Stealth and Sabotage, Cytomegalovirus and Solid Organ Transplantation

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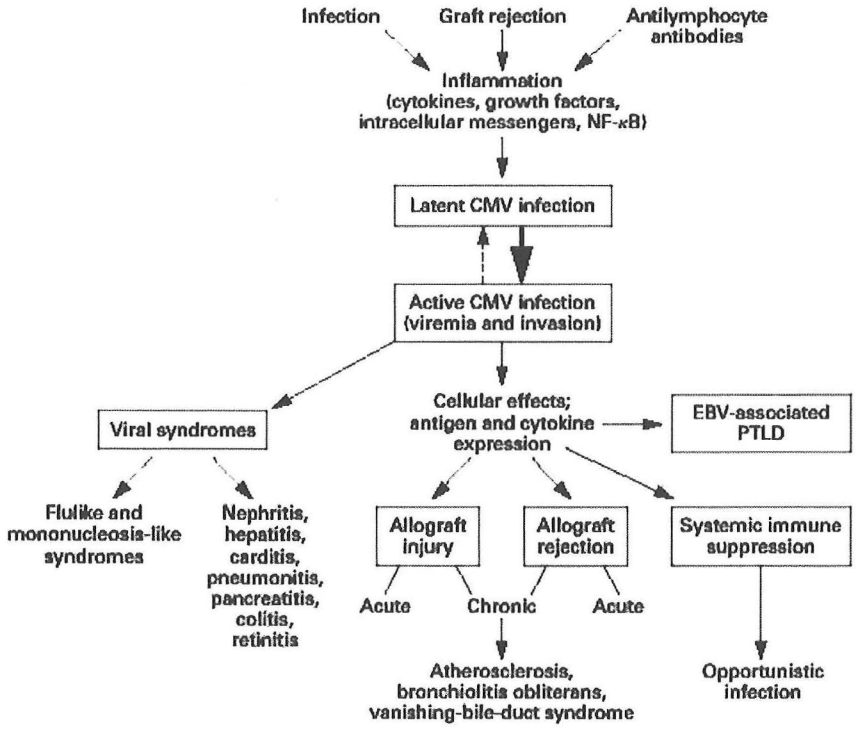

Fishman. *NEJM* 338:1741, 1998

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Regulation of the immune response in the placenta and neonate as a models of transplant tolerance.

Clinical Interests: Transplant nephrology

Infections by cytomegalovirus (CMV) are common, occurring in up to 60-70% of urban Americans. Symptoms vary widely: there is no disease in most normal individuals; infectious mononucleosis occurs in young adults; infections of pregnant women may result in congenital infection that may be often fatal or result in long-term sequallae; severe disease occurs in patients with acquired immunodeficiency syndrome or organ transplants (1). Thus, CMV is both an important problem for physicians responsible for immunosuppressed patients, and a fascinating problem for students of immunology.

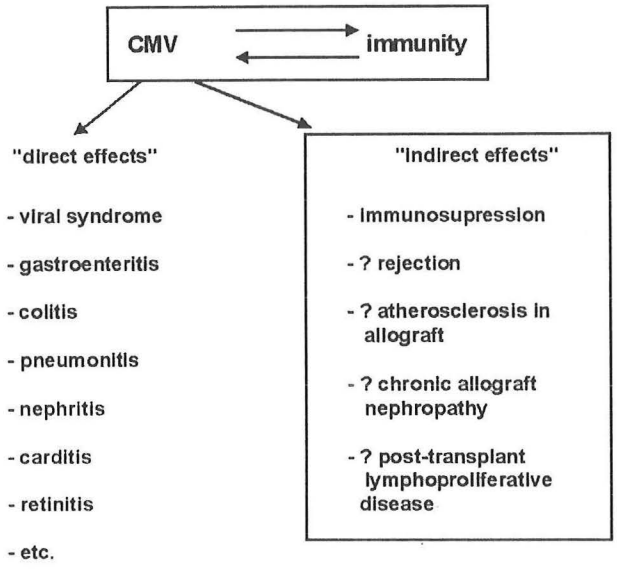

Figure 1: Direct vs indirect effects of CMV.

I will focus on CMV in recipients of solid organ transplants, particularly renal transplants. The major clinical issues are summarized in the diagram on the title page (2). At the bottom of the diagram are the clinical manifestations. These may be divided into "direct" effects of the viral infection - a flu-like syndrome that is the most common presentation, gastroenteritis, colitis, nephritis, hepatitis, carditis, pneumonitis, pancreatitis, retinitis, etc. In addition, the virus has "indirect" effects. It injures the allograft, and suppresses the immune response such that opportunistic infections may occur. This manuscript will focus on the interaction of CMV with the immune system, and on the "indirect" effects of the virus.

The Figure above represents a new way of thinking about CMV. Previously, most authors divided CMV into CMV infection without symptoms and CMV disease. The latter represented the "direct" effects of the virus. The former was thought to be benign. Long term follow up of CMV infections suggests that there may be no benign CMV infections for solid organ recipients. Instead, viral infection may result in allograft loss, and opportunistic infections.

My discussion will address the following issues: Why has this virus been so successful in propagating itself in so many normal individuals? What is the role of transplantation itself and anti-rejection therapy in causing disease? By "transplantation itself", I mean the interaction between the host immune response, and the allograft. We will see that immunosuppression itself is not sufficient to completely explain some clinical syndromes associated with CMV.

I will begin by reviewing how this virus interacts with host cells, how it subverts the immune system in order to survive, how it remains in equilibrium with its host through cycles of latency and reactivation. This initial review will form the basis for a discussion of therapy.

Definitions:

CMV is a beta herpes virus. The genome of some isolates has been completely sequenced, and could potentially encode over 200 proteins. The size of this genome earns CMV the distinction of being the largest virus to infect humans. Some of these genes have "housekeeping" functions which allow infection and propagation of virus in human cells. These genes encode the enzymes required for viral DNA synthesis, and the proteins of the three layers surrounding the DNA "core" of the completed viron. The inner layer is the icosahedral "capsid". This is surrounded by a "tegument" or "matrix"; many of the "matrix" proteins also participate in the regulation of host and viral gene activation. The outermost layer is an "envelop" consisting of glycoproteins. In addition to these housekeeping genes, other genes protect virus growing in an patient by subverting his/ her anti-virus immune response; deletion of these "subversion" genes does not prevent CMV from propagating in cultured eucaryotic cells. [See review (3).] As we will discuss later, other CMV genes change the behavior of host cells such that there is a maladaptive response to vascular and other injury

The CMV genome is a linear DNA duplex. It is organized into a unique long (UL) and a unique short (US) sequence of DNA separated by short sequences which have inverted repeats at the ends of the linear duplex. These short sequences are designated Terminal Repeat Long (TRL), Interior Repeat Long (IRL), Terminal Repeat Short (TRS), and Interior Repeat Short (TRS). CMV genes are identified according to their transcriptional start sites in these regions. For example, the gene encoding pp65, which is the antigen detected by the "pp65" diagnostic test for CMV used at UTSWMC, is designated UL83 (3).

The various CMV genes may be divided into three families based upon when the genes are activated during the viral life cycle. The "immediate-early" or "α" genes are the first viral genes transcribed after infection. These genes are transcription factors that activate other viral genes. The "early" or "β" genes are then transcribed. Many of these viral genes control the viral DNA polymerase and other proteins necessary for replication of the viral DNA. These include the gene products which are the targets for the anti-viral drugs acyclovir, ganciclovir, foscarnet, and ciofovir. The "late" or "γ" genes are transcribed last. They encode structural components of the viral capsid, tegument, and envelop (3,4).

Active infection - two phases, involvement of both viral and host transcription factors.

Active infection is divided into two phases: In the first phase, the virus enters host cells, proliferates, and infects neighboring cells. In the second phase, immune cells are recruited to the initial site of infection and then migrate to other tissues, thus spreading the virus throughout the body. Note that that immune cells have two different roles during CMV infection. On the one hand they disseminate the virus from one tissue to another; on the

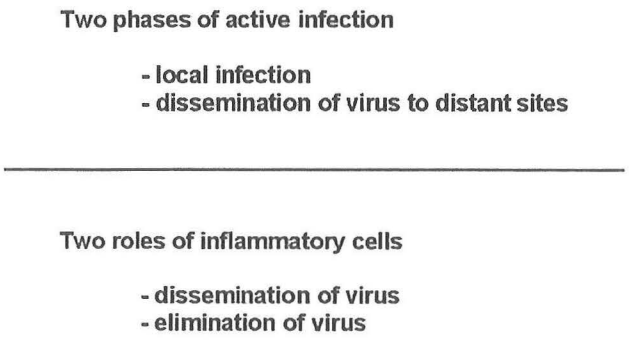

Figure 2

other hand some leukocytes are important in the anti-viral defense. In addition to infections of cells which produce new virions, persistently infected cells may make some viral proteins or be driven to produce cellular proteins which are conducive to survival and spread of CMV, and latently infected cells are apparently normal but harbor some viral DNA and may produce small amounts of viral "latency" proteins.

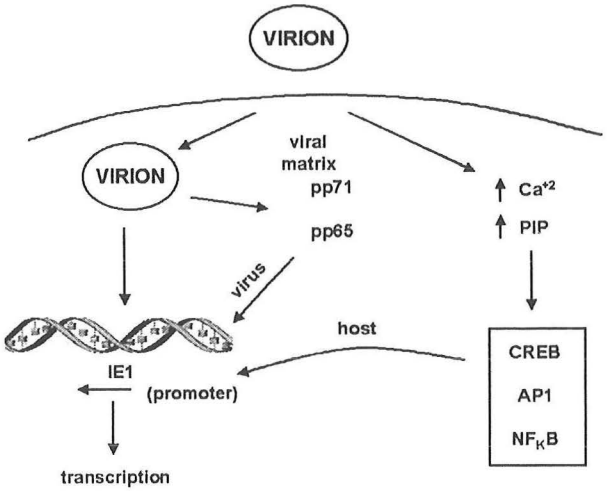

Figure 3: Infection is controlled by both viral and host factors.

