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SYMPOSIUM: Herpesvirus Infections of the Nervous System

Polymerase Chain Reaction as a Diagnostic Adjunct in Herpesvirus Infections of the Nervous System

B. K. Kleinschmidt-DeMasters, M.D.[1,2], Roberta L. DeBiasi, M.D.[2,3], Kenneth L. Tyler, M.D.[2,4,5]

Departments of [1]Pathology, [2]Neurology, [3]Pediatrics, [4]Microbiology, and [5]Immunology and Medicine, University of Colorado Health Sciences Center and The Denver Veterans Administration Hospital, Denver, Colorado, USA

Polymerase chain reaction (PCR) is a powerful technique that allows detection of minute quantities of DNA or RNA in cerebrospinal fluid (CSF), vesicle and endoneurial fluids, blood, fresh-frozen, and even formalin-fixed tissues. Various infectious agents can be detected with high specificity and sensitivity, including bacteria, parasites, rickettsia and viruses. PCR analysis of CSF has revolutionized the diagnosis of nervous system viral infections, particularly those caused by human herpesviruses (HHV), and has now replaced brain biopsy as the gold standard for diagnosis of herpes simplex virus (HSV) encephalitis. PCR analysis of both CSF and nervous system tissues has also broadened our understanding of the spectrum of disease caused by HSV-1 and -2, cytomegalovirus (CMV), Epstein-Barr virus (EBV), varicella zoster virus (VZV), and HHV-6. Nonetheless, positive tissue PCR results must be interpreted cautiously, especially in cases that lack corroborating clinical and neuropathologic evidence of infection. Moreover, positive PCR results from tissues do not distinguish latent from productive (lytic) viral infections. In several neurological diseases, negative PCR results have provided strong evidence against a role for herpesviruses as the causative agents. This review focuses on the use of PCR tests to diagnose HSV and VZV infections of the nervous system.

Introduction

Polymerase chain reaction (PCR) can be applied to the diagnosis of any disease in which nucleic acids (*e.g.* DNA, RNA) or their expression as messenger RNA (mRNA) play a role. PCR is useful to study congenital diseases, malignancies, autoimmune disorders, and infections (23); PCR aids in diagnosis, therapy, disease classification, epidemiology, and basic research. In infectious disorders, PCR is ideally suited for identifying fastidious organisms that may be difficult, or impossible, to culture (24). The technique can be performed rapidly and inexpensively, and test results are typically available faster than with standard culture or serological methods. Nucleic acids of bacteria, mycobacteria, rickettsia, parasites, treponemes, and viruses can be identified by PCR analysis of any body secretion, fluid, or tissue. The widespread availability of an in-house test in most hospitals, or ready access to a reference laboratory, has led to the incorporation of PCR testing on CSF and body fluids into medical practice in the United States and other developed countries. Unfortunately, the rapid proliferation of testing has led to substantial variation among laboratories in terms of quality control, techniques and procedures.

Despite the wide application of PCR to CSF and other body fluids, such as vesicle or endoneurial fluids, PCR analysis of central (CNS) and peripheral (PNS) nervous system tissues remains primarily a research tool and is not available at all institutions. PCR on tissues and CSF, sometimes in conjunction with immunohistochemistry or *in situ* hybridization techniques, have furthered our understanding of the role of herpesvirus in cases of encephalitis, meningitis, meningoencephalitis, myelitis, ventriculoencephalitis, polymyeloradiculitis, and brainstem infections. PCR of cerebral arteries has established a direct role for VZV in cases of waxing and waning vasculitis (36), and PCR of temporal arteries has negated a role for VZV in giant cell arteritis (66).

Interpretation of positive PCR amplification of herpesvirus genome in brain tissues from patients with neurological disorders of uncertain etiology can be problematic. Since amplification of viral nucleic acid does

Corresponding author:
B.K. Kleinschmidt-DeMasters, Department of Pathology B216, University of Colorado Health Sciences Center, 4200 East Ninth Avenue, Denver, CO 80262; Tel.: (303) 315-7298; Fax (303) 315-6721; E-mail: BK.DeMasters@UCHSC.edu

not distinguish between genomic fragments, latent infection, low-grade persistent infection, or active (lytic) infection. The interpretation of a positive result may depend on the precise gene or genes being amplified, and whether the methods used are designed to detect genomic DNA, RNA, or mRNA. Herpesviruses are generally classified as either neurotropic (HSV, VZV) or lymphotropic (EBV, HHV-6, HHV-7, HHV-8) based on their tissue location during latency; defined as "the persistence of the virus in a host in the absence of clinically apparent infection," with the capacity to reactivate (31). Considerable information is available about the physical state, viral nucleic acids and proteins during latency of neurotropic herpesviruses (see companion article by Cohrs *et al.*) Numerous studies have also documented latency of herpesviruses in cells other than those of the nervous system, including peripheral blood mononuclear cells (78), B-lymphocytes, and T-lymphocytes.

