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Polymerase chain reaction analysis of human herpesvirus-6 sequences in the sera and cerebrospinal fluid of patients with multiple sclerosis

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Several studies have suggested a possible association of human herpesvirus-6 (HHV-6) with multiple sclerosis (MS), a demyelinating disease with a variable course and progression. To determine whether HHV-6 could be detected in the sera of CSF of patients with different subtypes of MS, we performed nested polymerase chain reaction (PCR) on samples obtained from MS patients as well as samples from normal adults or individuals with other neurological diseases. Ninety-six serum samples from 24 patients with MS, including 13 individuals with relapsing remitting MS, one individual with primary progressive MS, seven individuals with secondary progressive MS and three individuals with an unspecified type were analyzed. Multiple serum samples were examined from individuals over varying periods of time and included samples obtained during exacerbations, remissions, and at different stages of progressive disease. HHV-6 DNA was detected only in one out of 15 serum samples that were collected over a number of years from one individual with secondary progressive MS. No HHV-6 DNA was detected in CSF from six patients with MS or 14 patients with other neurologic disease. These results indicate that the presence of HHV-6 DNA in the serum or CSF of patients with MS is not a common phenomenon, at least within the limits of the sensitivity of our assay.

Keywords: HHV-6; multiple sclerosis; PCR amplification

Introduction

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system with a variable course and progression. The search for the underlying cause or causes of MS has encompassed extensive investigations of infectious (Cook *et al*, 1996), genetic (Hillert, 1996; Ebers *et al*, 1996; Sawcer *et al*, 1996; Haines *et al*, 1996, 1998) and environmental etiologies (Pryse-Phillips, 1996; Weinshenker, 1996), without resolution. Recent studies have proposed a relationship between MS and human herpesvirus-6 (HHV-6), and enveloped double stranded DNA virus with close homology to cytomegalovirus (CMV). HHV-6 is the etiologic agent of childhood febrile and exanthematous illnesses, hepatitis, and infectious mononucleosis-like illnesses, and is known to infect CD4+ T-cells (Lusso *et al*, 1988) and, at least *in vitro*, several CNS cell types including astrocytes, oligodendrocytes, and microglia (He *et al*, 1996; Albright *et al*, 1998). In addition to MS, HHV-6 has been implicated in other disorders such as chronic myelopathy, chronic fatigue and lymphoproliferative malignancies (Leach *et al*, 1992; Mackenzie *et al*, 1995).

Primary HHV-6 infection may be either asymptomatic or result in non-specific symptoms, but PCR studies have indicated that the virus establishes a latent CNS infection in a large proportion of individuals with no known neurological disease (Luppi *et al*, 1994). The relationship between HHV-6 and MS was first suggested by experiments where a DNA sequence nearly identical to the major DNA binding protein of HHV-6 was identified from one MS brain using representational display analysis (Challoner *et al*, 1995). Immunohistochemical studies showed that oligodendrocytes of MS patients, but not those of control patients, expressed

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HHV-6 antigens, and that HHV-6-expressing oligodendrocytes were primarily associated with the regions around the plaques in those MS brains (Challoner *et al*, 1995).

Serological studies analyzing the immune response of MS patients to HHV-6 have been equivocal, with some reports demonstrating an elevated antibody titer in comparison to controls, and others showing no significant differences (Sola *et al*, 1993; Nielson *et al*, 1997; Soldan *et al*, 1997). One group found that IgM directed against an early antigen of HHV-6 is increased in relapsing remitting MS in comparison with patients with chronic progressive MS or normal controls (Soldan *et al*, 1997). However, serum antibody titer for several viruses is higher in MS than in controls (Cook *et al*, 1996). Similarly a recent study showed a statistically significant difference between the frequency of HHV-6 DNA amplified from serum of MS patients in comparison to negative controls (Soldan *et al*, 1997). Other studies using PCR for HHV-6 in MS have not shown a similar statistically significant association (Martin *et al*, 1997; Liedtke *et al*, 1995; Wilborn *et al*, 1994; Merelli *et al*, 1997).

