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Human Cytomegalovirus (CMV) DNA in Plasma Reflects Quantity of CMV DNA Present in Leukocytes

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A quantitative DNA amplification assay for human cytomegalovirus (CMV) DNA has been used to evaluate the relationship between quantities of CMV DNA in plasma and those in infected leukocytes (WBC) from human immunodeficiency virus-infected patients. The target sequence for DNA amplification was a region of the immediate-early 1 gene of CMV. The quantitation assay uses an internal control that is coamplified with each patient sample DNA and contains a sequence for detection by colorimetric hybridization with the same bases, but in different order than in the CMV immediate-early 1 region used for hybridization of amplified patient sample DNA. Results showed that patients with CMV disease had more CMV DNA in both WBC and plasma than those without disease. However, in this study, copy numbers of CMV DNA in WBC were higher than those in plasma. The gB and gH variants were the same in plasma and WBC.

The detection of human cytomegalovirus (CMV) DNA in the plasma of immunocompromised hosts has been reported (1, 10, 15, 17, 21). This observation is of interest because it suggests that (i) there may be an alternate mechanism for dissemination of infectious virus within the host in addition to cell-to-cell transmission and (ii) plasma may be a convenient source of patient material for detecting a disseminated CMV infection. Although the presence of CMV DNA in peripheral leukocytes (WBC) is a sensitive marker for virus dissemination (9, 11, 13, 16, 22), detection of CMV DNA in plasma may be useful when counts of circulating WBC are low, such as in immunocompromised hosts. A positive correlation between detection of infectious CMV in WBC and CMV DNA in plasma has been reported (1, 21). Since the highest levels of CMV DNA are usually seen in patients with symptomatic disease, most often detected as retinitis (19), it is possible that the presence of CMV DNA in plasma is reflecting the quantity in WBC. The purpose of this study was to compare quantities of CMV DNA in WBC and in plasma and to see how they relate to symptomatic CMV disease in human immunodeficiency virus (HIV)-infected patients.

MATERIALS AND METHODS

Patient population. HIV-seropositive patients were enrolled randomly from among the participants in various HIV treatment trials at the Center for AIDS Research at the Stanford University School of Medicine and its affiliated subunit at Kaiser Permanente Medical Center, San Francisco, Calif. CD4 cell counts for the patients in this study were quantitated by fluorescence-activated cell sorting analysis. CD4 cell counts were available for each patient within 30 days of sample acquisition. Forty-nine patients with CD4 counts of <100 (mean ± standard deviation = 32 ± 27) were free of any known CMV disease. CMV retinitis (n = 22) was diagnosed ophthalmologically by the typical clinical appearance of retinal hemorrhage and exudate. CD4 cell counts in patients with retinitis were 39 ± 25 (mean ± standard deviation). CMV gastrointestinal disease in the symptomatic disease group (n = 1) was diagnosed by both sigmoidoscopy and the presence of typical intranuclear and intracytoplasmic inclusions in stained tissue samples obtained by biopsy of the intestinal wall.

Patient participation was with the approval of the Stanford University Committee on Human Subjects, and informed consent was obtained from all patients in this study.

Sample preparation. WBC were separated from whole, citrated blood by sedimentation of the erythrocytes for 30 min at room temperature in an equal volume of a 3% dextran T500 solution in 0.9% NaCl (19). The plasma, diluted 1:1, was withdrawn and frozen. Seminal fluid and nonspermatozoal cells were obtained from ejaculate as previously described (18).

DNA was extracted from all samples with a commercial kit (QIAamp Blood Kit 29104; Qiagen Inc., Chatsworth, Calif.). For WBC and semen, DNA was extracted from 1×10^6 to 5×10^6 cells, and DNA yields were quantitated spectrophotometrically. The DNA yields obtained with the QIAamp Blood Kit were comparable to those obtained by standard phenol-chloroform extraction. Yields of DNA from cellular samples were comparable to those reported by the manufacturer. In addition, when multiple aliquots of the same cellular samples were used, the yields were found to be comparable. For dextran-diluted plasma and undiluted seminal fluid, 200 μl was used for DNA extraction; elution in 200 μl of 10 mM Tris-HCl followed. DNA was extracted from plasma to avoid inhibition of the PCR, as is frequently encountered in this type of sample (12). DNA yields from these samples were too low for quantitation by standard spectrophotometric methods.

