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Quantitative Analyses of Cytomegalovirus Genome in Aqueous Humor of Patients with Cytomegalovirus Retinitis

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Purpose: To investigate copy numbers of cytomegalovirus (CMV) in CMV retinitis patients during ganciclovir treatment using real-time polymerase chain reaction (PCR).

Methods: Thirteen aqueous humor samples obtained from 6 patients with clinically diagnosed CMV retinitis were analyzed. As controls, aqueous humor samples were obtained at the time of surgery from patients with senile cataracts.

Results: The CMV genome was detected in the range from 10^1 to 10^4 copies/μL of aqueous humor before antiviral treatment. The samples obtained from retinitis patients showing widespread retinal changes contained much higher copy numbers than those from patients with focal lesions. After treatment, the copy number decreased to one hundredth of that observed prior to treatment, but the CMV genome was detectable for 4 to 8 weeks after ganciclovir administration to 4 patients.

Conclusion: These results revealed the correlation between the copy numbers of the CMV genome and the extent of the area affected by CMV retinitis before antiviral treatment, and the prolonged retention of CMV genome after antiviral treatment. Quantitation of the viral genome after the start of therapy will be of value in determining whether to continue or intensify the dosage of antiviral agents. **Jpn J Ophthalmol 2002;46:254–260** © 2002 Japanese Ophthalmological Society

Key Words: Aqueous humor, cytomegalovirus, polymerase chain reaction, retinitis.

Introduction

Cytomegalovirus (CMV) retinitis is frequently observed in patients with organ transplants, malignancies, or HIV infection and is caused by the latent virus, which is reactivated in these immunocompromised patients.[1] To prevent blindness, CMV retinitis must be diagnosed and treated as quickly as possible. CMV retinitis is usually clinically diagnosed based on the patient's history, including the state of the immune system and the fundoscopic appearance.[1] Occasionally, other pathogens, such as herpes simplex virus,[2,3] varicella-zoster virus,[4] *toxoplasma gondii*,[5] and fungi, causing similar fundoscopic changes, must be eliminated as causative agents.

For confirmation of the clinical diagnosis, several laboratory techniques have been applied, such as virus culture, antibody tests, antigenemia tests, and nucleic acid detection.[5] Isolation of CMV in tissue culture usually takes 2 or 3 weeks and is inadequate for prompt diagnosis. Seroconversion and local antibody response in the aqueous humor are not reliable for diagnosis because of the poor immune response in immunocompromised hosts.[6]

The most reliable and rapid test is to detect the viral genome by polymerase chain reaction (PCR) analysis.[7–21] This sensitive technique has been used for samples obtained from the aqueous humor and vitreous fluid; however, latent and reactivated CMV

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must be differentiated. To solve this problem, quantitation of viral genomes in these samples is attempted using competitive PCR and real-time PCR methods.[22–26] The former method is complicated and time-consuming, while the latter is rapid and has a wide dynamic range. In this study, we analyzed the copy numbers of the CMV genome in the aqueous humor of patients with CMV retinitis by real-time PCR using the LightCycler instrument to reveal the sequential change in the copy number during antiviral treatment for the disease. We measured the amount of fluorescent dye bound to the amplified double-stranded DNA product during each step of the PCR. In addition, we also monitored the cell numbers in the aqueous humor by quantitation of the human β-globin gene.

Materials and Methods

Patients and Samples

Patients with CMV retinitis and senile cataracts who were at least 20 years of age were eligible for this study. The diagnosis of CMV retinitis was based on the characteristic fundoscopic findings and the immune conditions of the hosts. Of the 6 patients with CMV retinitis, 2 patients had already been treated with ganciclovir. Aqueous humor samples were aspirated from the other 4 patients before ganciclovir treatment.

