The objective was to compare the use of the polymerase chain reaction (PCR), virus culture, and immunostaining of alveolar cells used alone and in combination as diagnostic methods for the rapid diagnosis of cytomegalovirus (CMV) pneumonia in marrow transplant recipients. Seventy-five marrow transplant recipients with clinical and radiological evidence of pneumonitis were used as subjects. Bronchoalveolar lavage was performed on all patients to obtain material for conventional and/or rapid CMV culture, immunostaining of alveolar cells with monoclonal antibodies (MoAbs), and amplification of CMV-DNA by PCR. Assay results were then prospectively correlated with clinical outcome. Seven of the 75 patients (9.3%) had CMV pneumonitis, and 6 patients (8%) had CMV infection without pneumonia. PCR is the most sensitive assay for the detection of CMV in bronchoalveolar lavage fluid. For the diagnosis of CMV pneumonitis, the sensitivity of alveolar cell immunostaining and PCR were both 100%. The sensitivity of virus culture was 85.7%. The positive predictive value for each test, used alone, for the identification of CMV pneumonitis was low. However, when the result of the PCR assay was assessed in combination with CMV immunostaining of alveolar cells, the sensitivity, specificity, positive, and negative predictive value of this strategy was 100%. The concomitant use of PCR and the rapid immunostaining of alveolar cells for CMV has facilitated the development of a sensitive and specific diagnostic algorithm for the detection and early treatment of CMV pneumonitis in transplant recipients.

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MATERIALS AND METHODS

Patients. From March 1989 through June 1991, 84 bronchoalveolar lavage specimens from 75 patients were evaluated as part of a diagnostic evaluation for symptoms of pulmonary dysfunction and radiologic evidence of pneumonitis, after marrow transplantation. All patients had received an allogeneic (60 patients) or an autologous marrow transplant (15 patients) as treatment of acute or chronic leukemia, lymphoma, or aplastic anemia. Bronchoscopy was performed according to previously published procedures. All therapeutic and diagnostic procedures were performed on Institutional Review Board approved protocols and with signed informed consent. Only 1 of the 75 patients studied had received prior treatment for CMV infection. Clinical, laboratory, and if applicable, autopsy data for all 75 patients were collected prospectively for a minimum follow-up period of 3 months from the time of bronchoalveolar lavage.

Direct immunostaining of alveolar cells and virus culture. The detection of CMV early and late proteins in alveolar macrophages and epithelial cells or in lung tissue obtained by biopsy or autopsy, was performed using a pool of murine MoAbs as previously described. These MoAbs are freely available and will be provided to other investigators, on request. Briefly, a minimum of 5 × 10⁶ cells obtained by bronchoalveolar lavage were cytopsioned on a clean glass slide, fixed in 100% acetone for 10 minutes at 4°C, and then exposed to a pool of six murine MoAbs to early and late CMV proteins for 1 hour at 37°C. The cells were then washed and exposed to a human adsorbed fluorescein-conjugated goat Fab2 anti-murine IgG antibody for 1 hour at 4°C, washed again, and the cells examined by fluorescence microscopy for evidence of nuclear and/or cytoplasmic fluorescence, and the number of positive cells assessed. Positive and negative controls using CMV infected and uninfected alveolar cells and non-specific, subclass-matched, MoAbs, were always performed in parallel. A specimen was considered inadequate for direct immunostaining if less than 5 × 10⁶ cells/slide were available.

CMV was isolated from bronchoalveolar lavage fluid or lung tissue by standard virus culture on human embryonic fibroblasts in culture tubes, in parallel with rapid immunoperoxidase staining for the major CMV immediate early protein in infected human embryonic fibroblasts using previously described techniques.

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DNA extraction. Bronchoalveolar lavage fluid was centrifuged in a 1.5-mL freezing tube (Sarstedt Inc. Newton, NC), the supernatant removed, and the pellet stored at −20°C when not immediately processed. The cell pellet was incubated for 1 hour at 50 to 60°C in 100 µL of extraction buffer (50 mmol/L KCl, 10 mmol/L Tris·HCl [pH 8.3] 2.5 mmol/L MgCl2, 0.01 mg/mL gelatin, 0.45% NP40, 0.45% Tween 20) containing 6 µg of Proteinase K (Boehringer Mannheim, Indianapolis, IN) that was subsequently inactivated by heating for 10 minutes at 95°C. The extracted DNA was stored at −20°C.

