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Case report

The evidence of human herpesvirus 6 infection in the lymph nodes of Hodgkin's disease

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Abstract. Human herpesvirus 6 (HHV–6), the causative agent of exanthem subitum, has been implicated in other diseases. Recently HHV–6-specific sequences have been detected by Southern blot analysis and polymerase chain reaction in the lymph nodes of three patients with Hodgkin's disease. The pathological localization of HHV–6, however, is still unknown. In order to study the pathological role of HHV–6 in Hodgkin's disease, we investigated, by immunohistochemical and molecular methods, two lymph node biopsies taken from a 7-year-old boy with Hodgkin's disease during the course of disease evolution. Although the histopathological findings of the first biopsy differed from those of the second, HHV–6 antigens and sequences could be detected in both lymph nodes by immunohistochemistry and in situ hybridization, respectively. HHV–6 was localized in macrophages, predominantly in lymphoid follicles, but not in Reed–Sternberg cells. Antibody titres to HHV–6 were consistent with reactivation of latency. Neither cytomegalovirus nor Epstein–Barr virus was present. Our data suggest a role for HHV–6 in the pathogenesis of Hodgkin's disease.

Key words: Human herpesvirus 6 – Epstein–Barr virus – Hodgkin's disease – In situ hybridization – Immunohistochemistry

Introduction

Human herpesvirus 6 (HHV–6), originally isolated from patients with lymphoproliferative disorders (Ablashi et al. 1987), was identified in 1988 as the causative agent of exanthem subitum, an acute self-limiting febrile disease of infants (Yamanishi et al. 1988). Primary HHV–6 infection in adults has been associated with various clinical outcomes, such as subacute necrotizing lymphadenitis (Eizuru et al. 1989), chronic fatigue syndrome (Krueger et al. 1987; Komaroff et al. 1988), and hepatitis (Dubedat and Kappagoda 1989; Irving and Cumingham 1990; Tajiri et al. 1990). Like other herpesviruses, HHV–6 persists in a latent state after primary infection, probably for the life-time of the host (Kondo et al. 1991). Presently the clinical manifestations of HHV–6 reactivation are unknown.

Hodgkin's disease (HD) is a neoplastic disorder characterized histopathologically by the presence of Reed–Sternberg cells and Hodgkin cells (Lukes and Butler 1966). The specific histological appearance of HD is variable and four subtypes have been identified (Lukes 1971). Because of this histological heterogeneity and the biphasic onset of disease (one during adolescence and the other among the elderly) (Gutensohn and Cole 1980), the aetiology of HD may not be homogeneous. Previous epidemiological studies suggest that some viruses may contribute to the pathogenesis of HD (Gutensohn and Cole 1980). Several recently published reports indicate a relationship between HD and Epstein–Barr virus (EBV) infection (Lawrence et al. 1989; Wu et al. 1990; Jarrett et al. 1991; Pallesen et al. 1991). Also, HHV–6 sequences have been detected in the lymph nodes of three HD patients by polymerase chain reaction (PCR) and Southern blot analysis (Torelli et al. 1991). We present here a patient with HD in whom HHV–6 specific antigens and nucleic acid sequences were detected in the lymph nodes. This is the first report demonstrating the histopathological localization of HHV–6 in the lymph nodes of HD.

Case report

The patient, a 7-year-old boy, was admitted to Kochi Medical School Hospital on March 13, 1991, complaining of remittent fever