In permissive cells, the CMV virus binds to and penetrates into the host, releases its DNA into the nucleus, and then begins transcribing the immediate early genes. These events are not understood in detail. However, it is clear that the host cell must actively participate in any viral infection. Activation of the "immediate-early" genes requires the host transcription factors such as NF kappa B, p107, Spl 1; these are activated by calcium fluxes and increased concentrations of cAMP, cGMP, etc that are induced by contact of the virion with the cell surface. In addition, viral matix proteins such as UL 89 may also contribute to activation of the immediate-early viral genes (4,5).

The products of the immediate-early genes further increase the transcription of these genes, and thus provide a positive feedback loop. CMV has four immediate early genes whose gene products control the expression of several hundred "early" and "late" viral genes. Flexibility in the regulation of these genes occurs via the participation of various host and other viral transcription factors as well as alternative splicing of mRNA produced by these immediate early genes (4).

After the initial infection, the virus may become latent. Then, at various times, it may become reactivated and, depending upon the circumstances, cause problems for the host. Reactivation will be discussed in more detail in a later section.

Viral gene products that subvert the immune response and allow viral propagation in patients.

The genome of clinical isolates of human CMV contains over 15 kbp that are not present in some laboratory strains of virus. Despite the deletion of a large number of genes the laboratory strains propagate perfectly well in tissue culture. However, they would not survive in a patient because the deleted genes are necessary to defend the virus against the host immune response (6). Over twenty viral genes allow CMV to propagate in patients. The functions of some of these genes are known and will be discussed below.

The effective immune response against CMV involves CD8 CTL (cytotoxic T lymphocytes) and natural killer (NK) cells that kill infected cells, and CD4 T cells that "help" B cells make anti-CMV antibodies that opsonize virions for phagocytosis and destruction by polymorphonuclear cells and that may also participate in complement-dependent lysis of virally infected cells. The immune response

against CMV consists of redundant mechanisms. As we discuss below, such redundancy evolved because the virus may subvert some mechanisms.

Interleukin 10

Cellular interleukin 10 is a cytokine that either inhibits or stimulates immune responses depending on the clinical situation (7-10). Host cells infected by CMV produce a viral gene product (cmv IL10) which is a homolog of cellular IL10 (11). Although cmv IL10 does interact with IL10 receptors on immune cells, exactly which functions are activated or inhibited remain to be defined. It is possible, that like the IL10 homologue produced by EBV, which is understood in some detail, the cmv IL10 will only inhibit immune responses. We will briefly discuss EBV IL10 to illustrate the principle that a Herpesvirus may not only have a gene for a generally immunosuppressive cytokine, but mutate that cytokine, so that it is a more potent immunosuppressant that the original cellular cytokine. The amino acid sequences of cellular IL10 and its viral homologues are similar but not identical. In the case of EBV IL10, changing a single alanine of cellular IL10 at position 87 to isoleucine, results in a cytokine which has only immunosuppressive properties (12). Thus, EBV IL10, but not cellular IL10, is an effective immunosuppressant when transfected into solid organ transplants in animal models.

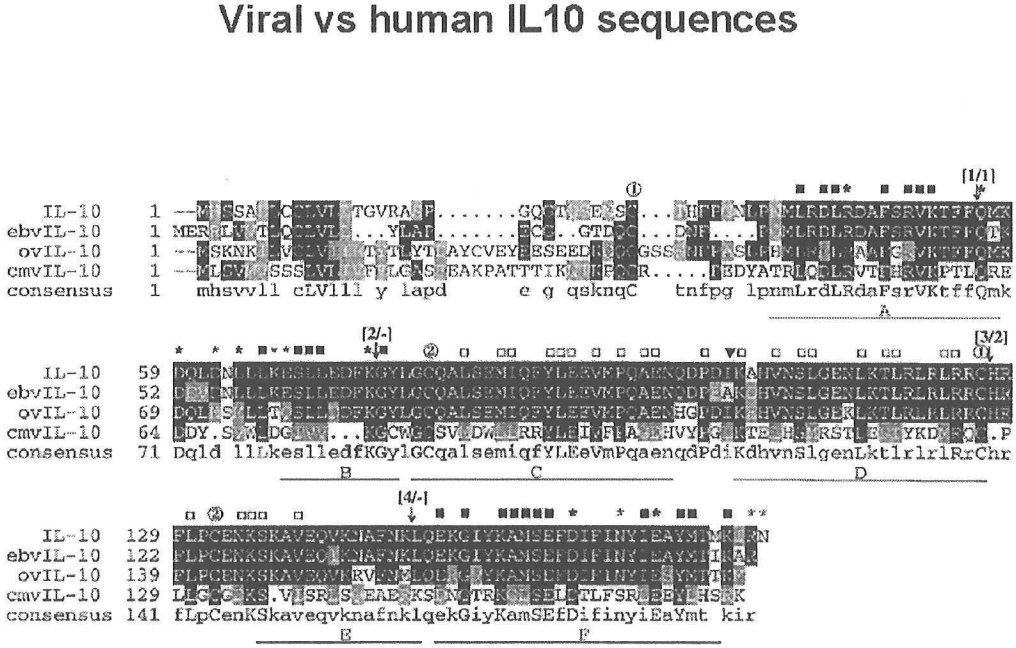

Figure 4: Ref: Kotenko. *PNAS* 94:1695. 2000.

Chemokines and chemokine receptors - two roles of leukocyte traffic during CMV infections.

Leukocyte traffic has two roles in CMV infections. First, the appropriate types of leukocytes must arrive at the site of infection to destroy the virus. Second, the virus uses leukocytes to disseminate infection; the virus would like to recruit the subsets of leukocytes that do not destroy virus, infect these leukocytes, and cause the infected leukocytes to disseminate throughout the body and thus disseminate

the infection. Thus, chemokines and their receptors regulate leukocyte traffic (13) and thus are potentially important in both the control and the dissemination of infection.

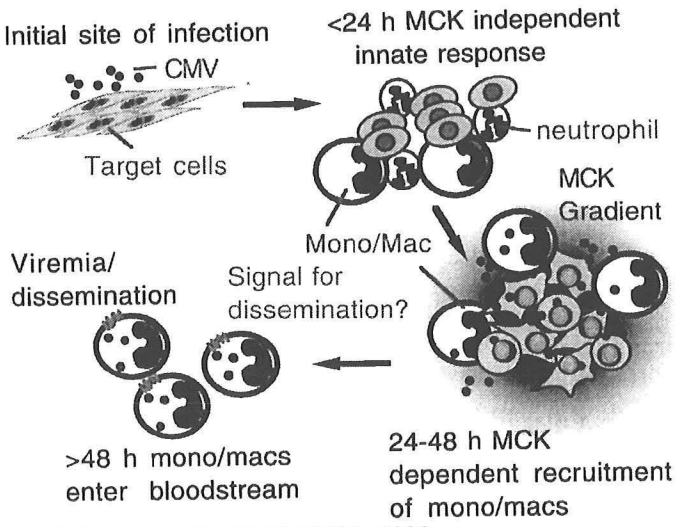

Figure 5: Ref. Saederup. *PNAS* 96:10881. 1999.
Viral cytokine facilitates dissemination by mononuclear cells.

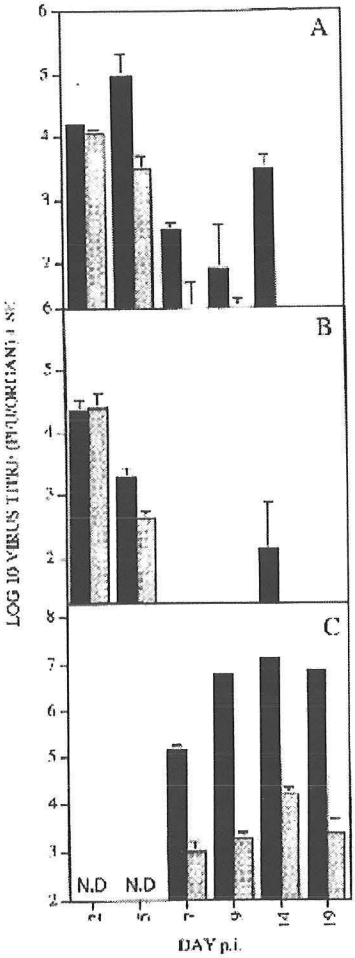

Figure 6: Ref. Fleming. *J Virol* 73:6800. 1999.
Wildtype (solid bars) or chemokine mutant (open bars) virus was measured in murine spleens (A), livers (B), or salivary (C). Note two stages of infection. An initial infection in the spleen and liver after intra-peritoneal injection of virus, and a secondary peak of infection in the salivary gland.

A number of large DNA viruses, including CMV (6), may produce chemokine homologues (14). In human CMV, the chemokine homologues UL146 and UL147 are late gene products (15). In mice[1], these CMV chemokine homologues recruit, to the site of infection, a subset of leukocytes that cannot eliminate the virus; instead these leukocytes themselves become infected and disseminate the virus by migrating from the initial sites of infection to other tissues. Other leukocytes, NK cells and CTL that would eliminate virus, are excluded from sites of infection by the viral chemokine homologues. CMV with mutations which inactivate these chemokine homologues are less virulent in mice (16,17). Mutations in these CMV chemokines decrease the number of infected mononuclear cells in peripheral blood. As shown in the Figure 6, these mutations thus prevent dissemination of the virus via the blood. Furthermore, CMV inhibits the production of authentic chemokine, MCP 1, from infected cells (18,18).

