As an approach to understanding these virus/cell interactions, an accurate in vitro model would be very useful.

II. 7. CMV/Endothelial Cell Interactions: In Vitro Models

There have been only a few published attempts at evaluating CMV/endothelial cell interactions in vitro. Results of these studies have been somewhat inconsistent, and have often been difficult to resolve with clinical observations.

Friedman et al.³⁵ were unable to detect CMV infection in cultured endothelial cells from either human umbilical vein or bovine thoracic aorta. Cultures were inoculated with the Davis strain (see II. 8.) of CMV (0.3 MOI^a), observed for the development of CPE, and stained by immunofluorescence for CMV-specific antigens. In addition, inoculated cells were harvested and assayed for infectious virus production by inoculation onto fibroblast monolayers. Although the endothelial cells were susceptible to infection by many of the other viruses evaluated in this study (HSV 1 and a variety of respiratory and enteroviruses), no evidence for CMV infection was found.

^a MOI (multiplicity of infection) = ratio of the titer of the inoculum to the number of cells within the inoculated monolayer.

Ho et al.³⁶ reported successful infection of human umbilical vein endothelial cells with CMV AD169 (see II. 8.) and two clinical isolates. The cultures were inoculated with virus at various MOI (0.01-1.0) and evaluated for evidence of infection using basically the same strategies employed by Friedman et al.,³⁵ as described above. Induction of cytopathic changes by AD169 was observed only at the highest input titer (1.0 MOI), which may explain Friedman's failure to demonstrate CMV infection. The clinical isolates, however, produced CPE in the endothelial cells even when inoculated at an MOI of 0.1. Results of immunofluorescent staining were consistent with the above findings, and infectious virus production was demonstrated by virus yield assays of sonicated infected cells.

More recently Smiley et al.³⁷ demonstrated productive CMV infection in human umbilical vein endothelial cells. Cultures were inoculated with low passage (P₃₆₋₃₉) or high passage (P₁₂₄) Towne strain (see II. 8.), or strain K9V (isolated from a Kaposi's sarcoma cell line¹⁵⁰), at input titers of 2.5-12.5 plaque forming units (PFU)/cell. Human embryonic lung fibroblasts (HEL or MRC-5) were similarly inoculated for comparative purposes. Cultures were observed for the development of cytopathic changes, and harvested at intervals (up to 10 days) for quantitation of infectious virus production by plaque assay. As an additional

indication of viral reproductive activity, DNA dot blot hybridizations were performed on nucleic acid extracts at various intervals post-inoculation, using radiolabelled Towne strain DNA as a probe. Results of these experiments demonstrated equivalent production of all CMV strains. However, the levels of infectious virus produced in endothelial cells were quite low as compared with those produced in fibroblasts. Curiously, active CMV replication was observed in endothelial cells in the absence of accompanying cytopathic effect.

In another recent investigation, Waldman et al.¹⁵¹ demonstrated enhanced endothelial cytopathogenicity induced by infection with a CMV isolate which had been propagated in human umbilical vein endothelial cells, as compared with that induced by AD169. Cultures were inoculated with each strain of virus at 10^{-2} -1.0 MOI, and observed for the development of cytopathic changes. Inoculated cultures were further assayed by *in situ* hybridization using biotinylated CMV-specific DNA probes, and by immunofluorescent staining for CMV antigen expression. In addition, viral reproduction in endothelial cells and similarly inoculated fibroblasts was quantified by plaque assay of culture supernatants. Results of these experiments revealed dramatic differences in the infectious characteristics of the two strains. The endothelial-raised isolate was shown to exhibit greatly

enhanced endothelial cytopathic potential, and replicated more efficiently in endothelial cells than did AD169.

Taken collectively, these results suggest the specific nature of the CMV/endothelial cell interaction to be a function of the particular virus strain and/or the in vitro propagation history of the isolate. This point is particularly exemplified by the studies of Waldman et al.,¹⁵¹ and by Ho's³⁶ findings of apparent attenuated infectivity AD169 as compared with that of recent CMV isolates. Indeed both Bruggeman et al.¹⁵² and Radsak et al.¹⁵³ have reported very low infection efficiency in endothelial cells inoculated with Towne strain,¹⁵² AD169, or Kerr strain,¹⁵¹ even at high input titers (5-10 M.O.I.).

II. 8. Propagation of CMV In Vitro: A Key Issue

Isolation and in vitro propagation of CMV was first accomplished in 1956-57 by three independent research teams.^{93 94 95} CMV AD169 was isolated by Rowe et al.⁹⁴ from adenoid tissue of a 7-year old girl with no reported signs of CMV disease. The virus was propagated in fibroblast culture, and although for the earliest passages the cultures were not pure, all cytopathic changes reported were observed in fibroblast cells. Progression of cytopathology was at first very slow, requiring 6-8 weeks for total monolayer destruction. However, with each passage of the infected cells onto fresh cultures, CPE appeared more

quickly and cytopathic destruction of the cells was accelerated. Another effect that evolved with continued passage of the virus was a progressive increase in titer of infectious virus released into the culture supernatant. Since its isolation, AD169 has proceeded through many generations in various fibroblast cell lines,^b and has been widely used as an in vitro model in studies of CMV/cellular interaction, genomic analysis, as well as many other types of molecular and biochemical experiments.

In 1957 Weller et al.⁹⁵ reported on the isolation and in vitro propagation of three strains of CMV which have also come to be used extensively in vitro. The Davis strain was derived from a liver biopsy of a 3-month old microcephalic infant with jaundice and hepatosplenomegaly, as well as other clinical signs of disseminated congenital CMV disease. Strain Kerr was propagated from a urine specimen of a 5-day old neonate who showed evidence of liver disease within 8 hours after birth. The Esp strain was similarly derived from a urine specimen of a 3-month old infant with hepatosplenomegaly, periventricular cerebral calcification, and progressive chorioretinitis. As with AD169, propagation of all three strains was accomplished by serial passage through fibroblast cultures, resulting in the same pattern of in vitro evolution as that observed by Rowe et al.⁹⁶

^b See Appendix D.

This process has come to be known as fibroblast-adaption, and has been exhibited by all CMV strains propagated to date.¹⁵⁴

Another very commonly used strain of the virus, the Towne strain, was isolated in 1970¹⁵⁵ from the urine of a 2-month old microcephalic infant with hepatosplenomegaly. The virus has subsequently been passaged well over 100 times in fibroblast culture (WI-38), and thus, as described above, has undergone the process of fibroblast-adaption.

Many other CMV strains have been isolated, and all have been propagated in fibroblasts, as these cells seem to be the most permissive for virus reproduction. Similarly, most of the knowledge regarding the nature of the virus is based upon data generated by fibroblast-based studies. A few investigators have reported in vitro CMV infection in other cell types, such as monocytes,¹⁵⁶ brain astrocytes,¹⁵⁷ epithelioid cells,^{158 159 160} kidney mesangial cells,¹⁶¹ and endothelial cells (see II. 7.), however in all cases, the inocula have been exclusively fibroblast-raised.

Although fibroblast-adaption has proved an effective means for generating large quantities of virus for study, it seems reasonable that extensive propagation of the virus in this cell type may result in a selection process, restricting the host range of the resulting progeny, while limiting its spectrum of cytopathic potential, as alluded to by Weller.³⁸ This concept bears strong analogy to the

process of serial in vitro culture of prokaryotic or eukaryotic cells, during which extracellular environmental conditions can dramatically affect cellular characteristics, resulting in a progeny with very different properties than those of the tissue from which the specimen was derived. This adaptive response may bear particular relevance in the case of CMV propagation; infected fibroblasts are rarely observed in vivo and, thus, may represent a permissive, yet unnatural host for the virus. Indeed, as described above, fibroblast-adapted progeny behave quite differently than does freshly isolated virus.

II. 9. Summary

Endothelial involvement in human CMV disease has been well documented. Studies of tissue sections obtained by biopsy and at autopsy have shown evidence of CMV infection in endothelial cells within most organ systems. Most often, infected cells have exhibited classical CMV-associated morphologic derangements (cytomegaly, nuclear and cytoplasmic inclusions). However, results of in vitro studies of CMV/endothelial cell interactions have frequently been difficult to resolve with clinical observations, possibly due to the exclusive use of fibroblast-adapted virus in these experiments. In order to accurately model the virus/cell interactions as they occur in vivo, an

alternative approach involving an endothelial-raised CMV strain might be beneficial.

CHAPTER III

GENERAL METHODS 1: CELL CULTURE

Underlying all experiments performed throughout the course of this investigation was an extensive battery of tissue culture techniques. Procedures described in this chapter include cell isolation and culture maintenance, culture expansion, cell enumeration, and cryopreservation.

III. 1. Cell Isolation/Primary Culture

In this section, methods of tissue dissociation and isolation particular to the cell lineages used in these studies are detailed. Primary culture maintenance is also discussed.

III. 1. 1. Primary Culture: Neonatal Human Dermal Fibroblasts (NHDF)

Neonatal human dermal fibroblast cells (NHDF) were derived from human foreskins as follows. Foreskins acquired from normal neonatal circumcisions were washed thoroughly in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl,

8.1 mM Na_2HPO_4 , 1.5 mM KH_2PO_4),^a supplemented with penicillin (100 U/ml), streptomycin (100 ug/ml), and amphotericin B (250 ng/ml). Foreskins were then minced in the presence of several drops of 0.01% trypsin.^b The minced tissue was suspended in 5 ml Eagle's MEM (Gibco), modified by the addition of essential and non-essential amino acids and vitamins (heretofore designated MEM₉),^c and supplemented with 10% fetal bovine serum (Hyclone), penicillin/streptomycin and amphotericin B (as above). After allowing the tissue to settle for 5 minutes, the supernatant was aspirated and the tissue was resuspended in 4 ml fresh complete MEM₉, and plated into a 25 cm² plastic tissue culture flask (Corning).

The primary culture was incubated at 37° C in a humidified environment of 5% CO₂/95% air, with growth medium changes at 48 hour intervals. Just prior to confluence, the primary culture was passed (as described below in III. 2.) at a ratio of 1:2. Cultures were expanded by continued propagation, with medium changes at 48-hour intervals,^d and serial passage at ratios of 1:2-1:4 when cells reached confluence.

^a For detailed formula, see Appendix A.

^b For formula, see Appendix A.

^c For detailed formula, see Appendix A.

^d For replacement medium volumes corresponding to specific flask sizes, see Appendix C.

III. 1. 2. Primary Culture: Human Umbilical Vein Endothelial Cells (HUVE)

Human umbilical vein endothelial cells (HUVE) were isolated from vessels by a modification¹⁶² of the techniques of Jaffe et al.¹⁶³ and Gimbrone et al.¹⁶⁴ Umbilical cords collected from patients undergoing normal vaginal delivery or Caesarean section were transported to the laboratory in PBS. The vein was cannulated and flushed with PBS to remove residual blood, then distended with 0.1% collagenase (Type Ia, Sigma) in PBS for 30 minutes. Following gentle kneading, the collagenase/cell suspension was drained into a 50 ml polypropylene centrifuge tube (Corning). To retrieve residual endothelial cells, the vein was flushed with 30 ml of medium M-199 (Gibco), which was also collected in the centrifuge tube. The cells were pelleted by centrifugation (350 x g, 7 minutes, 4° C), then resuspended in 6 ml of endothelial cell growth medium* (ECGM), consisting of M-199 (Gibco) supplemented with 20% fetal bovine serum (Hyclone), 100 ug/ml endothelial cell growth supplement[†] (ECGS, prepared according to the methods of Maciag et al.¹⁶⁵), 12 U/ml sodium heparin (Sigma), and 20 mM HEPES buffer (Sigma).

* For detailed formula, see Appendix A.

† For details of preparation, see Appendix B.

To prepare for plating of primary cultures, two 35-mm wells of a 6-well plastic tissue culture plate (Corning) were treated for 45 minutes with human fibronectin (25 ug/ml in PBS). Excess fibronectin was removed and the cell suspension was divided between the two treated wells (3 ml/well). Primary cultures were incubated at 37° C in a humidified environment of 5% CO₂/95% air. 16-24 hours after plating, medium was removed and cells were gently washed with M-199 to remove residual erythrocytes and debris before addition of fresh ECGM. Thereafter, medium was changed at 48-hour intervals until cultures reached confluence (2-5 days), at which time cells were passed (as described below in III. 2.) into fibronectin-treated 25 cm² plastic tissue culture flasks (Corning), one flask per confluent well.

Cultures were expanded by continued propagation, with medium changes at 48-hour intervals,⁹ and serial passage at ratios of 1:2 or 1:3 when cells reached confluence.

III. 2. Passage of Cell Cultures

The procedure for passage of confluent cultures was basically the same for both HUVE and NHDF cells. Growth medium was removed and monolayers were washed twice with

⁹ For replacement medium volumes corresponding to specific flask sizes, see Appendix C.

PBS. Cells were released from the substrate by addition of 0.01% trypsin/0.02% EDTA^h (1:1).

Following addition of trypsin/EDTA, cultures were incubated at 37° C (3-5 minutes for NHDF, 1-2 minutes for HUVE) to promote the limited proteolytic activity required for cell release. To free the entire monolayer, flasks were physically agitated when necessary. Proteolytic action was then stopped by suspending cells in their respective complete media, at a volume of at least 10 times that of the trypsin. Final volumes of cell suspensions were dictated by the passage ratio.

Following suspension and thorough trituration, cells were plated into fresh culture vessels (flasks, plates, chamber slides, etc.) according to the particular experimental protocol. Specific plating volumes corresponding to each culture vessel type and size are listed in Appendix C.

III. 3. Cryopreservation of Cell Cultures

Low passage stock cultures of HUVE and NHDF cells were cryopreserved by the following methods. Confluent monolayers were washed twice with PBS and trypsinized as described above (III. 2.). Released cells were suspended in their respective culture media, then pelleted by centrifugation (350 x g, 5 minutes). Pellets were resuspended in

^h For formula, see Appendix A. For specific volumes as a function of monolayer surface area, see Appendix C.

their respective media supplemented with 10% dimethylsulfoxide (DMSO), at a concentration of 10^6 cells/ml. 1-ml aliquots were transferred to 1.8 ml cryovials (Nalgene), and samples were cryopreserved by progressively decreasing temperature over a 48 hour period (-40°C : 24 hours, -80°C : 24 hours) before storing in liquid nitrogen. Cultures were revived by rapidly thawing in warm water ($37-40^{\circ}\text{C}$), followed immediately by resuspension of the samples in their respective culture media. In order to minimize DMSO exposure, the newly thawed cells were pelleted by centrifugation (as above) and resuspended in fresh growth medium before plating in 25 cm^2 flasks.

III. 4. Cell Enumeration

The principal method of cell enumeration employed in this investigation was the standard hemocytometer counting technique. A sample of monodispersed cells was loaded into the hemocytometer, and cells lying within 5 large squares were counted. If necessary, suspensions were diluted in order to yield counts of approximately 100-200 cells. The number of cells in 5 squares was multiplied by 2×10^3 (then corrected for the dilution, if necessary) to calculate cells/ml of the original suspension. Viability was determined by preparing a 1:1 dilution of cell suspension in trypan blue vital dye (0.4% aqueous), then counting the live (non-stained) and dead (stained) cells. The ratio of

live cells/total cells (live + dead) x 100, yields the per cent viability of the sample. Cell loss upon passage was consistently negligible, with viability generally ranging 98-99%. Based on these data, cell suspensions were adjusted to yield the appropriate concentrations of viable cells, as dictated by the particular experimental protocols.

CHAPTER IV

GENERAL METHODS 2: VIRUS PROPAGATION AND TITRATION

This chapter provides a detailed description of the techniques employed in propagation of the several virus strains studied in the course of this investigation. Sources of the isolates along with methods of cryopreservation and titration of virus stocks are described. The section concludes with a description of the preparation of sham-inocula, which served as negative controls for experiments described in subsequent chapters.

IV. 1. Virus Propagation

In this section, sources of virus isolates and methods employed for their propagation are described. All reagents mentioned herein are as described in Chapter III and Appendix A. It should be noted, however, that growth media used in all virus-inoculated cultures were supplemented with 100 ug/ml gentamicin sulfate (Elkins-Sinn) and 250 ng/ml amphotericin B (Fungizone, Squibb).

IV. 1. 1. CMV AD169

CMV strain AD169, isolated by Rowe et al.^a (see II. 8), was obtained from the American Type Culture Collection.^a The 1 ml sample was diluted to 10 ml in serum-free MEM₂ and inoculated onto a confluent 150 cm² NHDF monolayer (in a plastic tissue culture flask) which had been washed twice with PBS. Following 1.5 hours adsorption at 35.5° C, 15 ml of complete MEM₂ (supplemented with gentamicin and amphotericin B, as described above) was added to the flask. The culture was incubated and growth medium was replaced at 3-day intervals. When >90% of the cells showed advanced cytopathic changes (10 days post-inoculation [PI]), the culture was trypsinized, suspended in 40 ml complete medium, and inoculated onto 4 fresh confluent 150 cm² NHDF monolayers. Cultures were again incubated until extensive cytopathology developed (>90%), then harvested for cryopreservation as described below (IV. 2).

IV. 1. 2. OSU CMV 3

OSU CMV 3, a fibroblast-raised clinical isolate, was obtained from Dr. Ronald Glaser.^b Stocks of this strain

^a For complete description of specimen, see ATCC specification sheet, Appendix D.

^b Department of Medical Microbiology & Immunology, The Ohio State University, Columbus, Ohio.

were expanded using methods identical to those described for AD169 above (IV. 1. 1.).

