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Human Cytomegalovirus (HCMV) Late-mRNA Detection in Peripheral Blood of AIDS Patients: Diagnostic Value for HCMV Disease Compared with Those of Viral Culture and HCMV DNA Detection

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A reverse transcriptase polymerase chain reaction (RT-PCR) was used to detect a human cytomegalovirus (HCMV) late mRNA in peripheral blood leukocytes (PBL) of 102 human immunodeficiency virus-infected individuals. The clinical value of this new technique for the diagnosis of acute HCMV disease was evaluated in comparison with viral culture and direct amplification of viral DNA (PCR). The sensitivity of the RT-PCR was slightly lower than that of the two other methods, but its specificity was 94%, compared to 55 and 32% for culture and PCR, respectively. Transcription of this late mRNA is linked to viral replication, and its detection in PBL confirms that these cells can support a complete viral cycle. The relationship between complete replicative cycles and HCMV disease makes RT-PCR a useful clinical tool.

Human cytomegalovirus (HCMV) is the main opportunistic viral pathogen in AIDS patients, causing frequently life-threatening disease, including retinitis, gastrointestinal conditions, pneumonia, and neurological disorders (7). Detection of HCMV in peripheral blood leukocytes (PBL) or viremia is the hallmark of disseminated infection and can easily be performed by viral culture or rapid detection of viral antigens. However, the results of these techniques do not necessarily correspond to HCMV disease status. Indeed, if viremia is predictive for subsequent HCMV organ involvement (15), it is not a basis for therapeutic decision-making, since AIDS patients can be viremic without HCMV disease. Direct detection of HCMV early antigen in polymorphonuclear cells has recently been described (8, 20), with quantification by counting antigen-bearing cells. A positive correlation between HCMV disease and a large number of positive cells was reported (8, 12) but requires confirmation. Detection of viral nucleic acids in PBL, initially performed by dot-slot hybridization (18) or in situ hybridization (5), is now generally done by using the polymerase chain reaction (PCR) technique in AIDS patients (for examples, see references 6, 8, 16, and 21). However, the high sensitivity of enzymatic amplification makes this technique unusable for the diagnosis of HCMV disease (6).

A reliable blood test for likely HCMV organ involvement would thus be valuable in this setting. We have previously developed a reverse transcriptase polymerase chain reaction method (RT-PCR) for indirect detection of late viral mRNA by initially converting it into a cDNA which is then amplified by means of PCR (9). We confirmed in vitro the late kinetics of the chosen mRNA (encoding for the major capsid protein of HCMV) and the link between its transcription and viral replication (9). RT-PCR appeared to be a specific and sensitive method (a single infected cell could be detected) with potential clinical applications.

The objective of the present work was to study the clinical value of the RT-PCR method for the diagnosis of HCMV disease in AIDS patients, in comparison with viral culture and direct PCR detection of viral genomic DNA from PBL.

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Peripheral blood samples were obtained from 102 human immunodeficiency virus-infected individuals (95 men and 7 women; mean age, 39 years), classified as follows: 13 asymptomatic patients or patients with AIDS-related complex with CD4 cell counts of >200/μl (mean: 395/μl) and no suspicion of HCMV disease (group I); 47 symptomatic patients (10 with AIDS-related complex, 37 with AIDS) with CD4 cell counts of <200/μl (mean: 34/μl) for whom HCMV disease was later ruled out on the basis of the clinical course or negative complementary test results (group 2); and 42 AIDS patients (mean CD4 cell count: 19/μl) with proven HCMV organ involvement before starting specific therapy (group 3). Forty healthy control subjects, 23 of whom were HCMV seropositive, were also studied. Patients who had received anti-HCMV treatment less than 3 months before the blood tests were excluded.

HCMV organ involvement was diagnosed in the 42 group 3 patients as follows. Retinitis (n = 18) was diagnosed by routine ophthalmologic examination (white areas, perivascular exudation, and hemorrhage); gastrointestinal illness (n = 14) was diagnosed on the basis of the gross endoscopic aspect (ulcerative colitis, n = 4; esophagitis, n = 4; gastritis, n = 6) and confirmed by viral culture or histological examination of biopsy specimens; HCMV-related neurological disease (n = 6) was diagnosed if the patient had a compatible neurological disorder (myeloradiculitis, n = 2; encephalitis, n = 3; peripheral neuropathy, n = 1) and confirmed either by viral culture or positive PCR (10) from cerebrospinal fluid or by autopsy findings; HCMV-related pneumonia (n = 2) was diagnosed by positive histologic examination of the lung parenchyma concomitant to unexplained interstitial pneumonia; HCMV-related hepatitis (n = 1) or adrenalitis (n = 1) was diagnosed in patients with liver or adrenal dysfunction