Aqueous humor samples were obtained using a sterile 27G needle following topical anesthesia and were kept at −80°C. The underlying diseases of these patients were HIV infection (n = 1), malignant lymphoma (n = 2) for whom bone marrow transplant had been performed, leukemia (n = 2), and common variable immunodeficiency (n = 1) (Table 1). For 5 of the 6 patients, aqueous humor samples were taken during both the acute and the convalescent stages. The total number of samples was 13. As controls, aqueous humor samples were obtained at the time of surgery from patients with senile cataracts. Informed consent was obtained from all patients.

The activity and extent of retinitis were determined based on the fundoscopic findings on photographs, and the retinitis was described as active or inactive, with the number of quadrants involved. The grades of aqueous chamber cells were determined by slit-lamp microscope analysis.

DNA Extraction from Aqueous Humor

Aqueous humor samples were digested with Proteinase K (Roche Diagnostics, Indianapolis, IN, USA) in a lysing buffer containing 50 mM Tris HCl (pH 8.0), 2% sodium dodecyl sulfate, and 50 mM EDTA. DNA was extracted using a phenol/chloroform method, precipitated in 2 volumes of ethanol in the presence of 0.1 M sodium chloride and resuspended in distilled water.

Quantitation of CMV Genome in Aqueous Humor Samples

A LightCycler instrument (Roche) was used to amplify and monitor the amplified product of targeted nucleic acid at each cycle of the PCR. Monitoring is based on the binding of a double strand-specific fluorescent dye, SYBR Green I (S-7567; Molecular Probes, Eugene, OR, USA), which binds amplicons. For each assay, a 2 μL extracted DNA sample was added to 18 μL of PCR mixture in a reaction capillary, according to the manufacturer's instructions. The PCR mixture contained 4 mM $MgCl_2$ and 0.7 μM of the primers. For amplification of the CMV genome, we used a pair of primers targeting the glycoprotein B (gB) sequence.[26] The forward and reverse primers were 5′GAG GAC AAC GAA ATC CTG TTG GGC A′3 and 5′GTC GAC GGT GGA GAT ACT GCT GAG G′3, respectively. After amplification, the sizes of amplicons were confirmed by 2% agarose gel electrophoresis. The samples were also subjected to nested PCR analysis using a primer pair targeting the internal region of the gB sequence: 5′ACC ACC GCA CTG AGG AAT GTC AG′3 and 5′TCA ATC ATG CGT TTG AGG AGG TA′3. For amplification of the human β-globin gene we used the PC03 and PC04 pair of primers (Takara, Kyoto): 5′ACA CAA CTG TGT TCA CTA GC′3 and 5′CAA CTT CAT CCA CGT TCA CC′3. Amplification was performed using the following protocol: 95°C for 2 minutes for one cycle, followed by DNA denaturation at 95°C, 5 seconds of annealing at 60°C, and 12 seconds of primer extension at 72°C for 45 cycles.

For quantitation, prediluted standard DNA templates from 10^1 to 10^5 copies, were prepared for CMV genome and β-globin gene assays. For these assays, the whole sequence of ORF UL55 coding the CMV gB and the sequence of human β-globin gene were cloned into pGEM-T vector (Promega, Madison, WI, USA) by a TA-cloning method. The gB sequence of CMV, 1952 bp in length, was amplified with a primer pair (5′ATC ATG CTG TCG ACG GTG GAG ATA′3 and 5′GGG ATC CAC ATG GAA TCC AGG ATC TGG′3) using a CMV AD169 strain as a template. That of human β-globin gene, 110 bp in length, was obtained with the pair of PC03

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Table 1. Clinical Details of All Subjects with Cytomegalovirus Retinitis

