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Cytomegalovirus

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ABSTRACT Cytomegalovirus (CMV), the largest of the herpesviruses, causes a wide range of clinical syndromes, from asymptomatic infection to severe disease in immunocompromised hosts. Laboratory methods for diagnosis include molecular testing, antigenemia, culture, serology, and histopathology. Treatment of CMV infection and disease is indicated in selected immunocompromised hosts, and preventive approaches are indicated in high-risk groups. This chapter reviews the epidemiology, clinical aspects, and the laboratory diagnosis and management of CMV in immunocompromised hosts.

VIROLOGY

Cytomegalovirus (CMV), the fifth member of the human herpesvirus family, is one of the largest viruses known to cause clinical disease. It is a double-stranded DNA virus that belongs to the beta-herpesvirus subfamily, along with human herpesviruses 6A, 6B, and 7. CMV was first associated with an infectious mononucleosis-like illness in healthy individuals in 1965 (1). Currently, it is known to cause a wide range of clinical syndromes, from asymptomatic infection in healthy hosts, to severe and even fatal disease in immunocompromised individuals, such as transplant recipients.

CMV has an icosahedral shape measuring 150 to 200 nm in diameter, and has four fundamental structural elements: an outer lipid envelope, tegument, a nucleocapsid, and an internal nucleoprotein core that contains its genome. The viral envelope contains lipoproteins and at least 33 structural proteins, including those involved in viral entry into cells. The tegument is composed of structural proteins, including the pp65 antigen, which is a major target for diagnosis testing (2). The genome is a 64-nm linear double-stranded DNA molecule that contains nonoverlapping open-reading frames for over 230 proteins. One of the proteins is a DNA polymerase, which plays an integral role in viral replication and serves as the main target for all currently approved antiviral drugs.

EPIDEMIOLOGY AND CLINICAL MANIFESTATIONS

A survey in the United States reported an overall CMV seroprevalence rate of 50.4%. The prevalence increases with age; in children ages 1 to 5, it may be as low as 20.7%, but it approaches 100% in older adults in developing countries (3, 4). CMV seroprevalence rate varies widely with (a) geographic location, with higher rates in developing countries; (b) age, with the rate increasing directly with older age; and (c) socioeconomic status, with highest seroprevalence in crowded and economically challenged populations.

CMV is acquired most commonly early in life, during childhood to early adulthood, through exposure to saliva, tears, urine, stool, breast milk, semen, and other bodily secretions from infected individuals. The virus has been shown to retain viability for up to 6 hours on certain surfaces, and therefore, transmission via fomites is possible (5). It can also be transmitted efficiently via organ and tissue transplantation and blood transfusions (6–9); leukoreduction of blood products have markedly reduced the risk of transfusion-transmitted CMV infections (7, 9, 10).

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In the immunocompetent healthy host, primary CMV infection is usually asymptomatic, although it may also present as a nonspecific febrile illness, or an infectious mononucleosis-like syndrome characterized by fever, lymphadenopathy, and lymphocytosis (11). After a self-limited course, CMV establishes latency in a wide variety of cells, including endothelial cells, epithelial cells, smooth-muscle cells, and fibroblasts, where the virus can multiply and may be carried by peripheral monocytes and circulating endothelial cells to reach distant sites of the body (12). The initial infection leads to production of CMV-specific IgM and, later, IgG antibody that persists for life (13).

The clinical presentation of CMV infection is highly influenced by the immune fitness of the host (14). Its reactivation in healthy immunocompetent hosts, which occurs intermittently throughout life, triggers immunologic memory that leads to effective control of viral replication (15). On the other hand, the loss of CMV-specific CD4+ and CD8+ T-cells in the immunocompromised host, such as those with human immunodeficiency virus (HIV) infection, recipients of solid-organ transplant (SOT), or hematopoietic stem-cell transplant (HSCT), may permit uncontrolled viral replication, leading subsequently to clinical disease (16, 17) (Table 1).

CMV in Patients with AIDS

CMV disease in acquired immunodeficiency syndrome (AIDS) patients is most commonly manifested as sight-threatening retinitis, typically occurring when CD4+ T-cell count falls below 50 cells/mm^3 (18). It causes a complete-thickness infection of retinal cells, which if left untreated, results in a subacute progressive retinal destruction that leads to irreversible blindness. The diagnosis is made by ophthalmologic examination that reveals pathognomonic white fluffy infiltrates with or without areas of hemorrhage. The diagnosis can be confirmed by demonstrating CMV DNA using nucleic-acid test (NAT) of aqueous humor (19). CMV may also involve other organ systems, including the central nervous system (CNS) to cause polyradiculopathy and meningoencephalitis; the lungs leading to pneumonitis (often with coinfections with *Pneumocystis jirovecii*, *Aspergillus fumigatus*, or other pathogens); and the gastrointestinal (GI) tract causing esophagitis, gastritis, ileitis, colitis, pancreatitis, and hepatitis. All of these clinical syndromes have become less common in the current era of combination antiretroviral therapy that allows an almost complete recovery of immunologic function (as measured by CD4+ T-cells) (20, 21). However, opportunistic CMV infection and disease among HIV-infected patients continues to be a challenge in resource-limited areas, where the prevalence ranges from less than 5% to over 30% (22).

CMV in Solid-Organ Transplant Recipients

SOT recipients can develop either primary or secondary (reactivation) CMV infection and disease. Primary infection occurs when a CMV-seronegative (R-) individual receives an allograft from a CMV-seropositive donor (D+) (23). CMV-seronegative recipients lack pre-existing CMV-specific humoral and cell-mediated immunity and,

TABLE 1 Risk factors and clinical manifestations of cytomegalovirus disease in immunocompromised patients

Patient population	Major risk factors	Clinical disease
Patients with AIDS	CD4 count less than 50 cells/mm^3	CMV retinitis is the most common manifestation Other organ systems may be involved to cause polyradiculopathy, hepatitis, pneumonitis, gastrointestinal disease
Solid-organ transplant patients	Lack of pre-existing CMV-specific immunity in recipients Immunosuppressive drugs Donor transmission of CMV Allograft rejection	CMV syndrome of fever and myelosuppression Tissue-invasive disease, which is most commonly gastrointestinal CMV disease. Transplanted allografts are particularly at risk. Numerous indirect effects such as acute and chronic allograft rejection, increased risk of opportunistic infections, and mortality
Hematopoietic stem-cell transplant recipients	Lack of pre-existing CMV-specific immunity in donors and recipients Intensity of immunosuppression Degree of T-cell depletion Graft-versus-host disease Unrelated, umbilical cord, or mismatched donors	Fever Tissue-invasive disease, which is most commonly gastrointestinal CMV disease. CMV pneumonitis can be severe and fatal. Numerous indirect effects such as delayed engraftment, graft-versus-host disease, increased risk of opportunistic infections, and mortality
Newborns	Immature immune system	Congenital CMV disease manifested as cytomegalic inclusion disease and characterized by jaundice, petechial rash, microcephaly, hepatosplenomegaly, chorioretinitis, cerebral calcifications, hearing defects, lethargy, and seizures. Sensorineural hearing defect is most common.

in the presence of drug-induced immunosuppression, this results in the inability to control primary infection (8, 24). This CMV "donor positive-recipient negative" (D+/R-) mismatch constitutes the highest-risk scenario for CMV disease after SOT.

Secondary CMV disease may occur in CMV-seropositive (R+) SOT recipients, either as reactivation (of endogenous latent CMV in the recipient) or superinfection (with CMV transmitted by transplantation) (23). Allostimulation, allograft rejection, and intense pharmacologic immunosuppression, particularly with antithymocyte-immunoglobulin therapy, combine to provide an environment that permits CMV reactivation after SOT (25). During superinfection, the circulating CMV consists of both donor-transmitted and recipient-endogenous CMV, although some data indicate predominance of donor-transmitted strains (23).

CMV infection after SOT can be classified as asymptomatic (subclinical), whereby viral replication is detected in the blood in a patient without clinical signs and symptoms, or symptomatic CMV disease, which can be further categorized into CMV syndrome or tissue-invasive disease. CMV syndrome is characterized by fever, malaise, and some degree of myelosuppression. CMV could also invade various organ systems leading to tissue-invasive CMV disease. The most common organ affected by CMV is the GI tract, accounting for over 70% of tissue-invasive CMV disease cases. CMV has a predilection to infect the transplanted allograft, where it may manifest as hepatitis, pneumonitis, myocarditis, pancreatitis, or nephritis among liver, lung, heart, pancreas, and kidney recipients, respectively. Rarely, it can cause retinitis, meningoencephalitis, and polyradiculopathy after transplantation (23, 26).

CMV has numerous indirect effects after SOT, including an increased predisposition to develop other opportunistic infections, such as bacteremia, invasive fungal disease, and Epstein-Barr virus infection leading to posttransplant lymphoproliferative disease. CMV infection has also been associated with acute and chronic allograft injury, manifesting as allograft nephropathy, coronary vasculopathy, and bronchiolitis obliterans after kidney, heart, and lung transplantation, respectively (23, 27).

CMV in Hematopoietic Stem-Cell Transplant Recipients

Allogeneic stimulation and the use of immunosuppressive drugs to treat or prevent graft-versus-host disease after HSCT increase the risk of CMV disease. Allogeneic-HSCT recipients are at higher risk of CMV disease compared to autologous-HSCT recipients (28). Like SOT recipients, CMV in HSCT patients can either be a primary or reactivation disease. The clinical manifestations are similar to those of SOT patients, and can be classified as asymptomatic infection or CMV disease. In contrast to the SOT population, CMV syndrome is not a well-defined entity after HSCT since these patients often have underlying myelosuppression and elevated hepatic transaminases from multiple causes other than CMV. Allogeneic-HSCT recipients are particularly at high risk of CMV pneumonia, with an incidence of approximately 10% to 30%; the rate of CMV pneumonia is lower (1% to 6%) after autologous HSCT (29). On chest radiographs, CMV pneumonitis is typically manifested as diffuse interstitial infiltrates, although a nodular pattern may be observed. CMV pneumonia has an acute onset, rapid clinical course, and sometimes fatal outcome. Other organ systems may be involved, including the GI tract, liver, and the CNS. With aggressive surveillance and preemptive strategy, the overall incidence of CMV disease after HSCT has decreased to 5% to 8% (30, 31). Among the indirect effects of CMV after HSCT are delayed engraftment, higher incidence of bacterial and fungal infections, and graft-versus-host disease (32).

In contrast to SOT recipients, the risk for CMV infection and disease among HSCT recipients is highest with CMV R+ status, where the incidence approaches 70%, especially when the donor is seronegative (reverse D-/R+ mismatch) (33). Indeed, recipient CMV R+ seropositivity is perhaps one of the most important risk factors for CMV reactivation after HSCT, and it has been suggested as a marker for higher mortality (28, 34).

