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Molecular Evidence and Clinical Significance of Herpesvirus Coinfection in the Central Nervous System

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A total of 60 cerebrospinal fluid (CSF) specimens from patients manifesting symptoms resembling viral central nervous system (CNS) disease were examined for the presence of herpes simplex virus (HSV), human herpesvirus 6 (HHV-6), Epstein-Barr virus (EBV), cytomegalovirus, varicella-zoster virus, *Borrelia burgdorferi*, and *Tropheryma whippelii* DNA by PCR. Of 30 specimens which were selected on the basis of HSV DNA positivity, 2 were concomitantly positive for HHV-6 DNA and 1 was positive for EBV DNA. In the three specimens positive for more than one herpesvirus, amplicons generated with virus-specific primer sets hybridized specifically to the corresponding virus-specific probe. Sequence analysis of the two amplified DNA fragments demonstrated that they were derived from distinct herpesviruses. Of 22 patients with clinically diagnosed encephalitis, 2 of 3 patients coinfected with HSV and HHV-6 died, compared to 1 of 19 (5%) patients infected with only HSV. Of 30 CSF specimens that were negative for HSV DNA, EBV DNA was detected in one sample. These data indicated the presence of DNA specific for two distinct herpesviruses in the same CSF specimen, providing molecular evidence that coinfection with this group of viruses may occur in the CNS.

Herpesviruses are large, DNA-containing, enveloped viruses and include herpes simplex virus type 1 (HSV-1); HSV-2; Epstein-Barr virus (EBV); varicella-zoster virus (VZV); cytomegalovirus (CMV); and human herpesviruses 6 (HHV-6), -7, and -8. Herpesviruses are an important cause of central nervous system (CNS) infections (17, 19). Among these viruses, HSV accounts for approximately 2 to 19% of all cases of encephalitis and 20 to 75% of all cases of necrotizing encephalitis (40, 46).

Conventional laboratory diagnosis of CNS infections caused by these viruses has not been productive. HSV is rarely recovered in cell cultures from cerebrospinal fluid (CSF). In contrast, a brain biopsy specimen from the temporal lobe may yield the virus by culture techniques; however, the procedure is controversial when performed solely to collect specimens for diagnostic purposes. The sensitivity of the HSV antigen or antibody for rapid diagnosis of central nervous system (CNS) infections is very low (23). For example, with evidence of increasing titers in CSF of HSV antibodies, a diagnosis can be established retrospectively, but in only 80% of cases. Antibodies may appear in the CSF as the consequence of a breakdown in the blood-brain barrier, leading to a false-positive result (30).

As an alternative to these techniques, demonstration of the presence of HSV DNA in CSF of patients with CNS disease and subsequent reports of the excellent performance characteristics of the test has suggested that PCR be considered the new standard for the diagnosis of this infection (26, 38). Experience in testing for herpesvirus DNA in CSF has rapidly expanded to specific molecular assays for other herpesviruses (CMV, EBV, VZV, and HHV-6) (1, 5, 9).

Individual cases of mixed herpesvirus infections in CNS disease (HSV plus EBV and CMV plus EBV), as determined by PCR, have been reported (27, 42). Because HSV causes a wide spectrum of clinical features in patients with CNS disease, we wanted to determine the incidence of coinfection of this virus with several other agents that commonly cause these manifestations. In this study, we performed PCR for the detection of five herpesviruses (HSV, CMV, EBV, VZV, and HHV-6) and two bacterial pathogens, *Borrelia burgdorferi* and *Tropheryma whippelii*, in CSF specimens previously determined to be positive or negative for HSV DNA.

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MATERIALS AND METHODS

Samples. A total of 60 original CSF specimens submitted to the Molecular Microbiology Laboratory at the Mayo Clinic for the diagnosis of CNS disease were included in the study. Of these samples, 30 were HSV DNA positive (age, 44.1 ± 19.8 years; male/female ratio, 3:7) and were randomly chosen from available CSF specimens stored at −70°C for the past 3 years. The same number of specimens that were HSV DNA negative (matched for age and gender of HSV DNA-positive specimens) were chosen as controls. All 60 specimens were tested by PCR for DNA specific for CMV, EBV, VZV, HHV-6, *B. burgdorferi*, and *T. whippelii.*

Extraction of nucleic acids. The IsoQuick extraction procedure (Microprobe Corp., Bothell, Wash.) for extraction of DNA from CSF samples was performed according to the manufacturer's instructions.

