CONCISE COMMUNICATIONS

Heterogeneity of Viral IL-6 Expression in HHV-8–Associated Diseases

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In order to characterize the expression of the viral interleukin-6 (vIL-6) homologue in various human herpesvirus 8 (HHV-8)–associated diseases, in situ hybridization and immunohistochemistry were applied to formalin-fixed specimens. These assays showed consistent expression of vIL-6 in primary effusion lymphomas and in a case of human immunodeficiency virus (HIV)–associated lymphadenopathy with a Castleman's disease–like appearance. In contrast, Kaposi's sarcoma specimens showed marked differences among specimens. In a consecutive series of specimens from the Johns Hopkins archives, vIL-6 expression was demonstrated in one of 13 cases. However, among 7 specimens selected from the AIDS Malignancy Bank because of their high levels of the T1.1 lytic transcript and virion production, vIL-6 expression was consistently demonstrated in infiltrating mononuclear cells and occasional spindle-shaped cells. Thus vIL-6 expression in clinical specimens correlates with other measures of the lytic viral cycle. Both assays generally give congruent results and are consistent with the possibility that vIL-6 expression plays a role in the pathogenesis of a variety of HHV-8–associated diseases.

Interleukin-6 (IL-6) has been implicated in the pathogenesis of a variety of proliferative disorders, including lymphoma, multiple myeloma, Kaposi's sarcoma (KS), and Castleman's disease [1–3]. The discovery of a homologue of IL-6 in human herpesvirus 8 (HHV-8) with 24.8% amino acid identity suggested the possibility that this viral cytokine might play a role in the pathogenesis of HHV-8–associated diseases [4, 5]. Similar to its human counterpart, viral IL-6 (vIL-6) promotes growth and proliferation of the mouse plasmacytoma B9 cell line [4]. Previous investigations have demonstrated vIL-6 expression in a primary effusion lymphoma, in a lymph node of a patient with KS, and in a case of human immunodeficiency virus (HIV)–negative Castleman's disease, but not in a series of KS lesions [5, 6]. Here we apply immunohistochemistry complemented by in situ hybridization to detect vIL-6 expression in a variety of lesions, including primary effusion lymphoma, HIV-associated lymphadenopathy with Castleman's disease–like appearance, and KS.

Materials and Methods

Cell lines and diseased tissue specimens. Formalin-fixed primary effusion lymphoma specimens were obtained from the pathology archives of Johns Hopkins Hospital, New York Hospital, and Nashville Veterans Administration Hospital. A hyperplastic lymph node with Castleman's disease–like features was obtained from the Johns Hopkins Hospital. Formalin-fixed, paraffin-embedded archival biopsy specimens of KS tumors were obtained from Johns Hopkins dermatology archives and the National Cancer Institute–funded AIDS Malignancy Bank. GenBank accession numbers for HHV-8 subtype variants at open-reading frame (ORF) K1 include the following: AF133038(A1), AF133039(A4), AF133040(B), and AF133042(C3).

In situ hybridization and immunocytochemistry. In situ hybridization for vIL-6 was performed, as described elsewhere [7]. The full-length vIL-6 coding sequence was cloned into the pSK (bluescript) vector (Stratagene, La Jolla, CA) between the T7 and T3 RNA polymerase promoters. The T7 RNA polymerase was used to transcribe the antisense strands. Plasmids were linearized and

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transcribed *in vitro* by use of digoxigenin-UTP (Boehringer Mannheim, Indianapolis, IN) to generate sense and antisense riboprobes.