Patient No.	Sample No.	Age (y)	Sex	Underlying Diseases	Laterality (Sampling)	Activity of Retinitis	Paracentesis from Onset	Ongoing Treatment of Ganciclovir	Visual Acuity	Grades of Anterior Chamber Cells	Extent of Retinitis at Initial Visit or Admission (Quadrant)
1	1	57	M	Non-Hodgkin's lymphoma under chemotherapy	Bilateral (left)	Active	6 weeks	−	1.0	3+	2
	2					Active	14 weeks	+	0.4	1+	
	3					Inactive	8 months	−	0.3	0	
2	4	40	M	AIDS	Right	Active	2 weeks	−	1.2	2+	2
	5					Active*	5 weeks	+	1.2	2+	
3	6	36	M	CML, post-BMT	Right	Active	4 weeks	−	0.8	3+	1
	7					Inactive	8 weeks	+	1.2	1+	
4	8	50	M	CML, post-BMT	Right	Active	1 week	+	0.2	3+	4
5	9	38	M	Hodgkin's Lymphoma, post-BMT	Right	Active	3 months	+	0.5	2+	3
	10					Active*	8 months	+	0.2	1+	
6	11	26	M	Common variable immunodeficiency	Bilateral (left)	Active	8 weeks	−	0.9	3+	4
	12					Active	10 weeks	+	0.9	2+	
	13					Inactive	14 weeks	+	0.7	1+	

*In a convalescing condition with focal active changes.

and PC04 using an extracted DNA from human blood cells.

Results

Establishment of Quantitation Methods of CMV Genome and Human β-globin Sequence by Real-Time PCR

To determine the sensitivity and reliability of the LightCycler (LC)-PCR, serially diluted pGEM-T-CMV gB and pGEM-T-human β-globin were assayed and standard curves of cycle number versus log of the copy number were constructed. For both CMV genome and human β-globin sequence the standard curves were linear from 10^1 copies to 10^5 copies of the plasmid DNA (Figure 1). To confirm the specificity of the primer pair for the CMV genome, we subjected the amplified product to agarose gel electrophoresis and found it to be the expected size, 150 bp in length (Figure 2).

Analysis of Clinical Samples for CMV Genome

Next, we determined the copy numbers of the CMV genome in the aqueous humor samples obtained from the patients with CMV retinitis and with senile cataracts using LC-PCR. Sample DNAs from the CMV retinitis patients contained 21–15 885 copies of the CMV genome/μL of aqueous humor samples obtained from the initial paracentesis before antiviral treatment. In contrast, less than 10 copies/μL were detected in the samples from patients with inactive retinitis (Table 2). No viral genome was detected in the samples from senile cataract patients. To further confirm the presence of CMV in the aqueous humor samples, nested PCR analysis was performed on the samples which were positive detectable and undetectable in the LC-PCR analysis. All the samples from the retinitis patients were positive but those from the cataract patients were negative.

Correlation of Copy Number of CMV Genome and Activity of CMV Retinitis

Aqueous humor samples from the 4 untreated patients with CMV retinitis were used to determine whether there is a relationship between the copy number of the CMV genome and the number of retinal quadrants involved. The aqueous humor samples seemed to contain higher copy numbers of the CMV genome if more retinal quadrants were involved (Figure 3A). No linearity between copy number and retinal quadrant involvement was observed for the 2 patients who had already been treated with ganciclovir.

For the 4 previously untreated patients, the copy numbers of the CMV genome in the aqueous humor samples were monitored during ganciclovir treat-

