Quantitation of Cytomegalovirus (CMV) DNA in Leukocytes of Human Immunodeficiency Virus-Infected Subjects with and without CMV Disease by Using PCR and the SHARP Signal Detection System

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Received 3 September 1996/Returned for modification 16 October 1996/Accepted 18 November 1996

We report the development of a simple and rapid PCR assay for quantification of the cytomegalovirus (CMV) DNA load in polymorphonuclear leukocytes. Using this system, a very good correlation was found between a high number of CMV copies in the blood and the presence of CMV disease in subjects with AIDS.

PCR has been shown to be an extremely sensitive method for detecting small amounts of cytomegalovirus (CMV) DNA in polymorphonuclear leukocytes (PMNL) of immunocompromised subjects (3, 12, 14). However, interpretation of a positive test is often difficult because the detection of CMV by PCR alone may not be associated with symptomatic CMV infections (7, 14). Quantitative-competitive methodologies have been developed in order to increase the specificity of a PCR test. However, these assays are not of immediate clinical use because of expensive and cumbersome detection procedures (1, 6, 15). We report a rapid, sensitive, and reproducible quantitative PCR protocol using a commercially available detection kit, the SHARP Signal System.

PMNL from human immunodeficiency virus (HIV)-infected, CMV-seropositive individuals were obtained after dextran separation of EDTA-treated blood. An aliquot of 105 PMNL was dedicated to the pp65 antigenemia (AG) assay (IC3 clone; Argene, Parc Technologique Delta Sud, France). For PCR, a second aliquot of PMNL was mixed with a lysis solution (1× PCR buffer [Promega Corporation, Madison, Wis.] and proteinase K [ICN Biomedical Inc., Aurora, Ohio] [final concentration, 120 μg/ml]) to obtain a concentration of 106 PMNL/μl. The mixture was incubated 1 h at 56°C followed by proteinase K inactivation for 10 min at 95°C. Each 100-μl PCR mixture contained DNA from 105 PMNL, 1× PCR buffer (Promega), 1.5 mM MgCl2 (Promega), 50 pmol of each primer, 200 μM each deoxyribonucleoside triphosphate, and 2.5 U of Taq DNA polymerase (Promega). Primer sequences were as follows: 5′-TGT CCT CCC GCT CTC C-3′ (forward) and 5′-biot in-ATG AAG GTC TTT GCC CAG TA-3′ (reverse). Samples were denatured 5 min at 94°C and thermocycled for 30 cycles (1 min at 94°C, 2 min at 56°C, 2 min at 72°C) with a final extension of 2 min at 72°C. PMNL DNA samples from CMV-seronegative blood donors were selected as negative controls. DNA samples from cells infected with the CMV AD-169 strain and a plasmid containing the major immediate-early (MIE) region of the CMV Towne strain (Digene Diagnostics Inc., Silver Spring, Md.) served as positive controls. All negative specimens for CMV were also tested for cellular integrity and the presence of PCR inhibitors by amplification of the β-actin gene (4).

The PCR products were detected by using the SHARP Signal System (Digene Diagnostics Inc, Silver Spring, Md.) as recommended by the manufacturer. Briefly, the biotinylated PCR fragments were denatured and hybridized with a specific single-stranded RNA probe containing the entire coding sequence and introns of the CMV MIE gene. The RNA:DNA hybrids were then captured through biotin onto the surface of streptavidin-coated microwells. Immobilized hybrids were first reacted with an alkaline phosphatase-conjugated antibody specific for RNA:DNA hybrids and then detected with the addition of a colorimetric substrate. The optical density (OD) at 405 nm was read after different incubation times of the substrate (0.5, 1, 2, and 24 h) on a conventional microplate reader. The positive cutoff value (at 24 h) was the greater of [(2 × mean NPC) + 0.080] or 0.100 OD unit, where NPC is the negative PCR control. In each experiment, serial dilutions of the CMV plasmid DNA (25 to 25,000 copies) were amplified in presence of DNA extracted from 105 PMNL of CMV-seronegative donors. Standard curves of each PCR assay were obtained by plotting the OD values read after different incubation times of the substrate against the number of input CMV copies. The original number of CMV copies present in 105 PMNL from patients was determined by interpolating the OD values into the standard curves. Over the range of results obtained (from 25 to 25,000 copies), this assay was found to have an intra-assay variability of 15% and an interassay variability of 24%. Statistical analyses were done after square-root transformation of the raw data. Comparison between the three groups of patients was performed by using an analysis of variance. Correlation between the PCR and the AG assay was done with the Pearson correlation test.

