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Human herpesvirus 6 polymerase chain reaction findings in human immunodeficiency virus associated neurological disease and multiple sclerosis

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A role for human herpesvirus 6 (HHV6) in the neurological complications associated with infection by human immunodeficiency virus (neuro-AIDS) and during multiple sclerosis (MS) is not known. For the present study, an improved PCR and immunofluorescence serology method were applied to sera and cerebrospinal fluid (CSF) from 27 neuro-AIDS, 36 MS and 24 non-inflammatory control patients. HHV6 DNA was present in 30–40% of the cellular CSF from all groups. In the acellular CSF, HHV6 could be detected in four of 36 MS, 2 of 27 neuro-AIDS and none of the control patients. HHV6 IgG was present in one of 27 neuro-AIDS, and one of 36 MS patients. HHV6 IgG was present in all patients. There was no correlation between clinical features and HHV6 PCR findings or HHV6 antibodies. The significance of the present documentation of HHV6 DNA in the acellular CSF from a minority of MS and neuro-AIDS patients remains to be determined.

Keywords: MeSH: HHV6; PCR; neuro-AIDS; encephalomyelitis disseminata (ED); cerebrospinal fluid (CSF); immunofluorescence serology

Introduction

Human herpesvirus 6 (HHV6), a member of the β-herpesvirus family, was first isolated in 1986 from peripheral blood mononuclear cells (PBMC) of six patients with lymphoproliferative disorders, two of whom were seropositive for human immunodeficiency virus (HIV) (Salahuddin *et al*, 1986). The tropism of HHV6 *in vitro* has been defined as mainly CD4+ cell-related, but among others, B-lymphocytes and glial cells are also susceptible (Ablashi *et al*, 1987, Ablashi *et al*, 1988, Lusso *et al*, 1988, Takahashi *et al*, 1989). In 1988, a causal role of HHV6 in exanthem subitum in children was reported (Yamanishi *et al*, 1988). HHV6 infection has been associated with chronic fatigue syndrome in adults (Buchwald *et al*, 1992).

Febrile convulsions and encephalitis/encephalopathy in exanthem subitum patients have pointed towards a possible role for HHV6 in nervous system disease (Kondo *et al*, 1993, Suga *et al*, 1993). In addition, HHV6 DNA has been amplified from the CSF of such patients (Yamanishi *et al*, 1992; Yoshikawa *et al*, 1992). It has been hypothesized by these authors that HHV6 establishes latency in glial cells after primary infection and is subsequently involved in the pathogenesis of recurrent febrile convulsion. In recent reports, HHV6 antibody titers in patients with multiple sclerosis (MS) and Guillain-Barré-Syndrome were higher than those of controls (Merelli *et al*, 1992; Sola *et al*, 1993). HHV6 DNA could be amplified from CSF of MS patients and a potential role of HHV6 in this disease was hypothesized (Wilborn *et al*, 1994).

HHV6 induces CD4 surface expression and HIV-1 susceptibility *in vitro* in cells that were previously CD4 negative and HIV-1 resistant (Lusso *et al*, 1991). HHV6 can also be cytotoxic for such cells (Lusso *et al*, 1993). On the other hand, HIV-1 replication in PBMC was suppressed by HHV6 coinfection (Carrigan *et al*, 1990). In neurological complications of patients infected with HIV-1 (neuro-AIDS), crucial issues remain to be resolved. For

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Table 1 HHV6 PCR findings in cellular and acellular CSF of three different patient groups

Patient groups and P-values	*HIV*	*MS*	*Ctrl*	P_{HIV}*	P_{MS}*
Patient number	27	36	24		
PCR + cellular sample	11/27	14/36	7/24	0.58	0.62
PCR + acellular sample	2/27	4/36	0/24	0.52	0.24

* *P* values for χ^2 test for comparison of neuro-AIDS with control patients (P_{HIV}) and MS with control patients (P_{MS})
HIV: neuro-AIDS; MS: multiple sclerosis; Ctrl: non-inflammatory controls

Table 2 HHV6 immunofluorescence antibodies (IgG and IgM) in three different patient groups

Patient groups and P-values	*HIV*	*MS*	*Ctrl*	P_{HIV}*	P_{MS}*
HHV6 IgG 1/10++	27/27	36/36	24/24		
HHV6 IgG 1/50+	20/27	18/36	16/24		
HHV6 IgG 1/50++	4/27	14/36	5/24	0.86	0.23
HHV6 IgM 1/20+	1/27	1/36	0/24		

