

## Drug Resistance due to Mutation in UL 97 and Certain Other Specific Genes in Cytomegalovirus Strains: An Overview

Sunil Kumar and Sanjay Mishra\*

School of Biotechnology, IFTM University, Lodhipur Rajput, Delhi Road (NH 24), Moradabad 244102, U.P., India.

\*Corresponding Author: sanjaymishra@iftmuniversity.ac.in

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**Abstract:** The study of human cytomegalovirus (HCMV) antiviral drug resistance has enhanced knowledge of the virological targets and the mechanisms of antiviral activity. The currently approved drugs, ganciclovir (GCV), foscarnet (FOS), and cidofovir (CDV), target the viral DNA polymerase. The widespread use of ganciclovir (GCV) to treat cytomegalovirus (CMV) infections in immuno-suppressed patients has led to the development of drug resistance. GCV anabolism also requires phosphorylation by the virus-encoded UL97 kinase. GCV resistance mutations have been identified in both genes, while FOS and CDV mutations occur only in the DNA polymerase gene. Confirmation of resistance mutations requires genotypic assays based on sequencing provide more rapid results but are dependent on prior validation by phenotypic methods. Recombinant phenotyping methods performed in a few research laboratories have resolved some of the conflicting results. Treatment options for drug-resistant HCMV infections are complex and have not been subjected to controlled clinical trials, although consensus guidelines have been proposed.

**Keywords:** Cytomegalovirus, antiviral activity, drug-resistance, ganciclovir, foscarnet, cidofovir, viral DNA polymerase.

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### I. Introduction

Cytomegalovirus (from the Greek cyto-, "cell", and -megalo-, "large") is a viral genus of the viral family known as *Herpesviridae* or *herpesviruses*. It is typically abbreviated as CMV. The species that infects humans is commonly known as human CMV (HCMV) or human herpesvirus-5 (HHV-5), and is the most studied of all cytomegaloviruses (Ryan and Ray, 2004). Within *Herpesviridae*, CMV belongs to the *Betaherpesvirinae* subfamily, which also includes the genera *Muromegalovirus* and *Roseolovirus* (HHV-6 and HHV-7) (Koichi et al., 2007). It is related to other herpesviruses within the subfamilies of *Alphaherpesvirinae* that includes herpes simplex viruses (HSV)-1 and -2 and varicella-zoster virus (VZV), and the *Gammapherpesvirinae* subfamily that includes Epstein-Barr virus (Ryan and Ray 2004). All herpesviruses share a characteristic ability to remain latent within the body over long periods. Although they may be found throughout the body, CMV infections are frequently associated with the salivary glands in humans and other mammals (Koichiet al., 2007). Other CMV viruses are found in several mammal species, but species isolated from animals differ from HCMV in terms of genomic structure, and have not been reported to cause human disease. HCMV is strictly species specific, as are the other cytomegalovirus.

CMV was derived from the observation of a characteristic swollen or "cytomegalic" appearance of cells first noted in infants who died of various causes and is a common viral cause of congenital defects and becomes particularly prominent as a pathogen in immunocompromised patients, mental retardation and nonhereditary sensorineural deafness in children (Porath et al., 1990). It also is a significant cause of transfusion acquired infections and is perhaps the most infection that occurs in transplant recipients and those with acquired immune deficiency syndrome (AIDS) (Weller et al., 1993).

CMV is associated with leukocytes and may be transmitted with a blood transfusion (Lerner and Sampliner, 1977) or transplanted organ (Ho et al., 1975). HCMV infection causes by reactivation of the latent virus months or years later. Primary infection with HCMV is usually not diagnosed, because it is asymptomatic or associated with a mild influenza like illness in the normal host. Symptomatic primary HCMV may occur in surgical patients who acquire the virus from blood transfusion and it is one of the causes of the post cardiectomy syndrome after open-heart surgery. The second major clinical problem caused by HCMV is congenital or intrauterine infection, estimated at 1:100 infants by viral isolation from urine at birth. Congenital infection is classically associated with hepatosplenomegaly, thrombocytopenia, hemolytic anemia, chorioretinitis and encephalitis (Porath et al., 1990). In children and adults symptomatic CMV infection is characterized by fever, heterophile agglutinin- negative mononucleosis, and subclinical hepatitis (Klemola et al., 1970). Although CMV infection is quite common, CMV disease is rare. The majority of infection is asymptomatic. In symptomatic infection, the clinical manifestations vary with the age of the affected individual, and the severity ranges from mild to fetal illness. A variety clinical syndromes resulting from involvement of various organs are being

increasingly recognized and reported. Examples are neonatal infection, mononucleosis, post perfusion syndrome, hepatitis, pneumonia, chorioretinitis, conjunctivitis and other ocular manifestation, gastrointestinal syndromes, meningoencephalitis, polyneuritis, thrombocytopenic purpura, hemolytic anemia, vasculitis, carditis, arthritis, cystitis, nephritis and periodontitis (Naraqi, 1991).

