

## PROPAGATION OF A HUMAN HERPESVIRUS FROM AIDS-ASSOCIATED KAPOSI'S SARCOMA

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**ABSTRACT**

**Background** Although unique DNA sequences related to gammaherpesviruses have been found in Kaposi's sarcoma lesions, it is uncertain whether this DNA encodes a virus that is able to reproduce.

**Methods** We isolated and propagated a filterable agent whose DNA sequences were found to be identical to those of the Kaposi's sarcoma-associated herpesvirus (KSHV). We obtained early-passage spindle cells from skin lesions of patients with the acquired immunodeficiency syndrome (AIDS) who had Kaposi's sarcoma and cultured them with cells of the human embryonal-kidney epithelial-cell line 293. We characterized the virus according to its effects on cellular morphology and viral replication and its appearance on electron microscopy.

**Results** KSHV was cytotoxic to 293 cells and was detected by the polymerase chain reaction (PCR) in infected cells but not uninfected ones. Cytotoxicity and positive PCR signals were consistently maintained with viral titers of 1 million per milliliter or higher for about 20 serial infections of 293 cells. The viral copy number was relatively low (1 to 10 copies per cell). Viral replication was confirmed by Southern blot analysis of DNA isolated from the enriched nuclear fraction of infected cells and by a semiquantitative PCR using dilutions of the lysates of infected cells to detect the 233-bp viral DNA fragment originally described in association with Kaposi's lesions. Electron microscopy revealed herpesvirus-like particles in about 1 percent of cells from infected cultures, as compared with none in cells from uninfected cultures.

**Conclusions** A herpesvirus with DNA sequences identical to those of KSHV can be propagated from skin lesions of patients with AIDS-associated Kaposi's sarcoma. (N Engl J Med 1997;336:163-71.)

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**K**APOSI'S sarcoma was originally described in the late 1800s as a rare neoplasm predominantly affecting elderly men of Jewish or Mediterranean descent. Currently, Kaposi's sarcoma is the most common cancer in patients with the acquired immunodeficiency syndrome (AIDS), affecting approximately 20 percent of persons with human immunodeficiency virus type 1 (HIV-1) infection.<sup>1-3</sup> Epidemiologic data have suggested that an infectious agent could spread the disease through sexual contact,<sup>4</sup> but the etiologic agent has not been identified. Several viruses, including cy-

tomegalovirus (CMV), hepatitis B virus, and human papillomavirus, have been found in patients with Kaposi's sarcoma. Recently, Chang et al. found that over 90 percent of the tissue samples they studied from patients with AIDS-associated Kaposi's sarcoma were positive for herpesvirus-like DNA sequences.<sup>5</sup> These sequences were homologous to, but distinct from, minor capsid and tegument proteins of Epstein-Barr virus (EBV) and herpesvirus saimiri.<sup>5</sup> They defined a putative new member of the gamma-herpesvirus family, referred to as Kaposi's sarcoma-associated herpesvirus (KSHV) or human herpesvirus 8 (HHV-8). Since then, investigators in several laboratories have found these DNA sequences in patients with four types of Kaposi's sarcoma — the classic one, the one endemic in Africa, the iatrogenic one, and the one associated with AIDS<sup>6-8</sup> — as well as in patients with body-cavity-based lymphomas.<sup>9-11</sup>

It is uncertain whether these DNA sequences encode a replication-competent virus or represent a replication-defective, adventitious virus present in patients with Kaposi's sarcoma. Although B-cell lines that carry the virus have been identified,<sup>10,11</sup> neither viral replication in vitro nor serial propagation of the virus in cell lines has been demonstrated. In this study we demonstrate the propagation of a human herpesvirus from AIDS-associated Kaposi's sarcoma.

**METHODS****Cell Culture, Isolation of DNA, and Analysis by the Polymerase Chain Reaction**

Kaposi's sarcoma cell lines were isolated independently from specimens of Kaposi's sarcoma skin lesions obtained at biopsy from five HIV-infected patients,<sup>12</sup> all of whom had multiple lesions, diminished CD4 cell counts, and other signs of AIDS. For some patients, two biopsy specimens were evaluated. The cells were plated in tissue-culture dishes coated with microvascular endothelial-cell attachment factor (Cell Systems, Kirkland, Wash.) and were maintained as previously described,<sup>12,13</sup> but without the

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**TABLE 1.** PRESENCE OF KSHV AND OTHER DNA SEQUENCES IN ISOLATED CELL LINES.\*

SAMPLE TESTED	KSHV	HHV-6	HSV-1, HSV-2	EBV		CMV
				A	B	
Isolated KS cells						
KS-2 (Patient 1)	+	-	-	-	-	-
KS-3 (Patient 1)	+	-	-	-	-	-
KS-4 (Patient 2)	+	-	-	-	-	-
KS-5 (Patient 3)	+	-	-	-	-	-
KS-6 (Patient 3)	+	-	-	-	-	-
KS-7 (Patient 4)	+	-	-	-	-	-
KS-8 (Patient 5)	+	ND	ND	ND	ND	-
Specimens from patients						
Pulmonary KS						
Sample 1	+	+	-	-	-	-
Sample 2	+	+	-	-	-	-
Psoriasis	-	-	ND	ND	ND	-
Controls						
Human umbilical-vein endothelial cells	-	-	-	-	-	-
293 cells	-	-	-	-	-	-

\*DNA was isolated from the indicated cell lines from the designated subjects (Patients 1 through 5), and tissue samples were analyzed by PCR (see the Methods section). Plus and minus signs indicate the presence and absence, respectively, of fragments of the relevant size on ethidium bromide staining of agarose gels (range of assay sensitivity, approximately 0.1 to 1 fg). Positive control reactions for each type of virus were performed with the same concentration of DNA (1  $\mu$ g) that was used in the test reactions. Positive-control DNA samples were isolated from biopsy specimens obtained from another patient, who had pulmonary Kaposi's sarcoma. KS denotes Kaposi's sarcoma; ND, not determined; and 293 cells, cells of the human embryonal-kidney epithelial-cell line 293. EBV A and B refer to viral subtypes distinguished by their genomic organization and transforming potential.

addition of medium conditioned by cells infected with the human T-cell lymphotropic virus type II (HTLV-II).

