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Development and Clinical Significance of a Diagnostic Assay Based on the Polymerase Chain Reaction for Detection of Human Cytomegalovirus DNA in Blood Samples from Immunocompromised Patients

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The presence of human cytomegalovirus (HCMV) DNA in blood was investigated by the polymerase chain reaction (PCR) in 293 blood samples from 86 immunocompromised patients. Of the 86 patients, 23 underwent clinical and virologic follow-up for HCMV infection. In parallel, blood samples were examined for viremia and antigenemia. Concordant results between PCR and assays for viremia and antigenemia were obtained on 124 positive and 110 negative samples, with an overall concordance of 79.8%, while 59 samples (most from patients with HCMV infection) were positive by PCR alone. PCR is a new powerful tool for detection of HCMV infections in blood samples from immunocompromised patients. However, its clinical significance appears to be restricted to the indication of a risk of reactivation of HCMV infection.

Human cytomegalovirus (HCMV) viremia is a hallmark of disseminated infection in immunocompromised patients. Among leukocytes, polymorphonuclear leukocytes (PMNLs) appear to be the major carrier of virus in blood (2). Procedures thus far available for HCMV detection in blood include determination of viremia (12, 17), antigenemia (12, 17), and DNAemia by hybridization procedures (13, 15) and, recently, by DNA amplification through the polymerase chain reaction (PCR) (4, 8, 14). The sensitivity as well as the clinical significance of results provided by PCR remains substantially undefined.

The present study addresses this problem through simultaneous determination of viremia, antigenemia, and DNAemia (as determined by PCR) in sequential blood samples from immunocompromised patients.

A total of 293 blood samples from 86 immunocompromised patients were examined for HCMV viremia, antigenemia, and DNAemia, including samples from 33 heart transplant recipients (HTRs), 32 AIDS patients, 18 human immunodeficiency virus-seropositive subjects, 2 infants with congenital HCMV infection, and 1 kidney transplant recipient. Of these 86 patients, 19 HTRs and 4 AIDS patients underwent virologic follow-up for variable periods of time. In addition, single blood samples from 2 HCMV-seronegative and 10 HCMV-seropositive healthy subjects were examined as controls. Ganciclovir was administered intravenously at a standard dosage of 10 mg/kg of body weight per day for 14 days to 8 HTRs with primary or recurrent infections, when levels of viremia or antigenemia were >50 infected PMNLs of 2×10^5 cells examined in the presence of HCMV-related clinical symptoms.

Aliquots of 2×10^5 PMNLs, separated by sedimentation of heparinized blood on a 6% dextran solution (blood and dextran, 5/1 [volume]), were used for PCR (4), as well as for quantitative determination of viremia and antigenemia, as previously reported (12, 18).

Two pairs of oligonucleotide primers were chosen for DNA amplification in two regions of the HCMV genome coding for the major immediate-early antigen (IE1) (1, 16) and for a structural matrix phosphoprotein (pp150) (9, 10), respectively (4). The primer specificity and sensitivity for HCMV DNA detection have been recently established in our laboratory (19). Both pairs of primers could detect all wild-type viral isolates tested so far and gave consistently negative results when the assay was performed on DNA of related human herpesviruses (herpes simplex virus types 1 and 2, Epstein-Barr virus, varicella-zoster virus, and human herpesvirus 6).

Samples of PMNLs for PCR analysis were suspended in PCR buffer with nonionic detergents and proteinase K (Boehringer, Mannheim, Germany) (7) and subjected to DNA amplification, following proteinase K inactivation, as previously reported (4, 19). Each sample was tested with both pairs of primers in two different reactions. The PCR reaction was performed in a Thermal Cycler (Perkin-Elmer Cetus) for 50 cycles (94°C for 1 min, 55°C for 2 min, and 72°C for 3 min). Twenty microliters of each sample was then subjected to electrophoresis on a 10% polyacrylamide gel in Tris-borate-EDTA (TBE) buffer, stained with ethidium bromide and photographed. Thirty microliters of each sample was also analyzed by liquid hybridization (19) with 0.2 pmol of ^{32}P-labeled specific oligonucleotide probe (4). The samples were then electrophoretically separated on an 8% polyacrylamide gel in TBE buffer, and the gel was finally autoradiographed with Hyperfilm-MP film (Amersham International, Amersham, United Kingdom) at −80°C for 3 to 5 h.

