Novel DNA Assay for Cytomegalovirus Detection: Comparison with Conventional Culture and pp65 Antigenemia Assay

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We compared conventional cytomegalovirus (CMV) isolation, rapid viral culture, a CMV pp65 antigenemia assay, and a novel CMV DNA hybrid capture system (HCS). A total of 309 blood samples from individuals in different risk groups were assessed by at least two of the methods mentioned above. Leukocytes were recovered either after centrifugation in Leucosep tubes containing 1.080 Ficoll for pp65 assay or after simple differential lysis steps for DNA detection. HCS was based on DNA hybridization with a CMV RNA probe and its capture by antibodies to DNA-RNA hybrids. The CMV pp65 lower matrix protein was detected by fluorescence with c10-c11 monoclonal antibody in formalin-fixed leukocytes. Concordant results were observed for 92.9, 78.3, and 82.7% of the patients when comparing (i) viral culture and the pp65 antigenemia assay, (ii) viral culture and HCS, and (iii) the pp65 antigenemia assay and HCS, respectively. Discordant results were observed between a positive HCS result and negative culture and/or pp65 results. These results were associated with relatively low DNA levels (<20 pg/106 cells) and positive viruria. In conclusion, the pp65 antigenemia assay is a rapid and reliable method of detecting CMV and is preferable to culture, but the Murex HCS appears to be more sensitive for CMV detection.

Cytomegalovirus (CMV) infection is widespread in humans, with a 50 to 60% prevalence in rich countries and a 90 to 100% prevalence in poor countries. CMV infections are a major cause of mortality and morbidity in immunocompromised patients, such as organ or bone marrow transplant recipients, persons with AIDS, neonates, and older patients (1, 6, 13, 14). A prompt diagnosis is essential for efficient antiviral treatment in patients with severe or life-threatening CMV disease. Important efforts have been deployed to improve CMV detection. Consequently, several tests for CMV are now available, including PCR for CMV DNA detection (4), in situ hybridization (3), and monoclonal antibody (MAb)-based detection of CMV antigens (16, 18). Routinely, common methods such as cell culture for viral isolation and immunoglobulin M (IgM) serology are still used. Nevertheless, none of the current methods is entirely satisfactory in terms of sensitivity, specificity, and rapidity.

In our laboratory, diagnosis of CMV infection is achieved by the detection of CMV antigens in a rapid (48 h) cell culture for virus in which the culture is inoculated with patient leukocytes and is confirmed by conventional virus isolation. The aim of this study is to compare our diagnostic methods with (i) a pp65 antigenemia assay and (ii) a new, commercially available CMV DNA hybrid capture system (HCS; Murex Diagnostics Ltd., Dartford, United Kingdom).

MATERIALS AND METHODS

Patients. A total number of 198 patients with or at high risk of CMV disease were included in this study. Sixty-nine patients were from the Infectious Diseases Center (patients suffering from AIDS), 41 were from the Nephrology Center (kidney transplant recipients), 30 were from the Hematology Center (leukemic patients and bone marrow recipients), and 58 were suspected of having CMV disease. One of the AIDS patients who was undergoing antiviral (ganciclovir) therapy was monitored for 3 weeks for kinetic studies in terms of CMV pp65 antigen and CMV DNA levels in leukocytes. The IgG and IgM anti-CMV serological status of these patients was determined by enzyme immunoassay (CMV Enzygnost [Behring, Marburg, Germany] and CMV IMX [Abbott, North Chicago, Ill.]).

Blood samples. We analyzed 309 blood samples from the 198 patients. Peripheral blood samples were obtained in heparinized or EDTA-containing tubes, depending on the test method. Two cell culture-based methods were used (conventional CMV isolation and rapid viral culture), as were a pp65 antigenemia assay (254 samples) and a CMV DNA test (157 samples). When sufficient blood volumes were available, the last two tests were performed (133 samples). Blood samples were processed within 2 h following collection to minimize artificial results (11). Blood cells were counted with an automatic cell counter (Technicon H.2 system; Bayer Diagnostics, Tarrytown, N.Y.).