The polymerase chain reaction (PCR) was performed with KSHV-specific primers.<sup>5</sup> Either genomic DNA or Hirt supernatants — that is, episomal DNA from infected cells — were isolated from tissue-culture cells as previously described.<sup>14,16</sup> PCR was performed as previously described to detect human herpesvirus 6 (HHV-6), EBV, and herpes simplex virus types 1 and 2 (HSV-1 and HSV-2).<sup>17-19</sup> CMV was detected with the following primers from the intron of an immediate early gene, under standard conditions: 5'CCAAGCTTCCACGCTGTTTGGACCTCCATAGA3' (sense) and 5'CCAAGCTTCTGTGTCAGCTATTATGCTGGTG-GC3' (antisense); a 908-bp product was generated. Positive controls for these known herpesviruses were obtained from paraffin-embedded tissue samples and from the EBV-transformed B-cell line M16B. The sensitivity of the PCR was 0.1 to 1 fg of DNA.

In a semiquantitative PCR, the 233-bp fragment of viral DNA originally described in association with Kaposi's sarcoma lesions<sup>5</sup> (Genbank accession number U18551, base pairs 987 to 1219) was quantitated by electrophoresis with ethidium-stained agarose gel and spectroscopy in serial dilutions. PCR was performed, followed by Southern blot hybridization with a form of the same 233-bp DNA fragment that was radiolabeled with phosphorus-32. Each PCR used DNA from 100,000 cells, purified by phenol-chloroform extraction. The range of the DNA concentration measurable in the assay was 0.001 to 1 fg (4 to 4000 copies).

#### Propagation of Virus from Cocultures and Serial Passage

Cells from primary Kaposi's sarcoma lesions (200,000 cells, passaged no more than three times after the cells from the biopsy

samples were plated) were incubated with 2 million cells of the human embryonal-kidney epithelial-cell line 293 (293 cells) in 35-mm six-well Costar plates, either stimulated before culture with tumor necrosis factor  $\alpha$  (200 IU per milliliter) or unstimulated. The 293 cells (provided by Dr. Garry Nolan) were grown in Dulbecco's modified Eagle's medium supplemented with fetal-calf serum (10 percent), antibiotics, and *L*-glutamine. To release viral particles, cell-free lysates from the 293 cells were prepared by three cycles of freezing and thawing in dry ice and ethanol and a water bath at 37°C three to five days after the initial coculture with the Kaposi's sarcoma cells. After lysis of the cells, the extracts were centrifuged at 10,000 $\times g$  for 10 minutes, then filtered through a 0.45- $\mu$ m membrane.

For the serial propagation of virus, cell-free lysates were prepared from infected 293 cells by this method on day 3 after infection, while a duplicate cell culture was maintained to confirm subsequent cytotoxicity. No viable cells were detected in the lysates by staining with trypan blue, and positivity for KSHV DNA was confirmed in the lysates by PCR. Virus was propagated serially in an equivalent number of 293 cells for approximately 20 passages. Viral titers were estimated by incubating the infected lysates in progressive dilutions (10<sup>-2</sup> to 10<sup>-10</sup>) with 293 cells. Human umbilical-vein endothelial cells were propagated as previously described.<sup>20</sup>

#### Treatment of Extracellular Virions with Nuclease

To confirm that viral DNA was protected by components of the viral structure, isolated virions were digested with RNase-free DNase (1 U per milliliter; Promega) and pronase (1 mg per milliliter), which together should degrade free or protein-associated DNA. Some virions were incubated in addition with a nonionic detergent, 1 percent Nonidet P-40 (NP40), which disrupts the lipid bilayer of the viral envelope<sup>11,21</sup> and allows DNase and pronase to degrade virion-associated DNA. The supernatants of infected cultures of 293 cells were centrifuged for 10 minutes at 3000 rpm and filtered through a 0.45- $\mu$ m membrane. To prevent the potential carryover of viral DNA, the supernatants were ultracentrifuged (25,000 $\times g$ ) at 4°C (SW 28 rotor, Beckman) to pellet the viral particles. Before analysis, the particles were washed once with phosphate-buffered saline (PBS).