Plasmid construction. External (p162) and internal (p128) control plasmids were constructed as shown in Fig. 1A on the basis of the sequence of the highly conserved immediate-early 1 (IE1) gene (5). The probe sequences for both plasmids are shown in the heavily shaded area between the primers. Oligonucleotide hybridization probes based on these sequences were synthesized for use in a colorimetric hybridization assay to quantitate amplified DNA (19). The p162 plasmid (Fig. 1A), containing CMV DNA from the 162-bp region of the IE1 gene, widely used for CMV detection by PCR (20), was constructed by amplifying the region from CMV AD169-infected cells and cloning into plasmid pCR (TA Cloning Kit; Invitrogen, San Diego, Calif.), following the manufacturer's instructions. p162 was used as a template for further PCR reactions involved in constructing the p128 internal control plasmid.

The p128 plasmid was constructed to contain (i) the same upstream and downstream primer target sequences that were present on p162 and (ii) an internal nucleotide sequence (probe p128) distinct from the one in p162 but with a similar base content. The region, designated p162 probe, was replaced with a scrambled version of the p162 probe, using the series of amplifications shown in Fig. 1A. The new sequence was introduced in order to detect the p128 with a probe different from the one used to detect the CMV IE PCR product. PCR products containing the 128 probe sequence were made in two separate reactions, using the SP6 and L128 primers in one reaction to yield LL128 and the R128 and T7 primers in the other reaction to yield RR128 (step 1). These new products were extended in additional PCR reactions with primers SP6 and S128 and T7 and p128 probe (step 2). The resulting products, EXT-LL128 and EXT-RR128, were mixed, denatured, and reannealed (step 3). To allow the formation of the hybrid product, the molecules were denatured at 94°C for 5 min and then the temperature was slowly lowered to 50°C over a 15-min period. After 2 min of annealing at 50°C, the temperature was raised to 72°C and kept there for 5 min to allow extension of the annealed products (8). This product was then amplified for 30 cycles, as follows: 94°C for 40 s, 45°C for 30 s, and 72°C for 30 s. All PCR reactions for plasmid construction consisted of 10 pmol of CMV IE primers, 2 U

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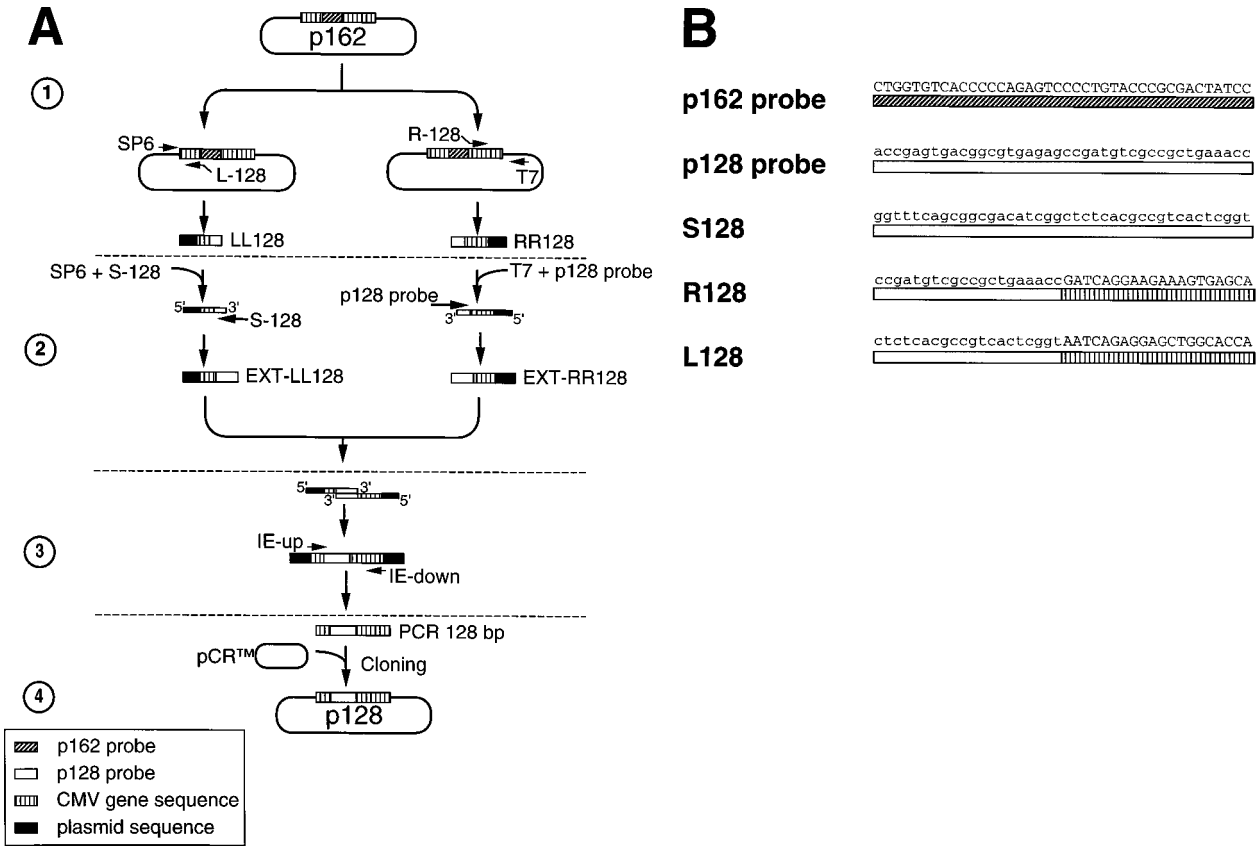

