Monitoring of Human Cytomegalovirus Infections in Pediatric Bone Marrow Transplant Recipients by Nucleic Acid Sequence–Based Amplification

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In the diagnosis of human cytomegalovirus (HCMV) infection, it is very important to distinguish symptomatic from asymptomatic infection. The nucleic acid sequence–based amplification (NASBA) technique was compared with single and nested polymerase chain reaction (PCR) methods. For NASBA detection, the $\beta_{2.7}$ transcript was chosen as a target because of its abundant active HCMV-specific expression. Of 20 pediatric bone marrow transplant (BMT) recipients, 8 developed HCMV-related clinical symptoms. The clinical sensitivities and specificities were 50% and 100% for single PCR, 100% and 67% for nested PCR, and 100% and 83% for NASBA, respectively. Follow-up of HCMV infections in pediatric BMT recipients showed that NASBA could both detect viral transcript prior to the onset of clinical symptoms and reflect clinical improvement due to antiviral therapy. These data suggest that NASBA should be useful for both predicting HCMV disease development and monitoring the effect of antiviral therapy.

Human cytomegalovirus (HCMV) is a significant cause of morbidity and mortality in immunocompromised patients, such as organ or bone marrow transplant (BMT) recipients, AIDS patients, and developing neonates [1–3]. Some antiviral drugs, such as ganciclovir [4] and foscarnet [5], are useful for treating HCMV disease, but these drugs must be used carefully because of their side effects. Therefore, it is important to establish a sensitive system by which to monitor HCMV infection.

Classical viral culture from peripheral blood mononuclear cells is not a very sensitive technique, and it is time-consuming. Serologic methods are generally useful but often inaccurate in the case of immunocompromised patients because of hyperimmune globulin therapy and defective immune responses. The HCMV antigenemia assay has been shown to have good clinical specificity for symptomatic HCMV infection [6, 7], but it is complicated and demands a large number of cells from patients, including pediatric BMT recipients and neonates. The detection of HCMV genomic DNA by polymerase chain reaction (PCR) is highly sensitive [8–10] but not always helpful in HCMV infection because it cannot distinguish latent from active infection.

mRNA is a good target for active HCMV-specific detection, and the usefulness of reverse-transcription PCR (RT-PCR) has been reported [11–15]. However, the genomic DNA must be completely removed because RT-PCR would amplify not only the target RNA but the genomic DNA unless the target gene has an appropriate intron sequence.

In the present study, we used the nucleic acid sequence–based amplification (NASBA) technique, which is highly suited for the amplification of RNA sequences [16, 17]. As a target RNA, we chose the $\beta_{2.7}$ transcript, the most abundant viral transcript, accounting for >20% of total viral transcripts made during infection [18–20]. Using this NASBA method, HCMV activities in the blood of BMT recipients were monitored and analyzed.

Materials and Methods

Clinical samples. Whole blood samples ($n = 233$) were collected weekly from 20 children who had received bone marrow transplants. Whole blood samples from healthy individuals ($n = 100$) were also obtained for examination of the false-positive rate in each amplification method. An aliquot (1 mL) of whole blood was mixed with 9 mL of lysis buffer (50 m*M* Tris-HCl [pH 6.4], 1.3% Triton X-100, 5.25 *M* guanidinium thiocyanate, and 20 m*M* EDTA) and stored at −80°C until use.

Total nucleic acid isolation. Total nucleic acid was isolated from whole blood, essentially according to Boom et al. [21]. One milliliter of lysed whole blood in lysis buffer (equivalent to 100 μL of whole blood) was transferred to a microcentrifugation tube. The nucleic acids were bound to activated silica (50 μL of a 1 g/mL suspension in 0.1 *N* HCl; Sigma, St. Louis), which was added to the lysis mixture. The silica was washed and dried, and the nucleic acid was eluted with 50 μL of RNase-free H_2O and stored at −80°C.