Primers. Two sets of oligonucleotide primers complimentary to sequences of the major immediate early gene of human CMV were synthesized on a Model 380 DNA Synthesizer (Applied Biosystems, Foster City, CA) and were used in this study. From March 1989 through March 1991, primers IER-1 and IER-2 were used. To evaluate the sensitivity of the PCR procedure using these primers, known amounts of plasmid DNA containing the cloned EcoRl fragment J of human CMV strain AD169 (kindly provided by Dr B. Fleckenstein, Erlangen, Germany) were amplified. Fifty to 200 target molecules could be detected after hybridization of the PCR product with the 32P end-labeled oligonucleotide probe designated IER-P, after alkaline transfer of the amplified DNA to a nylon filter (data not shown).

After publication of two reports using primers IE-A and IE-B in which the investigators reported being able to detect from 20 to 60 target molecules without Southern blot hybridization, these primers were evaluated in our laboratory.8,9 Using known amounts of plasmid CMV-DNA, 50 target molecules were consistently detectable, without Southern blotting, on ethidium-bromide-stained agarose gels. Because primers IE-A and IE-B provided equivalent sensitivity without hybridization, when compared with primers IER-1 and IER-2 with hybridization, from March 1991, all clinical samples were assayed using primers IE-A and IE-B, without Southern blot oligonucleotide probe hybridization (Fig 1).

Primers IER-1 and IER-2 flank a 421-bp fragment of the immediate early 1 gene of strain AD169.13 The sequences of the primers and probe and their relative positions within the IE1 gene are given below: IER-1 GTC TGA TGA TGG GAG AC 1215-1243; IER-2 GGC ATT CTA ACA TTG GGA AGC 1615-1636; IER-P GTG GAC ATG GTG CCG CAT AGA ATC. The primers IE-A and IE-B flank a 147-bp fragment of DNA between positions 1767 and 1913 of the fourth exon of the CMV immediate early 1 gene of strain AD169.8 The sequences of the primers and their positions within the IE1 gene are given below: IE-A AGC TGC ATG ATG TGA GCA AG 1767-1786; IE-B GAA GGC TGA GGG CAT GGT AA 1894-1913.

PCR technique. To minimize the well-documented risks of contamination associated with PCR technology, amplification of extracted DNA was physically separated from the laboratory area where extraction was performed. In addition, separate, designated laboratory instruments were used for each step in the procedure. Each PCR experiment with clinical materials always included human embryonic fibroblasts infected with a clinical isolate of human CMV and with the laboratory strain AD 169, as positive controls. Uninfected human embryonic fibroblasts and PCR buffer containing no DNA, were always included as negative controls, which were randomly distributed among patient samples. All assays were discounted and repeated, in the event of false-positive negative controls or false-negative positive controls.

The extracted DNA (5 µL) was amplified in a 50 µL reaction (1 µmol/L of each primer, 10 mmol/L Tris·HCl [pH 8.3], 50 mmol/L KCl, 2.0 mmol/L MgCl2, 0.01% gelatin, 200 µmol/L of each deoxyribonucleotide, and 1.25 U of Taq DNA Polymerase, [Perkin Elmer-Cetus, Emeryville, CA]) overlaid with two drops of mineral oil. Forty cycles were performed in a thermal cycler (Perkin Elmer-Cetus, Emeryville, CA) with the following cycle characteristics: denaturation for 1 minute at 94°C, re-annealing for 45 seconds at 55°C, and extension for 1 minute at 72°C. A final extension step of 7 minutes at 72°C was used for the last cycle. About 20% of the PCR-product was run on a 1.5% agarose gel containing ethidium bromide and visualized with UV light and the gel photographed. When primer pair IER1/IER2 were used, alkaline transfer of the DNA to a nylon filter was performed and the filters hybridized in 0.5 mol/L phosphate buffer (pH 7.2), 7% SDS, 1% BSA, 1 mmol/L EDTA, for 6 to 16 hours with 32P end-labeled IER-P probe. After washing, the filters were autoradiographed at 70°C using XAR-5 film (Kodak, Rochester, NY), with one intensifying screen.

