Lymphotropic herpes virus (EBV, HHV-6, HHV-8) DNA sequences in HIV negative Castleman's disease

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Abstract

Aim—To evaluate the possible involvement of lymphotropic herpes viruses in Castleman's disease.

Methods—Archival formalin fixed, paraffin wax embedded biopsy specimens from 16 HIV negative patients (11 with localised and five of multicentric disease) were studied. Epstein-Barr virus (EBV), human herpes virus-6 (HHV-6) and human herpes virus-8 (HHV-8) DNA was detected using PCR. PCR was also used to characterise the EBV genomes and the clonal status of the lesions.

Results—EBV sequences were identified in nine (56%) cases. The main EBV genotype detected was type 1. Two (12%) cases were positive for both HHV-6 and EBV sequences. HHV-8 sequences were detected in one case of localised Castleman's disease, the sequence of which differed from that of the HHV-8 prototype. No clonal immunoglobulin gene rearrangements were found.

Conclusions—EBV DNA was detected in a substantial proportion of cases, suggesting that it may have a role in the pathogenesis of Castleman's disease, unlike HHV-6 which was detected rarely. This is the first report of HHV-8 specific sequences in the localised form of the disease.

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Castleman's disease, also commonly referred to as angiofollicular or giant lymph node hyperplasia, is a group of morphologically and clinically heterogeneous lymphoproliferative disorders of unknown aetiology. Two histological types, hyaline vascular and plasma cell type, each with different clinical characteristics, have been recognised. These histological types coexist occasionally (mixed type).¹ The much more common localised form occurs as a solitary mass, is asymptomatic and is usually curable surgically. The less common multicentric form (MCD) has been reported as an atypical lymphoproliferative disorder with more generalised lymph node involvement and an aggressive clinical course. Two types of recurrent malignancies, lymphoma and Kaposi

sarcoma, have been documented to occur in 18% and 13% of patients with MCD, respectively.²

Previous studies of Castleman's disease have suggested that viruses, such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV) may be implicated in its aetiology.³ Recently, Kaposi sarcoma associated herpes virus (KSHV) or HHV-8⁴ has been identified in a large number of pathological lymph nodes as well as in the peripheral blood mononuclear cells (PBMCs) of HIV positive and negative patients with MCD, suggesting that HHV-8 also may play a role in the pathogenesis of Castleman's disease,^{5,6} but apparently not in the localised form.⁷

Methods

Sixteen cases of Castleman's disease were retrieved from the archives of the Centre for Experimental Haematology, Modena, Italy. The diagnosis in all cases had been confirmed both clinically and histologically. Eleven patients presented with the localised and five with the MCD form of the disease. The histological features in the involved sites were characterised by hyalinised and hypervascular germinal centres (hyaline vascular type) in 11 cases, while five cases presented with the mixed type. All patients were HIV negative as determined by enzyme linked immunosorbent assay (table 1).

PCR was carried out on 10 µl of crude extracts from single 5 µm formalin fixed, paraffin wax sections prepared as described by Luppi *et al.*⁸

The primer pair used in the HHV-6 PCR assay was derived from the 8.7 kilobase (kb) HHV-6 sequence inserted into the pZVH14 plasmid. These primers amplify a 186 base pair (bp) segment.⁹ To confirm the viral origin of the amplified DNA template, one fifth of the PCR product was subjected to electrophoresis on a 1.6% agarose gel and transferred to nylon membranes (Biotechnology Systems, NEN Research Products). Filters were hybridised with an internal oligonucleotide probe and labelled with γ -³²P-ATP, as described by Torelli *et al.*⁹ DNA extracted from HHV-6 infected and uninfected HSB-2 cell lines, respectively, served as positive and negative controls.¹⁰

Detection of HHV-8 using PCR resulted in the amplification of a 233 nucleotide segment derived from the KS330₂₃₃ sequence, as described by Ambroziak *et al.*¹¹ The results

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Table 1 Clinical and molecular features of the patients studied

