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Herpesvirus type 1-8 in sinus aspirates from HIV-infected patients and immunocompetent individuals*

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SUMMARY

Sinusitis is frequently occurring in HIV-infected patients, but in a substantial number of cases the etiology is unknown. The purpose of this study was by PCR 1) to determine the prevalence of the eight human herpesviruses in sinus aspirates from 24 HIV-positive/AIDS patients with sinusitis 2) to relate the presence of herpesvirus DNA to clinical and immunological parameters and 3) to compare the prevalence of herpesvirus DNA in sinus aspirates from HIV-infected patients with the prevalence observed in 50 immunocompetent patients with sinusitis. DNA from HSV-1, EBV, CMV and HHV-8 was detected in 8 (33%) of the sinus aspirates from HIV-infected patients. In the immunocompetent patients, one of the herpesviruses, HHV-6, was found in one sinus aspirate. These data indicate that herpesviruses are frequently found in sinus aspirates from HIV-infected patients with sinusitis, whereas they do not seem to be related to clinical signs of sinusitis in immunocompetent individuals. The cause of these discrepancies may be due to uncontrolled reactivation of herpesviruses, which is known to occur in immunocompromised individuals. It remains to be established whether the herpesviruses play a pathogenic role in the development of sinusitis in HIV-infected patients.

Key words: Herpesvirus, sinusitis, HIV/AIDS, PCR, Kaposi's sarcoma (KS).

INTRODUCTION

The prevalence of sinusitis has been shown to be higher in patients infected with Human Immunodeficiency Virus 1 (HIV) than in the general population (Lamprecht and Wiedbrauck, 1988). The etiologic agents of sinusitis in the HIV-infected patients are in most cases equivalent to those observed in immunocompetent patients: *Streptococcus pneumoniae, Haemophilus influenzae* and *Staphylococcus aureus,* but atypical organisms as *Legionella pneumophila, Mycobacterium avium* and *M. kansasii*, *Cytomegalovirus, Aspergillus flavus* and A. *fumigatus*, and parasites as *Microsporidium*, *Cryptosporidium* and *Acanthamoeba* species have been reported (Schlanger et al., 1984; Naguib et al., 1994; Dunand et al., 1997; Iwen et al., 1997; Yoskovitch and Cantrell, 1998). However, in a substantial number of the sinus aspirates no microorganisms are demonstrable (Tarp et al., unpublished data). In these patients sinusitis may be caused by non-infectious conditions as impaired mucociliary clearance or allergic rhinitis. The lack of demonstrable microorganisms may also be due to the use of insensitive methods in the detection of the agents or due to viral infections.

The purpose of this prospective study was 1) to determine the prevalence of the eight human herpesviruses in sinus aspirates from HIV-positive/AIDS patients with sinusitis 2) to relate the presence of herpesvirus DNA to clinical and immunological parameters 3) to compare the prevalence of herpesvirus DNA in sinus aspirates from HIV-infected patients with the prevalence observed in immunocompetent patients with sinusitis.

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MATERIALS AND METHODS

Patients

HIV-infected patients undergoing sinus puncture for sinusitis during the period from May 1996 to June 1998 at the Department of Infectious Diseases, Marselisborg Hospital, Aarhus University Hospital, Denmark were prospectively included. At inclusion the patients completed a questionnaire commenting on the presence of (1) allergy, (2) current symptoms of sinusitis, (3) previous events of sinusitis, (4) cold more than twice annually and (5) smoking habits. Clinical examination was performed and blood samples were drawn and immediately analyzed. The diagnosis of sinusitis was verified by magnetic resonance imaging (MRI) as previously described (Tarp et al., 2000).

Sinus aspirates from immunocompetent patients with sinusitis undergoing sinus puncture during the period from February to December 1998 at a practice of Otorhinolaryngology, Aarhus, Denmark, were prospectively included. At inclusion the age and sex of the patient and the date of sinus puncture were recorded. The diagnosis of sinusitis in this population was based on sinus x-rays, computed tomography (CT) or symptoms and clinical signs of sinusitis.

Sample preparation

DNA was extracted from 300 μl of sinus aspirate according to the manufacturer's description for the Puregene® DNA Isolation Kit (Gentra Systems Inc, Minneapolis, MN, USA). Water was extracted following each third sample. The integrity of the extracted DNA was confirmed by PCR for the gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (Kelsen et al., 1999). To the samples, being negative to amplification, 1 μl of the PCR negative sample was mixed with 1 μl of a PCR positive sample and PCR repeated to control for inhibition.