Definitions. CMV pneumonitis was diagnosed when a minimum of 4 of the following 5 criteria were fulfilled: (1) clinical and radiologic evidence of interstitial pneumonitis; (2) detection of CMV in bronchoalveolar lavage fluid by virus culture; (3) immunohistochemical detection of CMV with CMV-specific MoAb, in alveolar macrophages or epithelial cells, obtained by bronchoalveolar lavage; (4) definitive response to therapy with Ganciclovir and high-dose IgG; (5) absence of other pathogens in the lavage sample, known to cause interstitial pneumonitis.

CMV infection was defined as (1) a positive virus culture from bronchoalveolar lavage fluid without any further evidence for CMV pneumonitis; (2) histologically proven extrapulmonary CMV disease at the time of lavage; or (3) a positive virus culture from an extrapulmonary site at the time of bronchoalveolar lavage.

Sensitivity is the ratio of true-positive tests to the sum of the true-positive plus false-negative tests, expressed as a percentage.14 Specificity is the ratio of the true-negative tests to the sum of the true-negative plus the false-positive tests, expressed as a percentage.14 Positive predictive value is the proportion of times that a patient will be
confirmed positive for infection or disease when the test is positive.\textsuperscript{14} Negative predictive value of a test is the proportion of times that a patient will be confirmed negative for infection or disease when the test is negative.\textsuperscript{14}

RESULTS

Detection of CMV in bronchoalveolar lavage (BAL) material by culture, PCR, and direct immunostaining. Data on all patients with either a positive CMV culture, CMV-PCR assay, and/or direct immunostaining of alveolar cells from BAL material is given in Table 1. Eighty-four specimens were examined from 75 patients. Nine patients had two bronchoalveolar lavage procedures. Five of these patients had a second procedure performed within 2 weeks, either because of inadequate initial sampling or because of progressive interstitial pneumonitis without a diagnosis. Four patients had two episodes of interstitial pneumonia, separated by 3 to 18 months. Performance of a second lavage in any of these patients did not change the incidence of either CMV pneumonitis or CMV infection given in Table 1.

CMV was detected by either routine or rapid culture in 10 of 84 (11.9%) of bronchoalveolar lavage samples. Twelve of 84 samples (14.3%) were positive by PCR assay. Nine samples were synchronously positive by PCR and virus culture. One sample, positive by virus culture, was negative by PCR, and three samples positive by PCR, were negative by culture.

Rapid immunostaining for CMV early and late proteins in alveolar cells was performed on 78 of the 84 samples (92.9%). Six samples could not be tested because of paucity of cells. Eleven of 78 (14.1%) specimens were positive. All these specimens had from 1% to 5% of cells positive, significantly above the threshold previously established in our laboratory, to be correlated with a diagnosis of CMV pneumonia.\textsuperscript{7} Four patients had positive cellular immunostaining and a negative PCR, 5 patients were negative by immunostaining but positive by PCR, and 7 were positive by immunostaining and PCR. Four samples were positive by culture and negative by immunostaining, 5 samples were negative by culture and positive by immunostaining, and 6 samples were positive by culture and immunostaining.

Clinical outcome. Clinical follow-up data for all patients with a positive virus culture, PCR, and immunostaining assay from a bronchoalveolar lavage specimen is given in Table 1. According to the diagnostic criteria given in Materials and Methods and predefined for this study, seven patients (9.3%), all recipients of allogeneic marrow transplants, had CMV pneumonitis. In contrast, six patients (8%), had CMV infection without evidence for CMV pneumonia. Four of these patients had CMV cultured from the bronchoalveolar lavage fluid, and two had either histopathological evidence for extrapulmonary CMV disease (CMV adrenalitis) or had a positive CMV culture from an extrapulmonary site (urine) at the same time as the bronchoalveolar lavage.