Like other DNA viruses, CMV produces several chemokine receptors. There functions are not well defined (19). Human CMV U28, an early gene (20), may to be a cofactor for entry of HIV into cells (21). The viral chemokine receptors are important for virulence in rodents. Mutant virus that do not

[1]Murine and human CMV have genomes that are approximately 70% homologues. Although similar the evasive tactics used by the two viruses are different when analyzed in detail. In this manuscript, murine CMV is discussed to illustrate "proof of principle" of certain immunosuppressive strategies where experiments on humans are not possible.

have functional receptors do not efficiently infect particular target tissues such as the salivary glands of the rat, but the mechanism is not known (22). In one in vitro system, UL28 produced by infected cells sequester RANTES, MCP 1, MIP 1β, and MCP 3 and thus would diminish immune responses (23). Furthermore, expression of UL28 on smooth muscle cells may contribute to atherosclerosis (24) as we discuss in a later section.

Sabotage of the Class I MHC pathway of antigen presentation to Cytotoxic T Lymphocytes (CTL's).

CMV and other viruses infect the inside of cells. A fundamental problem for the host defense against viruses is the delivery of viral proteins from the cell interior to the cell surface so that lymphocytes become aware that the cell has a viral infection. This is accomplished by the Class I MHC and associated proteins. The viral proteins are produced in the cytosol. The host proteasome degrades the viral proteins, the host TAP then transports the viral peptides into the endoplasmic reticulum where they are loaded onto the Class I MHC molecules. The Class-I-MHC viral peptide complexes are then transported through the Golgi apparatus to the outer surface of the plasma membrane where they are recognized by CD8+ CTL's. This entire process is called "antigen-presentation" or "antigen processing." (25).

CMV interdicts antigen processing at a number of steps. The CMV gene products US2 and US11 cause Class I MHC molecules to dislocate into the cytoplasm where they are destroyed by proteasomes (step 1, Figure below) (26,27). As we discuss in the section on "latency", the transcription of viral genes is regulated by the host cell's response to its environment. This is the case for the US11 gene whose transcription requires the CMV immediate early (IE) and UL82 gene products, as well as host cell CREB and ATF transcription factors (28). Thus, production of US11 depends on both the virus and which transcription factors the cell has activated in response to its environment. The CMV pp65 phosphorylates the immediate early protein (IE) and prevents its processing by proteosomes (29) (step 2). CMV US6 prevents the TAP transporter from bringing cytosolic peptides into the endoplasmic reticulum (30) (step 4). CMV US3 prevent the peptide-Class I MHC from leaving the ER (30) (step 6). Other viruses also escape the immune response by interdicting Class I MHC antigen processing. Herpes simplex ICP47 prevents TAP from binding peptides on its cytosolic

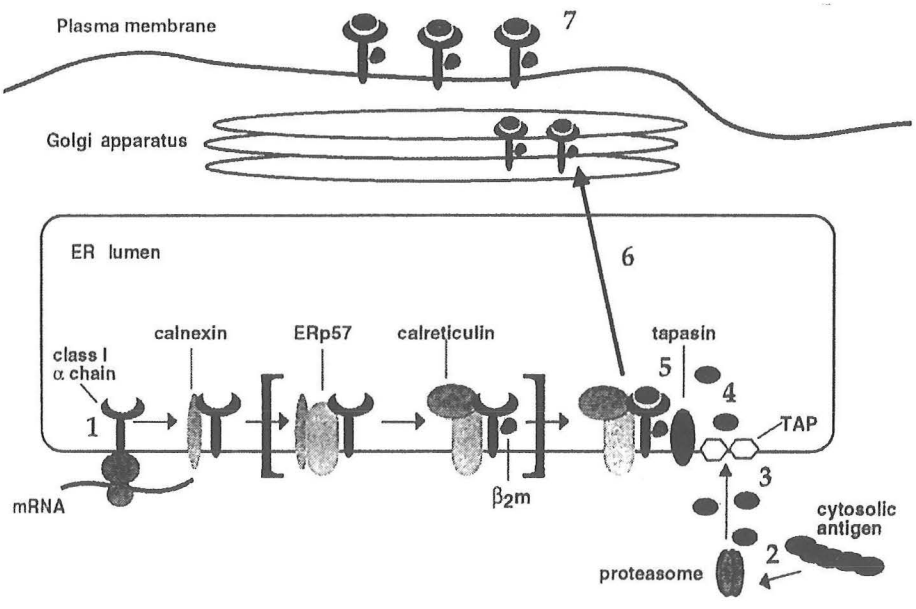

Figure 5: Ref. Brodsky. *Immuol Rev* 168:199. 1999
CMV interdicts antigen-processing.

face (step 3); Adenovirus E19 prevents the association of Class I MHC with TAP (step 5); and the HIV Nef causes internalization of the Class-I MHC-peptide complexes from the cell surface (step 7) (31).

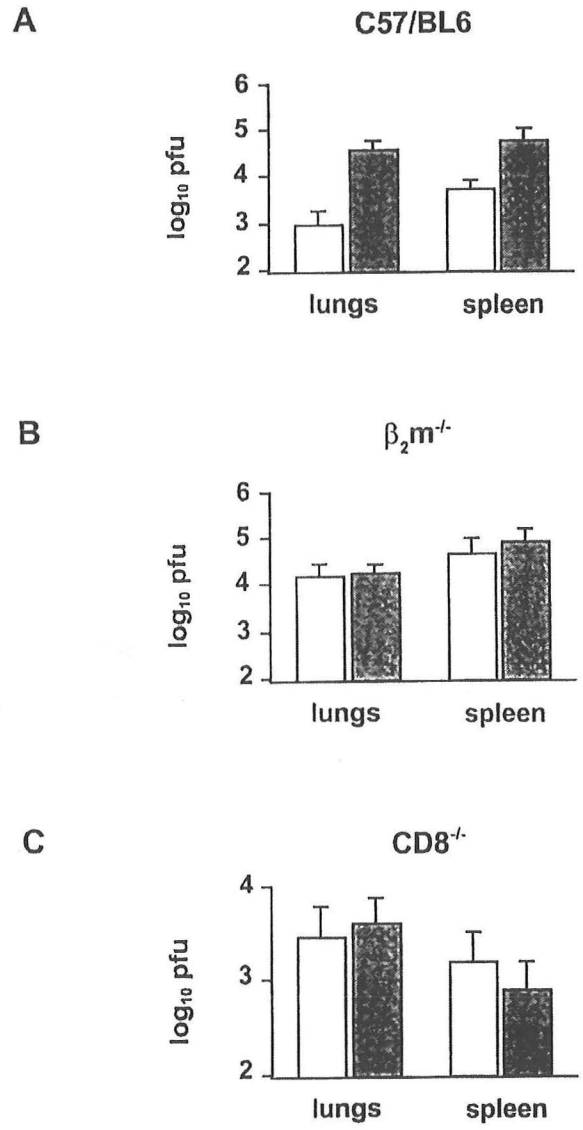

Figure 8: Ref. Hengel. *Immunol Rev* 168:167. 1999. Open bars - growth of CMV mutant which does not inhibit Class I MHC processing. Grey bars - growth of wild-type CMV.

One would predict that by preventing antigen processing, CMV would escape the immune response. As shown in the Figure below, mutant murine CMV which cannot prevent processing of antigen via Class I MHC are less virulent than wild-type CMV. If this pathway of antigen processing in the mice was "knocked out" by transgenic techniques instead of a wild-type CMV gene, the virulence of the mutant was restored. The loss of virulence was due to an inability to activate CD8+ CTL's because inactivating these cells by transgenic knockout allowed the mutant virus to become as virulent as wild-type virus.

Natural killer cells (NK) in the defense against CMV and other viruses.

Many viruses, in addition to CMV, interdict the Class I MHC pathway of antigen processing. To defend against these viruses the immune system evolved a defense based on NK cells. NK cells have "killer inhibitory receptors" (KIR) on their plasma membranes. When these receptors interact with MHC Class I on the surfaces of normal cells, the NK cells are turned off. However, if NK cells interact with virally infected cells which do not have MHC Class I, the virally infected cells are killed.

The monomorphic Class I MHC HLA-E inhibits NK cells via KIR and has a special function. HLA-E binds the leader sequence of most other MHC Class I molecules. If the number of Class I MHC molecules decreases, there are less leader sequences to bind the HLA-E; HLA-E is unstable without a leader sequence in its binding groove and will disintegrate. In the absence of HLA-E, NK cells

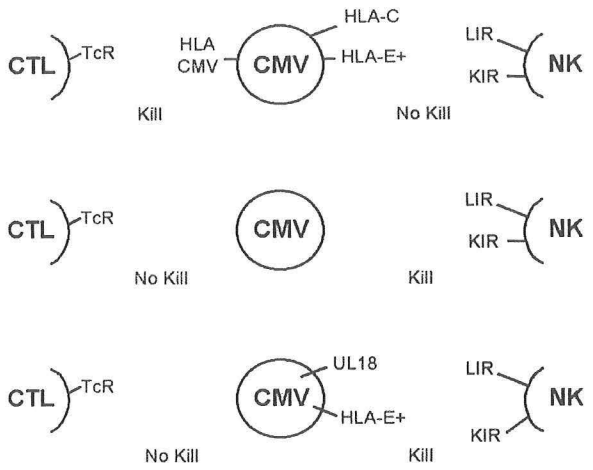

Figure 9: Killing of CMV-infected cells by CTL vs NK cells.

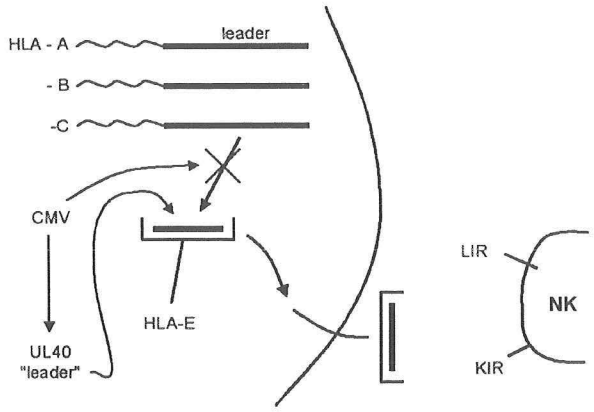

Figure 10: Ref. Tomasec. *Science* 287:1031. 2000. HLA-E prevents MK killing.

will tend to kill. Although CMV decreases the number of Class I MHC I (HLA) molecules, the virus produces a protein gpUL40 that maintains contains the HLA leader sequence and thus maintains the number of cell surface HLA-E molecules (32).