Recent studies have implicated mammalian viruses in the pathogenesis of human periodontitis (Contreras and Slots, 1996; Parra and Slots, 1996). HCMV is frequently detected in human periodontitis lesion. However, no information is available on the types of gingival cells infected by herpes viruses. Gingivitis is usually a precursor to periodontitis but not all gingivitis progresses to periodontitis. Periodontitis result from a host response to microbial plaque; however, mechanisms for its initiation and progression are still being studied. Periodontitis have four major types included: adult, early-onset (prepuberal), refractory (Juvenile), and periodontitis associated with systemic diseases. HCMV in the pathogenesis of human periodontitis can infect a variety of inflammatory cells. It is important to determine which inflammatory cells of the periodontium harbor herpes viruses. A significantly higher prevalence of HCMV was detected in the gingival cervical fluid of advanced periodontitis patients than that of gingivitis patients (Parra and Slots, 1996) and in deep periodontal pockets compared with shallow periodontal sites within individual adult periodontitis patients (Contreras and Slots, 1996). Periodontal breakdown in children is often associated with some fault in host response (as in Down' Syndrome), but early destructive periodontal disease i.e. Juvenile periodontitis (JP), has been reported by Cogen et al. (1992) in healthy Alabama children. Periodontitis in adults, there is considerable paleontological evidence of periodontal disease in early humans, and past epidemiological studies have emphasized the general prevalence of the disease.

## **II. Human Cytomegalovirus**

### **○ Virus structure and genome organization**

HCMV has a linear double-stranded DNA genome of approximately 230 kbp encoding more than 200 proteins. The virion, which is 150-200 nm in diameter, consists of an icosahedral capsid surrounded by a tegument or matrix, which is further surrounded by a lipid-protein envelope. The viral genome is divided into two unique components, unique long (UL) and unique short (US) regions. The unique regions are flanked by terminal (TR) and inverted repeat (IR) sequences, UL by TRL and IRL, and US by IRS and TRS, respectively. DNA sequence based analyses demonstrate a great genomic heterogeneity within HCMV worldwide. The capsid of the virion is composed of seven proteins. The major (MCP) and minor capsid proteins (mCP) are encoded by UL86 and UL85, respectively. The minor capsid binding protein (mC-BP) is encoded by UL46 and the smallest capsid protein (SCP) by UL48.5. Three assembly protein-related proteins associate with capsids and are the products of UL80, UL80a and UL80.5 genes. A tegument contains at least 25 proteins, of which many are phosphorylated. The basic phosphoprotein (pp150) encoded by UL32 and lower matrix protein (pp65) encoded by UL83, are the most abundant proteins expressed during virus replication. The other prominent tegument proteins are ppUL99 (pp28), ppUL82 (pp71) and ppUL48 (huge tegument protein). Several transcriptional transactivator proteins have also been localized to the tegument. The envelope consists of two prominent glycoprotein complexes, a complex of dimers of glycoprotein B (gB), encoded by UL55, and a complex of the gH, gL, and gO, encoded by UL75, UL115 and UL74, respectively. These glycoprotein complexes are assumed to play an important role in virus entry.

### **○ Virus replication**

HCMV exhibits a restricted host range in cell culture. Although in vitro the most commonly used cells are primary differentiated human fibroblasts derived from skin or lung, HCMV can also replicate in other cell types including endothelial and epithelial cells, smooth muscle cells and leukocytes (Sinzger and Jahn, 1996). After the attachment of the virus to the cell surface, fusion of the virion envelope and the cell membrane occurs (Mocarski and Courcelle, 2001). The cellular receptors for HCMV are still unclear, but it has been suggested to be widely distributed in cells (Mocarski and Courcelle, 2001). Epidermal growth factor receptor has recently been suggested to serve as a receptor for HCMV (Wang et al., 2003). The virus capsid is rapidly transported to the cell nucleus and, after the proteolysis of the capsid proteins; the viral DNA is released into the nucleus.

