Newer Methods for Diagnosis of Cytomegalovirus Infection

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Recently developed techniques have greatly increased the sensitivity and speed of detection of CMV and of host antibody responses to it. Newer serologic assays such as enzyme immunoassay or latex agglutination assay are accurate and efficient for screening donors and recipients and for determining susceptibility to primary infection. Available IgM antibody assays have occasional utility in recognition of recent infection. The slow process of isolating CMV in cell culture has prompted development of effective rapid techniques that utilize CMV-specific monoclonal antibodies and DNA sequences. Immediate-early viral antigen can be detected in infected cell cultures within hours of specimen inoculation. CMV antigens can also be detected directly in cells within clinical specimens. DNA hybridization has been used for CMV analysis in dot-blot, Southern blot, and in situ hybridization assays; the use of the latter is increasing for the detection of virus in fixed, paraffin-embedded tissue sections. Antigen or nucleic acid detection procedures, when applied directly to relevant clinical specimens, aid in the recognition of tissue invasive disease for which antiviral therapy might be considered. DNA amplification, using the polymerase chain reaction, achieves new levels of sensitivity in viral detection and should be useful for clinical diagnosis and for investigation of CMV pathogenesis and latency.

The increase in the number of patients with AIDS or organ transplants has resulted in an increase in opportunities to consider the diagnosis of cytomegalovirus (CMV) infection, and the possibility of effective therapy has heightened the need for accurate and rapid laboratory methods for diagnosis of viral infection. In addition, the well-established epidemiology of CMV transmission from blood transfusions [1] and donor organs [2, 3] has created a continuing need for serologic methods suitable for screening donors and recipients in efforts to avoid adverse clinical outcomes in immunodeficient recipients.

Traditional methods for CMV diagnosis are limited by such problems as insensitivity of complement-fixation assays and slow development of CMV cytopathology in cell culture. Also, the options for direct detection of virus in clinical material in the past were few. Most of the new approaches to rapid viral diagnosis that have been developed over the past decade have been applied to CMV, and a number of them were pioneered with this virus. As a result, many more options have become available for serologic and virologic diagnosis of CMV.

Because only a minority of cases of CMV infection are symptomatic, the ability to diagnose tissue-invasive CMV infection has become more important in the era of antiviral agents. It is necessary to distinguish viral shedding or viremia, which establishes the fact of infection, from CMV disease syndromes such as pneumonia, retinitis, or gastrointestinal lesions, for which therapy might be warranted.

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Serology

Depending on the population surveyed, prevalence of CMV seropositivity ranges from ~30% to 100% [2]. CMV antibody generally is detectable within a few weeks of onset of viral shedding in primary infection and persists indefinitely. Prior antibody confers some protection against subsequent CMV disease but, as with all herpesviruses, does not protect against reactivation of endogenous latent virus or reinfection with additional, exogenous strains of CMV. Thus, the two main reasons for the clinical use of serologic tests for CMV are for determining susceptibility to primary infection (which is more apt to be symptomatic) and for screening blood and organ donors for previous exposure to CMV and therefore for the potential for transmitting latent CMV. Seroconversion (seronegative to seropositive) remains a reliable means of diagnosing primary CMV infection but is usually practical only for closely moni-

tored patient populations such as transplant recipients, for whom pre- and postinfection sera are readily available.

Currently, most serologic assays are based on antigen extracted from fibroblasts infected with a laboratory strain of CMV (e.g., AD169). The method of extraction of antigen affects the assay performance, and the development of glycine-extracted CF antigen [4] some years ago was an important technical advance, increasing sensitivity of the assay. Unfortunately, even today some CF antigens are not of optimal quality, and the CF assay has had variable results in different clinical laboratories. Questions have also been raised about the ability of an antigen extracted from one strain of CMV to represent the wide variety of strains known to exist [5]. However, wide cross-reactivity of human sera to different CMV strains is the rule [6].

Alternatives to CF Assays

Accuracy, speed, and efficiency are important considerations in assaying for CMV antibody. Traditional CF technology does not fulfill any of these requirements very well, although its ability to detect seropositivity can be quite respectable [7, 8] when high-quality antigens are used. More recently, immunofluorescence, ELISA, and latex agglutination assays have been used in many laboratories as replacements for the CF assay [9–15]. Radioimmunoassays and indirect hemagglutination assays are also available. Each of these methods has potential advantages and pitfalls.