PCR amplification

PCR was performed for each of the eight human herpesviruses: herpes simplex virus type 1 & 2 (HSV-1 & HSV-2), varicella zoster virus (VZV), Epstein-Barr virus (EBV), cytomegalovirus (CMV), human herpes virus type 6 - 8 (HHV-6, HHV-7, HHV-8). DNA was amplified from 2 μl of extracted DNA in a final volume of 21 μl. The reaction mixture contained 0.5 U Amplitaq Gold™ (PE Applied Biosystems,), 2 μl $MgCl_2$ 25 mmol/l, 2 μl dNTP 500 mmol/l, 2 μl PCR Buffer II (PE Applied Biosystems) and 20 pmol of each primer (the primers were synthesized by DNA Technology ApS, Aarhus, Denmark). A negative control was run following each fifth sample minimizing the risk of false-positive reactions due to contamination, and nine samples were flanked by positive controls of the DNA concerned. The primer sequences and the references of published studies, in which the primer sequences have been used, are given in Table 1.

The amplification reaction was done in a GeneAmp™, PCR System 9700 thermal cycler (PE Applied Biosystems). In all PCR assays initial denaturation were performed with "Hot start" 10 min at 95 °C and followed by an amplification programme for the different herpesvirus types as shown in Table 1.

For herpes simplex virus type 1 & 2 a primer set common for HSV-1 & HSV-2 (Kimberlin et al., 1996) was run and positive samples were subsequently assayed with specific primers for HSV-1 and HSV-2 (Read et al., 1997)

A 10 μl aliquot of the PCR product was analyzed by electrophoresis in a 2% agarose gel containing 0.5 μg/ml ethidium bromi-

Table 1. Primer sequences and amplification programmes.

Virus	Primer sequence	Amplification programme: Denaturation	Annealing	Extension	Number of cycles	Size of PCR product
HSV-1 &	5: [illegible]	30 s at 95°C	[illegible]	30 s at 72°C	40	[illegible]
HSV-2 [21]	3: [illegible]			7 min at 72°C		
HSV-1	5: [illegible]	30 s at 95°C	30 s at [illegible]	[illegible]	35	[illegible]
[33]	3: [illegible]			7 min at 72°C		
HSV-2	5: [illegible]	30 s at 95°C	20 s at 55°C	[illegible] at 72°C	[illegible]	[illegible]
[33]	3: [illegible]			7 min at 72°C		
VZV	5: [illegible]	[illegible] at 95°C	[illegible]	[illegible] at 72°C	40	[illegible]
[illegible]	3: [illegible]			10 min at 72°C		
EBV	5: [illegible]	[illegible] at 95°C	[illegible]	[illegible] at 72°C	[illegible]	[illegible]
[illegible]	3: [illegible]			7 min at 72°C		
CMV	5: [illegible]	[illegible] at 95°C	[illegible] at 65°C	[illegible] at 72°C	[illegible]	[illegible]
[15]	3: [illegible]			7 min at 72°C		
HHV-6	5: [illegible]	[illegible]	[illegible]	[illegible] at 72°C	[illegible]	[illegible]
[illegible]	3: [illegible]			10 min at 72°C		
HHV-7	5: [illegible]	[illegible] at 95°C	[illegible] at 57°C	[illegible]	[illegible]	[illegible]
[illegible]	3: [illegible]			7 min at 72°C		
HHV-8	5: [illegible]	[illegible] at 95°C	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	3: [illegible]			[illegible] at 72°C		

*: Final extension. § Plus 10 s per cycle. ¶Plus 5 s per cycle.

de and visualized under UV light. Assays with suboptimal sensitivity of the positive controls were repeated. At all PCR positive samples, DNA re-extraction was performed on another aliquot of the original sinus aspirate, to exclude cross-contamination. If consecutive samples in the PCR run were positive, they were repeated in separate assays. DNA extraction, mixing of PCR reactions and amplification were done in three separate laboratories.

Determination of the detection limits of the PCR assays

Bands were excised from the agarose gel and the PCR products purified according to the QIAquick Gel Extraction Kit (QIAGEN Gmbh, Hilden, Germany). The PCR products were cloned by the use of pGEM®-T Vector System II (Promega Corporation, Madison WI, USA) according to the manufactures description. Essentially, the PCR products were ligated into the pGEM®-T Vector and transformed into JM109 competent cells (Promega Corporation). Plasmids were purified by QIAprep Spin Miniprep Kit Protocol (QIAGEN Gmbh) and the amount of plasmid DNA measured on a GeneQuant *pro* RNA/DNA Calculator (Pharmacia Biotech (Biochrom) Ltd., Cambridge, UK). The number of plasmids was calculated and from dilution series the detection limits of the PCR assays were estimated. The detection limits of the nine PCR assays were < 10 genome copies.