DNA replication, formation of capsid and packaging of viral DNA occur in the nucleus. Subsequently, nucleocapsids acquire a primary envelopment by budding at the nuclear membrane. They further mature through a de-envelopment/re-envelopment process in the cytoplasm before leaving the cell via an exocytic like pathway (Mettenleiter, 2002; Mocarski and Courcelle, 2001). The whole replication cycle of human CMV is slow, requiring approximately 48-72 hours. The cytopathic effect in response to HCMV is characteristically cell enlargement with intranuclear inclusions.

### **Pathogenesis**

It has been suggested that epithelial cells of the upper alimentary, respiratory and genito-urinary tracts can be the site of primary replication after the direct contact of infectious virus and mucosal surface (Pass, 2001). In addition, it has been suggested that a fetus can be infected hematogenously (Halwachs-Baumann et al., 1998; Hemmings et al., 1998). In any case, once HCMV infection is established, leukocyte-associated and endothelial cell-associated viremia appears to play an important role in the passage of the virus between the various tissues of the host. Viremia may last for several weeks during the primary infection. Hematogenous spreading typically results in the infection of ductal epithelial cells at the initial site. HCMV can infect a wide range of tissues including salivary glands, gastrointestinal tract, lung, liver, brain, kidney, spleen, pancreas, eye, heart, adrenals, thyroid and genital tract and then replicate in diverse cell types, including fibroblasts, epithelial cells, macrophages, vascular smooth muscle and endothelial cells. HCMV is also suggested to infect the vascular wall and play a role in the development of atherosclerosis.

In immunocompetent individuals, primary infection (and also reactivation/reinfection) usually remains asymptomatic or results in benign clinical symptoms such as fever, elevated levels of transaminases and/or mononucleosis syndrome. Severe clinical symptoms are observed in congenital infections and among immunocompromised patients (such as transplant recipients and AIDS-patients)(Griffiths and Emery, 1997), in whom HCMV infections are an important factor for developing HCMV-associated disease.

### **Epidemiology and transmission**

HCMV infects humans of all ages. Acquisition of the virus in the general population usually occurs early in life, mainly during the first two decades, and often already during the first year. Based on sero-epidemiological studies, the seroprevalence varies between 30% to 100% in different countries. The great seroprevalence and also early acquisition of the virus have been associated with lower socioeconomic circumstances, developing countries and countries with crowded populations (Ho, 1990). However, in Scandinavian countries seroprevalence is as high as 60-80 %, and in Finland even 70-80 %, despite good hygiene and a high socioeconomic level.

After the primary infection, which is usually asymptomatic or very mild in immunocompetent individuals, the virus will remain in a lifelong latent state in the host. Later in life, the virus can reactivate or an individual can have a reinfection. Transmission of the virus can occur vertically from mother to fetus or newborn, or horizontally from one individual to another (Griffiths and Emery, 1997). During primary infection, the virus is secreted into the urine, saliva, semen and cervical secretions for weeks to months, as well as intermittently during reactivations and reinfection. Vertical transmission can occur transplacentally or prenatally. An intra-uterine infection is the result of maternal viremia and prenatally acquired infection can occur at the time of delivery from infected maternal genital secretions or later via breast milk. Later in life, infection usually occurs by close contact via infected bodily fluids, e.g. from child to child or from child to susceptible adult via saliva or urine. In the adult population, a major route of infection is sexual transmission. HCMV can also be transmitted by blood transfusions or by organ or bone marrow transplantations.

### **III. Laboratory Diagnosis of HCMV**

An accurate and rapid diagnosis of HCMV in transplant patients is of utmost importance. Different laboratory methods based on either serology or identification of virus/virus components in clinical specimens are now available. However, in recent years PCR methods, especially with quantitative modifications of PCR, and also with other nucleic acid amplification techniques are well authenticated and established.