CMV in Newborns and Infants

CMV infection during pregnancy can lead to intrauterine fetal infection and congenital CMV disease. Congenital CMV infection occurs most commonly among infants born to mothers who developed primary CMV infection during pregnancy. In this situation, transmission of infection has been described to occur in approximately 40% of cases (35). The risk of transmission is highest if CMV infection occurs during the first half of pregnancy, although it may occur at any stage. Less commonly, transmission of CMV may occur among infants born to CMV-immune women, when the mother is superinfected with a different strain of CMV, since preconceptional immunity provides only partial protection (36).

Congenital CMV infection may manifest as clinical disease in only about 10% to 15% of cases. The clinical

manifestations can be mild, nonspecific findings to severe, multiple-organ system involvement. Congenital CMV disease has manifested as petechial rash, jaundice with hepatosplenomegaly, neurologic abnormalities such as microcephaly and lethargy, chorioretinitis and optic-nerve atrophy, and prematurity and low birth weight (37). About 10% to 15% of infants with congenital CMV disease may manifest solely with sensorineural hearing loss (38).

CMV in Other Immunocompromised Hosts

CMV disease may also occur in other immunocompromised patients, such as patients with leukemia, lymphoma, other malignancies, or those on immunosuppressive treatment. For example, CMV disease is common among patients receiving alemtuzumab for the treatment of lymphoma (39). The manifestations of CMV disease in this population are similar to those observed in transplant patients.

CMV colitis is increasingly recognized among patients with inflammatory-bowel disease such as ulcerative colitis, but it remains debated whether the virus exacerbates the disease or simply appears as a bystander of a severe underlying disease (40). It has been estimated that about 10% of patients with ulcerative colitis have CMV infection as diagnosed by histopathology (41). It is unclear which subset of patient would benefit from antiviral therapy, although treatment is suggested for patients with histologic evidence of CMV infection before attempting to enhance immunosuppression or consideration for colectomy (42).

THERAPEUTIC CONSIDERATIONS

Therapeutic Options

Intravenous ganciclovir and oral valganciclovir are the drugs of choice for the treatment of CMV disease (43), and reduction of immunosuppressive therapy should be considered, whenever possible (23). The duration of therapy should be guided by clinical and virologic response, as measured by CMV quantitative nucleic-acid tests (QNAT) or, alternatively, pp65 antigenemia on a weekly basis. Viral suppression has been shown to predict a successful clinical response, and should be achieved and maintained for at least 2 consecutive weeks prior to discontinuation of antiviral therapy (44). Persistent viremia after treatment correlates with disease relapse (45).

Foscarnet and cidofovir are considered second-line agents because of their associated toxicities and are used mainly when there is clinical and virologic failure of ganciclovir (23). For the purpose of treatment, ganciclovir should be administered intravenously. Oral ganciclovir should not be used for treatment of established disease since the oral drug has poor bioavailability and the systemic ganciclovir levels that are required to halt CMV replication are not achieved. Valganciclovir, the valine ester of ganciclovir, is available in oral formulation, has high bioavailability and attains high systemic levels, and can be used for mild to moderate disease (14, 43, 46). The major toxicity of ganciclovir is myelosuppression, and this usually resolves upon the discontinuation of the drug. The major limiting toxicity of foscarnet and cidofovir is nephrotoxicity, and this has relegated their use as alternative agents. Electrolyte abnormalities are common with foscarnet use.

Brincidofovir (CMX001) is a novel investigational oral prodrug that produces high intracellular levels of cidofovir diphosphate (47). It is currently undergoing evaluation for prevention of CMV infection in HSCT recipients. Diarrhea was the major limiting toxicity of brincidofovir, and notably, no myelosuppression or nephrotoxicity were observed (48). Another novel investigational drug, letermovir, acts via a new mechanism by inhibiting the UL 56 viral terminase. It has been evaluated in kidney-transplant recipients in a phase 2a study, and efficacy, safety, and pharmacokinetics were favorable (49). A randomized placebo-controlled trial to evaluate safety, tolerability, and antiviral activity of this drug in HSCT has recently been completed (clinicaltrials.gov NCT01063829). Future studies are needed to determine clinical utility of these drugs. Maribavir, another investigational drug with potent *in vitro* activity against CMV, was well tolerated but was not found to be adequate for the prevention of CMV disease in HSCT and liver-transplant recipients at high risk for CMV disease (50).

The role of IV immunoglobulin (Ig) or CMV Ig, as adjunct to antiviral therapy, remains unclear and debated, but it is recommended for use in patients with severe, refractory, and life-threatening disease, such as CMV pneumonitis (23).

LABORATORY METHODS FOR THE DETECTION OF CYTOMEGALOVIRUS

A variety of methods are available for the laboratory diagnosis of CMV (Table 2). Generally, the methods can be classified into nonmolecular and molecular tests (2). The nonmolecular techniques include (1) the isolation or growth of virus from blood, urine, or other body fluids (viral culture); (2) the demonstration of CMV-specific

TABLE 2 Laboratory methods for the diagnosis of CMV infection[a,b]

Method	Principle	Sample processing	Turn-around time	Results and clinical utility	Advantages	Disadvantages
Nonmolecular methods						
Serology	Detection of antibody against CMV (IgG, IgM)	Requires serum samples	6 hours	CMV-IgG indicates past CMV infection CMV-IgM implies acute or recent infection	Prognostication of patients prior to transplant Screening posttransplantation for evidence of infection Diagnosis of acute congenital CMV disease	May require paired acute- and convalescent-phase sera for complete interpretation Not helpful in immune-compromised patients who have an attenuated and delayed antibody production
Histopathology	Histologic detection of CMV-infected cells	Requires tissue specimens Microscopy	24–48 hours	Detection of CMV-infected cells indicates the presence of active tissue-invasive disease	Confirmatory test for tissue-invasive disease Highly specific	Need for invasive method to obtain tissue specimen
Virus cultures						
Tube culture	Viral growth and CPE	Cell-culture facility Light microscopy	2–4 weeks	Detection of characteristic CPE indicates presence of virus	Specific for CMV infection The viral isolate can be tested for phenotypic-susceptibility	Prolonged processing time is not clinically useful in real-time Poor sensitivity Requires viable CMV Very slow CPE
Shell-vial assay	Viral growth	Cell-culture facility Immunofluorescence detection	16–48 hours	Infectious foci detected by monoclonal antibody directed to immediate early antigen of CMV	Specific for CMV infection More sensitive and rapid than conventional tube cultures	Relatively low sensitivity compared to molecular methods Rapid decrease of CMV activity in clinical specimens
Antigenemia	Detection of pp65 antigen	Recovery of PMN within 4–6 hours Cytospin Light microscopy or immunofluorescence	6 hours	Number of CMV-infected cells per total (e.g., 5×10^4) cells evaluated Early detection of CMV replication	Rapid diagnosis of CMV infection Quantification used as guide for preemptive therapy	Subjective interpretation of results Requires rapid processing Not useful in leukopenic patients Lack of standardization
Molecular methods: nucleic acid tests						
Various assays (commercial or laboratory developed tests)	PCR (most common) and non-PCR amplification and detection of CMV DNA	Plasma, whole-blood, PMN samples Others (cerebrospinal fluid, bronchoalveolar fluid, vitreous fluid, others)	1–4 hours	Reported as CMV copies per ml of sample (lowest limit of detection varies by test) Rapid detection of CMV infection Monitor CMV DNA decline during therapy Surrogate marker for antiviral-drug resistance	Highly sensitive for CMV infection Highly specific for CMV infection Monitor response to therapy Rapid turn-around time	No widely accepted threshold for predicting CMV disease Standardization may help in this regard Quantitative ability is useful for prognosis, and assessing risk and disease severity Molecular target varies by assay, and can be DNA or RNA

[a]The examples noted in this Table are representative of multiple assays and are not intended to be comprehensive.
[b]CMV, cytomegalovirus; CPE, cytopathic effects; PMN, polymorphonuclear cells; PCR, polymerase chain reaction; DNA, deoxyribonucleic acid; RNA, ribonucleic acid.

IgG and IgM antibodies (serology); (3) the detection of virion components in leukocytes, such as the pp65 antigenemia assay; and (4) demonstration of characteristic nuclear inclusion-bearing cells (histopathology). Viral nucleic-acid amplification and detection (also termed NAT), most commonly using the polymerase-chain reaction (PCR), constitutes the major molecular method for CMV detection, although non-PCR molecular methods are also available.

Viral Culture

The detection of CMV by culture techniques, either through conventional-tube cell culture or shell-vial assay, was the primary laboratory method to confirm the clinical diagnosis of CMV for many decades (2). Clinical specimens, such as blood, urine, respiratory secretions, cerebrospinal fluid, or other body fluids, are inoculated into conventional-tube cell cultures, such as MRC-5 human embryonic lung fibroblasts. After incubation, the presence of cytopathic effects (CPE), which are indicated by large and rounded infected cells that contain cytoplasmic "ground-glass"-appearing inclusions, are observed (Fig. 1). Once CPE is observed, the identity of the specific viral isolate is confirmed by immunofluorescence with the use of specific antisera (such as antisera against CMV) (Fig. 2). Cell-culture assays are highly predictive of CMV disease and they are relatively specific, especially if a monoclonal anti-CMV antibody reagent is used (51–53). The major drawback of viral culture is its poor sensitivity. In a study of 47 liver-biopsy specimens obtained from patients with histopathologically proven CMV, the sensitivity of cell culture was only 52% (54). The slow growth of CMV in human-fibroblast cultures is another major limitation of culture. Conventional-culture techniques take at least a week and may take up to 4 weeks for CPE to be observed. The slow turnaround time and the poor sensitivity (even if highly specific) limit the clinical utility of conventional culture for diagnostic and therapeutic decision-making processes for a viral disease that could have rapid clinical progression.

FIGURE 1 Cytomegalovirus-induced cytopathic effects. Unstained preparation; 100X magnification.

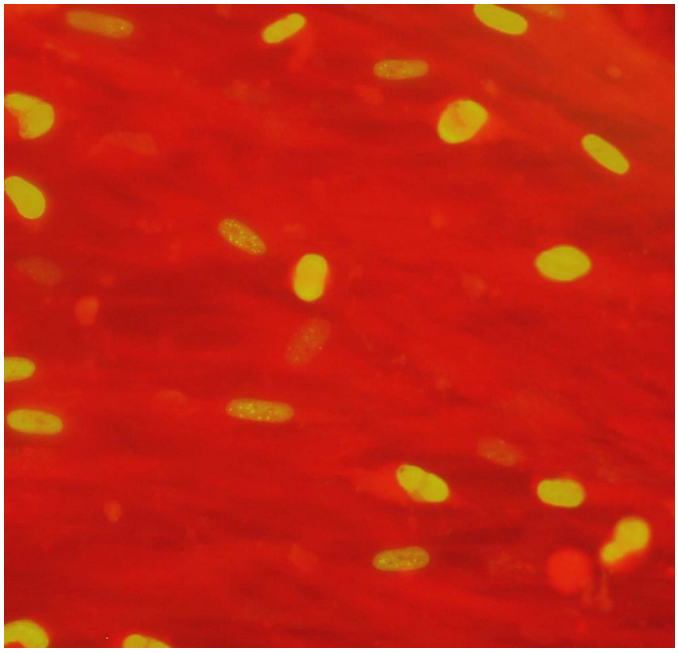

FIGURE 2 Detection of CMV antigens in the nucleic acid of infected MCR-5 cells. Following shell-vial culture, cells are stained with fluorescently-labeled antibodies which detect CMV immediate early antigen. Magnification 200X.

