Cytomegalovirus-Antigenemia-Positive and Polymerase-Chain-Reaction-Negative Transplant Patient

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Clinical History
Patient
38-year-old female with chronic myelogenous leukemia (CML).

History of Present Illness
This 38-year-old woman presented to her primary physician with complaints of back pain. A bone marrow (BM) examination revealed a hypercellular marrow with increased myeloid precursors. Fluorescence in situ hybridization and reverse transcriptase (RT)-polymerase chain reaction (PCR) analysis of BM myeloid cells confirmed the diagnosis of CML by demonstrating the presence of the bcr-abl fusion gene and transcript. She began Gleevec therapy but experienced significant side effects and failed to achieve cytogenetic remission. After 3 years, she received a matched related-donor allogeneic stem-cell transplant and was treated with immunosuppressive therapy. Routine follow-up after transplant revealed the results listed below.

Principal Laboratory Findings
Cytomegalovirus (CMV) pp65 antigenemia: 400 positive cells/50,000 leukocytes; real-time PCR for CMV (glycoprotein B gene): < lower limit of detection (LLD) (1,000 copies/mL); real-time PCR for CMV (UL54 gene): 133,000 copies/mL.

Questions
1. What is CMV, and why is CMV infection significant in transplant recipients?
2. What laboratory tests are used for the detection of active CMV infection?
3. What is pp65 antigenemia?
4. What is real-time PCR?
5. What are the advantages of using real-time PCR over other methods for detecting (eg, viral culture) and/or quantifying viral DNA?
6. How do you explain the patient’s test results?
7. What assay(s) would you use to definitively explain the test results?

Possible Answers
1. Cytomegalovirus (CMV) is a member of the Beta-herpesvirinae genus in the subfamily of Herperviridae. It is a ubiquitous virus, and most individuals are infected with CMV at some time during their lifetime. In immunocompetent individuals, it is usually an asymptomatic infection, or, less commonly, infection presents as a mononucleosis syndrome. The virus, however, can cause life-threatening complications in immunocompromised individuals. Infection is of particular concern in patients with HIV or after solid-organ or bone-marrow transplant. Cytomegalovirus infections can contribute to vanishing bile duct syndrome in liver-transplant patients, accelerated coronary or renal artery stenosis in heart and renal transplant patients, graft rejection in solid-organ transplant recipients, and exacerbation of graft-versus-host disease. Pneumonitis is a common problem seen in bone-marrow transplant individuals with active CMV infection. Moreover, active CMV infection in transplanted patients can predispose them to more aggressive bacterial and fungal infections.

2. Traditional laboratory tests used for the detection of CMV include viral culture and serological methods; however, these methods lack the ability to differentiate an active infection from latent persistence and provide little information about the status of the patient. Moreover, antiviral prophylactic treatment protocols carry risks associated with drug exposure, namely drug toxicity and resistance. The decision whether to treat is based on the clinical data and the ability to predict CMV disease in immunocompromised hosts. Thus, newer tests such as pp65 antigenemia and real-time polymerase chain reaction (PCR) are more helpful for clinicians considering patient treatment options, as they are better markers for viral replication and allow for quantification of viral organisms.

3. The pp65 antigenemia assay uses an antibody cocktail that specifically binds to CMV pp65. The pp65 antigen is present in early viral replication and can be detected in CMV-positive polymorphonuclear cells (PMNs). Indirect immuno- fluorescence is used for the detection and quantification of CMV pp65 positive PMNs. The number of positive PMNs per duplicate stain is counted, and the results are reported as positive cells per 50,000 leukocytes. Antigenemia is a good marker for predicting CMV disease. The cutoff value for instigating prophylactic treatment varies among different patient groups. Ten positive cells per 2 × 10^5 is the recommended threshold for beginning therapy in patients who have received solid-organ transplants or stem-cell transplants. A lesser value of 2 positive cells is recommended for bone-marrow transplant patients. The test is rather labor-intensive, and the patient must have an acceptable leukocyte count for optimal test performance. Moreover, samples must be processed quickly (within 6 hours) for optimal test results. This patient had 400 positive cells per 50,000 leukocytes.