To determine whether HHV-6 can be amplified from the sera and/or the cerebrospinal fluid (CSF) of patients with MS we have performed a retrospective analysis on patients with MS, patients with other neurologic diseases, and normal adults using nested PCR. Serum from MS patients was obtained from patients with relapsing remitting, primary progressive, and secondary progressive types of MS (Lublin and Reingold, 1996). Serial serum samples from individuals with MS were obtained during both exacerbations and remissions in patients with relapsing remitting MS and other various periods of time in patients with primary or secondary progressive MS. Our results do not support an association between acute relapses or progressive MS and the presence of HHV-6 DNA in either the serum or CSF.

Results

The sensitivity of the PCR reaction was determined by using known amounts of pCRII plasmid containing HHV-6 DNA. The first round PCR performed with the oligonucleotides EX1 and EX2 and plasmid detected 1 pg of plasmid containing HHV-6. Nested PCR with the oligonucleotides IN3 and IN4 and PCR product from the first round PCR resulted in an increase in sensitivity to 0.01 fg (two copies) of plasmid containing HHV-6 (Figure 1A). Cell-free supernatant from HHV-6 infected Molt 3 cells served as a positive control during all PCR reactions. Nested PCR was capable of detecting a 10^{-4}-fold dilution of stock cell free supernatant from HHV-6 infected Molt 3 cells (Figure 1B).

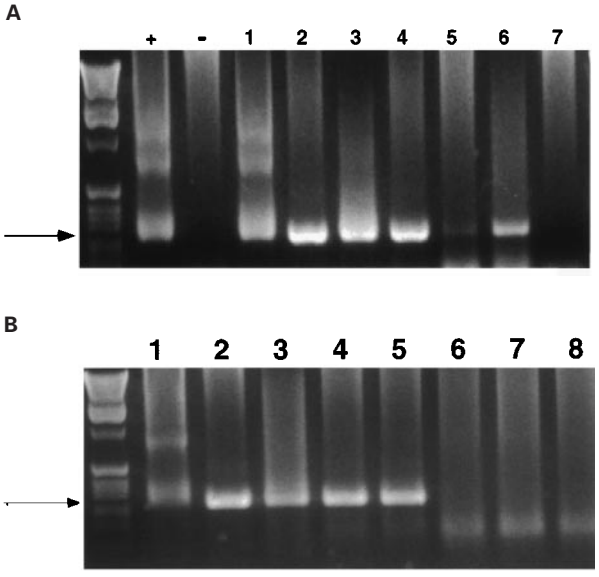

Figure 1 Determination of the sensitivity of the PCR reaction. (**A**) Plasmid. Agarose gel electrophoresis of nested PCR amplification of dilutions of pCRII plasmid containing an HHV-6 genomic sequence. Arrow: expected mobility of the 258 bp amplicon using a 1 kb ladder (Gibco-BRL). '+' denotes cell free supernatant from HHV-6 infected Molt 3 cells; '−' denotes negative control (no template DNA). The numbers 1−7 indicate varying amounts of pCRII plasmid containing an HHV-6 genomic sequence. 1=1000 fg; 2=100 fg; 3=10 fg; 4=1 fg; 5=0.1 fg; 6=0.01 fg; 7=0.001 fg. The nested PCR reaction could detect 0.01 fg (two copies) of plasmid. (**B**) HHV-6 Infected Cells. Dilutions of cell-free supernatant from HHV-6 infected Molt 3 cells were added to normal serum and DNA was purified using Elu-Quik protocol as described in Materials and methods. Nested PCR was then performed on the purified DNA. Arrow: expected mobility of the 258 bp amplicon. Lanes numbered 1−6 denote serial dilutions of cell-free supernatant from HHV-6 infected Molt 3 cells, ranging from 10^0-10^5, respectively. Lane numbered 7 depicts normal serum as a negative control; Lane numbered 8 depicts an additional negative control (no template DNA). The nested PCR was capable of detecting a 10^{-4}-fold dilution of cell-free supernatant from HHV-6 infected Molt 3 cells (lane 5).