ELISAs for detection of CMV antibody are available from several manufacturers, and a number of in-house assays have also been developed. Numerous papers document the comparative performance of the assays [6–13]. The ELISAs give higher antibody titers and are at least as accurate as CF assays in determining serologic status, are much easier to perform, and eliminate the problem of anticomplementary sera. Results are typically available in a few hours. A single serum dilution is adequate to screen for presence of antibody, and the most widely used ELISAs are licensed for this type of use only. This restriction causes some problems for those wishing to evaluate changes in endpoint titer over time, determinations often done with CF assays. Other potential problems with commercial ELISAs include nonspecific reactivity of some sera with uninfected cell antigens—giving rise to false-positive results—or a lack of sensitivity if the positive cutoff level is set high enough to avoid such results. Performance of ELISAs can be improved by repetitive testing and by testing each specimen against control antigen from uninfected cells [7, 12], precautions that are not included in most commercial assays.

Latex agglutination has become an important method of screening blood and organ donors for CMV antibody [12–15]. The assay provides results within minutes and is reasonably accurate despite its ~5% rate of errors (involving mostly false-negative results) and a significant element of subjectivity in the reading of results [12]. This assay is licensed for both single-dilution screening applications and titration.

IgM Assays

Most of the commercial assays for CMV antibody measure total antibody (IgG and IgM), but IgG is the predominant antibody in most seropositive individuals. ELISAs for CMV-specific IgM are now commercially available [16–18]. As with all IgM assays, interference by rheumatoid factor and competition for antigen by IgG antibody are potential problems. Solutions to these problems include removal of IgG antibody by adsorption to a solid phase or by the complexing of IgG by anti-IgG antibody [19]. When these specimen pretreatments are used and repeated freezing and thawing of sera are avoided, IgM assays can be fairly reproducible and specific.

Although the majority of primary infections in transplant recipients are associated with an IgM antibody response, some immunocompromised patients with severe or fatal primary infection may have no detectable antibody response of any kind [20]. Pregnant women represent another group at risk for complications from primary CMV infection. IgM was detected by a radioimmunoassay in 55% of 29 women with primary CMV infection (or six of seven who were within 4 months of seroconversion) but in none of 18 women with secondary infection [21]. In another study, 73% of sera from pregnant women with primary infection and 69% of sera from congenitally infected babies had IgM antibody detectable by ELISA [17]; these figures could have been artificially low because the assays were performed with sera that had been stored at −20°C and possibly frozen and thawed several times. IgM antibody responses frequently are seen in secondary infections (reactivation of endogenous latent virus or exoge-

nous reinfection). For example, we found that 46% of 91 kidney recipients who were CMV seropositive at the time of transplantation had detectable IgM antibody after transplantation [18]. In pregnant women with secondary CMV infection, 11% of 43 had detectable IgM [17]. In a survey of sera from homosexual men, the prevalence of CMV IgM was high, presumably a reflection of the presence of secondary infections [22]. Presence of IgM antibody in nonprimary infections detracts from its diagnostic value, since most secondary infections are asymptomatic and do not warrant therapy.

The use of IgM assays as a rapid means of diagnosing active infection has been eclipsed by wide availability of rapid methods for detecting CMV shedding (discussed below). Detection of virus (e.g., in blood or urine) is much more reliable than IgM assays for transplant recipients with primary infection, who are likely to be viremic well before they exhibit a detectable antibody response [23]. Likewise, detection of viruria is preferable for diagnosis of congenital CMV infection.

IgM assays have been proposed as a means of identifying infected blood or organ donors. Although it is plausible that persons with recent CMV infection are likely to be infectious [24], it is clear that latent, transmissible virus often is present in CMV-seropositive donors who have no IgM antibody or any other evidence of recent infection. For example, we found no detectable IgM in four kidney donors who were known to have transmitted virus to eight recipients [18]. Whether exclusion of the relatively few IgM-positive individuals among the overall population of CMV-seropositive donors will have any significant impact on transmission of CMV remains to be seen.

Neutralizing Antibody

Usual CMV serologic assays measure the ability of serum antibody to bind to extracted viral antigen. It is also possible to assay for antibody that neutralizes the infectivity of live virus. Serum neutralizing antibodies are usually present at relatively low titers (seldom >1:2,000), require serum complement for optimal activity, and are technically difficult to measure. Typically, standardized viral inocula are mixed with dilutions of serum and complement, and the residual infective virus that remains after a period of incubation is assayed by adsorption onto fibroblast monolayers and by culture under semisolid medium [25]. Plaques representing foci of cytopathology that develop from individual infectious particles are counted after ≥7 days. Recently available reagents have enabled the scoring of neutralization assays within 24 hours [26], but the procedure remains labor-intensive. Neutralization titers of individual sera have varied depending on the virus strain used [27]. This variability could reflect antigenic variation among strains but could also be related to the effects of in vitro culture; generally, repeated passage of clinical CMV strains in vitro is necessary to obtain cell-free virus stock of reasonable infectivity and plaque-forming ability. This requirement makes the routine determination of neutralizing titers against specific infecting strains impractical.