IV. 1. 3. CMV VHL/E

CMV strain VHL was isolated in 1988 (Ohio State University Hospitals, Columbus, Ohio) from a duodenal biopsy of a bone marrow transplant recipient with histologically verified CMV infection of the duodenum. Following initial diagnostic culture of the specimen in fibroblasts, the virus was inoculated onto a confluent, low passage HUVE monolayer in a 25 cm² plastic culture flask as follows. The diagnostic tube culture (exhibiting extensive CPE) was trypsinized (0.1 ml 0.01% trypsin, 0.1 ml 0.02% EDTA) and suspended in 2 ml of complete MEM₉. 800 ul of the suspension, diluted to 1.5 ml with serum-free MEM₉, was inoculated onto the HUVE cell monolayer, which had been washed twice with PBS. (The remainder of the suspension was reserved for standard propagation in fibroblast culture. (See IV. 1. 4. below.)

Adsorption was permitted for 1.5 hours at 35.5° C following which, 4 ml of ECGM, supplemented with gentamicin and amphotericin B, was added to the flask. The culture was incubated as above and provided with fresh medium at 3 day intervals. Obvious cytopathic changes were visible by day 5 PI. When >75% of the cells exhibited marked CPE, the culture was washed twice with PBS, trypsinized, suspended

in 4 ml of growth medium, and inoculated onto a PBS-washed HUVE monolayer in a 75 cm² flask. Following adsorption, 10 ml of additional medium was added and the culture was incubated as described above, then passed again when >75% of the cells showed cytopathology.

In this manner, the sub-strain was propagated through 20 passages in endothelial cells (VHL/E₂₀). Although the initial inoculation of the clinical diagnostic culture introduced a small number of fibroblasts into the endothelial culture, these cells deteriorated rapidly and, by the second passage of the infected HUVE cells, no evidence for the persistence of fibroblasts was apparent, as determined by both morphologic criteria and staining for von Willebrand Factor (see V. 3.).

IV. 1. 4. CMV VHL/F

This sub-strain was propagated from the VHL isolate described above (IV. 1. 3.) by inoculation of the specimen onto NHDF cells as follows. 1.2 ml of cell suspension from the original diagnostic culture was inoculated onto 3 fresh confluent NHDF tube cultures (0.4 ml/tube). Following 1 hr adsorption (35.5° C), 1 ml of complete MEM₉ (supplemented with gentamicin and amphotericin B) was added to each tube and cultures were incubated as above. When extensive cytopathic changes became evident (7 days PI), cultures were trypsinized, pooled, suspended in 5 ml of complete

MEM₉ (with gentamicin and Amphotericin B), and inoculated onto a confluent 75 cm² NHDF monolayer. Following 1 hour adsorption, 10 ml of fresh medium was added, and the culture was incubated. Growth medium was replaced at 3 day intervals.

When >90% of the cells exhibited CPE, the culture was trypsinized, suspended in growth medium, and inoculated onto fresh NHDF monolayers. Thus, by continued serial passage, this sub-strain was propagated through 20 passages in fibroblast cells (VHL/F₂₀).

IV. 2. Cryopreservation of Virus Stocks

Frozen stocks of CMV AD169 and OSU CMV 3 were prepared from heavily infected NHDF cultures (>90% CPE). Stocks of CMV VHL/E and VHL/F were prepared at approximately 5-passage intervals (through passage 20), from equivalently cytopathic HUVE and NHDF cultures respectively. For cryopreservation, growth medium was discarded and cells were washed twice with PBS, then released from the substrate by the addition trypsin/EDTA. Cells were resuspended in virus storage medium consisting of 40% MEM₉, 10% fetal bovine serum, and 50% sucrose phosphate buffer^c, transferred to 1.8 ml cryovials (Nalgene) or 12 x 75 mm polystyrene culture tubes (VWR), and rapidly frozen over

^c For formula, see Appendix A.

liquid nitrogen. Frozen stocks were stored in liquid nitrogen vapors. Alternatively, some stocks were deposited directly into a -80°C freezer. Both methods of storage were found to provide stocks of reproducible titer while effectively killing the viable cells within the preparation.

IV. 3. Titration of Virus Stocks

Concentrations of virus stocks were determined by plaque assay titration on fibroblast monolayers,^d according to a protocol based on the method of Wentworth and French.¹⁶⁶ For each virus stock, a sample was thawed and serially diluted in serum-free MEM₉. In order to insure production of interpretable data, assays of a given stock were performed with 3 different 10-fold dilutions of that stock (10^{-4} - 10^{-6} for AD169 and CMV 3, 10^{-2} - 10^{-4} for VHL/E and VHL/F).

To prepare for inoculation, NHDF cells were grown to confluence in 35 mm wells of 6-well plastic culture plates (Corning). Medium was removed from the wells and monolayers were washed twice with PBS, then inoculated with virus suspensions (6-10 wells/dilution). Following a 2-hour adsorption period (35.5°C), the inocula were removed and cells were washed free of residual viral suspension with 2

^d Virus used in preliminary experiments was titrated by the terminal dilution method. See Appendix E.

washes of PBS. Inoculated cultures were then overlaid with complete MEM, supplemented with gentamicin, amphotericin B, and 0.5% agarose* (SeaKem LE, FMC), 3 ml/well. The mixture was allowed to gel at room temperature and the plates were inverted and incubated at 35.5° C.

After 7 days, a second agarose-medium overlay was applied and plates were incubated for an additional 7 days. 14 days after inoculation, cultures were fixed by addition of 10% buffered formalin (3 ml/well, 30-45 minutes). The agar overlays were removed and fixed monolayers were air dried, then stained by covering with a saturated crystal violet solution for 1 minute. Crystal violet was removed and the monolayers were freed of residual stain by several washes with distilled water.

Plaques (Plate I) were counted under a low power dissecting microscope. Titers of each virus stock, expressed in plaque-forming units (PFU)/ml, were derived from counts of 6-10 replicate wells.†

IV. 4. Preparation of Sham-Inocula

In order to provide all experiments with appropriate negative controls, sham-inocula were prepared to test all

* For complete description of agarose-supplemented medium, see Appendix A.

† Titers of AD169 and CMV 3 stocks generally ranged between 10^5 - 10^6 PFU/ml, while titers of VHL/E and VHL/F averaged approximately 10^4 PFU/ml.

PLATE I: Photomicrograph of fixed, stained plaque generated during plaque assay titration of CMV VHL/E₂₀ (original magnification: 50x).

components of stock virus suspension except the virus itself. NHDF and HUVE cells were grown separately to confluence in culture flasks under standard conditions, as previously described (III. 1.). Cultures were trypsinized, then suspended in virus storage medium (IV. 2. above). Cryopreservation methods were identical to those described for virus stocks above.

In this manner, two separate stocks of sham-inocula were maintained. The NHDF preparation was used as a negative control for all experiments involving inoculation with fibroblast-derived virus (AD169, CMV 3, VHL/F); while the HUVE preparation served as a negative control for experiments involving inoculation with the endothelial-derived VHL/E.

CHAPTER V

GENERAL METHODS 3: HYBRIDIZATION AND STAINING TECHNIQUES

In this chapter, methods of detecting acute viral infection in vitro are detailed, including special culture techniques such as viral inoculation, adsorption and incubation, and culture fixation. In situ hybridization methodology is described, as well as immunohistochemical staining techniques for demonstration of viral antigens and von Willebrand Factor.

V. 1. Acute Infection of Cultured Cells

For acute infection studies, HUVE cells were plated in fibronectin-treated glass or plastic chamber slides (Nunc, Lab-Tek), while NHDF cells were plated in plastic ones.^a Cultures were grown to confluence, washed twice with PBS and inoculated with virus suspension diluted to the appropriate titer in serum-free MEM.^b To verify staining specificity, each experiment included cultures which were

^a In early studies, cells were inoculated in tissue culture flasks and prepared for staining by the drop-slide method. See Appendix F.

^b For specific volumes of inocula, as a function of culture vessel type and size, see Appendix C.

sham-inoculated, as well cultures inoculated with herpes simplex virus (HSV 1, strain KOS^c).

Following 1.5 hours adsorption at 35.5° C, inocula were removed and cultures were washed twice with PBS before addition of virus-free, complete growth medium (supplemented with gentamicin and amphotericin B as described in IV. 1.). Slide cultures were incubated for various intervals (1-20 days, with medium changes at 48-72 hour intervals) at 35.5° C, then washed twice with PBS and fixed in cold acetone (4° C) for 10 minutes for staining. In addition, growing cultures were observed throughout the course of infection for the appearance of cytopathology.

V. 2. In Situ Hybridization: Visualization of Viral DNA

In situ hybridization was performed according to a protocol based on the methods of Brigati et al.,¹⁶⁷ Unger et al.,¹⁶⁸ and Roberts et al.¹⁶⁹ (Figure 3). Biotinylated CMV-specific or HSV-specific DNA probes (ENZO Biochem)^d were suspended in a hybridization solution consisting of deionized formamide (62.5%), dextran sulfate (2.5% w/v), 375 mM sodium chloride, and 37.5 mM sodium citrate.¹⁶⁹

^c Gift of Dr. Marshall V. Williams, Department of Medical Microbiology & Immunology, The Ohio State University, Columbus, Ohio.

^d For full description of probes, see Appendix G.

**IN SITU HYBRIDIZATION EMPLOYING A BIOTINYLATED DNA PROBE
AND AN AVIDIN-PEROXIDASE/AMINOETHYLCARBAZOLE COMPLEX**

FIGURE 3: Diagram summarizing the in situ hybridization protocol for detection of intracellular CMV DNA.

Fixed culture slides were rehydrated for 10 minutes in PBS. Probe preparations were applied, and slides were incubated first at 85° C for 11 minutes to allow denaturation of DNA, then at 37° C for 2 hours for hybridization. Unbound probe was removed by washing in dilute Triton X 100 (0.05% in PBS). Hybridization stringency was regulated by a 10-minute immersion of slides in 50% formamide (in PBS), followed by two final 5-minute washes in 0.05% Triton X.

To visualize bound probe, slides were incubated for 20 minutes at 37° C with horseradish peroxidase-conjugated avidin D (Vector), diluted 1:500 in Tris-buffered saline (TBS: 0.05 M Tris HCl in 0.85% NaCl, pH 7.6).^{*} Following removal of unbound avidin complex by PBS rinse, slides were immersed in acetate buffer (0.02 M sodium acetate/acetic acid, pH 5.2) for 5 minutes, then developed for 2-3 minutes in 0.024% aminoethylcarbazole (0.24% 3-amino-9-ethylcarbazole [Sigma] in N,N-dimethylformamide [J.T. Baker], diluted 1:10 in acetate buffer, 0.0045% H₂O₂). The reaction was stopped by immersion of slides in acetate buffer. Slides were then washed in warm tap water and briefly counterstained with Gill's III hematoxylin (Sigma) before coverslips were applied. Slides which were additionally stained for von Willebrand Factor, as described below, were

* In early studies, visualization was with a streptavidin-peroxidase conjugate (Detek SA-HRP, ENZO), diluted 1:200 in buffer supplied by manufacturer.

not counterstained. (For examples of results see Plates XI, XII, XIII, XX, XXI.)

V. 3. Immunohistochemical Demonstration of von Willebrand Factor Expression

In order to verify the endothelial identity of infected cells, a sampling of fixed culture slides was stained for von Willebrand Factor (VIII) prior to in situ hybridization, by an immuno-alkaline phosphatase assay based on the methods of Mukai et al.,¹⁷⁰ as described previously¹⁶⁹ (Figure 4).

Fixed slide cultures were washed for 1 minute in 0.1% bovine serum albumin (BSA, Sigma) in PBS. The primary antibody, a polyclonal rabbit anti-human Factor VIII (DAKO), diluted 1:1000 in 0.1% BSA/PBS, was applied and slides were incubated 30 minutes at 37° C. After two 5-minute washes in BSA/PBS, slides were incubated (30 minutes, 37° C) with alkaline phosphatase-conjugated sheep anti-rabbit secondary antibody (Sigma), diluted 1:150 in BSA/PBS. Unbound secondary was removed by additional washing (BSA/PBS) and slides were developed by the application of alkaline phosphatase substrate (Vector), prepared according to the manufacturer's instructions.[†] The reaction was stopped following 5-10 minutes development by

[†] 80 ul of each kit reagent + 5 ml tris-buffered saline (0.1 M Tris-HCl, 0.85% NaCl, pH 8.20).

FIGURE 4: Diagram summarizing the immuno-alkaline phosphatase method for demonstration of the intracellular presence of von Willebrand Factor.

washing in PBS. No counterstain was applied. (For examples of results, see Plates XI, XII.)

V. 4. Immunohistochemical Demonstration of Viral Antigen Expression

As additional confirmation of CMV infection, fixed culture slides were stained for CMV-specific antigens by an immunoperoxidase technique based on methods described by Sharma et al.¹⁷¹ (Figure 5). Primary CMV-specific antibodies, originally developed by Shuster et al.,¹⁷² included mouse monoclonal antibodies (DuPont) directed against 1) CMV early nuclear protein (2H2.4, mw 72 Kd) and, 2) CMV late nuclear protein (2F3.0, mw 68 Kd). Additionally, a pool of 3 mouse monoclonal antibodies to HSV (Bartel) was used as a specificity control. Primary antibodies were diluted in TBS (1:100 for anti-HSV and anti-CMV early, 1:150 for anti-CMV late).

Fixed slides were rehydrated for 5 minutes in TBS then immersed in 20% horse serum (Gibco) in TBS for 10 minutes. Primary antibody dilutions were applied and slides were incubated 45 minutes at 37° C in a humidified atmosphere. Following incubation slides were washed in TBS, briefly immersed in 20% horse serum, then rewashed in TBS. Slides were incubated 15 minutes (37° C) with a secondary antibody preparation consisting of biotinylated horse anti-mouse IgG (Vector) diluted 1:200 in TBS supplemented with 0.2%

FIGURE 5: Diagram summarizing the immunoperoxidase staining protocol for demonstration of CMV-specific antigen expression.

gelatin. Slides were then washed in TBS, and bound antibody complexes were visualized following 15 minutes incubation (37° C) with peroxidase-labelled avidin D by development in 0.024% aminoethylcarbazole, as described above (Section V. 2.). Slides were briefly counterstained in Gill's III hematoxylin before coverslips were applied. (For examples of results, see Plates XV, XVI, XXIII, XXIV.)

CHAPTER VI

ENHANCED ENDOTHELIAL CYTOPATHOGENICITY INDUCED BY A CYTOMEGALOVIRUS STRAIN PROPAGATED IN ENDOTHELIAL CELLS^a

VI. 1. Abstract

Endothelial involvement has been implicated in cytomegalovirus infection, a source of major complications in immunosuppressed individuals (e.g. AIDS and organ transplants). Traditionally CMV has been grown in fibroblasts; however, propagation in these cells may alter characteristics of the virus. In developing an in vitro model system of CMV/endothelial cell interaction, this issue has been addressed by propagating a clinical isolate, CMV VHL, in human umbilical vein endothelial cells (HUVE) by serial cocultivation of heavily infected cultures with fresh HUVE monolayers. The infectious properties of this isolate have been compared with those of the fibroblast-raised strains, CMV AD169 and OSU CMV 3. In situ hybridization using a biotinylated DNA probe, as well as immunohistochemical staining for CMV-specific antigens, has confirmed infection of HUVE cells inoculated with all three

^a Previously published in modified form.¹⁵¹

strains of the virus. Infection of HUVE by VHL was accompanied by dramatic cytopathology not observed in AD169- or CMV 3-infected cells. Plaque assay of culture supernatants revealed greater virus production in VHL-infected HUVE as compared to equivalently inoculated fibroblasts. In contrast, AD169 production in inoculated fibroblasts exceeded that in HUVE. These studies demonstrate the suitability of cultured endothelial cells as a substrate for CMV propagation, and suggest that a strain of virus thus propagated may offer an accurate model of CMV/endothelial cell interaction in human disease.

VI. 2. Introduction

Cytomegalovirus is a leading cause of death in immunosuppressed patients.¹⁹ Although this is not a new observation,²⁰ it gains new consequence with the growth in numbers of immunosuppressed individuals that has occurred in the last decade.^{21 22 23 104 173} Individuals with impaired cellular immunity, attributable to either iatrogenic manipulation or disease process are at extreme risk of disseminated and frequently fatal infection with this virus. Since the 1960's CMV has overwhelmed both recipients of organ transplants and, recently, patients with the acquired immune deficiency syndrome. Although pulmonary infection is most common,²⁴ the virus manifests itself in nearly all organs. Careful studies have indicated that the

endothelial cell is a common site of infection regardless of the organ involved.^{24 26 27 28 29 31 32 33 34 174}

In vitro studies of CMV/endothelial cell interactions have yielded varying results. Friedman et al.³⁵ reported a failure to infect endothelial cells (both bovine and human) with the Davis strain of CMV, as determined by the absence of cytopathic changes or CMV nuclear antigens in inoculated cells, as well as by the absence of detectable levels of infectious virus in the corresponding culture supernatants. Ho et al.³⁶ described infection of human endothelial cells with CMV AD169 and two clinical isolates, as indicated by the development of cytopathic effect (CPE), the expression of CMV antigens, and the production of infectious progeny virus in the inoculated cultures. In that study, enhanced CPE was observed in cells inoculated with the clinical isolates as compared with that observed in AD169-infected cultures. In a more recent investigation, Smiley et al.³⁷ reported productive infection of human umbilical vein endothelial cells with the Towne strain of CMV without accompanying morphologic cytopathology. Thus the specific nature of the CMV/endothelial cell interaction appears to be both qualitatively and quantitatively dependent upon virus strain and/or the in vitro propagation history of the virus.