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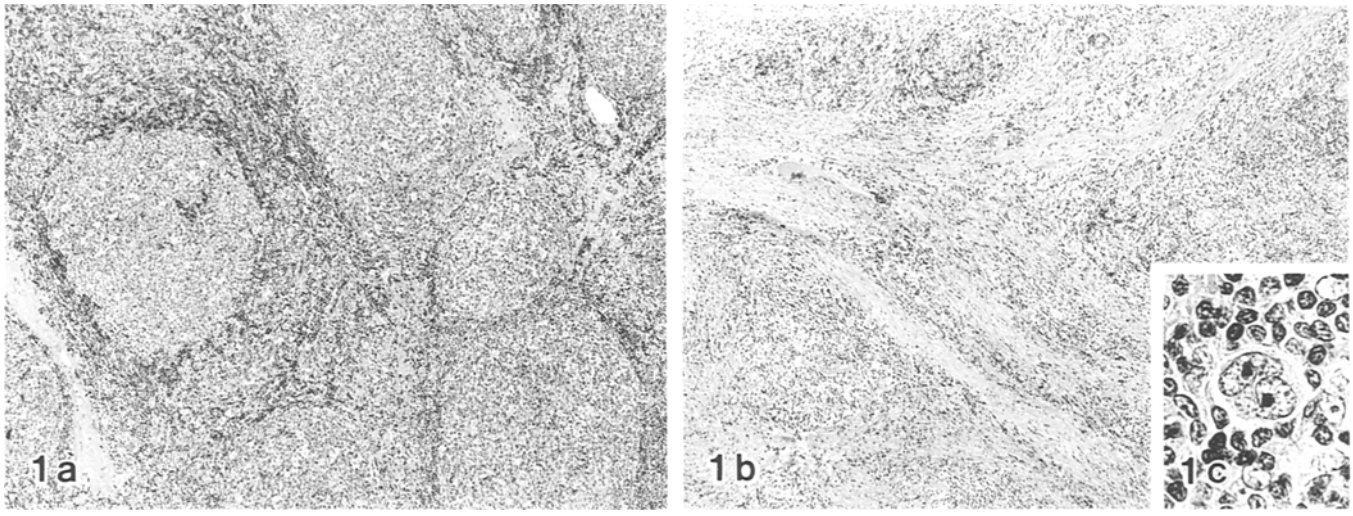

Fig. 1. Histopathology of axillary lymph node taken in April 1991 revealing non-specific features consistent with reactive hyperplasia (**a**). Biopsy of the supraclavicular lymph node in August 1991 showed Reed–Sternberg cells (**c**) and fibrosis, consistent with a diagnosis of Hodgkin's disease, nodular sclerosis/lymphocytic depletion subtype, according to Lukes' classification (**b**). a, b: × 40 c: × 400

up to 38° C for 7 days, and lymphadenopathy. He had a past history of exanthem subitum in infancy, and his grandmother had died of malignant lymphoma. The initial physical examination revealed swelling of the left axillary (3 cm in diameter) and supraclavicular lymph nodes (1 cm), hard and rubbery in consistency without tenderness or local heat. An elevated level of C-reactive protein (20.8 mg/dl) and high erythrocyte sedimentation rate (105 mm/h) suggested inflammation and hypergammaglobulinaemia was present. Biopsy of an axillary lymph node, performed on April 4, showed non-specific pathological changes consistent with a diagnosis of reactive hyperplasia (Fig. 1a). Despite sequential treatment with antibiotics (amoxycillin, minocycline, clindamycin, fosfomycin), 20 mg/kg/day of acetyl salicylic acid, and 40 mg/day of prednisolone, his condition remained unimproved, with persistent fever and enlarging supraclavicular lymphadenopathy, and he developed anaemia. On August 6, a second biopsy, of the supraclavicular lymph node, revealed HD of the nodular sclerosis/lymphocyte depletion (NS–LD) type (Fig. 1b, c). Gallium scintigram at the time of the second biopsy showed abnormal accumulations in the enlarged lymph nodes, which had not been present at the time of the first biopsy. The patient received combined chemotherapy, consisting of mechlorethamine, vincristine, predonisolone, procarbazine, doxorubicin, bleomycin, vinblastine, and dacarbazine resulting in the induction of a complete remission. The patient has remained in excellent health for the past 14 months, without evidence of relapse.

Materials and methods

Paraffin-embedded specimens of the two lymph node biopsies, obtained in April and August, were investigated for the presence of antigens and nucleic acids of HHV–6, human cytomegalovirus (HCMV), and EBV, since these herpes viruses might remain latent in peripheral blood and become reactivated in lymph nodes. A portion of the second lymph node biopsy, which was stored at −80° C, was subjected to virus isolation.

Paraffin-embedded, 4 μm thick sections were stained by the avidin-biotin complex (ABC) immunoperoxidase method (Sata et al. 1986), using monoclonal antibodies to HHV–6 recognizing glycoproteins (OHV1 and OHV3) (Okuno et al. 1990, 1992), and a rabbit antiserum against HCMV. Primary antibodies were incubated overnight at 4° C, and for colour development, a substrate, consisting of 0.02% 3,3-diaminobenzidine (Dohjin, Kumamoto, Japan) and 0.015% hydrogen peroxide, was employed.