Slides were deparaffinized in xylene and were rehydrated in decreasing concentrations of ethanol. Cells were made permeable with 0.3% Triton X-100 and were digested with proteinase K (20 μg/mL) in 100 m*M* Tris-HCl and 50 n*M* EDTA at pH 8.0 for 7 min at 37°C. The denatured digoxigenin-labeled riboprobes were applied to slides in a hybridization mix containing 50% formamide, 10% dextran sulfate, 1% polyvinylpyrrolidine, 5× Denhardt's solution, 0.5% SDS, and 100 μg/mL salmon sperm DNA in 5× subacute sclerosing panencephalitis (SSPE; 0.9 *M* NaCl, 50 m*M* NaH_2PO_4, and 5 m*M* EDTA at pH 7.4). Specimens were hybridized for 16 h at 55°C in a sealed humidified chamber. Slides were washed once each in 2× SSPE with 0.1% SDS and 0.1× SSPE with 0.1% SDS for 5 min at room temperature and were incubated in RNase A (Boehringer Mannheim) at 10 μg/mL for 10 min. Hybridization was detected by use of an antidigoxigenin antibody–alkaline phosphatase conjugate, according to the manufacturer's instructions (Boehringer Mannheim).

Immunohistochemical detection of vIL-6 was performed, as described elsewhere [5]. Polyclonal rabbit antiserum raised against vIL-6 peptides was made available by Y. Chang (Columbia University, New York). This vIL-6 antiserum does not cross-react with human IL-6, as determined by immunohistochemical staining. Briefly, formalin-fixed, paraffin-embedded cell pellets and tissue specimens were deparaffinized, incubated in citrate buffer (10 m*M,* at pH 6.0) for antigen retrieval, and quenched for 30 min in 0.03% H_20_2 in PBS. Polyclonal antiserum for vIL-6 was applied at a 1 : 2500 dilution in blocking solution (10% normal goat serum, 1% bovine serum albumin, and 0.05% Tween-20 in PBS) and incubated for 18 hr. Immunostaining of vIL-6 was conducted with an avidin-phosphatase conjugate, followed by Vector Red phosphate chromagens for development (Vector Laboratories, Burlingame, CA). Immunohistochemical staining of KS tissue with a monoclonal antibody (MAb) specific to CD34 (QB-END 10; Seratech, Oxford, UK) was used to identify KS cells of endothelial origin and to confirm protein preservation. The CD34 antibody was applied at a dilution of 1 : 1000 and incubated for 1–2 h. Detection of CD34 was carried out with the diamino-benzidine chromagen (Dako, Carpinteria, CA).

Sequencing of HHV-8 DNA. Procedures used for library preparation, subcloning, and polymerase chain reaction (PCR) sequencing have been described elsewhere [8]. Determination of subtype designation for these samples was based on the analysis of direct first-round PCR products of the VR-2 subdomain of ORF K1, using primers LGH 2507 (nt 589–606) and LGH 2508 (nt 840–864). The sequences of these primers are 5′-CGTCTCGCCTGTCAAATC-3′ and 5′-AGATACCACACATGGTT-3′, respectively.

Results

In situ hybridization and immunohistochemistry vIL-6 detection assays were applied to 3 primary effusion lymphoma specimens from lymphomas that had appeared in HIV-positive homosexual men. Two of these were "classic" primary effusion lymphomas of B cell lineage. The third was $CD8^+$, suggesting a T cell lineage. All 3 effusion lymphomas were dually infected with Epstein-Barr virus (EBV) and HHV-8. Immunohistochemistry with polyclonal rabbit vIL-6 antiserum showed cytoplasmic staining of vIL-6 in at least 60% of the B-cell lymphoma cells of the primary effusion lymphomas in each case (figure 1*A*). In situ hybridization of cells pelleted from a malignant effusion lymphoma with a full-length vIL-6 antisense riboprobe showed vIL-6 transcript in ~25%–40% of the cells in the 2 tumors of B-cell lineage (figure 1*B*). Control human lymphoblastoid cell lines that did not harbor HHV-8, but did produce human IL-6, showed no vIL-6 signal by either in situ hybridization or immunohistochemistry (data not shown).

An enlarged lymph node and massively enlarged spleen from an HIV-positive homosexual man who experienced weight loss, nightsweats, and fevers also showed vIL-6 expression by in situ hybridization and immunohistochemistry. Immunohistochemistry of vIL-6 protein showed strong cytoplasmic staining in lymphocytic cells of an HIV-positive Castleman's disease–like lesion, and vIL-6 was found in scattered lymphocytic cells in the mantle zone of both tissues (figure 1*C*). The inset shows in situ hybridization with an antisense digoxigenin–labeled vIL-6 riboprobe.