FIG. 1. Construction of internal control p128 plasmid from parent plasmid p162 (A). Arrows indicate the primers used, the sequences of which are shown in panel B. Patterns of the horizontal bars in both panels A and B indicate the corresponding DNA regions. Thick bars represent double-stranded DNA, and thin bars represent single-stranded DNA. SP6 and T7 are plasmid primer sequences. CMV primers were named L128, R128, S128, and p128 probe. EXT indicates extended subfragments. IE-up and IE-down are primers used for CMV amplification, as described in Materials and Methods.

of *Taq* polymerase (Perkin-Elmer, Norwalk, Conn.), 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, and 2.5 mM (each) deoxynucleoside triphosphate (dNTP). The resulting 128-bp PCR product was cloned into vector pCR. The plasmid containing the correct sequences was characterized and verified by restriction enzyme analysis and PCR and designated p128.

The sequences of the probes and primers depicted in Fig. 1A are shown in Fig. 1B.

Amplification and quantitation of CMV DNA. For amplification of intracellular CMV DNA, 1 μg of extracted DNA was used. For amplification of CMV DNA extracted from plasma, 50 μl of eluted DNA was used, corresponding to 25 μl of the original undiluted plasma sample. In our study, 1 μg of DNA could routinely be extracted from 200,000 cells, which corresponds to the WBC count in 40 μl of whole blood. The 25 μl of plasma corresponds to approximately 40 μl of whole blood, because the plasma yield is approximately two-thirds of the initial blood volume. Therefore, the quantities of the plasma and WBC samples analyzed in this study are directly comparable since they are derived from an equivalent blood volume.

Each DNA sample was coamplified with 100 copies of p128. The reaction mixture was in a 100-μl volume containing 30 pmol of upstream primer and 30 pmol of biotinylated downstream primer, 2 U of *Taq* polymerase (Perkin-Elmer), 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, and 2.5 mM (each) dNTP. As a reference, half-log dilutions of the p162 plasmid, ranging from 3,333 to 3 copies, were coamplified with 100 copies of plasmid p128 in the presence of 1 μg of human placental DNA.

The PCR reactions for amplification of CMV DNA sequences were performed for 45 cycles, as follows: 30 s at 94°C, 30 s at 65°C, and 30 s plus 5 s per cycle at 72°C.