4. Polymerase chain reaction is a method of logarithmic amplification of short segments of DNA from within a larger molecule of double-stranded DNA. The technology requires the use of primers, which are short lengths of nucleotides complementary to the length of the DNA in question. DNA
polymerase is used to extend these primers once they have annealed to target DNA. Thus, complementary copies of the DNA in question are generated. The primers can be used again in multiple rounds of amplification. In end-point PCR, after a set number of cycles, the product is analyzed by an additional method, such as gel electrophoresis. Real-time PCR generates a fluorescent signal from amplification products. This fluorescent signal can be generated using a sequence-specific, fluorescently labeled probe located between the PCR primers or non-sequence-specific fluorescent dyes. One example of a sequence-specific fluorescently labeled probe is the TaqMan category of probes. These probes have a fluorescent molecule on one end of the probe (FAM) and a quencher molecule (BHQ) on the other end of the probe. The close proximity of the quencher molecule to the fluorescent molecule prevents the FAM molecule from fluorescing when excited by a laser. During extension of the primers, the exonuclease activity of the DNA polymerase (ie, Taq polymerase) cleaves the nucleotide containing the quencher molecule away from the probe, causing the FAM molecule to fluoresce. An animation of this process is available online. The amount of fluorescence produced and the cycle number in which the fluorescence crosses the threshold (or baseline) is directly proportional to the amount of target nucleic acid in the patient specimen. When combined with calibrators, real-time PCR is quantitative.

5. Real-time PCR assays are sensitive and can accurately quantify viral DNA in specimens containing hundreds of viral DNA copies/mL to millions of copies/mL depending on the range of the laboratory’s standard curve. Results using these assays are reproducible, and the turnaround time is substantially improved when compared with traditional methods such as viral culture. Unlike the pp65 antigenemia assay, the viral DNA required for analysis using real-time PCR does not require intact cells.

6. Remember that in PCR, oligonucleotide primers are used to amplify a specific target sequence within a particular region of the genome. When mutations occur within the primer binding sites of the gene, the primers cannot bind to the template DNA, and no amplification can occur. Similarly, if a nucleic acid change is present at the binding site for the TaqMan probe, then the PCR product may not be detectable. In this particular case, the initial PCR assay used primers specific for the CMV glycoprotein B gene (ie, forward primer 5'-AAGTACCCCTATCGCGTGTG; reverse 5'-ATGATGCCCTC[A/G]TCCA[A/G]GTG; TaqMan probe 5'-FAM TGGCCCAGGG- TACGGATCTTATTCG-BHQ) and was performed prior to pp65 antigenemia testing. The positive pp65 antigenemia result was surprising given the initial negative PCR result. Suspecting that there was a mutation within the primer or TaqMan probe binding region of the viral genome, PCR was repeated using another PCR assay with a primer set targeting a different gene (ie, the UL54 gene [primer/probe sequences are proprietary; Roche Diagnostics, Indianapolis, IN]). This case illustrates that discrepancies can occur between PCR and classic methods of viral detection (eg, pp65 antigenemia assay). These discrepancies can be resolved by using a primer set directed toward a second gene in the viral DNA genome.

7. Several experimental approaches can be used to detect the presence of a point mutation in the primer binding site for the CMV glycoprotein B gene. First, the annealing temperature of the PCR reaction could be lowered, thus allowing less stringent hybridization of the primer to the target area resulting in a PCR product. Second, a separate set of primers that would yield a larger PCR product (ie, containing the original PCR sequence within it) would permit further analysis using high resolution melting-curve analysis. A change in the melting temperature of this patient’s sample compared with control CMV samples would indicate that the sequence was different; however, this approach may not detect nucleic acid changes that do not affect the melting temperature of the PCR product. Finally, the most definitive method would be to perform sequence analysis on the larger PCR product that includes the region of the gene amplified by the original PCR reaction. Once the sequence data is obtained, the primer and TaqMan probe binding sites of the original PCR would be compared with the control CMV sequence. IM

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