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TABLE 1. Primers and probes for DNA and mRNA detection

Primer or probe[a]	Location[b]	Sequence (5' to 3')	Product length
CMCP1	1198–1217	GTGATCCGACTGGGCGAAAA	263 (mRNA orDNA)
CMCP2	1461–1442	GAGCGCGTCCACAAAGTCTA	
CMCP3 (probe)	1326–1364	GCCCGAGGATCGCGGTTACACCACGGTGGAAAGCAAAGT	
IES1	338–357	AACGAGTGACCGAGGATTGC	240 (mRNA)
IES2	571–552	ACTTCATCTCCTCGAAAGGC	395 (DNA)
IES3 (probe)	424–453	GTGCGGCATAGAATCAAGGAGCACATGCTG	

[a] CMCP, major capsid protein-encoding gene; IES, immediate-early 1-encoding gene, adapted from Buffone et al. (3).
[b] Numbered from the presumed start site of the mRNA; sequence data from references 13 and 14.

and positive virological or histological study of biopsy or autopsy specimens.

The detection of early antigen foci (DEAF) or viremia was performed on the buffy coat by a rapid culture technique, using a monoclonal antibody (clone E13; Clonatec Biosoft, Paris, France), in a classical immunoperoxidase reaction described elsewhere (1).

The detection of viral nucleic acids was performed on PBL preparation isolated by Histopaque 1119 gradient centrifugation (Sigma, L'isle d'Abeau Chesnes, France) from 10 ml of heparinized blood. Two sets of oligonucleotides were synthesized (Table 1). The first one, located in the late gene encoding the major capsid protein (4), was used in all the samples for DNA and late-mRNA detection. The second, located in the major immediate-early 1 (IE1) antigen-encoding gene (19) and flanking the exon 3 to 4 splice junction site, was used in a subgroup of 27 patients for the detection of DNA and IE mRNA.

The PCR and RT-PCR for detection of DNA and mRNA were performed as described before (9). Briefly, DNA was extracted from 5×10^5 cells after a rapid lysis in the presence of Nonidet P-40, Tween 20, and proteinase K and amplified in the following reaction mix: 50 pmol of primers, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, and 1.25 U of cloned *Taq* polymerase (Perkin Elmer Cetus, Emeryville, Calif.). The samples were covered with 100 μl of paraffin oil, denatured for 5 min at 94°C, and amplified for 40 cycles in a thermal cycler as follows: 1 min of denaturation at 94°C, 1 min of annealing at 60°C, and 1 min of polymerization at 72°C. This last step was extended to 10 min in the last cycle to ensure complete amplification.

Total RNA was extracted from the same number of cells with the RNAzol RNA extraction system (Bioprobe, Paris, France), and cDNA was synthesized with 100 U of murine Moloney RT (BRL, Bethesda, Md.) and 50 pmol of antisense primer (CMCP2 or IES2). Thereafter, cDNA was amplified by the same PCR procedure as described above, following the addition of 50 pmol of the second primer (CMCP1 or IES1) and 1.25 U of *Taq* polymerase.

The products were analyzed by electrophoresis on a 2% agarose gel, Southern blotting, and probing with the ^{32}P-kinase-labeled oligoprobe (CMCP3 or IES3).

Controls. All the recommended precautions (11) to avoid contamination by PCR product carryover were followed, including physical separation of the pre- and post-PCR steps and the systematic use of filtered tips. Furthermore, two negative and positive controls were included in each experiment. To determine whether cell specimens were suitable for DNA amplification, all of the negative samples were controlled by amplification with primers flanking the β-globin gene as described elsewhere (14). We also checked that the RNA extract was free of contaminating DNA by amplifying directly the RNA sample without the RT step.

