

Human Herpesvirus-8/Kaposi's Sarcoma-associated Herpesvirus Is a New Transmissible Virus That Infects B Cells

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Summary

Herpesviral DNA fragments isolated from AIDS-associated Kaposi's sarcoma (KS) tissue (KSHV-DNA) share homology with two lymphotropic oncogenic γ -herpesviruses, Epstein-Barr virus and Herpesvirus saimiri, and are present in the lesions of more than 95% of HIV and non-HIV-associated forms of KS, AIDS-related body cavity-based lymphomas, and AIDS-related multicentric Castleman's disease. Here we show that BC-1, a KSHV-DNA-positive, body cavity-based lymphoma cell line, produces infective herpesviral particles carrying a linear 270-kb genome that specifically transmits KSHV-DNA to CD19⁺ B cells. Transmission of KSHV-DNA is dependent upon a biologically active, replicating virus, since it is blocked by UV irradiation and foscarnet, an inhibitor of viral DNA-polymerase. This study represents the first isolation and transmission of the human herpesvirus-8/KS-associated herpesvirus.

The herpesviruses (family *Herpesviridae*) are double-stranded DNA enveloped viruses widely distributed in nature that are implicated in many human diseases (1). Herpesviral DNA fragments recently identified in AIDS-associated Kaposi's sarcoma (KS) tissue (2-4) (KS herpesviral-like DNA [KSHV-DNA]) (5) share homology with two lymphotropic oncogenic γ -herpesviruses, EBV (6) and Herpesvirus saimiri (HVS) (7). EBV is a B cell-immortalizing virus that is involved in the pathogenesis of malignant lymphomas (6). HVS is a T cell-immortalizing virus that can cause fulminant T cell lymphomas (7). The homology of KSHV-DNA with these viruses suggests that the newly identified sequences may belong to the genome of a new member of the herpesvirus family (1). Although these sequences were first identified in KS tissue (5, 8, 9), and more recently in other proliferative diseases (10, 11), the role of the putative new herpesvirus in the pathogenesis of these disorders remains to be defined. Primary to this question is the isolation and characterization of the putative KSHV-DNA virus. Here we show the first isolation of infective herpesviral particles (human herpesvirus-8 [HHV-8/KSHV]) that specifically transmit KSHV-DNA to normal circulating human B cells.

E.A. Mesri dedicates this paper to his mother, Frida Schlaen (1937-1982).

Materials and Methods

Viral Preparation. Viral pellets were obtained as described in (12). Briefly, BC-1 cells (13) were collected at low-speed centrifugation, resuspended in 1:20 vol of media, and snap-frozen (cell-associated virus extract). To obtain supernatant virus pellets, postculture-conditioned media from BC-1 cells was cleared by centrifugation at 1,400 g 4°C filtered through a 0.45- μ m membrane and centrifuged 2 h at 23,000 g at 4°C. Pellets were resuspended in 1:50 vol of media and snap-frozen in dry ice/ethanol. Both extracts (cell or supernatant virus) were thawed and frozen and thawed once more, sonicated 4 \times 40 s in a cup sonicator (Heat Systems-Ultrasonics, Inc., Plainview, NY), and cleared by centrifugation at 10,000 g at 4°C. To concentrate and further purify the viral preparations (14), they were loaded over a 25% sucrose/PBS (Ca²⁺, Mg²⁺) cushion and pelleted by overnight centrifugation at 70,000 g at 4°C. Viable BC-1 cells that might contaminate the preparation were excluded by passaging through a 0.45- μ m filter (supernatant virus), triple freezing and thawing in the absence of freezing agents, and extensive sonication and centrifugation at 10,000 g. Furthermore, the viral isolates were examined for the presence of any contaminating cell by light and electron microscopy, by culture in rich medium, and sensitivity to DNase degradation of the cellular gene *p53* (see Fig. 1 A) matched with a control of viable BC-1 cells (not shown).

Negative-staining Electron Microscopy. Viral pellets were resuspended in PBS (Ca²⁺, Mg²⁺), and the virus was absorbed onto parlodion/carbon-coated grids using the agar diffusion method,

stained with 2% phosphotungstic acid, pH 6.8, and viewed with an electron microscope (JEOL U.S.A. Inc., Peabody, MA).