A total of 13 formalin-fixed, paraffin-embedded KS biopsy specimens from the Johns Hopkins dermatology archives were studied (table 1), and vIL-6 immunohistochemistry and in situ hybridization were detected in only a single case. However, using a CD34-specific MAb, we were able to verify by immunohistochemistry that all the KS samples expressed the endothelial cell marker CD34, confirming that the KS tissue was well-preserved and intact. The integrity of the mRNA in all KS specimens was confirmed by in situ hybridization with a β-actin digoxigenin–labeled riboprobe (data not shown).

Intrigued by a report of lytic infection in KS tissue [9], we obtained specimens from the AIDS Malignancy Bank that had been previously identified as showing foci of lytic viral infection, as evidenced by electron microscopy and T1.1 in situ hybridization.

Each of these KS lesion specimens showed vIL-6 expression by immunohistochemistry and in situ hybridization, predominantly in infiltrating lymphocytes (figure 1*D*) but also in occasional spindle-shaped cells with elongated nuclei, characteristic of KS cells (figure 1*F*). In situ hybridization confirmed the presence of vIL-6 RNA in these specimens (figure 1*E*). The heterogeneity observed in vIL-6 expression among KS specimens prompted us to consider the possibility that different strains of virus might be associated with different tendencies to viral lytic-cycle expression. Analysis of the hypervariable VR-2 region (amino acids 199–227) of the ORF K1 gene for each strain by PCR showed that 4 subtypes of virus were represented among the KS specimens studied (table 1). The subtype patterns found show some preferential associations with the patient's ethnic and geographic backgrounds, which are consistent with more extensive analyses [8]. However, there is no