The shell-vial assay, which utilizes low-speed centrifugation and monoclonal antibodies directed against early antigens of replicating CMV, accelerates the viral-culture process (2, 52, 53). Some examples of techniques that detect CMV in shell-vial culture systems are direct and indirect fluorescent monoclonal-antibody staining and *in situ* hybridization with a biotinylated DNA probe or a horseradish peroxidase-labeled probe directly linked to a DNA molecule (55, 56). In comparative studies, the shell-vial assay was more sensitive than conventional cultures (51). However, when compared to antigenemia

and molecular assays (discussed below), the culture-based systems, including the shell-vial, generally suffer from a relatively poor sensitivity. The labor-intensive procedure, slow turn-around time, and poor sensitivity all combine to limit the use of viral culture in contemporary clinical practice. Accordingly, viral culture is no longer recommended as the first-line method for laboratory diagnosis of CMV infection, and has been supplanted mainly by more sensitive, rapid, quantitative, and real-time tests for this purpose.

Serology

The detection of CMV-IgG and -IgM antibody responses has been used to diagnose acute or previous CMV infection (2). In principle, an assay utilizes latex particles or beads that are coated with CMV antigens that are recognized by the CMV antibody present in a patient's serum or plasma. The bound patient antibodies are detected by antihuman immunoglobulins conjugated with horseradish peroxidase. Clinically, the presence of CMV-IgM or a ratio of ≥4 in paired serum CMV-IgG titers is indicative of acute or recent CMV infection. Detecting CMV-IgM in umbilical-cord blood suggests acute intrauterine CMV infection. However, the presence of CMV-IgM may not always be indicative of acute primary infection as some conditions may cause false-positive results. In addition, IgM may persist for months in some individuals, and it could also be produced during CMV reactivation. The acuity of CMV infection may be differentiated by the use of IgG-avidity testing, which distinguishes primary CMV infection from reactivation (57). In using this test, patients with low-avidity IgG are believed to have acute CMV infection. Low-avidity IgG persists for approximately 17 weeks following acute infection, and its full maturation takes approximately 25 weeks after the onset of clinical symptoms (58).

There are important limitations to the clinical application of serology for the diagnosis of acute CMV infection in immunocompromised individuals. First, there is a time lag between the onset of clinical illness and the appearance of CMV-IgM, and as a result, the diagnosis and treatment of primary CMV disease may be delayed if the diagnosis is based solely on the detection of CMV-IgM. Second, some individuals may have CMV-IgM antibody persist long after the resolution of acute clinical illness and thus, detection of CMV-IgM does not necessarily indicate active infection. Third, some immunocompromised individuals fail to develop serologic response or may have a markedly delayed or attenuated response (leading to false-negative results) (2). Hence, CMV-IgM and -IgG serology testing is not recommended for real-time diagnosis of CMV infection in immunocompromised individuals (2).

In immunocompromised individuals, particularly transplant recipients, the most important clinical use of CMV serology is in the assessment of risk and susceptibility after transplantation. Specifically, CMV serology is useful in assessing prior CMV exposure during the pretransplant evaluation of transplant candidates (and their donors) in order to determine the risk of either primary or reactivation CMV infection after transplantation. Likewise, serology is useful in assessing the CMV status of an individual with AIDS as it will guide clinicians in prognosticating an individual's risk of developing CMV retinitis or other CMV-related diseases when the level of immunodeficiency is severe (i.e., CD4 count <50).

CMV-Specific T-cell Assays

CMV infection elicits a strong virus-specific CD4+ and CD8+ T-cell response, and there is ongoing effort in assessing the utility of measuring cell-mediated immunity response to predict risk of CMV infection or disease after transplantation. There are numerous ways to define cell-mediated response to CMV, and these include peptide-major histocompatibility complex (MHC) multimer for identification and enumeration of CMV-specific T-cells, or functional assays such as enzyme-linked immunospot (ELISPOT) and flow-cytometric intracellular-cytokine staining, which enable the detection of interferon (INF)-γ (Quantiferon-CMV assay) or other cytokine-secreting cells in response to *in vitro* antigen stimulation (59, 60). Numerous studies have assessed assays of immune function to determine risk of CMV disease after transplantation. In one study, where CMV-specific cell-mediated immunity was assessed by the Quantiferon-CMV assay, there was a lower incidence of CMV disease among transplant patients with INF-γ secretion by CD8+ T-cells (61). Another study looked at the cell-mediated immunity shortly after the onset of CMV viremia, and found that transplant patients with CMV-specific T-cell immunity were associated with spontaneous clearance, while those lacking T-cells developed progression to clinical disease (62). Assessing cell-mediated immunity at baseline and over time may also be useful in predicting transplant patients at low, intermediate, or high risk of developing subsequent CMV disease after prophylaxis (63, 64). Similar observations have been reported in HSCT recipients, wherein a failure to reconstitute CMV-specific immunity soon after the onset of CMV viremia was associated with higher peak viral loads and a more complicated clinical course (65).

Antigenemia

Direct detection of CMV antigens in neutrophils using monoclonal antibodies against CMV matrix-protein pp65 is useful for the diagnosis of CMV in immunocompromised individuals (2). In this assay, cytospin preparations of a patient's peripheral-blood mononuclear cells are fixed and permeabilized, and the presence of CMV-specific matrix-protein pp65 is demonstrated by immunofluorescence, immunoperoxidase, and other antigen-detection methods (2). Various antigen-detection methods are commercially available and have comparable sensitivity, specificity, and performance characteristics (66). One example is the demonstration of CMV pp65-positive cells by immunoperoxidase staining with monoclonal antibody. Generally, the number of positive cells are counted and reported per fixed number of leukocytes (i.e., for every 50,000–250,000 leukocytes) on the cytocentrifuge preparation (67). The quantitative assessment of antigen-positive cells is a marker of the severity of CMV infection and thus assists clinicians in risk stratification (i.e., higher number of positive cells indicate higher risk of clinical disease progression) and in monitoring therapeutic responses (i.e., number of positive cells decline with effective antiviral therapy). Across many centers, the pp65-antigenemia assay is used to determine the risk of subsequent disease in transplant patients, to diagnose acute CMV infection in patients with AIDS, and to monitor response to antiviral treatment (2). Studies have shown that pp65 antigenemia is much more sensitive than shell-vial and tube-culture systems (68). Others have demonstrated that the performance of pp65-antigenemia assay is comparable to molecular methods (discussed below).

The major limitation of pp65-antigenemia assay is its lack of standardization across laboratories and its subjective and operator-dependent nature (2). The comparative evaluation of pp65 antigenemia among four laboratory sites, however, did not demonstrate significant differences in assay performance (66). Another limitation of this assay is the need to have sufficient numbers of neutrophils so that the test can be performed. This may not always be possible in some individuals, such as leukopenic patients undergoing myeloablative and cytotoxic chemotherapy, including HSCT recipients. Moreover, since the assay detects virus-associated cells, the presence of free viruses in biological fluids, such as plasma, is not detected. Some experts argue that the presence of virus in cell-free environment, such as plasma, is more indicative of active viral replication (69, 70). Another limitation of the pp65-antigenemia assay is the need to process the clinical specimens shortly after collection because it is dependent on the lifespan of neutrophils *ex vivo* (i.e., within 6–8 hours). This limits the utility of this assay in major referral laboratories (that processes samples shipped from distant sites). Hence, a long hands-on time, the subjective nature of quantification, the need for adequate number of cells, and the immediate sample-processing time, combine to make CMV pp65-antigenemia assay a less useful test for many referral laboratories that perform large-scale testing of clinical samples (71).

Molecular Methods for CMV Detection—Nucleic Acid Testing

NAT has emerged as the preferred method for the rapid diagnosis of CMV in immunocompromised hosts. In principle, the NAT assays are based on the detection and/or amplification of CMV nucleic acids in clinical samples. However, CMV persists in latent form in many nucleated cells; therefore, NAT has the risk of detecting inactive nonreplicating CMV. Generally, molecular methods have higher sensitivity than nonmolecular methods. Among them, CMV PCR is the most widely used methodology. The basic principle of the CMV PCR is to generate a large number of target CMV gene-sequence copies that can be easily detected. The amount of CMV DNA theoretically doubles for every PCR cycle, resulting in an exponential increase in the quantity of amplified DNA. The amplification process, combined with its ability to detect nucleic acids from nonviable virus (hence, it can be useful during the course of antiviral therapy), markedly increases its sensitivity compared to nonmolecular methods. Molecular assays have the ability to detect infection even at early stages, when the viral burden is still small, and even prior to antibody production (i.e., serologic conversion) (72). In the past, the major drawback to molecular assays is the potential for contamination. Amplified large volume of nucleic acids may spill over into the laboratory work space and equipment leading to potential contamination of uninfected clinical samples. This limitation has been addressed with closed PCR systems, and is no longer a major concern in most laboratories.

In most CMV NAT assays, the target is DNA, although a few have been developed to detect RNA through reverse transcriptase-PCR (2, 73). Studies have demonstrated that NAT detecting CMV DNA is highly sensitive for CMV infection (2, 72). CMV DNA is stable in clinical specimens over time, and delayed sample processing has not been associated with any major impact on CMV-DNA concentration (74). One study demonstrated the stability of the CMV DNA in

ethylenediaminetetraacetic acid (EDTA)-blood sample that had been stored at 4°C for 14 days (75). While it is a highly sensitive indicator for CMV in clinical samples, the detection of CMV DNA is a relatively less-specific indicator (compared to RNA testing) of active CMV replication; a highly sensitive CMV-DNA test may detect inactive latent viral DNA (72). Several CMV DNA targets have been used in various NATs, including DNA-polymerase gene and glycoprotein B gene, among others. The amplification efficiency of these DNA targets varies, resulting in noncomparable viral load results (76). The concern for detecting latent CMV DNA has led to the development of assays that detect viral RNA targets. Because RNA intermediates are produced mainly during CMV replication, and serve as the biologic link between the CMV genome and gene expression, their detection is more specific and indicative of active CMV infection (77–81). Reverse-transcriptase PCR is the method used to selectively detect viral mRNA transcripts in blood and other clinical specimens. However, RNA molecules are readily degraded, and their degradation *in vitro* can lead to false-negative results (80, 81). Hence, compared to NAT detecting CMV DNA, the sensitivity of CMV RNA testing is lower (73).