In the CNS, nucleic acid hybridization first detected HSV viral genome in human brains in 1979 (73) and again in 1981 (32). PCR on human brain revealed HSV DNA in brainstem, olfactory bulbs and limbic areas (7). Since HSV has never been isolated from normal human brain tissue, the presence of the viral genome does not definitively signal latent viral infection or the capacity for reactivation, and instead might represent random viral sequences or fragments of virus. Although the viral transcripts associated with latency (LATS) have been identified in a mouse model of HSV infection (27), their presence has not been definitively demonstrated in human brain tissue (see accompanying article by Cohrs *et al.*). Several laboratories have also reported detection of viral genomic material of HHV-6, HHV-7, and HHV-8 in normal autopsy human brain (15, 21). It remains to be proven whether these viruses establish latency within brain. In contrast, VZV DNA has not been detected in normal human brain in most studies, and its presence on tissue PCR usually indicates productive infection of CNS.

This review summarizes the principles of PCR testing and the role of CSF PCR in the diagnosis and therapeutic management of herpesvirus infections of the nervous system, particularly HSV and VZV. Problems inherent in interpretation of positive tissue PCR for viruses that may become latent in the nervous system are also discussed.

Principles of PCR testing

PCR techniques allow the *in vitro* synthesis of millions of copies of a specific gene segment, and in turn,

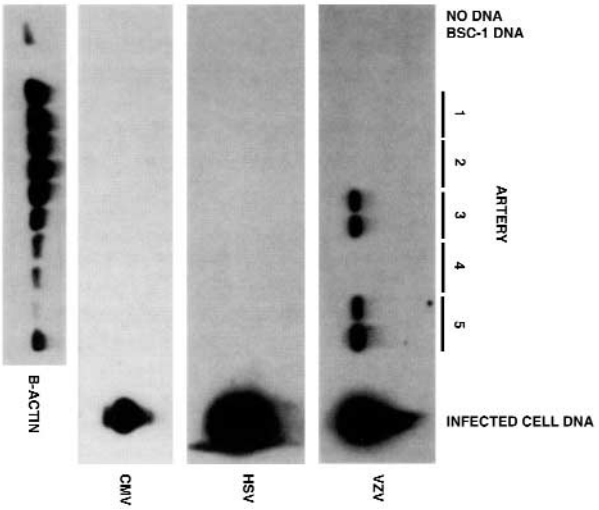

Figure 1. *PCR gel.* PCR detection of VZV DNA in cerebral artery sections from a patient with waxing/waning vasculitis (see ref 36). Total DNA was extracted from: BSC-1 cells; sections of multiple cerebral arteries; VZV- or HSV-1-infected BSC-1 cells; and CMV-infected human WI38 cells. Template DNA was omitted from one of the reaction tubes (no DNA). One ng of DNA from uninfected and virus-infected cells, and 10 μl of duplicate samples of DNA from sections of right middle cerebral artery (1), anterior cerebral artery (2), basilar artery (3), right vertebral artery (4), and right posterior cerebral artery (5) were PCR-amplified, using primers specific for VZV gene 29, HSV-1 gene UL30, genes corresponding to the CMV major immediate early genes, or human β-actin. Figure reproduced with permission, American Academy of Neurology.

the rapid detection of even a few copies of the target nucleic acid (see refs. 23 and 24 for reviews). DNA is often the target nucleic acid, although RNA can also be detected by reverse transcription (RT-PCR).

PCR uses the heat-stable DNA-synthesizing enzyme, DNA polymerase, to extend synthetic DNA fragments onto oligonucleotide primers designed to bind to target DNA segments. Oligonucleotide primers have sequences complementary to each strand of DNA to be amplified and are (usually) 20 to (rarely) 40 bases in length. Primer design is crucial for efficient and accurate PCR analysis. In the past, primer sequences were generated manually. Currently, software programs that facilitate optimal primer design are available *(http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi)* to address issues such as guanine-cytosine content, melting points that match for both primer sequences, and the reduction of unwanted primer-primer formation.

The first step in PCR is the denaturation of the double-stranded target DNA by heating the sample to 95°C. During heating, the DNA polymerase remains intact.