To avoid contamination by PCR product carryover and false-positive results, samples were prepared in a different laboratory from that in which the amplification reactions were performed. In addition, reagent aliquots, positive dis-

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TABLE 1. Correlation between PCR, HCMV quantitation in PMNLs, and clinical symptoms in four HTRs

Patient no.	Patient age (yr)	Type of HCMV infection	No. of days after transplantation	PCR result	HCMV quantitation in PMNLs[a]		Presence of HCMV-related clinical symptoms[b]
					Antigenemia	Viremia	
1	36	Primary	12–30	Negative	0	0	−
			33	Positive	0	0	−
			40	Positive	69	80	+
			44	Positive	63	83	+
			51	Positive	72	80	+
			55[c]	Positive	36	40	+
			56	Positive	5	0	−
			58–82	Positive	0	0	−
			111–162	Negative	0	0	−
2	38	Recurrent	6	Positive	0	3	−
			9	Positive	2	5	−
			14	Positive	49	31	−
			20	Positive	142	100	+
			21[c]	Positive	215	200	+
			24	Positive	16	0	−
			36–120	Negative	0	0	−
3	54	Recurrent	4–7	Negative	0	0	−
			19	Positive	0	0	−
			25	Positive	1	3	−
			47	Positive	3	5	−
			62–92	Positive	0	0	−
			125–131	Negative	0	0	−
4	53	Recurrent	5	Negative	0	0	−
			11–15	Positive	0	0	−
			22	Positive	4	1	−
			25	Positive	3	1	−
			29	Positive	2	0	−
			32	Positive	1	1	−
			36–57	Positive	0	0	−
			64–102	Negative	0	0	−

[a] Number of HCMV-positive PMNLs per 2×10^5 cells examined by direct antigen detection in PMNLs (antigenemia) or by immediate-early antigen detection in HELF cell cultures (viremia).

[b] −, absent; +, present.

[c] Initial day of ganciclovir administration, which was continued for 14 days at a dosage of 10 mg/kg/day. In patient 1, treatment was delayed because of a temporary unavailability of the drug. Patients 3 and 4 were not given ganciclovir.

placement pipettes, and all other recommended precautions (11) were used. Furthermore, every set of reactions included a control with no DNA ("0" DNA), a negative control consisting of uninfected HELF cells, and a positive control of 100 to 1,000 HCMV-infected HELF cells, with an average of one negative control for every five samples examined. Each sample was tested twice in two different experiments, and only samples giving positive results with both pairs of primers were scored positive.

PCR sample quality is known to be largely dependent on the number of contaminating erythrocytes (RBCs) and on the procedure adopted for DNA extraction (3, 6). In this study, treatment of samples with NH_4Cl solution was a fast and effective method for removal of undesired RBCs and could also be accomplished on stored frozen samples without significant loss of material. Prior to introduction of routine RBC lysis, less than 2% of positive (as determined by positive viremia and antigenemia results) samples examined were resistant to amplification. The use of a "one-tube" method of template preparation, such as the procedure using nonionic detergents and proteinase K (7), appeared superior to conventional DNA separation procedures by sodium dodecyl sulfate lysis and organic solvent extraction, when handling samples with low numbers of cells. Reproducible results were obtained by PCR upon satisfaction of these requirements and establishment of optimal reaction parameters. Contaminations were observed only sporadically, and the test could be completed in 12 h. The sensitivity of the assay was striking, and although samples contained, on average, less than 1 infected cell in 10,000, most samples could be evaluated directly by UV transillumination of polyacrylamide gels. However, hybridization analysis was also performed, mainly by liquid hybridization, which in our experience was superior to solid-phase hybridization techniques both in execution time and sensitivity.