Primers and probes. All oligonucleotides were synthesized with a DNA synthesizer (model 391; Applied Biosystems, Foster City, CA) and purified FPLC system (Pharmacia, Uppsala, Sweden).

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Informed consent was obtained from patients and their parents and guardians.

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Table 1. Comparison of sensitivity of single and nested polymerase chain reaction (PCR) and nucleic acid sequence–based amplification (NASBA) methods using in vitro–synthesized nucleic acid.

	No. of target molecules (copies/test)				
Assay	2×10^3	2×10^2	2×10^1	2×10^0	0
Single PCR	5/5	4/5	0/5	0/5	0/5
Nested PCR	5/5	5/5	5/5	3/5	0/5
NASBA	5/5	5/5	1/5	0/5	0/5

NOTE. Data are no. positive/no. tested.

The detection probes were labeled with alkaline phosphatase (Boehringer Mannheim, Mannheim, Germany), according to the method of Jablonski et al. [22].

Controls. For the positive control, 2×10^2 copies of in vitro–transcribed RNA derived from plasmid pCMV-β2.7 (for NASBA) and linearized plasmid pCMV-POL DNA (for PCR) were used in each experiment.

NASBA. NASBA amplification reactions were performed according to a reported procedure [16], with some modifications. The nucleic acid sample (5 μL) was mixed with 10 μL of 2× reaction mixture containing 80 m*M* Tris-HCl (pH 8.5), 24 m*M* $MgCl_2$, 140 m*M* KCl, 10 m*M* dithiothreitol, 30% DMSO, 2 m*M* each of the four dNTPs, 4 m*M* ATP, CTP, and UTP, 3 m*M* GTP, 1 m*M* ITP, each 0.2 μ*M* primer 1 (C-NAS-P1: 5′-AATTCTAATACGACTCACTATAGGGAGGAGGGAATCGTCGACTTTGAATTCTTCGA [nucleotides 344–318]). The underlined sequence represents the T7 RNA polymerase promoter and spacer sequence) and primer 2 (C-NAS-P2: 5′-TCCTTTCCTTAATCTCGGATTATCA [nt 198–222]). The reaction mixtures were incubated at 65°C for 5 min and then cooled to 41°C. The amplification reaction was started by the addition of 5 μL of enzyme mixture (6.4 U of AMV reverse transcriptase [Seikagaku, Tokyo], 0.08 U of RNase H [Pharmacia], 32 U of T7 RNA polymerase [Pharmacia], and 0.1 g/mL bovine serum albumin). The reaction mixtures were incubated at 41°C for 90 min and stopped by placing the samples on ice.

PCR. Primers and probes for PCR were chosen from the coding region of the DNA polymerase gene. PCR amplification reactions were essentially according to Saiki et al. [23]. For single PCR, 5 μL of nucleic acid sample was amplified in 50 μL of reaction mixture containing 10 m*M* Tris-HCl (pH 8.9), 80 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.5 mg/mL bovine serum albumin, 0.1% sodium cholate, 0.1% Triton X-100, and 0.5 μ*M* each of primers C-PCR-F1 (5′-ATGCATGGCCAAGACTAACT [nt 2126–2145]) and C-PCR-R1 (5′-CACCGTCGTACCTTTGCT [nt 2480–2463]). The PCR was done in a thermal cycler for 40 cycles (94°C for 1 min, 55°C for 2 min, and 75°C for 1.5 min). Aliquots (0.5 μL) of each amplification product were subjected to nested PCR with nested primer pairs, primers C-PCR-F2 (5′-CGGCAACGCAAGGATGACCTGTC [nt 2187–2209]) and C-PCR-R2 (5′-TGTAGATACGGATCTGCTG [nt 2409–2391]) under the same conditions as for the first-round PCR.