* *P* values for χ^2 test for comparison of neuro-AIDS with control patients (P_{HIV}) and MS with control patients (P_{MS})
HIV: neuro-AIDS; MS: multiple sclerosis; Ctrl: non-inflammatory controls

example, the mode of entry of HIV-1 into the central nervous system, the pathogenesis of declining immunocompetence within the CNS evidenced by opportunistic CNS infections and the pathogenesis of AIDS dementia complex (ADC) remain to be clarified (Elder and Sever, 1988). A virus with features attributed to HHV6 (eg CD4 surface expression and induction of HIV-1 susceptibility, cytotoxicity and neurotropism), represents an attractive candidate viral cofactor in neuro-AIDS, particularly ADC. Theoretically, HHV6 might facilitate the entry of HIV into the CNS. In the invaded CNS, HHV6 could act synergistically with HIV-1 to accelerate HIV-1 cytotoxicity.

Hence, the present study has examined a role for HHV6 in neuro-AIDS and MS by polymerase chain reaction (PCR) and immunocytochemistry. Cellular and acellular CSF samples from 27 neuro-AIDS patients, 36 MS patients and 24 non-inflammatory controls were examined by a highly sensitive nested PCR with a non-radioactive magnetic particle-based detection system. In addition, sera from the same patients were examined for the presence of HHV6 antibodies by an indirect immunofluorescence assay (IFT).

Results

Table 1 shows HHV6 PCR findings in cellular and acellular CSF. No statistically significant differences between neuro-AIDS and control patients or between MS and control patients could be observed by χ^2 analysis (minimal $P = 0.24$). In addition, no statistically significant differences between neuro-AIDS patients and non-inflammatory control patients and also between MS patients and non-inflammatory control patients could be obtained by the Mann–Whitney U test of the background corrected OD values of each patient's PCR product (see

Table 3 HHV6 PCR findings and AIDS dementia complex grade in neuro-AIDS patients

#	*cPCR*	*acPCR*	*Sex*	*Infection*	*HIV+since*	*AIDS*	*ADC grade*	*CD4 count*	*CSF cell#*
1	-	-	M	Homo	3/85	PCP	2	500	4
2	-	+	F	Drug	89		2	300	2
3	+	-	M	Homo	?		2	nd	2
4	+	-	M	Homo	6/85	PCP	1	nd	1
5	+	-	M	Homo	6/88		2	250	2
6	-	-	M	Homo	6/89	PCP	2	100	2
7	+	-	M	Homo	6/89	PCP	2	30	8
8	-	-	M	Drug	85	Candida	1	250	4
9	-	-	M	Bisexual	10/90	Kaposi; PCP	2	20	0
10	-	-	M	Homo	6/91	PCP	2	15	1
11	-	-	M	Homo	6/85	PCP	1	15	6
12	-	+	M	Homo	3/93	Crypto	0	154	40

All patients suffering from ADC are listed. Patient #12 did not suffer from ADC and was positive for HHV6 PCR in his acellular CSF. Patients #2 and #12 were the only neuro-AIDS patients with HHV6 PCR+ in acellular CSF: *First row*: cPCR: HHV6 PCR in CSF cells; acPCR: HHV6 PCR in acellular CSF supernatant; Infection: mode of HIV acquisition; AIDS: manifestations of HIV infection outside the nervous system and opportunistic infections; ADC grade: grading of ADC as reported (Price and Brew, 1988): 1 — light, 2 — moderate, 3 — severe, 4 — endstage; CSF cell#: CSF cell count in number of cells μl^{-1} (normal 0–4). *In the table*: Homo: homosexual; drug: intravenous drug abuser; PCP: pneumocystis carinii pneumonia; Candida: recurrent systemic candida infections; Crypto: meningitis due to cryptococcus neoformans; nd: not determined

Material and methods): $P_{HIV} = 0.50$, $P_{MS} = 0.47$. Patients with positive PCR amplification from acellular CSF had CSF cells which were PCR negative. In cellular CSF, HHV6 DNA sequences were detectable in seven of 24 (29%) non-inflammatory controls, in 11 of 27 (41%) neuro-AIDS and in 14 of 36 (38%) MS patients.

Table 2 shows the IFT IgG and IgM findings from the patient groups. No statistically significant differences were detectable between neuro-AIDS and non-inflammatory control patients or between MS and non-inflammatory control patients (minimum $P = 0.23$; χ^2 test). However, there was a tendency towards higher IgG titers in the sera of MS patients: 14 of 36 MS sera had an IgG titer of 1/50 rated '++'; for comparison the figure in neuro-AIDS patients was 4 of 27 and 5 of 24 in non-inflammatory controls. The HHV6 IgM positive patients were PCR negative and did not reveal unusual clinical signs.