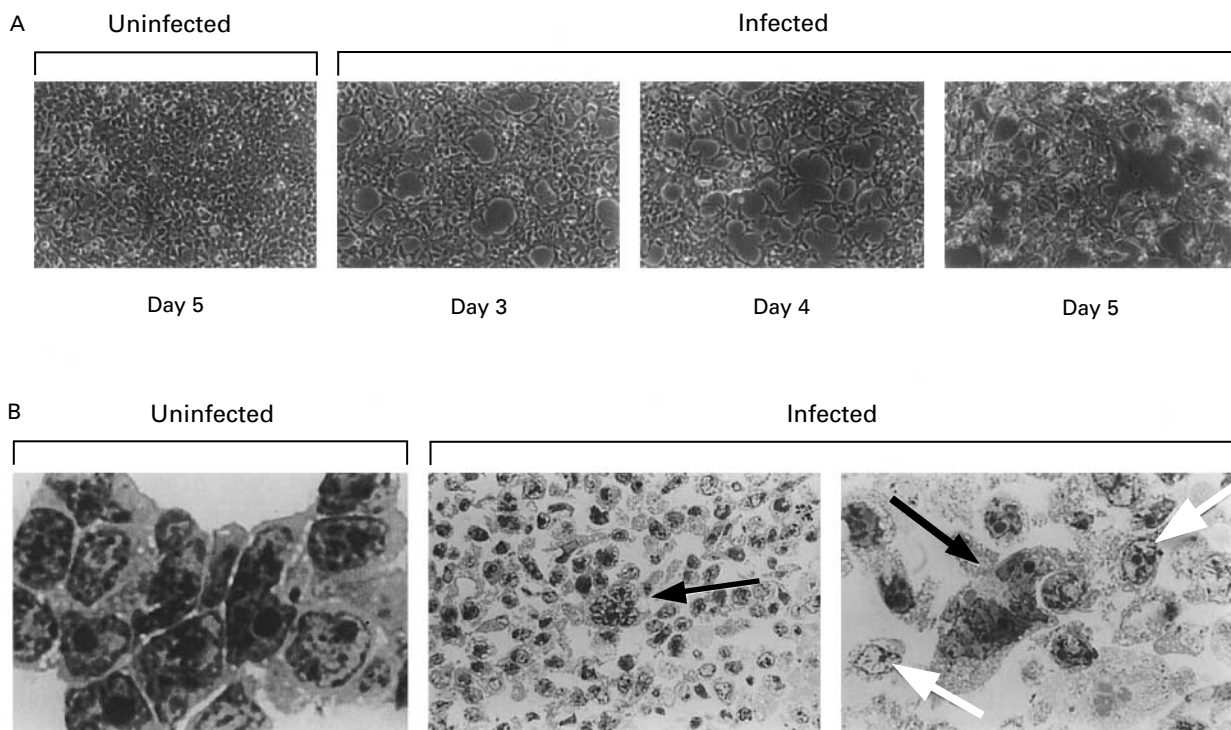
#### Electron Microscopy

Cells from the in vitro cultures described above were rinsed with PBS, and monolayers were fixed in 2.5 percent glutaraldehyde in PBS (pH 7.4) for five minutes at 4°C. The cells were then resuspended, centrifuged into pellets at 300 $\times g$ , and incubated for two hours at 4°C. The samples were rinsed, dehydrated, and embedded in epon. The specimens were sectioned, stained with uranyl acetate and lead citrate, and examined with a Zeiss 109 transmission electron microscope. In some instances, the cells were incubated for 12 hours with brefeldin A (0.3  $\mu$ g per milliliter), an inhibitor of Golgi transport, added 24 hours after infection. This treatment improved the visualization of virions.

## RESULTS

#### Characterization of KSHV DNA in Isolated Kaposi's Sarcoma Cells

To establish Kaposi's sarcoma cell lines in vitro, we developed conditions for cell culture that did not require the presence of medium conditioned with HTLV-II-infected cells, unlike the culture conditions in previous studies.<sup>12,13</sup> Kaposi's sarcoma cell lines grew rapidly after the initiation of the cultures. Each was positive for clotting factor XIIIa and vascular-cell adhesion molecule 1 on immunohistochemical staining and negative for factor XIIIb,



**Figure 1.** Cytotoxic Effect on 293 Cells of Virus Propagated in Vitro.

Panel A shows uninfected 293 cells and cells exposed to virus (infected) after incubation in vitro for the numbers of days shown, as analyzed by phase-contrast microscopy. The degree of cytotoxicity observed in the infected cells was graded as + on day 3, ++ on day 4, and +++ on day 5 ( $\times 10$ ).

Panel B shows the typical light-microscopical appearance of 293 cells before exposure to lysate and five days thereafter. The uninfected cells appear uniformly viable, with typical epithelial differentiation, but the infected cells have undergone degeneration, with the formation of multinucleated giant cells (black arrows) and the alteration of chromatin typical of infection by a virus of the herpes group (white arrows) (left,  $\times 400$ ; middle,  $\times 200$ ; right,  $\times 400$ ).

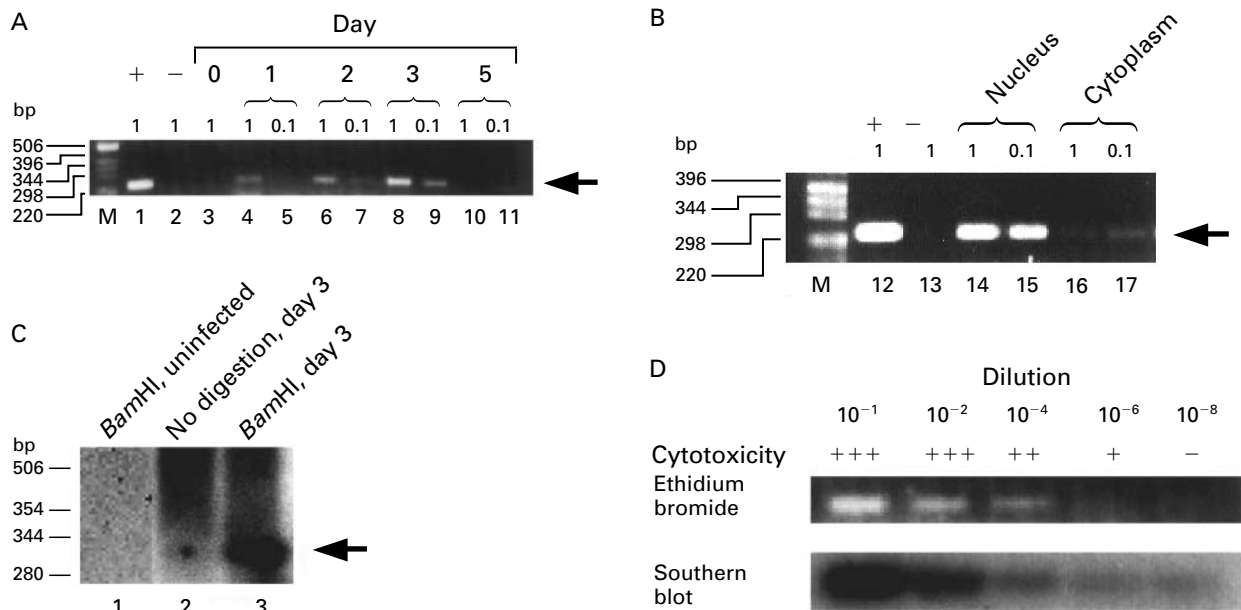
E-selectin, CD31, factor VIII, and CD34 (data not shown), as is consistent with the phenotype of isolated Kaposi's sarcoma cells studied under different conditions of culture.<sup>12,22</sup>

PCR was used to determine whether the proliferating Kaposi's sarcoma cells contained recently described herpesvirus-like DNA sequences.<sup>5</sup> Early-passage, isolated Kaposi's sarcoma cell lines from six of the seven specimens obtained were positive for these DNA sequences (Table 1). The positive signal was present in the cell lines until passage 2 or 3 and could not be detected thereafter. The 233-bp PCR product was also found in DNA isolated from two paraffin-embedded samples of tissue from a different patient who had pulmonary Kaposi's sarcoma, as has been described in other patients.<sup>5,6</sup> No evidence of KSHV DNA sequences was detected in human umbilical-vein endothelial cells, 293 cells, or DNA isolated from paraffin-embedded tissue from a patient with psoriasis (Table 1). To determine whether other herpesviruses were present in these samples, PCR was performed to detect viral sequences of HHV-6, HSV-1, HSV-2, CMV, and EBV. DNA from the

samples of Kaposi's sarcoma cells was negative for each of these sequences (Table 1). In addition, there was no evidence of mycoplasma, HIV, or adenovirus in freshly derived Kaposi's sarcoma cells or viral isolates (data not shown).