Traditionally, CMV has been propagated in fibroblasts, as these cells seem to be the most permissive for virus

reproduction. Although this has proved an effective means for generating large quantities of virus for study, the recent emergence of the role of the endothelial cell in human CMV infection has prompted the development of an alternative approach. It is therefore proposed that a strain of CMV raised in the endothelial cells themselves may accurately model the CMV/endothelial cell interactions observed *in vivo*. In pursuing this line of reasoning, a recent clinical isolate of CMV has been propagated through multiple passages in human endothelial cells, and its infectious properties have been investigated and compared with those of the fibroblast-raised laboratory strains, CMV AD169 and OSU CMV 3.

VI. 3. Materials and Methods

VI. 3. 1. Cell Culture

Neonatal human dermal fibroblasts (NHDF), isolated from human foreskins, were propagated in Eagle's MEM (Gibco) modified by the addition of essential and non-essential amino acids and vitamins,^b and supplemented with 10% fetal bovine serum (Hyclone). Cultures were incubated at 37° C in a humidified environment of 5% CO₂/95% air, and were passed weekly at a ratio of 1:2. Cultures used in experiments were of passage 10-20.

^b As detailed in Appendix A.

Human umbilical vein endothelial cells (HUVE) were isolated from vessels by a modification¹⁶² of the techniques of Jaffe et al.¹⁶³ and Gimbrone et al.,¹⁶⁴ as described in III. 1. 1. Cultures were propagated in endothelial cell growth medium (ECGM),^c consisting of M-199 (Gibco) supplemented with 10% fetal bovine serum, 100 ug/ml endothelial cell growth supplement^d (ECGS, prepared according to the methods of Maciag et al.¹⁶⁵), 12 U/ml sodium heparin, 110 ug/ml sodium pyruvate, 2 mM L-glutamine, and 20 mM Hepes buffer. All growing surfaces for HUVE cells were pretreated with fibronectin. Cells were passed weekly at a ratio of 1:2. Cultures of passage 6-12 were used in experiments.

VI. 3. 2. Virus Propagation

CMV strain AD169 was obtained from the American Type Culture Collection and propagated in NHDF cells. OSU CMV 3, a fibroblast-raised clinical isolate, was obtained from Dr. Ronald Glaser (see IV. 1.).

CMV strain VHL, obtained from a duodenal biopsy of a bone marrow transplant recipient, was propagated in HUVE cells (VHL/E), as described in IV. 1. 3.

^c See Appendix A.

^d See Appendix B.

Frozen stocks of CMV AD169 were prepared from heavily infected NHDF cultures (>90% CPE), while stocks of CMV VHL were prepared from equivalently cytopathic HUVE cultures (see IV. 2.).

Concentrations of virus stocks, in terms of plaque forming units (PFU)/ml, were determined by plaque assay titration on NHDF monolayers in 6-well culture plates (Corning), according to a protocol based on the method of Wentworth and French¹⁶⁶ (see IV. 3.). Titers of each viral strain were derived from counts of 10 replicate wells.

VI. 3. 3. Acute Infection of Endothelial Cells

For acute infection studies, HUVE cells were grown to confluence in plastic chamber slides (Lab-Tek), washed twice with PBS and inoculated with either CMV AD169, OSU CMV 3, or CMV VHL/E₆, at titers ranging from 10^{-3} to 10^0 PFU/ml. Sham inoculated cultures were included in each experiment. Following 1.5 hours adsorption at 35.5° C, inocula were removed and cultures were washed twice with PBS before addition of virus-free growth medium. Slide cultures were incubated for various intervals (1-20 days), then washed twice with PBS and fixed in cold acetone (4° C) for 10 minutes for staining. In addition, growing cultures were observed throughout the course of infection for the appearance of cytopathology.

VI. 3. 4. Detection of CMV DNA by In Situ Hybridization

In situ hybridization^e was performed according to a protocol based on the methods of Brigati et al.¹⁶⁷ and Unger et al.,¹⁶⁸ as described previously¹⁶⁹ (see V. 2.). Briefly, fixed culture slides were treated with 6% H₂O₂/ methanol for 20 minutes to quench endogenous peroxidase. A biotinylated CMV-specific DNA probe^f (ENZO Biochem) was applied, and slides were incubated first at 85° C for 11 minutes to allow denaturation of DNA, then at 37° C for 2 hours for hybridization. The bound probe was visualized following 20 minutes incubation (37° C) with a streptavidin peroxidase conjugate (ENZO), by development in 0.05 % aminoethylcarbazole.

VI. 3. 5. Demonstration of von Willebrand Factor by Immunocytochemistry

In order to verify the endothelial identity of infected cells, fixed culture slides were stained for von Willebrand Factor^g prior to in situ hybridization, by an immuno-alkaline phosphatase assay based on the methods of Mukai et al.,¹⁷⁰ as described previously¹⁶⁹ (see V. 3.). Briefly, slides were incubated for 30 minutes (37° C) with rabbit

^e See V. 2.

^f See Appendix G.

^g See V. 3.

anti-Factor VIII primary antibody (DAKO), followed by washing, and subsequent incubation (30 minutes, 37° C) with alkaline phosphatase-conjugated sheep anti-rabbit secondary (Sigma). Slides were developed by the application of alkaline phosphatase substrate (Vector).

VI. 3. 6. Demonstration of CMV-Specific Antigen Expression by Immunocytochemistry

Expression of CMV-specific antigens in infected cells was detected by immunocytochemical staining techniques based on the methods of Sharma et al.,¹⁷¹ as described in V. 4. Briefly, fixed slides were incubated for 45 minutes (37° C) with primary antibodies (DuPont) directed against either early or late viral proteins.¹⁷² After washing, slides were incubated with a biotinylated secondary antibody (Vector) for 15 minutes (37° C). Bound antibody complexes were visualized following 15 minutes incubation with peroxidase-conjugated avidin D (Vector) by development in 0.024% aminoethylcarbazole.

VI. 3. 7. Production of Infectious Virus

Virus production by infected cells was quantified by plaque assay of culture supernatants at various intervals over the course of infection. HUVE cells were grown to confluence in 25 cm² plastic culture flasks and inoculated

with either a virus-free preparation (sham inoculum^h), CMV AD169 (10^0 , 10^{-1} , or 10^{-2} PFU/cell), or CMV VHL/E (10^{-2} or 10^{-3} PFU/cell).ⁱ Following 1.5 hours adsorption, cultures were washed three times with PBS and once with serum-free medium, supplied with virus-free complete medium (5 ml/flask), and incubated. The final rinse was reserved for a baseline (day 0) plaque assay. Confluent NHDF cultures were inoculated in an identical manner for comparative purposes.

At various intervals, culture supernatants were withdrawn and reserved for plaque assay, and infected cultures were provided with fresh, virus-free medium.

Plaque assays were performed by a modification of the methods of Wentworth and French.¹⁶⁶ Confluent NHDF monolayers in 6-well culture plates were inoculated in triplicate with appropriate dilutions of supernatants from infected HUVE or NHDF flasks, as well as from sham-inoculated cultures, and assays were carried out as described in IV. 3.

^h See V. 4.

ⁱ Since AD169 is a well established, better characterized representative of fibroblast-raised CMV, OSU CMV 3 was omitted from these studies.

VI. 4. Results

VI. 4. 1. Morphologic Cytopathology in CMV-Infected Endothelial Cells

No obvious cytopathology was observed in endothelial cells infected with CMV AD169 or OSU CMV 3 at any time throughout the course of infection up to 30 days post-inoculation (Plates II, III, IV). Cells in which viral infection was verified both by the presence of CMV DNA (Plates XI, XII) and by the expression of CMV-specific antigens (Plate XV) appeared morphologically unperturbed at the light microscope level.

In contrast to CMV AD169 and CMV 3, the clinical strain, VHL/E, induced marked cytopathic changes in inoculated endothelial cells as early as 3 days PI, even at titers 2 orders of magnitude lower than that of the highest AD169 inoculum. Lesions in the HUVE monolayer initiated as small regions of apparent cell fusion, followed by increasing refractility and rounding of the multicellular mass, as well as by the appearance of satellite lesions about the initial cytopathic focus. Advancing cytopathology progressed to ultimately involve the entire monolayer (Plate V).

Infection of NHDF cells with CMV VHL/E resulted in typical CMV/fibroblast cytopathology qualitatively similar to that induced by AD169 and other strains of CMV (Plates VI, VII, VIII, IX).

PLATE II: Phase contrast micrograph of human umbilical vein endothelial cell culture 20 days following sham-inoculation (original magnification: 50x).

PLATE III: Phase contrast micrograph of human umbilical vein endothelial cell culture 30 days post-inoculation with CMV AD169, 1.0 PFU/cell (original magnification: 50x).

PLATE IV: Phase contrast micrograph of human umbilical vein endothelial cell culture 30 days post-inoculation with OSU CMV 3, 1.0 PFU/cell (original magnification: 50x).

PLATE V: Phase contrast micrograph of human umbilical vein endothelial cell culture 20 days post-inoculation with CMV VHL/E₆, 10^{-2} PFU/cell (original magnification: 50x).

PLATE VI: Phase contrast micrograph of neonatal human dermal fibroblast culture following sham-inoculation (original magnification: 50x).

PLATE VII: Phase contrast micrograph of neonatal human dermal fibroblast culture showing cytopathic effect induced by CMV AD169 infection (original magnification: 25x).

PLATE VIII: Phase contrast micrograph of neonatal human dermal fibroblast culture showing cytopathic effect induced by OSU CMV 3 infection (original magnification: 25x).

PLATE IX: Phase contrast micrograph of neonatal human dermal fibroblast culture showing cytopathic effect induced by CMV VHL/E₈ infection (original magnification: 50x).

VI. 4. 2. Demonstration of CMV DNA and CMV-Specific Antigen Expression in Infected Endothelial Cells

In situ hybridization studies demonstrated the presence of CMV DNA in cells within the inoculated cultures (Plates XI, XII, XIII). Sham-inoculated cells were consistently negative for CMV DNA staining (Plate X). The presence of von Willebrand Factor, as detected by immunocytochemical staining, within CMV-positive cells verified their endothelial identity (Plates XI, XII, and as demonstrated previously¹⁶⁹). In cells staining strongly for CMV DNA, implying advanced stages of cellular infection, a decline in von Willebrand Factor expression was apparent. Similar observations have been noted by Bruggeman et al.¹⁵³

Infection of HUVE by AD169 and VHL/E was additionally confirmed by positive immunocytochemical staining for CMV-specific antigens (Plates XIV, XV, XVI).

PLATE X: Photomicrograph of sham-inoculated human umbilical vein endothelial cells stained for the presence of CMV DNA by *in situ* hybridization using a biotinylated CMV-specific DNA probe, and an avidin-peroxidase/aminoethylcarbazole complex for visualization (original magnification: 64x).

PLATE XI: Photomicrograph of AD169-infected human umbilical vein endothelial cells dual-stained for 1) the presence of nuclear CMV DNA by *in situ* hybridization using a biotinylated CMV-specific DNA probe, and an avidin-peroxidase/aminoethyl-carbazole complex for visualization, and 2) the cytoplasmic expression of von Willebrand Factor by immuno-alkaline phosphatase (original magnification: 64x).

PLATE XII: Photomicrograph of CMV 3-infected human umbilical vein endothelial cells dual-stained for 1) the presence of nuclear CMV DNA by in situ hybridization using a biotinylated CMV-specific DNA probe, and an avidin-peroxidase/ aminoethylcarbazole complex for visualization, and 2) the cytoplasmic expression of von Willebrand Factor by immuno-alkaline phosphatase (original magnification: 64x).

PLATE XIII: Photomicrograph of VHL/E₆-infected human umbilical vein endothelial cells stained for the presence of CMV DNA by in situ hybridization using a biotinylated CMV-specific DNA probe, and an avidin-peroxidase/aminoethyl-carbazole complex for visualization. Cells were briefly counterstained with hematoxylin (original magnification: 64x).

PLATE XIV: Photomicrograph of sham-inoculated human umbilical vein endothelial cells stained by immunoperoxidase using a primary antibody directed against CMV early nuclear antigen, a biotinylated secondary antibody, and an avidin-peroxidase/aminoethylcarbazole complex for visualization. Cells were briefly counterstained with hematoxylin (original magnification: 64x).

PLATE XV: Photomicrograph of AD169-infected human umbilical vein endothelial cells stained for the presence of CMV early nuclear antigen by immunoperoxidase, using a primary antibody directed against CMV early nuclear antigen, a biotinylated secondary antibody, and an avidin-peroxidase/aminoethylcarbazole complex for visualization. Cells were briefly counterstained with hematoxylin (original magnification: 64x).

PLATE XVI: Photomicrograph of VHL/E₆-infected human umbilical vein endothelial cells stained for the presence of CMV late protein by immunoperoxidase using a primary antibody directed against CMV late nuclear antigen, a biotinylated secondary antibody, and an avidin-peroxidase/aminocethyl-carbazole complex for visualization. Cells were briefly counterstained with hematoxylin (original magnification: 64x).

VI. 4. 3. Quantitation of Infectious Virus Production

Plaque assays of culture supernatants indicated that infectious virus was produced by cells inoculated with either strain of CMV. As shown in Figures 6 and 7, the level of virus production in AD169-infected endothelial cells was considerably lower than in equivalently inoculated fibroblasts. In addition, release of detectable amounts of infectious virus into the culture medium seemed to be delayed in inoculated HUVE cells, as compared to the infected NHDF cultures. No plaques were ever produced by supernatants from sham-inoculated cultures. It should be noted that, once again, HUVE cells productively infected with AD169 were morphologically indistinguishable from their sham-inoculated counterparts.

In contrast to the above findings, CMV VHL/E infection resulted in an opposite effect. In this case, the infected endothelial cells ultimately released more virus into the medium than did the equivalently inoculated NHDF cells (Figure 8). In fact, as is apparent in the figure, levels of infectious virus released by inoculated NHDF were barely detectable even as late as day 20 PI. Productive infection in VHL-inoculated HUVE cultures was accompanied by progressive advance of cytopathic lesions within the monolayers, as described above.

Although these studies demonstrate productive infection in cells inoculated with either strain of the virus, levels

FIGURE 6: Infectious virus production in neonatal human dermal fibroblasts (NHDF) and human umbilical vein endothelial cells (HUVE) inoculated on day 0 with CMV AD169, 1.0 PFU/cell, expressed in PFU/ml of culture supernatant, as determined by plaque assay on fibroblast monolayers (error bars represent standard deviation).

FIGURE 7: Infectious virus production in neonatal human dermal fibroblasts (NHDF) and human umbilical vein endothelial cells (HUVE) inoculated on day 0 with CMV AD169, 10^{-2} PFU/cell, expressed in PFU/ml of culture supernatant, as determined by plaque assay on fibroblast monolayers (error bars represent standard deviation).

FIGURE 6

FIGURE 7

FIGURE 8

FIGURE 8: Infectious virus production in neonatal human dermal fibroblasts (NHDF) and human umbilical vein endothelial cells (HUVE) inoculated on day 0 with CMV VHL/E₆, 10^{-2} PFU/cell, expressed in PFU/ml of culture supernatant, as determined by plaque assay on fibroblast monolayers (error bars represent standard deviation).

of productivity were dramatically lower in VHL-inoculated cultures of both NHDF and HUVE, as compared to those inoculated with AD169. This is apparently a result of the lower titers of the VHL inocula available for these experiments (10^{-2} - 10^{-3} PFU/cell), as limited by the recency of its isolation. However, the apparent lower infectivity of the VHL strain for fibroblasts raises some question as to the validity of the fibroblast-based plaque assay as a quantitative indicator of the initial inocula, as well as of VHL reproduction in general.

VI. 5. Discussion

CMV infects human endothelial cells in clinical disease, as shown by studies of tissue obtained by biopsy and at autopsy. Evidence for CMV infection has been found in endothelial cells within most organ systems, including lung,²⁶ skin,²⁷ retina,²⁸ and gastrointestinal tract,^{29 30 31 32 33} as well as spleen and pancreas,²⁴ and peritoneum.³⁴ These findings suggest the need for an in vitro model of CMV/endothelial cell interaction. However, several recent in vitro investigations of endothelial cell infection by CMV have yielded conflicting results. While Friedman et al.³⁵ were unable to demonstrate infection of human or bovine endothelial cells with the Davis strain, experiments by Ho et al.³⁶ and Smiley et al.³⁷ resulted in successful human endothelial cell infection by AD169 and

Towne strain respectively. It has been somewhat curious, however, that in studies using laboratory-adapted strains of the virus, productive infection has been demonstrated in the absence of obvious morphologic cytopathology. Previous results from this laboratory,¹⁶⁹ as well as experiments with CMV AD169 described in this report have also shown production of infectious virus by endothelial cells morphologically indistinguishable from sham-inoculated controls.

In contrast, productive infection in human umbilical vein endothelial cells inoculated with the clinical isolate, CMV VHL/E, was accompanied by dramatic cytopathology. Another distinguishing feature of this strain was its higher level of reproduction in endothelial cells than in equivalently inoculated fibroblasts. This behavior is again, in contrast to that of AD169, as described in this report, as well as that of the Towne strain, which was found to reproduce more efficiently in fibroblasts.³⁷ Collectively, these findings indicate significant strain-related differences in virus/cell interaction on both the qualitative and quantitative levels.