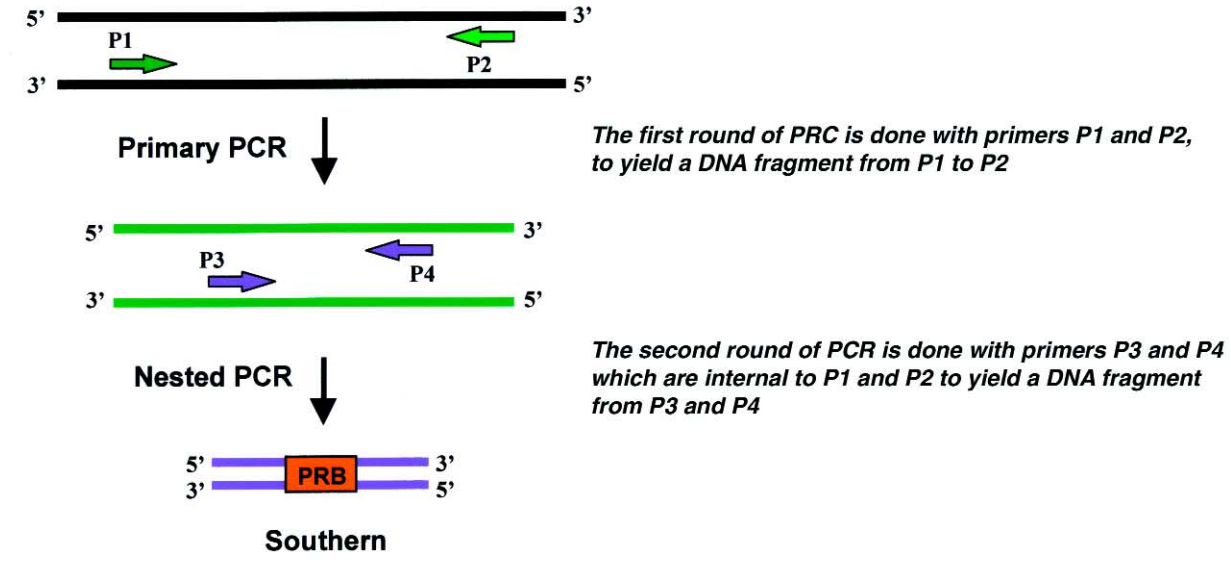

Figure 2. *Schematic of Nested PCR.*

Oligonucleotide primers are supplied in considerable excess to ensure that more primers rather than the original (*de novo*) complementary strand of DNA are available for binding. As the sample containing the denatured DNA, primers, and DNA polymerase is cooled to 55-70°C, the primers anneal (bind) to their respective complementary template strands. The nucleotide building blocks adenine, guanine, thymidine, and cytosine are also supplied in molar excess. Using these nucleotides, the DNA polymerase (usually *Taq*) "reads" the target DNA strand in a 5′ to 3′ direction and forms a new, complementary DNA sequence downstream from the primer. This results in two complete double-stranded DNA pairs concluding the first cycle of PCR testing. Multiple cycles (30-40) of denaturing, annealing of new primers and strand elongation amplify DNA into 10^6-10^9 copies over 2-3 hours.

The resulting PCR product is visualized and identified based on its migration in an electrophoretic agarose gel containing ethidium bromide. The generation of a PCR product (amplicon) of the appropriate size, as predicted from the target gene and primer set used is the first indication of a positive reaction. However, Southern blotting is usually performed to ensure that the products generated are specific. The amplified fragment is transferred from the gel and analyzed using a radioactively or fluorescently labeled probe that is complementary to the expected target gene (Figure 1). Failure of the probe to bind to the amplicon indicates a false-positive PCR product due to mispriming or other technical issues, whereas confirmation of the specificity of amplified PCR products by Southern blot ensures that the product is derived from the target gene. Direct sequencing of the amplified product is also used for PCR product confirmation; however, this approach is limited primarily to the research setting since it is too labor-intensive for routine use.

Nested PCR is a modification that increases the specificity and especially the sensitivity of PCR. Nested PCR involves a second round of PCR in which an additional set of new primers internal to the original primers is utilized (Figure 2). A fragment smaller than the original target fragment becomes the second target. While nested PCR can increase the sensitivity of PCR, it also increases the likelihood of false-positive reactions. Results from studies using nested PCR may vary considerably from those that utilize non-nested PCR.

Quantitative PCR (using Taqman) is increasingly being applied to CSF, and occasionally to tissues. The technique involves the use of internal oligonucleotides — labeled at one end with a fluorescent dye and at the other end with a quencher dye — along with the PCR primers during amplification. Fluorescence emitted during PCR is measured and directly correlated to the number of copies of the target.

The ability of PCR to generate and amplify a high number of DNA strand copies from the original sample, which may contain as few as 1 to 10 copies of the target DNA, is both its greatest virtue and greatest limitation. The exquisite sensitivity of the test can easily lead to false-positive results from amplification of minute amounts of contaminating DNA. This problem is well-recognized by experienced laboratories, and rigorous controls are run simultaneously with the clinical samples, using all reagents except the unknown specimen to insure that the reagents do not harbor contaminants. Dedicated work areas and separate reagents used exclusively for PCR testing, frequent glove changes, and testing in laminar flow hoods alleviate false-positives. False-negative test results due to deterioration of the original sample specimen, incorrect heating of the sample, or use of incorrect proportions of the various mixture reagents are more easily rectified.

PCR works very well on biopsy or autopsy tissues frozen soon after removal from the body; DNA can be amplified by PCR even when tissues are stored for several years at -80°C. Although one of the greatest assets of PCR is that it can also be used on archival, formalin-fixed, paraffin-embedded tissue samples, yields of extracted DNA are usually less from fixed than from fresh tissues. Extraction of target DNA involves removal of the wax with several rounds of xylene immersion and centrifugation, and the procedure itself may slightly diminish the DNA yield from fixed tissues. The DNA yield from fixed tissues may also be reduced due to cross-linking after prolonged fixation (several years), or in association with use of osmic acid or other fixatives. Both fresh-frozen and fixed tissues must be digested with proteinase K prior to performing the PCR techniques described above. The protocol for extraction of DNA is described in the commercial kit literature (DNeasy Tissue Kit Handbook, Qiagen, 28159 Avenue Stanford, Valencia, CA, 91355, USA).