Pulse Field Gel Electrophoresis (PFGE) Analysis. Cells (25×10^6 /ml final) or cell viral pellets (corresponding to 2×10^8 cells) were molded into 0.9% NaCl low melting point agarose plugs. The plugs were incubated for 2 d at 50°C in lysis buffer (0.5 M EDTA, pH 8.0, 1% sarkosyl, 1 mg/ml proteinase K), and washed and stored in 0.5 M EDTA at 4°C. PFGE was performed using a CHEF-DR II system (Bio Rad Laboratories, Richmond, CA) following the manufacturer's instructions. Samples were run on 1% PFGE-certified agarose (Bio Rad) gels in $0.5 \times$ Tris-borate-EDTA buffer at 200 V, initial A time = 3 s, final A time = 30 s, for 23 h at 14°C. PFGE Marker I ladder molecular weight marker plugs were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Southern Blot for KSHV-DNA or EBV Sequences. Gels were transferred to nitrocellulose filters and probed with random prime 32 P-labeled probes for KSHV-DNA (KSBam330 fragment [5]) or the EBV terminal repeat region (10, 13).

PCR Detection of KSHV-DNA and p53. Cells were pelleted and resuspended in 3 μ l PBS. Resuspended cells, or viral extracts (3 μ l), were treated with 30 μ l of Genereleaser (Bio-Ventures, Mufreesboro, TN). The DNA was released in a thermocycler following the manufacturer's protocol. Primers for KSBam 330₂₃₃ or for p53 (5) were added to one third of the DNA, and a PCR reaction was carried out in a thermocycler as described (5).

DNase Treatment of the Viral Isolates. Before PCR, viral extracts were treated with RNase-free DNase I (Boehringer Mannheim) for 15 min following the manufacturer's conditions.

Infection Studies. Umbilical cord blood mononuclear cells (CBMC) obtained by Ficoll centrifugation were incubated for 40 min at room temperature with viral extracts at a cell density of 10^7 cells/ml, washed once with media to remove excess virus, resuspended in RPMI, 20% FCS, 20% CD34-derived spindle cell (Rafii, S., E.A. Mesri, and A.S. Asch, unpublished data) conditioned media (RC-20 medium) at a density of 10^6 cells/ml, and incubated during a week. For PCR, one-third of the cells were harvested, washed once with media, and treated with Genereleaser.

UV Irradiation of the Viral Preparations. Viral pellets resuspended in PBS or media were exposed to a dose of 9 mJ of UV light using a Stratalinker (Stratagene, La Jolla, CA).

Enrichment in CD19 Cells and Immunodepletion of CD19 Cells. B cells were immunoselected from CBMC using an anti-CD19 mAb coupled to magnetic beads (Dynabeads; Dynal, Lake Success, NY) following the manufacturer's conditions. A similar procedure repeated three times was used to deplete the CBMC of B cells.

Effect of Foscarnet. Infected cells were incubated in the presence of different doses of Foscarnet (Foscavir; Astra, Westborough, MA).

Results

We used methods for herpesviral preparation and concentration to obtain viral particles from BC-1 cells, an AIDS-related, body cavity-based lymphoma (BCBL) cell line that stably carries KSHV-DNA (13). We obtained sucrose cushion purified cell-free pellets (see Materials and Methods) that were KSHV-DNA-positive by PCR from both BC-1 cells and their supernatants (Fig. 1 A). KSHV-DNA present in the viral preparations within a viral capsid should be resistant to DNase degradation, whereas free

DNA should be sensitive. Fig. 1 A shows the results of the DNase sensitivity assay of KSHV-DNA from viral isolates. As an internal control for free (nonencapsidated) DNA, we used the detection of the cellular gene p53, present in free BC-1 cellular genomic DNA which is also found in the viral preparations. When the pellets were treated with DNase, the p53 gene could no longer be detected by PCR. In contrast, KSHV-DNA in the viral isolates is DNase resistant and can be amplified by PCR after DNase treatment (Fig. 1), indicating that the KSHV-DNA is protected, quite possibly inside a viral capsid. To show that DNase protection of viral genes was not limited to this KSHV-DNA fragment (KS330Bam) and its immediately adjacent region, we also carried out a similar analysis with a viral gene adjacent to the other reported KSHV fragment (KS631Bam; [5] that was recently cloned and is distant from KSHV330Bam (Cesarman, E., R. Nador, and D.M. Knowles, manuscript in preparation). We found that this KSHV gene was also protected from DNase degradation (data not shown).