In addition to gpUL40, CMV produces another molecule UL18 which is similar to Class I MHC; instead of binding to KIR, UL18 binds leukocyte immunoglobulin-like receptor 1 (LIR-1) on NK cells, dendritic cells, some T cells, and monocytes. The cytoplasmic domain of LIR-1 contains a sequence of amino acids (the ITIM motif); other transmembrane molecules with this motif activate intracellular signaling sequences which inhibit leukocyte activation. Thus, although the function of human UL18 is controversial, many expect that it will activate LIR-1 on leukocytes and inhibit leukocyte activation. Overexpressing UL18 in some cells inhibits their being killed by NK cells (33-36), but this is not a universal finding [See review (37)]. Murine CMV has a protein, m144 that is a homolog of human UL18. As shown below, murine CMV without m144 fail to propagate in vivo. This data plus in vitro experiments suggest that m144 inhibits NK killing, and thus allows dissemination of the virus in vivo. It is possible that human UL18 has a similar role; in addition, because dendritic cells, monocytes, and some T cells express the UL18 counter ligand, LIR-1, UL18 may also inhibit functions of these cells (38).

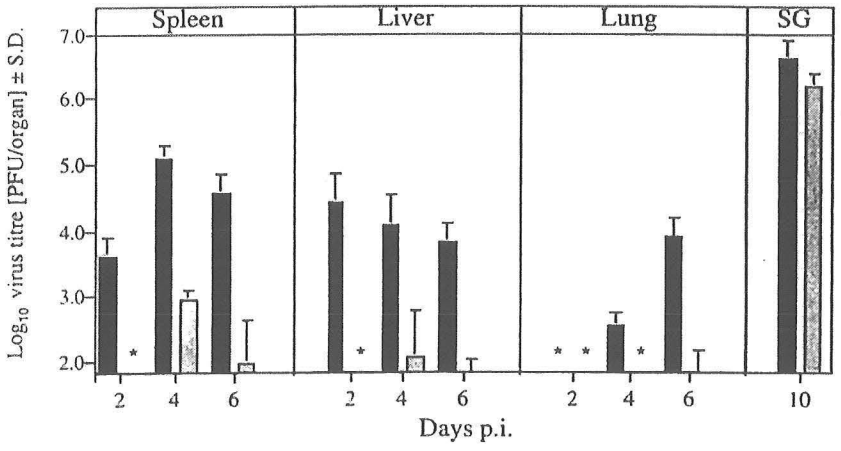

Figure 11: Ref. Farrell. *Immunol Rev* 168:187. 1999.
Inhibition of NK lysis by m144 (UL18) is required for infection in vivo.

Other immunosuppressive effects of CMV.

An effective immune response against viruses requires activation of CD4+ Tcells. Their cytokines "help" B cells make anti-viral antibodies and CD8+ cells differentiate into CTL's. Activation of CD4+ T cells requires their interaction with antigen-presenting cells with CMV peptide bound the Class II MHC. CMV US2 inhibits such antigen-presentation and thus activation of CD4+ cells (39).

During a viral infection, cells respond to interferon alpha and gamma by increasing MHC expression and inhibiting viral gene expression. CMV inhibits the response of cells to these interferons by inhibiting intracellular signalling via the JAK/ STAT pathways (40-43).

HCMV incorporates host complement inhibitory proteins into the virion membrane, and this protects the virion from antibody-complement mediated damage. The virus also increases increases the expression of CD46 and CD55 in the host cell, and thus makes the infected cell resistant to complement mediated lysis. [See review (44)].

The immunosuppressive effect of CMV is clinically important.

In addition to allowing survival of CMV in the host, the immunosuppressive effects of CMV have other clinical implications. In mice, the immunosuppressive effects of CMV result in an increased mortality after Candidal infections (45). The above theoretical concepts predict that post-transplant patients who develop CMV should later have an increased incidence of severe infections. This is indeed the case. As shown in the Table below, there is a higher incidence of serious fungal infections in those patients with CMV (46-48).

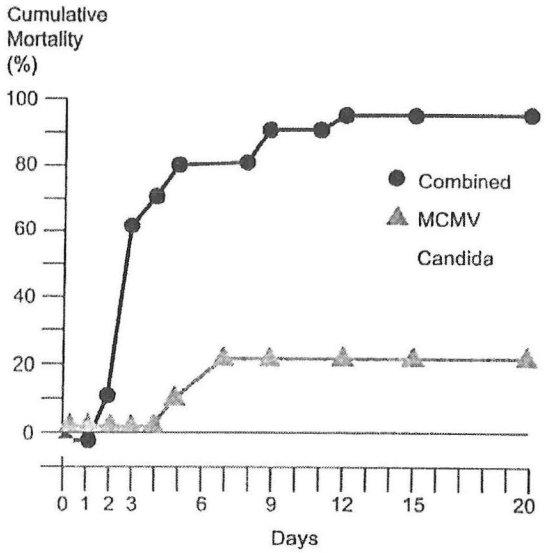

Figure 12: Ref. Hamilton. *Infect Immun* 14:982. 1976.
CMV increases mortality from candida in mice.

CMV and fungal disease

organ transplanted	deep fungal infections	
	p CMV	w/o CMV
liver	36%	8%
heart	27%	7%

Figure 13: Refs. George. *Am J Med* 103:106. 1997 and Wagner. *Transplantation* 60:1473. 1995.

Latency - reactivation: cytokines, and stress.

Given the formidable immunosuppressive armamentarium of CMV, it is fortunate that the virus enters a latent phase after acute infection in the normal host.

Although CMV infects many different cell types, including fibroblasts, endothelial cells, and epithelial cells, latent infections are best understood in cells of the myeloid lineage. Latent CMV may be found in a small percentage (0.001%) of peripheral blood cells of healthy people. These cells are the myeloid precursors of macrophages, monocytes, granulocytes, and dendritic cells (49). Like other herpesviruses, latent human CMV exist in circular form; in contrast, productive or persistently infected cells have the CMV genome in both linear structures, or large complex forms such as branched structures or concatemers (50). Unlike EBV, where the latently infected cells may express particular viral membrane proteins (EBNA-1, and latent membrane protein-1 [LMP-1] (44)), CMV is not known to have such proteins. Some the latently infected cells translate alternatively spliced variants of the immediate early gene IE1 (51), others do not have any transcribe or translate any known viral genes (52).

Reactivation of the latent virus does not occur by chance. During latent infection of the murine lung, for,example, occasional cells with transcripts of the IE1 immediate early gene are found; however, transcripts of later immediate early or late genes are never found as they should if reactivation occurred by chance (53). Reactivating the CMV by total body gamma irradiation resulted in transcription of late genes (54).

Human myeloid precursors with latent CMV will reactivate their infections when stimulated in vitro with allogeneic cells (55) or the following cytokines - TNFα, interferon gamma, interleukin 4, or graulocyte-macrophage colony-stimulating factor (56).

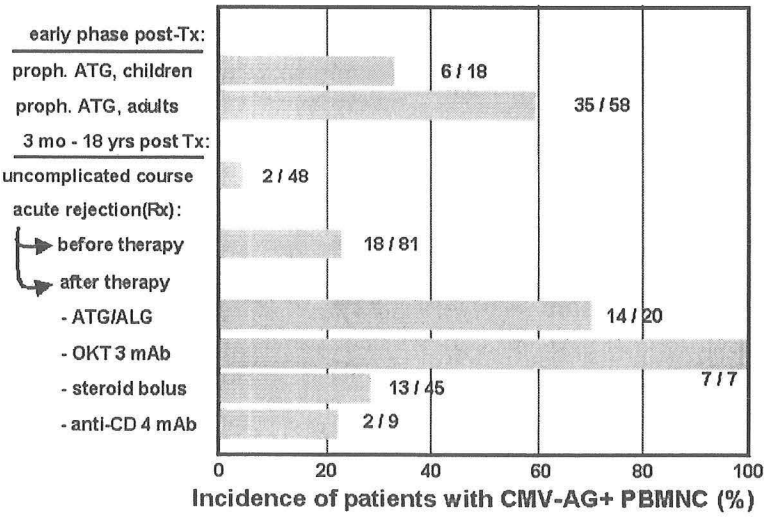

Figure 14: Ref. Fietze. *Transplantation.* 58:675. 1994.
Stress reactivates CMV.

In vivo reactivation of human CMV has been studied by Volk and his colleagues (57). They found that CMV-infected mononuclear cells appeared in the blood 6-12 days after renal transplant patients were treated prophylactically with OKT3, anti-CD4, or anti-lymphocyte polyclonal antibodies, or received high dose steroids for rejection episodes. All of these events, activated mononuclear cells produce TNFα. Indeed, closer examination of the data did show an association between high serum TNFα levels and reactivation of CMV. Other clinical situations where TNFα levels are elevated such as septic shock,

Relationship between elevated TNF plasma levels and the detection of CMV antigen-positive PBMNC[a,b]

Group of patients	TNF plasma peak levels (pg/ml)	Proportion of CMV antigen-positive patients
I	<10	6%
II	10-100	32%
III	>100	88%

Figure 15: Ref. Fietze. *Transplantation* 58:675. 1999.

myocardial infarction, and autoimmune diseases are also associated with reactivation (58-60)}.

There are no data available in humans on the effect of monoclonal anti-TNFα antibodies on CMV reactivation in the above clinical circumstances. However, in mice, inactivating TNFα with antibodies does prevent reactivation (61).

Altogether, the above data indicate that CMV lives in equilibrium with the host. An immune response consisting of CD4 , CD8, gamma-delta T cells, natural killer cells, and antibodies is elicited by the primary CMV infection (62-68). In response to its own internal signals, and to the anti-CMV immunity, the virus becomes latent after the initial infection. Occasionally, when the host responses to stress by producing cytokines such as TNFα, the CMV is reactivated. The immune response is then itself reactivated, and the CMV is again controlled. Recurrent subclinical cycles of viral reactivation and immunity may result in the unexpected high number of CMV-specific CD8 T cells which may result from multiple subclinical episodes of CMV reactivation (69). In an immunosuppressed patient such viral reactivations may not be controlled, and disease results..