#### Propagation of Lytic Virus

Early-passage Kaposi's sarcoma cells were cocultured for two to seven days with various types of cells, including human epithelial-cell lines (HeLa, 293, and A431 cells), human umbilical-vein endothelial cells, porcine endothelial cells, and human or porcine vascular smooth-muscle primary cells. Genomic DNA was isolated from the cocultures and analyzed for KSHV DNA sequences by PCR. Consistently strong PCR signals were found on day 2 in DNA isolated from cocultures of Kaposi's sarcoma cells with 293 cells, whereas the DNA from the cocultures with other types of cells was negative. In the 293 cells, the PCR signal was detected as Kaposi's sarcoma cells were lost from the culture (on days 4 and 5) and was accompanied by progressively increasing signs of cytotoxicity in the cell culture (Fig. 1). Light-



**Figure 2.** Detection of KSHV DNA Sequences in 293 Cells after Coculture with Kaposi's Sarcoma Cells.

Viral DNA was detected by PCR in Hirt supernatants of 293 cells (Panel A) on the indicated days after infection, but not in uninfected cells, and with DNA isolated from the enriched subcellular fractions on day 3 (Panel B). In both panels, the arrows indicate the specific 233-bp DNA fragment; the plus signs, a positive control sample from a Kaposi's sarcoma lesion; and the minus signs, DNA from uninfected 293 cells. M denotes molecular-size marker. The amount of DNA used in each PCR analysis (1 or 0.1  $\mu\text{g}$ ) is shown.

Panel C shows the results of Southern blot analysis of genomic DNA (10  $\mu\text{g}$ ) isolated from uninfected and infected 293 cells. Viral DNA sequences were detected without PCR amplification from uninfected cells (lane 1) and from cells three days after infection with passage 5 virus (lanes 2 and 3), probed either without digestion (lane 2) or after digestion with *Bam*HI (lanes 1 and 3). The arrow indicates the 330-bp product expected after digestion with *Bam*HI.

Panel D shows an analysis of the cytotoxicity and PCR reactivity of 293 cells after incubation with cell-free lysates (passage 5) at the dilutions shown. In the study of reactivity, ethidium bromide staining and Southern blot hybridization were used. No signal was detected by Southern blot analysis of the PCR product in uninfected cells (see Panel A, lane 2, and Panel B, lane 13). The degree of cellular cytotoxicity was determined by light microscopy and graded as in Figure 1. PCR was performed with 1  $\mu\text{g}$  of DNA isolated on day 3 from the enriched nuclear fraction of infected cells. PCR fragments and genomic DNA were transferred to nylon membranes (Genescreen Plus, Dupont) and hybridized to oligolabeled probe derived from the 233-bp PCR fragment under standard conditions.

microscopical analysis of uninfected 293 cells showed viable epithelial cells with round nuclei that contained evenly distributed chromatin and prominent nucleoli (Fig. 1B). In contrast, after exposure to the infected-cell lysate, the 293 cells showed substantial cytopathic changes, beginning as early as day 2. Groups of infected 293 cells contained multinucleated giant cells (Fig. 1B). In addition, certain cells had nuclear molding or dense, chromatin-like material at the margin of the nucleus, and the chromatin was dissolving, with residual chromatin forming a margin along the nuclear membrane (Fig. 1B). By day 5, virtually all the cells underwent condensation of chromatin and other morphologic changes consistent with cell death (Fig. 1). These findings suggested that the positive PCR signals were probably due to transmission of virus rather than to persistent DNA sequences from the primary Kaposi's sarcoma cell line. PCR signals were variably observed in the human umbilical-vein endothelial cells, but there was no consistent cytopathic effect. We

therefore focused our efforts on the 293 cells as host cells for viral replication.

#### Propagation of Virus by Serial Infection of 293 Cells

To propagate the virus further, we prepared cell-free lysates from cocultures of Kaposi's sarcoma cells with 293 cells at various times after incubation, and the 293 cells were infected both in the presence and in the absence of tumor necrosis factor  $\alpha$ . No PCR signals were detected in the uninfected cells, but signals were readily detected in the infected 293 cells within one day after infection (Fig. 2A). Signals were detected more readily in the nuclear fraction and were weaker in the cytoplasm (Fig. 2B). Hirt supernatants — that is, episomal DNA isolated from infected cells — contained KSHV DNA sequences by PCR. The signals were maximal three days after the viral challenge, diminishing by day 5 (Fig. 2A).

Although virus could be detected in the cell-culture supernatants and passaged serially, the cell ly-

sates consistently had viral titers two to three orders of magnitude higher than the supernatants (data not shown), and the lysates were therefore used in further studies. With the PCR we could also amplify regions of the thymidine kinase gene, the putative principal capsid protein, and glycoprotein H. These regions were visualized by staining with ethidium bromide after 30 cycles in at least three separate experiments (data not shown). No PCR signals from other known herpesviruses were detected in serially passaged viral isolates, although they were detected readily in positive control tissues and cell lines infected with the relevant viruses. In particular, there was no PCR evidence of EBV in either the primary early-passage Kaposi's sarcoma cell lines or subsequent serially passaged virus (with as many as 20 passages) (data not shown).

Southern blot analysis of DNA prepared from enriched nuclei isolated from 293 cells confirmed that in passage 5 virus, viral DNA was amplified by a factor of 1000 or more after infection (Fig. 2C), as was consistent with the results of semiquantitative PCR (Fig. 2A and 2B). Moreover, when 293 cells were incubated with infected-cell lysates at increasing dilutions, the development of cytotoxicity correlated with the magnitude of the signal for KSHV that could be detected by Southern blot analysis of the PCR product (Fig. 2D). Cytotoxicity and positive PCR signals for KSHV were consistently maintained with viral titers of 1 million per milliliter or more, as determined by the dilution at which no cytotoxicity was seen, for approximately 20 serial infections of 293 cells. The transfer of the cytotoxic effect among the cell cultures and the PCR signals after serial passage were maintained consistently after the infected-cell lysates were filtered through a 0.45- $\mu$ m membrane, but these characteristics disappeared after inactivation by heat (data not shown). Under identical culture conditions, serial passages of KSHV derived from body-cavity-based lymphomas were unsuccessful, and no cytotoxicity was seen (unpublished data).