Oligonucleotide primers and probes. Oligonucleotide primers for DNA gene targets of viruses and bacteria were prepared with a DNA synthesizer (Applied Biosystems) at the Mayo Clinic Molecular Core Facility, Rochester, Minn. The primers and probes used in PCR for HSV, CMV, EBV, *B. burgdorferi*, and *T. whippelii* have been reported previously (4, 12, 33, 37, 41). Primers (sense, 5′-ATC TCG ATT CCG TTC AGT CT-3′; antisense, 5′-CTT TTT CTT CAG GTG TCT CA-3′) spanning the region of the structural protein genome corresponding to nucleotide positions 2881 to 3120 (31) and internal probes (sense, 5′-CCG TTT AGG CTA CCG CAT GC-3′; antisense, 5′-ACC TTC ATT AAT TGC GCA GG-3′) were designed to amplify HHV-6 DNA by PCR. Primers (sense, 5′-ATT ATG GAC TAC GGC TTT TAC T-3′; antisense, 5′-CTT GGT CGG ATA GGG TGG TTT C-3′) and probes (sense, 5′-TTA GTC CGC GCG GCC ATG AAT C-3′; antisense, 5′-ATA AAA CCT CCT CTA GGA CAT G-3′) designated to cover the 53730-to-54016 portion of the gene 29 major binding protein (11) were used for VZV DNA amplification.

PCR amplification. The PCR mixture contained the following: 200 μM (each) deoxyribonucleoside triphosphates, 10× buffer (500 mM KCl, 100 mM Tris-Cl [pH 8.3], 15 mM $MgCl_2$, 2.5 mg of bovine serum albumin per ml), 100 pmol (each) of the appropriate primer, 25 μg of isopsoralen-10 (HRI Associates, Concord, Calif.) per ml, and 1.25 U of *Taq* polymerase. Each reaction tube received 45 μl of the reaction mixture, 2 drops of mineral oil, and 5 μl of target DNA. For *B. burgdorferi*, an additional 3.5 μl of 25 mM PE $MgCl_2$ was added, and the concentration of isopsoralen-10 was 100 μg per ml. A no-target control reaction received 45 μl of master mix 2 drops of mineral oil, plus 5 μl of H_2O from the reaction mixture only. Reactions were amplified in a DNA thermal

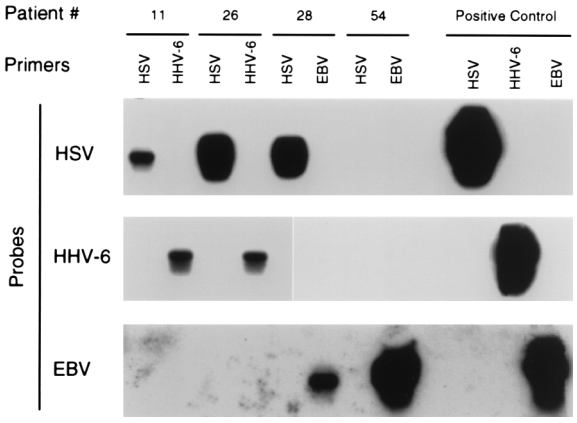

FIG. 1. Specificity of herpesvirus DNA amplified by virus-specific primers in CSF specimens. DNA extracted from the CSF specimen from patients 11, 26, and 28 was amplified individually by HSV-, HHV-6-, or EBV-specific primers and then transferred to the membrane by Southern blotting. The membrane was hybridized separately to HSV-, HHV-6-, or EBV-specific probes. Positive controls for HSV, HHV-6, and EBV DNA hybridization were included.

cycler (model 480; Perkin-Elmer Cetus) by a two-step protocol: 2 min of denaturation at 94°C for one cycle and 1 min of denaturation at 94°C, 1 min of annealing at 60°C, and 1 min of extension at 72°C for 50 cycles (12). For *B. burgdorferi*, an annealing temperature of 50°C and a 7-min extension at 72°C after 50 cycles were used (33). Amplicon products were inactivated by placing the reaction tube in an UV transilluminator (HRI-100; HRI Associates) at 4°C for 15 min before further analysis of amplification products.