#### **○ Histological methods**

Traditionally the recognition of cytomegalic inclusion bodies in histological specimens has been used for the diagnosis. In organ specific HCMV infection, such as HCMV pneumonitis or hepatitis, characteristic viral inclusions may be seen. The large inclusions are intranuclear and have a characteristic owl-eye appearance in haematoxylin and eosin stained tissue specimens. The positive results correlate well with active HCMV infection of the organ, e. g. hepatitis, but the sensitivity of the histopathological finding is relatively low (Colina et al., 1995; Paya et al., 1989). Immunostaining with specific polyclonal or monoclonal antibodies against HCMV antigens has increased the sensitivity of the method compared to conventional staining (Barkholt et al., 1994; Colina et al., 1995; Paya et al., 1990).

However, false-negative results may occur because of the focal and scarce distribution of HCMV positive cells in tissue samples (Colina et al., 1995). ISH methods, described for the identification of viral DNA in infected cells, have also been employed to improve the histological diagnosis of infection (Barkholt et al., 1994; Colina et al., 1995; Einsele et al., 1989; Espy et al., 1991; Musiani et al., 1996; Paya et al., 1990). HCMV specific probe, usually labeled by biotin, is used in these assays. Although the histological methods may have an important role in detecting HCMV organ involvement, they are not suitable for the early diagnosis of HCMV

infection. In addition, these assays are very laborious, and biopsy specimens are only taken when an end-organ disease is suspected.

○ **Serology**

Humeral response to primary HCMV infection is manifested by the production of IgG and IgM antibodies. Thus a diagnosis of HCMV infection can be obtained indirectly through serology. A variety of laboratory tests with different degrees of sensitivity have been described for the measurement of HCMV antibodies in human sera. The methods include complement fixation, indirect hemagglutination, latex agglutination, radioimmunoassay, immunofluorescence and enzyme immunoassay (Mendez et al., 1999a; Sia and Patel, 2000). In enzyme-linked immunosorbent (ELISA) assays many different antigens have successfully been used as targets for detecting specific antibody production (Monte et al., 1996).

○ **Viral culture**

Infectious virus can be isolated from various clinical materials, such as bodily secretions, tissue or peripheral blood, in cell cultures which are permissive for HCMV replication. Human fibroblast cell cultures, obtained from the foreskin or from the embryonic lung tissues, have been used for conventional and shell vial methods for the isolation of HCMV (Mendez et al., 1999a). In conventional viral isolation methods, commonly used in earlier times, determination of viral replication is based on typical cytopathic effects (CPE) produced by HCMV. The time required for the development of CPE usually varies from 2 to 4 weeks, even up to six weeks.

Several less time-consuming methods have been developed for the isolation of infectious virus (Mendez et al., 1999a; Sia and Patel, 2000). One of the most commonly used rapid methods is the shell vial culture (Gleaves et al., 1984). In this method, for the improvement of absorption of the virus, the specimen is centrifuged onto the cell culture. Fibroblasts monolayer cultured in vials containing cover slips are used. These shell vial culture methods utilize indirect immunofluorescence to detect the immediate-early (IE) viral antigen after incubation of culture for one to three days (Lautenschlager et al., 1989). The isolation of HCMV from the blood or target organ specimen by cell culture methods has a high correlation with disease. However, the relatively low sensitivity of detection of the virus in blood samples compared to nucleic acid-based and antigenemia methods, limits the use of these assays in the diagnosis and monitoring of HCMV infection in transplant patients (Badley et al., 1996; Evans et al., 1999; Van der Bij et al., 1988; Wirgart et al., 1996).

○ **Antigen detection**

The antigenemia assay has been a major advance in the diagnosis of HCMV infection in transplant patients. In this test, monoclonal antibodies to pp65 (UL83) (Depto and Stenberg, 1989; Gerna et al., 1992; Grefte et al., 1992), the lower matrix protein, are used for the direct immunostaining of blood polymorphonuclear leukocytes (PMNL). However, it has been shown that the pp65 antigen in PMNLs is not a direct indication of virus replication *in vivo*, since the virus and viral material detected in PMNLs are transferred from other infected cells, e.g. endothelial cells, mainly by microfusion events (Gerna et al., 2000). Nevertheless, the pp65 antigenemia assay has been successfully used in the diagnosis of HCMV in transplant patients since the late 1980's.

The antigenemia assay is more sensitive than cell culture methods in detecting HCMV in the blood (Landry and Ferguson, 1993; van den Berg et al., 1991; Van der Bij et al., 1988). It is also highly specific for HCMV but a positive result does not always predict the onset of symptomatic infection (Landry and Ferguson, 1993; Sia and Patel, 2000; Tanabe et al., 1997). Nevertheless, when using the test in a quantitative manner, the assay can be used to predict and diagnose HCMV infection: at least, a higher degree of antigenemia is correlated with symptomatic disease, as has been shown by many groups (Hadaya et al., 2003; Humar et al., 1999; Kim et al., 2003).