The quantitative PCR assay was compared to the AG assay using PMNL of HIV-infected subjects with asymptomatic CMV infection (group 1 CD4 counts, 100 to 250/mm3; group 2 CD4 counts, <100/mm3) and symptomatic untreated CMV disease (group 3) (Table 1). A good correlation was found between the number of CMV copies in PMNL and the number of pp65-positive cells when all groups were combined (r² = 0.74, P < 0.01). There was a statistically significant difference...
TABLE 1. Number of CMV copies and pp65-positive cells in PMNL of HIV-infected subjects

<table>
<thead>
<tr>
<th>Group(s)</th>
<th>No. of positive samples/no. of samples (% positive)</th>
<th>Mean/median copy no.</th>
<th>No. of positive samples/no. of samples (% positive)</th>
<th>Mean/median no. of positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23/74 (31.1)</td>
<td>38/80</td>
<td>6/85 (7.0)</td>
<td>1.5/0</td>
</tr>
<tr>
<td>2</td>
<td>67/164 (40.8)</td>
<td>603.60</td>
<td>32/218 (14.7)</td>
<td>2.7/0</td>
</tr>
<tr>
<td>1 + 2</td>
<td>90/238 (37.8)</td>
<td>428.00</td>
<td>38/303 (12.5)</td>
<td>2.4/0</td>
</tr>
<tr>
<td>3</td>
<td>16/16 (100.0)</td>
<td>20,452.9/5</td>
<td>25/26 (96.2)</td>
<td>435.5/50</td>
</tr>
</tbody>
</table>

*Per 10^5 PMNL.

*A P < 0.01 compared to groups 1 + 2.

in the mean CMV DNA load (P < 0.01) and the mean CMV antigenic load (P < 0.01) between asymptomatic subjects (groups 1 and 2 combined) and patients with CMV disease (group 3). For diagnosis of CMV disease, PCR and AG assays had sensitivity (SE), specificity (SP), positive predictive (PP), and negative predictive (NP) values of 100.0 and 96.1%, 62.4 and 87.4%, 15.1 and 39.7%, and 100.0 and 99.6%, respectively. By using a cutoff of 1,000 copies in the PCR assay and 5 pp65-positive cells in the AG assay (per 10^5 PMNL), these values became 87.5 and 84.6%, 96.2 and 94.0%, 60.9 and 55.0%, and 99.1 and 98.6%, respectively. In that context, 44 and 50% of false-positive results obtained with PCR and AG, respectively, were from asymptomatic patients who subsequently developed CMV disease (within 1 year of testing).

We designed a simple, rapid, and reproducible quantitative PCR protocol for CMV that is particularly suitable for analysis of large series of samples without the need for sophisticated equipment. For detection of the amplified products, we used the Digene SHARP Signal System that consists of a nonisotopic hybridization and an enzyme-linked immunosorbent assay in a microplate format. The sensitivity of this system for the detection of CMV DNA in blood was shown to be comparable to that of Southern blot hybridization (5). Contrasting with previous studies (9, 13), the reproducibility of our results over a large dynamic range (25 to 25,000 copies) by using the Sharp Signal System was excellent. This can be explained, in part, by careful optimization of the PCR conditions and detection of the amplicons at various time points. As previously reported (10), we found higher amounts of CMV DNA in PMNL of HIV-infected patients with CMV disease than in subjects with similar CD4 counts but asymptomatic CMV infection (Table 1). However, we and others have shown that the correlation between the circulating viral load and the development of CMV disease is not perfect, indicating that other factors are also important in the pathogenesis of this disease (2, 11). Nevertheless, the PCR assay described here appears particularly promising in confirming nonretinal CMV disease, monitoring the effects of antiviral therapy (2, 8), and selecting the candidates for preemptive therapy. In that regard, almost 50% of asymptomatic subjects who had >1,000 CMV copies per 10^5 PMNL went on to develop CMV disease within 1 year of testing.

We also found a good correlation between our quantitative PCR and the AG assay. The two methodologies showed similar sensitivity and specificity for the diagnosis of CMV disease when optimal threshold values were selected. Advantages of our PCR system include (i) lesser need for rapid recovery of PMNL from blood (alternatively, frozen plasma can also be substituted; unpublished data), (ii) greater potential for batch testing, and (iii) larger dynamic range (samples with a high number of CMV copies can be diluted and retested). On the other hand, the AG assay gives more-rapid results (6 h versus 24 h) and requires less-stringent laboratory procedures such as dedicated rooms and materials. Prospective studies are now needed to validate the use of the blood CMV DNA load as a surrogate marker for assessing the risk of CMV disease in HIV-infected subjects.

This work was supported by a grant (no. 6605-4309) from the National Health and Research Development Program (Canada). Guy Boivin is a scholar of the Medical Research Council of Canada.

We thank the following physicians for submitting blood samples from their patients: Hélène Senay, Louise Côté, Sylvie Trottier, Michel Morissette, Alain Martel (all in Quebec City), Réjean Delisle (Chicoutimi), and Jean-Pierre Routy (Montreal).

REFERENCES


