CMV retinitis in HIV-positive patients in the pre-HAART era
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CHAPTER V

CYTOMEGALOVIRUS GLYCOPROTEIN B GENOTYPING IN OCULAR FLUIDS AND BLOOD OF AIDS PATIENTS WITH CYTOMEGALOVIRUS RETINITIS
PURPOSE: To determine the frequency of cytomegalovirus glycoprotein B (gB) genotypes in clinical samples of ocular fluids of patients with acquired immune deficiency syndrome (AIDS) who have cytomegalovirus retinitis and to compare these with the cytomegalovirus gB genotype in paired peripheral blood leukocytes.

Methods: Glycoprotein B genotypes of cytomegalovirus genomic DNA were determined in 29 ocular and 9 paired blood samples of 27 patients, by polymerase chain reaction amplification followed by restriction fragment length polymorphism analysis. Results: In the 29 ocular samples, 30 gB genotypes were determined: Glycoprotein B1 was found in 8 samples (27%), gB2 in 9 samples (30%), gB3 in 6 samples (20%), and gB4 in 3 samples (10%). In one sample, a mixed genotype was observed. In addition to these previously characterized gB genotypes, a new gB variant was observed in the ocular fluid of four patients. Partial sequence analysis revealed that this new gB genotype is closely related to gB3, and it was therefore named gB3'. In the blood samples, only gB1, gB2, and gB3 genotypes were observed. In the nine paired samples of ocular fluid and blood, four showed a difference in gB genotype between these compartments.

Conclusions: The distribution of cytomegalovirus glycoprotein B genotypes gB1-gB4 in ocular fluids of patients with AIDS who have cytomegalovirus retinitis was determined in this study. The predominance of gB2, as described by others, was not confirmed. The glycoprotein B genotype in the eye can be different from the genotype found in the blood of the same patient. A new gB variant, gB3', was found in the ocular samples of 4 of 27 patients, but not in the blood samples tested.
Cytomegalovirus infection is the most frequently observed opportunistic ocular viral infection in patients infected with human immunodeficiency virus (HIV). The incidence of cytomegalovirus retinitis in patients with acquired immune deficiency syndrome (AIDS) is reported to be between 24% and 30% and is associated with a low CD4+ T-lymphocyte count. It has been suggested that cytomegalovirus infection accelerates the progression of HIV-related immunodeficiency. Several studies have shown that a person may harbor multiple cytomegalovirus strains, simultaneously and serially, at different sites and even at the same site. Because different cytomegalovirus strains may display widely distinct biologic behavior, including tissue tropism, virulence, drug resistance, and the ability to stimulate HIV replication, we studied the variation in a region coding for cytomegalovirus envelope glycoprotein B (gB). The gB protein is involved in virus-cell interactions and is a major target for virus-neutralizing antibody during the host immunologic response. Using polymerase chain reaction (PCR), followed by restriction fragment length polymorphism of the variable part of the gB coding sequence, four discrete gB genotypes (gB1-gB4) have been described. Recently, a new gB1-related genotype (gB-New) was described in a patient infected with HIV. The presence of the cytomegalovirus gB2 genotype in the blood of HIV-infected patients has been reported to increase the incidence of cytomegalovirus retinitis. To our knowledge, the distribution of cytomegalovirus gB genotypes in ocular fluids has not been published. Determining the cytomegalovirus gB genotype in the eyes of patients with retinitis would be interesting in view of possible differences in cytomegalovirus strain composition between the blood and the eye and the reported association of retinitis and the presence of cytomegalovirus gB2 in the blood. In this study, we determined the frequency distribution in the eye of the cytomegalovirus gB genotype and the differences in cytomegalovirus gB genotype in paired clinical samples of the eye and the blood of HIV-positive patients with cytomegalovirus retinitis.