Detection of amplified product. Both NASBA- and PCR-amplified products were detected by a microtiter plate–based sandwich hybridization assay with a capture probe (C-NAS-CAP for NASBA: 5′-TTTCCCTCTCCTACCTACCACGAA [nt 223–246], C-PCR-CAP for PCR: 5′-GTTCAACACCATTAATTTTCACTACG [nt 2305–2330]) immobilized to the plate and a detection

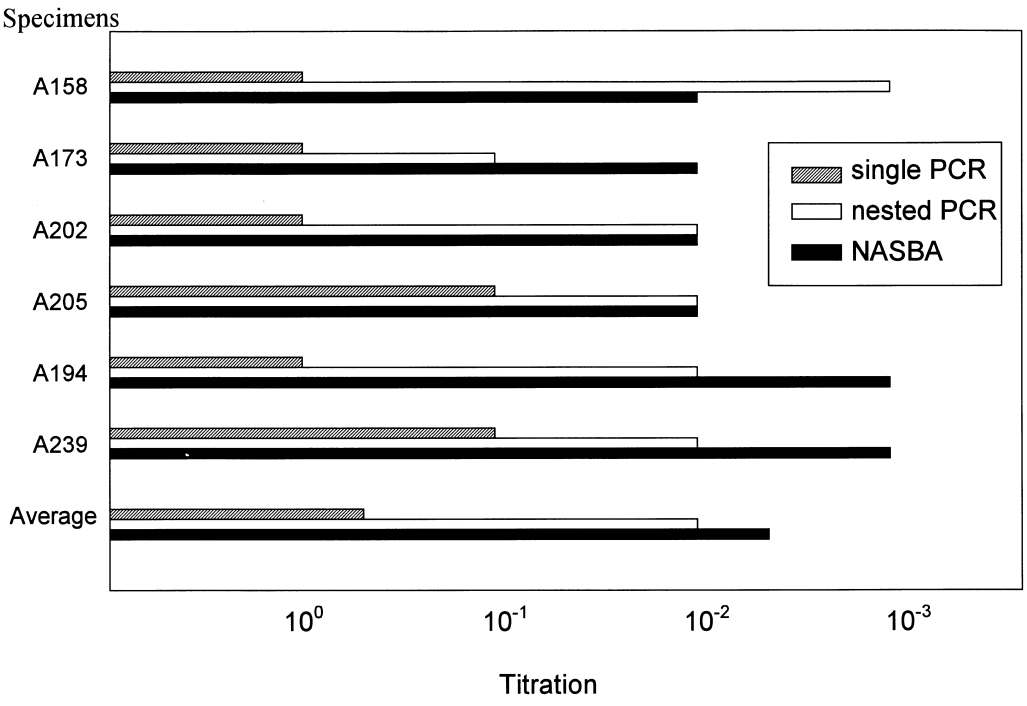

Figure 1. Detection of end-point titration of 6 HCMV-positive clinical specimens by single polymerase chain reaction (PCR), nested PCR, and nucleic acid sequence–based amplification.

probe (C-NAS-ALP for NASBA: 5′-CGCAGATGATAAACAA-GAGGGTAA [nt 248–271], C-PCR-ALP for PCR: 5′-ATTC-CGTTGCGGCGTGTCATCTTT [nt 2361–2384]) labeled with alkaline phosphatase. Aliquots (10 μL) of each amplified product were mixed with 10 μL of 0.1 *N* NaOH for denaturing, and then 10 μL of the mixture was applied to the plate with 90 μL of hybridization buffer. The hybridization reaction between an amplicate and a capture probe was carried out for 15 min at 50°C with shaking. After the reaction mixture was removed, 100 μL of a second hybridization mixture containing 200 fmol of detection probe was added, incubated in the same conditions, and then washed twice. After incubation with 100 μL of Lumi-Phos 480 (Wako, Osaka, Japan) for 15 min at 37°C, chemiluminescence was measured by a photon-counting head (Hamamatsu Photonics, Shizuoka, Japan).

Diagnosis of HCMV infection. HCMV infection was defined by IgG seroconversion with or without development of IgM antibodies, detection of HCMV DNA, or isolation of HCMV from any site. Symptomatic infections were defined as HCMV infections that were associated with unexplained fever combined with leukopenia, thrombocytopenia, appearance of atypical lymphocytes, liver dysfunction, interstitial pneumonia, retinitis, peptic ulcer, nephritis, or pancreatitis. The diagnosis of retinitis was confirmed by an experienced ophthalmologist.