A positive PCR was neither associated with CSF pleocytosis nor with a higher serum HHV6 IgG antibody titer. In neuro-AIDS, a positive HHV6 PCR or a high serum antibody titer were not associated with a low CD4+ count. In addition, there was no association between the occurrence of ADC and positive HHV6 PCR (cellular and acellular CSF) or elevated antibody titers. Table 3 shows features of neuro-AIDS patients with ADC. In MS, a positive HHV6 PCR or a high serum antibody titer were not associated with severity (as measured by expanded disability scoring scale (EDSS) (Kurtzke,1970), an investigator-based test to assess disease-induced disability), or duration of the disease (data not shown).

Discussion

The present PCR study for HHV6 sequences in the CSF of neuro-AIDS, MS and non-inflammatory control patients does not substantiate a role for HHV6 as a cofactor or as an etiopathogenetic agent in these diseases.

The present PCR method combined high sensitivity and non-radioactive detection. Due to the nested approach, the sensitivity of the assay was improved by a factor of 5 as compared to the original non-nested PCR of Gopal (Gopal, 1990), thus lowering the sensitivity level of detection to 12 virions. The present non-radioactive detection system for HHV6 PCR products combined rapidity, reliability, numeric measurement of PCR product detection, increased detection sensitivity by a factor of 1×10^1 as compared to standard agarose gel/southern blot probe hybridization assay, safety due to absence of radio- and hazardous chemicals and simultaneous detection and confirmation of the PCR product. The importance of separating CSF before PCR of lymphotropic viruses into cells and acellular supernatant is highlighted by our results (Table 1).

Due to the increased sensitivity of the applied PCR assay, the present results should be interpreted with caution. For example, in acellular CSF from neuro-AIDS and MS, HHV6 sequences could be amplified from a minority of patients (two of 27 neuro-AIDS, four of 36 MS and none of the non-inflammatory controls) (Table 1). The biological significance of these findings remains to be proven. It might be argued that these results were due to increased cellular trafficking through the blood–brain barrier. This is not supported by the HHV6 PCR findings from the cellular CSF (Table 1): there were no significant differences between 11/27 positive samples in neuro-AIDS patients, 14/36 in MS and 7/24 in non-inflammatory control patients with none of these control patients showing CSF pleocytosis. Furthermore, there was no correlation between the HHV6 PCR findings and CSF cell count. This was also true for acellular PCR findings and CSF cell count (Table 3): patient #2 had a CSF cell count of 2 per µl and patient #12 40 cells per µl. One might speculate that despite attempts to avoid cellular contamination of the acellular CSF, the HHV6 sequences were amplified from lysed CSF cells. The absence of amplifiable HHV6 sequences in the corresponding cellular CSF argued against this possibility but did not rule it out. The only virus-harboring cells might have been lysed with a resultant positive PCR in the acellular CSF and negative amplification in the cellular sample. Amplifiable HHV6 sequences in the cellular CSF of 29% of the non-inflammatory control patients demonstrated the sensitivity of the applied PCR. Given an initial amount of a maximum of 1×10^4 cells in control CSF and the sensitivity of our PCR assay, this meant that at least 1 cell per 10^4 CSF cells harbored amplifiable HHV6 genome in 29% of non-inflammatory control patients. On the other hand, it might be hypothesized that our HHV6 PCR findings reflect host immune control within the CNS. With regard to MS, this would mean a dysregulation of immune surveillance because MS typically shows unregulated CNS immunoreactivity. With regard to neuro-AIDS and ADC, no association between CD4 cell count and HHV6 PCR findings could be established (Table 3). However, CD4 cell count is considered only one marker for the decline of systemic, but not local CNS based immunocompetence. Therefore, it might be meaningfully speculated that in a minority of 2/27 neuro-AIDS patients, HHV6 CNS infection might have prevailed in the absence of clinical or laboratory changes. HHV6 infection in these cases would indicate insufficient control of the viral infection by the host immune system. In the remaining patients, HHV6 infection was presumably controlled by the host immune system.

With regard to clinical findings, there was no association between HHV6 PCR findings and the occurrence of ADC in neuro-AIDS patients; in MS, no association could be found between findings and

disease duration or disease severity as measured by EDSS.

Conflicting data on brain viral burden in AIDS have been obtained so far: consistent detection of HHV6 DNA sequences was reported with no accompanying organ specific pathology in the central nervous system of AIDS autopsy cases (Corbellino *et al*, 1993): five of five brains from AIDS patients harbored HHV6, whereas none of two controls did so. Contrarily, a recent investigation on brain viral burden in HIV infection demonstrated PCR-amplified HIV DNA in 70% and cytomegalovirus DNA in 50% of autopsied brains of HIV infected patients (Achim *et al*, 1994). In only one of these 45 brains could HHV6 DNA be amplified from the basal ganglia.