PCR of CSF

One of the most successful applications of CSF PCR is in the diagnosis of viral nervous system infections (8, 67, 88). PCR is preferable to serological testing, which usually requires 2-4 weeks after acute infection to reveal an antibody response. In contrast, CSF PCR yields positive results during acute infections, when the amount of replicating virus is maximal. Unlike traditional culture methods that may show negative results after the patient receives even small doses of antimicrobial drugs, CSF PCR retains its sensitivity after short courses of antiviral therapy (24). This allows the rapid start of empiric therapy when the patient initially presents with suspected meningitis or encephalitis, without potentially compromising the definitive diagnostic test. CSF PCR is also ideal for patients in whom brain biopsy is contraindicated clinically, such as patients with advanced acquired immunodeficiency syndrome (AIDS) (22).

Positive CSF PCR testing indicates the presence of viral DNA and is a marker of recent or ongoing active viral infection. Despite the high prevalence of seropositivity to HSV in the population and the fact that the virus becomes latent in multiple ganglia of most humans, HSV DNA is not detected in CSF of HSV-seropositive individuals without neurological disease or with other types of inflammatory non-HSV CNS infections (82). In general, a positive CSF PCR for viral DNA indicates a CNS infection by that particular pathogen, especially in an immunocompetent individual (82). A possible exception might arise from a breakdown of the blood-brain barrier (e.g., in severe bacterial meningitis), or contamination of the CSF with blood, resulting in a positive CSF PCR in the absence of viral infection (82). However, in one very recent study that addressed this problem, false-positive CSF PCR results for herpesviruses were not seen in any of 10 control patients with bacterial meningitis, despite high CSF white cell counts (75).

Replicating virus and viral DNA do not persist indefinitely, so that the CSF PCR test becomes negative over time, especially in immunocompetent patients. While most CSF testing in the clinical setting of suspected meningitis or meningoencephalomyelitis is performed within 1-2 days following onset of neurological symptoms, positive test results at least 2 and up to 4 weeks after onset of clinical disease have been recorded (88). False-negative tests can occur if PCR inhibitors are present in the CSF, such as hemoglobin products resulting from breakdown of red blood cells. However, modest xanthochromia does not negatively impact CSF PCR testing, nor does high CSF protein level or white blood cell count (24).

CSF PCR testing is optimally performed on fresh samples. However, DNA is more stable than RNA, and usually refrigeration for a few hours or even days does

not appear to significantly reduce the yield of the test although this has not been rigorously studied (24). In order to avoid cell lysis that liberates viral DNA into the supernatant and effectively dilutes the sample, CSF and body fluids for PCR should not be frozen prior to shipping. Optimally, samples are shipped overnight at room temperature, centrifuged, and the pellet examined for virus-specific DNA. The exquisite sensitivity of the technique implies the need for only small volumes of CSF for analysis (*i.e.* 30 μl) although laboratories generally request at least 0.5 ml.

Occasional immunocompetent or immunocompromised patients reveal more than one herpesvirus on CSF PCR, and EBV has been the most frequent agent associated with a dual positive result (75). In a large series of 662 patients, mostly immunocompetent, detection of HHV-6 and EBV by CSF PCR did not correlate clinically in several individuals with the presence of a CNS infection known to be caused by that virus (75). In contrast, detection of HSV-1, HSV-2, CMV, and VZV DNA correlated strongly with specific clinical syndromes of encephalitis/myelitis and meningitis (75). Similarly, in a large study of HIV-infected individuals, conducted to assess the diagnostic reliability of CSF PCR by comparison with biopsy or autopsy diagnoses, the most frequent false-positive herpesvirus detected was HHV-6 (19). In this study, seven of the 219 patients with corresponding tissue samples lacked histological evidence of a CNS disease that could be attributed to their HHV-6 DNA detected by CSF PCR (19). Additional large studies are necessary to determine the extent of false-positive PCR results for herpesviruses, especially HHV-6 and EBV.

Occasional false-negative and false-positive CSF PCR results for CMV have been seen in AIDS patients. In AIDS patients, CSF PCR detected HSV-1 or HSV-2 in all six cases (100%) of histologically confirmed HSV encephalitis, compared to 37 of 45 cases (82%) with CMV infections of CNS (19). An earlier study had suggested that a CSF PCR positive for CMV DNA correlated strongly with systemic CMV infections but not with CMV brain infections in AIDS patients at autopsy (1). Recent studies employing quantitative CSF PCR for CMV have indicated that AIDS patients with autopsy-confirmed CMV encephalitis harbor significantly higher levels of viral genome than do those with symptoms unrelated to CMV (91). These studies suggest the usefulness of quantitation in clarifying the clinical relevance of a positive CSF PCR result for CMV in AIDS patients (6). Despite these difficulties, CSF PCR for CMV has been used to monitor the efficacy of antimicrobial therapies and clearance of CMV viral burden in AIDS patients (16). Overall, CSF PCR testing has been crucial in diagnosing herpesviruses nervous system infections in AIDS patients and a positive CSF PCR result for herpesvirus in the majority of patients corresponds to CNS infection by that pathogen, just as it does in immunocompetent patients. CSF PCR has the potential to identify infections in AIDS patients who may not mount a classical serological response to viral infection or demonstrate protracted clinical manifestations (37).