In order to determine the effect of DNA extraction on the ability of the PCR to detect HHV-6 DNA, a known amount of plasmid was extracted from normal serum using the Elu-Quik protocol. Analysis of PCR product indicated that greater than 200 pg of plasmid must be present in 250 μL of serum in order to visualize an amplicon with agarose gel electrophoresis after one step PCR. When the products of the first PCR reaction were purified and amplified with and additional set of nested primers, the assay could detect as little as 20 fg (4.1×10^3 copies) of plasmid in 250 μL of serum (see Materials and methods).

In order to determine whether there is an association between HHV-6 and MS, PCR was performed on serum and CSF of MS patients. A total of 96 serum samples from 24 MS patients with relapsing remitting, primary progressive, and secondary progressive disease were analyzed by PCR

Table 1(A) PCR positive individuals/total numbers of individuals with multiple sclerosis (MS), other neurological disease (OND), or normal adults (N)

MS subtype	*MS (serum) pos/total*	*OND (serum) pos/total*	*N (serum) pos/total*	*MS (CSF) pos/total*	*OND CSF pos/total*
Unspecified	0/3	NA	NA	0/0	NA
Relapsing remitting	0/13	NA	NA	0/5	NA
Primary progressive	0/1	NA	NA	0/0	NA
Secondary progressive	1/7	NA	NA	0/1	NA
Total	1/24	0/16	0/14	0/6	0/14

NA=not applicable

(B) Total number of samples analyzed from 24 MS patients

MS disease activity	*Serum*	*CSF*
Stable/remission	34	1
Acute relapse	25	4
1 week–1 month after acute relapse	10	0
Primary progressive	2	0
Secondary progressive	21	1
Unspecified	4	0
Total	96	6

as described above (Table 1A). HHV-6 DNA was detected by nested PCR in one sample, from one secondary progressive MS patient (Table 1B and Figure 2). However, no HHV-6 DNA was detected by PCR in 15 other samples from this patient. HHV-6 DNA was not detected by nested PCR in the other 80 serum samples from individuals with MS. The MS and normal patient serum samples that were analyzed multiple times to serve as an internal control for reliability were all negative. A representative photograph of PCR products from serum of MS patients, individuals with other neurologic disease, and normal controls is shown (Figure 2). No HHV-6 DNA was detected in the CSF from six MS patients or the 14 patients with other neurologic disease (data not shown).

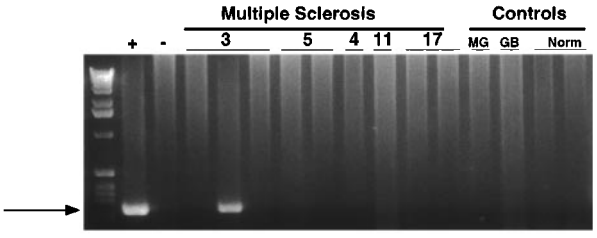

Figure 2 Nested PCR of serum samples from MS patients, patients with other neurologic diseases, and normal adults. Arrow: expected mobility of the 258 bp amplicon. The '+' indicates the positive control consisting of a 1/1000 dilution of cell-free supernatant from HHV-6 infected Molt 3 cells; The '−' indicates the negative control in which no template was used in the PCR reaction; The remaining lanes indicate reactions with sera obtained from patients 3, 5, 4, 11 and 17, as well as controls with either no neurologic disease or other neurological problems unrelated to MS. Three samples from patient 3 (secondary progressive MS) correspond to sera obtained on 2/24/93, 6/9/93, and 8/25/93; two samples from patient 5 were obtained during either exacerbation or remission; patient 4 had primary progressive MS; patient 11 had secondary progressive MS, and two samples from patient 17 were obtained during either exacerbation or remission. Controls included a patient with myasthenia gravis (MG); a patient with Guillain-Barré Syndrome (GB), and serum from the same normal individual (Norm) obtained on two different dates.