NAT can be a qualitative (reported as positive or negative) or quantitative (reported as amount of virus, typically normalized to volume of input specimen) assay. Qualitative CMV-DNA tests are highly sensitive for the diagnosis of CMV infection (2, 82). However, the specificity of qualitative NAT is modest, and its positive-predictive value is low compared to quantitative assays (47% versus 68%) (83). Qualitative CMV-DNA tests do not reliably distinguish latent DNA from active viral replication, and are not able to stratify the severity of active infection (2, 82). Qualitative tests have limited clinical utility in real-time monitoring of antiviral-treatment responses (2, 23, 82). To improve the clinical utility and increase the specificity of CMV-DNA tests, quantitative NATs (QNATs) have been developed, which commonly report results in absolute values per volume of specimen or per PCR reaction (2). Quantitation of CMV DNA has allowed the correlation of disease and infection severity with the degree of viral replication (viral load) (2, 23, 24, 45, 84–87). Active CMV disease is indicated by high-absolute viral-load values or a rising trend in viral load, while low-level viral load may indicate detection of latent viral DNA or subclinical infection (2, 23, 24, 45, 84–87).

NAT can detect CMV nucleic acid in various clinical specimens, although it is most commonly performed on blood samples (2, 75, 88–90). Different compartments of blood have been used, including unfractionated whole blood, or leukocyte preparations, plasma, and serum (2, 75, 88–90). Overall, studies have demonstrated that whole blood and leukocyte samples have the highest sensitivity for CMV-DNA detection, compared to plasma and serum (89, 91–94). Whole blood is easy to process since it does not require complex sample preparation compared to leukocyte subpopulations (89). Some have advocated whole blood for CMV-DNA detection due to its higher sensitivity and its ability to detect low-copy viral DNA. In a study that compared 170 plasma and whole-blood samples obtained from 61 transplant recipients, 14% of the samples had discordant results (positive viral load in whole blood, but negative in plasma) (93). The majority of the discordant samples were at the low viral-load copy levels, implying the higher sensitivity of whole blood in detecting low-level viral load (93). Some have suggested that using a highly sensitive sample can identify CMV disease in patients with low viral load, but specificity of low-level CMV DNA in whole blood for predicting CMV disease is only modest (as some may detect latent virus). Moreover, many patients with low viral-load values may have transient viremia that resolves spontaneously, and their detection may lead to unnecessary treatment. The use of highly sensitive whole-blood PCR tests may also lead to a longer course of antiviral therapy since treatment is usually continued until CMV DNA is undetectable (93). Because latent CMV may be detected and amplified in leukocyte-containing blood samples, the use of cell-free plasma or serum has been advocated by some as more indicative of active CMV infection (95–97). Several studies have shown a direct correlation between CMV infection and the viral load in cell-free serum (95, 96) or plasma (89, 91, 98, 99), but to date, there is no consensus on which blood compartment to use.

NAT can be performed on cerebrospinal fluid (CSF) for the diagnosis of encephalitis, meningitis, polyradiculopathy, and other neurologic illness. CSF is a relatively acellular specimen and the detection of CMV DNA, either by qualitative or quantitative assay, is highly suggestive of CNS disease (100–102). However, there should be cautious interpretation of CMV DNA result using a qualitative test, since significant pleocytosis (from inflammatory causes other than CMV disease) may result in falsely positive results due to detection of latent CMV in CSF leukocytes. CMV NAT on aqueous- and vitreous-humor fluid may be needed to confirm the diagnosis of CMV retinitis. A detailed funduscopic examination by an

experienced ophthalmologist can reliably diagnose CMV retinitis, which is characterized by retinal hemorrhages with a whitish granular appearance to the retina, even in the absence of NAT testing. However, the detection of CMV DNA in aqueous and vitreous fluid in these patients confirms the clinical diagnosis (103, 104). The detection of CMV in bronchoalveolar lavage (BAL) fluid has been proposed for the diagnosis of CMV pneumonia (105–111). However, shedding of CMV in saliva and respiratory secretions is not uncommon, and the demonstration of CMV DNA in these respiratory samples in the absence of compatible clinical signs and symptoms (or in the absence of biopsy confirmation) is of unclear significance (and may represent shedding or contamination), and does not necessarily indicate CMV pneumonia (107). However, a study of 76 simultaneously collected BAL and throat-wash samples from lung-transplant recipients indicates that such contamination is unlikely, and that demonstration of CMV DNA in the BAL fluid is highly representative of virus replication in the lung (110). In the presence of compatible clinical symptoms, the demonstration of CMV DNA in BAL may be helpful and may obviate the need for risky lung biopsy in certain situations. In a study of 27 lung-transplant recipients, those with CMV load higher than 500,000 copies/ml of BAL fluid was highly correlated with biopsy-proven CMV pneumonitis (107). CMV can also be detected in urine and stool, although these are generally not recommended as samples for CMV-disease diagnosis (23, 112, 113). Approximately 50% of transplant recipients excrete CMV in body secretions, such as urine and stool, at some stage after transplantation (114).

Until recently, the major drawback of CMV NAT was the lack of standardization. Different molecular assays amplify different targets and use different types of samples, reagents, and platforms, thereby limiting direct comparison of results (76, 84). The vast majority of CMV NAT assays were developed in-house (laboratory-developed tests [LDTs]). LDTs are developed, optimized, and validated by each performing laboratory, and each has unique assay characteristics, such as the upper and lower limits of detection, linear range of detection, precision, and accuracy. The protocols for CMV NATs differ in many other aspects, including specimen types (blood, urine, CSF, BAL fluid, others), blood-sample preparations (whole blood, plasma, serum, leukocytes), nucleic acid-extraction methods, primers and targets (various CMV genes [DNA-polymerase gene, glycoprotein B gene, immediate-early gene, major immediate-early gene, UL83, others], DNA versus RNA), quantitation standards and controls (versus qualitative assays), reaction and amplification protocols (e.g., number of cycles), signal-generation systems, and methods for calculating copy numbers and reporting of results (76, 84). In other words, all available CMV NAT assays were not created similarly, and their results are not interchangeable in the absence of standardization. Numerous investigators have highlighted the disparity of various CMV-DNA tests, as exemplified by a few studies discussed here (2, 88, 115, 116). A comparative study of TaqMan-based assay and another real-time commercial PCR assay (COBAS AMPLICOR CMV Monitor [Roche]) in 27 kidney- and liver-transplant patients demonstrated that, while results of the two assays were highly correlated, the TaqMan assay was more sensitive (92% versus 80% detection of all positive samples), and yielded higher viral-load results (117). Another study compared the commercial COBAS AMPLICOR CMV Monitor (targeting CMV DNA-polymerase gene) and a LDT using LightCycler (targeting glycoprotein B gene) and observed that viral load values from the LightCycler assay were significantly higher (118). In contrast, another group of investigators compared the same PCR systems, but used a different target for the LDT LightCycler system, and they observed higher viral-load values with COBAS AMPLICOR CMV Monitor (88). The significant inter-assay and inter-laboratory variability in viral-load detection and reporting was highlighted by recent multicenter trials that compared various CMV NATs (76, 119). In a multicenter study conducted across 33 laboratories in Europe and North America, variability in viral-load results for individual samples ranged from 2.0 $\log_{10}$ copies/ml to 4.3 $\log_{10}$ copies/ml (119). Likewise, another multisite assessment of CMV NATs in 23 laboratories (including 22 which used LDTs on a wide variety of platforms) showed significant inter-assay quantitative variability in viral-load reporting (120). Ten of the laboratories reported viral-load values that were significantly different compared to the expected values (with bias ranging from -0.82 to 1.4 logs) (120). These studies indicated that standardization of NAT methodologies and the presence of a common CMV-DNA reference standard are needed to allow laboratories to achieve comparable numeric results (119, 120).

Based on the findings that the variability in viral-load reporting is due largely to the variability in calibration standards, two reference materials have been made available for use—the World Health Organization (WHO) released the first International Reference Standard (NIBSC 09/162) (121) and the United States National Institute of Standards and Technology Standard Reference Material (SRM 2366) (122). Availability of

these standards should allow common calibration of commercially developed CMV NAT and LDTs. Several LDTs and commercially available CMV NATs have been calibrated to these standards, including the COBAS AmpliPrep/COBAS TaqMan CMV Test (CAP/CTM CMV Test, Roche Molecular Diagnostics) (44). In addition, secondary commercial standards are also available for clinical use, with varying degrees of agreement and commutability (123). In a study that compared the performance of the CAP/CTM CMV Test across five centers in the United States and Europe, there was high level of quantitative agreement in the reported viral load across different test centers (84). However, there was high quantitative variability observed for the CAP/CTM CMV test at the lower viral-load values, at or near the lower limit of detection (i.e., 2.8 $\log_{10}$ copies/ml) (84). The most stable viral-load results, with lowest interlaboratory variability, were those within the middle range of the assay (84). Another study compared the performance of an LDT and a commercially available RealTime CMV assay (Abbott) in 513 samples obtained from 37 transplant patients (124). There was significant correlation between the two assays, but despite standardized reporting in international units using the WHO reference standard, there were discordant results in 23% of samples (positive by the Abbott assay and negative by the LDT). These studies emphasize that even in the presence of an international reference standard, there are still potential differences in the viral-load test results based on other variables, such as the assay's performance characteristics and limits of detection (124). Indeed, while the availability of the international reference materials may significantly harmonize viral-load reporting (i.e., in IU/ml), there remains assay-specific variability due to other differences in test characteristics. Differences in nucleic acid-extraction methods, type and volume of clinical samples, selection of primers and probes, target-specific amplification efficiencies, detection chemistries and reagents, instrumentation, and operator-dependent variability, may independently or collectively account for assay-specific variability. Furthermore, the lack of commutability when using the WHO standard with various assays suggests the need for further work into the attainment of true consensus (123). Thus, standardization of quantitative calibrators, while a great step forward, has not completely eliminated the variability of viral-load results. Other issues will need to be addressed over time to ensure uniformity in CMV viral-load reporting, including preanalytical conditions, such as specimen selection and volume, collection, storage, and transport (76). Several other test characteristics are important to consider, including the upper and lower limits of detection and quantification (i.e., the highest and lowest concentration of DNA that can be detected and quantified in 95% of replicates, respectively), linear range, precision, and accuracy. Finally, variations in patient populations being studied and their level of immunosuppression may account for viral-load variability, and thus, viral-load threshold recommendations may need to be specific for every organ transplant, risk strata, and level of immunosuppression (23).