PCR products were quantified by colorimetric hybridization (19). Briefly, amplification products were hybridized on microtiter plates with the appropriate detector probe. The amplification products, containing a 5′-biotinylated strand, were hybridized to the corresponding specific detector probe bound to wells and quantified spectrophotometrically after conversion of substrate at an optical density at 490 nm (OD_{490}). For each concentration of reference DNA standard, a number corresponding to the OD_{490} ratio of p162 to p128 was calculated and used to construct a reference curve. The same ratio was calculated for each sample and used to extrapolate the copy number from the reference curve (11, 12, 23).

Analysis of gB and gH gene variants by DNA amplification followed by restriction endonuclease digestion. Patient samples with evidence of CMV IE1 DNA were amplified for gp UL55 (gB) and gp UL75 (gH) glycoprotein variant groups (3, 4, 6, 7), using nested DNA amplification. For gB amplification, primers gB1868 (6, 7) and gB829 (TCTCCTTTCTACAACGGAAC) were used as outer primers (2) and gB1319 and gB1604 were used as nested inner primers (3). For gH amplification, outer primers were gH459 and gH175 and inner primers were gH203 and gH172 (6). Ten microliters of the outer PCR reaction (40 cycles) was amplified in the inner PCR reaction for an additional 40 cycles. For identification of gB groups, samples were digested with *Rsa*I and *Hin*fI (New England BioLabs, Beverly, Mass.), and for gH, digestion was with *Hha*I (New England BioLabs). Strains were identified on the basis of migration patterns of the restriction endonuclease digestion products in ethidium bromide-stained agarose gels.

RESULTS

Quantitative reference curve and its variability. A representative reference curve, along with the OD_{490} ratios used to construct the curve, is shown in Fig. 2. The variability of the reference curve was analyzed by calculating the mean and the standard deviation of the interpolated copy numbers of 19 reference curves obtained from different experiments over a period of 6 months. For 100 copies and more, the average variability (calculated as percentage of the standard deviation around the mean) was 31.6%, compared with a variability of 58.4% for less than 100 copies. The lowest copy number detected in the assay was positive when it was greater than the mean of the OD_{490} ratio of the negative controls plus 2 stan-

Amplified copy number	p162 OD_{490}	p128 OD_{490}	p162 /p128	Extrapolated copy number
3333	0.8165	0.2525	3.2337	2748
1000	0.7515	0.3530	2.1289	1218
333	0.6640	0.5640	1.1773	385
100	0.4705	0.7770	0.6055	105
33	0.1550	0.5605	0.2765	23
10	0.0945	0.4705	0.1983	12
3	0.0670	0.6960	0.0963	3
0	0.0085	1.1445	0.0074	0

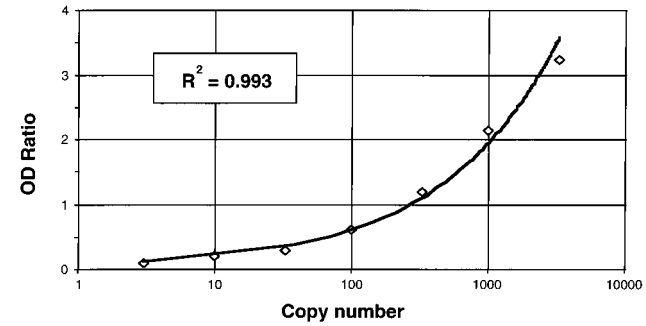

FIG. 2. Reference curve. The OD_{490} values obtained for each point (representing the average reading of two wells), along with the calculated ratio, are reported at the top. The reference curve was obtained by calculating the least-squares fit through points, using the equation $y = ax^b$ (in the example, $a = 0.0553$ and $b = 0.5138$). The reverse function of this equation was used to obtain the copy number (x) corresponding to the OD_{490} ratio value (y) measured in patient samples.

dard deviations; this value was usually 0. However, because of the high variability of quantitating copy numbers of <10 (64.4%), only values that were ≥10 were considered positive.