Detection of early antigen foci, DNA (direct PCR), and late mRNA (RT-PCR) in PBL of HIV-infected individuals and healthy controls (Table 2). Markers of HCMV infection were undetectable in group 1 patients. In group 2, viremia was detected in 21 patients, DNA was detected in 32 patients, and late mRNA was detected in 3 patients. These last three patients did not develop HCMV disease (follow-ups at 9, 4, and 2 months). In group 3, viremia was detected in all cases except two (one case of retinitis and one of esophagitis); HCMV DNA was also detected in all but two patients (both with gastrointestinal disease), and β-globin gene amplification was also negative in these last samples. Late mRNA was detected in 34 patients (three cases of retinitis and five cases of gastrointestinal disease were missed).

None of the 40 healthy controls had detectable HCMV.

To investigate the relevance of the three methods used for the diagnosis of HCMV disease, their sensitivity and specificity were calculated for the patients (groups 2 and 3) in whom HCMV disease was suspected. The sensitivity and specificity were, respectively, 95.2 and 55.3% for DEAF, 95.2 and 31.9% for DNA detection, and 81 and 93.6% for late mRNA detection. The positive predictive value of late mRNA detection was 91.9%, compared to 65.6 and 55.6%, respectively, for DEAF and DNA detection.

Detection of IE mRNA in viremic patients. To determine whether the good specificity of the mRNA detection technique was due to the late kinetics of the transcript chosen, we used the same RT-PCR method to detect an IE transcript of HCMV in a subgroup of 27 patients. All of the patients (n = 18) with positive viremia or HCMV DNA test results had detectable IE HCMV mRNA in their PBL, including those (n = 12) with no HCMV disease and no detectable late mRNA. In contrast, the IE mRNA was not detected in the nonviremic patients (n = 9).

We describe here the clinical application of the detection of HCMV mRNA by means of RT-PCR. The primers used were located in the major capsid protein-encoding gene, and the late kinetics of the transcript chosen means that its

TABLE 2. Distribution of the three HCMV markers in the different HIV-positive populations

Test	No. (%) of positive patients		
	Group 1 (n = 13)	Group 2 (n = 47)	Group 3 (n = 42)
Viremia	0 (0)	21 (44.7)	40 (95.2)
DNA	0 (0)	32 (68)	40 (95.2)
Late mRNA	0 (0)	3 (6.4)	34 (81)

presence in peripheral blood indicates effective viral replication (9). Most of the patients with disseminated infection and acute HCMV disease had detectable late viral mRNA in their PBL, suggesting that these cells can support a complete viral cycle. This is in keeping with a previous study (5), in which in situ hybridization was used to detect the transcription of IE, early, and late HCMV mRNA in polymorphonuclear cells from viremic patients. Moreover, Spector et al. (17) recently showed the presence of free HCMV DNA in plasma from patients with AIDS and HCMV visceral disease, suggesting the release of virus particles by infected leukocytes. In contrast, when disseminated disease was absent, the late mRNA was seldom detected, even in viremic patients in whom transcription of an IE transcript was detectable. This pattern of restricted transcription is reminiscent of the restricted expression of IE gene products observed after infection of mononuclear cells in vitro (13). PBL thus appear to be an important vector of virus dissemination, especially when abortive infection switches to a complete and lytic cycle, allowing new infectious particles to reach other targets. The factors controlling this steady-state situation are unknown, but the relationship between visceral disease and the detection of late transcripts in PBL may be useful for diagnosing HCMV disease in AIDS patients. By comparison with DEAF and direct PCR for genomic viral DNA, the detection of late mRNA by RT-PCR was highly specific for the diagnosis of HCMV visceral disease.

The sensitivity of RT-PCR was less satisfactory, as some patients with HCMV disease escaped detection. The detection limit for mRNA in our RT-PCR technique is about 100 fg of total RNA extracted from infected cells in vitro (9), and this could be insufficient in some clinical situations in which weak transcription occurs. Alternatively, RNA is very unstable and its degradation in vitro, despite the precautions taken to avoid it, could also explain these false-negative results. Finally, HCMV visceral disease without systemic viral replication may be possible. Nevertheless, the excellent specificity of the RT-PCR method makes it a potentially useful tool for the diagnosis of HCMV disease, particularly in clinical situations in which diagnosis is difficult, e.g., central nervous system disease, pneumonia, hepatitis, and adrenal gland involvement. It has now to be compared with other methods which have been correlated with symptomatic HCMV disease, such as quantitative pp65 antigenemia testing and DNA PCR on serum or plasma (2, 17). The value of RT-PCR for monitoring asymptomatic viremic patients with a view to early treatment or prophylaxis of HCMV disease remains to be determined.

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