#### Replication of Viral DNA in Infected Cells and Sensitivity of Virus to Treatment with Nuclease

To passage the virus serially, we prepared cell lysates from infected 293 cells on day 3, before the generation of cytotoxicity. Although tumor necrosis factor  $\alpha$  enhanced viral replication during the coculture of primary Kaposi's sarcoma cells and 293 cells, it did not increase cytotoxicity or viral titers during serial passage of 293 cells and thus was not used further. Replication of viral DNA was confirmed by semiquantitative PCR. With this technique, as little as about 0.001 fg of viral DNA (or about one copy per 100,000 infected cells) could be detected. After one hour of incubation with infected-cell lysates from passage 15 (a  $10^{-2}$  dilution, containing 1000 to 10,000 viral-genome equivalents), followed by

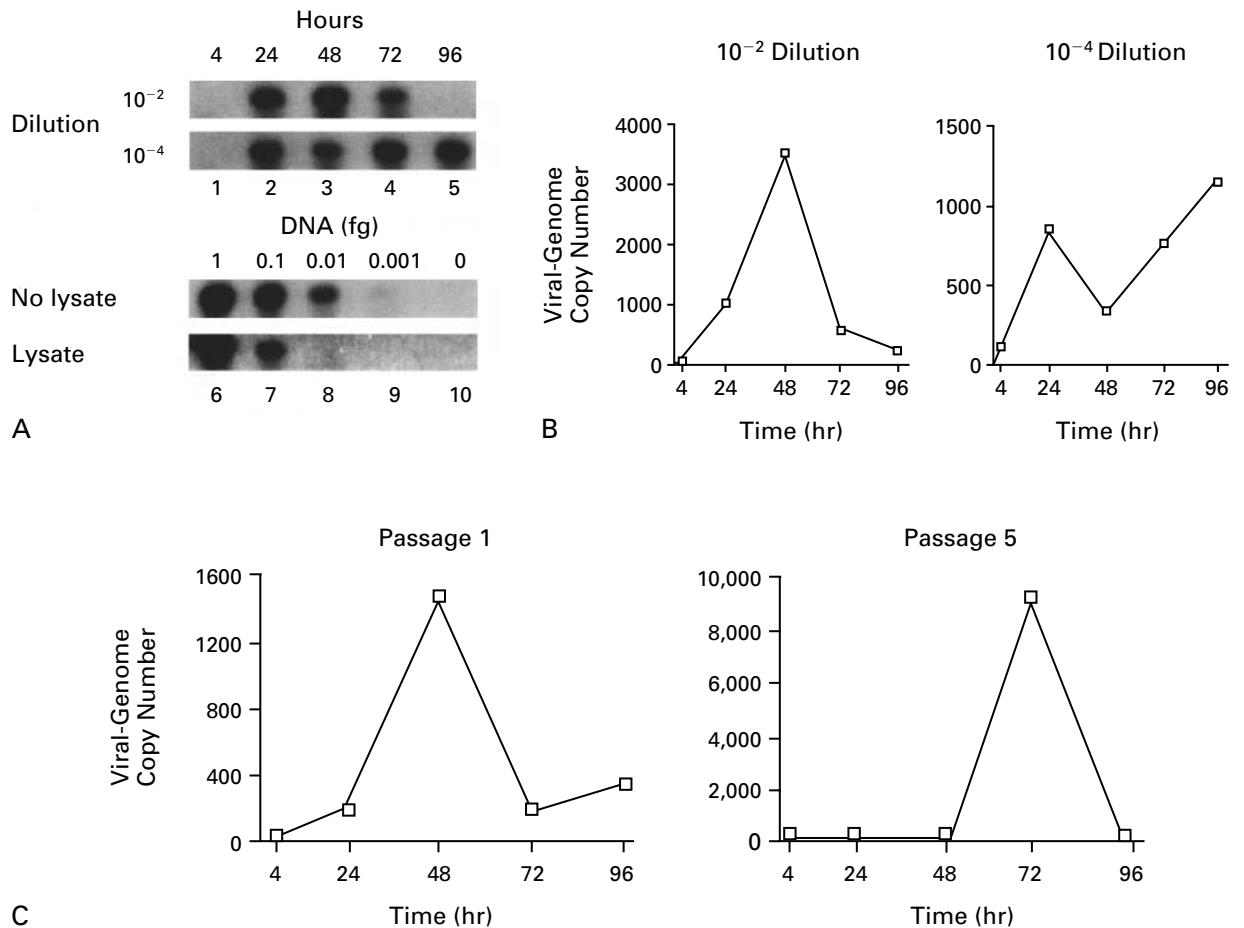
extensive washing with Dulbecco's modified Eagle's medium, viral DNA was barely detectable in the infected cells four hours after the removal of the viral supernatant. By 24 hours, a definite signal was detected. The peak replication was observed two days after infection, with amplification of viral DNA increased by at least three orders of magnitude.

Exposure to a smaller quantity of lysate in a  $10^{-4}$  dilution (containing 10 to 100 viral-genome equivalents) revealed sustained but decreased peak replication of viral DNA (Fig. 3A and 3B), which may have been due to a slower spread in the infected cell culture and to reduced cytotoxicity. No viral DNA was detected in uninfected cells (Fig. 2A and 3A). Thus, for the cultures of 293 cells exposed to infected lysates (passage 15), there was a period in which the viral titer was minimal, followed by one in which it increased exponentially, providing evidence that this virus is competent to replicate. Analysis of passage 1 and passage 5 virus showed a similar increase in the synthesis of viral DNA in infected 293 cells (Fig. 3C). These results are representative of those obtained with at least six infections in which virus from different passages (from passage 2 to passage 22) was studied by PCR. At least three primary isolates of the virus with similar properties have now been obtained and propagated (data not shown). Quantitation of the Southern blot signal suggested that there was a relatively low copy number of viral genomes in each cell (on average, 1 to 10 copies per cellular genome; data not shown), as is consistent with results of quantitative PCR and electron-microscopical data.

To determine whether the virus particles released from infected cells contained KSHV DNA, pelleted virus was subjected to treatment with DNase and pronase,<sup>11</sup> with or without prior exposure to NP40, and was then analyzed by PCR and Southern blotting. The KSHV DNA from passage 15 in the preparation treated with pronase and DNase was resistant to nuclease digestion. In contrast, no PCR signal was detected in the preparation exposed to NP40 before treatment with DNase and pronase (Fig. 4A). This finding provides evidence that the DNA is protected from the combined action of a protease and DNase by a virus-associated membrane. Furthermore, transmission of KSHV DNA in 293 cells was demonstrated at passage 22 after treatment with DNase alone (Fig. 4B), indicating that filtered and DNase-treated viral particles retained their infectivity. In this experiment, but not routinely, the preparation of virions was also treated with RNase-free DNase (1 U per milliliter, Promega). Similar results were observed with passage 15 virus.