Chemiluminescence probe. PCR products were visualized with UV light by staining with ethidium bromide after agarose gel electrophoresis (3% agarose gel with 1.5% NuSieve [FMC, Rockland, Maine] and 1.5% agarose [Bethesda Research Laboratories, Gaithersburg, Md.]). The products were then transferred to a nylon membrane (Magnagraph; Fisher) by Southern blotting. Membranes were prehybridized for 30 min at 42°C, with an ECL (enhanced chemiluminescence) hybridization solution purchased from Amersham (Arlington Heights, Ill.). The appropriate probe was developed by first amplifying a sequence internal to the initial primer set. These internal amplicons were then labeled for chemiluminescence with the enhanced chemiluminescence kit from Amersham. The labeled probes were added and allowed to hybridize to the membrane for 4 h. The membranes were washed, placed in 40 ml of detection reagent for 1 min at room temperature, and then exposed to X-ray film for 1 h (13). The film was then developed by a Kodak X-Omat X-ray film processor.

PCR fragment sequencing and analysis. DNA fragments amplified by PCR as described above, were purified with QIAquick PCR purification kits (QIAGEN, Inc., Chatsworth, Calif.). A reaction mixture including approximately 200 ng of purified DNA fragment and 2 to 3 pmol of primer was used for cycle sequencing with a DNA sequencing system (model 373A; Applied Biosystems). Multiple-sequence alignment was carried out with the software program Pileup/Gap included in the Wisconsin Package (14).

RESULTS

Herpesvirus coinfection in CSF. Of 30 HSV-positive CSF samples, 2 specimens (from patients 11 and 26) were also positive for HHV-6 DNA, and 1 specimen (patient 28) was positive for EBV by PCR. One sample (patient 54), of 30 CSF specimens that were HSV DNA negative by PCR, was positive for EBV. CMV, VZV, *B. burgdorferi*, or *T. whippelii* DNA was not detected in any of the study (HSV-positive) or control (HSV-negative) CSF specimens.

The specificity of the amplified DNA products representing coinfections in CNS disease was determined by probe and by sequence analysis. Amplified products generated by the HSV, HHV-6, and EBV primer pairs were separated electrophoretically and transferred to nylon membranes by Southern blotting. Probes specific for each virus reacted only with homologous target DNA (Fig. 1).

To confirm that the amplicons derived with different virus-specific primer sets came from distinct herpesviruses, the amplified DNA fragments were sequenced and compared to the known sequences of HHV-6 and EBV listed in GenBank (3, 31, 32, 45) (Table 1). The sequence of the amplified DNA fragments from CSF obtained from patients 11, 26, and 28 had a high similarity (99.1, 97.7, and 98.6%, respectively) to the target nucleotide sequence of HSV-1 DNA polymerase (43). In contrast, the DNA sequences amplified by either HHV-6 or EBV-specific primers from the same samples had a marked low similarity to the sequence of HSV. The sequence of DNA fragments amplified by HHV-6 (patients 11 and 26)- or EBV (patient 28)-specific primers had more than 90% similarity to the sequence of the corresponding virus genomes (3, 32). However, the sequence in the same samples amplified by the HSV-specific primer possessed only about 40% similarity to the sequence of the HHV-6 or EBV genomes. These data demonstrated that the DNA fragments amplified by HSV-, HHV-6-, or EBV-specific primers in the same CSF samples were derived from different herpesvirus genomes, suggesting the presence of more than one herpesvirus in the CSF specimens.

