Evidence for a Multiclonal Origin of Multicentric Advanced Lesions of Kaposi Sarcoma


Kaposi sarcoma (KS) is a highly vascularized tumor that primarily affects the skin. It can also disseminate to lymph nodes and viscera during disease progression (1–3). Four clinicopathologic forms of KS have been described and designated as classic, endemic, iatrogenic, and epidemic (the epidemic form is due to human immunodeficiency virus [HIV] infection). In several African regions, epidemic-KS is the most frequently diagnosed tumor, and, in 2002, the number of KS cases worldwide was estimated to be approximately 65,000, or 1% of all diagnosed cancers (4).

Lesions of the four KS forms contain three major cell types: endothelial cells, spindle cells, and infiltrating inflammatory cells. Spindle cells derive from lymphatic endothelial cells and are the main cell type present in late-stage (i.e., nodular) lesions that form during disease progression (1–3). Four clinicoepidemiologic forms of KS can affect the skin. It can also disseminate to lymph nodes and viscera. Kaposi sarcoma (KS) is a highly vascularized tumor that primarily affects the skin. It can also disseminate to lymph nodes and viscera during disease progression (1–3). Four clinicoepidemiologic forms of KS have been described and designated as classic, endemic, iatrogenic, and epidemic (the epidemic form is due to human immunodeficiency virus [HIV] infection). In several African regions, epidemic-KS is the most frequently diagnosed tumor, and, in 2002, the number of KS cases worldwide was estimated to be approximately 65,000, or 1% of all diagnosed cancers (4).

Lesions of the four KS forms contain three major cell types: endothelial cells, spindle cells, and infiltrating inflammatory cells. Spindle cells derive from lymphatic endothelial cells and are the main cell type present in late-stage (i.e., nodular) lesions that form well-defined fascicles (5–8).

Human herpesvirus 8, also known as KS-associated herpesvirus (HHV-8/KSHV), is present in spindle cells at all KS stages (9,10). Discovered in a biopsy of a patient with epidemic-KS, HHV-8/KSHV is the only known human γ2-herpesvirus (11). Its genome consists of a 140-kb region that contains more than 90 genes and is flanked by multiple GC-rich terminal repeat (TR) sequences of approximately 65,000, or 1% of all diagnosed cancers (4).

Methods A series of 139 KS biopsies (from skin, lymph node, or tonsil) was obtained from 98 patients, with 59 biopsies from 18 patients with disseminated multicentric KS skin lesions. The degree of spindle cell infiltration in biopsies was established by direct observation of hematoxylin-eosin–stained sections, and HHV-8 viral load was quantified by real-time polymerase chain reaction. To determine cellular clonality, the size heterogeneity of the HHV-8-fused terminal repeat (TR) region was determined by probing of electrophoresed restricted genomic DNA from KS biopsies for the HHV-8 TR sequence.

Results HHV-8 clonality analysis was performed on the 62 samples for which sufficient DNA was obtained. Most samples corresponded to histologically nodular lesions with high spindle cell infiltration and high viral load. A clonal HHV-8 pattern was determined for 59 samples; 11 were found to be monoclonal and 48 were oligoclonal. The informative samples that were from disseminated KS skin lesions (n = 26, from six patients) were either monoclonal or oligoclonal, and the size of HHV-8 episomes varied between these samples.

Conclusion Although some tumor KS lesions were monoclonal expansions of HHV-8–infected spindle cells, most advanced lesions were oligoclonal proliferations. Furthermore, individual KS disseminated tumor skin lesions were found to represent distinct expansions of HHV-8–infected spindle cells. Thus, our results suggest that KS lesions, especially in patients with advanced skin tumors, are reactive proliferations rather than true malignancies with metastatic dissemination.
803 bp each. Although several hypotheses as to the individual roles of particular HHV-8 genes in KS pathogenesis have been proposed, the exact role of each HHV-8 gene in the initiation and progression of KS is still under investigation (9,12–15). The expression of HHV-8 latent genes (LANA-1, v-cyclin, v-FLIP) in nearly all tumor spindle cells and the small fraction (about 1%) of these cells that express markers of HHV-8 lytic replication (14,16) suggest that HHV-8 is in a latent state in KS.

The great majority of the human cancers are clonal pathologies, meaning that the tumor cells derive from a unique somatic cell following a process that involves immortalization and/or transformation. Thus, tumor cells exhibit identical markers, which can be different for each cancer. Analysis of these markers allows us to distinguish between a true cancerous lesion and one derived from a nonneoplastic expansion of cells. Clonality studies are thus valuable for understanding cancer pathogenesis and in the diagnostic and follow-up of the pathology (17,18). To date, the assessment of clonality in KS, using cellular clonality markers, has yielded conflicting results (19–24). Still to be resolved is whether multicentric clonality in KS, using cellular clonality markers, has yielded consistent results (19–24).