Probes for the detection of herpes virus sequences consisted of a *Bam*HI fragment (6.9 kb) derived from the Z29 strain of HHV–6 (kindly supplied by Dr. Pellett P. Division of Viral and Rickettsial Disease, National Center for Infectious Diseases, Centers for Disease Control and Prevention), a mixture of *Eco*RI–B, D, F, I, K fragments (approximately 50 kb altogether) from the AD169 strain of HCMV (Greenaway et al. 1982), and *Bam*HI–W fragment (3.1 kb) of EBV (kindly supplied by Dr. Takada K. Yamaguchi University School of Medicine) (Arrand et al. 1981). All these were cloned DNA fragments inserted into plasmid. As verified by Southern blot analysis, the probes were specific for their respective herpes virus. The cloned herpes virus DNA and plasmid DNA, which was used as a negative control, were biotinylated by the random priming method (Sambrook et al. 1989).

In situ hybridization (ISH) was performed as described previously (Brigati et al. 1983) with minor modifications. Briefly, paraffin sections, mounted on slides coated with 3-aminopropyltriethoxysilane (Sigma, St. Louis, Mo., USA), were initially deparaffinized, treated with 10 μg/ml proteinase K (Boehringer Mannheim, FRG) for 30 min at 37° C, then fixed in 4% paraformaldehyde/0.05 M TRIS-HCl pH 7.4 and dehydrated. Thereafter each section was covered with a hybridization mixture consisting of 10 ng biotinylated probe, 20% formamide, 5 × sodium chloride, sodium citrate solution (SSC) (0.75 M sodium chloride, 75 mM sodium citrate), 50 mM HEPES pH 7.0, 40 μg/ml salmon sperm DNA, and 0.5 × Denhardt's solution, then denatured at 98° C for 10 min, cooled on ice and incubated at 42° C for 14 h. Following hybridization, sections were washed three times with 0.2 × SSC at 42° C, blocked with 2% skim milk, and the biotinylated probes were detected by streptavidin-alkaline phosphatase complex according to the manufacturer's instructions (In situ hybridization detection kit, Dakopatts, Carpinteria, Calif., USA).

To determine the localization of HHV–6 sequences and the types of target cells in lymph nodes, ISH and immunostaining were performed on the same section. Cell surface antigens were initially stained by the ABC method using monoclonal antibodies against human macrophage (HAM56, Dako, Carpinteria, Calif., USA; dilution 1/100) (Gown et al. 1986), human B cell (L26, Dako; dilution 1/200) (Smith et al. 1986), and human T cell (UCHL–1, Dako; dilution 1/200) (Ishii et al. 1986), followed by ISH for HHV–6. To block biotin residues, a solution of 0.005% biotin solution, prepared in 0.05 M TRIS-HCl pH 7.4, was applied to the sections for 1 h before hybridization (Wood and Warnke 1981).

Titres of anti-HHV–6 antibodies in sera collected at 1–4 month intervals from April 1991 to January 1992, were determined by the

