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Human Herpesvirus 6-A, 6-B and 7 in Vitreous Fluid Samples

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Abstract

Human herpesvirus 6 and 7 (HHV-6, HHV-7) have been associated with several neurologic syndromes and have been detected in nervous tissue from healthy persons; however, only two cases of HHV-6A have been reported to be associated with intraocular inflammatory disease. Vitreous fluid was tested from 101 patients, including 69 samples from patients with ocular inflammation including CMV retinitis, idiopathic retinitis, iritis, and vitritis, for HHV-6A, HHV-6B, and HHV-7 DNA by PCR. HHV-6A DNA (4,950 copies per ml) was detected in vitreous fluid from one patient with CMV retinitis, HHV-6B DNA (10,140 copies per ml) was detected in vitreous fluid from one patient with idiopathic ocular inflammation in the absence of CMV DNA, and HHV-7 was not detected any of the vitreous samples. HHV-6A, HHV-6B, and HHV-7 DNA are detectable in less than 2% of vitreous samples in patients with ocular inflammation.

Keywords

HHV-6A; HHV-6B; HHV-7; retinitis; iritis; vitritis

INTRODUCTION

Human herpesvirus 6 and 7 (HHV-6 and HHV-7; family Herpesviridae, subfamily Betaherpesvirinae, genus Roseolovirus, species Human herpesvirus 6 and 7) are neurotropic viruses that reactivate frequently in immunocompromised persons [Cohen 2010]. Most adults in the United States are infected with both of these viruses. Two variants of HHV-6 have been identified; HHV-6A has a greater predilection to infect neural cells than HHV-6B, but is less often associated with disease than HHV-6B. HHV-6B, and less frequently HHV-7, cause exanthem subitum (roseola). While HHV-6 DNA has been detected in the brain from 43% to 74% of persons at autopsy [Challoner, et al., 1995; Sanders et al., 1996], it is not known how frequently HHV-6 and HHV-7 DNA are detectable in the eye. Vitreous fluid was assayed from 101 patients with ocular inflammation for HHV-6A, HHV-6B, and HHV-7 DNA to ascertain the frequency of these viruses in the eye, and if these viruses are present in the absence of CMV or other pathogens in vitreous fluid.

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MATERIALS AND METHODS

Patients and Samples

Polymerase chain reaction (PCR) was performed for HHV-6A, HHV-6B, and HHV-7 DNA in 101 vitreous fluids obtained by ocular paracentesis from persons with various diseases, including diabetic retinopathy, CMV retinitis, ocular malignancies, idiopathic vitreal hemorrhage, retinal detachment, vitritis, iritis, and retinitis. All samples had been obtained previously for other diagnostic testing and were frozen at -80°C ; each was coded before PCR testing for HHV-6 and HHV-7. All experiments were performed in compliance with relevant laws and institutional guidelines and in accordance with the ethical standards of the Declaration of Helsinki. This research was reviewed by the Office of Human Subjects Research at NIH and determined to be exempt from IRB Approval.

Quantitative PCR

DNA was extracted from vitreous samples using the NucliSens nucleic acid isolation kit as recommended by the manufacturer (BioMerieux). For most samples (67%, 70/105) 100 μl of vitreous fluid was used; however, 200 μl of vitreous sample was used for 25% (26/105), and in the remainder (9/105) 50 to 150 μl of vitreous sample was used. All samples were eluted with 50 μl of elution buffer. HHV6 DNA was quantified using primers HHV6A/B.forward (5'-GTGGTGTGTTGATTTTCARAGTTTGTATCC-3') and HHV6A/B.reverse (5'-ATAAAGATGCTATCCGTATCACCATARATTAC-3') that amplify a portion of the viral DNA polymerase gene based on previously described sequences [Johnson et al., 2000] with minor modifications. Fluorescent resonance energy transfer (FRET) probes [Safronetz et al., 2003] were labeled with Red640 and distinguish HHV-6A from -6B by binding over a single base-pair mismatch. HHV-7 DNA was quantified using primers HHV7 forward 5'-GTAGTTTTTGTATTTCCAAAGTTTGTATCC-3' and HHV7 reverse 5'-ACAAAAAGACTGTCAGTATCACCATAAATCAC-3'. FRET probes were HHV7 FITC: 5'-GAAAATGCAGTAATTGGTTTACATGCAGATG-Fluor-3' and HHV7.RD640: 5'-Red640-CATTCTCACTGTGCATGTTGGACCTGTAA-Phos -3'.