To further characterize the viral preparation, we performed negative-staining electron microscopy that showed the presence of several virus-like particles (Fig. 1 B). While none of these particles allows unequivocal identification as a herpesvirus, they have features of the herpesviruses and similarly appearing particles are characteristic of herpesviral preparations (15). No intact cells or other cellular organelles were seen in these preparations.

PFGE of the viral preparations (Fig. 1 C) showed that KSHV-DNA belongs to a linear 270-kb genome present both in BC-1 cells (16) and in the viral pellet. In contrast, EBV, also produced by BC-1 cells (13), hybridizes with a 170-kb genome. Our results show that in the BC-1 viral isolates, KSHV-DNA is contained in a viral genome that is distinct from the EBV genome. The DNase protection of distant segments of the 270-kb KSHV-DNA viral genome, in contrast with the DNase sensitivity of nonvirally associated genes and the presence of herpesviral-like particles in the isolates, are consistent with encapsidation of the KSHV-DNA viral genome.

To determine whether the virus carrying KSHV-DNA was infective, we tested the viral preparations for their capacity to infect CBMC. Fig. 2 A shows viral transmission of KSHV-DNA to CBMC after 1 wk of exposure to the viral isolates. The figure further shows that UV irradiation of the viral extracts abolishes KSHV-DNA transmission without modifying its ability to act as a PCR template. Since this dose of UV irradiation is known to inactivate herpesviral DNA replication and transcription, our results indicate that transmission of KSHV-DNA is accomplished by transmission in an active herpesvirus, HHV-8/KSHV.

A recent report notes that KSHV-DNA is not present in AIDS-KS spindle cell lines, but is found in the CD19 B cell compartment of KS patients (17). This finding and the fact that KSHV-DNA is present in the neoplastic cells of BCBL (10, 13) strongly suggest that HHV-8/KSHV may be a lymphotropic virus possibly targeting the B cell compartment. To establish whether HHV-8/KSHV is a B cell-specific virus, we used immunoselection techniques to positively