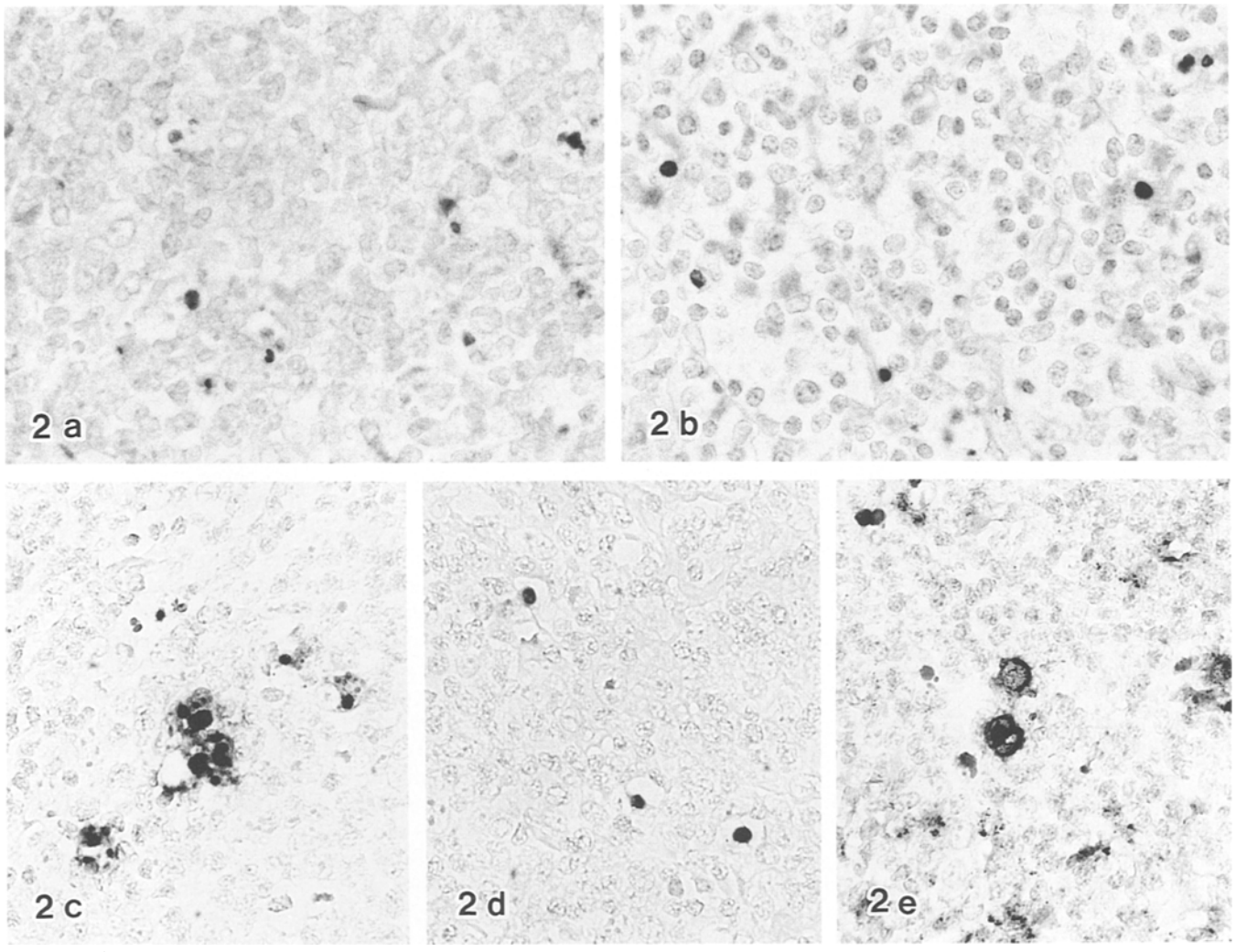

Fig. 2. Immunohistochemistry and in situ hybridization of first (**a, c, e**) and second (**b, d**) lymph node biopsies. Tissue sections from both biopsies revealed HHV–6-specific antigens by immunostaining using monoclonal antibodies against HHV–6 (OHV1 and OHV3), (**a, b**) and HHV–6-specific DNA sequences by in situ hybridization (**c, d**). HHV–6-positive cells were identified as macrophages as determined by a double-labelling technique which combines in situ hybridization for HHV–6-sequences with immunohistochemistry using a monoclonal antibody against human macrophages (**e**). Positive hybridization *(purple)* is present in the nucleus of the lower cell which is stained brown, indicating it is of macrophage lineage. By contrast, the cell, which exhibits cell surface staining but without nuclear hybridization, is considered to be as uninfected macrophage. ×400

indirect immunofluorescent antibody technique, as described previously (Kondo et al. 1990). Briefly, serial dilutions of each serum were incubated for 1 h at 37° C with acetone-fixed HHV–6 (Hashimoto strain), infected and uninfected HPB–ALL cells, spotted on 12-well slides. For the detection of IgM, serum was treated with *Streptococcus pyogenes*. Fluorescein isothiocyanate-conjugated $F(ab')_2$ antibody to human IgG or IgM (Tago, Burlingame, Calif., USA) was employed as the secondary antibody for 30 min at 37° C. The titres of IgG and IgM anti-HHV–6 antibodies were expressed as the reciprocal of the highest serum dilution yielding virus-specific fluorescence.

A homogenate of the lymph node biopsy of August 1991 was co-cultivated with cord blood lymphocytes for isolation of HHV–6 (Yamanishi et al. 1988). The cells were observed by microscopy for 8 weeks. Finally, evidence of HHV–6 infection was determined by indirect immunofluorescent method using monoclonal antibodies to HHV–6 (OHV1 and OHV3).

Results

Staining for OHV1 and OHV3 was seen in sections of both lymph nodes, in the cytoplasm and nuclei of macrophages located predominantly in follicles, and in some lymphocytes lying outside follicles. (Fig. 2a, b). Similarly, HHV–6 DNA sequences were localized by ISH in the nuclei of macrophages and in fragments of nuclei within their cytoplasm, and in lymphocytes (Fig. 2c, d). Comparatively many more cells bearing HHV–6-specific antigens and genomic sequences were detected in the first, than in the second, lymph node biopsy. Neither HHV–6 antigens nor sequences were found in 12 Reed–Sternberg cells and 45 Hodgkin cells in the second biopsy specimen. As verification of the lineage of HHV–6-infected cells, double–staining, combining ISH for HHV–6 sequences and immunostaining for macrophages with HAM56,

Table 1. Serum antibody titres to HHV–6 by immunofluorescent assay

Date of serum	Anti-HHV–6 antibody titres	
	IgG	IgM
April 4, 1991	40	<20
August 6	80	<20
September 6	20	<20
January 20, 1992	40	<20

revealed that macrophage harboured HHV–6 (Fig. 2e). Antigens and genomic sequences of HCMV and EBV could not be detected in either of the lymph node specimens. In addition, no hybridization was found with the labelled plasmid vector.