Statistical analysis. Statistical analysis was done using Fisher's exact test. $P < .05$ was considered significant.

Results

Basic performance of NASBA. To compare the performances of NASBA and PCR, serial dilutions of in vitro RNA and plasmid pCMV-POL DNA (each 2×10^0, 2×10^1, 2×10^2, and 2×10^3 copies) were amplified in the presence of cellular total nucleic acid extracted from 100 μL of whole blood from HCMV-seronegative persons. As shown in table 1, the minimum copy numbers of NASBA detection were nearly equal to those from the single PCR and less than those from the nested PCR. To reproduce the in vivo condition as closely as possible, serial dilutions of 6 HCMV-positive clinical specimens were also amplified by NASBA or PCR. As illustrated in figure 1, the end-point titration of the clinical specimens by the NASBA (average $10^{-2.3}$) was equal to or higher than by nested PCR. The false-positive rate, which is a large problem in the sensitive detection of HCMV infection, was also measured using 100 specimens from 13 HCMV-seropositive healthy persons. No positive signal was obtained from the NASBA and single PCR (false-positive rate = 0%), whereas three positive signals (from 2 persons) were seen from the nested PCR (false-positive rate = 3%).

Clinical relevance of three amplification methods. Symptomatic HCMV infection was diagnosed in 8 (40%) of 20 patients using standard methodologies (table 2). The clinical values of each test are summarized in table 3. The single PCR had a positive predictive value of 100% for the onset of symptomatic HCMV infection but showed poor clinical sensitivity and a moderate negative predictive value of 50% and 75%, respectively. Nested PCR was positive for all 8 symptomatic patients. However, the clinical specificity of nested PCR was only 67%, since 4 patients had DNA detected but had no symptoms. The NASBA had both high clinical sensitivity and positive predictive value of 100% and 80%, respectively. In two cases of discrepancy with standard methodologies and NASBA, the results of the nested PCR agreed with those from the NASBA.

Table 2. Laboratory detection of HCMV infection and clinical status in pediatric bone marrow transplant recipients.

Patient	Sex	Age	Underlying disease	Serologic status (donor/recipient)	GVHD	Single PCR	Nested PCR	NASBA	Clinical symptoms
1	F	1	ALL	+/+	Acute	−	−	−	None
2	M	9	AML	+/+	Acute	−	−	−	None
3	F	15	Rhabdomyosarcoma	Auto/−	No (auto)	−	−	−	None
4	M	3	Rhabdomyosarcoma	Auto/+	No (auto)	−	+	+	Fever
5	F	5	Ewing's tumor	Auto/+	No (auto)	−	−	−	None
6	F	1	Severe combined immunodeficiency	ND/+	Chronic	+	+	+	Interstitial pneumonia, encephalitis
7	F	9	AMMoL	+/+	Acute	−	−	−	None
8	M	15	ALL	ND/+	Chronic	−	+	+	Retinitis
9	F	2	Fanconi syndrome	+/+	No	−	+	+	Fever, pancytopenia
10	M	10	ALL	+/+	No	−	−	−	None
11	M	15	AML	+/+	Acute	−	+	+	None
12	M	14	Myelodysplasia syndrome	−/+	Acute	−	+	−	None
13	M	8	ALL	−/+	Chronic	−	+	+	Fever, interstitial pneumonia
14	M	5	Hurler's syndrome	−/+	No	−	+	+	None
15	M	5	ALL	+/+	No	+	+	+	Fever, interstitial pneumonia
16	F	3	JCML	+/−	Acute	−	−	−	None
17	M	4	Medulloblastoma	+/+	Acute	+	+	+	Retinitis
18	M	2	JCML	ND/+	Acute	−	−	−	None
19	F	14	Germ cell tumor	Auto/+	No (auto)	−	+	−	None
20	M	7	ALL	ND/+	Acute	+	+	+	Fever, liver dysfunction

NOTE. F, female; M, male; PCR, polymerase chain reaction; NASBA, nucleic acid sequence–based amplification; Auto, autologous; GVHD, graft-versus-host disease; ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; AMMoL, acute myelomonocytic leukemia; JCML, juvenile chronic myeloblastic leukemia; ND, not determined.