Determination of the serologic antibody response against EBV, CMV, and HSV-1 was performed at the Department of Clinical Microbiology, Aarhus University Hospital, Aarhus. Antibody test of HHV-8 was not performed.

Statistical analysis

SPSS package for personal computers was used. Univariate analysis was calculated by using odds ratios and χ^2 test. Level of significance was 5%.

Ethics

The study was approved by the local ethical commitee.

RESULTS

Twenty-four sinus aspirates from HIV-infected patients and 51 sinus aspirates from immunocompetent patients were examined. No samples showed inhibition in the PCR but in one sinus aspirate from an immunocompetent patient GAPDH was not detectable and the sample was excluded.

The HIV-infected patients

Twenty-four HIV-infected patients with 24 evaluable episodes of sinusitis were consecutively included. Twenty (83.3%) were men and 4 women with a median age of 36 years (range 19-59). The median CD4 cell count was 185 x 10^6/l (range 10-570). The median length of time since the diagnosis of HIV infection was 8 years (range 2 month to 12 years). Fourteen (58.3%) of the patients met the criteria for the diagnosis of AIDS.

By PCR EBV was detected in 3 cases, HHV-8 and CMV in 2 cases each, and HSV-1 in one case (Table 2). *Haemophilus influenzae* and *Streptococcus pneumoniae* were simultaneously demonstrated in 5 of these patients. Among the remaining 16 patients, *Streptococcus pneumoniae* were found in 3 cases, *Mycobacterium avium* in 2 cases, and in 11 patients no bacteriologic or other etiologic agents were found.

Table 2. Herpesvirus type 1-8 detected in sinus aspirates of HIV-positive/HIV-negative patients with sinusitus.

Virus	HIV positive patients (n=24)	HIV negative patients (n=51)
HSV-1	1	0
HSV-2	0	0
VZV	0	0
EBV	3	0
CMV	2	0
HHV-6	0	1
HHV-7	0	0
HHV-8	2	0

By the serological antibody test, none of the 6 patients with respectively, HSV-1, EBV, and CMV DNA in the sinus aspirates, showed sign of having a primary infection with the herpesvirus concerned. They all had antibody responses consistent with having had a previous infection.

All of the eight patients with herpesvirus DNA in the sinus aspirate had morphological changes corresponding to acute sinusitis (Tarp et al., 2000). They all had symptoms consistent with sinusitis and there was no difference in the duration of symptoms between the group with and without herpesvirus. There was no association between previous events of sinusitis, suffering from allergy and/or asthma, suffering from cold more than twice annually, and having herpesvirus DNA in the aspirate.

The eight patients all had CD4/CD8 ratios < 0.5, but the prevalence of herpesvirus DNA was not higher in the group of patients with CD4 counts < 200 x 10^6/l, in patients with known HIV infection ≥ 5 years, or in patients with the AIDS diagnosis. Five of the 8 patients with demonstrable herpesvirus DNA were in antiretroviral treatment and 5 received prophylaxis of *Pneumocystis carinii* pneumonia.

One of the patients with CMV had the diagnosis of Kaposi's sarcoma. The 2 patients with demonstrable HHV-8 had no clinical signs of Kaposi's sarcoma and they have not developed this malignancy during the following 3 years of follow-up. Both patients originated from Denmark. In one of the patients with EBV, a high-grade malignant Non-Hodgkin's Lymphoma (NHL) of B-cell origin (Burkitt-like type) was diagnosed in the left sphenoid sinus.

The immunocompetent patients

Fifty patients with 50 evaluable episodes of sinusitis were consecutively included. Sixteen (32%) were men and 34 women with a median age of 41 years (range 21-80).

HHV-6 was detected in the sinus aspirate from one of these patients (Table 2). In the remaining 49 immunocompetent patients none of the eight human herpesviruses was detected.

Thus, the odds of detecting herpesvirus DNA in sinus aspirates from HIV-infected patients is significantly higher than in aspirates from immunocompetent patients (odds ratio = 24.5 (2.8-211.2)).

DISCUSSION

Most of the human herpesviruses cause common childhood infections which persist in the host following primary infection and have the potential to cause disease upon reactivation, particularly in the immunocompromised host (Drew, 1992; Chan et al., 1997).

We found herpesvirus in 33% of the sinus aspirates from HIV-infected patients but in only 2% of the aspirates from immunocompetent patients. Different viruses other than herpesvirus have previously been demonstrated in 15% of sinus aspirates from immunocompetent patients with sinusitis (Malov and Creticos, 1989; Gwaltney et al., 1992). To the authors' knowledge, this is the first study investigating sinus aspirates for all of the eight human herpesviruses.