In considering the basis for such variation in behavior, it might be useful to examine the origins of the different strains. CMV AD169 was isolated in 1956 by Rowe et al.⁹⁴ from adenoid tissue acquired following adenoidectomy of a 7-year old girl with no reported CMV-related

disease, and has been propagated in fibroblasts since its isolation. The VHL strain, on the other hand, was isolated in 1988 from a duodenal ulcer biopsy of an iatrogenically immunosuppressed bone marrow transplant recipient showing histologic evidence of clinical CMV disease, and propagated since that time by serial subculture in human endothelial cells. It seems plausible that the extensively repeated subculture of AD169 in fibroblasts over so many generations may have resulted in a selection process that has restricted the host range of the virus and attenuated its spectrum of cytopathic properties. This concept was alluded to as early as 1970 by Weller,³⁵ and has been the basis of an approach to development of anti-CMV vaccination employing the Towne strain.¹⁷⁵ Indeed, Ho et al.³⁶ observed that recent clinical isolates of CMV showed more extensive cytopathic effect in endothelial cells than did AD169. Also consistent with this proposition are the findings of Furukawa¹⁷⁶ and Ogura et al.,¹⁷⁷ in which CMV variants were recovered from persistently infected cells 9-12 months after inoculation with Towne strain. In both cases these variants showed structural alterations in their genomes, as demonstrated by restriction fragment analysis, when compared with the original inocula.

In summary, an endothelial cell-propagated strain of CMV has been described, which shows greater cytopathic effects in these cells than does a well established

fibroblast-raised strain. In light of the accumulating evidence for a central role of the endothelial cell in disseminated CMV disease, it is proposed that a strain of the virus propagated in endothelial cells offers a closer model of this important aspect of human CMV infection.

CHAPTER VII

PRESERVATION OF NATURAL ENDOTHELIAL CYTOPATHOGENICITY OF CYTOMEGALOVIRUS BY PROPAGATION IN ENDOTHELIAL CELLS^a

VII. 1. Abstract

Cytomegalovirus (CMV) is a source of major complications in immunosuppressed individuals. Endothelial involvement in CMV infection is well documented, prompting the need for an accurate in vitro model. Traditionally the virus is propagated in fibroblasts; however this process may alter its characteristics. Thus we have propagated a recent isolate (CMV VHL) through multiple passages in human umbilical vein endothelial cells (HUVE) and, separately in neonatal human dermal fibroblasts (NHDF). Infection of HUVE inoculated with either sub-strain of the virus was confirmed by CMV-specific in situ hybridization and by immunocytochemical staining for CMV antigens. Whereas infection of HUVE by VHL/E (endothelial-raised) was accompanied by dramatic cytopathology resembling that observed clinically, the endothelial cytopathic potential of VHL/F (fibroblast-raised) was lost by its 20th passage

^a Submitted in modified form to J Virol, February, 1990.

in NHDF. Similarly, the ability of VHL/F to initiate sustained productive infection in HUVE was severely attenuated; plaque assay of culture supernatants and infected cell fractions, as well as virus-specific polymerase assay of cell lysates, demonstrated progressive viral reproductive activity in VHL/E-inoculated HUVE, whereas VHL/F reproduction was barely detectable. Since properties of VHL/F bear strong resemblance to those of the fibroblast-raised AD169, these studies suggest that the process of fibroblast adaption commonly employed in the propagation of CMV restricts the host range of the virus while attenuating its spectrum of cytopathic potential. Endothelial-based propagation, on the other hand, preserves the natural endothelial cytopathogenicity of the original isolate.

VII. 2. Introduction

Cytomegalovirus (CMV) has received increasing attention in recent years as a prominent cause of morbidity and mortality among the immunosuppressed population.¹⁹ Although the potential consequences of CMV infection in the immunocompromised host have been known for some time,²⁰ the magnitude of the problem has increased significantly with the growth in numbers of immunosuppressed individuals that has occurred in the last decade.^{21 22 23 104 173} The impaired cellular immunity induced by immunosuppressive therapy has

put organ transplant recipients at considerable risk of developing serious, often life-threatening, CMV-associated complications.^{20 31 102 105 110 178 179 180} In addition, the loss of immune system integrity that occurs in the acquired immune deficiency syndrome leaves AIDS patients particularly vulnerable to frequently fatal disseminated infection with this virus.^{23 32 114 174} Although pulmonary infection is most common,²⁴ the virus manifests itself in nearly all organs. Evidence continues to accumulate implicating the endothelial cell as a common target of infection regardless of the organ involved.^{24 26 27 28 29 31 32 33 34 87 174}

Results of the several in vitro studies of CMV/endothelial cell interaction published to date have been difficult to interpret with respect to clinical observations. Friedman et al.³⁵ reported a failure to infect endothelial cells (both bovine and human) with the Davis strain of CMV, as determined by the absence of cytopathic changes or CMV nuclear antigens in inoculated cells, as well as by the absence of detectable levels of infectious virus in the corresponding culture supernatants. Ho et al.³⁶ described infection of human endothelial cells with CMV AD169 and two clinical isolates, as indicated by the development of cytopathic effect (CPE), the expression of CMV antigens, and the production of infectious progeny virus in the inoculated cultures. In that study, enhanced CPE was observed in cells inoculated with the clinical

isolates as compared with that observed in AD169-infected cultures. More recently, Smiley et al.³⁷ reported infection of human umbilical vein endothelial cells with the Towne strain of CMV without accompanying morphologic cytopathology. Although this infection was associated with production of infectious virus, the reproductive efficiency was considerably lower than in similarly inoculated fibroblasts. Indeed both Radsak et al.¹⁵² and Bruggeman et al.¹⁵³ have reported very low infection efficiency in endothelial cells inoculated with Towne strain¹⁵², AD169, or Kerr strain,¹⁵³ even at high input titers (5-10 M.O.I.). Collectively, these findings suggest the specific nature of the CMV/endothelial cell interaction to be both qualitatively and quantitatively dependent upon virus strain and/or the in vitro propagation history of the virus.

Traditionally, CMV has been propagated in fibroblasts, as these cells seem to be the most permissive for virus reproduction. Although this has proved an effective means for generating large quantities of virus for study, the recent emergence of the role of the endothelial cell in human CMV infection prompted an alternative approach. In an effort to develop an accurate in vitro model of CMV/endothelial cell interaction, and to determine the impact of fibroblast-adaption on the resulting viral progeny, a recent CMV isolate has been propagated through multiple passages in human umbilical vein endothelial cells, and,

separately in neonatal human dermal fibroblasts. Results of earlier studies with the endothelial-raised strain^b indicated significantly enhanced endothelial cytopathic potential, as compared with CMV AD169,¹⁵¹ suggesting that continuous propagation in fibroblasts may alter characteristics of the virus. These findings can now be extended by reporting dramatic diversification in the cytopathic and reproductive properties of the VHL isolate as a function of the host system in which the virus was raised. It is clearly demonstrated herein that the process of fibroblast-adaption restricts the host range of CMV and attenuates its cytopathic potential, whereas endothelial-based propagation produces a progeny that retains the endothelial cytopathogenicity of the original isolate.

VII. 3. Materials & Methods

VII. 3. 1. Cell Culture

Neonatal human dermal fibroblasts (NHDF), isolated from human foreskins, were propagated in as described in III. 1. 1., and used for experiments at passage 10-20. Human umbilical vein endothelial cells (HUVE), isolated from vessels and propagated as described in III. 1. 2., were used for experiments at passage 4-8.

^b See Chapter VI.

VII. 3. 2. Virus Propagation

CMV strain AD169 was obtained from the American Type Culture Collection and propagated in NHDF cells as previously described (IV. 1. 1.).

CMV strain VHL was isolated from a duodenal biopsy of a bone marrow transplant recipient with histologically verified CMV infection of the duodenum. The initial diagnostic fibroblast culture was divided into two aliquots and propagated in endothelial cells and, separately, in fibroblasts, as detailed in IV. 1. 3. and IV. 1. 4. (see also reference 151). The virus stocks used in these experiments consisted of VHL progeny generated following 20 passages in HUVE cells (VHL/E₂₀) or in NHDF cells (VHL/F₂₀).

Frozen stocks of CMV AD169 and CMV VHL/F were prepared from heavily infected NHDF cultures (>90% CPE), while stocks of VHL/E were prepared from equivalently cytopathic HUVE cultures, as described in IV. 2. (see also reference 151).

Concentrations of virus stocks, in terms of PFU/ml, were determined by plaque assay titration on NHDF monolayers, according to a protocol based on the method of Wentworth and French,¹⁶⁶ as detailed in IV. 3. Titers of each viral strain were derived from counts of 6-10 replicate wells.

VII. 3. 3. Acute Infection of Endothelial Cells and Fibroblasts

For acute infection studies, HUVE cells were plated in fibronectin-treated glass chamber slides (Nunc), while NHDF cells were plated in plastic ones. Cultures were grown to confluence, washed twice with PBS and inoculated with either CMV VHL/E or CMV VHL/F, at titers ranging from 10^{-2} to 10^{-1} PFU/cell. To verify staining specificity, each experiment included cultures which were sham inoculated, as well as cultures inoculated with Herpes simplex (HSV 1, strain KOS). Following 1.5 hours adsorption at 35.5° C, inocula were removed and cultures were washed twice with PBS before addition of virus-free growth medium supplemented with gentamicin and amphotericin B. Slide cultures were incubated for various intervals (1-20 days) at 35.5° C, then washed twice with PBS and fixed in cold acetone (4° C) for 10 minutes for staining. In addition, growing cultures were observed throughout the course of infection for the appearance of cytopathology.

VII. 3. 4. Detection of CMV DNA by In Situ Hybridization

In situ hybridization was performed according to a protocol based on the methods of Brigati et al.,¹⁶⁷ Unger et al.,¹⁶⁸ and Roberts et al.,¹⁶⁹ as described in V. 2. Briefly, slides were incubated with biotinylated virus-specific

DNA probes,^c first at 85° C for 11 minutes to allow denaturation of DNA, then at 37° C for 2 hours for hybridization. To visualize bound probe, slides were incubated for 20 minutes at 37° C with horseradish peroxidase-conjugated avidin D, then developed in 0.024% aminoethylcarbazole.

VII. 3. 5. Demonstration of Viral Antigen Expression by Immunocytochemistry

As further confirmation of CMV infection, fixed culture slides were stained for CMV-specific antigens by an immunoperoxidase technique based on methods described by Sharma et al.,¹⁷¹ as detailed in V. 4. Briefly, slides were incubated for 45 minutes with virus-specific primary antibodies at 37° C, washed, and incubated 15 minutes with a biotin-conjugated secondary. Bound antibody complexes were visualized following 15 minutes incubation (37° C) with peroxidase-labelled avidin D by development in 0.024% aminoethylcarbazole.

VII. 3. 6. Plaque Assay of Infectious Virus Production

Virus production by infected cells was quantified by plaque assay of culture supernatants and, separately, of cell harvests (cell fraction), at various intervals over

^c See Appendix G.

the course of infection. HUVE cells were grown to confluence in fibronectin-treated 25 cm² plastic culture flasks and inoculated with either a virus-free preparation (sham inoculum^d), CMV VHL/E, or CMV VHL/F, at a titer of 10² PFU/cell. Following 1.5 hours adsorption, cultures were washed three times with PBS and once with serum-free medium, supplied with virus-free, (gentamicin and amphotericin B supplemented) complete medium (5 ml/flask), and incubated at 35.5° C as described above. The final rinse was reserved for a baseline (day 0) supernatant plaque assay. In addition, the corresponding monolayer was harvested for cell fraction plaque assay as described below. Confluent NHDF cultures were inoculated in an identical manner for comparative purposes.

At various intervals throughout the course of infection (30-35 days) cultures were harvested for assay as follows. Supernatant was withdrawn and reserved for plaque assay. The corresponding monolayer was washed twice with PBS, released from the substrate by addition of trypsin/EDTA,^e then resuspended in virus storage medium^f (40% MEM, 10% fetal bovine serum, 50% sucrose phosphate buffer [188 mM sucrose, 12mM K₂HPO₄, 7.5 mM KH₂PO₄]). This cell fraction

^d See IV. 4.

^e As described in III. 2. and Appendix C.

^f See Appendix A.

was then prepared for plaque assay by the rapid freeze-thaw method. Remaining unharvested cultures were supplied with fresh growth medium.

Plaque assays were performed by a modification of the methods of Wentworth and French,¹⁶⁶ as described in IV. 3. and VI. 7. Confluent NHDF monolayers in 6-well culture plates were inoculated in triplicate with appropriate dilutions of supernatants or cell fractions from infected HUVE or NHDF flasks, as well as from sham-inoculated cultures. Plaque assays were carried out as described in IV. 3.

VII. 3. 7. Assay of Viral DNA Polymerase Activity

As an additional determination of viral reproductive activity, CMV-specific DNA polymerase assays were performed on infected cell cultures at various intervals following inoculation. HUVE and NHDF cells were grown to confluence in 75 cm² plastic tissue culture flasks (Corning) and inoculated with either CMV AD169, CMV VHL/E, CMV VHL/F (10⁻² PFU/cell), or a sham preparation, according to the protocol described above. At various post-inoculation intervals (corresponding to those selected for plaque assay), replicate infected cell fractions were harvested for polymerase assay as follows.

Supernatant was removed and cells were washed twice with PBS, then released from the substrate by the addition

Supernatant was removed and cells were washed twice with PBS, then released from the substrate by the addition of trypsin/EDTA.^g Cells were suspended in 10 ml complete MEM,^h then centrifuged at 500 x g for 10 minutes. Following aspiration of the supernatant, pellets were washed in PBS, then re-sedimented by centrifugation. The supernatant was again aspirated and pellets were cryo-preserved at -80° C until assayed.

CMV-specific DNA polymerase assay protocol was based on previously established techniques.^{75 181 182 183} Frozen cell pellets were thawed, suspended in 0.5 ml extraction bufferⁱ (50 mM Tris-HCl [pH 7.5], 3 mM dithiothreitol, 200 mM KCL, 2 mM ATP-Mg), and disrupted by sonication (Branson Sonifier, output setting of 6, duty cycle 40%, 15 pulses/sample). The resulting homogenates were clarified by 2 minutes centrifugation (Fisher Model 235A microfuge).

Each DNA polymerase reaction mixture^j contained, in a total volume of 0.1 ml: 70 mM Tris-HCl (pH 8.0), 0.2 M KCl, 4 mM MgCl₂, 70 ug bovine serum albumin, 0.7 mM dithiothreitol, 2% (v/v) glycerol, 100 uM each of dATP, dCTP, dGTP, 1.6 uM [³H]-dTTP (500 uCi/umol), 13 ug of activated

^g See III. 2. and Appendix C.

^h See Appendix A.

ⁱ For details of preparation, see Appendix A.

^j For detailed formula, see Appendix A.

calf thymus DNA,^k and 10 ul cell lysate.^{182 183} Assays were incubated at 37° C in a shaking water bath for times ranging from 15 minutes to 2 hours (depending on inherent activities of the samples). Reactions were terminated after spotting on Whatman 3MM discs (50 ul/disc), by two 5-minute washes in cold 5% TCA/1% sodium pyrophosphate.¹⁸¹ Discs were dried under infra-red light following a final ethanol wash, immersed in toluene scintillation cocktail^l and counted for 1 minute in a Beckman LS 8100 scintillation counter.

Each sample was assayed at least in triplicate, and each series included 0-time reaction controls, sham-inoculated negative controls, high activity positive controls, and unwashed discs as indicators of total available label activity. Polymerase activity was expressed as nmol [³H]-dTTP incorporated into acid-insoluble material/ml/hour.^m

VII. 3. 8. Assay of Sample Protein Content

To facilitate determination of viral DNA polymerase specific activity, total protein content was measured for each sample lysate by the Comassie blue dye binding assay (Bio-Rad Laboratories, microassay procedure). To generate a

^k For details of preparation, see Appendix H.

^l See Appendix A.

^m For calculation of polymerase activity, see Appendix I.

standard curve, a solution of bovine serum albumin (BSA, 0.1463 mg/ml) was prepared in the extraction buffer described in VII. 3. 7. above. Each reaction mixture contained 700 ul distilled water, BSA solution (0, 10, 20, 40, 60, 80, or 100 ul, each in duplicate), and extraction buffer to bring the total volume to 800 ul. Lastly, to each reaction vessel was added 200 ul Comassie blue dye reagent (Bio-Rad). Assays were incubated 15 minutes at room temperature with periodic gentle agitation, then transferred to polystyrene semi-micro cuvettes (Kew Scientific). Optical density at 595 nm (OD_{595}) for each sample was read 15-60 minutes following dye reagent addition with a Zeiss Spektralphotometer model PM6.

OD_{595} values for each dilution were plotted against their corresponding protein contents (0-14.63 ug/ml). The first order regression curve derived from these data was then used to determine protein content of the experimental samples from their OD_{595} values. A new standard curve was generated for each series of experimental samples assayed.

Protein contents of all lysates (from which polymerase data were derived) were determined by this method. The procedure was basically as described above. Each lysate was assayed in quadruplicate: 2 assays with 10 ul of sample, and 2 assays with 20 ul of sample. Lysates often required additional dilution (up to 10^{-1}) in order to generate OD values within the limits of the standard curve.

Total protein values thus determined were used in conjunction with the [^3H]-TTP incorporation data generated by DNA polymerase assays described in VII. 3. 7. above, to calculate enzyme specific activity.ⁿ

VII. 4. Results

VII. 4. 1. Morphologic Cytopathology in CMV-Infected Endothelial Cells

Cytopathic changes induced in HUVE cells by CMV VHL/E upon initial isolation of the virus and at early passage have been previously described in VI. 4. 1. (see also reference 151). Identical cytopathology was evident in endothelial cell cultures inoculated with this strain following 20 passages in HUVE (Plate XVII).