Table 3. Clinical relevance of single and nested polymerase chain reaction (PCR) and nucleic acid sequence–based amplification (NASBA) methods for diagnosis of CMV infection.

	%				
Assay	Sensitivity	Specificity	PPV	NPV	*P*
Single PCR	50 (4/8)	100 (12/12)	100 (4/4)	75 (12/16)	.01
Nested PCR	100 (8/8)	67 (8/12)	67 (8/12)	100 (8/8)	<.01
NASBA	100 (8/8)	83 (10/12)	80 (8/10)	100 (10/10)	<.01

NOTE. PPV, positive predictive value; NPV, negative predictive value.

Monitoring of pediatric BMT recipients during antiviral therapy by NASBA. Follow-up of 2 pediatric child bone marrow transplant recipients with symptomatic HCMV is shown in figures 2 and 3. In patient 20, the NASBA became positive 7 days before onset of HCMV disease and PCR detection. Clinical improvement was accompanied by a reduction in the NASBA signal. Symptomatic recurrence (thrombocytopenia) occurred on day 74 in this patient. NASBA and nested PCR gave positive results 7 days before this clinical syndrome, and the single PCR became positive 8 days later. Patient 15 developed HCMV disease just after BMT. Mild interstitial pneumonia occurred on days 10–21. The NASBA gave positive results 2 days before transplantation and 12 days before the onset of interstitial pneumonia. The NASBA signal was obtained from <1000/μL leukocytes.

Discussion

HCMV infection remains a major problem following BMT or other transplants, yet a diagnostic method that is accurate and clinically relevant for HCMV infection has not been established. In this study we have shown that the detection of HCMV β2.7 transcript by NASBA is a sensitive and specific test for the diagnosis of HCMV infection.

Although detection of HCMV antigenemia is specific and rapid for the diagnosis of HCMV infection, this method has its drawbacks. For this assay, >5 mL of blood must be collected for leukocyte preparation. Because it was very difficult to obtain enough leukocytes from pediatric transplant recipients, we abandoned a direct comparison between the NASBA and the antigenemia assays. Moreover, cytospin preparation of separated blood must be done within 24 h because the pp65 antigen seems to degrade rapidly in antigen-bearing cells. Meyer-König et al. [13] reported that the number of pp65 antigen positive nuclei per 2×10^5 cells at 24 h after sampling was only 24% at 1 h, and no positive cells could be detected at 29 h. It is a large problem because a positive result in this assay is judged by a few positive cells on the slide.

PCR is a powerful tool for the diagnosis of infectious diseases. However, with HCMV, the sensitive detection of genomic DNA by PCR has poor clinical specificity and positive pre-