Susceptibility and Resistance Testing

The two general methods for testing the susceptibilities of viral isolates to antiviral drugs are genotypic and phenotypic methods. The standard phenotypic method for detection of antiviral-drug resistance is the plaque-reduction assay (125). This method requires a lengthy viral-propagation process in order to obtain sufficient infectivity. Cell monolayers are inoculated with a number of infected cells in a medium containing varying concentrations of antiviral drugs such as ganciclovir. After a period of incubation, the 50% inhibitory concentration (IC_{50}), which is the concentration of drug producing a 50% reduction in the number of plaques, is determined. For ganciclovir, an IC_{50} value $\leq$6 μM indicates sensitivity, while >6 μM indicates resistance. The slow growth of cell-associated clinical isolates and the subjectivity of the assay limit its value for real-time therapeutic decisions. Moreover, certain genotypic mutations confer replication inefficiency, thereby slowing the diagnostic process (126, 127). Other phenotypic assays, which require less time, include detection of the CMV immediate-early antigen by flow cytometry and detection of viral DNA by DNA hybridization. Nonetheless, these assays still require weeks of cell culture to produce the initial CMV inoculum for the assay. Hence, phenotypic assays do not generally offer a rapid turnaround time for real-time guidance in the clinical management of patients (125).

The most common method for testing drug susceptibility is genotypic testing. Genetic mutations that confer resistance to one or all of the three currently available antiviral drugs (ganciclovir, foscarnet, and cidofovir) have been described. In general, and compared to the phenotypic testing, genotypic assays provide a rapid measure that could guide clinical decision-making process. Genotypic methods use PCR primers that are designed to amplify the region containing the drug-resistance mutations. PCR products are subsequently sequenced and analyzed to identify mutations asso-

ciated with drug resistance. Currently, CMV strains that are resistant to ganciclovir, foscarnet, and cidofovir exhibit mutations in the UL97 (ganciclovir resistance) and UL54 genes (ganciclovir, foscarnet, and/or cidofovir resistance) (128). The CMV UL97 gene is an important viral target for genotypic assays because all documented mutations conferring ganciclovir resistance have been found at one of three sites within the coding region for the C-terminal half of the phosphotransferase. Most commonly, CMV UL97 mutations associated with ganciclovir resistance are observed at codons 460 and close to 600 (129). Studies comparing genotypic and phenotypic studies suggest the higher sensitivity of genotypic assays. In one study, genotypically detected ganciclovir resistance always preceded phenotypically detected resistance (126).

Histopathology

For the diagnosis of tissue-invasive disease, examination of biopsy specimens for the presence of CMV and its tissue damage is essential. The specimens are obtained with the use of fine-needle aspiration or through open-surgical approaches. The most common specimens in the evaluation of tissue-invasive CMV disease are lung, GI, and liver tissues. The choice of tissue specimen is dictated by clinical presentation of the patient. For example, when a patient presents with diarrhea and abdominal pain, upper GI endoscopy and colonoscopy are performed to examine for the presence of characteristic mucosal ulcers, hyperemia, and inflammation associated with GI CMV disease. At the same time, GI tissue is obtained to demonstrate the presence of tissue-invasive CMV disease. The histologic criteria for the diagnosis of tissue-invasive CMV disease vary widely from the demonstration of viral-inclusion disease to the demonstration of CMV-specific antigens or DNA from tissue specimens by *in situ* techniques. Biopsy specimens may be stained with hematoxylin-eosin stain to evaluate histological alterations and to demonstrate the presence of giant cells with typical intracellular viral inclusions (130) (Fig. 3). Immunohistochemical studies may be performed to detect CMV antigens in tissue specimens (Fig. 4). For example, the presence of viral antigens in biopsy specimens may be demonstrated by indirect immunoperoxidase staining with monoclonal antibody against CMV antigens, such as the viral matrix-protein pp65 (67). To confirm the presence of CMV in tissue, CMV DNA may be demonstrated by *in situ* hybridization such as with the use of biotinylated DNA-probe or CMV PCR done on formalin-fixed, paraffin-embedded tissue samples (55, 67, 131, 132).

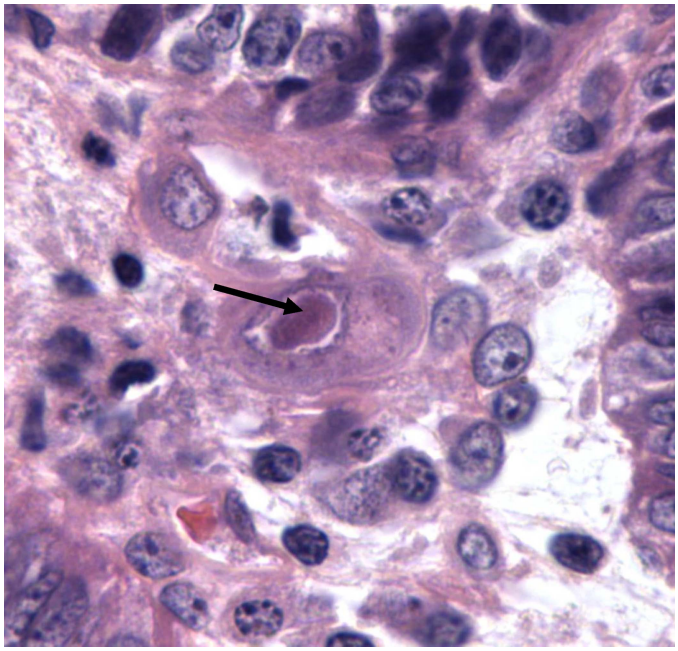

FIGURE 3 This biopsy specimen from a patient with cytomegalovirus colitis shows a classic "owl-eye" intranuclear inclusion (arrow) and intracytoplasmic inclusions. The dense intranuclear inclusion with surrounding halo is formed when the mass of viral particles shrinks away from the nuclear membrane during fixation. While herpes simplex virus intranuclear inclusions can have a similar appearance, CMV is the only member of the herpesviridae family that contains both intranuclear and intracytoplasmic inclusions. Hematoxylin and eosin stain, 1000x oil immersion.

GOALS AND ALGORITHMS OF LABORATORY TESTING FOR CYTOMEGALOVIRUS INFECTION

The various assays for CMV detection, as discussed above, can be used in the different aspects of CMV management in the immunocompromised patient. Specifically, these assays may be utilized for surveillance and screening, for prognostication and assessment of risk, for rapid and accurate diagnosis, for evaluation of therapeutic response, for assessment of the risk of relapse, and for the detection of antiviral-drug resistance. An illustration of the utility of the various assays in the management of CMV disease in immunocompromised hosts is depicted in Fig. 5–7.

Screening, Surveillance, and Prevention

One of the most common indications for laboratory testing of CMV is the assessment of disease risk. Depending on the clinical situation, serology, antigenemia, and molecular testing may be utilized. A typical situation for a transplant recipient is depicted in Fig. 5. CMV

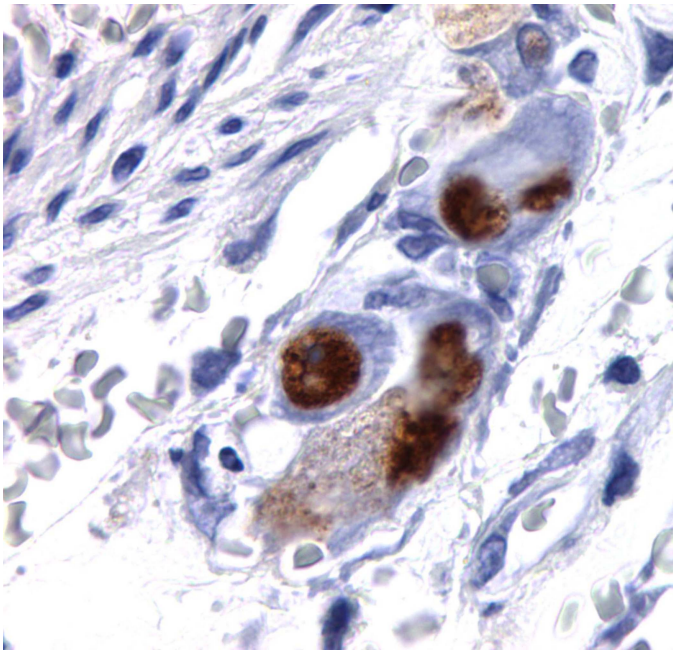

FIGURE 4 Immunoperoxidase staining of CMV antigens in a biopsy from a patient with CMV colitis. Viral inclusions stain brown (blue counterstain, 1000x oil immersion).

serology is used to determine the risk of primary infection in pregnant women or of reactivation in AIDS patients. In the field of transplantation, CMV serology is performed on blood samples from the prospective organ donor and recipient to determine the risk of primary infection or reactivation of disease. The knowledge of these risks influences the type of preventive efforts for each patient population. Specifically, a CMV D+/R- mismatch SOT recipient has the highest risk of primary CMV disease, and therefore may benefit from antiviral prophylaxis or aggressive CMV surveillance and preemptive therapy. Some centers have also used serology to determine ongoing risk of primary CMV disease after transplantation since seroconversion would suggest protection from subsequent CMV disease (133).

Serial surveillance for CMV replication, with the use of antigenemia or NAT, is a common practice during the early period after transplantation. This diagnostic approach is an integral component in the strategy of preemptive therapy against CMV disease (Fig. 6). Using this strategy, blood samples from transplant patients are collected on a weekly basis during the first 12 weeks after transplantation and tested for the presence of CMV, either by NAT or pp65 antigenemia. If CMV is detected above a predefined threshold associated with high risk of subsequent CMV disease, antiviral treatment is initiated preemptively to stop its progression to clinical disease. The success of this preventive approach is highly dependent on the performance characteristics of the diagnostic test, which should ideally be highly sensitive, highly specific, and offer high predictive characteristics. In this context, both the PCR and antigenemia assays have been tested and compared. Currently, however, the choice of which assay (PCR versus antigenemia) to use is much debated, and should be guided by the resources available in every center (66, 88, 134–137). Both pp65 antigenemia and molecular methods have demonstrated outstanding clinical utility in detecting CMV and in guiding preemptive therapy (82, 137). It is generally recommended that each center optimize their diagnostic approach according to their clinical practices and patient population. The main advantages of the molecular assays are the sensitive detection of CMV with quantitative results, rapid turn-around time, and the ability to perform the assay on stored and shipped specimens with potentially nonviable virus. The pp65 antigenemia, on the other hand, also provides rapid measure of viral replication; however, it is labor-intensive, limited by subjectivity, and it requires immediate specimen processing, and thus would be logistically difficult for samples that will need shipping (such as in major referral laboratories).