Case	Age/Sex	HIV serology	Histology	Form of Castleman's disease	Systemic symptoms	Ig clonality	EBV (type 1/2)	HHV-6	HHV-8
C1	41/F	-	HV	Localised	-	GL	-	-	-
C2	45/M	-	HV	Localised	-	GL	1	-	-
C3	37/M	-	HV	Localised	-	GL	1	-	-
C4	30/M	-	HV	Localised	-	GL	2	-	-
C5	46/F	-	HV	Localised	-	GL	1	-	-
C6	52/F	-	HV	Localised	-	GL	-	-	-
C7	35/M	-	HV	Localised	-	GL	1	-	-
C8	45/M	-	HV	Localised	-	GL	-	-	-
C9	16/F	-	HV	Localised	-	GL	1	-	+
C10	61/F	-	HV	Localised	-	GL	+	-	-
C11	21/M	-	HV	Localised	-	GL	-	-	-
C12	46/M	-	HV/PL	Multicentric	+	GL	-	-	-
C13	38/M	-	HV/PL	Multicentric	+	GL	+	+	-
C14	42/M	-	HV/PL	Multicentric	+	GL	+	+	-
C15	57/M	-	HV/PL	Multicentric	+	GL	-	-	-
C16	53/M	-	HV/PL	Multicentric	+	GL	-	-	-

HV = hyaline vascular type; HV/PL = mixed type; GL = germline configuration.

were evaluated after 35 amplification cycles and hybridisation with an internal probe. The positive control comprised the crude extract obtained from a HHV-8 positive biopsy specimen from a patient with AIDS and Kaposi sarcoma.¹² The analysis of MCD cases for HHV-8 has been reported in part by Luppi *et al.*¹³

The specific primers used in the EBV PCR assay were derived from the Bam W region of the EBV genome and amplify a 122 bp segment.⁸ To define the subtypes of EBV, EBNA2 gene 1 and gene 2 primers were used as the outer primers in a nested reaction with subtype specific primers in the second step, as reported by Borisch *et al.*¹⁴ Probes were applied each time to confirm the results. The positive controls comprised DNA extracted from the B95.8 infected cell line for subtype 1 (product size 497 bp), and from the AG 876 infected cell line for subtype 2 (product size 150 bp).

All standard recommended procedures were performed in order to avoid false positive results. Primers for the DQ gene were used as a positive control of amplification.⁸

The PCR product of the HHV-8 positive Castleman's disease case was subjected to direct sequence analysis by cycling sequencing,¹⁵ a variation of the classic dideoxy chain termination technique.¹⁶ Briefly, DNA bands were cut from a 1% low-melting agarose gel and purified further using a commercial kit (PCR preps DNA Purification System, Promega, Madison, Wisconsin, USA). The cycle sequencing reaction was performed in a total

volume of 8 µl containing 50 mM KCl, 50 mM Tris-HCl (pH 8.3) 2.5 mM MgCl₂, 10 µM each of dATP, dTTP, and dCTP, 20 µM 7-deaza-dGTP, 0.4 pmol ³²P labelled primer, 0.2 units Taq polymerase, 100 fmol purified PCR product, and 60 µM ddGTP, 400 µM ddATP, 600 µM ddTTP, or 200 µM ddCTP. PCR conditions were as follows: one minute at 95°C, one minute at 55°C and one minute at 72°C for 20 cycles. The reaction was stopped by the addition of 4 µl 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.02% xylene cyanol. The sequence product was analysed on a 7 M urea, 6% polyacrylamide sequencing gel. The two strands and two independent PCR products were sequenced to exclude mismatches.

Molecular analysis of B cell clonality was possible in all 16 cases.¹⁷ The immunoglobulin gene rearrangements were also investigated using Southern blot analysis of lymph node DNA from eight cases. DNA was extracted using a modification of the technique described by Enrietto *et al.*¹⁸ DNA samples were digested with EcoRI, HindIII and BamHI, transferred to nylon membranes and hybridised with a probe corresponding to the immunoglobulin heavy chain genes, as described elsewhere.⁹

Results

Nine (56%) of the 16 samples were EBV positive (seven of localised and two of MCD type) (table 1). On characterisation of the genotype of the six cases for which EBV positive lymph nodes were available, type 1 was detected in five and type 2 in one (table 1; fig 1).

HHV-6 sequences were detected in two (12%) of the 16 cases analysed, both of which were of MCD type (table 1). These two cases were also EBV positive.