METHODS

Patient Selection

The 27 patients with AIDS studied in this report were seen between 1988 and 1996. Twenty-six patients were men. Three patients were intravenous drug users; all others belonged to the homosexual risk group. Mean age at diagnosis of cytomegalovirus retinitis was 41 years (range, 30-58 years). All had a positive signal for PCR amplification of part of the cytomegalovirus immediate early 1 (IE-1) gene DNA in the ocular fluid.
Paired peripheral blood leukocytes (PBLs) obtained from nine patients could be tested for cytomegalovirus gB genotype. The blood samples were obtained at the same time as the ocular samples, unless stated otherwise. The tenets of the Declaration of Helsinki were followed, and institutional human experimentation committee approval was granted.

**Isolation of Viral DNA, Polymerase Chain Reaction Amplification, and Cytomegalovirus Glycoprotein B Genotyping**

Venous blood samples were placed in a tube with EDTA to obtain PBLs. Erythrocytes were lysed by adding three times the volume of the venous blood sample of lysis buffer (150 mM NH₄Cl, 10 mM KHCO₃, and 1 mM EDTA). After lysis of the erythrocytes, the sample was centrifuged at 450g, and the pellet of PBLs was washed with phosphate-buffered saline (PBS). DNA was isolated from aqueous humor (19 samples) or vitreous (10 samples) and PBLs, essentially as described.

Briefly, samples of PBLs (10⁶ cells in 100 : 1 PBS), 100 : 1 vitreous fluid and 100 : 1 aqueous humor were lysed in 0.1 M Tris, 10 M guanidinium thiocyanate, 0.036 M EDTA (pH 8), 2.6% Triton X-100, and incubated with 40 : 1 silicon dioxide beads. Subsequently, the suspension was washed twice with 0.1 M Tris, 10 M guanidinium thiocyanate, then twice with 70% ethyl alcohol and once with acetone. After washing, the DNA was eluted from the beads in distilled water, while shaking at 56°C for 10 minutes, using 20 : 1 more than the volume from which it originated.

The presence of amplifiable cytomegalovirus DNA was tested by PCR amplification of part of the cytomegalovirus IE/7 DNA. The major region of gB sequence variability was amplified using 2.5 : 1 DNA solution in a nested PCR, which resulted in the amplification of sequences between nucleotides 1232 and 1750 of the gB gene. The amplified gB sequence was digested with endonuclease MaeIII (Boehringer Mannheim, Mannheim, Germany) and subjected to 3% agarose gel electrophoresis. The cytomegalovirus gB genotype was determined by analyzing the generated fragments characteristic of the known four different gB genotypes.

**Cloning and Sequence Analysis of the Glycoprotein B3' Polymerase Chain Reaction Fragment**

The gB3' PCR fragment was ligated into pGEMT-easy (Promega, Madison, WI). Clones were sequenced using the dideoxy chain termination method. Glycoprotein B3' was compared with gB1-gB4 and gB-NeW by sequence alignment. The sequence of gB3' is present as an unpublished entry in GenBank accession number U88696.
RESULTS

DNA was isolated from 29 ocular fluid samples of 27 patients with AIDS who had cytomegalovirus retinitis. The gB genotype frequency distribution was determined by PCR amplification of the gB gene region between nucleotides 1232 and 1750, followed by restriction fragment length polymorphism analysis. DNA fragment patterns of 23 patients, resulting in 23 gB genotypes, could be readily assigned to one of the four known cytomegalovirus gB genotypes (Fig. 1, lanes gB1-gB4). Eight (27%) belonged to the gB group 1, nine (30%) belonged to gB group 2, six (20%) belonged to gB group 3, and three (10%) belonged to gB group 4. However, four patients (13%) showed a restriction fragment length polymorphism different from gB1-gB4 (Fig. 2).

False-positive results for cytomegalovirus DNA amplification probably do not account for the detection of cytomegalovirus DNA in ocular fluids of HIV-positive patients with cytomegalovirus retinitis. In patients without ocular infection (n = 38) and in patients with such intraocular infections as acute retinal necrosis (n = 16) or Toxoplasma chorioretinitis (n=22), cytomegalovirus DNA could not be detected in ocular fluids by PCR amplification of the IE-1 sequence. Results of polymerase chain reaction analysis of cytomegalovirus DNA in four patients with AIDS with typical Toxoplasma chorioretinitis also proved negative (results not shown).