CMV infection and vascular injury.

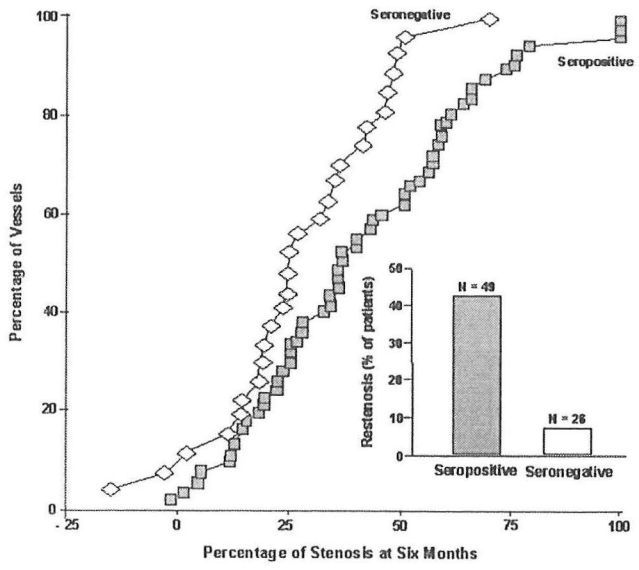

Figure 16: Ref. Zhou. *NEJM* 335:624. 1996.
Post angioplasty restenosis is increased by CMV.

Atherosclerosis may be considered an inflammatory process in response to injury (70). Prior infection by CMV, as evidenced by positive IgG but negative IgM antibodies in the serum, may exacerbate a particular type of vascular injury. This injury occurs when atherosclerotic coronary artery disease is treated by angioplasty. As shown in the Figure, the odds ratio for restenosis within 6 months of angioplasty is nine times higher in CMV seropositive patients as opposed to seronegative patients (71). A similar conclusion was reached by an independent study (72). CMV immediate early antigen is found in the restenosis lesions (73,74). A similar lesion is exacerbated by acute CMV infection after

balloon injury of the rat carotid artery; injury and presumably the associated local cytokines was required for CMV infection of the vascular wall (75,76).

CMV within the vascular wall may have direct effects that result in restenosis [see review (70,77)]. CMV infects and facilitates smooth muscle cell proliferation, migration, activation, and LDL uptake. The CMV immediate early antigen (IE84) binds to p53 and prevents this tumor suppressor gene from inhibiting cell cycle progression (73,78,79). CMV IE proteins also prevent apoptosis via the p53 pathway (78,80). In addition, the viral UL37 (vMIA) protein inhibits Fas-mediated apoptosis downstream of caspase 8 but upstream of cytochrome c release. Like Bcl-2, UL37 is a mitochrondrial protein, but is not identical structurally to Bcl-2 (81). CMV-infected vascular smooth muscle cells express the chemokine receptor US28 have increased migration in response to the chemokines RANTES or MCP 1 (24). The virus also activates intracellular NF_{κ} B and production of superoxide via a G-protein signalling pathway (82). Chlamydia pneumoniae also may contribute to atherosclerosis and may transactivate the CMV immediate early gene (83). The CMV IE protein increases the expression of scavenger receptors for LDL (84).

Potential Direct Effects of CMV on Atherosclerosis

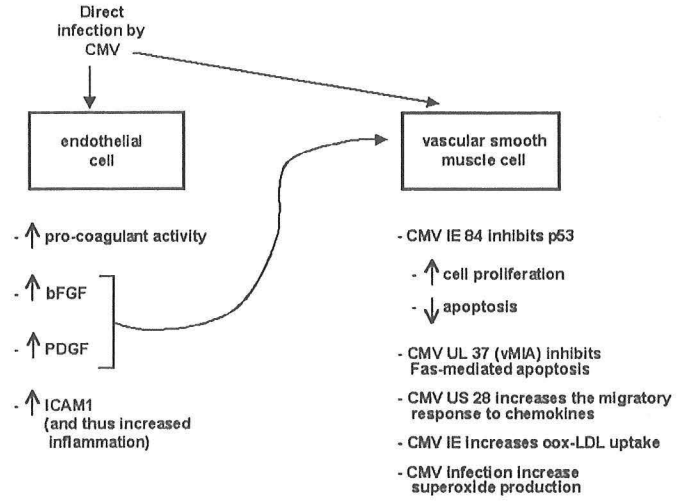

Figure 17

CMV also infects endothelial cells. These cells then increase their production of bFGF and PDGF, and these would increase vascular smooth muscle cell proliferation. CMV infection increases endothelial cell pro-coagulant activity (85).

In a rat model of carotid artery balloon injury, acute CMV infection exacerbated the injury, but no CMV gene expression was detected in the vessel by RT PCR. CMV infection was detected in salivary gland and spleen. These experiments suggest that infection distant from the injured vascular wall could exacerbate vascular injury (76). Increased interleukin 2 and 4 serum levels were found, and these might exacerbate the local vascular injury by further activating the local endothelial adhesion molecules and chemokines (76). Another possibility is that immunity develops against heat shock proteins expressed by the CMV-infected cells, and that there is also an autoimmune mechanism against heat shock proteins expressed by the damaged vascular wall (70,86).

A role for a virus such as CMV in atherosclerosis in humans and rodents would be similar to the role of herpesvirus in the pathogenesis of Marek's disease herpesvirus in chickens (87).

In light of the above arguments for a role for CMV in restenosis of atherosclerotic lesions, the hypothesis has been made that CMV might participate in atherosclerosis in the absence of angioplasty.

This is a controversial area that includes the broader issue of whether infections by other pathogens such as Chlamydia pneumoniae and Helicobacter pylori contribute to atherosclerosis. This area is beyond the scope of this manuscript; the reader is referred to several recent reviews on this controversial subject (70,88-93).

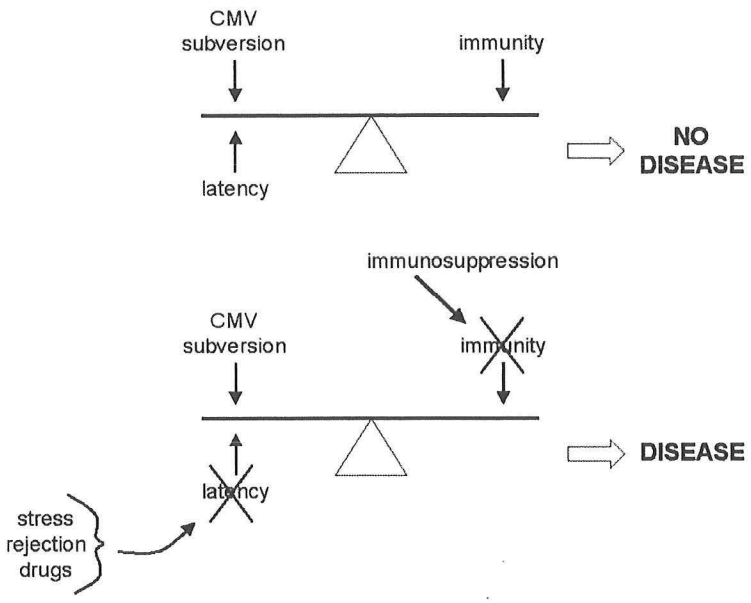

Figure 18: Factors determining CMV disease.

A model of CMV infection, reactivation, and latency: implications for allograft damage by CMV.

The Figure summarizes the equilibrium which may exist between CMV and the host immune system. Infection is aggravated by tissue injury and the associated cytokines, these may trigger reactivation. The severity of the viral infection after reactivation will depend upon a vigorous immune response that can overcome the numerous mechanisms CMV uses to defeat this response. Finally, we have discussed how the expression of viral genes depend upon the host cell's response to its microenvironment, and how in addition to latency or a productive infection, the function of a persistently infected host cell that expresses some viral genes may contribute to atherosclerosis and subversion of the immune response. We will now apply these concepts to the detrimental effects of CMV on allograft organ function.

CMV and allograft organ damage in the clinical setting.

CMV infection is associated with increased loss of heart, lung, liver, and renal allografts (2). In many patients, the allografts are lost through a chronic process. This is cardiac allograft vasculopathy, chronic bronchiolitis obliterans, vanishing bile duct syndrome, and chronic renal allograft nephropathy (94). In other patients, CMV may be associated with a higher incidence of allograft rejection. In still others, there may be direct cytopathic effects of the virus on the allograft.

CMV and cardiac allograft vasculopathy.