HHV6 DNA has been amplified from the CSF of three of 21 MS patients (Wilborn *et al*, 1994). However, CSF amplification from seven patients with Guillain Barré Syndrome and 19 patients with facial palsy was negative. In comparison to the above report (Wilborn *et al*, 1994), the present study applied a more sensitive PCR product detection method, but the major difference between the current and the previous investigation was the method of sample preparation. Wilborn and colleagues treated the CSF with proteinase K, centrifuged the sample and used the supernatant, whereas in the present study, the CSF was first separated into cellular and acellular components and then processed.

With regard to HHV6 antibody, no statistically significant differences could be detected between patient groups (Table 2). However, MS sera did tend to have elevated HHV6 IgG titers. This tendency was in accord with previous findings (Sola *et al*, 1993, Wilborn *et al*, 1994). As the previous authors hypothesized, the elevated IgG titers in MS might be due to immune dysregulation in this condition. The patients with positive IgM antibody (one in MS, one in HIV), revealed no outstanding particular clinical signs. The CSF of both of them was HHV6 PCR negative. There was no association between elevated serum HHV6 antibody titer and positive HHV6 PCR findings.

Taken together, no etiopathogenetic role of HHV6 could be substantiated for neuro-AIDS and MS.

Material and methods

Patients and samples

Patients with neurologic manifestations of HIV infection were hospitalized in the University Hospital of Essen, Germany, between October 1992 and August 1993. HIV-associated neurological diseases were diagnosed according to current criteria (Price and Brew, 1988; AAN AIDS Task Force, 1991). CSF and serum samples of 27 patients (24 male, three female, mean age 36.6 years) were examined. CSF pleocytosis (>4 cells μl^{-1} was present in 11 of 27 patients. Seventeen patients were homosexual, six intravenous drug abusers (ivda), one patient was bisexual and one patient a heterosexual prostitute. For two patients, we had no information about the route of infection. Eleven of the 27 patients suffered from ADC. These patients' features are summarized in Table 3. HIV infection was known for 4.9 years on average. The CD4+ cell count in peripheral blood cells was available from 20 patients and averaged 145 cells. CSF and serum samples of 36 MS patients (22 female, 14 male, mean age 35.9 years), hospitalized between 10/1992 and 8/1993, were obtained. Fifteen of 36 patients had a CSF pleocytosis (>4 cells μl^{-1}). All patients could be classified as 'laboratory supported MS' or 'clinically definite MS' (Poser *et al*, 1983). Mean EDSS (Kurtzke, 1970) was 3.8 and mean disease duration 4.2 years.

CSF and serum samples of 24 non-inflammatory neurological control patients (15 female, nine male, mean age 52.7 years) were included. Eleven of these suffered from lumbar disk herniation, eight from migraine, three from multiinfarct dementia, and two from normal pressure hydrocephalus. All routine CSF findings including CSF cell count and oligoclinical bands as well as HIV serum antibody in these patients were normal.

With regard to all patients involved, good clinical practice guidelines and human experimentation

Table 4 Nested HHV6 PCR

Cycle count: 25 (outer primer pair)/ 35 (inner primer pair)
Reaction volume: 50µl
Cycling parameters: 95°C (1')/65°C (1')/72°C (2')
Additional features: hot start, final 72°C 5', $MgCl_2$ 1.5 mmol, 10× PCR buffer supplied by Perkin Elmer (Perkin Elmer, Vaterstetten, Germany)
1.5 U Taq polymerase (Perkin Elmer)
Outer primer HHV6 01 5'CTCGAGTATGCCGAGACCCCTAATC3'
Outer primer HHV6 02 5'GCTTGCACAATGCCAAAAAACA3'
Inner primer HHV6 I1 5'-bio-TAGGATATACCGATGTGCGTGATC3'
Inner primer HHV6 I2 5'TTTGGCCGCATTCGTACA3'
Probe HHV6 PR 5'-dig-AACTGTCTGACTGGCAAAAACTT3'
Primers and modified primers supplied by TIB Molbiol Inc., Berlin, Germany

guidelines of the University of Essen were followed.