CSF PCR testing has played a critical role in establishing the frequency and distribution of herpesvirus infections in immunocompetent populations. In the study by Studahl *et al.*, 87% of patients with herpesvirus DNA detected in CSF were immunocompetent, with HSV-1 identified in 18 patients, EBV in 16, HSV-2 in 9, CMV in 7, HHV-6 in 6, and VZV in 6 of 69 positive patients (75).

The sensitivity and specificity of CSF PCR exceeds 95% for HSV encephalitis (55, 88). For HSV-1, the existing standards to which CSF PCR results are compared, such as brain biopsy, are actually *less* sensitive than PCR (55). Hence, CSF PCR has dramatically reduced the need for brain biopsy as a diagnostic test. CSF PCR has also led to the identification of mild or atypical forms of HSV encephalitis that were formerly attributed to other viruses, often in the absence of brain biopsy (26, 30), and that may account for 17% of total cases of HSV encephalitis (30). CSF PCR has established the diagnosis and role of HSV-1 in brainstem encephalitis (84), myelitis (75), multifocal or diffuse encephalitis without temporal lobe involvement in children (71), and neonatal encephalitis (75). HSV encephalitis in children may relapse after therapy, and CSF PCR has been used to identify the subset of children in which HSV viral DNA reappears (43, 49). Quantitative CSF PCR may also provide valuable information in cases of pediatric HSV encephalitis in monitoring response to antiviral drugs (4)

CSF PCR has helped clinicians to recognize that HSV-2 can cause aseptic meningitis even in the absence of genital herpetic lesions (72), and has established HSV-2 as the most common cause of benign recurrent lymphocytic meningitis, including many cases previously diagnosed as Mollaret's meningitis (79). Even when CSF viral cultures are negative, CSF PCR is positive in patients with recurrent episodes of meningitis following an initial episode of herpes simplex meningitis (79). CSF PCR has illustrated that immunocompetent adults may manifest HSV-2-induced meningoencephalitis as well as meningitis (75). CSF PCR has identified

rare cases of HSV-2 brainstem encephalitis and recurrent thoracic myelitis (65).

CSF PCR testing has been found to be nearly 100% specific and sensitive as a tumor marker for EBV-related CNS lymphoma (18, 19, 22, 87), and has changed the way in which clinicians diagnose CNS lymphoma in immunocompromised individuals. In one study of AIDS patients with CNS mass lesions, a positive EBV CSF PCR correctly identified all 17 CNS lymphoma cases and was positive in only 1 of 68 AIDS patients with non-CNS lymphoma mass lesions (18). CSF PCR for EBV is also positive during the acute phase of the illness in children with infectious mononucleosis and neurological complications such as transverse myelitis, meningoencephalitis, and aseptic meningitis (88).

A sensitivity of 82% and specificity of 99% of CSF PCR for detecting CMV CNS infections has been reported in AIDS patients (16). A sensitivity of 60% for CSF PCR is cited for congenital CMV infections, and positive results may correlate with poorer neurologic outcome in affected infants (81). CMV viral load has also been monitored in peripheral blood leukocytes as a method to predict which immunosuppressed patients might develop a systemic (and CNS) infection (9, 29).

CSF PCR testing has corroborated the role of HHV-6 in febrile seizures, meningitis, encephalitis, and encephalopathy in immunocompetent and immunocompromised individuals (48, 92). HHV-6 genome has been demonstrated in CSF from up to 57% of children younger than one year of age who have febrile seizures ,and has also been seen in children with recurrent febrile convulsions (92). The role of HHV-7 in neurological disease is unclear, although detection of HHV-7 DNA in CSF and serum of children with exanthem subitum and encephalopathy has been reported (80, 85). Encephalitis in immunocompromised individuals associated with HHV-8 has been described (68), but this awaits additional confirmation. HHV-8 DNA has been detected in primary CNS lymphomas in some studies (20) but not others (5). In the study in which HHV-8 was detected, the virus was surmised to play an indirect role in the development of primary CNS lymphoma, and was thought to be present in the adjacent non-neoplastic lymphocytes but not the lymphoma cells (20).

CSF PCR for VZV has considerably broadened the understanding of the neurologic complications due to this virus (3, 8). VZV, along with EBV, displays the most protean manifestations of nervous system infection of any of the herpesviruses (35, 50, 51). Serological techniques and CSF PCR for VZV have been particularly helpful in identifying cases of VZV CNS infections without associated rash (*sine herpete*) (8, 11). Since the virus can rarely be cultured from CSF, diagnosis of meningitis or meningoencephalitis previously depended on the presence of a characteristic vesicular erythematous rash before, during, or after CNS infection, and VZV-mediated neurologic diseases were under-recognized. CSF PCR for VZV has shown that aseptic meningitis and brainstem encephalitis due to this virus can occur in immunocompetent hosts (75). In a study of 514 consecutive HIV-positive patients, CSF PCR for VZV became negative in patients whose clinical conditions improved following antiviral therapy and remained positive despite appropriate therapy in several patients who subsequently died (17). Hence, CSF PCR for VZV DNA may have utility in monitoring therapeutic response and in predicting outcomes. In the same study, several patients with positive CSF PCR for VZV DNA, but without clinically recognizable VZV meningoencephalitis, were considered to have subclinical reactivation of VZV, treated with antiviral agents and survived. In those cases, positive CSF PCR was considered to antedate clinical disease and allowed effective use of prophylactic therapy (17).