Comparison of CMV DNA in WBC and plasma in individual samples. The data in Fig. 3 show the relationship between copy numbers of CMV DNA in paired samples of plasma and those in WBC. Patients were classified as having either no CMV disease ($n = 49$) or CMV disease ($n = 23$). Twenty-four of 72 samples had no detectable DNA (<10 copies) in either plasma or WBC; 5 of 24 (21%) samples showed CMV disease. Twenty-two of 72 samples were positive for CMV in both WBC and plasma; 11 of 22 patients had CMV disease (50%). Samples from 22 of 72 patients were plasma negative but WBC positive; 6 patients (27%) had CMV disease. Four of 72 samples were plasma positive but WBC negative; one patient (25%) had CMV disease. Most patients with CMV disease had CMV DNA in both plasma and WBC.

In order to compare the copy numbers present in WBC and plasma on an individual basis, copy numbers from 20 of the 22 samples, positive for both WBC and plasma and for which exact starting blood volume had been recorded, were normalized to 1 ml of whole blood. For each sample, the WBC count for 1 ml of blood and the yield of extracted DNA from WBC were used to calculate the WBC CMV DNA copy number per milliliter of blood. Similarly, the total plasma yield from the starting blood volume was used to calculate plasma copy number per milliliter of blood. The median values were 23,647 for WBC and 1,253 for plasma. The difference between medians was significant (Wilcoxon signed rank test for paired samples; $P < 0.01$). When quantities of CMV DNA in plasma were compared with copy numbers in WBC by linear regression analysis, there was a significant correlation between the two ($r^2 = 0.654$; $P < 0.01$).

Comparison of CMV DNA in WBC and plasma in patients with or without CMV disease. Figure 4 shows the quantitative relationship between CMV DNA in WBC (left) and plasma (right) in patients grouped according to CMV disease status. Forty-eight percent of the patients with CMV disease were negative for CMV DNA in plasma compared with 26% when WBC were tested. In the no-disease group, 71% had no detectable CMV DNA in plasma compared with 45% who were negative for CMV DNA in WBC. However, both patient groups (no CMV disease and CMV disease) were significantly different ($P < 0.05$) when the medians were compared (Mann-Whitney two-tailed U test).

Comparison of semen cells and seminal fluid for presence of CMV DNA. To determine the relationship of virus replication at sites other than peripheral WBC to the presence of CMV DNA in plasma, plasma from nine patients who were positive for CMV DNA in semen cells were studied. All nine patients had CD4 cell counts of >100/mm^3 but <10 copies of CMV DNA in WBC. CMV DNA was undetectable (<10 copies) in plasma from all of these patients.

To test the hypothesis that the presence of CMV DNA in body fluids reflects the presence of CMV DNA in the appropriate supporting or proximal cells, seminal fluid was analyzed for CMV DNA in 14 patients, 9 of whom were positive for CMV DNA in semen cells. The mean (± standard error) copy numbers of CMV DNA in semen cells was 6,274 ± 2,976 in the nine patients with CMV DNA in seminal fluid and 11 ± 3 in the five patients without detectable CMV DNA in seminal fluid. The copy numbers from semen cells of seminal fluid-positive patients was significantly higher than those from cells of seminal fluid-negative patients (Mann-Whitney U test; $P < 0.05$).

Correlation of gB and gH genotypes in plasma or seminal fluid with genotype of intracellular virus. We compared the gB and gH variants detected in plasma or seminal fluid with the types detected in proximal cells from the same patient by restriction endonuclease digestion of DNA amplification products to define four gB and two gH groups (6, 7). Sixteen patients who were both WBC and plasma positive and five patients who were both semen cell and seminal fluid positive were analyzed. Identical gB genotype composition was observed in 15 of 16 patients tested for gB in WBC and plasma and 5 of 5 patients tested for gB in semen cells and seminal fluid. A single patient had gB 2 and 3 in WBC but only gB 2 in

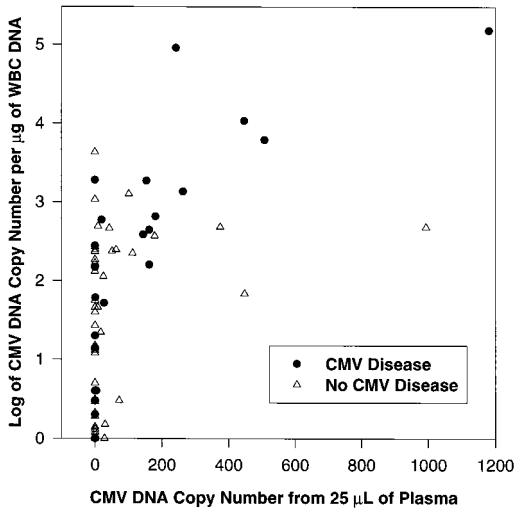

FIG. 3. Relationship between CMV DNA copy numbers in WBC and plasma of paired samples from patients with and without CMV disease.