One disadvantage of the antigenemia assay is that the blood samples should be processed within a certain time, preferably within six hours, for optimal results (Schafer et al., 1997). In addition, though there have been attempts to simplify the method (Gratacap-Cavallier et al., 2003; Ho et al., 1998), it is still quite time-consuming and laborious, at least with large specimen numbers. The automation of the test is also difficult because of the number of steps and the subjective evaluation of the infected leukocytes. Although there have been attempts to standardize the assay (Gerna et al., 1998; The et al., 1995; Verschuuren et al., 1999), there are a number of various in-house and commercial modifications available. This makes comparison of the results between different centers difficult. The clinically significant threshold of the number of positive leukocytes seems to vary also among different types of transplant populations (Baldanti et al., 1998; Razonable et al., 2002b; Sia and Patel, 2000). In any case, the pp65 antigenemia test has shown its clinical utility in the diagnosis of HCMV infection, in guiding of pre-emptive therapy, and also in the monitoring of response to antiviral treatment (Baldanti et al., 1998; Grossi et al., 1996; Kim et al., 2003; Kusne et al., 1999).

○ **Nucleic acid amplification methods**

The widespread introduction of the application of nucleic acid amplification technologies has been the most important improvement in HCMV diagnostics in the past decade. The most frequently used methods are based on PCR detecting HCMV DNA (or RNA) in whole blood or in different blood compartments. Both qualitative and quantitative assays have been used. However, understanding of the correlation between viral loads and clinical symptoms has diminished the significance of qualitative methods. Different kind of quantitative PCR assays, both in-house and commercial ones, have been employed. The in-house assays, based on competitive PCR, are usually technically complicated. Commercial and real-time based applications, which are more convenient and faster, are more widely used at present. Also other nucleic acid-based techniques, such as nucleic acid sequence based amplification (NASBA) and signal amplification (Hybrid Capture System) have successfully been used in the detection of viral mRNA or DNA, respectively.

○ **Other nucleic acid amplification methods**

It has been suggested that the detection of HCMV mRNA would correlate better with symptomatic HCMV infection than DNA, because of the ability of the virus to remain latent in the host cell. Previously, in-house RT-PCR methods were used for the detection of mainly immediate early or late transcripts in blood samples of transplant patients (Bitsch et al., 1993; Gaeta et al., 1997; Nelson et al., 1996). The sensitivities, specificities and correlations with HCMV disease of these tests varied considerably. Later, many applications based on NASBA technique have been employed. NASBA is an isothermal amplification process which involves the coordinated activities of three different enzymes: ribonuclease H, reverse transcriptase and DNA-dependent RNA polymerase (Compton, 1991). The final product of the process is a specific singlestranded RNA, instead of the dsDNA of PCR. The high sensitivity in detecting HCMV infection has been shown by the NASBA assay with the IE-1 target (Blok et al., 1999; Blok et al., 2000; Goossens et al., 1999; Oldenburg et al., 2000). In recent years, many applications of the NASBA technique based on the detection of pp67 mRNA have been developed and the test application is also commercially available (Gerna et al., 2003; Witt et al., 2000). These assays detect pp67 mRNA, which is transcribed late in the viral replicative cycle, in a qualitative manner. pp67 NASBA assay may have a clinical utility in both early diagnosis of symptomatic HCMV infection and monitoring transplant patients with active disease, as well as in guiding pre-emptive therapy (Caliendo et al., 2002; Gerna et al., 2003; Witt et al., 2000). Recently, the pp67 NASBA has also been combined with the quantitative detection of IE-1 mRNA, which may improve the clinical utility of this technique (Greijer et al., 2002).

HCMV DNA has also been detected by a signal amplification method, Hybrid Capture System, from blood samples of transplant patients (Aitken et al., 1999; Bhorade et al., 2001; Ho et al., 2000; Mazzulli et al., 1999; Norris et al., 2002). The Hybrid Capture System is a solution hybridization assay that involves amplified detection instead of amplification of the desired nucleic acid fragment. The assay uses a specific RNA probe which targets 17 % of the HCMV genome. The RNA-DNA hybrid is captured by a monoclonal antibody and is detected by a luminometer. Both qualitative and quantitative applications are commercially available. The quantitative form has clinical utility in the diagnosis and monitoring of HCMV infection (Aitken et al., 1999; Bhorade et al., 2001; Ho et al., 2000; Norris et al., 2002).