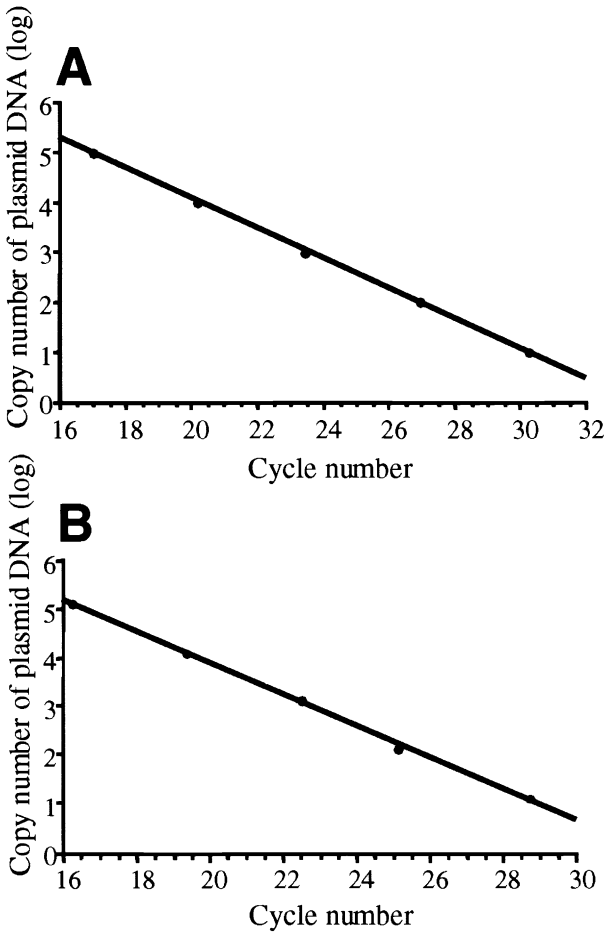

Figure 1. Standard curve for LightCycler-polymerase chain reaction (LC-PCR). Serially diluted plasmid DNAs were amplified and analyzed using an LC-PCR apparatus. Copy numbers of plasmid DNAs were plotted against threshold cycle numbers for the cytomegalovirus genome (A) and the β-globin gene (B). $R = 0.999$, $P < .0001$.

ment. Within 4 weeks of the commencement of antiviral treatment the copy numbers decreased to less than 1/100 of those observed prior to treatment. However, the CMV genome remained detectable for patients originally having a high copy number of the viral genome in the aqueous humor samples (Figure 3B).

Analysis of Clinical Samples for Copy Number of β-globin and Correlation of the Grades of Anterior Chamber Cells and the Extent of CMV Retinitis

Copy numbers of the β-globin gene were also determined to reveal the cell number in the aqueous humor samples and its correlation with the slit-lamp microscope analysis results and the retinal involvement. The copy number ranged from an undetectable level to 208 copies/μL of aqueous humor sample. The patients for whom more retinal quadrants were involved seemed to have higher copy numbers. The copy number of the β-globin gene might also correlate with the results of slit-lamp microscope analysis (Figure 4A) and with the copy numbers of the CMV genome (Figure 4B), although the sample numbers were too small for statistical analysis.

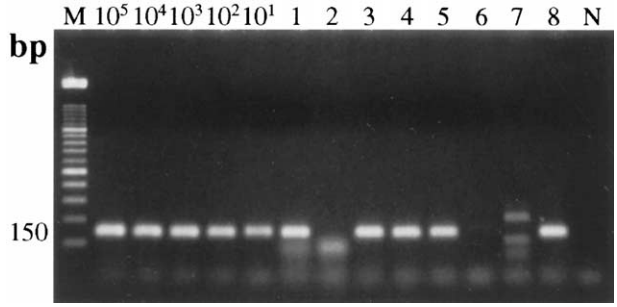

Figure 2. Confirmation of amplified products by 2% agarose gel electrophoresis. Serially diluted plasmid DNAs from 10^5 to 10^1 copies for titration (lanes 10^5 to 10^1) and samples 1 and 3 to 9 (lanes 1 to 8) were amplified for the cytomegalovirus genome in LightCycler-polymerase chain reaction (LC-PCR) and the amplified products were electrophoresed. The estimated size of the amplified product was 150 bp in length. Lane N was a negative control for the assay (no template added to the LC-PCR). Extra bands were observed for samples 2, 6, and 7, for which the copy numbers were under the detectable limit of the LC-PCR analysis.