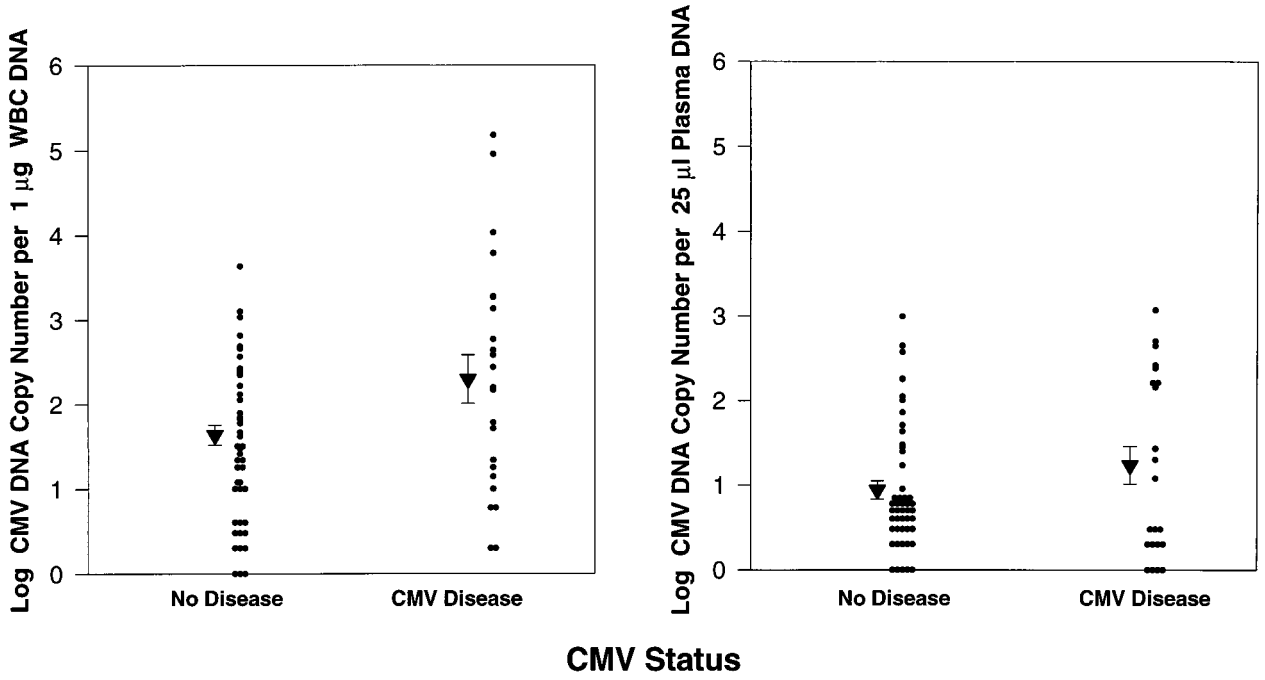

FIG. 4. CMV DNA copy numbers in WBC (left) and plasma (right) of HIV-infected patients. Patients had symptomatic CMV disease (retinitis, $n = 22$; CMV gastrointestinal disease with interstitial pneumonitis, $n = 1$) or no CMV disease ($n = 49$). All patients had <100 CD4 cells per mm^3. The geometric mean ± standard error is indicated for each group.

plasma. For gH, a similar identity of genotypes in cells and extracellular fluid was observed. However, one patient had both gH 1 and gH 2 in plasma but only gH 2 in WBC, and a second patient had both gH 1 and gH 2 in semen cells but only gH 1 in extracellular seminal fluid.

DISCUSSION

The data in this study have shown that the presence of CMV DNA in plasma or seminal fluid of patients with HIV infection usually reflects the presence of higher levels of CMV DNA in the corresponding cells.