Interest in neutralizing antibody is related to its possible correlation with protective immunity. However, accumulated data indicate that viral reactivation can occur in the presence of neutralizing antibody, even when its titer against the specific strain involved is relatively high. CMV antibody does appear to ameliorate severity of symptomatic disease [28, 29], but whether this effect is due specifically to virus-neutralizing activity is unclear.

Serologic Responses to Specific Viral Proteins

Increasing knowledge of the specific viral proteins encoded by CMV has resulted from development of a variety of monoclonal antibodies, gene probes, and sequences. These reagents have made possible the identification of at least three viral proteins relevant to neutralizing antibody [30–32] as well as a variety of other structural and regulatory proteins, many of which are recognized by human antisera. Comparative analysis of serologic responses indicates that there is considerable heterogeneity among different immune individuals. This is shown by relative intensity of recognition of different protein bands in CMV lysates on immunoblotting [33, 34] and by differences in neutralizing ability of sera with similar titers in conventional antigen-binding assays [8, 35, 36]. Whether these differences are predictive of clinical outcome is unknown. There is continuing interest in this area because responses to specific viral proteins appear to have some bearing on disease status in infections with Epstein-Barr virus [37] and human immunodeficiency virus [38]. If analogous findings are demonstrated with CMV, serologic responses would also have relevance in the assessment of protective immunity and to vaccine development.

Although antigens enriched for some specific component, e.g., early antigens [39, 40], have been used occasionally in an effort to define antigen-specific antibody responses in various groups of patients, most of this work has not involved pure, single proteins or peptides, and thus the interpretation of findings is difficult. With the present ability to define epitopes on a molecular level, it is likely that much more detailed information in this area will be available in the near future.

Detection of Virus in Clinical Specimens

Isolation of Virus

Isolation of CMV from a clinical specimen remains a definitive diagnostic procedure against which all newer assays must be compared. Traditionally, urine, blood, and throat specimens have been cultured, but during active infection a wide variety of specimens will yield virus. In most infected individuals, urine contains moderate to large amounts of infectious virus particles and is a convenient and reliable specimen for culture. However, viruria often represents asymptomatic infection and can be prolonged (for months to years). Viremia, demonstrated by culture of leukocytes, correlates better with active disease, although many viremic individuals also are asymptomatic [41]. Leukocytes, because of their increased toxicity to fibroblast monolayers, are technically more difficult to culture than urine. When separated leukocyte fractions are cultured, the polymorphonuclear fraction generally contains the most infectious virus [42].

The fundamental limitation of traditional isolation of virus is the prolonged interval required for development of visible cytopathology in cell cultures, which, on average, takes 1–2 weeks; however, when little virus is present in the specimen, recovery of virus can sometimes take ⩾6 weeks. CMV infectivity deteriorates rapidly under adverse conditions of specimen holding such as freezing, and isolation of virus is prevented by overgrowth of more rapidly growing microorganisms (e.g., herpes simplex virus or fungi), which cause early destruction of cell monolayers.

Early methods for rapid detection of CMV have included cytologic and electron microscopic methods [43], which tended to be inadequately sensitive, except possibly for use in neonatal infections (in which viruria is typically intense).

Antigen Detection

Important advances resulted from the development of monoclonal antibodies to the 72-kDa major immediate-early protein of CMV. These antibodies enable the detection of a nuclear antigen in fibroblasts within hours of infection [44–47], thus permitting the evaluation of cultures long before the appearance of identifiable cytopathology (figure 1). Unlike conventional cultures, which are usually performed in tubes containing fibroblast monolayers, cultures intended for monoclonal antibody staining are performed on flat monolayers, either on cover slips in shell vials or in 24-well cluster plates. Centrifugation of monolayers greatly assists absorption of virus [44], increasing apparent infectivity of the viral inoculum about fourfold in our experience [26].