○ **Antiviral susceptibility testing**

After the more widespread use of antiviral drugs, resistant HCMV strains have been reported also in the transplant patient population (Isada et al., 2002; Limaye, 2002; Lurain et al., 2002). The methods used for antiviral susceptibility testing can be divided into phenotypic and genotypic assays. Phenotypic assays rely on the suppression of viral growth in the presence of antiviral agents. Various modifications have been employed (McSharry et al., 1998) of which the plaque reduction assay is considered as a standard method. A major limiting factor of the phenotypic method is that they require viral culture from the clinical sample, followed by several passages to reach the necessary viral titers for the performance of the assay, which can take up 4-6 weeks. Because the assay is burdensome and it lacks standardization, it is not routinely undertaken in the majority of centers. The facts, that specific mutations in the UL97 and UL54 genes are associated with antiviral drug resistance, have led to the development of genetic methods. These are based on PCR amplification of the specific region of the genome followed by restriction enzyme analysis or direct sequencing of the amplification product/products (Alain et al., 1997; Lurain et al., 2002; Mendez et al., 1999b; Wolf et al., 1995). An obvious advantage of these assays is the possibility to use clinical specimens directly, rather than virus isolates, which significantly shortens the time required for performance of the test. In addition, mixtures of the different strains are not always detected in viral culture methods (Lurain et al., 2002). However, standardization and validation of these methods are needed for clinical use.

### **Treatment of the disease**

The three antiviral drugs that are currently licensed for the treatment of HCMV infection are ganciclovir (GCV), foscarnet and cidofovir. Ganciclovir is the drug of choice in the treatment of symptomatic infections. Ganciclovir is a specific nucleoside analog which first is phosphorylated before it gains antiviral activity (Littler et al., 1992; Sullivan et al., 1992). The initial phosphorylation is mediated by virus encoded phosphotransferase (product of UL97). The monophosphate form is further phosphorylated by the host's cellular kinases into the active triphosphate form, which inhibits viral DNA polymerase by competing with deoxyguanosine triphosphate (Noble and Faulds, 1998). For the treatment of HCMV disease, ganciclovir is administered intravenously (i.v.) for at least two weeks.

Intravenous foscarnet may be used under conditions of failure of ganciclovir treatment, ganciclovir resistance, or excessive side effects, such as leukopenia (Crumpacker, 1996; Noble and Faulds, 1998). Foscarnet is an inorganic pyrophosphate analog and directly inhibits viral DNA polymerase (Chrisp and Clissold, 1991; Crumpacker, 1992). However, foscarnet is nephrotoxic, which limits its use in kidney transplantation. Intravenous cidofovir may also be used, but experience of this agent in solid organ recipients is still very limited. Cidofovir is a cytosine analog which does not require the virus encoded phosphotransferase activity. Instead host cellular enzymes carry out the phosphorylations needed for activation (Lalezari et al., 1995; Snoeck et al, 1988).

Valganciclovir, a pro-drug of ganciclovir, is a new promising compound which is administered orally and has an increased bioavailability compared to that of oral ganciclovir (Pescovitz et al., 2000). Nowadays valganciclovir is used in therapy of HCMV disease. However, more clinical trials are needed. The HCMV hyperimmunoglobulin may also be used in some patient groups in combination with antiviral drugs, e.g. lung transplant patients with HCMV pneumonia (Patel and Paya, 1999; Zamora, 2001).

Two different strategies, prophylaxis and pre-emptive therapy, are used for the prevention of symptomatic HCMV infection after transplantation. In prophylaxis, antiviral drugs are administered before any evidence of the virus, and in pre-emptive therapy, antiviral drugs are administered when there is laboratory evidence of active but asymptomatic infection. The antiviral strategies for treatment and prevention of HCMV disease are outlined in more detail below.