CSF PCR testing for VZV has established a role for the virus in cases of stroke (granulomatous arteritis), multifocal infarcts, myelitis, and neuritis. CSF PCR has served as the diagnostic test for large vessel encephalitis (also called granulomatous arteritis, herpes zoster ophthalmicus with contralateral hemiplegia, or simply stroke with VZV) (62), small vessel vasculopathy (leukoencephalitis) (3), myelitis (34), and zoster *sine herpete* (39). Some of these complications had been previously linked to VZV but received full confirmation from PCR testing.

PCR testing of body fluids other than CSF

PCR testing of skin vesicle fluid from patients with varicella (chickenpox) and zoster (shingles) has identified VZV in 97% of patients; in traditional viral culture methods, virus was isolated in only 23% of these same patients (25). PCR for VZV DNA was positive for vesicles up to 14 days after the onset of the rash, even from crusted cutaneous lesions, compared to culture specimens that were positive only when taken within 5 days of rash onset (25).

PCR analysis of peripheral blood mononuclear cells and CSF has furthered our understanding of the pathogenic mechanism in postherpetic neuralgia, the most common complication of VZV reactivation from latency in the elderly. Postherpetic neuralgia is characterized by pain that persists for more than the 4-6 weeks asso-

ciated with zoster (shingles) (35). The cause of the persistent pain is unknown. Using PCR, VZV DNA was detected in blood mononuclear cells from 11 of 51 postherpetic neuralgia patients, but not in any of 19 zoster patients without the persistent pain syndrome or in any of 11 elderly control individuals without zoster (58). While it is not possible to examine dorsal root ganglia — the site of VZV latency (56) — during the life of these patients, it seems likely that the PCR results reflect the greater level of VZV present in these ganglia during pain episodes than during periods of latency. Blood mononuclear cells may traffic through ganglia during periods of putative ganglionitis and acquire viral DNA. Thus, PCR analysis of blood mononuclear cells provides a window to indirectly assess levels of VZV replication in deep ganglionic tissues of patients with postherpetic neuralgia.

PCR on both CSF and fluid from auricular vesicles has confirmed that VZV causes Ramsey-Hunt syndrome, the second most common cause of 7th nerve facial paralysis after Bell palsy (64). Ramsey-Hunt syndrome can be difficult to recognize since the rash is hidden in the ear or mouth, and rash may be delayed, particularly in pediatric patients (41). PCR on endoneurial fluids and posterior auricular muscle samples collected during decompressive facial nerve surgery for Bell palsy identified HSV-1 DNA in 79% of patients and suggested that neither EBV or VZV is an important cause of idiopathic Bell palsy (63). A subsequent study using PCR identified a subset of patients with acute peripheral facial palsy that have zoster *sine herpete* (33). Acute facial palsy due to VZV constituted a significant percentage of the overall patient population (29%) in that study, and an even higher incidence of VZV reactivation (88%) was responsible for the palsy in the patients who were HSV-seronegative (33). Hence, PCR has verified a role for herpesviruses in both of the common causes of facial nerve paralysis and distinguishes which virus is causative in clinically confusing cases.

PCR testing on blood mononuclear cells has also impacted patient care for transplant recipients, AIDS patients, and other populations at-risk for CMV infections or EBV-related lymphoproliferative disorders. Monitoring of CMV viral loads by PCR in blood has led to prophylactic therapy with antiviral agents in AIDS and transplant patients to prevent systemic (and CNS) viral infections (29).

Confirmation of herpesvirus etiology in nervous system infections by tissue PCR

PCR testing on fresh, frozen, or archival fixed and paraffin-embedded tissues has allowed assessment of viral presence, even from small or imperfectly preserved specimens. Tissue PCR of cerebral arteries has established a direct role for VZV in cases of large vessel (62) and small vessel vasculopathy. In a patient with waxing and waning VZV vasculitis, detection of VZV DNA by PCR led to the discovery that VZV antigen was also present, indicating a productive infection in blood vessels as the cause of disease (36). Brain tissues may be positive for VZV DNA by PCR even when virus is no longer detectable by other methods, such as light microscopy for viral inclusions, immunohistochemistry, or *in situ* hybridization (52).

VZV latency in human sensory ganglia was originally demonstrated with Southern blotting by Gilden *et al.* (38) and was later confirmed by PCR (59). More recently, PCR techniques have been used on dorsal root ganglionic tissues to address the important question of whether neurons, satellite cells, or both harbor latent virus. PCR combined with *in situ* hybridization on sorted neuronal and non-neuronal cells fractions showed that latent VZV resides primarily, if not exclusively, in neurons at a level of two to five viral copies per latently infected neuron (54).

Our laboratory has used PCR to address the role of subclinical reactivation of VZV. Understanding the extent of viral burden in transplant recipients may help in monitoring their response to the doses of prophylactic antiviral agents currently being utilized. Quantitative PCR applied to autopsy trigeminal ganglia to assess VZV DNA burden in transplant recipients at risk for reactivation demonstrated an average of 119 copies of VZV DNA/μg of total ganglionic DNA, compared to an average of 71 copies in controls (B.K. Kleinschmidt-DeMasters, R. Mahalingam, unpublished data). These results suggest an increased viral burden in transplant recipients despite "optimal" antiviral prophylactic therapies. Future studies are needed to determine the clinical and statistical significance of the increased viral load in a larger number of transplant recipients, as well as the extent of host inflammatory response and viral antigen production, if any.