Initially, assays used for determination of viremia, antigenemia, and DNAemia were found to give consistently negative results on peripheral blood PMNL samples from 2 HCMV-seronegative patients as well as from 10 HCMV-seropositive individuals. Subsequently, 293 blood samples from immunocompromised patients were examined. Of these blood samples, 78 gave concordant positive results and 110 gave concordant negative results. Thus, the overall concordance among viremia, antigenemia, and DNAemia was 188 of 293 (64.1%). However, since both viremia and antigenemia indicate the presence of circulating HCMV-infected PMNLs in blood, the overall concordance reaches 234 of 293 (79.8%), if samples positive by both (n = 78) and

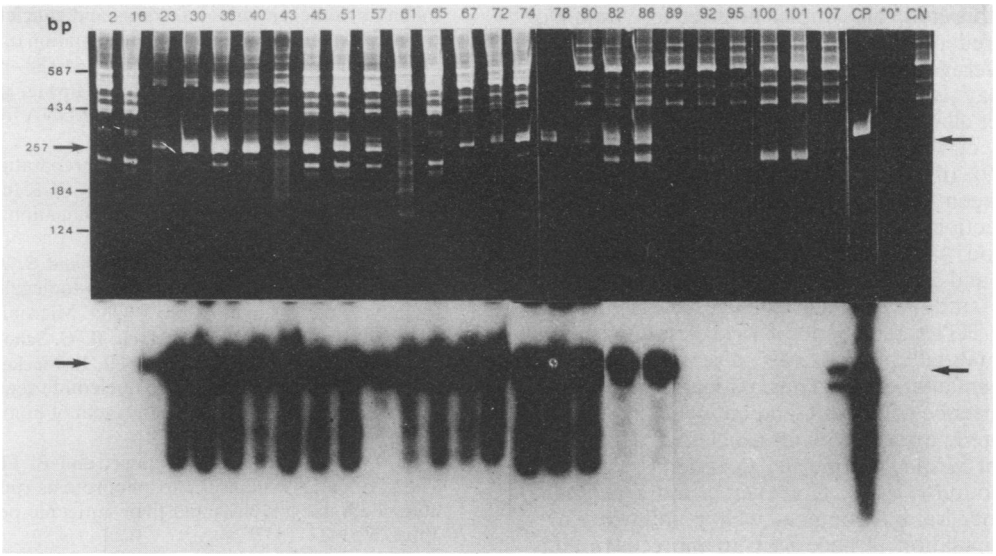

FIG. 1. Analysis of HCMV amplification products by PCR, using phosphoprotein pp150 primers, in sequential PMNL samples from a HTR. (Upper) Ethidium bromide (EtBr)-stained gel; (lower) liquid hybridization. Numbers at the top of the lanes indicate the number of days after transplantation. CP, positive control (HCMV-infected human fibroblasts); "0", control with no DNA; CN, negative control (uninfected human fibroblasts). Following the first blood sample (DNA negative at day 2), viral DNA was continuously detected in blood until day 89, when it disappeared; it reappeared on day 107. Extra bands in EtBr-stained gel are due to nonspecific PCR products that often arise when amplifying from a low number of starting template molecules; the multiple bands in the liquid hybridization gel are due to different conformations that labeled probe annealed to target may assume in gels when migrating under native conditions, being more evident in lanes corresponding to more-positive samples. The arrows in the upper part of the figure indicate the HCMV-specific amplification band, whereas in the lower part they indicate the most represented conformation.

either assay (n = 46) are combined. Dissociation between a positive antigenemia result and a negative viremia result was found in 29 samples. Thirteen of these were obtained during ganciclovir treatment, and 16 showed a number of antigen-positive PMNLs lower than 5 per 2×10^5 cells. In contrast, the opposite dissociation between a negative antigenemia result and a positive viremia result was observed in 17 samples, giving a value of immediate-early antigen-positive HELF cells lower than 5 per 2×10^5 PMNLs inoculated.

All 124 blood samples positive for direct antigen detection in PMNLs or virus isolation were also positive by PCR. In addition, as many as 59 blood samples were positive for HCMV DNA by PCR but were negative for both viremia and antigenemia. When considering all samples found positive by PCR (see below) to be true positives, comparison of direct antigen detection in PMNLs in combination with virus isolation in cell cultures versus PCR shows a specificity of 100% (110 samples negative of 110 true negatives), while the sensitivity is only 67.7% (124 positive of 183 true positives). In conclusion, about 30% of positive specimens were detected as positive for HCMV by PCR alone.