Discussion

MS is a demyelinating disease of the central nervous system with a variable clinical course and symptom severity, that has been classified into several subtypes based on distinct clinical courses and radiological features. Therefore, it is entirely possible that MS represents a group of conditions with different etiologies resulting in similar pathological and clinical characteristics. Under those circumstances only a subset of MS patients might exhibit an association with a specific pathogen such as HHV-6.

Our approach to correlating MS symptoms with the presence of HHV-6 DNA in serum was based on the findings of Soldan *et al* (1997), who demonstrated HHV-6 DNA in the sera of 30% of patients with relapsing remitting MS. Since MS is characterized by breakdown of the blood brain barrier in areas of demyelination and by a prominent perivascular lymphocytic infiltrate around the area of an MS lesion or plaque, it is reasonable to hypothesize that if HHV-6 plays a role in the development of the demyelinating lesion, some of the virus might spill into the peripheral circulation. Other viruses that cause encephalitis, for example cytomegalovirus (CMV) often result in virus being detected in the CSF of affected individuals (Aurelius *et al*, 1993).

For our study, we obtained samples from patients with all subtypes of MS, although most of the patients had relapsing remitting or secondary

progressive MS. Additionally, in order to determine whether detection of HHV-6 DNA varied within an individual and correlated with active disease, serial serum samples were obtained from several patients.

We were unable to detect HHV-6 DNA in 13 patients with relapsing-remitting MS, including 24 samples obtained during acute exacerbations. Additionally, no HHV-6 DNA was detected in the CSF of patients with relapsing-remitting MS, which included fluid obtained during four acute relapses. Among all of the 96 samples we had a single positive result in a serum sample stored for 5 years isolated from one patient with secondary progressive disease who was sampled a total of 16 times, including earlier in her course when she had relapsing remitting disease. Since none of the other samples obtained from this individual were positive, it is hard to ascribe great significance to this single positive result. HHV-6 DNA was not detected in serum in the other six patients with secondary progressive MS or in the CSF from one patient with secondary progressive MS.

Our results differ from those of Soldan *et al* (1997) even though the nested PCR assay was similar to that used in that study and others (Secchiero *et al*, 1995). We were able to detect 0.01 fg of plasmid, indicating that the sensitivity of our assay is as high as in those reports. However, the sensitivity of the assay decreased when multiple DNA purifications were performed; it is also possible that our samples had been stored for longer periods of time (some were stored for as long as 20 years) and some degradation of template may have occurred. However, some sera were examined within 1–2 years of sampling.

Studies that examine brain tissue of MS patients directly appear to be the most definitive way to further elucidate any possible association between HHV-6 and MS. The preponderance of data that suggests that HHV-6 is associated with MS is derived from histological studies that detected HHV-6 protein in areas of active demyelination in patients with MS (Challoner *et al*, 1995) and in several cases of acute, severe demyelinating diseases (Novoa *et al*, 1997; Carrigan *et al*, 1996). Both *in situ* PCR to directly detect viral DNA and immunohistochemistry to detect viral proteins would more definitively examine the association of HHV-6 with MS. Finally, it is possible that subsets of MS patients that have not yet been examined may harbor HHV-6 viral DNA in their serum or CSF during various stages of disease activity.