![Restriction fragment length polymorphism analysis of cytomegalovirus glycoprotein B (gB) genotypes observed in ocular samples. Polymerase chain reaction-amplified gB gene sequences were digested with restriction endonuclease MaelIII and subjected to agarose gel electrophoresis and ethidium bromide staining. Bands characteristic of the four known gB types (gB1-gB4) are shown next to the newly identified genotype gB3'. Numbers at the left indicate sizes (bp) of the molecular weight marker (M).](image-url)
Fig. 2. Frequency of glycoprotein B (gB) genotypes in ocular samples obtained from patients with AIDS who have cytomegalovirus retinitis. From 27 patients, 29 ocular samples were analyzed, in which a total of 30 gB genotypes could be scored.

Fig. 4. Glycoprotein gB genotyping of paired ocular (E) and peripheral blood leukocytes (B) samples of five patients (pat. 1–5). The second ocular sample of patient 3 (E?) was obtained after a 10-month interval. For the eye sample, aqueous humor of patients 1 through 4 and vitreous fluid for patient 5 were analyzed.
Fig. 3. Alignment of cytomegalovirus genomic DNA and deduced amino acid sequences for codon 463 through 532 of the five previously identified glycoprotein B (gB) genotypes (gB1-gB4 and gB-New²) and the newly identified genotype gB3³.
The new gB genotype, tentatively designated as gB3', has not been previously described in published studies. The DNA restriction pattern is characterized by a large *MaeIII* fragment, comigrating with the gB3-specific 362-bp fragment, and two smaller fragments of which one comigrates with the 139-bp fragment of gB4 and one unique fragment of approximately 50 bp (Fig. 1). Sequence analysis of the gB3' sequence coding for amino acids 463 through 532 revealed that gB3' is closely related to gB3. Nine of the 11 amino acid substitutions unique in gB3, (not found in gB1, gB2, gB4, and gB-New), are also present in the gB3' sequence (Fig. 3). In two patients, two serial samples of the same eye were available. One patient initially showed only a cytomegalovirus gB3 genotype in the aqueous humor and the blood; but after 10 months, a mixed gB3-gB1 genotype was detected in the aqueous humor of the same eye (Fig. 4, patient 3). In the aqueous humor of the other patient a switch from a gB4 to a gB3' genotype was observed within 5 months. Aqueous humor and vitreous collected at the same time from one eye of a patient showed a gB3 genotype in both ocular samples (not shown). Paired ocular and blood samples, collected at the same time from nine patients could be analyzed by PCR. When these samples were assayed for the cytomegalovirus gB genotype, it appeared that four patients displayed a different gB genotype in the blood, compared with expression in the aqueous humor sample (Fig. 4, patients 1 and 2), whereas the remaining five patients had identical gB genotypes in these two compartments (four aqueous humor and one vitreous; Fig. 4, patients 3, 4, and 5). In the nine blood samples, only gB1, gB2, and gB3 genotypes were observed. One sample contained a mixed gB2-gB1 genotype (Fig. 4, patient 2).

**DISCUSSION**

In this report, the cytomegalovirus gB genotype frequency distribution in the eye and the differences in cytomegalovirus gB genotype between ocular and blood samples of patients with AIDS who have cytomegalovirus retinitis is described. The distribution of gB1, gB2, gB3, and gB4 genotypes in ocular fluids from patients analyzed in the present study was 27%, 30%, 20%, and 10%, respectively, and was 13% for gB3'. Other laboratories report distributions of gB1-gB4 of 27%, 41%, 16%, and 16% in viral blood cultures of 44 HIV-positive patients (22) with CD4+ lymphocyte counts less than 100, and 8%, 56%, 30%, and 6% in leukocytes of 31 HIV-positive patients with retinitis and a CD4+ count less than 100.21 The high incidence of cytomegalovirus gB2 observed in these studies could not be confirmed by the gB frequency distribution in ocular samples determined in this study.
This discrepancy could result from differences in gB genotype between the eye and the blood of the same patient.