CMV DNA was quantitated in this study by amplification of patient DNA in the presence of a control plasmid; hybridization and quantitation of both the control and patient sample DNA followed. The introduced sequence in the plasmid control is a scrambled version of the parent IE1 sequence used for hybridization. This results in a molecule with thermodynamic properties that are very similar to the parent molecule and assures that amplification and quantitation of the plasmid control accurately reflect amplification and quantitation of the amplified DNA from patient samples. The addition of an internal control plasmid which can be amplified by the same primers as the parent CMV IE sequence but detected with a unique probe allows a simple quantitation of both the internal control plasmid and the patient CMV DNA within the same amplification reaction. The ratio of these values normalizes different amplification efficiencies among samples, giving an accurate copy number.

Data from two areas of investigation indicate that virus in body fluids reflects virus in the proximal cells. First, our quantitative data show that in most patients CMV DNA in extracellular fluid correlates with a high copy number of CMV DNA in the corresponding cells. These data are in general agreement with those published in a recent study (12). Also, there is a significant statistical relationship by linear regression between CMV DNA in WBC and that in plasma when copy numbers are expressed per unit volume of blood. In four patients we detected CMV DNA in plasma but not in WBC, but the plasma copy numbers were in the lower range of our assay (<100 copies for all four samples). Increased variability in this lower range may account for the lack of detection in WBC. Local virus replication in a more highly vascularized site such as the liver, spleen, or colon could possibly contribute to the presence of CMV DNA in plasma without infection of WBC. Second, the correspondence of the gB and gH variants in cells and in extracellular fluid supports the quantification data that indicate that extracellular DNA is derived from the supporting cells. The high degree of genetic conservation of the gB and gH genes (6, 7) supports the idea that the intracellular and extracellular CMV DNA is the same in most cases. When discrepancies in variant composition between the two compartments existed, they were probably the result of quantitative differences in the proportions of each variant present in the mixture. We realize, however, that the method for classifying variants only defines a limited region of those genes. It is possible that additional polymorphisms within the gB or gH genes, or even different viral genes, could be present.

Even though there is a quantitative, and possibly a genetic, relationship between the CMV DNA in cells and in body fluids, the dynamics of the interaction between these two compartments is not clear. One possibility is that virus is released from infected cells and circulates in the plasma as free virus or antigen-antibody complexes. The occasional ability to recover infectious virus from plasma (12, 21) supports this hypothesis. The finding that CMV DNA remains amplifiable in plasma even after filtration weakens the possibility that this CMV DNA is derived from circulating fragments of infected cells (12, 21). Last, there could be free CMV DNA from lysed

infected cells. Additional experiments are necessary to further understand the source of CMV DNA in plasma.

Quantitation of CMV DNA in WBC may have some advantage over detection of CMV DNA in plasma for evaluating risk of progression to CMV disease. CMV DNA in WBC can frequently be detected when plasma samples are negative. Also, quantitation of CMV DNA in plasma may be less accurate because copy numbers are usually quite low. Specifically, in our study, 63.9% of the plasma samples had <100 copies of viral DNA. We have shown that quantification of copy numbers within this range is less accurate due to the inherently high variability within the 10 to 100 copy number range. Finally, when copy numbers in WBC and plasma were normalized to 1 ml of whole blood, there were statistically higher copy numbers in WBC than in plasma. However, the relevance of CMV DNA quantity, in either plasma or WBC, to development of CMV symptomatic disease must be established in a longitudinal study.

Even with quantitation, we could not distinguish between patients with or without CMV disease by using a single sample. High copy numbers are found in some patients with no disease and low copy numbers are seen in some patients with CMV disease. The association of clinical disease with plasma or serum DNA in a single sample has been reported by some investigators (1, 14, 21), but others have not found this relationship (17). Differences in CMV DNA levels in plasma among laboratories (12, 17, 21) could be the result of both different sample handling procedures and quantitation protocols. For example, if some plasma DNA is derived from infected cells, the manner in which the sample is processed may result in release of variable quantities of CMV DNA into plasma. Standard procedures in sample handling and preparation as well as international standards for DNA quantitation must be defined to facilitate comparison of data among laboratories.

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