Real time PCR was performed using a LightCycler (LC) 1.5 instrument (Roche Diagnostics). PCR was performed in a 20 μl reaction consisting of 1X LightCycler FastStart DNA MasterHybProbe mixture containing FastStart Taq polymerase, reaction buffer dNTP mix (with dUTP instead of dTTP), and 1.0 mM MgCl_2 (Roche), an additional 3.0 mM MgCl_2 , 1.0 μM of each primer, 0.2 μM of each FRET probe, 1 U uracil-DNA-glycosylase (UNG), and 5 μl of extracted DNA. The reaction mixture was preincubated for 10 min at 30°C to activate UNG, DNA was denatured and UNG inactivated at 95°C for 10 min, and the template was amplified with 45 cycles of 10 sec at 95°C , 10 sec at 62°C , and 20 sec at 72°C . Melting curve analysis was then performed with one cycle of 95°C for 30 sec, 15 sec at 40°C , and 0 sec at 95°C (ramp, $0.2^{\circ}\text{C}/\text{sec}$, continuous acquisition). Positive samples were quantified using a standard curve (10-fold serial dilutions from 5×10^6 to 5×10^0 copies per reaction) with a plasmid containing the HHV-6B target region. HHV-6A and HHV-6B were differentiated by melting curve analysis with HHV-6A producing a melting point at 68.5°C and HHV-6B at 63°C .

Prior to extraction, each sample was spiked with an internal control (pBR322 plasmid DNA) to verify successful recovery of DNA and removal of PCR inhibitors. The internal control in extracted samples was detected by amplification in a separate qualitative LC real-time PCR as described above but using primers IC.forward (5'-ATTGCTAACGCAGTCAGGCACCG-3') and IC.reverse (5'-GCGAGCCCGATCTTCCCCAT-3') and FRET probes IC.FITC (5'-GATATCGTCCATTCCGACAGCATC-Fluor.-3') and IC.RD705 (5'-Red705-

CCAGTCACTATGGCGTGCTGC-TAG-Phos.-3'). The crossing point (Ct) for detection of the IC was defined as no more than three Cts greater than that obtained from the extracted negative control to be considered lacking significant inhibition.

To determine the number of human cellular equivalents in each PCR reaction, an additional aliquot of extracted DNA was analyzed by real-time PCR analysis for the human β -globin gene using the LC control DNA kit (Roche) according to the manufacturer's recommendations. The number of HHV-6 copies per cell was determined by dividing the number of HHV-6 copies/ml by half the number of copies of human β -globin DNA (since there are two chromosomes per cell).

RESULTS

The PCR assay was validated for vitreous fluid. Vitreous fluid from four patients was spiked with an internal plasmid control to evaluate the efficiency of DNA recovery and assess for the presence of PCR inhibition. Each vitreous sample tested positive for the plasmid DNA with no evidence of inhibition and negative for HHV-6 and HHV-7 DNA. Each sample was also tested for a cellular gene, beta-globin, and each was positive with values ranging from 10 cellular equivalents/ml of vitreous fluid to 1,754 cellular equivalents/ml of fluid. Another aliquot of each vitreous sample was spiked with HHV-6 and HHV-7 infected cell lysate, and PCR of the vitreous sample was positive for both viral DNAs. Thus, these results indicated that DNA could be extracted successfully from vitreous fluid, the fluid did not inhibit the PCR assay, and that HHV-6 and HHV-7 DNA could be detected by PCR in vitreous fluid.

PCR was performed for CMV DNA using coded vitreous samples from 33 patients that had been stored for several years at -80°C , including 11 samples that had previously tested positive for CMV DNA at the time they had been obtained, to determine if herpesvirus DNA was stable after prolonged storage. The other 22 samples were eyes of patients without inflammatory ocular disease (e.g. vitrectomy samples from diabetics, vitreous fluid from retinal detachment repairs, intraocular melanoma). One of these 22 samples which had initially tested negative for CMV DNA was CMV DNA positive in the assay. It is unclear if the positive sample had been incorrectly assayed as negative when tested originally or if the extraction and PCR procedures had improved in the 15 years since the sample was retested. Three samples which had initially tested negative gave faint signals which were below the level of the standard curve (<250 copies of CMV/ml). Ten of the 11 known CMV DNA PCR positive samples were again positive for CMV DNA PCR with 933 to $>250,000$ CMV DNA copies/ml (median 52,000 CMV DNA copies/ml); the one negative sample had been positive using one set of primers to the CMV major IE gene, but negative using another set of primers to the sample CMV gene when originally tested. These results indicated that herpesvirus DNA could still be detected by PCR in the stored samples.

An additional 68 vitreous samples were obtained from patients with non-inflammatory ocular disease (9 patients) ocular inflammation including patients with retinitis, vitritis, iritis (58 patients), and lymphoma (1 patient). Of these 68 samples, 6 were known to be CMV DNA positive, 1 was known to be HSV DNA positive, and 10 were from patients with HIV.

Of the 101 total vitreous samples, one was positive for HHV-6A DNA, 1 for HHV-6B DNA, and none for HHV-7 DNA (Table I). The HHV-6A DNA positive sample was from a patient with a detached retina and CMV retinitis. The sample contained 4,950 HHV-6A DNA copies per ml, was positive for CMV DNA (32,000 copies per ml), and negative by for HHV6-B, HHV-7, HSV, and VZV DNA by PCR. Based on detecting 33,000 human β -globin DNA copies per ml in the vitreous sample, there were approximately 0.30 copies of HHV-6A per cellular genome. The HHV-6B DNA positive sample was from a patient with

idiopathic ocular inflammation and contained 10,140 HHV-6B DNA copies per ml and was negative for HHV-6A, HHV-7, HSV, CMV, VZV, and toxoplasmosis DNA by PCR. Based on the 22,000 human β -globin DNA copies per ml in the vitreous fluid, there were an estimated 0.92 copies of HHV-6B per cellular genome.