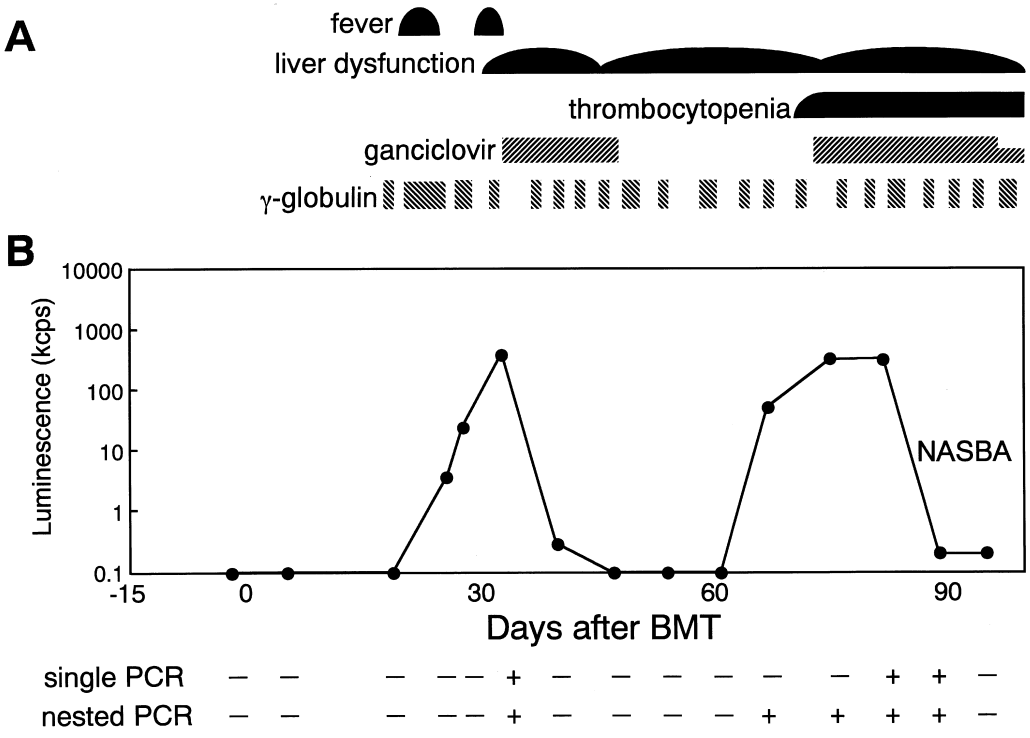

Figure 2. Follow-up of patient 20. **A**, Time course and activity of clinical disease associated with HCMV infection treated with ganciclovir. **B**, Results of single polymerase chain reaction (PCR), nested PCR, and nucleic acid sequence–based amplification (NASBA) detection of blood specimens. NASBA signal was detected on day 26 prior to other methods, disappeared on day 33, and reappeared on day 67.

dictive value [13] because DNA amplification also detects inactive HCMV. It is possible to make a more clinical symptom–specific PCR system, for example, by primer selection, controlling amplification conditions, using plasma or serum as a specimen [24], and quantitative PCR [25], but the sensitivity would decrease. The detection of HCMV mRNA by RT-PCR–targeted immediate-early and pp150 [13, 14] or major capsid protein [15] mRNA has higher clinical specificity and lower sensitivity as does the less-sensitive DNA PCR.

In this study, we used the NASBA technique and constructed the β2.7 transcript detection system with both high sensitivity and specificity. The NASBA method is optimized to amplify the target RNA, so it is not necessary to remove DNA before the amplification reaction. Moreover, this method has high amplification performance (>10^9-fold). On the other hand, the β2.7 transcript seems to be an ideal target molecule for the diagnosis of HCMV infection because of its abundant active HCMV-specific expression. This gene is dispensable, and the function of this gene product is not well characterized. However, at least for the sequences used, no interstrain variations were observed in 18 independent clinical isolates (data not shown).

In the experiment with a basic performance test using in vitro–synthesized target molecules, NASBA was inferior to PCR, but in the titration test of clinical specimens, which simulates the in vivo condition, NASBA performed well, as did the sensitive nested PCR. Moreover, the false-positive result commonly associated with sensitive HCMV detection was not observed in NASBA detection. These phenomena largely depend on the abundant active HCMV-specific expression of the β2.7 transcript. However, the RNA specificity of NASBA made it possible to select this intron-free transcript as a target.