Exclusion of herpesvirus etiology in nervous system disease by tissue PCR

Given the protean manifestations of herpesvirus-mediated infections of the central and peripheral nervous system, efforts have been made to detect these viruses by PCR on tissues from several disease entities characterized by arteritis and/or inflammation. PCR

testing of various tissue specimens has suggested that VZV is not the cause of giant cell arteritis (66) and has shown no role for VZV, CMV, EBV, or HSV in childhood multifocal encephalomalacia (89). PCR also suggests that HSV is not the cause of Meniere's disease (90). A survey of a variety of normal and disease peripheral nerve conditions, including inflammatory peripheral neuropathies, revealed no HSV DNA by PCR in peripheral nerves (76), thus excluding a role for this herpesvirus in several disorders with a plausible viral etiology.

Questionable herpesvirus etiology in nervous system disease despite positive tissue PCR

Positive PCR on tissues from patients without definite clinicopathologic evidence of infection is difficult to interpret. For example, the HSV genome is present in brain tissues from both controls and patients with a variety of neurologic diseases (7), so that assignment of a role for this herpesvirus in a disease condition is especially problematic. Positive PCR results on tissues have been most challenging to interpret in patients who might not manifest classic clinical features of infection, but who exhibit tissue inflammation, such as putative chronic herpes simplex encephalitis in children (47), Rasmussen encephalitis (46, 86), and multiple sclerosis (MS) (69). Also problematic are positive PCR results in tissues from certain other non-inflammatory seizure disorders (28, 70), Alzheimer's disease (44, 45, 60), and brain tumors (14, 21).

Jay *et al.* identified HSV DNA in surgical resection tissues of pediatric patients with remote, antecedent clinical histories of HSV-1 encephalitis, subsequent intractable seizure disorders, and chronic encephalitis (47). That study described 3 pediatric patients whose brains revealed microglial nodules, lymphocytic infiltrates, and gliosis, but negative immunohistochemistry and electron microscopy for virus (47), suggesting that the HSV genome was present but that infectious virus was not being produced or produced only at extremely low levels. It remains unclear whether the chronic inflammation was a result of persistent low-grade viral replication or an immune response to prior infection. HSV DNA detection might reflect either the presence of latent virus no longer responsible for the encephalitis or the presence of herpesviruses that "simply accumulate in CNS with the passage of time," a possible explanation for some positive tissue PCR results offered by Vinters *et al.* (86). Clinicopathologic features in encephalitic cases such as these do not help clarify the interpretation of positive PCR results.

Identification of viral genomes in patients with Rasmussen encephalitis has been similarly problematic. Jay *et al.* detected CMV and HSV genomic sequences in 10 patients with intractable seizures and chronic encephalitis, and several controls (46). In another study, PCR revealed small amounts of CMV and EBV DNA in 6 patients with Rasmussen encephalitis (86). Comparison of the PCR signal strength for the CMV and EBV DNA found in the Rasmussen encephalitis cases to that in controls (AIDS patients with documented CMV encephalitis or EBV-driven CNS lymphomas, respectively) revealed considerably lower amounts of viral nucleic acid in Rasmussen encephalitis patients than those in patients with diseases known to be caused by these viruses (86). The authors suggested that EBV and CMV did not directly cause Rasmussen encephalitis, but could not rule out an indirect role for these herpesviruses. The issue of viral etiology versus autoimmunity in Rasmussen encephalitis has recently been reviewed (40). Greenlee and Rose note that virus has never been cultured from brain tissues of Rasmussen encephalitis patients and conclude that "at the present time, there is no convincing evidence for a viral etiology" for the disease (40).

PCR has also identified HSV DNA in brain samples from other non-inflammatory types of epilepsy patients (70), but methodology and selection of controls in that study have been questioned (83). An additional report of HSV, CMV, and HHV-6 DNA in brain tissues from young seizure patients (28) awaits corroboration. A higher than expected prevalence of HSV genomic material in brain tissue from patients with Alzheimer's disease has been reported by some investigators (44, 45), but could not be confirmed by others (60).

Unlike the situation for HSV DNA, PCR studies have not demonstrated VZV DNA in autopsy brain tissues. Liedtke *et al.* detected VZV in only 1% of olfactory bulbs (56). VZV DNA was not detected by PCR of temporal lobe cortex from any of 8 schizophrenic patients, 8 non-schizophrenic suicide victims, or 8 normal control subjects (2). In a study using frozen brain tissue from 31 schizophrenic and 23 control subjects, no CMV, EBV, HSV-1, VZV, or HHV-6 was detected (77). No VZV DNA was found in brain from either Alzheimer's disease patients or normal age-matched controls (57). Only one group has reported detection of VZV DNA, as well as many other herpesviruses (HSV, EBV, CMV, HHV-6), in a significant percentage of both multiple sclerosis and control brains (69). Others have not reproduced these results.