In contrast, the endothelial cytopathic potential of VHL/F was lost after 20 passages in fibroblasts. No obvious cytopathology was observed in endothelial cells infected with this sub-strain at any time throughout the course of infection up to 35 days PI (Plates XVIII, XIX). Cells in which infection was verified both by the presence of CMV DNA (Plate XXI) and by the expression of CMV-specific antigens (Plate XXIV) appeared unperturbed at the light microscope level. Similarly, AD169-infected HUVE cells were

ⁿ See Appendix I.

PLATE XVII: Phase contrast micrograph of human umbilical vein endothelial cell culture 20 days post-inoculation with CMV VHL/E₂₀, 10^{-2} PFU/cell (original magnification: 50x).

PLATE XVIII: Phase contrast micrograph of human umbilical vein endothelial cell culture 20 days post-inoculation with CMV VHL/F₂₀, 10^{-2} PFU/cell (original magnification: 50x).

PLATE XIX: Phase contrast micrograph of human umbilical vein endothelial cell culture 20 days following sham-inoculation (original magnification: 50x).

morphologically indistinguishable from sham-inoculated controls (see Plate III and reference 151).

Infection of NHDF cells with CMV VHL/E or VHL/F resulted in typical CMV/fibroblast cytopathology qualitatively similar to that induced by AD169 and other strains of CMV (see Plates VII, VIII, IX).

VII. 4. 2. Demonstration of CMV DNA in Infected Endothelial Cells

In order to identify infected cells, and to confirm the identity of the virus, slide cultures were stained for CMV DNA by in situ hybridization. Sham-inoculated, and HSV 1-infected cultures were included in these experiments as specificity controls for the DNA probes.

Results of hybridization studies with VHL/E₂₀ confirmed the previous identification of this virus as a CMV strain. Cytopathic changes were again evident in endothelial cells staining positively for viral DNA (Plate XX). HUVE cultures inoculated with VHL/F₂₀ also contained CMV DNA-positive cells; however, these occurred with low frequency and without accompanying cytopathology (Plate XXI). The cellular response to VHL/F₂₀ infection therefore closely resembles the response to AD169 and OSU CMV 3 (see Plates XI, XII, and reference 151).

Attempts to hybridize HSV probes with CMV-infected cultures, or CMV probes with HSV-infected cultures all

PLATE XX: Photomicrograph of VHL/E₂₀-infected human umbilical vein endothelial cells stained for the presence of CMV DNA by *in situ* hybridization using a biotinylated CMV-specific DNA probe, and an avidin-peroxidase/aminoethylcarbazole complex for visualization. Cells were briefly counterstained with hematoxylin (original magnification: 64x).

PLATE XXI: Photomicrograph of VHL/F₂₀-infected human umbilical vein endothelial cells stained for the presence of CMV DNA by *in situ* hybridization using a biotinylated CMV-specific DNA probe, and an avidin-peroxidase/aminocethylcarbazole complex for visualization. Cells were briefly counterstained with hematoxylin (original magnification: 64x).

PLATE XXII: Photomicrograph of HSV 1-infected human umbilical vein endothelial cells stained by in situ hybridisation (to insure probe specificity) using a biotinylated CMV-specific DNA probe, and an avidin-peroxidase/aminocethylcarbazole complex for visualization. Cells were briefly counterstained with hematoxylin (original magnification: 64x).

yielded negative results (see Plate XXII for representative example), verifying the specificity of the probes, and ruling out the possibility of contamination of the CMV strains with HSV. Additionally, sham-inoculated cultures showed no evidence of hybridization with either probe.

Finally, it was noted that fibroblast cultures inoculated with each of the CMV strains exhibited positive hybridization staining within cytopathic foci (data not shown).

VII. 4. 3. Demonstration of CMV-Specific Antigen Expression in Infected Endothelial Cells

As further confirmation of CMV infection in inoculated HUVE cultures, slides were stained for either CMV early nuclear protein or CMV late nuclear protein using primary antibodies directed against each of these antigens.¹⁷² Sham-inoculated, and HSV-infected cultures were included in these experiments as specificity controls for the antibody reactions.

Results of immunohistochemical staining were consistent with those of the hybridization studies described above. While VHL/E-infected HUVE cells (as determined by positive staining for both CMV early and late nuclear proteins) exhibited obvious cytopathic changes (Plate XXIII), VHL/F-infected cells were few, and appeared morphologically unperturbed (Plate XXIV). No cross reactions were evident

PLATE XXIII: Photomicrograph of VHL/E₂₀-infected human umbilical vein endothelial cells stained for the presence of CMV early nuclear antigen by immunoperoxidase, using a primary antibody directed against CMV early nuclear antigen, a biotinylated secondary antibody, and an avidin-peroxidase/aminoethylcarbazole complex for visualization. Cells were briefly counterstained with hematoxylin (original magnification: 64x).

PLATE XXIV: Photomicrograph of VHL/F₂₀-infected human umbilical vein endothelial cells stained for the presence of CMV early nuclear antigen by immunoperoxidase, using a primary antibody directed against CMV early nuclear antigen, a biotinylated secondary antibody, and an avidin-peroxidase/aminocethylcarbazole complex for visualization. Cells were briefly counterstained with hematoxylin (original magnification: 64x).

PLATE XXV: Photomicrograph of HSV 1-infected human umbilical vein endothelial cells, immunoperoxidase-stained (to insure antibody specificity) using a primary antibody directed against CMV early nuclear antigen, a biotinylated secondary antibody, and an avidin-peroxidase/aminoethylcarbazole complex for visualization. Cells were briefly counterstained with hematoxylin (original magnification: 64x).

among CMV- and HSV-infected cultures and the corresponding primary antibodies (see Plate XV for representative example), nor did any of the antibodies show evidence of binding to sham-inoculated controls.

Staining of fibroblast cultures inoculated with each CMV strain also yielded results consistent with hybridization studies. Cells within or peripheral to cytopathic foci exhibited positive staining for both early and late antigens (data not shown).

VII. 4. 4. Quantitation of Infectious Virus Production

Earlier studies of the characteristics of VHL/E (at passage 6) indicated that this virus reproduced more efficiently in endothelial cells than in fibroblasts (see VI. 4. 3. and reference 151). This conclusion, however, was based on plaque assay of only the supernatant fraction of infected cultures. Visual observation of the progression of cytopathic changes in inoculated HUVE cultures suggested that data derived exclusively from supernatants may have underestimated the true extent of viral reproductive activity. Therefore, in this series of experiments, the plaque forming potential of supernatants and, separately, of cell fractions, from cultures inoculated with either VHL/E₂₀ or VHL/F₂₀, was quantified at various intervals PI.

Results of these studies demonstrate dramatic differences between the reproductive activities of VHL/E₂₀

and VHL/F₂₀. Whereas VHL/E has retained the ability to initiate sustained productive infection in endothelial cells, this potential has been severely attenuated in VHL/F following 20 passages in fibroblasts (Figure 9). Conversely, the efficiency of VHL/F₂₀ replication in fibroblast culture can be seen to exceed that of VHL/E₂₀ by several orders of magnitude (Figure 10). Comparing the virus production curves generated by each individual sub-strain in the two cell types (Figures 11, 12) with the curves generated in earlier experiments with supernatants from AD169-infected cultures (Figure 7), it is clear that VHL/F has come to resemble AD169 in its host preference.

Another distinction that has developed between the two VHL variants relates to the extent of release of free virions into the supernatant. In VHL/E-infected HUVE cultures, the greater plaque forming potential was found in the cell fraction as compared to that found in the supernatant (Figure 13). Such was also the case in VHL/E-infected NHDF cultures (Figure 14), suggesting retention of the tendency of newly isolated CMV to remain cell-associated.^{94 184} This likely accounts for previous underestimation of virus production when only supernatants were assayed (see VI. 4. 3. and reference 151). In contrast, VHL/F-infected fibroblasts released a greater

FIGURE 9: Infectious virus production in human umbilical vein endothelial cells (HUVE) inoculated on day 0 with CMV VHL/E₂₀ or VHL/F₂₀, 10⁻² PFU/cell, expressed as total PFU (supernatant + cell fraction) generated per inoculated flask, as determined by plaque assay on fibroblast monolayers (error bars represent standard error of the mean).

FIGURE 10: Infectious virus production in neonatal human dermal fibroblasts (NHDF) inoculated on day 0 with CMV VHL/E₂₀ or VHL/F₂₀, 10⁻² PFU/cell, expressed as total PFU (supernatant + cell fraction) generated per inoculated flask, as determined by plaque assay on fibroblast monolayers (error bars represent standard error of the mean).

FIGURE 9

FIGURE 10

FIGURE 11: Infectious virus production in neonatal human dermal fibroblasts (NHDF) and human umbilical vein endothelial cells (HUVE) inoculated on day 0 with CMV VHL/E₂₀, 10⁻² PFU/cell, expressed as total PFU (supernatant + cell fraction) generated per inoculated flask, as determined by plaque assay on fibroblast monolayers (replot of data presented in Figures 9 and 10; error bars represent standard error of the mean).

FIGURE 12: Infectious virus production in neonatal human dermal fibroblasts (NHDF) and human umbilical vein endothelial cells (HUVE) inoculated on day 0 with CMV VHL/F₂₀, 10⁻² PFU/cell, expressed as total PFU (supernatant + cell fraction) generated per inoculated flask, as determined by plaque assay on fibroblast monolayers (replot of data presented in Figures 9 and 10; error bars represent standard error of the mean).

FIGURE 11

FIGURE 12

FIGURE 13: Supernatant/cell fraction distribution of infectious virus produced in human umbilical vein endothelial cells (HUVE) inoculated on day 0 with CMV VHL/E₂₀, 10⁻² PFU/cell, expressed as total PFU generated by each fraction per inoculated flask, as determined by plaque assay on fibroblast monolayers (error bars represent standard deviation).

FIGURE 14: Supernatant/cell fraction distribution of infectious virus produced in neonatal human dermal fibroblasts (NHDF) inoculated on day 0 with CMV VHL/E₂₀, 10⁻² PFU/cell, expressed as total PFU generated by each fraction per inoculated flask, as determined by plaque assay on fibroblast monolayers (error bars represent standard deviation).

FIGURE 13

FIGURE 14

FIGURE 15

FIGURE 15: Supernatant/cell fraction distribution of infectious virus produced in neonatal human dermal fibroblasts (NHDF) inoculated on day 0 with CMV VHL/F₂₀, 10² PFU/cell, expressed as total PFU generated by each fraction per inoculated flask, as determined by plaque assay on fibroblast monolayers (error bars represent standard deviation).

quantity of infectious virus into the supernatant than was retained in the cell fraction (Figure 15).^o

Again, progressive advance of dramatic cytopathology was observed within VHL/E-infected HUVE monolayers, while no obvious changes occurred in response to VHL/F. NHDF cultures inoculated with each of these strains exhibited typical CMV cytopathic effect. Finally it should be noted that no plaques were ever induced by supernatants or cell fractions from sham-inoculated controls.

VII. 4. 5. CMV DNA Polymerase Activity

In an effort to strengthen the arguments set forth in the previous section, viral reproductive activity was quantitated on the biochemical level by determination of CMV-specific DNA polymerase activity in lysates of infected cells. Cultures of NHDF and HUVE cells were inoculated with each of the virus strains, then harvested for assay at post-inoculation intervals corresponding to those chosen for plaque assay. Data generated by polymerase assay of these harvests were plotted as [³H]-TTP incorporation (into activated DNA template) vs. time PI, and superimposed on plaque assay data (Figures 16-21). These graphs clearly show that trends in polymerase activity, as a function of

^o Data derived from VHL/F-inoculated HUVE were difficult to interpret in this regard, due to the minimal levels of virus production in this cell type (see Figure 20).

FIGURE 16: CMV DNA polymerase activity induced in neonatal human dermal fibroblasts (NHDF) inoculated on day 0 with CMV AD169, 10^{-2} PFU/cell, expressed as rate of [3 H]-TTP incorporation into activated DNA template. Polymerase activity is shown superimposed on plaque assay data to illustrate correspondence of results.

FIGURE 17: CMV DNA polymerase activity induced in neonatal human dermal fibroblasts (NHDF) inoculated on day 0 with CMV VHL/F₂₀, 10^{-2} PFU/cell, expressed as rate of [3 H]-TTP incorporation into activated DNA template. Polymerase activity is shown superimposed on plaque assay data to illustrate correspondence of results.

FIGURE 16

FIGURE 17

FIGURE 18: CMV DNA polymerase activity induced in neonatal human dermal fibroblasts (NHDF) inoculated on day 0 with CMV VHL/E₂₀, 10⁻² PFU/cell, expressed as rate of [³H]-TTP incorporation into activated DNA template. Polymerase activity is shown superimposed on plaque assay data to illustrate correspondence of results.

FIGURE 19: CMV DNA polymerase activity induced in human umbilical vein endothelial cells (HUVE) inoculated on day 0 with CMV AD169, 10⁻² PFU/cell, expressed as rate of [³H]-TTP incorporation into activated DNA template. Polymerase activity is shown superimposed on plaque assay data to illustrate correspondence of results.

FIGURE 18

FIGURE 19

FIGURE 20: CMV DNA polymerase activity induced in human umbilical vein endothelial cells (HUVE) inoculated on day 0 with CMV VHL/F₂₀'₃ 10⁻² PFU/cell, expressed as rate of [³H]-TTP incorporation into activated DNA template. Polymerase activity is shown superimposed on plaque assay data to illustrate correspondence of results.

FIGURE 21: CMV DNA polymerase activity induced in human umbilical vein endothelial cells (HUVE) inoculated on day 0 with CMV VHL/E₂₀'₃ 10⁻² PFU/cell, expressed as rate of [³H]-TTP incorporation into activated DNA template. Polymerase activity is shown superimposed on plaque assay data to illustrate correspondence of results.

FIGURE 20

FIGURE 21

time PI, closely parallel those of infectious virus production. In all experiments, polymerase activity was consistent with plaque forming potential. Correlation coefficients for each series, derived from first order regression plots of [^3H]-TTP incorporation vs. plaque forming potential, are listed in Table 1.

TABLE 1: Correlation of CMV-specific DNA polymerase activity and infectious virus production (expressed as R values derived from 1st order regression plots of [^3H]-TTP incorporation vs. plaque forming potential).

HOST CELL	VIRUS STRAIN		
	AD169	VHL/F	VHL/E
NHDF	0.565	0.836	0.916
HUVE	0.515	0.731	0.824

It should be noted that in the two AD169 series, [^3H]-TTP incorporation was plotted against supernatant plaque forming activity, rather than that of the cell fraction. This likely accounts for the lower coefficients of correlation (R values) generated by these experiments, as shown in Table 1.

In order to calculate specific activity of the enzyme, total protein content was determined for each sample lysate from which polymerase data were derived, by the Comassie blue dye binding method.^p Results of protein assays are shown in Figures 22-27. Based on these data and the corresponding [³H]-TTP incorporation rates presented above, specific activity of each sample was calculated^q and plotted vs. time PI. The resulting curves were superimposed on the corresponding plaque assay data as described above (Figures 28-33). Correlation coefficients for each series, derived from first order regression plots of polymerase specific activity vs. plaque forming potential, are listed in Table 2.

^p Standard curves for protein assays can be found in Appendix J.

^q See Appendix I.

FIGURE 22: Total protein content of crude lysates prepared from neonatal human dermal fibroblasts (NHDF) inoculated on day 0 with CMV AD169, 10^2 PFU/cell, expressed in mg protein/ml, as determined by Comassie blue dye binding assay.

FIGURE 23: Total protein content of crude lysates prepared from neonatal human dermal fibroblasts (NHDF) inoculated on day 0 with CMV VHL/F₂₀, 10^2 PFU/cell, expressed in mg protein/ml, as determined by Comassie blue dye binding assay.

FIGURE 22

FIGURE 23

FIGURE 24: Total protein content of crude lysates prepared from neonatal human dermal fibroblasts (NHDF) inoculated on day 0 with CMV VHL/E₂₀, 10⁻² PFU/cell, expressed in mg protein/ml, as determined by Comassie blue dye binding assay.

FIGURE 25: Total protein content of crude lysates prepared from human umbilical vein endothelial cells (HUVE) inoculated on day 0 with CMV AD169, 10⁻² PFU/cell, expressed in mg protein/ml, as determined by Comassie blue dye binding assay.

FIGURE 24

FIGURE 25

FIGURE 26: Total protein content of crude lysates prepared from human umbilical vein endothelial cells (HUVE) inoculated on day 0 with CMV VHL/F₂₀, 10⁻² PFU/cell, expressed in mg protein/ml, as determined by Comassie blue dye binding assay.

FIGURE 27: Total protein content of crude lysates prepared from human umbilical vein endothelial cells (HUVE) inoculated on day 0 with CMV VHL/E₂₀, 10⁻² PFU/cell, expressed in mg protein/ml, as determined by Comassie blue dye binding assay.

FIGURE 26

FIGURE 27

FIGURE 28: CMV DNA polymerase specific activity induced in neonatal human dermal fibroblasts (NHDF) inoculated on day 0 with CMV AD169, 10^{-2} PFU/cell, expressed as rate of [3 H]-TTP incorporation into activated DNA template per mg total protein. Polymerase activity is shown superimposed on plaque assay data to illustrate correspondence of results.

FIGURE 29: CMV DNA polymerase specific activity induced in neonatal human dermal fibroblasts (NHDF) inoculated on day 0 with CMV VHL/F_{20/3}, 10^{-2} PFU/cell, expressed as rate of [3 H]-TTP incorporation into activated DNA template per mg total protein. Polymerase activity is shown superimposed on plaque assay data to illustrate correspondence of results.