All of the eight patients with demonstrable HSV-1, EBV, CMV and HHV-8 DNA had CD4/CD8 ratios < 0.5. This corresponds with other studies, in which especially CMV and HHV-8 have been shown to occur with higher frequencies in severe immunocompromised patients, namely patients with low CD4 counts or with the diagnosis of AIDS, while other herpesviruses like HHV-6 and HHV-7 are mainly seen in patients with high CD4 counts or in non-HIV-infected (Fabio et al., 1997; Lucht et al., 1998). According to this we demonstrated HHV-6 DNA in the sinus aspirate from one of the immunocompetent individuals, but in none of the HIV-infected. The higher prevalence of HHV-6 and HHV-7 in immunocompetent individuals may be explained by the fact, that the CD4-cells are the main site of active viral replication of these viruses (Chan et al., 1997).

HSV-1 was found in the aspirate from one patient, who did not have any clinical signs of herpetic eruption. The presence of HSV-1 in sinus aspirates has not previously been described, but HSV-1 has been found in BAL fluids from immunocompromised patients (40% was AIDS patients) and in BAL fluids from patients with complicated respiratory tract infections (Prellner et al., 1992; Connolly et al., 1994). The role of HSV-1 DNA recovery in BAL fluids or sinus aspirates is still unclear as it is not possible to distinguish between active disease, oropharyngeal contamination, or asymptomatic shedding of the virus.

EBV was demonstrated in 3 (12.5%) of the aspirates. In one of the patients, the presence of EBV in the sinus aspirate was related to a high-grade malignant NHL of B cell origin (Burkitt-like type). The majority of AIDS-associated NHLs are B-cell neoplasms (Ioachim et al., 1991) probably induced by a carcinogenic potential of EBV (List et al., 1987; Pomilla et al., 1995; Blackbourn et al., 1998). NHL occurs 25-60 times more frequently in HIV-infected individuals than in the general population, but NHL involving the paranasal sinuses is rare in HIV-infected patients with only 20 cases, including our present case, identified in English-language articles (Del Forno et al., 1998). Most patients with HIV-associated NHL have serological evidence of EBV infection (Ziegler et al., 1982; Katz et al., 1992). A defect in T cell immunity to EBV has been documented for HIV-infected individual, who also have abnormally high numbers of circulating EBV-infected B-cells (Birx et al., 1986).

CMV is a major problem in HIV-infected individuals. More than 90% of patients with AIDS will develop CMV infection during the course of their illness (Drew, 1992). CMV has been found in saliva (Lucht et al., 1998) and in several studies CMV has been detected in BAL fluids from HIV-infected patients with pulmonary symptoms (Bower et al., 1990; Miles et al., 1990; Østergaard et al., 1995). From these studies it has been concluded that CMV does not participate in the pathogenesis of the pneumonia. In a few studies, however, CMV has been shown to be the etiologic agent of pneumonia or sinusitis in HIV-infected patients (Heurlin et al., 1991; Marks et al., 1996; Yoskovitch and Cantrell, 1998).

Since 1994 more studies have implicated HHV-8 as the etiologic agent of Kaposi's sarcoma (KS) (Chang et al., 1994; Moore and Chang, 1995). Detection of HHV-8 in BAL fluids from patients with pulmonary KS has been shown to be highly specific (95%) with a relatively high sensitivity (Benfield et al., 1997). In a prospective study of 100 BAL fluids Tamm et al., (1998) did only find one patient without clinical evidence of KS being HHV-8 DNA positive. This patient came from a region in central Africa with endemic occuring HHV-8 infection and KS. We found HHV-8 in the sinus aspirates of two Danish, heterosexual HIV-infected patients. These patients have been followed for 3 years after the sinus puncture and they have not developed AIDS or clinical KS. It can therefore be speculated that HHV-8 DNA may be detected prior to clinically visible KS or that the detection of HHV-8 in some patients is a manifestation of reactivation, like that of the other herpesviruses. However, it is of importance that both of these patients have been treated effectively with antiretroviral agents.

HHV-7 and VZV were not demonstrated, either in the HIV-infected or the immunocompetent patients. HHV-7 has never been demonstrated in sinus aspirates or BAL fluids and VZV has only once been found in a BAL fluid from an HIV-1 infected adult patient with the diagnosis of secondary varicella pneumonia (Fraisse et al., 1998).

In conclusion, the human herpesviruses HSV-1, EBV, CMV, and HHV-8 are frequently found in sinus aspirates from HIV-infected patients with sinusitis, whereas in immunocompetent individuals, these viruses do not seem to be related to clinical signs of sinusitis. The cause of these observed discrepancies is still unclear, but may be due to the uncontrolled reactivation of the herpesviruses, which occur in immunocompromised individuals. It remains to be established whether the herpesviruses play a pathogenic role in the development of sinusitis in HIV-infected patients.

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