PCR

Amplification of HHV6 specific sequences was performed on CSF samples of neuro-AIDS, MS and non-inflammatory control patients. Blood contaminated CSF was excluded from the present investigation. Only CSF obtained within 2 h of lumbar puncture was processed by immediate centrifugation for 10 min/1000g/4°C. The supernatant was processed as described by Aurelius (Aurelius *et al*, 1991) (briefly: boiling and 95% ethanol precipitation of DNA). Pelleted CSF cells resuspended in 20µl sterile saline were boiled for 5 min. All sample preparations were stored at -80°C. Two µl of CSF preparations were assayed using a nested PCR with outer primers and probe as described by Gopal (Gopal *et al*, 1990). The inner primers were designed according to the HHV6 genomic sequence as reported (Lawrence *et al*, 1990). Details of the assay are given in Table 4. Primer HHV6 I1 was biotinylated at its 5′ end and digoxigenin was linked to the probe at its 5′ end applying a commercially available labeling kit (Boehringer Mannheim, Mannheim, Germany). Detection of the HHV6 PCR product was accomplished by binding 20 µl of the biotinylated PCR product to 200 µg streptavidin conjugated superparamagnetic polystyrene beads (Dynabeads™, Dynal Inc., Hamburg, Germany) in Falcon flexible microtiterplates (Falcon, Becton and Dickinson, Heidelberg, Germany) for 30 min at room temperature. After denaturing in 0.1N NaOH solution, 200 fmol digoxigenized probe was hybridized for 1 h/42°C to the PCR product (hybridising solution: 50% formamide, 7% sodium dodecylsulfate, 0.1% laurylsarcosyl, 5 × saline sodium phosphate EDTA and 2% nucleic acid hybridization blocking reagent from Boehringer Mannheim) with subsequent stringent washing (at room temperature) down to 0.1 × saline sodium citrate. The beads were then incubated for 1 h/room temperature with anti-digoxigenin peroxidase conjugated antibody (Boehringer Mannheim) diluted 1/2000 in 1% ELISA blocking reagent (Boehringer Mannheim). A colorigenic dye, BM blue™ (Boehringer Mannheim), was added, the reaction stopped with 2N H_2SO_4 and the optical density (OD) measured at $\lambda = 450$ nm in a Titertek Multiscan microtiter plate photometer (Labortechink, Oehmen, Essen, Germany). Positive controls included DNA extracted from HHV6 positive saliva specimens, from HHV6 virions and from infected cell line J Jhan (kindly provided by Professors B Matz and KE Schneweis, Bonn, Germany). Negative controls were water, PCR reaction mix, DNA extracted from herpes simplex virus 1, Hep2 cell line and varicella zoster virus. All PCR assays were run in duplicate and all assays with >10% intersample difference repeated.

PCR products from negative control samples never resulted in an OD > 0.07. Samples were considered '+' when their OD ranged between 2× and 3×OD of the negative controls and '++' if their OD was >3×OD of the negative controls. Control experiments run in post-PCR detection consisted of water instead of the PCR product, assays without digoxigenized probe and without anti-digoxigenin antibody. Detection sensitivity in dilutional series was 1×10^1 lower as compared to standard ethidiumbromide agarose gel electrophoresis/Southern blot probe hybridization technique.

All samples were assayed under code. The intra- and interassay variation was never higher than 10%. PCR safety precautions were applied according to standard procedures (Dieffenbach and Dveksler, 1993). The specificity of this PCR assay is given through the outer primers and probe as described (Gopal *et al*, 1990). The reported sensitivity for this assay is less than 10 copies of HHV6 plasmid. The nested approach increases the sensitivity by a factor of 5 as determined by limiting dilution. The presence of human DNA in cellular CSF was verified by amplification of sequences from the tumor suppressor gene p53, exon (8+9) (Hensel *et al*, 1991).

Immunofluoresence antibody testing (IFT)

Antibody to HHV6 was determined in the sera diluted to 1/10 and 1/50 (IgG) or 1/20 (IgM). The assays were performed as described in detail elsewhere (Schneweis *et al*, 1990). Briefly, the sera were incubated with HHV6 infected J Jhan cells fixed to slides. After washing and secondary antibody incubation (Dako, Denmark), staining of cells was rated '–', '+' or '++'. As with PCR, investigation was performed under code.

Statistical analysis

Patient groups (neuro-AIDS, MS and non-inflammatory controls) were compared with regard to HHV6 PCR and HHV6 IFT findings by χ^2 analysis. In addition, neuro-AIDS and MS patients each were compared to non-inflammatory controls with regard to HHV6 PCR findings by Mann–Whitney *U* test: The background corrected optical densities (OD) of the PCR product detection assay were ranked and Sigmastat™ software was applied (Jandel, San Rafeal, CA).

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