Discussion

In this study we analyzed the copy number of the CMV genome and the β-globin sequence in the aqueous humor samples of patients with CMV retinitis using the LC-PCR analysis method. This method has the advantage of shortening the time required for analysis and can demonstrate the sequential changes in these copy numbers during antiviral treatment. A marked decrease in the copy number of the CMV genome after anti-viral treatments was quantitatively confirmed.

PCR analysis of aspirated aqueous humor or vitreous fluid samples has been used for the diagnosis of CMV retinitis, since Fox et al[11] and Fenner et al[12] first reported its use. The results of these analyses established it as a technique for the initial diagnosis of CMV retinitis and also circumvented the need for serological tests, which are unreliable.[8] However, it is difficult to monitor response to antiviral treatment by qualitative PCR analysis. To overcome this prob-

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Table 2. Quantitation of Cytomegalovirus (CMV) and β-Globin in the Aqueous Humor in CMV Retinitis Patients

Patient No.	Sample No.	Copy Numbers of CMV in 1 μL of Aqueous Humor					Copy Numbers of β-Globin in 1 μL of Aqueous Humor				
		1	2	3	Avg.	SD	1	2	3	Avg.	SD
1	1	812	3350	1156	1773	1052	7.3	9.8	<5	8.6	1.3
	2	<5	10.1	<5	<5–10	0	<5	<5	<5	<5	
	3	<5	<5	<5	<5*		<5	<5	<5	<5	
2	4	1647	3605	1073	2108	998	<5	6.2	5.3	5.8	0.5
	5	289	687	558	511	148	11.5	<5	5.4	8.5	3.1
3	6	24.1	31.7	6.5	20.8	10	9.2	<5	7.1	8.2	1.1
	7	<5	<5	<5	<5*		<5	<5	<5	<5	
4	8	<5	<5	<5	<5*		74.3	287.1	262.0	207.8	89.0
5	9	250.3	528	308	362	111	20.4	<5	10.5	15.5	5.0
	10	<5	7.5	<5	<5–7.5*		<5	<5	<5	<5	
6	11	16000	22500	9155	15885	4487	71.4	48.6	31.0	50.3	14.0
	12	6500	5500	5065	5688	541	18.9	23.1	12.4	18.1	3.8
	13	9.8	11.0	8.9	9.9	0.7	<5	6.0	6.7	6.4	0.4

*Positive in a nested polymerase chain reaction analysis.

lem, competitive PCR analysis was used, but it is rather complicated and time-consuming. Recently, a real-time fluorescence PCR was developed and two commercial systems (ABI PRISM 7700 SDS and Roche LC-PCR system) are available. Both have the advantages of decreasing the time required to accomplish PCR analysis and to measure the copy numbers of target sequences over a wide range from 10^0 to 10^9 copies. In the present study, the CMV genome was present at up to 1.6×10^4 copies/μL of aqueous humor sample obtained from patients with retinitis that involved the four quadrants. This copy number was similar to that reported by Gerna et al.[14] Thus, the CMV genome might be present on up to 10^5 copies per 1 μL of aqueous humor sample.

The copy numbers of CMV and retinal involvement were well correlated for the untreated patients, and also correlated with the number of infiltrating or desquamated cells in the aqueous humor. After antiviral treatment, this correlation was not observed. While the flow of CMV from the retina to the anterior chamber in CMV retinitis was not determined, CMV or CMV-infected cells might reach the anterior chamber by simple diffusion or active migration. A cytological study of aqueous humor would indicate whether the CMV genome was from a cell-free virus or a cell-associated one.

Gerna et al[14] reported the sequential change in CMV genome copy in the aqueous humor and blood cells during antiviral treatment. In their study, the viral genome copy number decreased from 1/5 to 1/100 of that prior to treatment after 3 weeks of intravenous foscarnet therapy. In the present study, the antiviral treatment reduced the CMV genome copy number to about 1/100 of that prior to treatment within 4 weeks, but the genome remained detectable by nested PCR analysis. In those patients originally having high copy numbers of the CMV genome, it was possible to detect the genome by LC-PCR even 4 weeks after ganciclovir treatment. Furthermore, quantitation of the viral genome in patients treated with antiviral therapy without verification of CMV retinitis by laboratory testing will be of value in deciding whether to continue or intensify the dosage of antiviral agents.