We will discuss cardiac allograft vasculopathy (CAV) first because some of the concepts discussed above relative to restenosis after coronary angioplasty may be applicable. CAV is the most common cause of death and retransplantation after heart transplant. Most data indicate that this is a form of chronic rejection. The lesions are limited to the allograft and occur in the great vessels up to but not beyond the suture line; the lesions are reproduced in allogeneic, but syngeneic, transplants in animals (95). Indirect clinical evidence suggests an association between CMV and CAV. For example, 28% of

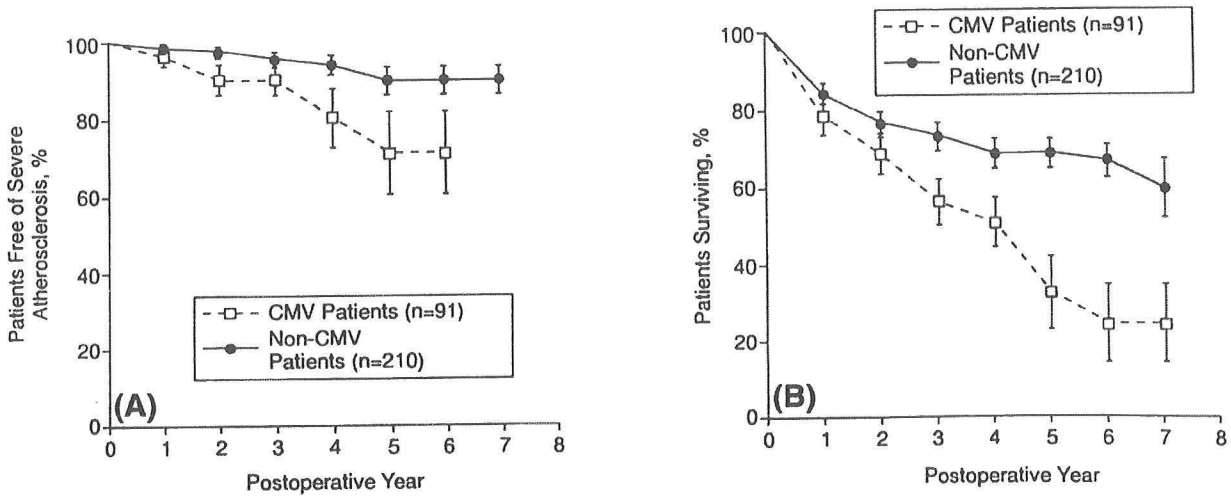

Figure 19: Ref. Grattan. *JAMA* 261:3561, 1989
Increased mortality and vasculopathy in CMV positive cardiac transplant recipients.

cytomegalovirus-infected, but only 10% of non-infected, cardiac transplant recipients developed CAV (96). See Figure. Many other studies confirmed this association, but others did not (see review (95). More recent data suggests that 4 months of CMV viremia (positive pp65) is required for a significant association of CMV with CAV (95). Although some investigators found evidence for CMV genes or antigen in the lesions of CAV, for example (97,98), others do not (99). The issue may be complicated by the facts that different strains of CMV have different potential to contribute to CAV (85), that 2 or more years may be required from CMV infection until the effect on CAV is clinically apparent (98), and that a small number of persistently CMV-infected smooth muscle cells might release growth factors which cause a large number of non-infected cells to form the atheroma (95).

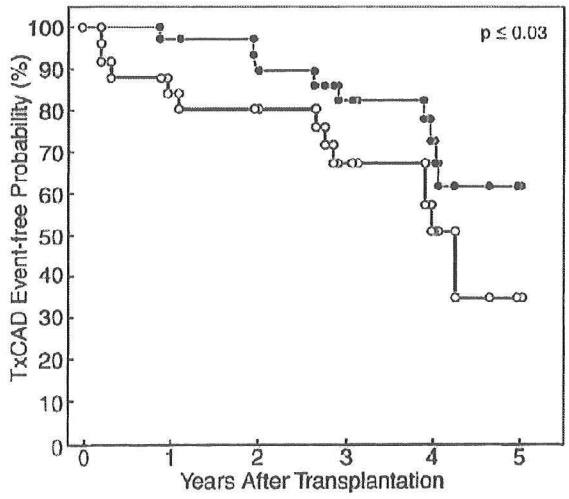

Figure 20: Ref. Valentine. *Circulation* 100:61. 1999.
Closed circles = post ganciclovir. Closed circles = no ganciclovir.
CMV prophylaxis prevents vasculopathy after heart transplant.

Additional data (Figure 20) in support of a contribution of CMV to CAV are a blinded study showing that a course of ganciclovir immediately after cardiac transplantation not only prevented CMV infection, but also decreased the incidence of transplant atherosclerosis from 62% to 11% in a cohort of 53 patients. However, this was a post hoc analysis and ganciclovir had this beneficial effect only in patients not taking calcium channel blockers (100).

A rat model of CMV and cardiac allograft vasculopathy.

When CMV is given to rat recipients of allogeneic aortic transplants, the aortas develop intimal inflammation and then smooth muscle proliferation. This does not occur when CMV is given to recipients of syngeneic grafts or to the native aorta. PDGF and TGFβ are found in the intimal lesions. The early inflammatory cells are unusual for allograft rejection in that they include NK cells in addition to T cells and macrophages (101); the possible significance of NK cells in the anti-CMV response was discussed previously. Similar intimal lesions are seen in the epicardial and subendocardial arteries of human cardiac transplant recipients who have CMV. In this rat model, two events are necessary for the intimal lesion - allograft rejection and CMV infection. If rejection is prevented with immunosuppression, the intimal lesion is ameliorated even though CMV proliferation in the spleen and salivary gland is increased. If the CMV is treated with ganciclovir and/or immunoglobulin, the intimal lesion is also ameliorated (98,101-105). This model is very similar to CMV infection of the rat carotid artery after balloon injury (75,76); in that case two events were necessary - vascular injury due to trauma instead of rejection, and CMV.

The effect of CMV on rat and murine cardiac allografts is exactly the same as on the aortic allografts discussed above (106-108).

CMV and renal allograft loss.

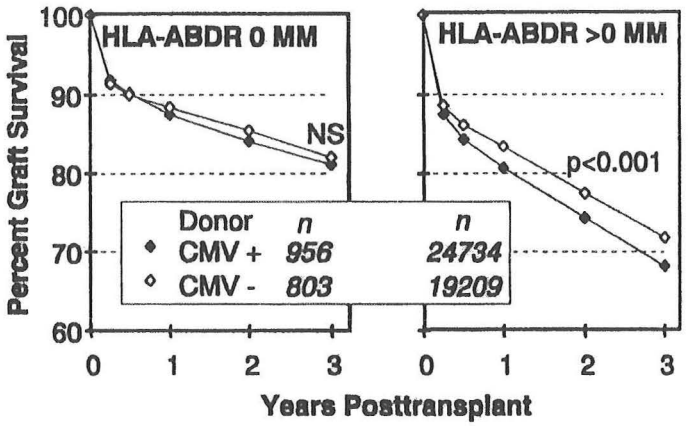

Figure 21: Ref. Hirata. *Transplantation* 62:34. 1996.

The Figure summaries the course of 47,146 renal transplants in the registry of the United Network for Organ Sharing (UNOS) from 1987-1994. Renal transplants from CMV-positive donors resulted in lower graft survival than transplants from CMV-negative donors, regardless of whether the recipient was CMV negative or positive. The greatest effect was in recipients who had more than one HLA A, B, DR mismatch.CMV positive donor was not a risk if the donor-recipient pair had not HLA A,B,DR mismatches (109).

The UNOS study examined only cadaver kidneys. An analysis of living donor transplants also indicates that CMV positive kidneys transplanted into seropositive recipients do less well (110). Other studies indicate a particular disadvantage if either the donor or recipient were HLA DR9 or 51 or HLA B13 (111). This may reflect the decreased ability of these particular HLA types to present viral peptides. Similar differential abilities of specific HLA alleles to present particular peptides of a pathogen has been described (112).

Close inspection of the UNOS data suggests that allograft survival disadvantage of a CMV positive kidney did not appear until after the first year post-transplant. This suggests an effect of CMV on

chronic allograft nephropathy or chronic rejection. In a rat model of CMV infection after renal transplant, the CMV did increase the severity of chronic arterial damage, and fibrosis in the kidney (113). Finally, a factor in the decreased survival of the CMV positive kidney was death of the recipient. The most common cause of death in these patients is cardiovascular disease, and one might speculate that the CMV may have aggravated any underlying atherosclerosis (see above).

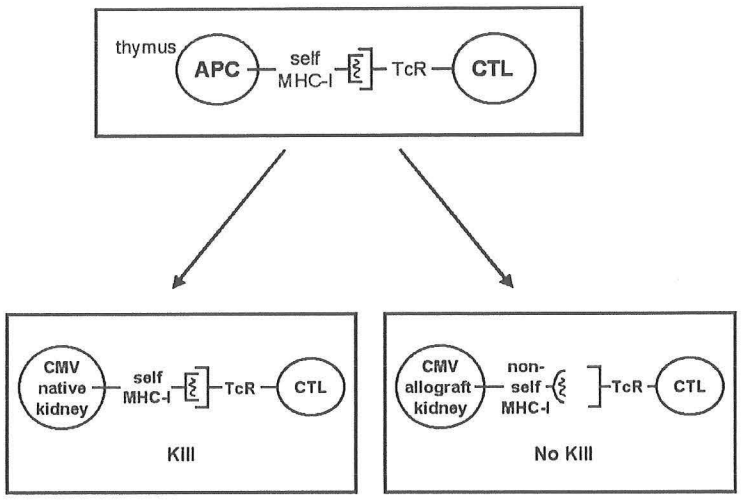

Figure 22: Privileged site

Why was HLA mis-matching associated with the detrimental effect of a CMV positive donor? Two issues may contribute to this observation. Rubin (114) has suggested that the allograft is a "privileged" site for CMV and other viruses (see Figure 22). A significant portion of the anti-viral response is performed by CD8+ CTL's. As shown in the Figure, such CTL's cells would differentiate in the recipient's thymus and learn to recognize viral peptides in the context of self HLA. These CTL's would not recognize viral peptides associated with non-self HLA. This is consistent with the clinical argument that the transplanted solid organ is the most severely afflicted by CMV. Thus, heart transplant recipients, but not renal, pancreas, liver, or lung transplant recipients develop cardiac allograft vasculopathy, for example. This formulation would also explain why the recipient CMV serology had no effect on the negative impact of a CMV positive donor. The clinical rule that CMV disease is worse in seronegative recipients of a seropositive kidney applys to direct CMV disease of recipient organs, not to the fate of the transplanted organ. The second issue is the possibility that acute rejection contribute to the allograft loss.

The question of whether CMV precipitates acute renal allograft rejection is controversial. Some have suggested that proinflammatory cytokines are generated in the allograft during CMV infection, and that these should facilitate rejection (2). As shown in Figures 23 and 24, a 90 day course of high dose valacylovir not only decreased CMV disease, but also allograft rejection in seronegative recipients (48). The dose of valacylovir used was sufficiently high that 6% of the treated patients experienced hallucinations. The rate of rejection in the control (no valacylcovir group) - 52% compared to the general experience with a similar immunosuppressive protocol of 35-40% (115). This trial also used "induction" with anti-T cell antibodies (ATGM or OKT3) which is less widely used now. The effect of valacyclovir combined with the newer immunosuppressive regimens of Tacrolimus plus mycophenolate, or tacrolimus plus rapamycin are not known. This study is consistent with others indicating that CMV may be associated with acute rejection (116,117). However, a meta-analysis of trials of prophylactic treatment to prevent CMV did not show a decreased incidence of rejection in the treated group; however, most of the included trials involved a shorter and less intense course of prophylaxis (118).