Prognostication and Risk Assessment

In immunocompromised hosts, such as transplant patients and those with AIDS, CMV pp65 antigenemia and NAT have been used to predict the development of CMV disease (138). Generally, a higher degree of CMV replication (as measured by the viral load) translates to a higher risk of progressing to clinical disease. However, a widely applicable clinically relevant CMV threshold that highly predicts the development of CMV disease is not defined. This difficulty in defining a widely applicable viral-load threshold is due to the variability of the different assays, clinical samples, and patient populations and their immunosuppressive regimen. In this regard, the kinetics of CMV replication for an individual patient measured on a weekly basis (i.e., trends in viral load) may be a better and more clinically useful measure for predicting CMV disease than the absolute number (86, 88, 90).

Diagnosis of CMV Infection

The diagnosis of CMV disease in the immunocompromised host requires the presence of clinical signs and symptoms and the demonstration of CMV in clinical specimens (Fig. 7). Traditionally, viral culture was the standard assay for the laboratory diagnosis of CMV infection. However, the poor sensitivity and slow turn-

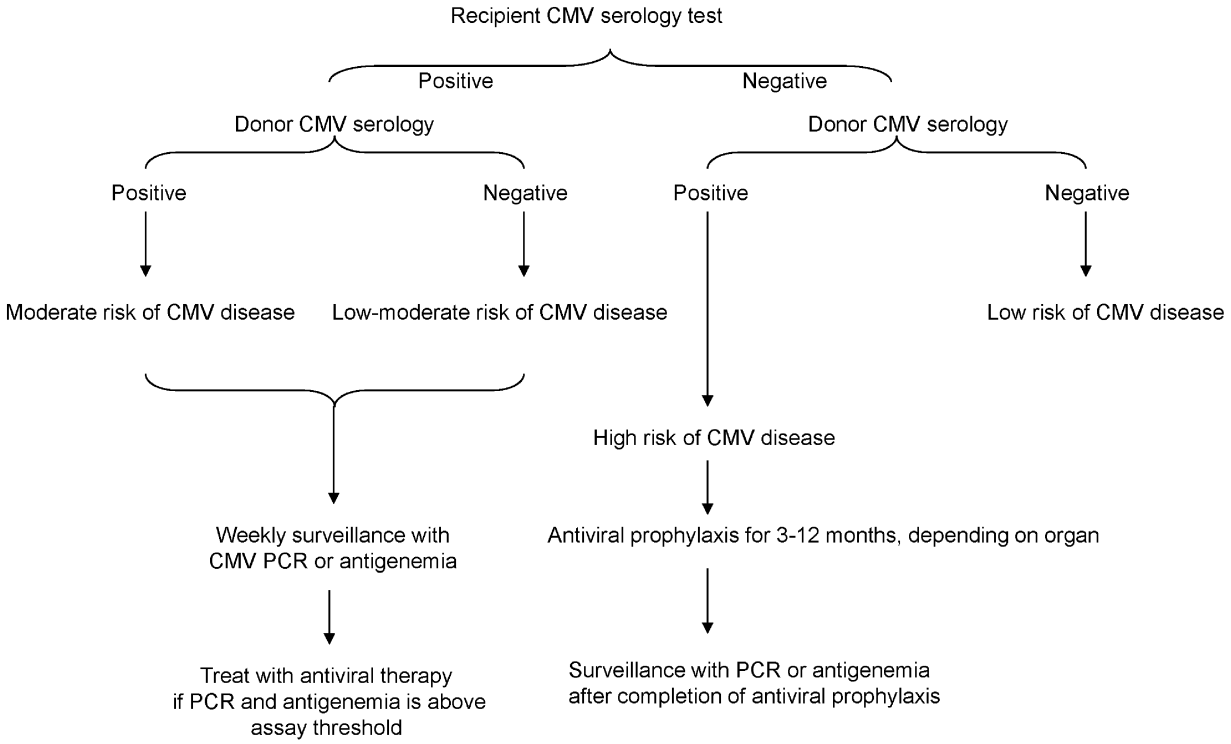

FIGURE 5 Diagnostic algorithm for transplant recipients and their donors with the use of CMV serology.

around time of viral-culture systems limited its clinical utility and led to the adaptation of more rapid and sensitive tests, such as the pp65 antigenemia and molecular assays (2). For example, molecular assays, such as PCR, are considered the method of choice for the detection of CMV in CSF of patients with CMV encephalitis or polyradiculopathy (139, 140). Congenital CMV infection can also be identified by testing for CMV DNA by PCR in neonatal dried blood on filter paper (known as Guthrie cards) (141). In one study, this test was found to be 100% sensitive and 99% specific for the diagnosis of congenital CMV infection in symptomatic and asymptomatic babies (141, 142). On the other hand, CMV serology is not usually used for the diagnosis of acute CMV in immunocompromised hosts. A negative CMV-serology assay does not completely rule out primary CMV infection. For example, CMV-IgM is not detectable in 10% to 30% of cord-blood sera from infants with CMV infection in the first week of life. Likewise, approximately 25% of pregnant women with primary CMV infection do not have detectable CMV-IgM within 2 months after onset of infection.

Monitoring Therapeutic Response

The quantitative results generated by pp65 antigenemia and the various molecular tests has allowed for their use to monitor for response to treatment, to individualize and assess duration of antiviral treatment, and to determine the risk of disease relapse. For example, the presence of detectable virus at the end of antiviral treatment indicates a higher risk of CMV disease relapse in transplant recipients (45, 88, 133, 143, 144). Certain observations with the molecular tests could indicate the emergence of a viral strain with drug resistance; for example, a rising viral load or its nondecline during antiviral treatment suggests a potentially drug-resistant CMV infection (2). Kinetics of viral load differ between those immunologically naïve to CMV versus those with immunity, with a significantly slower viral growth rate and viral doubling time in the latter group (24). In addition, viral-load decline after initiation of treatment may be slower in those with relapsed disease (87).

CLINICAL APPLICATION AND INTERPRETATION OF DATA

Cytomegalovirus in Patients with AIDS

CMV retinitis

CMV retinitis is diagnosed based on findings of characteristic fluffy yellow-white retinal lesions, with or without intraretinal hemorrhage on funduscopic examination, and has a 95% positive-predictive value in the

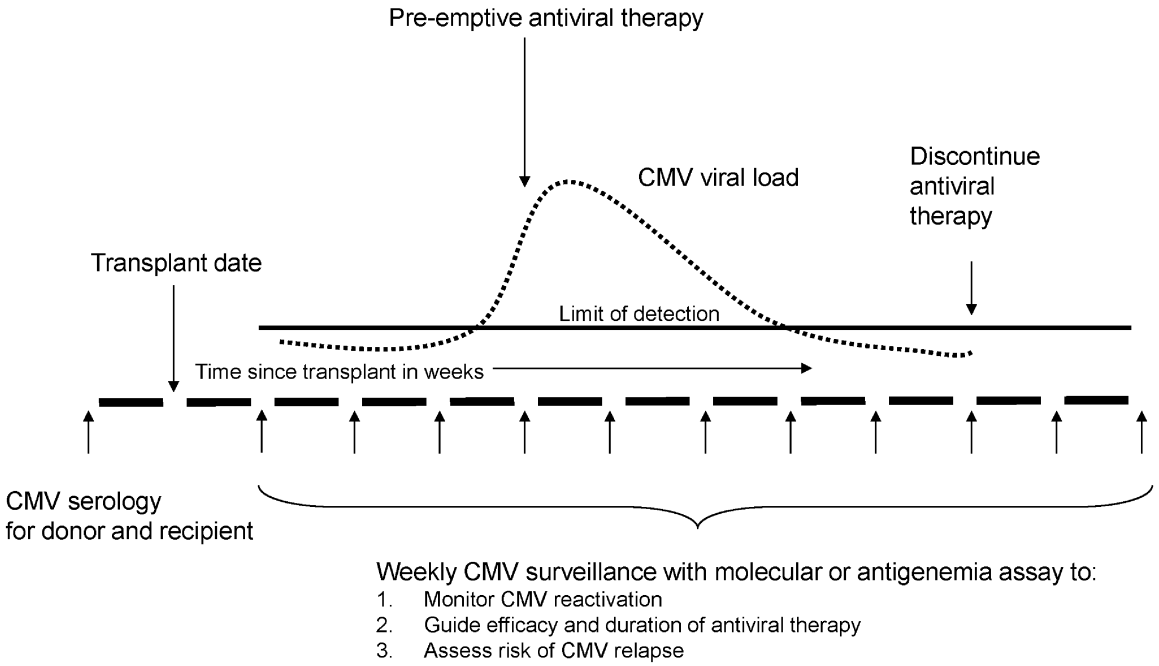

FIGURE 6 An illustrative algorithm utilizing various diagnostic assays in a transplant recipient prior to transplantation and during the period following transplantation.

hands of an experienced ophthalmologist. Therefore, based on this ophthalmologic finding alone, one can initiate anti-CMV therapy. If one needs to confirm the diagnosis, CMV can be demonstrated in aqueous humor either through viral culture or CMV PCR. In a study that compared CMV DNA in the aqueous humor of patients with active and inactive retinitis, CMV DNA was detected in 37 of 42 eyes (71%) with active CMV retinitis, but not in the eyes with inactive CMV retinitis. Following treatment with intravitreal ganciclovir, a decline in CMV DNA was observed in 29 of 37 eyes (78%) with active CMV retinitis. Hence, CMV-DNA detection in aqueous humor can be useful in differentiating active from inactive CMV retinitis, and in monitoring therapeutic response (145). Performing CMV PCR in blood may be used to predict, preceding by several months, the subsequent development of CMV retinitis (138, 146). The positive-predictive value of CMV PCR was 60% (138). Hence, serial monitoring of CMV DNA in the blood of patients at risk of CMV retinitis, such as those with CD4+ T-cell count <50 cells/mm^3 and high HIV viral load, could signal the need for preemptive therapy (138, 147). However, the impact of such approach in preventing CMV end-organ disease has been conflicting, and this approach is currently not encouraged (148, 149). Patients with CMV retinitis will have CMV DNA detected in blood in only 70% of cases, suggesting compartmentalized and localized disease (146). Treatment can be done with intraocular administration of antiviral agents, such as ganciclovir and foscarnet, in addition to systemic antiviral therapy. Systemic therapy is needed because of the multisystem nature of the disease and to reduce contralateral-eye involvement (150).

CMV polyradiculopathy and ventriculoencephalitis

Molecular-diagnostic assays offer a very sensitive and specific method for detecting CMV in CSF. These molecular tests are now considered standard for the diagnosis of CMV in the CNS (139, 140, 151). CMV polyradiculopathy is suspected in patients with low-back pain, urinary retention, progressive bilateral leg weakness, and a CSF characterized by high protein, low glucose, and lymphocytic pleocytosis. Quantitation of CMV DNA in CSF may be helpful in confirming the diagnosis, in evaluating disease severity, and for monitoring therapy (139, 140). Higher viral load in the CSF generally indicates a more severe disease. The sensitivity of PCR is over 90% for diagnosis of neurological CMV disease (139, 140).