Analysis of 59 blood samples positive by PCR alone with respect to follow-up of HCMV infection in single patients showed that as many as 54 (91.5%) were from patients with virologic or clinical signs of HCMV infection preceding or following blood collection. Results obtained by different methods in immunocompromised patients during follow-up (representative cases are shown in Table 1) led to the following conclusions. (i) The quantitative correlation previously demonstrated (12) between levels of antigenemia and viremia (patients 1 and 2 [Table 1]) was confirmed, thus showing that the specificity of antigenemia assay was comparable to that of virus isolation in cell cultures (except during ganciclovir treatment). (ii) HCMV-related clinical symptoms were generally observed when levels of viremia or antigenemia were >50 HCMV-infected PMNLs per 2×10^5 cells examined but could occur sporadically with levels of antigenemia or viremia of 10 to 50 per 2×10^5 cells (patients 1 and 2 [Table 1]). (iii) PCR detected the onset of HCMV infection in blood samples 1 to 2 weeks prior to other assays and revealed its resolution some weeks to some months later (patients 1, 3, and 4 [Table 1]). (iv) The detection of HCMV DNA by PCR alone was never associated with the presence of clinical symptoms.

However, the persistence of viral DNA in blood as revealed by PCR alone represented a risk of relapse of viral infection and HCMV disease. This event was observed in 6 of 13 (41.5%) HTRs undergoing prolonged virologic follow-up after transplantation as well as in all of the 4 AIDS patients studied. Such a risk is clearly demonstrated by follow-up of an HTR with a primary HCMV infection (Fig. 1). After a long period of positive DNAemia starting on day 16 after transplant, this patient presented a first peak of viremia or antigenemia associated with clinical symptoms (fever) at day 45. Following treatment with ganciclovir, viremia and antigenemia became negative at day 61, but PCR remained positive, and a second peak of viremia or antigenemia associated with fever appeared at day 82, when a second course of ganciclovir treatment was initiated. At day 92, PCR also became negative. However, DNA was detected again in blood samples at day 107, but no further appearance of clinical symptoms was observed in the following months.

Some important considerations may be drawn from analysis of PCR assay results. First, the assay is very sensitive. It was previously reported that in in vitro reconstruction experiments, PCR could detect one infected cell in one million (19). Results of the present study confirm this esti-

mate. In fact, in this study all samples positive for viremia or antigenemia scored positive with PCR, including samples containing an average of 1 positive PMNL of 10^4 to 10^5. Second, the assay is very specific, as shown by the (i) capacity to detect all of the wild-type HCMV isolates tested so far and lack of DNA amplification of related human herpesviruses (19), (ii) follow-up data demonstrating strong correlation between PCR-positive samples and clinical course of the infection, and (iii) consistently negative results obtained in blood samples from 10 HCMV-seropositive healthy subjects and 2 seronegative individuals.

In a previous study, we found that the persistence of HCMV DNA in HTRs represents a risk of recurrence of viremia and, potentially, of HCMV disease in the first months after transplantation (5). Thus, patients who are PCR positive in the absence of viremia and antigenemia must be carefully monitored, even following ganciclovir treatment, by using frequent virologic controls, in order to promptly detect virus infection relapse. However, a more extended study of recurrent viral infections as well as follow-up of a larger patient population is necessary to more accurately define clinical significance of HCMV DNAemia.

In conclusion, while determination of viremia and antigenemia is sufficient for correct clinical management of HCMV infections, PCR provides a new powerful tool for detection of systemic HCMV infections and for adoption of adequate monitoring measures in immunocompromised patients.