TABLE 1. Sequence similarity of HSV, EBV, and HHV-6 amplicons from CSF specimens

Patient no.	Specific primer	% Similarity to herpesvirus reference strain		
		HHV-6 Z29 (32)[a]	HSV KOS (43)	EBV B95-8 (3)
11	HHV-6	93.9	40.3	NA[b]
	HSV	37.3	99.1	NA
26	HHV-6	91.8	40.3	NA
	HSV	37.8	97.7	NA
28	EBV	NA	41.2	94.8
	HSV	NA	98.6	42.5

[a] Number in parentheses indicates the reference cited.
[b] NA, not analyzed.

Clinical features. The medical records of the 30 patients who had HSV DNA detected in the CSF were briefly reviewed, and information regarding clinical outcome, acyclovir administration, and previous human immunodeficiency virus (HIV) infection history was obtained. Eight of 30 patients were clinically diagnosed with meningitis, and all had a full recovery. Twenty-two patients presented clinically with viral encephalitis and were subsequently treated with acyclovir. Of 19 (5.3%) patients with only HSV DNA in CSF specimens, 1 died, 12 recovered partially, and 6 recovered fully; 2 of 3 patients who had mixed herpesvirus (HSV and HHV-6) infections died ($P = 0.037$; Fisher's exact test). The other patient with coinfection of HSV and EBV recovered, although psychiatric symptoms were still evident at a 6-month follow-up. Previous HIV infection was either denied by the caring physicians or serologically negative in all 30 patients.

Of the 29 HSV-negative control patients from whom clinical information was available, 13 had noninfectious CNS disease, 6 manifested generalized CNS symptoms, 5 had autoimmune diseases, 4 presented with aseptic meningitis, and 1 had a clinical diagnosis of meningoencephalitis. One patient in this control group had EBV sequences detected in the CSF; this individual had an underlying T-cell lymphoma. There were no deaths in these control patients.

DISCUSSION

The detection of CNS infections by PCR has provided a new level of laboratory diagnosis compared to conventional tech-

niques of cell culture and serology. Target genes of organisms responsible for CNS infections are generally present early in the acute phase of the disease; in contrast, CSF samples from patients without an infectious etiology do not contain these signature nucleic acid sequences (2, 32). Thus, the early concern that PCR would be too sensitive to be used to diagnose CNS infections due to common virus appears to have been unfounded. In genital HSV infection, PCR detects viral genome or genome fragments for several days after lesions become negative for infectious virus, and the clinical significance of this prolonged detection of HSV DNA is not known (10). HSV PCR has potential value for testing CSF from patients with suspected HSV encephalitis, but definitive studies with samples from patients with virologically proved disease have been limited (26, 35, 38).

In this study, we investigated a PCR-based DNA amplification technique for detecting herpesvirus DNA in the CSF samples of patients manifesting symptoms of viral encephalitis. Of 30 HSV-positive samples, 2 were also positive for HHV-6, and 1 was positive for EBV. The two DNA fragments amplified in the same sample by different virus-specific primers were probed by corresponding virus-specific probes. The sequence analysis of the two DNA fragments indicated they were derived from distinct herpesviruses. These results demonstrate that herpesvirus coinfection with HSV and HHV-6 may occur in CNS disease. This is the first demonstration of coinfection of the CNS with HSV and HHV-6.

Detection of more than one virus from any clinical specimen is uncommon. Respiratory sources yielded mixed viral infections diagnosed in cell cultures with frequencies ranging from 3 to 35% (44). Similarly, mixed viral infections of the CNS have been detected in AIDS patients. Coinfection with HIV-1 by other viruses such as JCV (progressive multifocal leukoencephalopathy), measles virus, CMV, VZV, and other herpesviruses has been established by PCR or by histological examination of brain biopsy or autopsy samples (28, 29). In these patients, spread of HIV-1 to the brain has been shown to be both an early event in this infection and to occur in the majority of symptomatic individuals (7). HIV infection of the CNS has been documented by PCR amplification of target RNA in CSF samples in almost 90% of both children and adults. Increased levels of HIV-1 are associated with CNS symptomatology (7, 34).