In this study, we compared NASBA with single and nested PCR, which had high specificity (100%)/low sensitivity (50%) and high sensitivity (100%)/low specificity (67%), respectively. Like viral culture, the single PCR is useful for final but not early diagnosis; the nested PCR, like other highly sensitive PCR methods, is useful for excluding the possibility of HCMV infection because of its high negative predictive value. In this light, the antigenemia assay, which seemed most clinically relevant for HCMV disease at the present time, has moderate sensitivity and specificity. Our aim was to develop a method for the diagnosis of HCMV that has both high sensitivity and specificity. mRNA would be an ideal target for this purpose because of the large copy numbers relative to genomic DNA and viral life cycle–specific expression. Our β2.7 transcript detection system by NASBA achieved both high sensitivity (100%) and specificity (83%). Because these results were obtained using only 0.01 mL of whole blood, NASBA is suitable for testing children and neonates. Moreover, the same results could be obtained at any time, because the RNA in our lysing buffer was stable for a long period in a deep freezer.

NASBA was also useful for monitoring BMT recipients. The

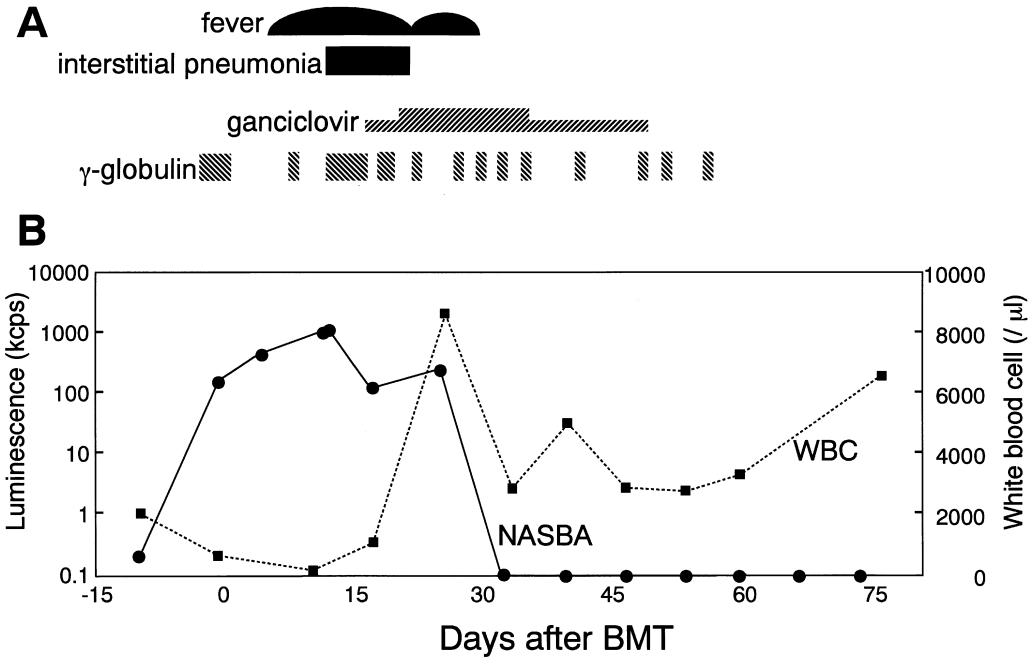

Figure 3. Follow-up of patient 15, who developed HCMV disease just after bone marrow transplant (BMT). **A**, Time course and activity of clinical disease associated with HCMV infection treated with ganciclovir. **B**, Results of nucleic acid sequence–based amplification (NASBA) detection of blood specimens. Even when white blood cell count was low (<1000/μL), NASBA signal was detected prior to appearance of clinical symptoms.

NASBA signal was detected prior to the appearance of clinical symptoms and disappeared with improvement due to antiviral therapy. Although the NASBA used in this study provided qualitative detection, the NASBA signal did not always reflect the copy numbers of the HCMV β2.7 transcript in blood. However, the NASBA signal was closely related to clinical status, and if necessary, quantitative NASBA detection [26] could be used to diagnose HCMV infection in the near future.

In conclusion, we have shown that detection of the β2.7 transcript by the NASBA is a sensitive and clinically relevant method for the diagnosis of HCMV infection. Our results suggest that monitoring HCMV activity by NASBA might make it possible to administer an antiviral drug in a timely manner and thus reduce the side effects of these drugs in immunocompromised patients.

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