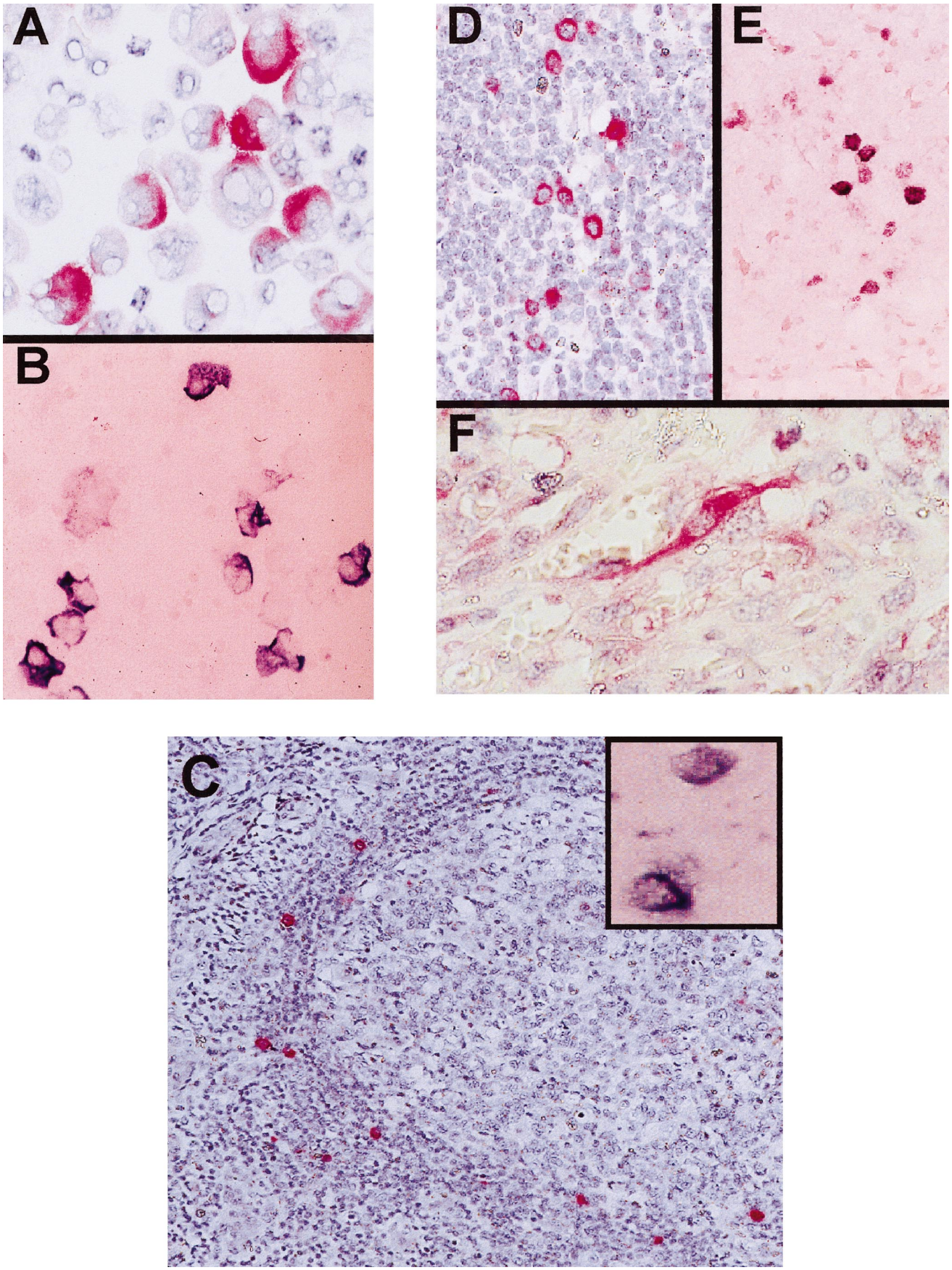

Figure 1. Detection of viral interleukin-6 (vIL-6) expression in human herpesvirus 8 (HHV-8)–associated diseases. See Results section for detailed explanation. Hematoxylin (*A, C, D,* and *F*) and eosin (*B,* inset of *C,* and *E*) counterstains were applied. Original magnification ×250 (*A, B*), ×64 (*C*), ×100 (*D, E*), and ×160 (*F,* inset of *C*).

Table 1. Summary of host factors, human herpesvirus 8 (HHV-8) strain, and viral interleukin-6 (vIL-6) expression.

KS specimen[a]	Age (years)	Sex	Race or ethnic group	HIV status	HHV-8 strain	vIL-6 expression
JHD						
1	65	M	White	ND	C-3	No
2	39	M	African American	+	B	No
3	37	M	White	+	A-4	No
4	37	M	White	+	A-2	No
5	37	F	White	ND	A-1	No
6	60	M	White	ND	C-2	No
7	52	M	White	−	A-4	No
8	78	M	White	ND	ND	No
9	39	M	White	ND	A-4	No
10	81	F	White	ND	ND	No
11	33	M	White	+	A-1	No
12	79	M	White	ND	ND	No
13	56	M	Black African	−	B	Yes
AMB						
1	34	F	Hispanic Mexican	+	B	Yes
2	47	M	Black Haitian	+	B	Yes
3	39	M	Hispanic Nicaraguan	+	A-4	Yes
4	34	M	White	+	A1	Yes
5	27	M	White	+	A4	Yes
6	29	F	African American	+	D	Yes
7	49	M	White	+	ND	Yes

NOTE. HIV, human immunodeficiency virus; ND, not determined.

[a] Johns Hopkins Dermatology archives (JHD) and the AIDS Malignancy Bank (AMB) were the sources of the Kaposi's sarcoma (KS) specimens.

clear association between HHV-8 subtypes and levels of vIL-6 expression in KS.

Discussion

Our results establish the applicability of in situ hybridization for the detection of vIL-6 in clinical specimens. These results demonstrate vIL-6 expression by immunohistochemistry and in situ hybridization in a variety of HHV-8–associated diseases.