Conventional CMV isolation. Confluent monolayers of MRC-5 cells (Biomedical, Lyon, France) in 25-cm2 flasks were inoculated with 1 ml of buffy coat cells isolated from 6 ml of heparinized blood samples. The estimated number of blood cells was 6 x 108/ml, which was higher than the recommended minimum number (2). After 1 h of absorption, the inoculum was replaced with 10 ml of minimum essential medium (MEM) containing 10% fetal calf serum (FCS). Cell cultures were maintained at 37°C for 3 weeks. The cultures were checked every other day for the appearance of the characteristic cytopathic effects of CMV.

Rapid viral culture. MRC-5 cells grown to confluency on coverslips in flat-bottom Bipou containers (Biobyx Sterilin Ltd., Stone, United Kingdom) were inoculated with 0.3 ml of the buffy coat cells mentioned above and were centrifuged at room temperature for 45 min at 600 g. The inoculum was then removed, and 1 ml of MEM containing 10% FCS was added to each tube. Cell cultures were placed in an incubator at 37°C with 5% CO2. After 48 h of incubation, the cells were fixed in acetone and the coverslips were transferred onto slides. Fifty microliters of a 100-fold-diluted murine MAb directed against CMV immediate-early antigens (E13 clone; Bioys, Compiègne, France) was added for 30 min at 37°C in a moist chamber. The cells were then washed with fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG and IgM (Bio-Source International, Camarillo, Calif.). A green fluorescent nuclear staining was observed for CMV-infected cells. This procedure for blood samples was also applied to urine samples.

pp65 antigenemia assay. The pp65 antigenemia assay is based on the direct detection of the CMV pp65 phosphoprotein, which is the viral lower matrix protein predominantly found in leukocytes during active CMV infections (17). Three milliliters of 1.080 Ficoll for polymorphonuclear cell isolation (Eurobio, Les Ulis, France) was added to a Leucosep tube (Esquire Chemie AG, Zurich, Switzerland). These tubes contained a porous membrane that covered the Ficoll after centrifugation. Approximately 6 ml of heparinized blood was poured onto the membrane, and the tubes were centrifuged at 800 x g for 15 min. The leukocytes were separated from the erythrocytes by the membrane and were easily recovered from the Ficoll upper layers by another centrifugation step.
The results of the culture methods was 88.4%. The 18 patients were concordant by these assays (data not shown), the assays with discordant results were under antiviral treatment (ganciclovir), and the numbers of pp65-positive cells were low (<50/10^6 cells), in accordance with Gerna et al. (8). A 78.3% concordance (κ = 0.441) was observed between culture methods and the CMV DNAemia assay by HCS (Table 2). Among the 34 samples with discordant results, 11 had very high DNA levels (>20 pg/10^6 cells, with an average value of 44.9 pg/10^6 cells), and 23 had lower DNA levels (<20 pg/10^6 cells, with an average value of 7 pg/10^6 cells). An 82.7% concordance (κ = 0.553) was observed between the pp65 antigenemia assay and the CMV DNAemia assay (Table 3). Among the 23 samples with discordant results, only 2 blood samples were CMV DNA negative, with a low number of pp65-positive cells (<5/10^6 cells). For the 21 pp65-negative samples, 4 had very high DNA levels (>20 pg/10^6 cells, with an average value of 31.7 pg/10^6 cells) and 17 had lower DNA levels (<20 pg/10^6 cells, with an average value of 8.4 pg/10^6 cells). These positive DNA samples with discordant results that were also negative by the blood culture methods were all positive for CMV, as determined by culture of a urine specimen taken at the same time (data not shown). Finally, the results of CMV IgM detection in this study were not concordant with either culture, pp65 antigenemia assay, or DNAemia assay results (61.7%; κ = 0.227; n = 47). Its sensitivity was 39% compared with the results of the other methods. Therefore, this serological marker cannot be used for the screening of replicative CMV in immunocompromised patients.