FIGURE 28

FIGURE 29

FIGURE 30: CMV DNA polymerase specific activity induced in neonatal human dermal fibroblasts (NHDF) inoculated on day 0 with CMV VHL/E₂₀, 10⁻² PFU/cell, expressed as rate of [³H]-TTP incorporation into activated DNA template per mg total protein. Polymerase activity is shown superimposed on plaque assay data to illustrate correspondence of results.

FIGURE 31: CMV DNA polymerase specific activity induced in human umbilical vein endothelial cells (HUVE) inoculated on day 0 with CMV AD169, 10⁻² PFU/cell, expressed as rate of [³H]-TTP incorporation into activated DNA template per mg total protein. Polymerase activity is shown superimposed on plaque assay data to illustrate correspondence of results.

FIGURE 30

FIGURE 31

FIGURE 32: CMV DNA polymerase specific activity induced in human umbilical vein endothelial cells (HUVE) inoculated on day 0 with CMV VHL/F_{20'}³ 10⁻² PFU/cell, expressed as rate of [³H]-TTP incorporation into activated DNA template per mg total protein. Polymerase activity is shown superimposed on plaque assay data to illustrate correspondence of results.

FIGURE 33: CMV DNA polymerase specific activity induced in human umbilical vein endothelial cells (HUVE) inoculated on day 0 with CMV VHL/E_{20'}³ 10⁻² PFU/cell, expressed as rate of [³H]-TTP incorporation into activated DNA template per mg total protein. Polymerase activity is shown superimposed on plaque assay data to illustrate correspondence of results.

FIGURE 32

FIGURE 33

TABLE 2: Correlation of CMV-specific DNA polymerase activity and infectious virus production (expressed as R values derived from 1st order regression plots of enzyme specific activity vs. plaque forming potential).

HOST CELL	VIRUS STRAIN		
	AD169	VHL/F	VHL/E
NHDF	0.792	0.673	0.863
HUVE	0.538	0.730	0.848

Although enzyme activity expressed in this manner still closely parallels infectious virus production as a function of time PI, the correlation is not as close in some cases as when polymerase activity is expressed in terms of [^3H]-TTP incorporation. This effect is apparent as well in the two corresponding series of figures above, and is clearly a result of the influence of sample protein content on enzyme specific activity values. Since total protein was observed to vary dramatically with time PI in some experiments (Figures 22-27), primarily as a function of monolayer condition, [^3H]-TTP incorporation rates seem to more accurately represent viral DNA polymerase activity under these experimental conditions.

VII. 5. Discussion

Endothelial involvement in human CMV disease has been well documented. Studies of tissue sections obtained by biopsy and at autopsy have shown evidence of CMV infection in endothelial cells within most organ systems, including lung,²⁷ skin,²⁷ retina,²⁸ and gastrointestinal tract,^{29 30 31 32 33} as well as spleen and pancreas,²⁴ and peritoneum.³⁴ Most often, infected cells have exhibited classical CMV-associated morphologic derangements (cytomegaly, nuclear and cytoplasmic inclusions). However, results of in vitro studies of CMV/endothelial cell interactions have frequently been difficult to resolve with clinical observations. While Friedman et al.³⁵ were unable to demonstrate infection of human or bovine endothelial cells with the Davis strain, experiments by Ho et al.³⁶ and Smiley et al.³⁷ resulted in successful human endothelial cell infection by AD169 and Towne strain respectively. Nonetheless, efficiency of endothelial cell infection by CMV in vitro has generally been quite low.^{152 153} It has also been somewhat perplexing that productive infection by laboratory-adapted strains has been demonstrated in the absence of obvious morphological cytopathic change. Previous results from this laboratory^{151 169} have also shown production of infectious virus by endothelial cells morphologically indistinguishable from sham-inoculated controls.

In an effort to develop an accurate in vitro model of CMV/endothelial cell interaction, clarification of these apparent discrepancies has been sought by propagating a single clinical isolate through multiple passages in human umbilical vein endothelial cells and, separately in neonatal human dermal fibroblasts. In an earlier comparison of the infectious properties of AD169 with those of the endothelial-raised strain, productive infection in HUVE cells (by VHL/E) was accompanied by dramatic cytopathology not observed in AD169-inoculated cultures (see Chapter VI and reference 151). In addition, quantitative, strain-specific differences in reproductive potential were noted. As CMV AD169, isolated in 1956,⁹⁴ has been extensively propagated in fibroblasts, the above results suggest that the process of fibroblast-adaption may significantly alter characteristics of the virus. However, since AD169 and VHL are of separate origins, it could be legitimately argued that their different behaviors in vitro are inherently strain-related, rather than a result of their modes of propagation.

To resolve this issue, the infectious properties of the VHL isolate have been directly compared, following 20 passages in endothelial cells (VHL/E₂₀), with those of the same isolate propagated through 20 passages in fibroblasts (VHL/F₂₀). Results of these experiments clearly show that dramatic diversification has occurred as a function of host

system. While VHL/E has retained its endothelial cytopathic potential, this ability was lost after 20 passages in fibroblasts. Additionally, the efficiency of acute endothelial cell infection by VHL/F, as indicated by the frequency of positive staining for CMV antigens or DNA, has been significantly attenuated. Similarly, the ability of VHL/F to initiate sustained productive infection in HUVE cultures has been nearly lost. Plaque assay of culture supernatants and cell fractions, as well as virus-specific polymerase assay of cell lysates, have demonstrated progressive viral reproductive activity in VHL/E-inoculated HUVE cultures, whereas VHL/F production was barely detectable. Yet another distinction that has developed between the two sub-strains relates to the relative distribution of viral progeny between the supernatant and cell fractions in actively productive cultures. Whereas newly replicated VHL/E remains primarily cell associated, the majority of plaque-forming activity of VHL/F is found in the supernatant. In particular, the data generated by virus production studies in inoculated NHDF cells clearly indicate that VHL/F has undergone the classical fibroblast-adaption process.^{94 184} Replication of this sub-strain in NHDF proceeds rapidly and efficiently, with abundant progeny being shed into the culture medium. In all of these respects, VHL/F has come to resemble AD169 and other laboratory-adapted CMV strains.

The idea of alteration of infectious properties in viruses as a result of extensive in vitro propagation is by no means new. With regard to CMV, this concept was alluded to as early as 1970 by Weller,³⁵ and has been the basis of an approach to development of anti-CMV vaccination employing the Towne strain.¹⁷⁵ Indeed, Ho et al.³⁶ observed that recent clinical isolates of CMV showed more extensive cytopathic effect in endothelial cells than did AD169. Einhorn and Ost¹⁵⁶ found that low passage CMV isolates regularly induced CMV early antigens in human leukocytes, while antigen-positive cells were rare in AD169-inoculated suspensions. Whereas early attempts to infect cultured human nasopharyngeal epithelium with Epstein-Barr virus released from established productively infected lymphoid cell lines failed,^{185 186} Sixbey et al.¹⁸⁷ succeeded using wild type virus from fresh oropharyngeal washings. Similarly, Carpenter & Chesebro¹⁸⁸ reported a dramatic shift in cell tropism of equine infectious anemia virus following repeated passage in equine dermal cell cultures. Infectivity for horse macrophages (the natural host cell in vivo) continually declined, while viral tropism for dermal cells was progressively augmented.

Regarding mechanisms responsible for the modifications in viral characteristics reported here and observed by others, several possibilities are worthy of consideration. Alterations in viral DNA has been demonstrated by both

Furukawa¹⁷⁶ and Ogura et al.¹⁷⁷ when CMV variants were recovered from persistently infected cells 9-12 months after inoculation with Towne strain. In both cases these variants showed structural alterations in their genomes, as demonstrated by restriction fragment analysis, when compared with the original inocula. A similar approach to distinguishing the two VHL sub-strains might prove fruitful; however, due to the relative enormity and complexity of the CMV genome, as well as its tendency to isomerize (see II. 3. 1. and Figure 1), a search for subtle polymorphisms may be exhaustive. Alternatively, modification of the viral proteins may account for the changes in infectivity. Since CMV obtains its envelope from virally modified host cell membrane components, a host-related alteration in protein profile would not be unexpected, and might explain the observed apparent shift in cell affinity. Resolution of these issues will require further detailed investigation. Nevertheless, it is clear that the host-specific intracellular environment profoundly influences the properties of the resulting progeny virus.

In summary, it has been demonstrated that the process of fibroblast-adaption commonly employed in the propagation of CMV restricts the host range of the virus while attenuating its spectrum of cytopathic potential; whereas endothelial-based propagation preserves the natural endothelial cytopathogenicity of the original isolate.

Considering the accumulating evidence for a central role of the endothelial cell in disseminated CMV disease, it is proposed that a strain of the virus propagated in endothelial cells provides a more accurate in vitro model of this important aspect of human CMV infection.

CHAPTER VIII

TRANSMISSION ELECTRON MICROSCOPY

VIII. 1. Introduction

The methods thus far described for demonstrating viral infection in cultured cells have been, in the strictest sense, indirect. Acute CMV infection has been implied by the demonstration of the presence of intracellular viral DNA by in situ hybridization, by immunohistochemical demonstration of CMV-specific antigen expression, and by observations of developing cellular cytopathic changes. Viral reproduction patterns have been traced by plaque assay of culture supernatants and cell fractions, as well as by virus-specific DNA polymerase assay of cell lysates.

None of these techniques, however, allow direct observation of the virus itself. Therefore, as final confirmation of CMV infection, intracellular Herpes-type virions have been directly visualized by transmission electron microscopy.

VIII. 2. Materials and Methods

HUVE and NHDF cultures were grown to confluence in 75 cm² culture flasks and inoculated, as described

previously, with AD169, VHL/F₂₀, VHL/E₂₀ (10⁻² PFU/cell), or a sham preparation. Cultures were incubated until cytopathic changes were widespread, then harvested and fixed as described below. AD169- or VHL/F-inoculated HUVE cultures, in which obvious cytopathic effect does not develop, were incubated 7 days.

To prepare for processing, cells were harvested by addition of trypsin/EDTA, suspended in growth medium, then pelleted by centrifugation (400 x g, 5 minutes). Cells were washed twice in PBS, and the final pellet was resuspended in 100 ul of PBS. Cells were fixed by the addition of 1 ml 2% phosphate buffered gluteraldehyde, then pelleted by centrifugation and stored at 4° C until processed (<7 days).

To prepare for embedding, specimens were post-fixed in 1% phosphate buffered osmium tetroxide (1 hour), then dehydrated by successive 30 minute washes in graded ethanols (50%, 70%, 95%, 100%). Specimens were embedded in Spur low viscosity embedding media (SEM), consisting of vinyl cyclohexene dioxide (ERL 4206, 15.0 g), DER 736 resin (10.5 g), nonenyl succinic anhydride (NSA, 39.0 g), and dimethyl aminoethanol (DMAE, 0.31 g). Blocks were cured for a minimum of 12 hours at 70° C.

Thin sections (approximately 100 nm) were cut from cured blocks using an ultramicrotome (LKB Nova), and mounted on 2-mm 200 mesh copper grids. Grids were heavy

metal-stained using a standard two-step uranyl acetate/alkaline lead salt technique. Grids were first immersed for 5 minutes in uranyl acetate solution (6.25% uranyl acetate, 25% ethanol), washed in 50% ethanol then in distilled water, and dried for one hour at 60° C. Dried grids were stained for 20 minutes in lead citrate solution (4.43% lead nitrate, 5.87% sodium citrate), washed in 0.2 N sodium hydroxide then in distilled water, and finally dried for two hours at 60° C.

Sections were examined and photographed using a Hitachi HU-12 transmission electron microscope employing a 50 kV electron beam.

VIII. 3. Results

Results of transmission electron microscopy studies are presented in Plates XXVI-XXXIX. As can be seen in Plates XXVIII-XXXIII, particles characteristic of Herpes-type capsids,¹⁸⁹ measuring approximately 100 nm in diameter,¹⁹⁰ are abundant in nuclei of NHDF cells inoculated with CMV AD169 (Plates XXVIII, XXIX), VHL/F₂₀ (Plates XXX, XXXI), or VHL/E₂₀ (Plates XXXII, XXXIII). In addition, complete enveloped virions (approximately 150 nm in diameter)¹⁸⁹ are visible in the cytoplasm (Plate XXXIV). Furthermore, pathologically clumped chromatin can be observed in cells showing evidence of advanced infection, as exemplified in Plate XXXII. Low and high power electron micrographs of an

PLATE XXVI: Transmission electron micrograph of neonatal human dermal fibroblast cell from sham-inoculated culture (final magnification: 6000x).

PLATE XXVII: Transmission electron micrograph of neonatal human dermal fibroblast cell from sham-inoculated culture (final magnification: 61,200x).

PLATE XXVIII: Transmission electron micrograph of AD169-infected neonatal human dermal fibroblast cell. Several clusters of viral capsids are visible within the nucleus (final magnification: 16,000x).

PLATE XXIX: Transmission electron micrograph of the nuclear region of an AD169-infected neonatal human dermal fibroblast cell. Several Herpes-type capsids, measuring approximately 100 nm in diameter, are distinctly visible (final magnification: 61,200x).

PLATE XXX: Transmission electron micrograph of VHL/F-infected neonatal human dermal fibroblast cell. A cluster of viral particles is visible within an electron-dense region adjacent to a nuclear segment (final magnification: 12,000x).

PLATE XXXI: Transmission electron micrograph of a nuclear region within a VHL/F-infected neonatal human dermal fibroblast cell (higher magnification of cell shown in Plate XXX). A collection of viral capsids (approximately 100 nm in diameter) is visible within an electron-dense area (final magnification: 61,200x).

PLATE XXXII: Transmission electron micrograph of VHL/E-infected neonatal human dermal fibroblast cell showing pathologically clumped chromatin. Several clusters of viral particles are visible within the nucleus (final magnification: 10,000x).

PLATE XXXIII: Transmission electron micrograph of a nuclear region within the VHL/E-infected neonatal human dermal fibroblast cell shown in Plate XXXII. Distinct Herpes-type capsids, approximately 100 nm in diameter, are abundant (final magnification: 61,200x).

PLATE XXXIV: Transmission electron micrograph of a peripheral cytoplasmic region within the VHL/E-infected neonatal human dermal fibroblast cell shown in Plates XXXII and XXXIII. Complete, enveloped Herpes-type virions, approximately 150 nm in diameter, are visible adjacent to the plasma membrane (final magnification: 61,200x).

uninfected fibroblast cell are presented for comparison (Plates XXVI, XXVII).

In contrast to results of fibroblast studies, an exhaustive search for viral particles within HUVE cells inoculated with AD169 or VHL/F₂₀ proved unsuccessful. Only VHL/E₂₀-infected endothelial cells showed evidence of viral proliferation (Plates XXXVII-XXXIX). Again, clumped chromatin is apparent in heavily infected cells (Plate XXXVII). Low and high power micrographs of an uninfected HUVE cell showing characteristic Weibel-Palade bodies are included for comparison (Plates XXXV, XXXVI).

PLATE XXXV: Transmission electron micrograph of human umbilical vein endothelial cell from sham-inoculated culture (final magnification: 8000x).

PLATE XXXVI: Transmission electron micrograph of human umbilical vein endothelial cell from sham-inoculated culture. Characteristic Weibel-Palade bodies are visible in the cytoplasm (final magnification: 30,000x).

PLATE XXXVII: Transmission electron micrograph of VHL/E-infected human umbilical vein endothelial cell showing pathologically clumped chromatin (final magnification: 8000x).

PLATE XXXVIII: Transmission electron micrograph of a portion of the nucleus of the VHL/E-infected human umbilical vein endothelial cell shown in Plate XXXVII. Herpes-type capsids are widely disseminated (final magnification: 24,000x).

PLATE XXXIX: Transmission electron micrograph of a nuclear region within the VHL/E-infected human umbilical vein endothelial cell shown in Plates XXXVII and XXXVIII. Distinct Herpes-type capsids, approximately 100 nm in diameter are apparent (final magnification: 61,200x).

VIII. 4. Discussion

Results of transmission electron microscopy studies described herein support conclusions generated by the experiments described in Chapters VI and VII. The abundance of viral particles observed within endothelial cells inoculated with VHL/E₂₀ confirm, by direct visualization, the infectivity and reproductive potential of this strain in HUVE culture. Conversely, the inability to locate even a single intracellular virion following inoculation of endothelial cells with either AD169 or VHL/F₂₀ is consistent with the negligible viral activity induced by these strains in HUVE cultures indicated by previous experiments. Finally, the ability of fibroblast cells to support replication of each of the viral strains has been reaffirmed.

CHAPTER IX

CONCLUSIONS AND DISCUSSION

IX. 1. Summary of Experimental Results: Conclusions

Endothelial involvement in human CMV disease has been well documented. Studies of tissue sections obtained by biopsy and at autopsy have shown evidence of CMV infection in endothelial cells within most organ systems, including lung,²⁶ skin,²⁷ retina,²⁸ and gastrointestinal tract,^{29 30 31 32 33} as well as spleen and pancreas,²⁴ and peritoneum.³⁴ Most often, infected cells have exhibited classical CMV-associated morphologic derangements (cytomegaly, nuclear and cytoplasmic inclusions). However, results of in vitro studies of CMV/endothelial cell interactions have frequently been difficult to resolve with clinical observations. While Friedman et al.³⁵ were unable to demonstrate infection of human or bovine endothelial cells with the Davis strain, experiments by Ho et al.³⁶ and Smiley et al.³⁷ resulted in successful human endothelial cell infection by AD169 and Towne strain respectively. Nonetheless, efficiency of endothelial cell infection by CMV in vitro has generally been quite low.^{152 153} It has also been somewhat perplexing that productive infection by

fibroblast-adapted laboratory strains has been demonstrated in the absence of obvious morphological cytopathic change.