Materials and methods

Samples

Serum samples from 24 different MS patients, serum samples from 16 patients with other neurologic diseases, and sera from 14 normal volunteers were obtained (Table 1) and stored at −70°C. The samples were coded before shipment at Wayne State University and analyzed in a blinded fashion at the University of Pennsylvania. Sera and CSF from 19 women and five men aged 18–44 with MS were included in the study. The MS patients included the following sub-classifications: three individuals with unspecified type of MS, 13 individuals with relapsing and remitting MS, one individual with primary progressive MS, and seven individuals with secondary progressive MS (see Lublin and Reingold (1996) for criteria). Three of the patients with secondary progressive MS had serum samples analyzed during both the relapsing remitting and secondary progressive phases of their illness. Multiple serum samples from 15 MS patients were obtained from various dates throughout the course of the illness and nine MS patients had one sample coded for analysis. A total of 96 MS serum samples from various states of disease activity were analyzed with the following classifications of disease: four serum samples were unspecified, two samples were characterized as primary progressive, 21 samples were from secondary progressive states, 25 samples were from acute relapses, ten samples ranged from 1 week to 1 month after relapse, and 34 samples occurred in stable MS patients (Table 1). One MS patient had three serum samples analyzed from the same date and four normal patients had four to six serial serum samples analyzed in order to have internal blinded controls for reliability of the assay. Additionally, CSF from six MS patients was analyzed from five men and one woman, five of whom were relapsing remitting (four acute relapses and one remission) and one was secondary progressive (Table 1). CSF from 14 patients with other neurologic disease served as controls (Table 1).

DNA extraction

DNA was extracted from either serum or CSF using the S & S Elu-Quik DNA Purification Kit (Schleicher and Schuell) with a modified protocol which included using 80 μL of lysis buffer, 160 μL of binding buffer, 50 μL of glass concentrate, and one elution in 25 μL water for the DNA isolation from 250 μL of sample. HHV-6 infected Molt 3 cells were used as positive control, and the DNA was extracted using a similar protocol.

Polymerase chain reaction

HHV-6 DNA amplification was performed in a two step reaction with nested primers. The outer primers EX1 and EX2 were derived from the major capsid protein gene and amplify a 520 bp fragment (Secchiero *et al*, 1995). The sequence of EX1 is 5′-GCGTTTTCAGTGTGTAGTTCGGCAG-3′ which differs by a G to C substitution at position 23 from the oligonucleotide sequence previously published (Secchiero *et al*, 1995). The sequence of EX2 is

5′-TGGCCGCATTCGTACAGATACGCAGG-3′. The inner primers, IN3 and IN4 amplify a 258 bp fragment within the 520 bp fragment (IN3: 5′-GCTAGAACGTATTTGCTGCAGAACG-3′; IN4 5′-ATCCGAAACAACTGTCTGACTGGCA-3′). DNAs extracted from either serum or CSF were centrifuged at 7000 r.p.m. × 1 min and 5 μL were added to 45 μL of reaction mix containing 1 × PCR buffer with 1.5 mM MgCl2 (Perkin-Elmer Cetus, Norwalk, CT, USA), 0.2 mM dNTP, 0.5 μM primers, and 6.25 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA). PCR parameters were as follows: (92°C × 1 min, 50°C × 1 min, 72°C × 1 min) for 35 cycles followed by 72°C × 10 min using a Hybaid OmniGene (Labnet) PCR apparatus. For all samples, the first round PCR product was purified with a QIAquick PCR purification kit (QIAGEN, Chatsworth, CA, USA) using the standard protocol and 5 μL of purified PCR product were then amplified with the internal primers using the same conditions. PCR products were analyzed by electrophoresis on 2% agarose gels containing ethidium bromide.

Plasmid
A diluted plasmid using the pCRII vector with the 520 bp amplicon derived from PCR with EX1 and EX2 oligonucleotides was used as a positive control to determine the sensitivity of the PCR. The plasmid was cloned and purified as described and was a gift from Dr Soldan (Viral Immunology Section, National Institute of Neurologic Diseases and Stroke). PCR was performed on a known amount of serially diluted plasmid with the outer oligonucleotides EX1 and EX2 and the product from this PCR was subjected to second round amplification with IN3 and IN4.

Sensitivity of nested PCR after DNA extraction
In order to determine the effect of Elu-Quik and QIAquick DNA purification protocols on the nested PCR sensitivity, diluted plasmid was added to normal serum to simulate the unknown serum samples, and HHV-6 DNA extracted as indicated above. The PCR products were analyzed in a 2% agarose gel stained with ethidium bromide.

All of the plasmid work was performed in a room separate from the PCR reagents and serum samples, and it was performed after amplification of the serum and CSF samples had been substantially completed.

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