We initially planned to use LC-PCR analyses for diagnosis and monitoring of treatment. From the results of the present study it is apparent that the CMV genome copy number is well correlated with the activity of CMV retinitis; however, it is difficult to determine when to cease antiviral treatment based on the results of LC-PCR analyses. Even in patients with inactive retinitis, a low number of copies of CMV genome was detectable in the aqueous humor samples. Because CMV retinitis is a disease frequently reactivated in immunocompromised patients and antiviral treatment only disrupts viral replication and does not eliminate the virus, it will be necessary to carefully use viral genome copy number as an index to decide treatment.

We also demonstrated a correlation between β-globin gene copy number and the grades of anterior chamber cells determined by slit-lamp microscope analysis. According to Nussenblatt et al,[27] the numbers of

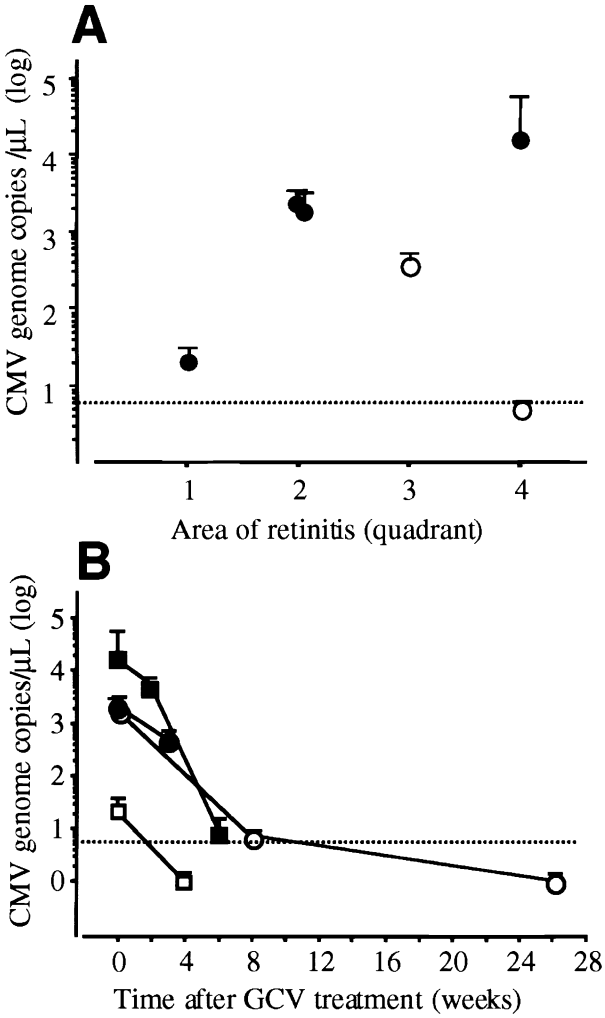

Figure 3. Clinical correlation of copy number of cytomegalovirus (CMV) genome. (**A**) Correlation of CMV genome copy numbers in 1 μL of aqueous humor sample and the extent of retinal involvement. Closed circles represent the samples from untreated patients and open circles represent those from ganciclovir-treated patients at admission. (**B**) Sequential changes of CMV genome copy numbers in 1 μL of aqueous humor samples during ganciclovir treatment in 4 patients. The results are expressed as mean ± SD for experiments performed in triplicate. A broken line at five copies/μL indicates the sensitivity of LC-PCR analysis for the CMV genome. ○: Patient 1, ●: Patient 2, □: Patient 3, ■: Patient 6.