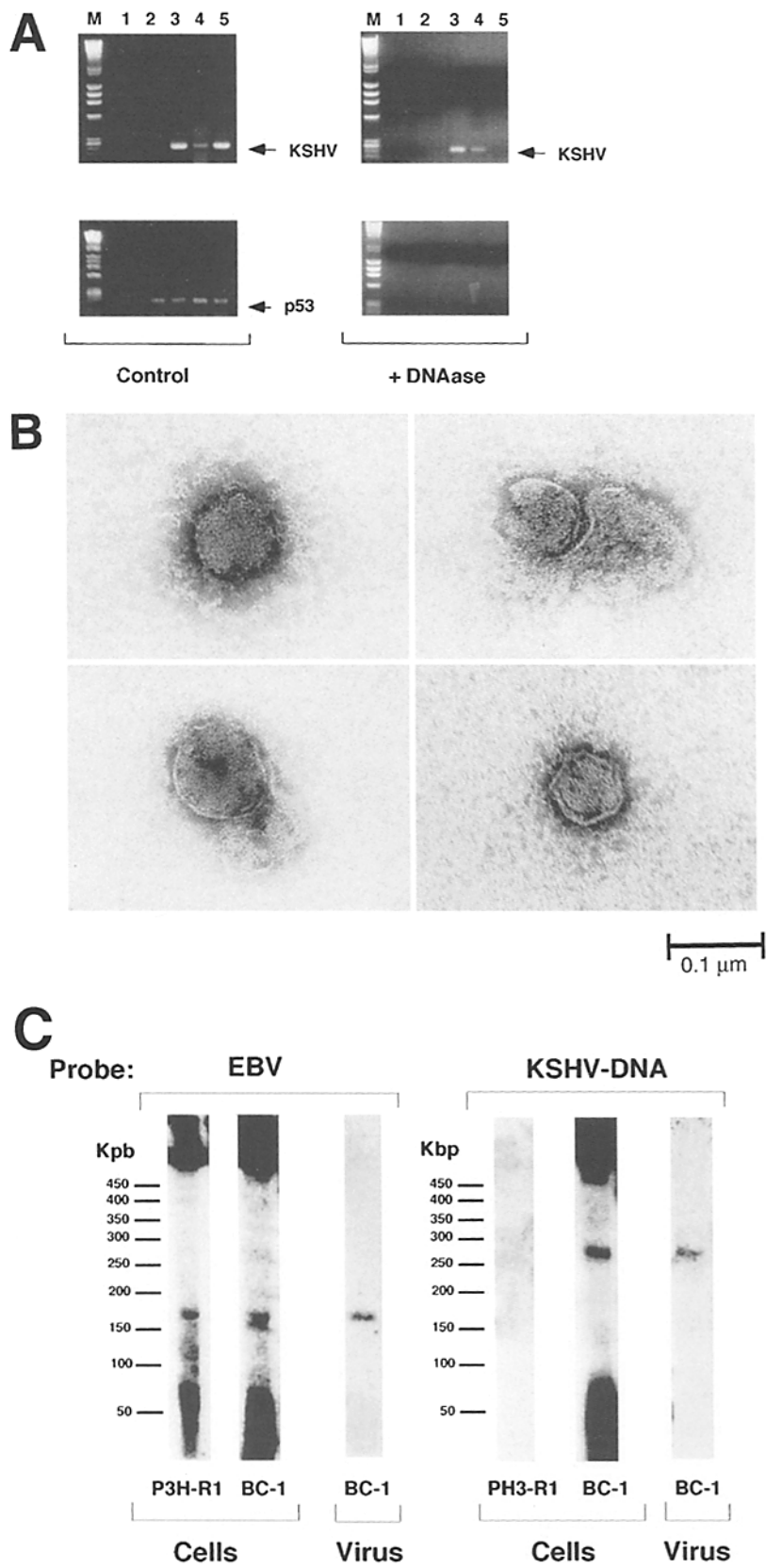


Figure 1. Characterization of the viral isolates obtained from BC-1 cells and supernatants. (A) DNAase protection analysis of KSHV-DNA: PCR analysis of the viral pellets to detect KSHV-DNA and p53. *Left panel*, before DNAase treatment; *right panel*, after incubation with DNAase I; lane 1, no cells added; lane 2, PH3-R1 cells (negative control); lane 3, BC-1 cell-associated viral pellet; lane 4, BC-1 supernatant viral pellet; lane 5, BC-1 DNA. The KSHV and p53 arrows indicate the positions of the KS330Bam PCR product of 233 bp (5) and the p53 PCR product, respectively (5). (B) Negative-staining EM of viral pellets shown at $\times 200,000$. (C) PFGE and Southern blot analysis of the viral isolate. High molecular weight DNA from BC-1 cells (EBV^+ , $KSHV-DNA^+$), P3H-R1 cells (EBV^+), and the cell viral pellet from BC-1 cells was separated by PFGE and analyzed by Southern blot for the presence of EBV or KSHV-DNA sequences.