### **Prophylaxis**

Prophylaxis consists of administration of antiviral drugs, immediately after transplantation, either to all transplant recipients or only to those who are at the highest risk for the HCMV disease, such as seronegative recipients receiving seropositive organ or patients who are treated with anti-T-lymphocyte antibodies. The practice and guidelines for prophylactic therapy vary widely, depending on the patient population and the transplant center (Batiuk et al., 2002; Patel et al., 1996). Also hyperimmunoglobulin in combination with ganciclovir has been used in certain patient groups, e.g. lung transplant recipients (Weill et al., 2003).

The first antiviral drug used for prophylaxis was high-dose oral acyclovir, an analog of deoxyguanosine. The efficacy of oral acyclovir has been demonstrated in studies showing reductions in the incidence of HCMV disease compared with no treatment (Balfour et al., 1989; Couchoud et al., 1998; Mollison et al., 1993). A pro-drug of acyclovir, valacyclovir, has been found to be more effective than acyclovir for prevention of HCMV disease (Fiddian et al., 2002; Lowance et al., 1999). Valacyclovir has increased oral bioavailability compared to that of acyclovir (Weller et al., 1993), and in vivo it is first converted to acyclovir. The action of acyclovir (valacyclovir) is dependent on phosphorylation, in which thymidine kinase is important. However, CMV does not encode this kinase (unlike herpes simplex and varicella zoster viruses), and the phosphorylation, which may be allowed by UL97 product (Talarico et al., 1999) is less specific. For this reason, these drugs are not potent enough for the treatment of HCMV infection.

Currently, the most widely used and specific drug for HCMV prophylaxis is ganciclovir, administered orally or intravenously. Intravenous ganciclovir has been proven to be a potent prophylactic agent in many studies (Merigan et al., 1992; Paya, 2001; Winston et al., 1995). The availability of the oral form has increased the use of this agent (Speich et al., 1999; Winston and Busuttill2004; Yango et al., 2003), although the bioavailability of oral ganciclovir is lower when compared to that of intravenous ganciclovir. A pro-drug of ganciclovir, valganciclovir, is a new promising option also for prophylaxis. The drug is effectively absorbed and it is nowadays widely used although the published reports are still limited (Lake, 2003; Paya et al., 2004).

### **Pre-emptive therapy**

In pre-emptive therapy only patients who have laboratory evidence of an active HCMV infection are treated with antiviral drugs. Therefore, the diagnostic test should predict impending symptomatic infection sufficiently early. In addition, because pre-emptive therapy requires frequent surveillance of all patients, the test should also be specific as well as rapid and easy to perform. Since viral load has been shown to be a major factor in the pathogenesis of HCMV (Emery, 1999; Hassan-Walker et al., 1999), quantitative measurements

have been considered to have greater clinical value in a pre-emptive therapy strategy. Various diagnostic methods, virological and/or DNA/ RNA-based, may be used for this purpose (Daly et al., 2002; Gerna et al., 2003; Grossi et al., 1996; Sia and Patel, 2000; Sagedal et al., 2003).

### **Drug resistance**

The initial studies of resistant laboratory and clinical CMV strains related ganciclovir resistance to inadequate intracellular phosphorylation of the compound in cells infected with ganciclovir-resistant viruses. Although experimental evidence at that time suggested that ganciclovir phosphorylation was controlled by a CMV-encoded function, the mechanism by which ganciclovir is phosphorylated in CMV-infected cells remained unresolved until 1992. In that year, two independent groups of investigators provided biochemical, immunological, and genetic evidence that the phosphorylation of ganciclovir in CMV-infected cells is controlled by a protein kinase homologue encoded by the UL97 open reading frame of the virus (Littler et al., 1992, Sullivan et al., 1992). In one study, a recombinant protein (UL97tr) was obtained by cloning a truncated part of the CMV UL97 open reading frame including sequences homologous to the catalytic domains of protein kinases into an expression vector. This protein was used to produce specific antiserum that were shown by Western blotting to react with UL97tr itself and with a protein obtained from CMV-infected cells that had a molecular weight similar to that predicted for UL97. In addition, extracts from bacteria expressing UL97tr efficiently phosphorylated ganciclovir compared with controls. Furthermore, this phosphorylating activity was neutralized by CMV seropositive serum but not by CMV seronegative serum (Littler et al., 1992). In another study, recombinant viruses containing fragments representing the complete UL97 sequence of the ganciclovir-resistant laboratory strain 759<sup>D</sup>100 prepared in marker transfer experiments were resistant to ganciclovir and unable to induce phosphorylation of ganciclovir in infected cells. Analysis of the DNA sequence of these recombinant viruses revealed a 12-bp deletion of UL97 codons 590 to 593, which resulted in a 4-amino-acid deletion (Ala-Ala-Cys-Arg) in a conserved region of the UL97 protein possibly implicated in substrate recognition (Sullivan et al., 1992). On the basis of these results, it was proposed that UL97 is responsible for the initial phosphorylation of ganciclovir in CMV-infected cells.