DISCUSSION

While HHV-6 DNA is frequently found in the nervous system in adults, these results indicate that viral DNA is rarely present in ocular fluid. One vitreous sample contained HHV-6A DNA, one had HHV-6B DNA, and none had HHV-7 DNA suggesting that HHV-6A and HHV-6B DNA is detectable in about 1% of vitreous samples from patients with ocular inflammation. In contrast, studies have detected HHV-6A DNA in the brain of 28% to 70% of persons [Chan et al., 2001; Cuomo et al., 2001], HHV-6B DNA in the brain of 30–75% persons [Chan et al., 2001; Cuomo et al., 2001], and HHV-7 DNA in the brain of 37% of persons at autopsy [Chan et al., 2000].

At least two possibilities might explain the presence of HHV-6 DNA in the eyes of the patients independent of HHV-6-induced disease. First, HHV-6 DNA has been detected in circulating T cells, monocytes, and polymorphonuclear leukocytes (Cohen, 2010; Palteau et al., 2006) and may simply have been carried into the eyes in the inflammatory cells. Second, HHV-6 DNA has been reported to be integrated into the chromosomes of 0.8% of normal blood donors and 2.9% of hospitalized patients [Leong et al., 2007], and detection of HHV-6 DNA in the vitreous fluid may have been due to virus integration in the host chromosomes. Unfortunately, samples were unavailable to directly test for evidence integrated HHV-6 DNA. Based on the number of copies of human β -globin DNA copies in the vitreous fluid, there were an estimated 0.30 copies of HHV-6A DNA per cellular genome in one eye and 0.92 copies of HHV-6B DNA per cellular genome in the other eye. These values are close to the 2 to 5 copies of HHV-6 DNA present per cell when viral DNA is integrated [Leong et al., 2007] and suggest that HHV-6 may have been integrated into the genome in these patients.

The HHV-6A DNA positive sample was from a patient with CMV retinitis. HHV-6 reactivation frequently accompanies CMV reactivation [Humar et al., 2000], and the presence of HHV-6A DNA in the eye may have simply reflected the immunocompromised state of the patient. The HHV-6B DNA positive sample was from a patient with idiopathic ocular inflammation. While there was no other apparent cause for this patient's eye disease, the presence of HHV-6 DNA in vitreous fluid alone is not sufficient to prove that HHV-6 was responsible for the ocular inflammation. Additional tests for HHV-6 RNA or protein in ocular tissue would have been more definite and provided evidence of HHV-6 replication; unfortunately, such samples were not available.

Prior studies of HHV-6 in the eye have yielded conflicting results. Qavi et al. (1995) reported that nearly 50% of eyes from patients with HIV had detectable HHV-6 antigen in the retina. Fillet et al. (1996) found CMV coinfection with HHV-6 in the retina in all the three patients who had AIDS-associated retinitis. While at least 17 of the patients had CMV DNA detectable by PCR in the eye, only one had HHV-6 DNA. None of 100 vitreous samples [Mitchell et al., 1994] and none of 102 aqueous humor samples [Vrioni et al., 2007] were positive for HHV-6 DNA. Sugita et al. (2008) used multiplex PCR to search for HHV-6 and HHV-7 in ocular fluids from 100 patients with uveitis and found no cases of HHV-7 DNA, but detected HHV-6A DNA in aqueous humor and vitreous fluid of one patient with severe unilateral uveitis. This patient also had antibody to *Toxocara canis* larva in the ocular fluid and it was uncertain as to whether *Toxocara canis* larva or HHV-6 was the predominant pathogen [Sugita et al., 2007]. A second case of HHV-6A associated uveitis

has also been reported [Maslin et al., 2007]. This is the first report of HHV-6B DNA detected in ocular fluid. In summary, despite a high frequency of detection of HHV-6A, HHV-6B, and HHV-7 DNA in the brain, these virus DNAs are rarely associated with ocular inflammation.

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TABLE I
HHV-6A and HHV-6B DNA positive vitreous fluid samples from 101 total samples

Sample	Copies of herpesvirus genome equivalents per ml of vitreous fluid						
	HHV-6A	HHV-6B	HHV-7	CMV	HSV	VZV	toxoplasma
1 (CM-25)	4,950	0	0	32,000	0	0	ND
2 (CM-58)	0	10,140	0	0	0	0	0

HHV-6=human herpesvirus-6, CMV=cytomegalovirus, HSV=herpes simplex virus, VZV=varicella-zoster virus, toxo=Toxoplasmosis gondii, ND=not done.