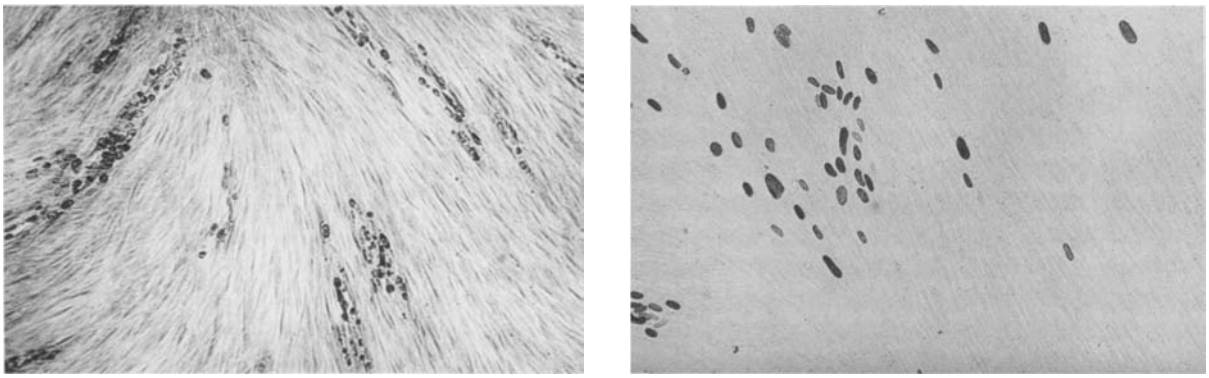

Figure 1. *Left*: Characteristic focal cytopathic effect due to CMV in conventional cell culture as seen at 1–2 weeks after infection. *Right*: Immediate-early nuclear antigen in fibroblasts, as visualized at 16 hours after infection by an immunoperoxidase procedure involving the use of a monoclonal antibody [26].

Urine and bronchoalveolar lavage specimens have given the best results with the rapid culture techniques; the sensitivity has been reported to exceed that of conventional culture [45, 47], probably because of the enhancement of infectivity provided by centrifugation. With buffy-coat specimens there has been some trouble with toxicity and with the loss of the monolayer after centrifugation, and the yield of positive results is somewhat diminished [47]. Therefore, conventional cultures may be needed as a backup.

Antibodies to early and late CMV antigens are also useful for direct visualization of infected cells in clinical specimens. This has been demonstrated with bronchoalveolar lavage cells [48, 49], biopsy specimens [50, 51], and leukocytes [52]. In these cases, proper technique is important, and special training of diagnostic laboratory staff may be necessary because of the skills required for the proper fixation of cells onto slides and the interpretation of the cell composition of the specimen.

Monoclonal antibodies generated to specific strains of CMV may not recognize the corresponding antigen in all clinical strains encountered. In a study of 21 distinct CMV strains, we found that two strains were not recognized by the immediate-early monoclonal antibody that we used [26]. We later found, however, that other commercially available antibodies to the same antigen did stain those strains. Thus, it is likely that a minority of strains may be missed by any single monoclonal antibody, and it may be necessary to use a mixture of antibodies for greatest sensitivity. Antibodies recognizing early and late antigens may be needed for adequate sensitivity of detection of infected cells in tissue sections and cytologic preparations [48].

Nucleic Acid Hybridization

Use of hybridization techniques for CMV diagnosis has been facilitated by the availability of a variety of cloned CMV sequences. A reasonable selection of probes is available commercially, and the increasing number of published sequences for human CMV, now amounting to at least one-third of the total genome, has made it possible for researchers to devise any number of synthetic probes representing genes of known function. Although some regions of the CMV genome are known to be homologous with human cellular sequences [53, 54], it is quite possible to avoid these causes of false-positive signals.

Initial applications of hybridization techniques involved dot-blot assays of extracts of urine [55–57] and leukocyte [57] specimens. Experience to date indicates that, with the use of relatively simple techniques of specimen processing, most urine specimens containing a moderate to large amount of CMV (e.g., >1,000 pfu/mL) will be detected by this technique, while those containing a smaller amount of virus will have signals that overlap those of culture-negative specimens. Studies correlating the results of dot-blot hybridization of leukocyte extracts with those of conventional culture indicate some discordance in parallel analyses. Most culture-positive specimens appear to be detected, but some culture-negative specimens give positive signals. This can be interpreted to mean either that hybridization yields some false-positive results or that culture techniques are not sensitive enough to detect virus. In a recent study, fractions of leukocytes from various patients were separately subjected to both culture and dot-blot hybridization [58]. It was found that, while the results of culture and hybridization correlated well in the polymorphonuclear fraction, there were more hybridization-positive specimens than culture-positive specimens in the mononuclear fraction. This was tentatively interpreted as an indication that some of the virus in the latter fraction was noninfective or was less so than the virus in the polymorphonuclear fraction. Although some work with biotin-labeled probes has been reported [59], the main limitation with dot-blot techniques is the requirement for highly radioactive probes for maximal sensitivity of detection, thus making them impractical for use in the diagnostic laboratory.