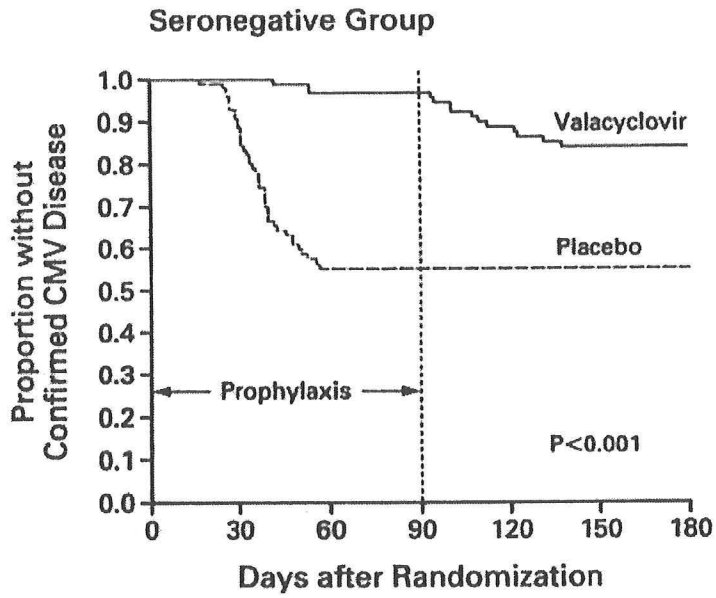

Figure 23: Ref. Lowrance. *NEJM* 340:1462. 1999.

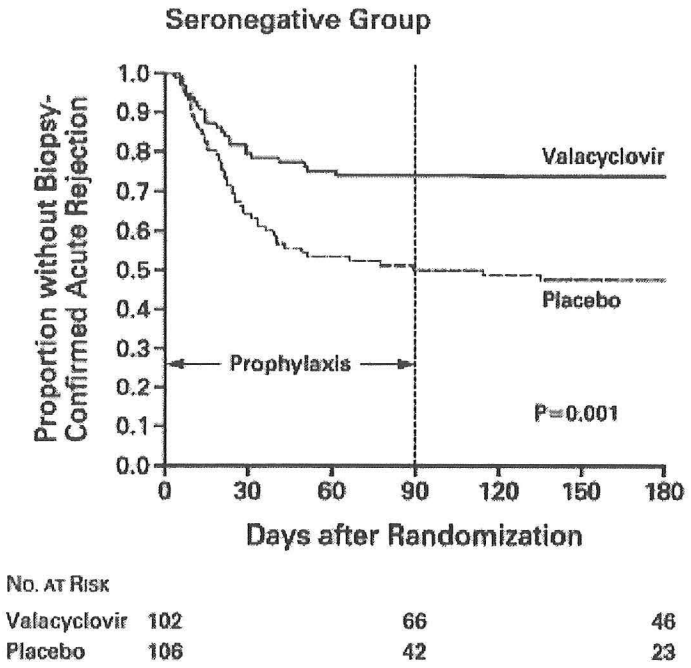

Figure 24: Ref. Lowrance. *NEJM* 340:1462. 1999.

It is also of note that in a rat model of renal transplantation, CMV did increase acute rejections (113).

Acute renal allograft rejection after the first 6 months has a poor prognosis (119). In one small study involving 21 patients with late acute rejections, a course of ganciclovir improved allograft function. None of the patients had symptoms of CMV disease; 82% were CMV positive by RT PCR and 42% were positive by the presence of IE antigen (120). It is not clear if the allograft dysfunction was misdiagnosed as rejection when it was actually CMV nephritis, or if the CMV precipitated rejection.

In another study, CMV disease itself did not negatively impact long-term renal allograft survival. However, it did further decrease the long-term survival of kidneys which had sustained acute rejection (121).

How might CMV precipitate acute allograft rejection? One possibility that, although infected cells are protected from CMV by the mechanisms discussed earlier in this manuscript, the injured and dying cells do stimulate the immune cells to produce cytokines. These may then increase HLA and adhesion molecule expression by non-infected allograft cells. This may predispose to rejection. (See Figure 25.)

In addition to exacerbating chronic renal allograft injury and increasing the incidence of acute rejection, CMV may also cause glomerulonephritis and interstitial nephritis in which the typical CMV inclusion bodies are seen in the

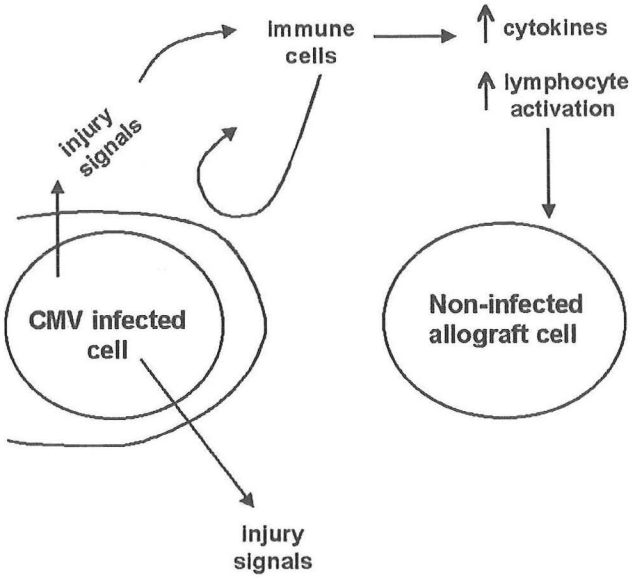

Figure 25: CMV and acute rejection.

kidney (122-127). Some have advocated decreasing the immunosuppression when CMV infections of the allograft is diagnosed.

CMV and loss of hepatic and pulmonary allografts.

CMV is also associated with chronic bronchiolitis obliterans in pulmonary transplants and a syndrome of vanishing bile ducts in liver transplants. The association between loss of liver transplants and CMV (128) may be due to CMV infection of the bile duct epithelium, hepatic artery and portal venous endothelium (129). As is the case for heart transplants, some CMV genotypes have a higher association with loss of the hepatic allografts (130). There is a model of acute and chronic lung rejection after rat CMV infections in the rat (131,132).

Post-transplant lymphoproliferative disease (PTLD); CMV as a co-factor.

Post-transplant lymphoproliferative disease is a heterogeneous group of lymphomas that occur after solid organ transplant and have a mortality of 50-80%. The disease is believed to result from the transplantation of an Epstein-Barr Virus (EBV) seropositive organ into a seronegative recipient. Although the EBV virus is thought to be the etiologic agent, the presence of CMV disease increases the risk of lymphoma by seven-fold (133,134). The role of CMV in the disease process is not understood. However, CMV does cause chromosomal breaks (135), and three different regions of the CMV gene are associated with the virus' ability to cause morphologic transformation of cultured cells (136).

Therapies -decrease immunosuppression and/or give anti-CMV agents.

There are a number of therapeutic strategies to treat or prevent CMV in organ transplantation. CMV infections are worse when a seronegative patient receives an organ from a seropositive donor. Ideally one would not transplant a CMV seropositive organ into a seronegative recipient; and the donor and recipient should have no HLA mismatches. Since only 20% of the population is seronegative, it is not practical for such patients to wait for a seronegative organ. Similarly, a zero mismatch occurs in only for less than 10% of all transplants, and waiting for such an organ is also not practical for most patients. The most practical therapies are to decrease immunosuppression or use anti-CMV agents.

The importance of using a minimal amount of immunosuppression cannot be overemphasized.

There are four classes of agents available for treatment:

- the purine analogues (ganciclovir and, for prophylaxis or preemptive therapy, acyclovir, and valacylovir),
- the pyrimidine analogue (Ciofovir),
- a direct inhibitor of the viral DNA polymerase (Foscarnet),
- an antisense agent (Fomivirsen) against CMV IE mRNA and used only for CMV retinitis,
- and immunoglobulin.

The most widely used therapeutic agent is ganciclovir, also known as 9-[1,3-dihydroxy-2-propxymethyl] or DHPG, or Cytovene. Ganciclovir is phosphorylated to a monophosphate by CMV UL97, and then further phosphorylated to the triphosphate by cellular enzymes. The ganciclovir triphosphate competitively inhibits deoxyguanosine triphosphate incorporation into viral more than host DNA. The drug is eliminated by the kidney and dose-adjustments must be made for patients with renal impairment. Myelosuppression, especially neutropenia, is the principle dose-limiting side-effect. However, CMV also causes neutopenia, and appropriate doses of ganciclovir may improve the neutropenia as the disease responds to treatment. In either case, the neutropenia may respond to G-CSF which can be used safely in transplant patients (137). CNS side-effects ranging from headache to psychosis occur in 5-15% of patients. Other complications include anemia, rash, liver function test abnormalities, immunosuppression, teratogenicity. (See reviews (1,138). Ganciclovir is poorly absorbed by the oral route. Valganciclovir is under study as a possible oral form of ganciclovir.