The high percentage of cells expressing vIL-6 in the primary effusion lymphomas is interesting from several perspectives. The likelihood that vIL-6, which can substitute for IL-6 as a B cell growth factor in vitro, contributes to primary effusion lymphoma pathogenesis and perhaps to maintenance of the malignant phenotype is high [4, 5]. However, it stands in striking contrast to the situation with cellular homologues found in another human tumor–associated gamma herpesvirus, EBV. The EBV proteins vIL-10 (*BCRF1*) and vBCL2 (*BHRF1*) might also be anticipated to play a role in lymphomagenesis [10, 11]. However, these lytic-cycle genes are expressed only in rare tumor cells or at very low levels in EBV-associated lymphoid malignancies. Although a background of lytic infection is well described in EBV malignancies, this is never the predominant form of infection in tumors, even in HIV-infected patients. The HHV-8 vIL-6 gene is also a lytic-cycle gene insofar as it is induced by phorbol esters and butyrate [12]. Its expression in a large percentage of primary effusion lymphoma cells suggests either ongoing lytic infection in many of these cells or a more complex regulation of its expression. We note, however, that when we culture one of the primary effusion lymphomas, the percentage of cells expressing vIL-6 decreases over time. In general, we have found the percentage of positively staining cells to be between 0.5% and 10% for a number of primary effusion lymphoma-derived cell lines, including BC-1, BC-2, BCBL-1, BCP-1, and JSC-1 (present authors, unpublished data).

Detection of vIL-6 expression in a Castleman's disease–like lesion from an HIV-infected patient raises many questions. HHV-8 infection and vIL-6 expression have previously been demonstrated in Castleman's disease tissues in other studies [6, 13]. There has been some uncertainty in the literature with regard to the appropriateness of a diagnosis of Castleman's disease in the setting of HIV infection, because of concerns that HIV infection itself might lead to similar changes. The demonstration of vIL-6 production in an HIV patient with all of the clinical features of Castleman's disease reinforces the suggestion that this may be a distinct entity and raises the possibility that detection of vIL-6 might be a useful tool to distinguish between other lymph node pathologies with a similar appearance and Castleman's disease.

In regard to KS, our results confirm those of Chang et al., that vIL-6 is generally not expressed at detectable levels in KS specimens [5]. The absence of vIL-6 expression is in contrast to v-cyclin D and kaposin, which are readily detected in spindle cells in KS tissues [14, 15]. However, detection by both in situ hybridization and immunohistochemistry in a subset of specimens highlights the possibility that vIL-6 expression may be important for KS pathogenesis in some settings. We note that the cases from the AIDS Malignancy Bank were deliberately selected because earlier studies of these same specimens showed foci of lytic infection [9]. To ensure that this selection, rather than technical differences between the Johns Hopkins specimens and the AIDS Malignancy Bank specimens, accounted for differences in vIL-6 expression, we studied 10 unselected specimens from the AIDS Malignancy Bank. Expression of vIL-6 was detected in only one of these specimens. This was similar to the one in 13 KS specimens from Johns Hopkins in which vIL-6 expression was detected. Thus, in specimens from both sources, it is clear that vIL-6 expression is the exception rather than the rule. The determinants of vIL-6 expression in KS lesions are not clear. Host factor data in table 1 do not hint at any relationship between vIL-6 expression and HIV status, age, race, or ethnic origin of patients. Similarly, no clear correlation exists with viral strain.

In conclusion, we confirm that vIL-6 is expressed in a variety of HHV-8–associated diseases: in a substantial fraction of tumor cells in primary effusion lymphomas, in cells found in the mantle zone of a case of Castleman's disease–like lymphadenopathy, and in a subset of KS lesions. It is in the KS lesions that our results differ from those of previous investigators. Detection of vIL-6 in these lesions confirms previous evidence of

lytic-cycle gene expression in these specimens [9]. The role, if any, of vIL-6 in KS pathogenesis remains to be determined.

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