All seven patients with CMV pneumonitis were positive by direct immunostaining and PCR assay. Six of the seven also had a positive virus culture. Three patients died of respiratory failure secondary to CMV pneumonia (AL, BT, DW). One patient (BT) was positive by direct immunostaining and PCR but was negative by culture. She was on Ganciclovir for 2 weeks before the lavage as treatment for symptomatic CMV viremia. Her pneumonitis failed to respond to Ganciclovir and high-dose Ig and she died of respiratory failure. An autopsy confirmed the presence of severe CMV pneumonitis by histopathology. The presence of CMV in the lung tissue was verified by indirect immunofluorescence assay with CMV specific MoAbs as well as by PCR. Two patients (AL, DW) had a partial response to therapy but eventually died of respiratory failure without an autopsy being performed in either case. Four patients had a complete clinical response after therapy with Ganciclovir and IVIg.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Direct Stain</th>
<th>PCR</th>
<th>Routine Culture</th>
<th>Rapid Culture</th>
<th>Diagnosis</th>
<th>Outcome</th>
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<tr>
<td>DC</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>CMV-P</td>
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</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>-</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>CMV-P</td>
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</tr>
<tr>
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<td>+</td>
<td>-</td>
<td>CMV-P</td>
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<tr>
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<td>+</td>
<td>-</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
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</table>

Abbreviations: CMV-P, cytomegalovirus pneumonia; ND, not done; +, positive; -, negative; PCP, Pneumocystis carinii pneumonia; IP, idiopathic pneumonia.
There were six patients with negative macrophage immunostaining and a positive CMV culture and/or PCR assay; i.e., they did not fulfill the diagnostic criteria for CMV pneumonitis and were not treated with Ganciclovir and Ig. None of these patients developed CMV pneumonitis within the 3-month follow-up period after the lavage. Two of these patients (AR, RD) had fungal pneumonia, 1 (GE) had pulmonary fibrosis with chronic graft-versus-host disease diagnosed by lung biopsy without histopathologic evidence of CMV pneumonitis, and 1 (LB) died of progressive pulmonary failure, secondary to disseminated toxoplasmosis, confirmed at autopsy. No CMV pneumonitis was apparent at autopsy, although there was histopathologic evidence of CMV adrenalitis. No specific diagnosis was made in two patients (SC, BD) with interstitial pneumonitis with a positive CMV culture or PCR assay as the only evidence of CMV infection. Both patients had resolution of their pneumonitis without receiving CMV-specific therapy. Before the era of therapy with Ganciclovir and Ig at Memorial Hospital, the mortality rate for CMV pneumonitis was greater than 95%. Five of six of these patients (83%) recovered from the acute episode of interstitial pneumonitis without receiving any CMV-specific therapy and the only patient to die from this group had disseminated toxoplasmosis at autopsy. Thus, it is unlikely that CMV was the primary cause of interstitial pulmonary infiltrates in this group of patients.

Four patients had positive alveolar cell immunostaining but were negative by PCR and virus culture. All these patients received antiviral therapy for 24 hours pending the results of PCR and rapid virus culture assays, and when these tests were negative, therapy was discontinued. These patients are assumed to have had false-positive immunostaining assays because no further evidence for CMV was found. All four patients were receiving either high-dose IVIg or antithymocyte globulin at the time of the lavage. We have previously noted occasional false-positive immunostaining with CMV-specific MoAbs, presumably caused by nonspecific binding to alveolar macrophages in these circumstances. One patient (XF) had Pneumocystis carinii pneumonia and recovered with specific therapy, and one (DS) had radiation fibrosis proven by open lung biopsy and improved with high-dose steroid therapy. Two patients (LG, LH), had idiopathic pneumonitis. Both of these patients died, without autopsies. Neither DS or XF developed CMV disease or infection in the next 3 months.

No patient with a “negative bronchoscopy study” (58 patients) developed either CMV disease or infection within 3 months following lavage.