In an effort to develop an accurate in vitro model of CMV/endothelial cell interaction, clarification of these apparent discrepancies has been sought by propagating a single clinical isolate through multiple passages in human umbilical vein endothelial cells and, separately in neonatal human dermal fibroblasts. In an initial comparison of the infectious properties of AD169 with those of the endothelial-raised strain, productive infection in HUVE cells (by VHL/E) was accompanied by dramatic cytopathology not observed in AD169-inoculated cultures¹⁵¹ (also see Chapter VI). In addition, quantitative, strain-specific differences in reproductive potential were noted. As CMV AD169, isolated in 1956,⁹⁴ has been extensively propagated in fibroblasts, the above results suggest that the process of fibroblast-adaption may significantly alter characteristics of the virus. However, since AD169 and VHL are of separate origins, it could be legitimately argued that their different behaviors in vitro are inherently strain-related, rather than a result of their modes of propagation.

To resolve this issue, the infectious properties of the VHL isolate have been directly compared, following 20 passages in endothelial cells (VHL/E₂₀), with those of the same isolate propagated through 20 passages in fibroblasts

(VHL/F₂₀). Results of these experiments clearly show that dramatic diversification has occurred as a function of host system (see Chapter VII). While VHL/E has retained its endothelial cytopathic potential, this ability was lost after 20 passages in fibroblasts. Additionally, the efficiency of acute endothelial cell infection by VHL/F, as indicated by the frequency of positive staining for CMV antigens or DNA, and as suggested by electron microscopy, has been significantly attenuated. Similarly, the ability of VHL/F to initiate sustained productive infection in HUVE cultures has been nearly lost. Plaque assay of culture supernatants and cell fractions, as well as virus-specific DNA polymerase assay of cell lysates, have demonstrated progressive viral reproductive activity in VHL/E-inoculated HUVE cultures, whereas VHL/F production was barely detectable.

Yet another distinction that has developed between the two sub-strains relates to the relative distribution of viral progeny between the supernatant and cell fractions in actively productive cultures. Whereas newly replicated VHL/E remains primarily cell associated, the majority of plaque-forming activity of VHL/F is found in the supernatant. In particular, the data generated by virus production studies in inoculated NHDF cells clearly indicate that VHL/F has undergone the classical fibroblast-adaption process.^{94 184} Replication of this sub-strain in NHDF

proceeds rapidly and efficiently, with abundant progeny being shed into the culture medium. In all of these respects, VHL/F has come to resemble AD169 and other laboratory-adapted CMV strains.

Regarding the hypotheses stated in Chapter I, these findings clearly demonstrate that viral properties, including expression of cytopathic potential, viral reproductive activity, and cell tropism, are influenced by the host system selected for in vitro propagation. In addition, results of this investigation show that endothelial cells are natural hosts for productive CMV infection, provided the characteristics of the original viral isolate are maintained in culture by endothelial-based propagation.

IX. 2. Basis for Sub-Strain Diversification

The idea of alteration of infectious properties in viruses as a result of extensive in vitro propagation is by no means new. Indeed this process bears strong analogy to serial in vitro culture of prokaryotic or eukaryotic cells, during which extracellular environmental conditions can dramatically affect characteristics of the resulting progeny. With regard to CMV, this concept was alluded to as early as 1970 by Weller,³⁶ and has been the basis of an approach to development of anti-CMV vaccination employing the Towne strain.¹⁷⁵ Indeed, Ho et al.³⁶ observed that recent

clinical isolates of CMV showed more extensive cytopathic effect in endothelial cells than did AD169. Einhorn and Ost¹⁵⁶ found that low passage CMV isolates regularly induced CMV early antigens in human leukocytes, while antigen-positive cells were rare in AD169-inoculated suspensions. Whereas early attempts to infect cultured human nasopharyngeal epithelium with Epstein-Barr virus released from established productively infected lymphoid cell lines failed,^{185 186} Sixbey et al.¹⁸⁷ succeeded using wild type virus from fresh oropharyngeal washings. Similarly, Carpenter & Chesebro¹⁸⁸ reported a dramatic shift in cell tropism of equine infectious anemia virus following repeated passage in equine dermal cell cultures. Infectivity for horse macrophages (the natural host cell *in vivo*) continually declined, while viral tropism for dermal cells was progressively augmented.

Regarding mechanisms responsible for the modifications in viral characteristics reported here and observed by others, several possibilities are worthy of consideration. Alterations in viral DNA has been demonstrated by both Furukawa¹⁷⁶ and Ogura et al.¹⁷⁷ when CMV variants were recovered from persistently infected cells 9-12 months after inoculation with Towne strain. In both cases these variants showed structural alterations in their genomes, as demonstrated by restriction fragment analysis, when compared with the original inocula. A similar approach to

distinguishing the two VHL sub-strains might prove fruitful; however, due to the relative enormity and complexity of the CMV genome, as well as its tendency to isomerize (see II. 3. 1. and Figure 1), a search for subtle polymorphisms may prove exhaustive. Alternatively, modification of the viral proteins may account for the changes in infectivity. Since CMV obtains its envelope from virally modified host cell membrane components, a host-related alteration in protein profile would not be unexpected, and might explain the observed apparent shift in cell affinity. Resolution of these issues will require further detailed investigation. Nevertheless, it is clear that the host-specific intracellular environment profoundly influences the properties of the resulting progeny virus.

IX. 3. VHL/E as a Model for CMV/Endothelial Cell Interaction: Critical Analysis

Nearly all available information regarding the structure and behavior of CMV has been derived from in vitro experiments involving virus which has been extensively propagated in fibroblasts (AD169, Towne, Davis, etc.). However the nature of the cellular interactions of fibroblast-adapted strains seems somewhat inconsistent with in vivo observations in human CMV disease. Indeed properties of these strains differ significantly from those of fresh clinical isolates in several important aspects.

While newly replicated virus tends to remain cell-associated in cultures of fresh isolates, the bulk of the progeny produced by established strains is released into the supernatant.⁸⁸ In addition, absolute titers of virus production increase dramatically following several passages in fibroblasts.^{93 94 95} Furthermore, fibroblast-adaption seems to limit the host range of the virus and attenuate its spectrum of cytopathic potential. Although evidence of CMV infection has been seen in many cell types *in vivo*, results of the paucity of *in vitro* experiments designed to evaluate the interaction of fibroblast-raised CMV with cells of differing lineages indicate a very narrow host range for the virus.^{37 157 159 161} Results of this investigation have indeed confirmed that fibroblast adaption severely restricts the infectivity of VHL in endothelial cells while nearly eliminating its endothelial cytopathogenicity.

Conversely, CMV VHL/E, through endothelial cell-based propagation, has retained properties of the original isolate. This strain continues to induce in endothelial cells (and in fibroblasts), marked cytopathology which often shares morphological characteristics with that observed *in vivo*. In addition, VHL/E is capable of initiating sustained productive infection in both cell types, which progresses to ultimately destroy the entire monolayer.

Although these characteristics have been stably retained over 20 passages in vitro and seem to qualify VHL/E as a superior model, several limitations of the system are worthy of attention. The most significant of these is the relatively low titer of infectious virus produced in endothelial cells. While high passage fibroblast-based propagation commonly generates stocks of 10^6 - 10^8 PFU/ml, VHL/E titers have remained at approximately 10^4 PFU/ml throughout its culture history. In addition, endothelial-based propagation is a slow process. Each passage requires approximately 15-20 days and results in only a two-fold increase in the quantity of infected cells. Whereas fibroblast-adaption results in a dramatic acceleration in propagation with increasing passage number, the rate of VHL/E production has remained fairly constant. Finally, economic issues must be considered. The requirements of cultured endothelial cells for higher serum concentrations, supplemental growth factors, and fibronectin treatment of growing surfaces drive the cost of endothelial-based CMV propagation far above that of traditional methods.

IX. 4. Directions for Further Research/Potential Applications for the VHL/E Model

Although the retention of natural endothelial cytopathogenic properties of VHL/E have been well documented in

the course of this investigation, it is of primary importance to rule out the possibility that these characteristics may simply represent a peculiarity of this particular isolate. Replication of these experimental results following similar propagation of additional clinical isolates would be useful in generalizing the model.

Also as suggested in the previous section, an obvious objective for continued experimentation might be the improvement of the VHL/E propagation system. Specifically, augmentation of infectious virus production would be advantageous. Several methods of boosting CMV production in vitro have been described. Dimethyl sulfoxide (DMSO) treatment has been shown to enhance infectivity and/or virus production in cultured fibroblasts¹⁹¹ and in a human rhabdomyosarcoma cell line.¹⁹² Dexamethasone has also been shown to increase CMV infectivity.¹⁹¹ Treatment of fibroblasts with 5-iodo-2'-deoxyuridine (IUdR) has resulted in augmentation of infectious virus yield accompanied by acceleration of virus replication.¹⁹³ Cortisol also has been shown to possess titer-boosting capability.¹⁹⁴ Finally, Radsak et al.¹⁵² have reported that temporary post-inoculation treatment of cultured human umbilical vein endothelial cells with sodium butyrate enhances CMV infectivity, as indicated by expression of viral antigens and synthesis of viral DNA.

Acceleration of VHL/E replication and augmentation of absolute titer of virus stocks would facilitate the design of experiments based on higher input virus concentrations. This would reduce the incubation time required to achieve widespread *in vitro* infection, thus hastening the experimental process and reducing the costs. In addition, high titer virus preparations would likely be necessary to permit a practical approach to the molecular and biochemical analyses of VHL sub-strain diversification suggested above (IX. 2.). Thus, enhancement of VHL/E production would clearly be a desirable goal. However, since the value of this model weighs heavily on its similarity to *in vivo* observations, the properties of progeny virus generated by chemical intervention would require careful evaluation to assure significant changes, which may reduce the accuracy of the system, have not occurred.

As an additional direction for further research, the availability of the two sub-strains of VHL provides a unique opportunity for study of the mechanisms of the diversification that has occurred (as suggested in IX. 2.). Molecular analysis in search of genomic alterations, as well as biochemical studies of protein profiles could help elucidate the basis for the host-dependent modifications in progeny characteristics which have been documented in the course of this investigation.

With regard to in vitro modelling of various aspects of human CMV disease, potential applications of the VHL/E system seem abundant. Whereas previous in vitro models employing fibroblast-adapted strains have depended upon data derived from a small population of infected cells, widespread endothelial cell infection can eventually be achieved with even a low titer VHL/E inoculation, permitting simpler, more thorough assessment of virally-induced effects. In addition, the apparent homology between VHL/E induced endothelial cytopathology and that observed in vivo may endow this system with greater relevancy with respect to cellular responses occurring in human CMV disease.

Bruggeman et al.,¹⁵³ for example, based their conclusion that CMV down-regulates von Willebrand Factor expression on data derived from the small fraction of endothelial cells that showed evidence of infection (10%). The large supply of infected cells provided by the VHL/E model might significantly improve this analysis, allowing for a correlation between degree of vWF loss and extent of cytopathic change. Similarly, this model could be exploited to assess other CMV-induced metabolic derangements, such as interference with the checks and balances of the thrombogenic/coagulation response, or the induction of aberrant angiogenic activity. Thus, the VHL/E system may provide insight into the precise role of CMV in atherosclerosis,

and may shed some light on the relationship CMV infection bears with Kaposi's sarcoma.

Another potential application of this model relates to the postulated role of CMV in allograft rejection. Although such rejection has been associated with HLA class II antigen expression by cells within the grafted organ,¹⁹⁵ neither van Dorp et al.¹⁴⁹ nor Sedmak et al.¹⁹⁶ were able to demonstrate induction of these antigens by established CMV strains in cultured endothelial cells. Owing to the more natural cellular interactions exhibited by VHL/E, experiments performed with this strain may yield very different results, and thus may help elucidate the mechanisms of CMV-associated transplant failure.

Finally, endothelial cell-based propagation may hold some promise in providing material for the development of protective vaccines. Although some progress has been made in this area using high passage Towne strain,¹⁷⁵ a satisfactory agent is not yet available. Since in all aspects addressed in this investigation, VHL/E has been found to resemble fresh clinical isolates more closely than does fibroblast-adapted virus, it is possible that the antigens of endothelial-raised CMV bear stronger homology to those present in vivo than do the antigens of traditional strains. Therefore, vaccination with components of VHL/E (or similarly propagated strains) may elicit a more effective immune response.

IX. 5. Summary

This investigation has demonstrated that the host system employed for propagation of CMV dramatically influences the infectious properties of the resulting progeny. While VHL/E has retained characteristics of the original isolate, the endothelial cytopathogenicity of VHL/F has been markedly reduced after 20 passages in fibroblasts. Similarly, the ability of VHL/F to initiate sustained productive infection in endothelial culture has been nearly lost. Since properties of VHL/F strongly resemble those of other fibroblast-raised strains (CMV AD169, OSU CMV 3), these findings suggest that the process of fibroblast adaption traditionally employed in the propagation of CMV restricts the host range of the virus while attenuating its spectrum of cytopathic potential.

These results may have profound implications not only for the study of CMV, but for virology research in general. Clearly, a primary objective of in vitro experimentation is the development of models which accurately reflect in vivo interactions. Since nearly all experimental protocols in virology depend upon propagation of viral specimens in culture to provide sufficient material for study, the choice of an appropriate host system may be crucial to the relevance of the outcome. Superior quantitative yield of infectious virus does not appear to be sufficient as the sole criterion for the acceptability of a given host cell

type. Results of this investigation indicate that careful qualitative evaluation of the resulting progeny should be undertaken, and that equal consideration be given to selection of a host system that promotes retention of the properties of the original clinical isolate.

APPENDIX A

FORMULARY

Culture Media

1. Modified Minimal Essential Medium (MEM_g, - 10 liters

B Medium (Gibco special formula MEM #78-5048, with

Earle's salts, 1.5 x essential amino acids,

2 x non-essential amino acids, L-glutamine,

1.5 x vitamins) - one 10-liter packet

sodium chloride (NaCl) - - - 8.35 g

sodium pyruvate - - - 1.10 g

sodium bicarbonate (Na_2HPO_4) - 15.00 g

water (triple distilled, deionized) 10.00 L

phenol red (1%) - - - 10.00 ml

Adjust pH to 7.2

Gas with CO_2 30 seconds

Filter sterilize (0.20 μm)

2. Medium M-199 (with Earle's salts and L-glutamine) -

5 liters

Medium M-199 (Gibco) - one 5-liter packet

sodium bicarbonate (Na_2HPO_4) - 2.20 g

water (triple distilled, deionized) 5.00 L

Adjust pH to 7.2

Filter sterilize (0.20 μm)

3. Endothelial Cell Growth Medium (ECGM)

Medium M-199 (see above) supplemented as follows:

fetal bovine serum (Hyclone) - 20 %

endothelial cell growth supplement (ECGS -

see Appendix B) - - 100 $\mu\text{g/ml}$

sodium heparin (Sigma) - - 12 U/ml

Hepes buffer - - - 20 mM

Phosphate Buffered Saline (PBS) - 10 liters

sodium chloride (NaCl) - - - 80.00 g

potassium chloride (KCl) - - - 2.00 g

sodium phosphate, dibasic (Na_2HPO_4) - 11.50 g

potassium phosphate, monobasic (KH_2PO_4) 2.00 g

water (triple distilled, deionized) - 10.00 L

Adjust pH to 7.2

Sterilize by autoclave

Trypsin (0.01%) - 1 liter

sodium chloride (NaCl)	-	-	-	-	8.00 g
potassium chloride (KCl)	-	-	-	-	0.20 g
sodium phosphate, dibasic (Na_2HPO_4)	-				1.15 g
potassium phosphate, monobasic (KH_2PO_4)					0.20 g
phenol red (1%)	-	-	-	-	1.00 ml
crystalline trypsin (Worthington)	-				0.10 g
water (triple distilled, deionized)	-				1.00 L

Adjust pH to 7.8

Filter sterilize (0.20 μm)

0.02% Ethylene-Diamine-Tetraacetate (EDTA) - 100 ml

EDTA, disodium salt (Sigma)	-	-	-	-	0.02 g
PBS (see above)	-	-	-	-	100.00 ml

Adjust pH to 7.2

Filter sterilize (0.20 μm)

Sucrose Phosphate Buffer (4-SP) - 1 liter

sucrose	-	-	-	-	-	64.46 g
potassium phosphate, monobasic (KH_2PO_4)						1.02 g
potassium phosphate, dibasic (K_2HPO_4)	-					2.02 g
water (triple distilled, deionized)	-					1.00 L

Filter sterilize (0.20 μm)

Virus Storage Medium

sucrose phosphate buffer (see above)	-	50.00%
MEM ₂ (see above)	- - - - -	40.00%
fetal bovine serum	- - - - -	10.00%
gentamicin sulfate (Elkins-Sinn)	-	100 ug/ml
amphotericin B (Fungizone, Squibb)		250 ng/ml

0.5% Agarose-Supplemented MEM (for plaque assay overlay)

1. 2x MEM₂: MEM₂ as described above, but prepared in 1/2 the volume of distilled water (phenol red is omitted), completed by the addition of:

fetal bovine serum	- - -	20.00%
gentamicin sulfate (Elkins-Sinn)	-	200 ug/ml
amphotericin B (Fungizone, Squibb)		500 ng/ml

Warm to 45° C

2. 1% Agarose Solution

agarose (SeaKem LE, FMC)	- -	1.00%
--------------------------	-----	-------

water (triple distilled, deionized)

Boil 1 hour

Cool to 45° C

3. Final Preparation of Overlay Medium

mix equal volumes of complete 2x MEM₈ and 1% agarose

(maintain 45° C temperature)

final concentrations of components:

MEM ₈	-	-	-	-	-	-	-	1x
fetal bovine serum				-	-	-		10.00%
gentamicin sulfate				-	-			100 ug/ml
amphotericin B				-	-	-		250 ng/ml
agarose	-	-	-	-	-	-		0.50%

Staining Reagents

1. 0.05 M Tris-Buffered Saline (TBS)

0.50 M Tris-HCl (pH 7.6)	-	-	-	10.00%
0.85% NaCl (normal saline)	-	-	-	90.00%

2. Aminoethylcarbazole (0.24%) - AEC stock solution

3-amino-9-ethylcarbazole (Sigma)	-	-	0.30 g
N, N'-dimethyl formamide (J.T. Baker)			125.00 ml

3. AEC (0.024%) Development Solution

AEC stock solution (0.24%)	-	-	-	5.00 ml
0.02 M sodium acetate/acetic acid				
(acetate buffer, pH 5.2)			-	45.00 ml
Filter (Whatman #1)				
hydrogen peroxide (H ₂ O ₂), 30%				
(added just prior to development)			-	7.50 ul

4. 3.0 M Sodium Chloride/0.3 M Sodium Citrate

(SCC, 20x)

sodium chloride	-	-	-	-	1.75 g
sodium citrate	-	-	-	-	0.88 g
water (triple distilled, deionized)				-	10.00 ml

5. Hybridization Solution

formamide, deionized (Mallinkrodt)				-	1.00 ml
dextran sulfate solution (10% w/v)				-	400 ul
SCC, 20x (see above)	-	-	-	-	200 ul

6. DNA Probe Mixture

hybridization mixture (see above)				-	400 ul
carrier DNA (salmon sperm, ENZO)	-			-	16 ul
specific DNA probe (ENZO)	-	-		-	80 ul

Final concentration of probe DNA: approx. 15 ug/ml

Polymerase Assay Reagents**1. Scintillation Cocktail - 1 liter**

toluene	-	-	-	-	-	-	600 ml
ethanol	-	-	-	-	-	-	400 ml
2, 5- diphenyloxazole (PPO)	-	-	-	-	-	-	4.0 g
1, 4-bis-(2-(5-phenyloxazolyl))benzene							
						(POPOP)	0.1 g

2. Extraction Buffer - 100 ml

<u>COMPONENT</u>	<u>VOLUME</u>	<u>FINAL CONC</u>
Tris-HCl, pH 7.5 (1.0 M)	5.0 ml	50 mM
KCl (2.0 M)	10.0 ml	0.2 M
dithiothreitol (1.0 M)	0.3 ml	3 mM
ATP-Mg (40.52 mM)	4.9 ml	2 mM
water (triple distilled, deionized)	79.8 ml	

3. DNA Polymerase Reaction Mixture - 70 ml

<u>COMPONENT</u>	<u>VOLUME</u>	<u>*FINAL CONC</u>
Tris-HCl, pH 8.0 (1.0 M)	3.899 ml	50 mM
KCl (2.0 M)	5.845 ml	150 mM
MgCl ₂ (1.0 M)	0.315 ml	4 mM
BSA (1.0 %)	0.612 ml	80.0 ug/ml
glycerol (50% v/v)	3.5 ml	2.25%
dithiothreitol (1.0 M)	77 ul	1.0 mM
dATP (Sigma, 13.8 mM)	0.56 ml	100 uM
dCTP (Sigma, 10.08 mM)	0.77 ml	100 uM
dGTP (Sigma, 5.26 mM)	1.477 ml	100 uM
dTTP (Sigma, 8.95 mM)	14 ul	1.6 uM
[³ H]-dTTP (ICN, 58 Ci/mMol, 1 mCi/ml)	0.35 ml	86.2 nM
(specific activity: 2.665 uCi/nMol)		
activated DNA template, 735 ug/ml (see Appendix H)	15.47 ml	132 ug/ml
water (triple distilled, deionized)	37.1105 ml	
*final concentrations of components under assay reaction conditions: 90 ul of reaction mix + 10 ul sample lysate		

APPENDIX B

PREPARATION OF ENDOTHELIAL CELL GROWTH SUPPLEMENT (ECGS)

Endothelial cell growth supplement was prepared from fresh whole bovine brain by the method of Maciag.¹⁶⁵ All reagents and tissues were maintained at 4° C throughout the procedure. Brains were washed in cold distilled water and blood clots and meninges were removed before obtaining a wet weight. The tissue was homogenized in cold 0.15 M NaCl (1250 ml/kg tissue) for 3-4 minutes in a Waring blender. The homogenate was stirred for 2 hours at 4° C, then centrifuged for 40 minutes at 13,000 x g, followed by careful decanting of the supernatant.

Streptomycin sulfate was prepared by dissolving 3 grams in 40 ml of distilled water (per 0.41 kg original brain tissue), adjusting the pH to 8.0, and cooling on ice. To precipitate soluble lipids, this solution was added dropwise (over approximately 5 minutes) to the decanted tissue extract with constant stirring. The extract was stirred for 20 minutes more, then allowed to stand at 4° C for at least 2 hours.

The extract was again clarified by centrifugation (as above), and protein content was determined using the

Comassie blue dye binding assay (Bio-Rad, see VII. 3. 8.). The final extract was aliquotted into glass serum vials, shell-frozen in a rotating dry ice/ethanol bath, then lyophilized for 24-48 hours before storing at -80° C.

For reconstitution, the lyophilized ECGS was combined with an appropriate amount of sodium heparin (as dictated by the ECGS protein content; see Appendix A) and a small volume of M-199 (15-20 ml). The mixture was dissolved and allowed to stand at room temperature for 15-30 minutes. The preparation was then clarified and sterilized with a 0.20 μ m syringe filter. (Pre-filtration using larger pore filters was often necessary to clear insoluble debris from the extract.)

As a quality control measure, each lot of ECGS was tested in endothelial cell culture before being released for general use. Several small aliquots of ECGM^a were prepared with different concentrations of ECGS (generally 50, 100, and 150 μ g/ml). Low passage HUVE cells were trypsinized, suspended in the various ECGM preparations, and plated at approximately 30-40% confluent density in fibronectin-treated 25 cm² culture flasks. Test cultures were incubated and observed for cell morphology and growth kinetics (approximate duration of lag and log phases, time to reach confluence). Based on these observations, the

^a See Appendix A.

lowest ECGS concentration giving satisfactory results was determined (generally 100 ug/ml).

APPENDIX C

TABLE 3: Specific volumes of culture reagents/inocula as a function of culture vessel type and size.

V O L U M E S			
<u>CULTURE VESSEL</u>	<u>INITIAL PLATING/ MEDIUM REPLACEMENT</u>	<u>TRYPsin/ EDTA (1:1)</u>	<u>VIRAL INOCULUM</u>
<u>Flasks</u>			
25 cm ² (T-25)	3-5 ml	1.0 ml	2-3 ml
75 cm ² (T-75)	10-12 ml	2.0 ml	5 ml
150 cm ² (T-150)	20-25 ml	3.0 ml	10 ml
<u>6-well Plates</u>			
vol/35-mm well	3 ml	0.5 ml	1 ml
<u>Chamber Slides</u> (vol/single well)			
2-well	1.5-2 ml	0.3 ml	0.5 ml
4-well	0.8 ml	0.2 ml	0.2 ml
8-well	0.3 ml	0.1 ml	0.1 ml

APPENDIX D

CMV AD169 SPECIFICATION SHEET (ATCC)

AMERICAN TYPE CULTURE COLLECTION
12301 Parklawn Drive, Rockville, Maryland 20852

COLLECTION OF ANIMAL VIRUSES

ATCC NO.: VR- 538 Permit Required None

Classification: Herpesvirus Strain: AD-169

Agent: Cytomegalovirus

Original Source: Tonsillectomy - adenoidectomy, 7 year old girl

Reference: Rowe, W., et al., Proc. Soc. Exp. Bio. Med. 22: 418, 1956.

Host of Choice: Human fibroblast cells

Effect: Cells rounding and eventual areas of sloughing

Host Range: Human fibroblast cells Incubation: 3-6 days, 36°C

Storage Temperature: -60°C or lower Vol./Ampule: 1.0 ml

Special Characteristics: Virus primarily cell associated. Passage best effected by transfer of infected cells. Stable at -60°C in form of glycerinated or DMSO treated cells for 6 months or for several years in liquid nitrogen.

PART A: FROZEN Virus Lot 18

Passage History: RU-1/24, FP/2, FSP/15, FS11/1, FS10/4, FS9/14, RU-1/4, HELF/10, WI-38/11, MRC-5/2

Preparation: Cells and fluid

Medium: L-15 + 10% FBS + 5% DMSO

Antibiotics: Gentamicin

Titer: 10^{4.5} TCID₅₀/0.2ml, MRC-5, 12 days Tested: 12/86

Original Preparation Contributed By: Dr. W. A. Chappell

PART B: FREEZE-DRIED Virus Lot

Passage History:

Preparation:

Medium:

Antibiotics:

Tested:

Titer:

Original Preparation Contributed By:

Reconstitute to original volume with distilled water and store at -60°C or lower.

Part C: REMARKS - For passage, rupture infected cells in supernatant fluid by vortexing with glass beads or by sonication. Adsorb 1.5 hours at 36°C.

APPENDIX E

VIRUS TITRATION BY TERMINAL DILUTION

Concentrations of virus stocks used in preliminary experiments were determined by terminal dilution titration. A sample of virus stock was thawed, and a series of 10-fold dilutions was prepared in serum-free MEM₉ (generally ranging from 10^{-1} - 10^{-10}). Confluent NHDF monolayers in glass culture tubes were washed twice with PBS, then inoculated in triplicate with each virus dilution (200 ul/tube). Following 1 hour adsorption (35.5° C), inocula were removed and monolayers were washed twice more with PBS before addition of complete MEM₉ (1 ml/tube).

Cultures were incubated 28 days at 35.5° C with weekly growth medium changes, while being observed at 3-day intervals for development of CPE. Tubes were scored as + or -, corresponding to presence or absence of CPE, respectively. Titer, expressed as tissue culture infectious dose for 50% of inoculated cultures (TCID₅₀), was determined from the final scoring (day 28 PI) by the method of Reed & Muench.¹⁹⁷

Titers determined by this technique represent approximations to within an order of magnitude and thus,

are inherently less accurate than those determined by plaque assay. Additionally, due to inevitable sloughing/deterioration of the NHDF monolayer over the 4-week duration of the assay, data was frequently lost, resulting in an inaccurate, irreproducible titration. Finally, the plaque assay is easily more convenient and more expedient (14 days) than the terminal dilution method. For these reasons it was decided very early in the investigation to abandon this technique in favor of the plaque assay.

APPENDIX F

DROP-SLIDE METHOD FOR ACUTE INFECTION STAINING

Cells were grown to confluence in tissue culture flasks (usually 25 cm²), washed twice with PBS, and inoculated with appropriate dilutions of viral suspensions. Following 1.5 hours adsorption (35.5° C), inocula were removed and monolayers were again washed twice with PBS before addition of virus-free, complete growth medium, supplemented with gentamicin and amphotericin B (as described in V. 1.). Flasks were incubated for various intervals (1-20 days) then harvested for slide preparation as follows.

Monolayers were washed twice with PBS and released from the substrate by addition of trypsin/EDTA (see III. 2.). Cells were suspended in complete medium (10 x trypsin volume), then pelleted by centrifugation (350 x g, 5 minutes). The supernatant was aspirated and cells were washed twice in PBS. The final cell pellet was resuspended in a small volume of PBS (200-400 ul). Small aliquots (10-20 ul) of this cell suspension were placed on acid-cleaned glass microscope slides. Slides were air-dried, then fixed in cold acetone (4° C) for 10 minutes, and stained as described in Chapter V.

This method has proved effective for demonstrating presence/absence of viral DNA, viral antigens, and von Willebrand Factor; however, since the fixed cells retain the globular configuration assumed in suspension, morphological cytopathic changes associated with infection are not well delineated. In addition, this technique does not facilitate accurate determination of the distribution/compartmentalization of DNA or antigens within the cells. On the other hand, as a means of initial screening of cells for susceptibility to viral infection, it is simple and convenient. Also, since many slides can be generated by a single inoculated flask, and chamber slides are not required, this procedure is less expensive than that described in Chapter V.

APPENDIX G

DESCRIPTION OF VIRUS-SPECIFIC DNA PROBES

The virus-specific biotinylated DNA probes used for in situ hybridization experiments were produced by ENZO Biochem, Inc. The CMV probe was prepared from a mixture of two Bam HI restriction fragments of CMV Towne, a 17.2 Kilobase (Kb) sequence from the U_L region (designated fragment B on the restriction map), and a 25.2 Kb sequence from the U_L region (fragment A).⁴⁶ The fragments were cloned in the Bam HI site of pBR322. These sequences are unique to CMV and, thus show no cross hybridization with other members of the Herpes group or with human genetic material.

The HSV probe was prepared from a mixture of fragments of HSV 1 and HSV 2, all cloned in the Bam HI site of pBR322. The two HSV 1 sequences included a 3.0 Kb Bam HI fragment from the 5'-terminus of the genome (fragment S), and an 8.0 Kb Bam HI fragment from the U_L region (fragment F).¹⁹⁸ The probe mixture also included a 16.0 Kb Bgl II restriction fragment from the U_L region of HSV 2 (fragment G).¹⁹⁹ This combination permits hybridization with either HSV 1 or 2, but not with other members of the Herpes group.

Probes were labelled by nick-translation in a reaction mixture which included, in place of thymidine triphosphate, biotin-11-deoxyuridine triphosphate^a (Bio-11-dUTP).^{167 200} This process results in the substitution of approximately 25-30% of the thymidine residues in the probe with the biotinylated nucleotide. The resulting probe preparation consists of double-stranded fragments in the range of 100-2000 base pairs (see ENZO Bio-Probe Product Specification Sheets).

^a 5-[N-(N-biotinyl-{-aminocaproyl}-3-aminoallyl)]-deoxyuridine triphosphate

APPENDIX H

PREPARATION OF ACTIVATED DNA TEMPLATE^a

To serve as a substrate for the DNA polymerization reaction in the enzyme assay, double-stranded calf thymus DNA is nicked at multiple sites using the following procedure. Pancreatic DNase I (Sigma) is dissolved in 0.01 M HCl to yield a final concentration of 0.5-1.0 mg/ml. The enzyme is activated by adding 50 ul of the DNase solution to 450 ul of activation buffer (10 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 1 mg/ml BSA) and incubating at 4° C for 2 hours.

Calf thymus DNA (Sigma) is dissolved in 445 ml distilled water + 30 ml 0.2 M MgCl₂ + 25 ml 0.1 M Tris-HCl, pH 7.5, by stirring over a period of several days. To nick the DNA, activated DNase is added to the DNA solution (10 ul DNase solution per mg/ml concentration of DNA), and the reaction is incubated at 37° C for 30-60 minutes. The mixture is then heated at 77° C for 5 minutes and placed immediately on ice. The quality of the preparation is

^a Protocol courtesy of Dr. Marshall V. Williams, Department of Medical Microbiology and Immunology, The Ohio State University, Columbus, Ohio.

assessed by using samples of the nicked DNA as templates in polymerase assays of standard concentrations of *E. coli* enzyme.

APPENDIX I

CALCULATION OF POLYMERASE ACTIVITY

Raw data generated by DNA polymerase assays consisted of triplicate scintillation counts of incorporated radioactivity (CPM) for each sample lysate (3 assays/sample), as well as triplicate counts of zero-time controls (background) and unwashed filters (total available label). Means were calculated for each set of 3 counts, and net incorporation for each sample (net CPM) was determined by subtracting the background level from the mean sample activity. Net total available activity (net total CPM) was similarly determined by subtracting background from mean total available label.

From these data, rate of [³H]-TTP incorporation was calculated for each sample according to the following formula:

INCORPORATION RATE (nMol/ml/hr) =

$$\frac{\text{Net CPM}}{\text{Net Total CPM}} \times 9 \text{ nMol} \times \frac{1}{\text{rxn incubation time (hrs)}} \times \frac{1000 \text{ ul}}{10 \text{ ul}^*}$$

*volume of sample assayed

Enzyme specific activity was then calculated for each sample from incorporation rates and sample protein values as follows:

SPECIFIC ACTIVITY (nMol/hr/mg total protein) =

$$\frac{[^3\text{H}]\text{-TTP INCORPORATION (nMol/hr/ml)}}{\text{SAMPLE PROTEIN CONTENT (mg/ml)}}$$

APPENDIX J

TOTAL PROTEIN ASSAY: STANDARD CURVES

First order regression curves generated from assay of protein standards (see VII. 3. 8.) are presented for each experimental series. Correlation coefficients (R) are included for each curve.

FIGURE 34

FIGURE 34: Standard curve for Series I protein assay (CMV AD169-infected neonatal human dermal fibroblasts [NHDF]).

FIGURE 35: Standard curve for Series II protein assay
(CMV VHL/ F_{20} -infected neonatal human dermal
fibroblasts [NHDF]).

FIGURE 36: Standard curve for Series III protein assay
(CMV VHL/ E_{20} -infected neonatal human dermal
fibroblasts [NHDF]).

FIGURE 35

FIGURE 36

**FIGURE 37: Standard curve for Series IV protein assay
(CMV AD169-infected human umbilical vein
endothelial cells [HUVE]).**

**FIGURE 38: Standard curve for Series V protein assay
(CMV VHL/F₂₀-infected human umbilical vein
endothelial cells [HUVE]).**

FIGURE 37

FIGURE 38

FIGURE 39

FIGURE 39: Standard curve for Series VI protein assay (CMV VHL/E₂₀-infected human umbilical vein endothelial cells [HUVE]).

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