Use of in situ hybridization for the detection of CMV in infected cells has increased. In contrast to the situation with dot-blot assays, the use of biotin-labeled probes on clinical specimens was adopted early on, thus placing the technique within reach of surgical pathology or cytology laboratories [60–62]. It is possible to detect CMV in formaldehyde-fixed, paraffin-embedded sections with the use of relatively straightforward techniques in cases where active infection is present in immunosuppressed hosts. Specimen preparation consists of methods for securing the specimen on a glass slide, rendering the cells permeable, and denaturing the DNA without detaching the cells or destroying morphologic identity. Use

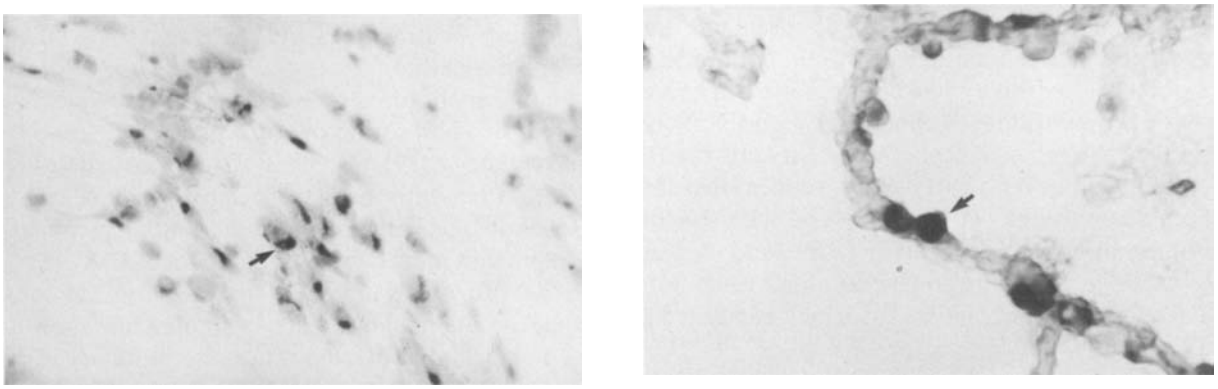

Figure 2. In situ hybridization, using a biotin-labeled CMV probe. *Left*: A fibroblast monolayer 4 days after infection with CMV showing intracellular inclusions (arrow). *Right*: Paraffin-embedded, formalin-fixed section of lung in a case of CMV pneumonia, showing inclusions (arrow) in infected cells.

of moderately high concentrations of biotin-labeled probes permits fairly short hybridization times, and posthybridization development is brief. Commercial kits are now available for accomplishing these steps. With in situ hybridization, viral inclusions can be detected in cultured cells or directly in infected tissue (figure 2). Applications include diagnosis of CMV pneumonia [63, 64], hepatitis [65, 66], and gastrointestinal lesions [67]. Unfortunately, the simple methods of in situ hybridization suitable for use in actively infected tissue are inadequate for the detection of the presence of latent virus in seropositive donor organs. More elaborate techniques, involving the use of radioactive probes, have suggested the presence of CMV sequences in mononuclear cells of some normal seropositive subjects [68].

Southern-blot hybridization has been used on clinical material primarily for comparative analysis of CMV strains [2, 69, 70] Use of subgenomic probes and blot hybridization permits a clearer visualization of restriction profiles as well as the recognition of unusual events such as the simultaneous shedding of multiple viral strains and genetic recombination among strains, information that is useful for molecular epidemiologic studies.