HHV-8 sequences were detected in one case of localised Castleman's disease (table 1; fig 2). The PCR product from this positive sample was sequenced and compared with the prototypic sequence originally derived from a genomic library made from a Kaposi sarcoma lesion (fig 3).⁴ The PCR product had five base substitutions at positions 46, 47, 69, 146, and 153). Base changes at positions 46 and 47 encode a proline to isoleucine substitution and

Figure 1 Ethidium bromide stained gel showing EBV subtype specific products. Lanes C2, C3, C5, C7, and C9, EBV subtype 1; lane C4, EBV subtype 2; NC, negative control; M, ϕ X 174, HaeIII molecular weight marker; B95.8, positive control for subtype 1; AG876, positive control for subtype 2.

Figure 2 Southern blot hybridisation of the HHV-8 PCR product (KS330₂₃₃) from different samples (lymph node, serum, PMBCs, faeces, urine, and saliva) of a patient with Castleman's disease (case 9) with isotope labelled KS330₂₃₃ sequence specific oligoprobe. KS, Kaposi sarcoma (positive control); NC, negative control.

the base change at position 146 encodes an aspartate to glycine substitution. The remaining base changes do not result in amino acid substitutions. Peripheral blood mononuclear cells collected from this patient one year after lymph node enlargement were positive for HHV-8 specific sequences (fig 2). Unexpectedly, HHV-8 sequences were also detected in the saliva sample taken at the same time, while samples of serum, faeces and urine were negative (fig 2). The PMBCs also harboured type 1 EBV sequences.

Clonal immunoglobulin gene rearrangements were not detected in any of the 16 cases studied. The absence of B cell clonality was confirmed by Southern blot analysis of the DNA extracted from the frozen lymph nodes of eight patients (data not shown).

	10	20	30	40
HHV-8	AGCCGAAAGG	ATTCCACCAT	TGTGCTCGAA	TCCAACGGAT
Case 9	AGCCGAAAGG	ATTCCACCAT	TGTGCTCGAA	TCCAACGGAT
	50	60	70	80
HHV-8	TTGACCCCGT	GTTCCCCATG	GTCGTGCCGC	AGCAACTGGG
Case 9	TTGAC <u>AT</u> CGT	GTTCCCCATG	GTCGTGCC <u>TC</u>	AGCAACTGGG
	90	100	110	120
HHV-8	GCACGCTATT	CTGCAGCAGC	TGTTGGTGTA	CCACATCTAC
Case 9	GCACGCTATT	CTGCAGCAGC	TGTTGGTGTA	CCACATCTAC
	130	140	150	160
HHV-8	TCCAAAATAT	CGGCCGGGGC	CCCGGATGAT	GTAAATATGG
Case 9	TCCAAAATAT	CGGCCGGGGC	CCCGG <u>G</u> TGAT	GT <u>CA</u> ATATGG
	170	180	190	200
HHV-8	CGGAACTTGA	TCTATATACC	ACCAATGTGT	CATTATGGG
Case 9	CGGAACTTGA	TCTATATACC	ACCAATGTGT	CATTATGGG
	210	220	220	
HHV-8	GCGCACATAT	CGTCTGGACG	TAGACAACAC	GGA
Case 9	GCGCACATAT	CGTCTGGACG	TAGACAACAC	GGA

Figure 3 Sequence of the KS330₂₃₃ PCR product produced on amplifying DNA from the HHV-8 positive patient with localised Castleman's disease (case 9). The PCR product differed from the prototype HHV-8 sequence at positions 46, 47, 69, 146, and 153 (substitutions are bold and underlined).

Discussion

The presence of EBV specific sequences in a substantial percentage of the cases of Castleman's disease (56%) examined in this study is in striking contrast to the previously reported low frequency of EBV DNA in hyperplastic and non-hyperplastic lymph nodes.⁸ Hanson *et al*⁸, using Southern blot analysis, detected EBV sequences in two of four cases of MCD, and in none of four cases of localised disease. The use of a much more sensitive technique in a larger series of patients may explain the different results obtained. Murray *et al*,¹⁹ using in situ hybridisation, detected non-coding EBV early RNAs (EBERs) in five of 12 cases of localised Castleman's disease. Our data also suggest that the frequency of EBV is higher in the localised form of Castleman's disease than in normal carriers.⁸ This finding is difficult to explain. The localised form of Castleman's disease is not characterised by immune dysfunction, the most obvious disorder underlying primary viral infection or reactivation. In fact, detection of HHV-6 DNA, commonly found patients with severe immunosuppression, is rare in Castleman's disease, even using a method as sensitive as PCR. However, we are well aware of the fact that the detection of viral sequences by PCR in a lesion is not proof, per se, that the virus is involved in its pathogenesis. Thus, the present findings are certainly not sufficient to determine whether EBV is involved in the pathogenesis of the localised form of Castleman's disease.