Gastrointestinal CMV disease

The GI tract is the most common extra-ocular site of CMV infection in AIDS patients (152). The clinical presentation of GI CMV disease, which depends on the site of infection, could be painful esophageal ulcers or extensive enterocolitis, and may present as an acute

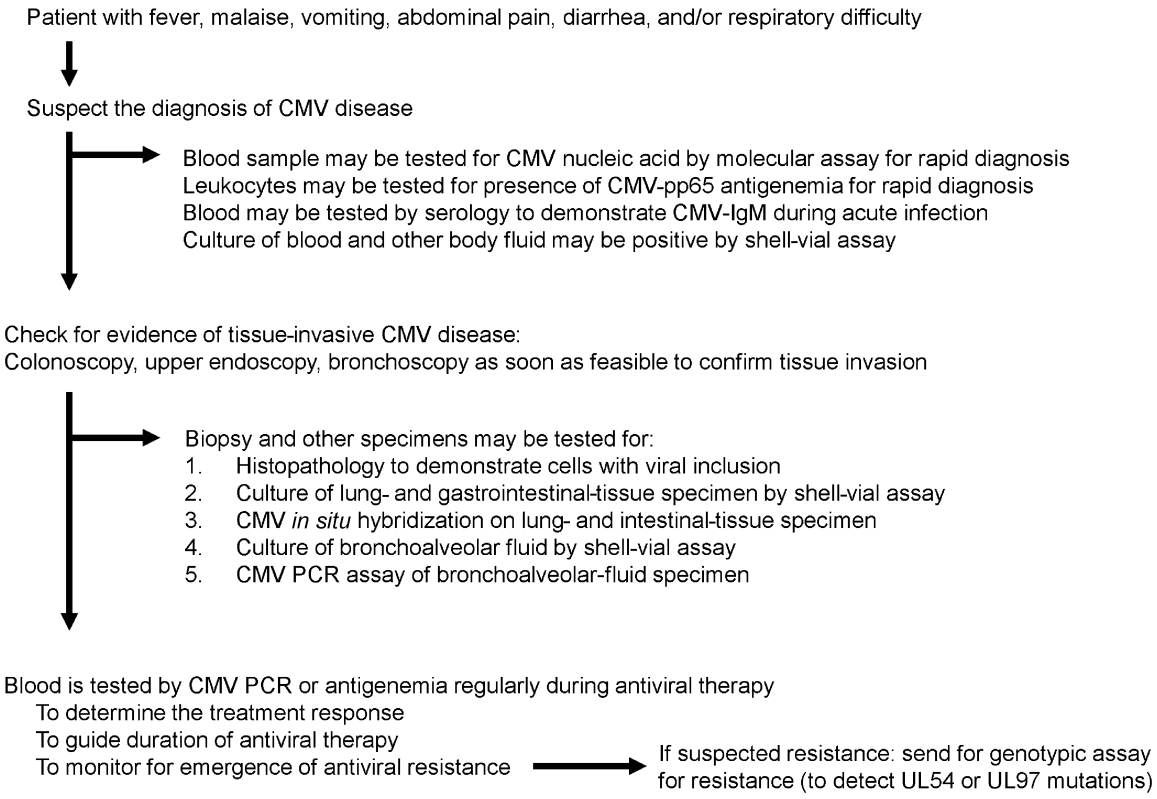

FIGURE 7 Algorithm for the diagnosis of CMV disease in an immunocompromised patient.

abdomen due to perforation. While CMV can often be detected in the blood of patients, this is not always the case, and thus, the diagnosis requires the demonstration of characteristic lesions on endoscopy and histopathology with cytomegalic inclusion, positive immunostaining, or *in situ* hybridization.

CMV pneumonitis

The diagnosis of CMV pneumonia is difficult to establish in AIDS patients because of the high prevalence of asymptomatic viral shedding, and frequent presence of other pulmonary pathogens. Isolation of CMV by culture or detection of CMV DNA from BAL fluid usually represents asymptomatic viral shedding, and should trigger a search for a more likely pathogen. If diagnosis is suspected, it should be confirmed by histopathologic exam of lung-tissue biopsy.

CYTOMEGALOVIRUS IN HEMATOPOIETIC STEM-CELL TRANSPLANT PATIENTS

Diagnosis of CMV Infection and Tissue-Invasive Disease

Molecular methods for CMV detection are currently the standard methods for laboratory diagnosis of CMV in HSCT recipients. CMV pp65 antigenemia is limited by the prolonged neutropenic phase of these patients; therefore, it has largely been replaced by NAT (153). Asymptomatic CMV infection is diagnosed by detection of CMV in blood in the absence of clinical symptoms. In some patients, CMV is accompanied by clinical manifestations of tissue-invasive disease. In the presence of cough and pulmonary infiltrates, demonstrating either intranuclear viral inclusions or detecting CMV by immunohistochemical staining or *in situ* hybridization in lung-biopsy specimens is the gold standard for diagnosis (154). However, obtaining tissue by biopsy is not always possible in HSCT patients who are often thrombocytopenic and at high risk of bleeding complications. Hence, measurement of viral load in BAL samples has been an attractive method, although this is not yet standardized and there are no defined viral thresholds for discriminating true disease from viral shedding (107).

CMV can affect any portion of the GI tract of HSCT recipients, and the clinical manifestations vary according to the site; odynophagia and retrosternal chest pain may be manifestations of esophageal disease; epigastric pain and nausea indicate gastric involvement; and diarrhea with abdominal pain should raise suspicion for intestinal disease. The definitive diagnosis of GI CMV disease

relies on histologic examination. Macroscopically, the mucosal lining may appear normal, or it may show scattered erosions or deep ulcerations with bleeding and inflammation (155). Detection of CMV DNA by PCR of tissue samples is not totally diagnostic, since it may represent viral shedding or latent virus. However, the gold standard for diagnosis relies on demonstrating tissue damage with cells that exhibit viral inclusions and with positive immunostains or *in situ* hybridization (154). Interestingly, GI involvement may not be accompanied by detectable CMV levels in blood; only about 50% of HSCT patients with GI involvement will have detectable viral replication (156).

CMV CNS disease is rare, but with a high mortality rate. It manifests as a ventriculoencephalitis or myelitis, and it is associated with prolonged, profound, and protracted T-cell immunodeficiency. Often, it occurs in patients with history of recurrent CMV viremia treated with multiple courses of antiviral therapy. Mortality may reach 100%, according to one report (157). Diagnosis of CMV CNS disease can be made by CSF analysis with detection of CMV DNA by PCR, compatible neurological symptoms, and characteristic changes in neuroimaging (139, 158). For retinitis, funduscopic examination that shows the characteristic fluffy yellow-white retinal lesions, with or without intraretinal hemorrhage, is the gold standard. However, detection of CMV DNA by PCR in vitreous fluid may be required for confirmation in cases with atypical clinical findings.

Surveillance and Preemptive Therapy

One of the most common clinical applications of the antigenemia and NAT in HSCT recipients is in the implementation of the strategy of preemptive therapy—an approach of CMV prevention whereby an antiviral drug is administered upon the detection of active CMV replication with the goal of halting its progression to clinical disease. For this to work, blood specimens should be processed immediately so that the results can be provided to the clinician in a timely manner, and the diagnostic surveillance should be performed frequently, which in most centers, is performed once or even twice weekly. Hence, the key to effective preemptive therapy is the availability of a rapid diagnostic test with high sensitivity and predictive qualities (7, 153, 159). In the current era, CMV QNAT has largely replaced pp65 antigenemia in this regard due to its higher sensitivity, faster turnaround time, and lack of limitation by neutropenia (160). Another major advantage of CMV QNAT is its quantitative nature, making it ideal for serially monitoring viral-load decline during antiviral therapy. One study comparing CMV QNAT and pp65 antigenemia showed similar rates of CMV disease prevention, without an increase in the proportion of patients receiving preemptive therapy or an increase in the risk of ganciclovir-related toxicities (31). The advantages of preemptive therapy over the other major approach for CMV disease prevention (i.e., antiviral prophylaxis) are the reduction in the number of patients receiving antiviral drugs, and hence, a reduction in the risk of antiviral resistance, adverse drug effects, and drug cost. CMV surveillance and preemptive therapy has been utilized by many centers; however, there is significant variability in CMV viral-load results among many QNATs, which hampers standardized recommendations for viral-threshold triggers for initiation and discontinuation of antiviral therapy (7, 119). For example, the viral-load threshold for preemptive therapy is 1,000 copies/ml of plasma in one center using a LDT, but is 10,000 copies/ml in another center using a different platform (161, 162). Standardization of QNAT should partly overcome this issue (163).

Monitoring Response to Treatment

The quantitative capability of molecular tests (QNAT), together with their sensitivity, makes them the preferred methods, over nonmolecular techniques (such as virus culture), for monitoring viral load during treatment of CMV disease in HSCT patients (7, 153). In a study of HSCT (and SOT) patients, there was a demonstrable decline in blood CMV-DNA level during intravenous ganciclovir treatment (144). The half-life of decline in CMV load (termed viral decay) was estimated to be 1–5 days (53, 86, 87, 144). The slope of decline correlated with therapeutic efficacy (144). Longer duration of CMV-DNA decline and longer antiviral therapy has been associated with CMV relapse (121, 144). In addition, patients with detectable viral loads at the end of treatment are at higher risk of relapse, and studies have found that it would be reasonable to continue antiviral treatment until after a negative PCR test is achieved (7, 87, 121). In general, most clinicians monitor for CMV-DNA decline during antiviral treatment and demonstrate serial (at least two) weekly CMV-DNA tests before discontinuing antiviral treatment. In some cases, viral-DNA monitoring can serve as an early indirect measure for antiviral-drug resistance (e.g., when there is failure of viral-load decline during antiviral therapy) (7). It should be noted, however, that there may be a delay (of up to 2 weeks) in the decline in viral load in some patients, especially those with relapsed disease and those who are immunologically CMV-naïve (24, 87).

CYTOMEGALOVIRUS IN SOLID-ORGAN TRANSPLANT PATIENTS

Diagnosis of CMV Syndrome and Tissue-Invasive Disease

Molecular methods are currently the standard methods for diagnosis of CMV infection and disease after SOT. The pp65-antigenemia test has several limitations, including lack of standardization, subjective interpretation, and the need to process the blood specimen within 6 to 8 hours of collection, and thus, has been replaced in most transplant centers by QNAT (164).