#### Electron Microscopy of Infected 293 Cells

To further document the presence of the virus after serial propagation, we performed additional anal-



**Figure 3.** Replication of Viral DNA in 293 Cells and Analysis of Extracellular Viral Particles.

Panel A shows the course of viral propagation over time in infected 293 cells, as estimated by semiquantitative PCR. A Southern blot analysis of the PCR product amplified from DNA extracted from cells (passage 15) after infection with two dilutions of virus ( $10^{-2}$  and  $10^{-4}$ ) is shown at the top. DNA from cultures of infected cells was isolated at various times, extracted, precipitated with ethanol, and amplified by PCR. For comparison, a known amount of DNA (the same 233-bp PCR product) was quantitated in the presence and absence of cellular DNA lysate isolated from 100,000 uninfected cells. Viral-genome equivalents were calculated with standards containing cellular DNA. The quantity of the viral PCR product was standardized initially by ethidium agarose-gel electrophoresis and spectrophotometry. DNA from cells infected with virus at two dilutions ( $10^{-2}$  and  $10^{-4}$ ) was extracted 4, 24, 48, 72, and 96 hours after infection (lanes 1 through 5). Positive controls containing various amounts of DNA (the 233-bp KSHV PCR product from a Kaposi's sarcoma lesion) are shown in lanes 6 through 9, and a negative control sample with buffer alone is shown in lane 10. Samples purified by phenol-chloroform extraction were subjected to PCR as described in the Methods section. Specific hybridization is shown of PCR products from the infected cell extracts and the positive control DNA to the  $^{32}\text{P}$ -labeled 233-bp PCR product from a Kaposi's sarcoma lesion after transfer of the gel to nylon membranes.

Panel B shows copy numbers of viral genomes estimated by semiquantitative PCR at various times after infection, as quantitated from Panel A by phosphor imaging. The copy numbers were calculated on the basis of image analysis by a PhosphorImager (Molecular Dynamics) and compared with the standards in Panel A.

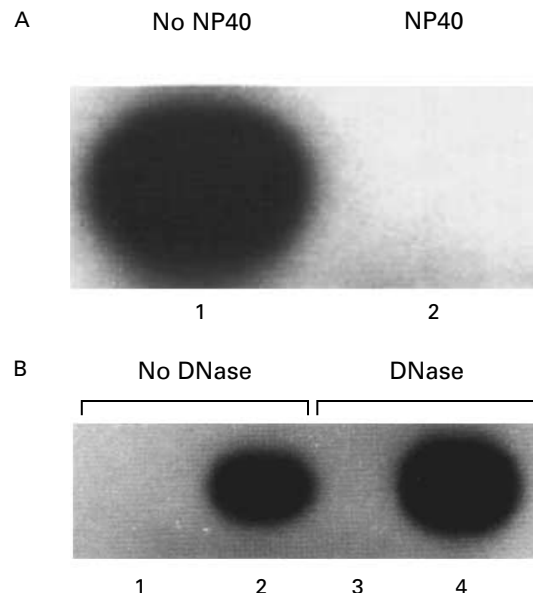
Panel C shows the kinetics of viral DNA synthesis in earlier-passage cells, derived from passages 1 and 5. In these infections, a  $10^{-2}$  dilution of cellular lysate prepared from approximately 2 million infected 293 cells was incubated with the same number of uninfected 293 cells.

yses using electron microscopy. In early-passage Kaposi's sarcoma cells, particles were found in the cytoplasm whose size and structure were consistent with those of herpes virions (Fig. 5A). Electron-microscopical analysis of 293 cells infected with viral lysate (passage 5) revealed multiple particles that resembled viral nucleocapsids (Fig. 5B). These viral nucleocapsids containing electron-dense cores (approximately 100 nm in diameter) were observed in nuclei of infected cells, but not those of uninfected cells, as soon as two days after exposure to lysate (Fig. 5C). Furthermore, enveloped virions (approximately 140 nm in diameter) were detected in the perinuclear cisternae and the cytoplasm of infected 293 cells (Fig. 5D and 5E). We estimated that about 1 percent of cells in the infected cultures contained herpesvirus-like particles, but none of the cells in the control cultures contained such particles (300 cells were analyzed in each group).

### DISCUSSION

Since DNA sequences associated with Kaposi's sarcoma were first described, several reports have either supported<sup>6-8,23-25</sup> or challenged<sup>26,27</sup> the notion that this novel DNA virus is important in Kaposi's sarcoma. We used early-passage cell lines from biopsy specimens of Kaposi's sarcoma skin lesions as a source of the virus and defined conditions that allowed it to be propagated in vitro. KSHV DNA sequences were detected by PCR in early passages of these cell lines (in six of seven primary cultures), and these sequences resembled previously described herpesvirus-like DNA sequences.<sup>28,29</sup> It is noteworthy that the PCR signal disappeared from the primary Kaposi's sarcoma cell lines by passage 3 or 4, suggesting that viral replication in these cultures was limited. In coculture experiments, the virus was transmitted to the embryonal-kidney epithelial-cell line 293 in a manner that correlated with the presence of the viral PCR signal. In addition, substantial cytotoxicity was observed, which could be due to the direct toxic effects of viral proteins on infected cells or, indirectly, to the release of cytokines, as noted in the case of EBV infections,<sup>30</sup> to the presence of soluble factors, or to the action of virus particles on uninfected cells in the culture. Because a small fraction of cells appear to be infected (0.1 to 1 percent) at the time cytotoxicity is observed, it is likely that this effect is largely indirect.

Independent evidence of the presence of the virus was obtained by analysis of viral DNA, studies of cellular morphology, and electron microscopy. Although replication of viral DNA was repeatedly demonstrated by PCR, the viral copy number was relatively low (1 to 10 copies per cell). For this reason, Southern blotting for the routine detection of virus was not feasible, although the virus was detect-