Lymphomas in immunocompetent patients are generally associated with infection with EBV type 1. In immunocompromised patients, however, infection with EBV type 2 predominates.²⁰ Thus, the frequency of one EBV type in lymphoproliferative disorders may provide some information about the immune status of the patients affected. EBV type 1 was detected in the majority of cases of localised Castleman's disease, suggesting that immune dysfunction is not the underlying cause of this condition.

In MCD, immunoregulatory abnormalities may contribute to excessive lymphoproliferation and it has been hypothesised that a virus may act as a cofactor, perhaps as a stimulus for cytokine production.^{2,3} In this regard, the presence of both the EBV and HHV-6 sequences in our cases of MCD is further evidence in support of underlying immune dysregulation. The association between MCD and Kaposi sarcoma, which occurs frequently in immunodeficiency states, and the frequency of opportunistic infections also lends weight to the above hypothesis. Cells latently infected with EBV and other herpes viruses may escape immune surveillance when T cell immunity is impaired. Thus, it is possible that EBV and HHV-6 DNA detected in patients with MCD may not be involved in the pathogenesis of the disease, but merely represents an associated finding related to defects in normal cellular control, which permits either viral replication or simply an increase of the number of the latently infected cells. The implication of co-infection with HHV-6 and EBV in two

patients with MCD remains unclear, and a possible interaction between the two herpes viruses is only matter of speculation.

Soulier *et al*⁶ identified HHV-8 sequences in excised lymph nodes of 14 patients with HIV associated MCD and in seven of 17 HIV negative patients with MCD. Moreover, Gessain *et al*⁷ documented the presence of HHV-8 sequences in three of four HIV positive and in one of six HIV negative patients with Castleman's disease. In our series, all five cases of HIV negative MCD were HHV-8 negative. Our findings, along with those of other authors, suggest that HHV-8 sequences are present only in a minority of HIV negative patients with Castleman's disease.

The most unexpected finding in the present study was the detection of HHV-8 DNA in a HIV negative patient with localised Castleman's disease. The viral subtype was characterised and was found to differ from the HHV-8 prototype, giving a sequence similar to that found in two patients with benign lymphadenopathy.¹³ The detection of HHV-8 sequences in the saliva sample of this patient is also a previously unreported finding, which raises the possibility that saliva may be a possible route of transmission of this virus. At the time of the study both the clinical status (absence of fever and constitutional symptoms) and the histological picture (hyaline vascular type) were consistent with the diagnosis of localised Castleman's disease. The spectrum of conditions associated with HHV-8 infection includes AIDS and non-AIDS associated Kaposi sarcoma,^{4 11 12 21} body-cavity-based lymphomas in HIV negative and positive patients,²² primary cerebral B-cell lymphoma in a HIV negative patient after prolonged treatment with steroids,²³ AIDS and non-AIDS associated MCD,⁵⁻⁷ and a benign lymphadenopathy characterised by giant germinal centre hyperplasia and increased vascularity in HIV negative patients.¹³ The detection of HHV-8 DNA in one case of localised Castleman's disease is the first evidence of this novel herpes virus in a benign lymphoproliferative disorder. Although it is accepted that the localised form of Castleman's disease is characterised by a benign clinical course, it is impossible to rule out the occurrence of malignant lymphoma in patients with Castleman's disease several years after the initial diagnosis.²⁴ It has been reported that the presence of HHV-8 DNA in the circulating PMBCs of HIV infected subjects might reflect a propensity to develop Kaposi sarcoma.^{25 26} Long term infection with HHV-8 may result in the transformation of a non-neoplastic condition, such as Castleman's disease, into a more malignant variant. Further studies are required to define the possible role of HHV-8 in the natural history of Castleman's disease.

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