Whereas PCR reveals an abundance of CMV and EBV DNA in brain from patients with CMV encephalitis and EBV-associated CNS lymphoma, the significance of low levels of these lymphotropic herpesviruses in brain tissues remains unclear, especially in AIDS patients. One quantitative PCR study of CMV genome in brains of AIDS patients with and without neuropathologic evidence of CMV encephalitis found significantly higher viral copy numbers in brains of patients with clinicopathologic evidence of productive viral infection than in non-encephalitic AIDS brains (53). Like quantitative CSF PCR, quantitative tissue PCR for CMV may identify which positive cases are due to productive lytic infection by the virus.

Viral DNA sequences of lymphotropic herpesviruses have been recently identified by PCR in normal brain tissue, including HHV-6 (21), HHV-7, and HHV-8 (15). HHV-6 genomic material was also detected in neoplastic brain tissues in 37% of 118 biopsies from primary (115) and metastatic (3) tumors, compared to 32% of normal controls (21). The presence of viral genome in brain tumors was considered to represent "reactivation from latency in immunocompromised patients," based on the detection of HHV-6 immediate early protein p41 by immunohistochemistry (21). Interestingly, peripheral blood lymphocytes from 7 of the HHV-6-positive tumor patients contained the same HHV-6 variant (A or B) as did their respective brain tumor sample. Chan *et al.* also demonstrated HHV-6 and HHV-7 sequences by PCR on tissues in 8.2% and 14.3%, respectively, of primary brain tumors (14). HHV-6 has been identified in oligodendrocytes in patients with progressive multifocal leukoencephalopathy, but genome has also been found in normal, AIDS, and other control brains, especially with increasing patient age (10). In cases of HHV-6 meningoencephalitis, lymphocytes, microglia, and some neurons are infected (42). However, latent virus need not reside in the same cells as those involved in productive, lytic infections. VZV, for example, can infect glia, neurons, and blood vessels in the CNS in cases of encephalitis (50), but has never been shown to become latent in these CNS sites. Further studies are needed to firmly establish which cells in the CNS, if any, harbor latent HHV-6, HHV-7, and HHV-8.

PCR for HHV-6 has been intensively applied to the study of multiple sclerosis (MS). In one of the best-known studies, HHV-6 DNA was identified in >70% of MS brains and controls by PCR (13). In contrast to tissue PCR, analysis of CSF with this technique has not always revealed HHV-6 in MS patients (61). HHV-6 antigens have been identified by immunohistochemistry in oligodendrocytes in MS brains, but not in normal controls (13), suggesting the presence of viral activation, especially within the demyelinative plaques. However, as noted by the authors themselves, the PCR (and immunohistochemical) data were insufficient to establish a causal link between HHV-6 and MS (13). The role of this virus in MS is a complex topic and is discussed in a recent review of controversies in neurologic infectious diseases (40). As noted by these authors, MS may not have a singular causation and even if an infectious organism such as HHV-6 is found, it may only be related to the disease in a subset of patients (40). Positive tissue PCR could be detecting an agent of no etiological importance, a commensal organism, or a virus that causes an infection that non-specifically precipitates a clinical relapse, but is not the direct cause of MS (40).

One possible explanation for some positive tissue PCR results for the blood-borne herpesviruses may be that lymphocytes and mononuclear cells harboring these viruses are entrapped within the lesional tissue. A recent study of a large number of arthritis patients has also raised the possibility that PCR on fluids and tissues might detect viruses in peripheral white blood cells that are migrating into inflamed tissues (74). In that study, PCR demonstrated CMV in 25, EBV in 12, and HSV DNA in 16 of 73 samples of synovial fluid or tissue (74). Based on analysis of several viruses from a large number of patients with a spectrum of arthritides and arthropathies, those investigators argued rather convincingly that inflammatory cells harboring viral DNA(s) migrate into damaged tissues but are not directly causative of arthritis. It remains uncertain whether similar mechanisms occur in some CNS disorders involving large numbers of lymphocytes or mononuclear cells and can explain some positive tissue PCR results.

In summary, tissue PCR is a valuable tool for specific diagnosis of VZV CNS infections, since the overwhelming majority of PCR studies on human brain do not reveal VZV genomic material in normal controls. When VZV is detected by PCR in tissues or CSF, the patient is likely to harbor a nervous system infection caused by VZV. Positive CSF PCR results for HSV-1 or HSV-2 in patients also usually correspond to clinical syndromes of meningitis, encephalitis, or myelitis. In contrast, HSV viral genomic material is present in a large percentage of brains from individuals without neurologic diseases, and positive PCR results on tissues do not equate with productive HSV infection. CSF PCR for EBV DNA is an excellent diagnostic adjunct for detection of CNS lymphomas in immunosuppressed patients.

The presence of low copy numbers of CMV and EBV in immunosuppressed patients and in patients with Rasmussen encephalitis suggests that quantitation of viral load in tissues or CSF may further assist in identifying disorders directly caused by productive infection by these viruses. Finally, the very recent identification by tissue PCR of HHV-6, HHV-7, and HHV-8 genomic sequences in normal and neoplastic brain awaits further studies to establish whether these lymphotropic viruses are latent in cells of the CNS.

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