Valacyclovir is the L-valyl ester of acyclovir, and is metabolized to the active parent drug after absorption by the GI tract. Valacyclovir has a greatly enhanced oral bioavailability compared to acyclovir. Like ganciclovir, acyclovir must be mono-phosphorylated by viral kinases, and then further phosphorylated to the triphosphate by cellular enzymes. The acyclovir triphosphate competitively inhibits the viral DNA polymerase, and some is incorporated into the viral DNA where it acts as a chain terminator. Acyclovir is efficiently phosphorylated by the thymidine kinase of Herpes simplex and is an extremely active against this virus. CMV does not have this thymidine kinase, but the UL97 which phosphorylates ganciclovir will also phosphorylate acyclovir and the acyclovir triphosphate does inhibit the DNA polymerase (139). The intracellular concentrations of ganciclovir triphosphate is more than 10x that of acyclovir triphosphate in infected cells. This may explain why CMV infected cells are so much more sensitive to ganciclovir. Thus, ganciclovir, not acyclovir or valacyclovir, is indicated for acute CMV infections. However, valacyclovir has been used successfully for prophylaxis (48). (See reviews (1,138).

Cidofovir ([S]-1-[3-hydroxy-2-phosphonylmethoxypropyl]cytosine) is an acyclic phosphonate nucleotide analogue of deoxycytidine monophosphate. It does not require metabolism by CMV UL97 and thus may be effective against resistant strains due to mutations in UL97. Cidofovir must be given intravenously for systemic CMV and has long intra-cellular half-life. It may be given every other week. It is eliminated by both glomerular filtration and tubular secretion. Nephrotoxicity is a major, common complication which may be ameliorated with vigorous saline administration and probenicid. (See reviews (1,138,140)

Foscarnet (trisodium phosphonoformate) will directly inhibit the viral DNA polymerase without any metabolism by viral enzymes. It is also excreted by the kidney. It must be given intravenously. Major toxicities include renal failure, and electrolyte abnormalities. Hypocalciumia due to chelation may cause serious arrhythmias and CNS problems. See review (138).

Fomivirsen is a 21 nucleotide phosphorothioate oligonucleotide that is an antisense to an immediate-early "IE2" mRNA of CMV. It is approved only for CMV retinitis and must be injected directly into the vitreous. It has a mechanism different from the inhibitors of CMV DNA polymerase, and is expected to be effective against strains of CMV that are resistant to those inhibitors. See review (138).

Immunoglobulin and CMV hyperimmune globulin have been used to prevent CMV and as adjunctive therapy for one of the anti-viral agents above. A monoclonal antibody against the gpH on the virion membrane is not effective.

The most effective anti-viral agents inhibit the viral DNA polymerase. Although this will prevent viral replication, it may not prevent other adverse effects of the virus which are caused by translation of other viral genes as discussed in previous sections of this manuscript. This includes the genes which subsert the immune response and those which may exacerbate atherosclerosis in the allograft.

Therapeutic strategies.

Three different therapeutic strategies are being used to treat CMV infections in solid organ transplant recipients: treatment of acute disease, prophylaxis, and preemptive (or deferred) treatment. The presentation, diagnosis, and treatment of acute CMV disease is well covered in recent reviews (1,2). Because of the serious effects of CMV disease, two strategies have been proposed to prevent the disease.

One strategy to prevent disease is prophylaxis. In renal transplantation, a Canadian group developed a set of clinical practice guidelines base on the published literature from 1976 through July, 1997 (141). The following recommendations were graded A - E. Grades A and B: use of intervention advised based on high or fair quality evidence respectively; grades D and E: intervention not advised; grade C: no recommendation pro or con.

Situation 1) Seropositive recipient; donor seropositive or seronegative; immunosuppression with antilymphocyte products. Prophylaxis recommended with an "A" grade.

Situation 2) Seronegative recipient; seropositive donor; immunosuppression with antilymphocyte products. Prophylaxis recommended with an "A" grade.

Situation 3) Seronegative recipient; seropositive donor; conventional immunosuppression (no antilymphocyte products). Prophylaxis recommended with a "B" grade.

Situation 4) Seronegative recipient; seronegative donor; any immunosuppressive regimen. No prophylaxis with antiviral therapy required (Grade D/E).

Situation 5) Seropositive recipient; donor seropositive or seronegative; conventional immunosuppression. Prophylaxis left to discrimination of physician (Grade C).

As we discussed in a previous section, anti-lymphocyte antibodies result in the release of TNFα which reactivates CMV. Thus, prophylaxis is indicated in situations 1 and 2. The epidemiology indicates that the risk of disease is high when a seronegative patient receives a kidney from a seropositive donor (situation 3). This confirms excellent data in humans, using restriction enzyme analysis of viral DNA, that CMV is transmitted from a seropositive donor to the recipient (142); this is substantiated by data in a rodent model where a kidney was transplanted from a latently infected rat into a CMV negative recipient and disease was transmitted (143). The authors were unable to determine which of the various prophylactic regiments - iv ganciclovir, oral ganciclovir, or acyclovir - was superior.

Since the above manuscript was published, a large double blinded study indicated that valacylovir prevented CMV disease in seronegative recipients of a kidney transplant (48).

A meta-analysis of all prophylactic trials in renal, heart, liver, and lung transplantation also supported the use of prophylaxis (118). However, CMV disease is more severe in heart, lung, and liver transplantation than in kidney transplantation. Antiviral prophylaxis with acyclovir or ganciclovir is helpful in most trials. Some groups report that the highest risk patients - those who are seronegative, or receive anti-lymphocyte products - are not protected by ganciclovir alone, and that the addition of immunoglobulin may be nessary (144).

Another strategy to prevent CMV disease is preemptive (or deferred) therapy. Not all transplant recipients will develop CMV disease in the absence of prophylaxis. For example, over 50% of seronegative renal transplant patients, who did not receive prophylaxis, not develop CMV disease in one study (48). In other words, 50% of the patients receiving antiviral prophylaxis were needlessly subjected to these medications and their complications. To avoid this problem, some groups have advocated preemptive therapy. These groups test the patients regularly for CMV using the newer pp65 antigenemia or RT-PCR techniques; as soon as the patients are positive, but before they become symptomatic, antiviral therapy is initiated (see review (144)), or the immunosuppression is decreased (58,145) .

The problem with this approach is that not all patients with a positive pp65 test will eventually develop disease. In a recent study, 153 renal transplant recipients had a positive antigen test. All of the patients with a low grade positive became antigen negative and never developed disease. 25% of patients with high grade antigenemia, not previously treated with OKT3, developed disease; the others became antigenemia negative. On the otherhand, 100% of the high grade antigenemia patients, who had previously received OKT3, developed CMV disease (146).

Resistance of CMV to the common anti-viral agents - ganciclovir, foscarnet, and cidofovir.

Most resistant strains of CMV have developed in patients with AIDS because of the larger viral burden and the prolonged therapy necessary to control the CMV disease. However, the increasing use of prolonged prophylaxis and frequent pre-emptive therapy, discussed in the preceding section, have

raised concerns that resistant strains will also become a problem for patients with solid organ transplantation.

Two types of resistance to ganciclovir has been described. Mutations in the UL97 kinase necessary to phosphorylate ganciclovir lead to resistance. There may also be mutations in the viral DNA polymerase. As shown in Figure 26, mutations in the DNA polymerase (UL54) may also result in resistance to ganciclovir, cidofovir, and foscarnet. DNA replication by CMV requires viral proteins in addition to UL54, and further mutations may be defined by later studies. See reviews (147,148).

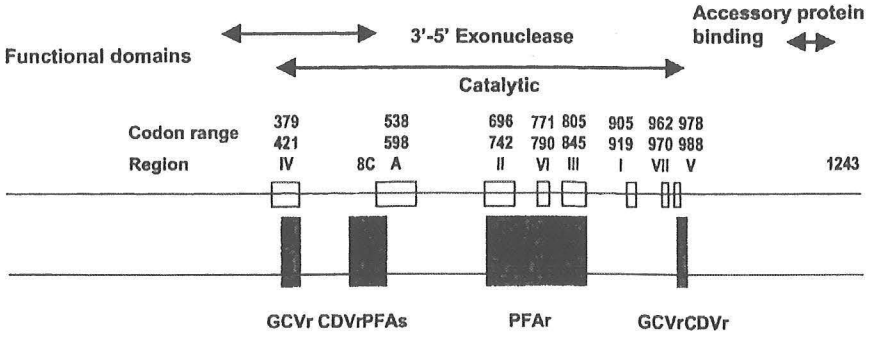

Figure 26: Ref. Crumpacker. *Mandel's Inf. Dis.*, 2000.
Mutations in the CMV polymerase which result in resistance to GCV (ganciclovir), cidofovir (CDV), and foscarnet (PFA).

Detection of resistance to the known antivirals is currently difficult because the standard plaque assay requires viral growth in the presence of the antiviral agents. This is too lengthy to be useful clinically. However, knowledge of the gene sequences of UL97 and UL54 and the location of the known mutations may allow the use of genetic techniques such as RT-PCR for rapid detection of drug resistance.

CMV vaccines in development.

The attenuated Towne strain of CMV has been used in ESRD patients prior to transplantation, and in seronegative mothers of childern secreting virus; the vaccine did not prevent infection. Trials with other attenuated viruses are underway. Immunization with the CMV surface glycoprotein B does elicit an antibody response, but it is not known if this will be protective. Recombinant canarypox virus are being developed which will produce the CMV surface glycoproteins, or the CMV pp65 and IE1 immediate early gene product. It is hoped that the nonvirulent recombinant canarypox will elicit antibody responses as well as T cell responses. The pp65 and IE1 proteins are the major antigens of anti-CMV T cell responses. Another strategy is to directly inject plasmids containing CMV genes as an immunogen (DNA vaccine) (149). See review (150).

Conclusion.

CMV has co-evolved with its human host. A number of viral genes subvert the anti-CMV immune response and allow the virus to propagate and then become latent. The host cells' response to its microenvironment determines the ability of the virus to propagate during the initial infection and if it will be reactivated. This microenvironment includes cytokines such as TNFα that appear in response to stress occurring during the clinical course of a transplant patient. This includes rejections, anti-rejection therapies, and vascular injury. The interaction of the virus with the vascular injury of

atherosclerosis may contribute to the chronic rejection and vasculopathy which complicates the long-term course of many patients. In view of the serious consequences of CMV infection several anti-viral agents have been developed and are employed in treatment strategies. Such strategies are directed at treatment of the acute disease, prophylaxis, and preemptive therapy.

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