These findings prompted the characterization of UL97 sequences in ganciclovir-resistant laboratory and clinical CMV strains. In one study, plaque-purified ganciclovir-resistant strains D1/3/4, D6/3/1, and D10/3/2 were obtained after sequential passages of the susceptible strain, AD169, in increasing concentrations of ganciclovir (Lurain et al., 1994). Cells infected with these ganciclovir-resistant viruses showed reduced levels of ganciclovir phosphorylation, suggesting the presence of mutations in the UL97 region of these viruses. Sequencing studies of this open reading frame demonstrated the same mutation (ATG to ATT), resulting in a methionine to isoleucine amino acid change at amino acid residue 460 (M460I mutation) of the UL97 protein in all three resistant strains (Lurain et al., 1994). A recombinant virus (R6HS) prepared after transfection of UL97 sequences from D6/3/1 into cells infected with AD169 was also resistant to ganciclovir and induced ganciclovir phosphorylation levels that were more than 10-fold lower than those in AD169-infected cells (Lurain et al., 1994).

The occurrences of antiviral drug resistant HCMV strains have been documented also among organ transplant patients (Isada et al., 2002; Limaye, 2002; Lurain et al., 2002). The major risk factors for drug resistance include the long duration of drug exposure and high amounts of viral load (Chou, 2001; Drew, 2000). These factors are most prevalent in patients with primary infection, i.e. in D+/R- population, in which resistance has most often been observed (Rosen et al., 1997). The mutations in both the viral phosphotransferase gene (UL97) and the viral polymerase gene (UL54) may confer antiviral drug resistance in HCMV. The mutations of the UL97 coding sequence, which may confer resistance only to ganciclovir, occur mainly in the region including codons 460-607 (Chou et al., 1995; Hanson et al., 1995; Lurain et al., 1994). The more rare mutations in the UL54 coding sequence may confer resistance to any or all of the three most commonly used drugs (ganciclovir, foscarnet or cidofovir) (Cihlar et al., 1998b; Mousavi-Jazi et al., 2003). Mutations in UL54 appear to occur in regions between codons 300 and 1000, and they are often accompanied by mutations in UL97, showing higher levels of resistance to ganciclovir with possible cross-resistance to foscarnet and/or cidofovir (Jabs et al., 2001).

Wellcharacterized (canonical) mutations in UL97 are found in GCV-resistant clinical strains, with one of the seven mutations M460V/I, H520Q, C592G, A594V, L595S, and C603W encountered in close to 90% of cases. Other UL97 mutations confer various degrees of GCV resistance, while mutations in the UL54 DNA polymerase gene sometimes evolve to increase resistance to GCV and confer cross-resistance to cidofovir and/or foscarnet (Lurain, N. S., and S. Chou., 2010).

#### IV. Conclusion

Resistance of CMV to antiviral agents is a well-documented complication of long-term antiviral therapy. This problem has been observed mostly in patients with AIDS and CMV retinitis in whom drug-resistant CMV infections have been associated with clinical progression and therapeutic failure. Current data suggest that the incidence of infections caused by drug-resistant CMV in bone marrow or solid-organ transplant recipients is low. Because of the routine use of antiviral agents for prophylaxis or pre-emptive therapy for CMV infections in bone marrow or solid-organ transplant recipients, surveillance antiviral susceptibility studies of CMV isolates from patients developing active CMV infections would be appropriate. CMV resistance studies have been limited by the difficulty and lack of standardization of antiviral susceptibility assays.

In contrast, significant advances have been made in recent years in understanding the mechanisms by which CMV becomes resistant to antiviral agents. The recognition that specific mutations in the UL97 and UL54 genes of CMV are associated with different antiviral susceptibility patterns has prompted the development of molecular laboratory methods for detection of mutant viral sequences in viral isolates and directly in clinical specimens.

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*Drug resistance due to mutation in UL 97 and certain other specific genes in Cytomegalovirus strains:*

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