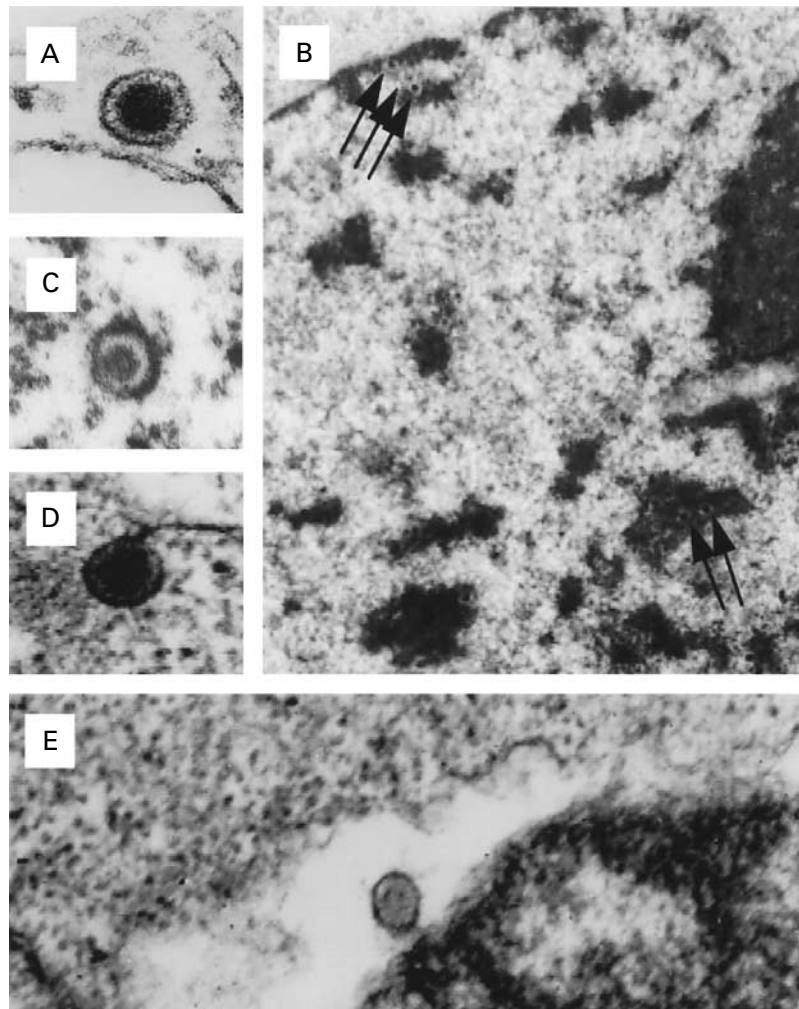
**Figure 4.** Nuclease Sensitivity Assays for Enveloped Virions Derived from Infected 293 Cells.

Panel A shows the results of an assay in which particles from the culture fluids (passage 15) of infected cells were concentrated and incubated both without NP40 (lane 1) and with NP40 (lane 2), as described.<sup>11,21</sup> Each preparation was then exposed to pronase and digested with DNase before the extraction of nucleic acid. The isolated DNA underwent PCR amplification, and specific hybridization of PCR products to the <sup>32</sup>P-labeled 233-bp KSHV DNA probe was performed as described in the Methods section.

Panel B shows the results of an assay in which particles from culture fluids (passage 22) of infected 293 cells were concentrated and incubated at 37°C for 30 minutes both with and without DNase. Equal numbers of 293 cells were exposed to each viral preparation, and DNA was extracted 4 hours (lanes 1 and 3) and 48 hours (lanes 2 and 4) after infection. Purified samples were amplified by PCR, and specific hybridization of PCR products was performed as described in the Methods section.

able by this technique in larger-scale preparations (Fig. 2C). The disappearance of viral DNA (Fig. 3B and 3C) was probably due to the cytotoxic effects observed in the culture, with the release of nucleases, proteases, and lipases that may degrade cell-associated viral DNA. Although KSHV appears to replicate with a low copy number, the features we observed are consistent with previous reports based on DNA analysis and in situ hybridization of Kaposi's sarcoma tissue.<sup>5,31</sup>

Renne et al.<sup>11</sup> have described a body-cavity-based lymphoma that contains KSHV. Contrary to Cesarman et al. in an earlier report,<sup>10</sup> they showed that a lymphoma that carries KSHV DNA is devoid of EBV sequences. Although the formation of virus was induced in a proportion of cells (5 to 10 percent) by treatment with phorbol 12-myristate 13-acetate,<sup>11</sup>



**Figure 5.** Electron-Microscopical Analyses of Cultures of Infected 293 Cells.

Panel A shows a herpesvirus-like particle in the cytoplasm of a passage 2 Kaposi's sarcoma cell ( $\times 70,000$ ). Panel B shows a 293 cell two days after exposure to infected cellular lysate. Margination of the chromatin is readily apparent, and nucleocapsids (arrows) are detectable within the nuclei ( $\times 12,900$ ). Panel C shows a higher magnification of a typical viral nucleocapsid with an electron-dense central core (approximately 100 nm,  $\times 67,840$ ). Panel D shows a representative enveloped virion in the cytoplasm of an infected 293 cell ( $\times 82,400$ ). Panel E shows an enveloped viral particle in the perinuclear cisternae of an infected 293 cell ( $\times 82,400$ ). The cells shown in Panels D and E were incubated with brefeldin A, as described in the Methods section.

serial passage of replication-competent virus was not demonstrated. Another report suggested that transient transmission of KSHV derived from an EBV-containing B-cell lymphoma line was observed, but that virus also could not be propagated serially.<sup>32</sup> We studied and propagated KSHV from primary Kaposi's sarcoma lesions. Further research is needed to clarify the relation between the viruses in body-cavity-based lymphomas and those in primary Kaposi's sarcoma lesions.

Despite our ability to propagate this virus *in vitro*

and its close association with the presence of Kaposi's sarcoma *in vivo*,<sup>33,34</sup> whether KSHV has a causal role in the disease remains uncertain. The ability to propagate KSHV should facilitate the development of diagnostic reagents, allow more detailed study of the association of the virus with Kaposi's sarcoma lesions, and improve our understanding of how people respond after exposure to the virus. Finally, it remains uncertain whether the pathogenesis of Kaposi's sarcoma derives from its ability to transform primary cells *in vivo* or whether the sarcoma repre-

sents an immune-mediated inflammatory and angiogenic response to virally infected cells. The fact that cloned viral isolates can be maintained and propagated should facilitate further study of the molecular regulation of the virus and help define its role in the causation and pathogenesis of Kaposi's sarcoma.