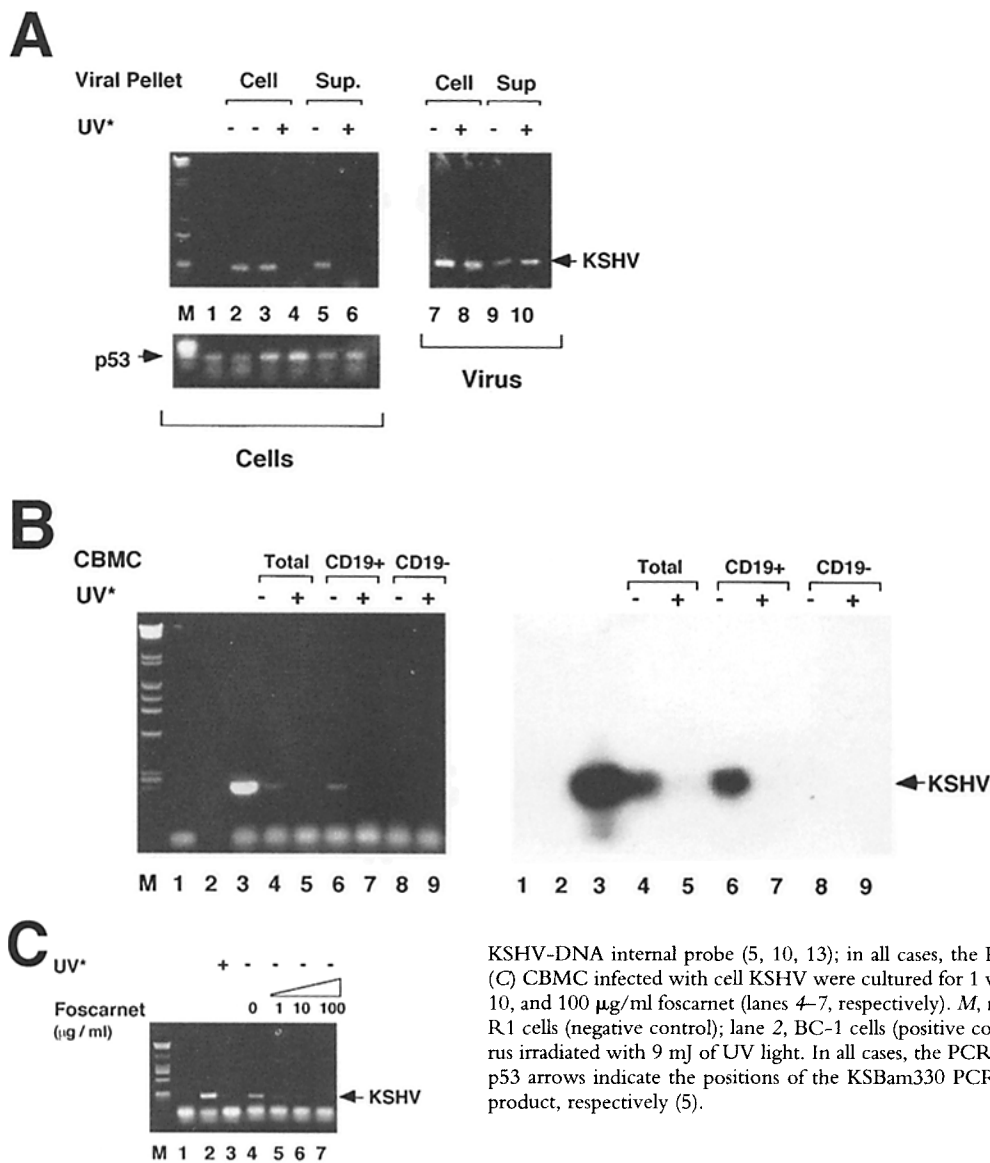


Figure 2. Viral transmission of KSHV-DNA to cord blood B cells: inhibition by UV irradiation and foscarnet. (A) Viral transmission of KSHV-DNA to cord blood mononuclear cells. Lane 1, mock-infected CBMC; lanes 2–6, CBMCs were infected with cell-associated (lanes 2–4) or supernatant (lanes 5 and 6) virus; lanes 3–6, cells were analyzed 1 wk after infection; lane 2, cells were analyzed 2 wk after infection; lanes 4 and 6, virus was irradiated with UV light; lanes 7–10, PCR of the viral isolates; lanes 7 and 8, cell-associated virus; lanes 9 and 10, supernatant virus; lanes 8 and 10, the viral extract was irradiated with UV before PCR. Upper panel, KSHV-DNA PCR, lower panel, p53 PCR. (B) Viral transmission of KSHV-DNA to CD19⁺ B cells. Lane 1, P3H-R1 cells (negative control); lane 2, Gene-releaser; lane 3, BC-1 cells (positive control); lanes 4 and 5, total CBMC; lanes 6 and 7, CD19⁺ cells; lanes 8 and 9, B cell-depleted CBMCs; lanes 4, 6, and 8, active virus; lanes 6, 7, and 9, virus irradiated with UV. The left panel shows the analysis of the PCR products on an agarose gel, the right panel shows the result of the Southern blot of the same gel hybridized with the

KSHV-DNA internal probe (5, 10, 13); in all cases, the PCR was positive for p53 (not shown). (C) CBMC infected with cell KSHV were cultured for 1 wk with or without the addition of 0, 1, 10, and 100 µg/ml foscarnet (lanes 4–7, respectively). M, molecular weight markers; lane 1, P3H-R1 cells (negative control); lane 2, BC-1 cells (positive control); lane 3, CBMC infected with virus irradiated with 9 mJ of UV light. In all cases, the PCR were positive for p53. The KSHV and p53 arrows indicate the positions of the KSBam330 PCR product of 233 bp and the p53 PCR product, respectively (5).

select CD19⁺ (Pan-B) cells from cord blood and to deplete cord blood mononuclear cells of CD19⁺ cells. The results in Fig. 2 B show that the preselected CD19⁺ CBMCs were infectable with KSHV, while CD19⁻ CBMC were not infected. These results indicate that HHV-8/KSHV is a B-lymphotropic herpesvirus.