For the pp65 antigenemia assay, the test with the 20-fold-diluted IC3-AYM1 Argene MAb appeared to be as good as the test with the Biotest Clonab MAb diluted 5-fold, with reduced background signals.

When comparing CMV pp65- and DNA-positive blood samples, we observed that low, mild, and high levels of pp65 antigenemia were positively correlated with the presence of CMV DNA (Fig. 1). The leukocyte count was well correlated after erythrocyte lysis in the HCS kit with the count obtained by automatic blood cell counter (Leader TM 50; MGM Instruments Inc., Hamden, Conn.) and is reported to 10^6 cells. For the 21 pp65-negative samples, 4 had very high DNA levels (>20 pg/10^6 cells, with an average value of 31.7 pg/10^6 cells) and 17 had lower DNA levels (<20 pg/10^6 cells, with an average value of 8.4 pg/10^6 cells). These positive DNA samples with discordant results that were also negative by the blood culture methods were all positive for CMV, as determined by culture of a urine specimen taken at the same time (data not shown). Finally, the results of CMV IgM detection in this study were not concordant with either culture, pp65 antigenemia assay, or DNAemia assay results (61.7%; κ = 0.227; n = 47). Its sensitivity was 39% compared with the results of the other methods. Therefore, this serological marker cannot be used for the screening of replicative CMV in immunocompromised patients.

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HCS detects a chemiluminescence intensity proportional to the quantity of CMV DNA. Thus, it allows an objective quantification of the viral load and avoids (i) the laborious screening of fluorescent cells either in the pp65 antigenemia assay or in rapid viral culture and (ii) the mistakes in counting the numbers of infected cells. We tried to evaluate the equivalence of the number of pp65-positive cells and the related amount of DNA detected by HCS. Discordant pp65 and DNA levels were observed for several reasons. First, fluorescent cell counts were only approximate, and the results could be given only in three intensity groups (low, mild, and high levels of antigenemia). Consequently, pp65 antigenemia levels may not be reliable. Second, the pp65 antigenemia assay evaluated the number of CMV-infected cells, i.e., the numbers of cells expressing pp65 antigens during active CMV infection, whereas HCS detected all CMV genomes from these cells. Since infected cells harbor greater or fewer numbers of CMV genomes, pp65 antigenemia levels can correspond to either very high or low DNA levels. Furthermore, HCS may detect defective CMV (15) which is not able to replicate in cell cultures. If pp65 antigenemia reflects active CMV infection and HCS detects both CMV replication and the latent form of CMV, then it is expected that HCS will not correlate with clinical symptoms. In fact, Lazzarotto et al. (12) found that the DNA level by HCS correlated as well as the level of pp65 antigenemia with clinical symptoms. According to Imbert-Marcille et al. (9), a DNA level below 23 pg/ml of blood was predictive of asymptomatic CMV infection in renal transplant recipients, whereas a higher viral load was always associated with CMV disease. However, these low DNA levels associated with the asymptomatic form of CMV infection did not exclude the possibility of active CMV replication. Indeed, all of our patients with samples with CMV DNA-positives but with negative results by both culture and the pp65 antigenemia assay were positive for viruria. The meaning of long-lasting positive viruria is still under discussion, but it may reflect low-level CMV replication in infected leukocytes, below the sensitivity of blood culture and the pp65 antigenemia assay. The specificity of CMV DNA detection in some of these patients was also assessed by positive CMV PCR results (data not shown), as indicated by Lazzarotto et al. (12).

In conclusion, the pp65 antigenemia assay is a more rapid and reliable method than CMV culture for CMV detection. Nevertheless, quantification of infected cells on the basis of the pp65-positive cell count is exhaustive for a large number of samples and is unsuitable for the precise measurement of the level of CMV in cells. On the other hand, the Murex CMV DNA HCS appears to be a promising method for CMV detection, in particular for patients with a low viral load. It is also well adapted for the therapeutic follow-up of patients under antiviral treatment. Indeed, for our AIDS patients on ganciclovir therapy, we observed a rapid decrease in the level of CMV DNAemia by day 4 and viral disappearance by day 12 using this method.
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