The CSF samples evaluated in our study were likely from patient populations with CNS disease not associated with HIV infection. For example, review of the medical records of 176 patients from the Mayo Clinic whose CSF samples were submitted for HSV detection showed that only 2 (1.1%) were AIDS patients (16). In 30 HSV-positive CSF samples, patients were either detected as negative or denied risk factors for HIV infection. Nevertheless, 3 of 30 (10%) HSV-positive CSF samples in our patient population showed mixed viral infections of the CNS.

Documented reports of viral coinfections in immunocompetent patients, determined by PCR, have also been uncommon. In one patient with associated extramural influenza A virus infection, CMV and EBV DNAs were detected in a CSF sample of an 8-year-old patient with CNS-related symptomatology (42). Similarly, HSV and EBV were detected by PCR in an 11-year-old boy with a clinical course typical for infectious mononucleosis. After several weeks, he developed clinical features of HSV encephalitis (27). These unique findings of mixed viral infection of the CNS in non-AIDS patients were most likely uncovered by the extensive laboratory experience of PCR testing in the United Kingdom (36). CSF specimens from 1,962 patients yielded 143 (6.6%) positive results, but no mixed infections were reported, with the exception of EBV DNA in the CSF samples of four AIDS patients. In the latter study, multiple targets (HSV, CMV, EBV, enterovirus, mumps, measles, and *B. burgdorferi*) were tested (36).

The pathogenesis of herpesvirus-associated encephalitis remains poorly understood. Limited studies with animal models indicated that both olfactory and trigeminal tracts can provide a neurological avenue for HSV to reach the CNS (20, 39). Herpesviruses, such as HSV, EBV, and HHV-6, have a marked tropism of cells of the peripheral and central nervous system (15). Several studies have revealed that the molecular basis for HSV neurotropism may include inverted repeats of the genome and the thymidine kinase gene; these sequences may convey enhanced neurovirulence. In addition, complex interactions between the virus and host cellular immune responses may be involved in neurovirulence (6, 8, 18). For example, interleukin-6 (IL-6) has been suggested to be involved in the pathogenesis of several diseases in humans, including inflammatory and autoimmune disorders as well as lymphoid malignancies (22). In the hyperthermia- and UV light-induced mouse models, treatment with anti-IL-6 antibodies results in significantly lower frequencies of ocular reactivation compared with those in mice treated with a control immunoglobulin (24). Herpesviruses, including HSV, CMV, EBV, and HHV-6, have been demonstrated to induce a concomitant release of IL-6, thereby disturbing immune homeostasis (15, 21, 25). This effect may itself be immunosuppressive, which could allow establishment or reactivation of other infectious agents within the host, thereby enhancing the occurrence of coinfection in the CNS.

An important question for the future is whether herpesvirus coinfection in the CNS influences the clinical features and disease course of the patient. One patient, who had both EBV and HSV-1 DNA detected in a CSF specimen, experienced a protracted recovery (27). Another 8-year-old girl who had both CMV and EBV detected in the CSF specimen was treated with acyclovir for 10 days and had an uneven recovery (42). Therapy with intravenous acyclovir has been proved to be very effective; the mortality of HSV encephalitis was reduced from 70% to 19 to 30% (40, 46). In the present study, among 30 patients who had positive HSV DNA detected in their CSF specimens, 22 were clinically diagnosed with viral encephalitis and appropriately treated with acyclovir. In 19 patients who were infected only with HSV, only 1 died, while 2 of 3 patients with CNS herpesvirus coinfection (HSV plus HHV-6) confirmed by molecular methods died, although these patients were treated with acyclovir. The other, who had both HSV and EBV DNA detected in CSF specimens had prolonged recovery with psychiatric sequelae. Importantly, no deaths occurred in the control study group (HSV negative). The significance of herpesvirus coinfection in the CNS and associated clinical manifestations requires further investigation.

Detection of viral (microbial) nucleic acid targets in CSF by PCR has brought a new dimension to the laboratory diagnosis of CNS infections. The results of our study suggest that a diagnosis compatible with HSV CNS disease can be confirmed by specific PCR testing; however, clinical and laboratory recognition and medical management of patients with mixed infections will need to be considered further.

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