CMV disease in SOT patients can be classified as an asymptomatic infection, whereby viral replication is detected in the absence of symptoms; or symptomatic CMV disease, which can be further classified into CMV syndrome or tissue-invasive CMV disease (23). CMV syndrome is characterized by fever, malaise, myelosuppression, and CMV DNA detected in blood. In general, diagnosis of tissue-invasive disease requires biopsy and histopathology for confirmation; it is indicated by cellular and nuclear enlargement (cytomegalic cells), positive CMV-specific immunoperoxidase stain, or positive *in situ* hybridization for CMV (165). Because of emerging data to suggest that detecting CMV in the blood and other body fluids may suffice for the probable diagnosis of tissue-invasive disease, some have deferred performing invasive procedures, such as biopsy, for diagnosis. In the case of CMV pneumonitis, for example, CMV QNAT in respiratory specimens such as BAL has been suggested as a useful test for diagnosis of CMV pneumonia, but this is still debated. CMV can be found in up to 50% of lung-transplant recipients without clinical or histopathologic findings of CMV pneumonia (termed CMV shedding) (166). It is noteworthy to emphasize, however, that there are some cases of tissue-invasive CMV diseases without accompanying viremia, hence the importance of performing biopsy as clinically indicated.

The majority of tissue-invasive CMV disease in SOT recipients involves the GI tract, and its diagnosis is suggested clinically by abdominal pain, nausea, vomiting, and diarrhea (165). Endoscopic examination may show patchy erythema, exudates, and micro erosions to edematous mucosa, multiple erosions, deep ulcers, and pseudo tumors (167). The diagnosis is supported by the detection of CMV in the peripheral blood (although this is not always present in CMV R+ transplant patients), and confirmed by the demonstration of CMV in tissue through *in situ* hybridization, culture, or PCR of tissue specimens. SOT patients with CMV hepatitis manifest prolonged fever, elevated bilirubin, and elevated liver enzymes; the presence of CMV in the blood of these patients may indicate CMV hepatitis, although the coexistence of other infection and allograft rejection is possible. Hence, liver biopsy is the only reliable way to distinguish rejection from CMV hepatitis. It is important to distinguish these two entities, since treatment is contrastingly different with rejection treated by increasing immunosuppression, and CMV treated by antiviral treatment and reduction in immunosuppression. In most cases of tissue-invasive CMV disease, the detection of CMV in blood with QNAT is suggestive. However, there are cases when CMV may not be detected in blood or is detected only transiently and in small quantities. Hence, histopathology remains as the cornerstone for its diagnosis.

Surveillance and Prevention of CMV Disease

There are two approaches for CMV prevention after SOT—universal prophylaxis and preemptive therapy. Universal prophylaxis consists of the administration of antivirals for varying duration, from as short as 3 months to as long as >12 months, for the prevention of CMV disease in patients at high risk (26). The main drawback with antiviral prophylaxis is the high incidence of late-onset CMV disease after discontinuation of prophylaxis and CMV D+/R- SOT recipients (164, 168). In the preemptive approach, serial monitoring of CMV replication, either by QNAT or pp65 antigenemia, is performed and thereafter, patients are treated with antiviral drugs once a predetermined threshold of viral replication is reached. As in the protocol for HSCT recipients, blood samples should be processed immediately so that the results can be provided to the clinician in a timely manner. The frequency of monitoring varies at different transplant centers from twice a week to every other week. The aim of this approach is to detect early viral replication and treat prior to progression to more severe disease. Currently, there are no universal predefined CMV QNAT thresholds to guide initiation of treatment, but the recent introduction of the first WHO International Reference Standard for CMV QNAT should facilitate comparable studies to define this threshold. Other centers use a hybrid approach to CMV prevention, whereby antiviral prophylaxis is followed by the preemptive approach, in the hope of reducing the incidence of late-onset disease, but this is only of modest efficacy (169).

Monitoring Response to Antiviral Treatment

Monitoring the efficacy of antiviral treatment can be done by pp65 antigenemia or CMV QNAT, but the

latter is preferred. Higher viral loads have been associated with longer time to resolution of CMV disease after SOT, and longer courses of antiviral treatment (87). Detectable viral DNA at the end of antiviral treatment was significantly associated with higher rates of CMV-disease relapse (45). A recent study found that CMV viral-load suppression to <137 IU/mL, as measured by a test calibrated to the WHO standard, is associated with faster time to resolution of clinical disease during antiviral therapy (44). Current-practice guidelines recommend continuation of antiviral therapy until viral replication is undetectable on at least two weekly NAT measurements.

CONGENITAL CYTOMEGALOVIRUS INFECTION

Primary CMV infection in the mother can be diagnosed by documenting positive serology in a woman previously known to be seronegative. CMV-specific IgM can be detected in women with primary infection; however, they may persist for 6 to 9 months after infection (38, 170). IgG-avidity assays can be utilized to distinguish primary from reactivation infections. A combination of CMV-IgM with IgG-avidity assays increases sensitivity for detecting a mother who could potentially transmit CMV to her offspring (171). The use of QNAT or pp65 antigenemia for diagnosis of primary maternal CMV infection is limited, since less than 50% of pregnant women have detectable viral replication (170).

Fetal infection can be diagnosed by detection of CMV DNA in the amniotic fluid by NAT, ideally after the 21st week of gestation and at least 6 weeks after the first positive maternal serology (38, 172). However, false-negative tests have been documented (173). Fetal imaging by ultrasound can show ascites, fetal-growth retardation, microcephaly, and structural abnormalities of the brain, which may indicate clinically significant congenital CMV infection (174).

The diagnosis of congenital infection is often demonstrated by the presence of CMV or viral antigens in urine or saliva within the first two weeks of life. Studies have investigated the utility of PCR techniques for detection of viral DNA in urine, serum, and saliva samples for the diagnosis of congenital CMV, with good sensitivity and specificity (175–177). More recently, a study comparing real-time PCR assays of liquid-saliva and dried-saliva specimens with rapid culture of saliva specimens obtained at birth showed again high sensitivity and specificity, and recommended these tests as potential screening tools (178). As mentioned earlier, congenital CMV infection has also been identified by testing for the presence of CMV DNA by PCR in neonatal DBS on filter paper (known as Guthrie cards) (141). In one study, this test was 100% sensitive and 99% specific for the diagnosis of congenital CMV infection in symptomatic and asymptomatic babies (141, 142). However, a large multicenter study comparing DBS PCR to saliva rapid culture showed that it could only detect less than 40% of congenitally infected infants. Due to its low sensitivity, its value is limited as a screening test (179).

Several trials have also looked into the role of antiviral drugs for prevention of end-organ CMV disease in infants with congenital CMV infection. Treatment of congenital CMV disease with intravenous ganciclovir is recommended for infants with evidence of CNS disease, including sensorineural-hearing loss, and in those with serious end-organ disease (hepatitis, pneumonia, thrombocytopenia) (180).

SUSCEPTIBILITY TESTING

The increasing use of antiviral drugs for prevention and treatment of CMV infection and disease has resulted in an emerging problem of antiviral drug-resistant CMV. Antiviral therapy selects for CMV gene mutations that confer antiviral-drug resistance. Reassuringly, CMV resistance remains low in incidence in the transplant population, and it has been reported to be anywhere from 0% to 2.2% (181, 182). Risk factors for drug resistance include history of prolonged exposure to low-dose antiviral prophylaxis, D+R- serostatus, increased intensity of immunosuppression, and lung transplantation (183). Drug-resistant CMV is associated with poor allograft and patient survival after transplantation. Among patients with AIDS, the presence of drug-resistant CMV has also been associated with poor outcome. Among 197 patients who received ganciclovir therapy, 18 (9.1%) patients developed genotypic resistance to ganciclovir. The presence of ganciclovir-resistant CMV was associated with a 4.17- to 5.61-fold increase in the odds of retinitis progression and greater loss of visual acuity (125). In a study of 87 patients with AIDS and CMV retinitis, sequence analysis of vitreous specimens showed that 15% of the patients had either a ganciclovir resistance-conferring mutation or a polymorphism in the CMV UL97 gene (184). In a study of 148 AIDS patients with CMV retinitis, the cumulative percentages of patients with UL97-mutant viruses at 3, 6, 12, and 18 months of antiviral treatment was 2.2%, 6.5%, 12.8%, and 15.3%, respectively (129). In a study of 23 CMV isolates from 10 immunocompromised

patients, there were eight isolates with a UL54 mutation that were correlated with resistance to ganciclovir, with four isolates cross-resistant to cidofovir (128).

In order to have activity against CMV, ganciclovir needs to undergo initial phosphorylation by UL97 viral kinase and subsequently by cellular kinases to its active form, ganciclovir-triphosphate. Ganciclovir-triphosphate will inhibit CMV replication by serving as competitive substrate for CMV-DNA polymerase, which is encoded by the viral gene UL54. Mutations in UL97, and less commonly to UL54 gene, may confer resistance to ganciclovir (185).

Treatment options for UL97 ganciclovir-resistant virus are foscarnet and cidofovir, both of which are associated with significant nephrotoxicity. UL97 is not required for the activation of foscarnet and cidofovir, hence they retain activity against UL97 mutants. However, like ganciclovir, both drugs inhibit CMV replication by acting on UL 54-encoded CMV-DNA polymerase. Hence, mutations affecting this UL54 gene may confer cross-resistance to all of these drugs. Therefore, genotypic assays are commonly used to guide treatment in this circumstance (23, 185). Drug resistance is often indicated by nondecline (or rise) in viral load over at least 2 weeks, especially in antiviral-experienced patients. In patients suspected to have ganciclovir resistance but with nonsevere clinical manifestations, increasing the dose of ganciclovir may be considered; however, this approach may be limited by development of toxicity (186). For those with progressive disease, a switch to foscarnet therapy is recommended. Once available, the results of the genotypic testing will guide the choices for antiviral therapies. There are several investigational drugs that are being evaluated for CMV disease, such as letermovir, maribavir, and brincidofovir, and their role for treatment of drug-resistant CMV is anticipated.

SUMMARY

CMV is an important pathogen that causes severe disease in immunocompromised hosts, including transplant patients, patients with AIDS, and immunologically immature newborns. Advances in diagnostic modalities over the past decade have revolutionized the clinical management of CMV disease in these patients. Currently, the clinical diagnosis of CMV can be rapidly confirmed in the laboratory, thereby facilitating the early initiation of treatment in an effort to curtail the morbid effects of CMV disease. Current methods have allowed for the early detection of CMV so that therapy can be instituted even prior to the onset of clinical disease. Monitoring responses to therapy has also been one of the major advances in management of CMV. Genotypic assays have evolved to rapidly diagnose drug-resistant virus, and guide therapeutic recommendations. There is also an emerging tool in assessment of CMV-specific T-cell immunity that could indicate risk of disease and its outcomes. Collectively, numerous diagnostic tests exist for the detection of CMV, from traditional methods of culture and serology to the more rapid and quantitative methods of antigenemia and NAT by PCR testing, with the latter being implemented in many laboratories worldwide.

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