Although in this study aqueous humor and vitreous were used as sources of ocular fluid, we assume that the cytomegalovirus genotype in these samples of the same eye are identical. The detection of the same cytomegalovirus gB genotype in aqueous humor and vitreous obtained from one eye argues in favor of this assumption. Remarkably, the gB3' genotype isolated from ocular samples of 4 of 27 patients in the present study was not observed in blood samples or in the large number of samples of other tissues analyzed in previous studies (as many as 161 samples from 128 patients). These findings could indicate that cytomegalovirus with the gB3' genotype has a distinct tropism for the eye and therefore may not have been detected in other tissues. However, we cannot exclude the presence of cytomegalovirus gB3' in low concentration or as a small component of an evolving infection. Furthermore, the prevalence of cytomegalovirus gB3' in other immunocompromised Dutch patient groups is unknown.

Because herpes simplex virus gB is involved in cell entry, cell fusion, and virulence, it is conceivable that the closely related cytomegalovirus gB protein has similar functions. This is supported by the observation that in HIV-positive patients, the distribution of gB genotypes is different in leukocytes and semen, which suggests distinct tissue tropism for different cytomegalovirus gB genotypes. Alternatively, the presence of relatively rare cytomegalovirus gB genotypes could be dependent on the geographical origin of the samples analyzed. For example, the gB-New variant was isolated from only two patients and has not been reported by other investigators.

Clinical observations such as CD4+ lymphocyte counts, time between diagnosis of AIDS and diagnosis of retinitis, presence of extra ocular cytomegalovirus disease, development of retinal detachment, localization and extent of retinitis, and survival time could not be related to the gB genotype distribution in this group of patients. However, the number of patients in each group is small. Recently a correlation between the presence of cytomegalovirus gB2 in the blood and the development of cytomegalovirus retinitis in patients with advanced HIV disease was reported. In view of the difference in genotype between blood-derived and eye-derived cytomegalovirus of paired clinical samples, linking of blood-derived cytomegalovirus genotypes to clinical manifestations in the eye may not be valid. Approximately half of the patients had the same gB genotype at both sites, which suggests that in these cases, blood-derived cytomegalovirus represents the cytomegalovirus genotype in the eye. In view of the small number of different gB genotypes, the presence of the
same gB genotype in two body compartments does not necessarily indicate an identical cytomegalovirus strain. A linkage study has shown that one gB genotype can be associated with different glycoprotein H genotypes.25

The presence of multiple cytomegalovirus strains, concurrently and serially, has been demonstrated in semen of HIV-positive men12 and was also shown in ocular cytomegalovirus in the present study. In one patient, the ocular gB genotype changed from gB4 to gB3' within 5 months. In another patient, only gB3 was detected initially, whereas after 10 months, the presence of gB3 and gB1 was demonstrated in the same eye.

The difference in cytomegalovirus strain distribution between the blood and the eye in samples drawn at the same time, as was seen in several patients, is somewhat puzzling. Cytomegalovirus is probably disseminated by the bloodstream to various organs by mononuclear cells and neutrophils.28 This hematogenous spread implies that the cytomegalovirus strain found in the eye must have been present in the blood. However, in several patients, the cytomegalovirus strain in the ocular sample was different from the strain detected in the blood sample. Possibly, the level of the ocular cytomegalovirus strain is below detection level in the blood, and the difference in strain distribution is established and maintained by tissue tropism or restoration of the blood-retinal barrier. Alternatively, the intraocular cytomegalovirus strain could originate from a previous viremia of a cytomegalovirus gB genotype that has already been cleared from the blood but that has remained in a latent state in ocular tissue. The differences in cytomegalovirus strain distribution between the eye and the blood could explain cases of progressive retinitis observed in patients treated with ganciclovir, despite normal sensitivity to this drug of the blood-derived cytomegalovirus strains.29 Furthermore, these differences argue in favor of using ocular samples in determining cytomegalovirus drug-resistance by PCR-based drug-sensitivity analysis in cytomegalovirus retinitis.30

REFERENCES

Cytomegalovirus glycoprotein B genotyping.