DNA Amplification

Recent introduction of the polymerase chain reaction for amplification of specific short segments of nucleic acid sequences has stimulated intense exploration of its utility in a wide variety of clinical settings, including viral diagnosis [71–73]. For the herpesviruses especially, the dramatic gains in sensitivity of viral detection made possible by this technique offered new hope for the detection of heretofore elusive latent genomes as well as more routine application in the detection of viremia or other active infection. Before the ultimate role of DNA amplification in CMV detection is known, some technical problems must be solved. These include a definition of the most suitable primers and the extent to which they cause amplification of sequences from diverse CMV strains, of nonspecific sequences of host origin, or of homologous sequences of other herpesvirus or unrelated organisms. Sensitive yet practical methods for verifying the authenticity of the amplified CMV sequence must be established. Cross-contamination of specimens with trace amounts of CMV sequences must be prevented. Most likely, CMV sequences will be detected in some specimens that are culture-negative or even in material from CMV-seronegative individuals, and corroborative epidemiologic and clinical data will have to be gathered to assess the clinical significance of these findings. For example, it will be important to determine if the presence of certain CMV sequences is sufficient to predict infectivity or ability to transmit virus to recipients of blood or donated organs. Finally, for the technique to be adopted by clinical laboratories, methods must be easily reproducible and cost-effective.

Diagnostic Methods for Invasive CMV Disease

Newer methods for detection of CMV antibody and of CMV viruria and viremia are essentially improvements or alternatives to traditional techniques, and the roles of such techniques are apparent. Less es-

tablished is the role of direct detection methods in making a diagnosis of tissue-invasive CMV infection. Here, criteria are being developed as newer techniques become available, and further clinical correlation is needed.

Reliable diagnosis of CMV pneumonia, by the least invasive procedure possible, has become an important goal for transplant recipients and patients with AIDS. Among transplant recipients, those who receive bone marrow have a high incidence of interstitial pneumonia with CMV as a major causative agent. In these recipients, antigen detection and in situ hybridization are of comparable sensitivity in detecting virus in tissue sections, while histopathologic techniques are less sensitive [63]. An advantage of in situ hybridization is the acceptability of formaldehyde-fixed, paraffin-embedded tissue, whereas for antigen detection fresh frozen sections generally are required for adequate sensitivity.

Bronchoalveolar lavage has become an accepted means of diagnosing pulmonary infections in compromised hosts [74, 75]. Culture of lavage fluid, with overnight detection of immediate-early antigen, has proven to be more reliable than the culture of tissue obtained at biopsy in identifying CMV infection [76]. However, the question remains whether recovery of infectious virus indicates the presence of CMV pneumonia and, if so, whether it does in all categories of patients. Information obtained for bone marrow transplant recipients suggests that recovery of virus correlates fairly closely with histopathologic evidence of pneumonia [76]. Viral antigens, sequences, or inclusions can be detected in cells recovered in bronchoalveolar lavage fluid from most marrow recipients for whom cultures of lavage fluid are positive [48, 64, 76] and would appear to offer additional support for a diagnosis of invasive pulmonary disease. Thus, a compatible clinical syndrome, confirmed by recovery of CMV from bronchial washings and/or by identification of CMV antigens or sequences in cytologic preparations of lavage fluid, is probably adequate for a presumptive diagnosis of CMV pneumonia for purposes of decisions about antiviral therapy for transplant recipients. The problem in patients with AIDS, who are commonly infected with multiple pulmonary pathogens, requires further investigation.

Support for a diagnosis of CMV hepatitis can be conveniently obtained by in situ hybridization with biopsy specimens fixed and processed in the manner usually used by surgical pathology laboratories, and favorable experiences with this technique have been reported [65, 66]. However, criteria for assessing severity of CMV hepatitis and suitability of antiviral therapy have not been established.

Evidence of CMV infection has been obtained by in situ hybridization or antigen detection for a variety of other organs, including gastrointestinal mucosa [67], nervous system tissue [77], and numerous other tissue specimens [78] in the disseminated CMV infection seen in patients with AIDS.

Conclusions

As a result of recent rapid progress, a delay in laboratory confirmation of CMV infection is no longer a serious obstacle in clinical practice. Likewise, efficient and accurate serologic procedures permit the rapid screening of donors and recipients so that transfer of cells from seropositive to seronegative individuals can be avoided, even in situations of some clinical urgency.

Remaining challenges for CMV diagnosis include (1) the development of a better definition of what constitutes invasive CMV disease requiring therapeutic intervention and (2) an improvement in the ability to detect the viral latency or low-grade persistence that seems responsible for transfusion- and transplantation-associated CMV infection as well as many cases of secondary CMV infection following immunosuppression. Clinical correlation will be needed to establish the value of new diagnostic approaches in these areas. It will also be important to standardize reagents and techniques so that clinical laboratories have a clear understanding of what sensitivity, specificity, and technical pitfalls to expect of each of the newer methods.

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