Foscarnet is a specific inhibitor of herpesviral DNA polymerase (18). Its potential benefit as a therapeutic agent for AIDS-KS may be of importance (19, 20). The results in Fig. 2 C show that foscarnet has an inhibitory effect on the transmission of KSHV-DNA to CBMC, further reinforcing the concept that transmission and persistence of KSHV-DNA depends on a replication-competent herpesvirus.

Discussion

Our data demonstrate that the herpesviral DNA fragments associated with KS and BCBL are part of a new

transmissible B-lymphotropic herpesvirus. We favor its denomination as human herpesvirus-8 instead of the former descriptive name of Kaposi's sarcoma-associated herpesvirus (5) because it is also associated with BCBL and AIDS-related multicentric Castleman's disease (10). Genetic studies show that HHV-8/KSHV is a virus of the subfamily *Gammaherpesvirinae*, and it is the first human virus of the genus *Rhadinovirus* (16); studies also show that KSHV-DNA is part of a viral genome that is collinear to Herpesvirus saimiri (Cesarman et al., manuscript in preparation).

Although it has been found in a few other tumors (4, 21), the very strong association found between KSHV-DNA and KS (5, 8, 9), KSHV-DNA and the unique effusion phenotype of BCBL AIDS lymphomas (10), and KSHV-DNA and AIDS-related multicentric Castleman's disease (11) suggests that an infectious, HHV-8/KSHV-related lymphoproliferative or inflammatory component could be common to these malignancies. The pattern of HHV-8/

KSHV gene expression (Cesarman et al., manuscript in preparation) and KSHV-DNA amplification found in AIDS KS and BCBL (5, 10) indicates a productive infection in the lesions suggesting that HHV-8/KSHV may play an active role in direct or immune-mediated pathogenesis. Moreover, a recent report shows that the appearance of HHV-8-infected cells in the peripheral blood predisposes for the development of KS in HIV-infected individuals (22). It is therefore possible that agents that can impede HHV-8/KSHV replication or infection (such as foscarnet)

might be useful in preventing or ameliorating the course of these disorders (19, 20). Our findings raise the possibility that HHV-8/KSHV-infected B cells play a role in the pathogenesis of KS. Further studies defining the biology of HHV-8/KSHV and how it can modify the phenotype of infected B cells in the context of HIV infection (3, 4, 23) would provide important insights on the pathophysiology of KS and other AIDS-associated malignancies, and they may establish the potential of HHV-8/KSHV as a therapeutic target for these disorders.