The titres of IgM antibody to HHV–6 in all sera were less than 20, while IgG antibody titres to HHV–6 ranged from 20 to 80 (Table 1).

Attempts to isolate HHV–6 from the second lymph node biopsy were unsuccessful. Cultures, which were maintained for 8 weeks, exhibited no evidence of HHV–6, as determined by the immunofluorescent antibody method using OHV1 and OHV3.

Discussion

This patient's initial clinical presentation and course was somewhat enigmatic. The absence of Reed–Sternberg cells in the first lymph node biopsy and the lack of abnormal accumulation in the enlarged lymph nodes in a gallium scintigram, together with the initial ineffectiveness of prednisolone, which is generally effective in HD (and became effective in this patient later) suggested a pre-neoplastic state at the time of his admission to hospital. However, HHV–6 specific antigens and genomic sequences were detected in both lymph nodes, suggesting active infection with HHV–6. As determined by double-labelling experiments using immunostaining for cell surface markers and ISH for HHV–6, we demonstrated that HHV–6 infected cells of macrophage lineage were present in the lymphoid follicles. Previously, in patients with necrotizing lymphadenitis, HHV–6 antigens have been found in the cortex and paracortex of lymph nodes (Kurata et al. 1990). Though the infected cell type in our patient was similar to that in patients with necrotizing lymphadenitis, the infected cells localized predominantly in the follicles rather than the cortex. Although Krueger et al. (1992) showed that HHV–6 could infect Hodgkin cells in vitro, neither nucleic acid sequences nor antigens of HHV–6 were found in Reed–Sternberg and Hodgkin cells in our case.

The patient had a past history of exanthem subitum during infancy. Serological studies revealed no IgM antibody to HHV–6 and HHV–6 specific IgG antibody titres remained relatively stable. Therefore, the demonstration of HHV–6 antigens and sequences of this patient probably reflects reactivation of latency rather than primary infection. The absence of significant alterations in antibody titres with time supports the notion that the viral infection was not systemic but was localized to the lymph nodes.

The active infection of HHV–6 found in both developing and tumour stages of lymph nodes of HD suggests some direct role of the virus in the pathogenesis of the disease or presence of factors reactivating latent virus. Although we failed to detect HHV–6 sequences in Reed–Sternberg cells, the possibility remains that the level of sensitivity of our ISH resulted in inability to detect HHV–6 in Reed–Sternberg cells. Alternatively, the oncogenic potential of HHV–6 DNA may be dissociated from maintenance of transformation (Razzaque 1990). The tropism of HHV–6 for CD4 positive mature T-lymphocytes (Takahashi et al. 1990) and subsequent viral replication may lead to cell death and the immune insufficiency that causes HD. Persistent active infection by HHV–6 can induce excessive lymphoid hyperplasia mimicking malignant transformation, the so-called atypical polyclonal lymphoproliferation (APL) (Krueger et al. 1988). The histological findings of initial lymph node biopsies may be implicated in APL, a condition of enhanced susceptibility to transformation resulting in HD. To investigate whether putative factors causing reactivation from latency were specific for HHV–6, we examined the biopsy specimens for EBV and HCMV, but neither was present in the lymph nodes. Thus, HHV–6 was the only herpes virus that proliferated selectively in the lymph nodes from this patient.

Torelli and his colleagues (1991) detected HHV–6 specific sequences in tumour tissues taken from 3 of 25 HD patients by PCR and Southern blot hybridization. The 3 HHV–6-positive cases shared some common features, such as their youth, the histopathological subtype (NS–LD) and the presence of pleural effusions or ascites. Interestingly, our patient also had some of these characteristics, but did not have significant effusions. Though we examined four more lymph node biopsies taken from adult patients with HD, who were all older and were not diagnosed as NS–LD type, there was no evidence suggesting the presence of HHV–6 specific antigens or nucleic acids in them (unpublished data). Based on the premise that the aetiology of HD is heterogeneous, HHV–6 may play a direct or indirect role in the pathogenesis of HD exhibiting the characteristics defined above.

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