Comparison of PCR, virus culture, and direct immunostaining of alveolar cells for the diagnosis of CMV pneumonitis and the detection of infection. The sensitivity, specificity, and predictive values of PCR, virus culture, and the direct immunostaining of alveolar cells for the diagnosis of CMV pneumonitis and the detection of CMV infection are provided in Table 2. The sensitivity and specificity of direct immunostaining and PCR for the diagnosis of CMV pneumonitis were 100%, 94.8%, and 100%, 93.5%, respectively. The sensitivity and specificity for the combination of the two virus culture techniques were 85.7% and 94.8%, respectively. The positive predictive value for each test applied to the diagnosis of CMV pneumonitis was 63.6% for direct immunostaining, 60% for virus culture, and 58.3% for PCR. The negative predictive values were 100% for immunostaining and PCR and 98.6% for virus culture.

When the results of immunostaining and PCR are evaluated concomitantly for the same patient, the positive predictive value of this strategy becomes 100%, namely that all patients with positive macrophage immunostaining and a positive PCR assay had CMV pneumonitis. All patients negative by immunostaining and PCR did not have CMV pneumonitis, i.e., the sensitivity and specificity of the combined results of PCR and immunostaining are 100% (Table 2).

The sensitivity, specificity, and positive and negative predictive values of direct immunostaining for the detection of CMV infection (with or without CMV pneumonitis) in bronchoalveolar lavage fluid were 53.3%, 94.4%, 63.6%, and 91.8%, respectively. Parallel values for the PCR assay were 92.3%, 100%, 100%, and 98.6%, respectively. The sensitivity of virus culture for the detection of CMV infection was lower than PCR (76.9% vs 92.3%). Two patients were PCR positive but culture negative. Both of these patients had concurrent extra-pulmonary CMV infections at the time of lavage (vi-ruria, adrenalitis). One patient was culture positive but PCR negative. Of the 9 patients who had CMV isolated from lavage fluid by conventional culture, 6 were concurrently positive by rapid immunoperoxidase assay, 2 were negative, and 1 did not have the assay performed.

**DISCUSSION**

The availability and efficacy of specific anti-viral therapy has made the accurate diagnosis of CMV pneumonitis a practical necessity. A number of strategies including rapid virus detection with MoAbs and in situ hybridization with cDNA probes, have been used to detect CMV in bronchoalveolar lavage fluid, alveolar macrophages, and lung tissue, with varying degrees of sensitivity and specificity. The diagnostic significance of detecting CMV in bronchoalveolar lavage fluid without concurrent evidence of tissue infection is controversial. The mere presence of CMV in bronchoal-
DIAGNOSIS OF CYTOMEGALOVIRUS PNEUMONIA

veolar lavage material from marrow transplant recipients does not imply that the virus is a pathogen of clinical relevance. CMV infection has to be differentiated from CMV disease. We, and others, have previously reported the high specificity of detecting CMV with MoAbs in alveolar cells from marrow transplant recipients and the presence of CMV pneumonitis. The immunodetection of CMV antigens in alveolar cells implies tissue infection, whereas the detection of CMV by PCR and culture only implies the presence of CMV-DNA and viable virus in the sample. Thus, these assays may have disparate relevance as tools for the diagnosis of CMV disease. In this study, a diagnosis of CMV pneumonitis was made only when a patient fulfilled at least 4 of 5 diagnostic criteria used at Memorial Hospital since 1985. All patients assigned a diagnosis of CMV pneumonitis in this study had (1) clinical and radiologic evidence of interstitial pneumonitis; (2) the presence of CMV in lavage fluid confirmed by either PCR or virus culture; (3) evidence of pulmonary tissue infection by demonstration of CMV-specific antigens by MoAbs in alveolar macrophages or epithelial cells; and (4) no other pathogens beside CMV known to cause interstitial pneumonitis in marrow transplant recipients. In addition, 4 of the 7 patients (DC, JG, CR, YT) had a complete response to the combination of Ganciclovir and IVIg and survived at least 3 months from the time of lavage. Six patients with CMV infection did not fulfill the diagnostic criteria for CMV pneumonitis, were not treated with Ganciclovir and Ig, and 5 of 6 survived. If these patients had true CMV pneumonitis, it is likely, based on our own historical data, that without specific therapy greater than 95% would have died. Histopathologic verification either by lung biopsy or at autopsy remains the "gold standard" for the diagnosis of CMV pneumonitis. One of the 7 patients with presumed CMV pneumonitis (BT) and 1 of 6 patients with CMV infection in lavage fluid (LB) had an autopsy performed in this study. CMV pneumonitis was confirmed in patient BT. Disseminated toxoplasmosis with toxoplasma pneumonia, and CMV adrenalitis without evidence of CMV pneumonitis, was shown in patient LB. Thus, verification of the diagnosis of CMV pneumonitis premortem will remain a difficult problem to resolve, as the trend over the past 3 years has been to forego tissue diagnosis in favor of rapid diagnostic assays and early therapeutic intervention.