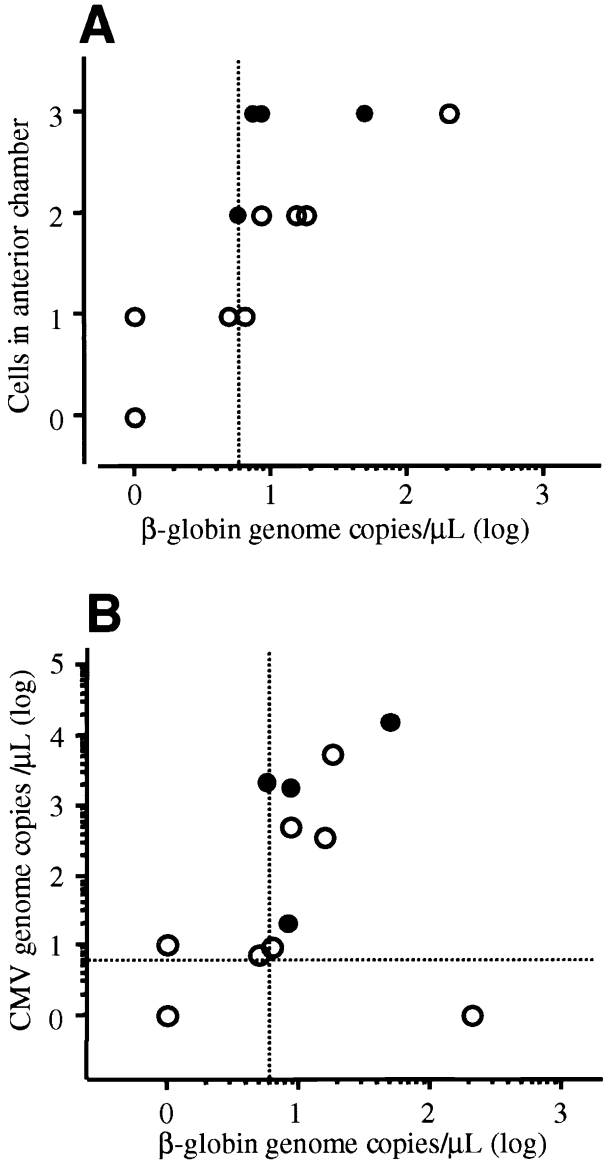

Figure 4. Correlation of β-globin gene copy numbers in 1 μL of aqueous humor samples with grades of anterior chamber cells (**A**) and with cytomegalovirus (CMV) genome copy number in 1 μL of aqueous humor sample (**B**). Closed circles represent the samples from untreated patients and open circles represent those from ganciclovir-treated patients. The broken lines at five copies/μL indicate the sensitivity of LightCycler-polymerase chain reaction analysis for the β-globin gene and CMV genome.

cells for grades 1–4 are 6–15, 16–25, 26–50 and more than 50 cells per slit field (1 × 1 mm), respectively. If the depth of the slit field was 3 mm and one cell contained two copies of β-globin gene, the number of cells would be 4–10, 12–16, and 18–32 cells for grades 1–3, respectively, based on the copy numbers of the β-globin gene in 1 μL of aqueous humor sample. The cell numbers obtained by slit-lamp microscope analysis tended to be less than those obtained by LC-PCR. This might be due to the detection of degraded cellular DNA detectable by LC-PCR.

In the LC-PCR analysis we used double-strand specific SYBR Green I, which binds not only amplified product, but also primer dimers and other non-

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specifically amplified fragments. Fortunately, nonspecific fragments were rarely observed for the samples from the aqueous humor. In addition, the number of primer dimers formed could be minimized by optimization of LC-PCR conditions. To decrease nonspecificity, performing the LC-PCR with hybridization probes will give more reliable results. However, the amount of CMV genome in the aqueous humor is usually enough for the present method at the time of initial examination.

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