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## REFERENCES

- Beral V, Peterman TA, Berkelman RL, Jaffe HW. Kaposi's sarcoma among persons with AIDS: a sexually transmitted infection? *Lancet* 1990; 335:123-8.
- Safai B, Johnson KG, Myskowski PL, et al. The natural history of Kaposi's sarcoma in the acquired immunodeficiency syndrome. *Ann Intern Med* 1985;103:744-50.
- Beral V, Jaffe H, Weiss R. Cancer surveys: cancer, HIV, and AIDS. *Eur J Cancer* 1991;27:1057-8.
- Beral V. Epidemiology of Kaposi's sarcoma. *Cancer Surv* 1991;10:5-22. [Erratum, *Cancer Surv* 1992;12:225a.]
- Chang Y, Cesarman E, Pessin MS, et al. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* 1994; 266:1865-9.
- Huang YQ, Li JJ, Kaplan MH, et al. Human herpesvirus-like nucleic acid in various forms of Kaposi's sarcoma. *Lancet* 1995;345:759-61.
- Moore PS, Chang Y. Detection of herpesvirus-like DNA sequences in Kaposi's sarcoma in patients with and those without HIV infection. *N Engl J Med* 1995;332:1181-5.
- Ambroziak JA, Blackburn DJ, Herndier BG, et al. Herpes-like sequences in HIV-infected and uninfected Kaposi's sarcoma patients. *Science* 1995;268:582-3.
- Cesarman E, Chang Y, Moore PS, Said JW, Knowles DM. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. *N Engl J Med* 1995;332:1186-91.
- Cesarman E, Moore PS, Rao PH, Inghirami G, Knowles DM, Chang Y. *In vitro* establishment and characterization of two acquired immunodeficiency syndrome-related lymphoma cell lines (BC-1 and BC-2) containing Kaposi's sarcoma-associated herpesvirus-like (KSHV) DNA sequences. *Blood* 1995;86:2708-14.
- Renne R, Zhong W, Herndier B, et al. Lytic growth of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in culture. *Nat Med* 1996;2:342-6.
- Huang YQ, Friedman-Kien AE, Li JJ, Nickoloff BJ. Cultured Kaposi's sarcoma cell lines express factor XIIIa, CD14, and VCAM-1, but not factor VIII or ELAM-1. *Arch Dermatol* 1993;129:1291-6. [Erratum, *Arch Dermatol* 1993;129:1622.]
- Yang J, Xu Y, Zhu C, Hagan MK, Lawley T, Offermann MK. Regulation of adhesion molecule expression in Kaposi's sarcoma cells. *J Immunol* 1994;152:361-73.
- Eizuru Y, Inagawa S, Minamishima Y. Application of "Hirt supernatant" DNA to the molecular epidemiology of cytomegalovirus infections. *J Clin Microbiol* 1984;20:1012-4.
- Pater MM, Hyman RW, Rapp F. Isolation of herpes simplex virus DNA from the "Hirt supernatant." *Virology* 1976;75:481-3.
- Coen DM. Quantitation of rare DNAs by PCR. In: Ausubel FM, Brent R, Kingston RE, et al., eds. *Current protocols in molecular biology*. Vol. 2. New York: Greene Publishing, 1992:15.3.1-15.3.8.
- Sa'Adu A, Thomson BJ, Bountiff L, Webster AD. Lymphotropic viruses in "common variable" immunodeficiency — PCR analysis of lymphocyte DNA for HIV-1 and HHV-6. *Clin Exp Immunol* 1993;91:50-3.
- Cao M, Xiao X, Egbert B, Darragh TM, Yen TS. Rapid detection of cutaneous herpes simplex virus infection with the polymerase chain reaction. *J Invest Dermatol* 1989;92:391-2.
- Apolloni A, Sculley TB. Detection of A-type and B-type Epstein-Barr virus in throat washings and lymphocytes. *Virology* 1994;202:978-81.
- Duckett CS, Perkins ND, Kowalik TF, et al. Dimerization of NF- $\kappa$ B2 with RelA(p65) regulates DNA binding, transcriptional activation, and inhibition by an I $\kappa$ B- $\alpha$  (MAD-3). *Mol Cell Biol* 1993;13:1315-22.
- Lenhoff RJ, Summers J. Coordinate regulation of replication and virus assembly by the large envelope protein of an avian hepadnavirus. *J Virol* 1994;68:4565-71.
- Naidu YM, Rosen EM, Zitnick R, et al. Role of scatter factor in the pathogenesis of AIDS-related Kaposi's sarcoma. *Proc Natl Acad Sci U S A* 1994;91:5281-5.
- Lebbe C, de Cremoux P, Rybojad M, Costa da Cunha C, Morel P, Calvo F. Kaposi's sarcoma and new herpesvirus. *Lancet* 1995;345:1180.
- Dupin N, Grandadam M, Calvez V, et al. Herpesvirus-like DNA sequences in patients with Mediterranean Kaposi's sarcoma. *Lancet* 1995; 345:761-2.
- Whitby D, Howard MR, Tenant-Flowers M, et al. Detection of Kaposi sarcoma associated herpesvirus in peripheral blood of HIV-infected individuals and progression to Kaposi's sarcoma. *Lancet* 1995;346:799-802.
- Rady PL, Yen A, Rollefson JL, et al. Herpesvirus-like DNA sequences in non-Kaposi's sarcoma skin lesions of transplant patients. *Lancet* 1995; 345:1339-40.
- Lin JC, Lin SC, Mar EC, et al. Is Kaposi's-sarcoma-associated herpesvirus detectable in semen of HIV-infected homosexual men? *Lancet* 1995; 346:1601-2.
- Finesmith TH, Shrum JP. Kaposi's sarcoma. *Int J Dermatol* 1994;33: 755-62.
- Ioachim HL, Adsay V, Giancotti FR, Dorsett B, Melamed J. Kaposi's sarcoma of internal organs: a multiparameter study of 86 cases. *Cancer* 1995;75:1376-85.
- Jones K, Rivera C, Sgadari C, et al. Infection of human endothelial cells with Epstein-Barr virus. *J Exp Med* 1995;182:1213-21.
- Boshoff C, Talbot S, Kennedy M, O'Leary J, Schulz T, Chang Y. HHV8 and skin cancers in immunosuppressed patients. *Lancet* 1996;347: 338-9. [Erratum, *Lancet* 1996;348:138.]
- Moore PS, Gao SJ, Dominguez G, et al. Primary characterization of a herpesvirus agent associated with Kaposi's sarcoma. *J Virol* 1996;70:549-58.
- Kedes DH, Operskalski E, Busch M, Kohn R, Flood J, Ganem D. The seroepidemiology of human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus): distribution of infection in KS risk groups and evidence for sexual transmission. *Nat Med* 1996;2:918-24.
- Gao SJ, Kingsley L, Li M, et al. KSHV antibodies among Americans, Italians and Ugandans with and without Kaposi's sarcoma. *Nat Med* 1996; 2:925-8.