The sensitivity of each of the 3 diagnostic methods used alone for the diagnosis of CMV pneumonitis ranges from 85.7% to 100%. The positive predictive value for each test used alone, as a means of predicting the presence of CMV disease was significantly lower, i.e. approximately 60%. However, if the results of direct alveolar cell immunostaining and PCR were evaluated in combination for the same sample, the positive and negative predictive value of this strategy was now 100%. In practice, this means that a patient with suspected CMV pneumonitis can be managed as outlined in Fig 2. Alveolar cell immunostaining with MoAbs is a rapid procedure providing a result in 2 to 3 hours. If this assay is negative, it is extremely unlikely that the patient has CMV pneumonitis. If the assay is positive, definitive therapy should be started, pending the results of the PCR assay usually available in 24 hours. If both direct immunostaining and the PCR are positive, it is very likely that the patient has CMV pneumonitis and treatment should be continued. If no virus is detected by PCR or culture, it is unlikely that the diagnosis is CMV pneumonitis and the potentially toxic and expensive therapy with Ganciclovir and high-dose Ig can be stopped.

The recognition that the presence of CMV in bronchoalveolar lavage fluid or blood is a significant risk factor for the development of CMV pneumonitis following marrow transplantation has facilitated the early and thereby successful prophylaxis of this infection. The presence of virus in bronchoalveolar lavage fluid by culture at day 35 posttransplant appears to have prognostic significance for the future development of CMV pneumonitis. Antiviral prophylaxis therapy with Ganciclovir significantly reduced the incidence of CMV pneumonitis compared with a nontreated control group. Nonetheless, in this study a number of patients developed CMV pneumonia without an antecedent positive culture, possibly indicating that virus culture may not be sensitive enough to detect low titers of virus present in the lung at the time of lavage. The PCR is the most sensitive and specific assay for the detection of CMV in lavage fluid. The high sensitivity of PCR as a means of detecting the presence of CMV-DNA in the lung provides an ideal means for the selection of patients for prophylactic antiviral therapy. We were unable to detect infection by PCR or culture in over 80% of samples tested, approximately half of which were from seropositive individuals. These data confirm that latent or "low level" active CMV infection is not routinely detectable in these patients.

PCR may have a significant advantage over virus culture methods for detecting CMV in those patients receiving Ganciclovir, Foscarnet, or other virus-suppressive drugs. The detection of virus by culture-related methods in these patients may be very difficult because of the very effective antiviral activity of these drugs, thereby reducing the titer of virus in the test material to very low levels. This is an issue of some practical importance as these agents are now being more widely used in clinical practice for prophylaxis of CMV infection often without a clear indication. Thus, studies are underway to compare PCR, rapid virus culture, and CMV antigen detection in leukocytes for
the early detection of CMV infection in blood, bone marrow, and lavage material of marrow and solid organ transplant recipients, before the development of CMV disease as means for selecting those patients most likely to benefit from prophylactic antiviral therapy.

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Rapid diagnosis of cytomegalovirus pneumonia in marrow transplant recipients by bronchoalveolar lavage using the polymerase chain reaction, virus culture, and the direct immunostaining of alveolar cells

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