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REFERENCES

1. **Akrigg, A., G. W. G. Wilkinson, and J. D. Oram.** 1985. The structure of the major immediate early gene of human cytomegalovirus strain AD169. Virus Res. **2**:107–121.
2. **Dankner, W. M., J. A. McCutchan, D. D. Richman, K. Hirata, and S. A. Spector.** 1990. Localization of human cytomegalovirus in peripheral blood leukocytes by in situ hybridization. J. Infect. Dis. **161**:31–36.
3. **De Franchis, R., N. C. P. Cross, N. S. Foulkes, and T. M. Cox.** 1988. A potent inhibitor of Taq-polymerase copurifies with human genomic DNA. Nucleic Acids Res. **16**:10355.
4. **Gerna, G., M. Parea, E. Percivalle, D. Zipeto, E. Silini, G. Barbarini, and G. Milanesi.** 1990. Human cytomegalovirus viremia in HIV-1 seropositive patients at various clinical stages of infection. AIDS **4**:1027–1031.
5. **Gerna, G., D. Zipeto, M. Parea, M. G. Revello, E. Silini, E. Percivalle, M. Zavattoni, and G. Milanesi.** 1991. Monitoring of human cytomegalovirus infections and ganciclovir treatment in heart transplant recipients by determination of viremia, antigenemia and DNAemia. J. Infect. Dis. **164**:488–498.
6. **Gustafson, S., J. A. Proper, E. J. W. Bowie, and S. S. Sommer.** 1987. Parameters affecting the yield of DNA from human blood. Anal. Biochem. **165**:294–299.
7. **Higuchi, R.** 1989. Simple and rapid preparation of samples for PCR, p. 31–38. *In* H. A. Erlich (ed.), PCR technology: principles and applications for DNA amplification. Stockton Press, New York.
8. **Hsia, K., D. K. Spector, J. Lawrie, and S. A. Spector.** 1989. Enzymatic amplification of human cytomegalovirus sequences by polymerase chain reaction. J. Clin. Microbiol. **27**:1802–1809.
9. **Jahn, G., T. Kouzarides, M. Mach, B.-C. Scholl, B. Plachter, B. Traupe, E. Preddie, S. C. Satchwell, B. Fleckenstein, and B. G. Barrell.** 1987. Map position and nucleotide sequence of the gene for the large structural phosphoprotein of human cytomegalovirus. J. Virol. **61**:1358–1367.
10. **Jahn, G., B.-C. Scholl, B. Traupe, and B. Fleckenstein.** 1987. The two major structural phosphoproteins (pp65 and pp150) of human cytomegalovirus and their antigenic properties. J. Gen. Virol. **68**:1327–1337.
11. **Kwok, S., and R. Higuchi.** 1989. Avoiding false positives with PCR. Nature (London) **339**:237–238.
12. **Revello, M. G., E. Percivalle, M. Zavattoni, M. Parea, P. Grossi, and G. Gerna.** 1989. Detection of human cytomegalovirus immediate-early antigen in leukocytes as a marker of viremia in immunocompromised patients. J. Med. Virol. **29**:88–93.
13. **Saltzman, R. L., M. R. Quirk, and M. C. Jordan.** 1988. Disseminated cytomegalovirus infection. J. Clin. Invest. **81**:75–81.
14. **Shibata, D., W. J. Martin, M. D. Appleman, D. M. Causey, J. M. Leedom, and N. Arnheim.** 1988. Detection of cytomegalovirus DNA in peripheral blood of patients infected with human immunodeficiency virus. J. Infect. Dis. **158**:1185–1192.
15. **Spector, A. S., J. A. Rua, D. H. Spector, and R. McMillan.** 1984. Detection of human cytomegalovirus in clinical specimens by DNA-DNA hybridization. J. Infect. Dis. **150**:121–126.
16. **Stenberg, R. M., D. R. Thomsen, and M. F. Stinski.** 1984. Structural analysis of the major immediate early gene of human cytomegalovirus. J. Virol. **49**:190–199.
17. **Van der Bij, W., J. Schirm, R. Torensma, W. J. Van Son, A. M. Tegzess, and T. H. The.** 1988. Comparison between viremia and antigenemia for detection of cytomegalovirus in blood. J. Clin. Microbiol. **26**:2531–2535.
18. **Van der Bij, W., R. Torensma, W. J. Van Son, J. Anema, J. Schirm, A. M. Tegzess, and T. H. The.** 1988. Rapid immunodiagnosis of active cytomegalovirus infection by monoclonal antibody staining of blood leukocytes. J. Med. Virol. **25**:179–188.
19. **Zipeto, D., E. Silini, M. Parea, E. Percivalle, M. Zavattoni, A. Di Matteo, G. Milanesi, and G. Gerna.** 1990. Identification of human cytomegalovirus isolates by the polymerase chain reaction. Microbiologica **13**:297–304.