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References

1. Roizman, B. 1993. The family *Herpesviridae*. In *The Human Herpesviruses*. B. Roizman, R.J. Whitley, and C. Lopez, editors. Raven Press, New York. p. 1.
2. Friedman-Kien, A.E. 1981. Disseminated Kaposi's sarcoma syndrome in young homosexual men. *J. Am. Acad. Dermatol.* 5:468-471.
3. Levine, A.M. 1993. AIDS-related malignancies: the emerging epidemic. *J. Natl. Cancer Inst.* 85:1382-1397.
4. Gallo, R.C. 1995. Human retroviruses in the second decade: a personal perspective. *Nature Med.* 1:753-759.
5. Chang, Y., E. Cesarman, M.S. Pessin, F. Lee, J. Culpepper, D. Knowles, and P. Moore. 1994. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science (Wash. DC)*. 266:1865-1869.
6. Liebowitz, D., and E. Kieff. 1993. Epstein-Barr virus. In *The Human Herpesviruses*. B. Roizman, R.J. Whitley, and C. Lopez, editors. Raven Press, New York. p. 107.
7. Fleckenstein, B., and R.C. Desrosiers. 1982. Herpesvirus saimiri and herpesvirus ateles. In *The Herpesviruses*. Vol 1. B. Roizman, editor. Plenum Publishing, New York. p. 253.
8. Moore, P.S., and Y. Chang. 1995. Detection of Herpesvirus-like DNA sequences in Kaposi's sarcoma lesions from persons with and without HIV infection. *N. Engl. J. Med.* 332:1181-1185.
9. Huang, Y., J.J. Li, M.H. Kaplan, B. Poiesz, E. Katabira, W.C. Zhang, D. Feiner, and A.E. Friedman-Kien. 1995. Human herpesvirus-like nucleic acid in various forms of Kaposi's sarcoma. *Lancet*. 345:759-761.
10. Cesarman, E., Y. Chang, P.S. Moore, J.W. Said, and D.M. Knowles. 1995. Kaposi's sarcoma-associated herpes virus-like DNA sequences are present in AIDS-related body cavity B-cell lymphomas. *N. Engl. J. Med.* 332:1186-1191.
11. Soulier, J., L. Grollet, E. Okenhendler, P. Cacoub, D. Cazals Hatem, P. Babinet, M. D'Agay, J. Clauvet, M. Raphael et al. 1995. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric castlemans disease. *Blood*. 86:1276-1280.
12. Dixon, F.J., and J. McLauchlan. 1993. Herpes simplex virus vectors. In *Molecular Virology*. A.J. Davison and R.M. Elliot, editors. IRL Press, Oxford, New York. 285-307.
13. Cesarman, E., P.S. Moore, G. Ingharami, D.M. Knowles, and Y. Chang. 1995. In vitro establishment and characterization of two AIDS-related body cavity based lymphoma cell lines containing the Kaposi's sarcoma-associated herpesvirus-like (KSHV) DNA sequences. *Blood*. 86:2708-2714.
14. Federoff, H.J., M.D. Geschwind, A.I. Geller, and J.A. Kessler. 1992. Expression of nerve growth factor in vivo from a defective herpes-simplex-1 vector prevents effects of axotomy on sympathetic ganglia. *Proc. Natl. Acad. Sci. USA*. 89: 1636-1640.
15. Madeley, C.R., and A.M. Field. 1988. *Virus Morphology*. Churchill-Livingstone, London. 50-69.
16. Moore, P.S., S. Gao, G. Dominguez, E. Cesarman, D.M. Knowles, R. Garber, P. Pellet, D. McGeoch, and Y. Chang. 1996. Primary characterization of a herpesvirus-like agent associated with Kaposi's sarcoma. *J. Virol.* 70:549-558.
17. Ambrosziak, J.A., D. Blackburn, B.G. Herndier, R.G. Glogau, J.H. Gullet, A.R. McDonald, E.T. Lennette, and J.A. Levy. 1995. Herpes-like sequences in HIV-infected and uninfected Kaposi's sarcoma patients. *Science (Wash. DC)*. 268:582-583.
18. Crumpacker, C.S. 1992. Mechanism of action of foscarnet against viral polymerases. *Am. J. Med.* 92:2A3S-2A7S.
19. Morfeldt, L., and J. Torssander. 1994. Long-term remission of Kaposi's sarcoma following foscarnet treatment in HIV-infected patients. *Scand. J. Infect. Dis.* 26:749-753.
20. Jones, J.L., D.L. Hanson, S.Y. Chu, J.W. Ward, and H.W. Jaffe. 1995. AIDS-associated Kaposi's sarcoma. *Science (Wash.*

- DC). 267:1078–1079.
21. Rady, P.L., A. Yen, J. Rollefson, I. Orengo, S. Bruce, T. Hughes and S.K. Tyring. 1995. Herpesvirus-like DNA sequences in non-Kaposi's sarcoma skin lesions of transplant patients. *Lancet*. 345:1339–1340.
 22. Whitby, D., M.R. Howard, M. Tenant-Flowers, N.S. Brink, A. Copas, C. Boshoff, T. Hatzioannou, F.E. A.Sugett, D.M. Aldam, A.S. Denton et al. 1995. Detection of Kaposi's sarcoma associated herpesvirus in peripheral blood of HIV-infected individuals and progression to Kaposi's sarcoma. *Lancet*. 346:799–802.
 23. Fauci, A.S. 1993. The multifactorial and multiphasic components of human immunodeficiency virus disease: implications for the design of therapeutic strategies. *Science (Wash. DC)*. 262:1011–1018.