In virally induced tumors in general, the demonstration of a particular viral clonal pattern has been instrumental in linking a given virus to a specific tumor. One illustration of the value of this approach is the work that linked the human oncoretrovirus HTLV-I with adult T-cell leukemia. Because HTLV-I integrates its provirus randomly into host chromosomal DNA, monoclonal integration of HTLV-I provirus indicates the clonal proliferation of HTLV-I–infected cells (25). Therefore, demonstration of clonality of HTLV-I proviral DNA is essential to diagnosis of adult T-cell leukemia and to support that HTLV-I is the causative agent of such a tumor cell proliferation. Furthermore, in the case of herpesviruses, an approach that used the size of the Epstein-Barr virus (EBV)–fused TR region as a molecular marker for clonality demonstrated that EBV-associated nasopharyngeal carcinomas are monoclonal and showed that EBV infection precedes monoclonal expansion of some non-Hodgkin lymphomas including Burkitt lymphoma (26,27). Using a similar approach to study the viral role in etiology, and to use viral markers to assess clonality, our group has studied the size heterogeneity of the HHV-8–fused TR region as a viral marker in HHV-8–infected tumors and cell lines (28). The heterogeneity arises from fusion of particular TR sequences at opposite extremes of the viral genome when the virus establishes latency. The size of the newly created TR region is specific for each virus and thus for each infected cell and its descendants. Our initial study was performed on a small series of tumor biopsies, mostly from epidemic-KS patients for whom we did not have any available information on the degree of progression of the lesion (this progression can be determined by quantifying the levels of the spindle cell infiltration and the lesion’s viral load). Furthermore, despite the identification of some monoclonal lesions supporting a causative role of latent HHV-8 in a tumor, in most of the cases, we were not able to determine the viral clonality status. This might have been due to a low level of spindle cell infiltration in the lesions and thus insufficient viral DNA for molecular characterization (28).

**CONTEXT AND CAVEATS**

**Prior knowledge**

Whether Kaposi sarcoma (KS) lesions arise from transformation and proliferation of a unique somatic cell that is infected with human herpesvirus 8 (HHV-8) or from a nonneoplastic expansion of infected cells has been debated.

**Study design**

Molecular diagnostic techniques were used to characterize HHV-8 DNA from KS tumor samples for heterogeneity.

**Contribution**

The study presented evidence that most advanced lesions are reactive proliferations of multiple cells after distinct infections rather than malignancies deriving from a single infected and transformed cell. Similarly, separate disseminated tumor skin lesions in the same patient were found to often represent distinct expansions of multiple cells after multiple infections.

**Implications**

The etiology of KS is different in kind from that of the majority of cancers, which arise from immortalization of a single cell.

**Limitations**

Much additional work will be needed to understand how human HHV-8 causes KS.

The main goal of this study was to clarify the clonality status of KS lesions using a viral marker. To accomplish this, we expanded our preliminary work to a larger series of KS samples originating from the four KS forms and from patients of both sexes. Furthermore, we evaluated whether disseminated KS lesions (a frequent feature of this disease) correspond to metastatic lesions or to proliferation of independent clones. Single or multiple biopsies of KS lesions from skin, lymph nodes, and tonsil from 98 patients were studied. To determine the progression of the lesions, we characterized the level of spindle cell infiltration and the HHV-8 viral load for all samples.

**Patients and Methods**

**Tumor Samples and Pathologic Study**

The investigations were performed with institutional approval of the hospitals and departments involved in the study. After written informed consent had been obtained from each of the 98 patients, who had sought dermatologic services and presented with clinically diagnosed KS, tissue samples from these patients were biopsied to obtain a histologic diagnosis. Skin lesions were sampled by punch biopsy, and lymph node and tonsil samples were removed surgically. The biopsy specimens were stored frozen (at −20 °C or below). The histologic KS diagnosis was based on the presence of three main features: neoangiogenesis with slit-like vascular spaces, infiltration by inflammatory cells, and proliferation of spindle cells. Furthermore, for most of the samples, different classical stainings, including the use of an antibody specific for latent nuclear antigen (LANA; encoded by open reading frame 73), were performed as previously described to confirm the diagnosis (especially in difficult cases) (29).
The frozen specimens were sectioned (thickness = 5 µm). One section was stained with hematoxylin–eosin. The degree of spindle cell infiltration was established on these sections by direct observation. Tumor biopsy sections were stained with hematoxylin–eosin. The degree of spindle cell infiltration was assessed on these sections by direct observation. For each sample, 30–40 sections (20-µm thick) were cut for DNA extraction.

**Human Herpesvirus 8 Viral Load Quantification**

High–molecular weight DNA was extracted from tumor KS biopsy sections as previously described (28). Briefly, tumor biopsy sections were resuspended in lysis buffer containing 10 mM Tris–HCl (pH 8.0), 5 mM EDTA, 50 mM NaCl, 0.5% sodium dodecyl sulfate, and 200 µg/mL proteinase K (Invitrogen, Cergy-Pontoise, France). Samples were incubated overnight in lysis buffer at 65 °C. The DNA was purified by three consecutive extractions (phenol, phenol/chloroform [1 : 1 vol/vol], and chloroform), precipitated with isopropanol, and resuspended in buffer containing 10 mM Tris (pH 8.0) and 1 mM EDTA.

HHV-8 viral load was determined by real-time quantitative polymerase chain reaction (PCR) on a fragment of the open reading frame 26 using a previously described technique (30). The primers used in amplification corresponded to a 104-bp well-conserved sequence of open reading frame 26 (3S: CGG CCG ATA TTT TGG AGT AGA T; 3AS: CGC TAT TCT GCA GCA GCT TCG TG and 2AS: CGG CCG ATA TTT TGG AGT AGA T). The internal probe (KSHVS: CGC TAT TCT GCA GCA GCT TTG GTT GTA CCA) contained a dual-label composed of a fluorescer dye (FAM) at the 5' end and a quencher dye (TAMRA) at the 3' end (Eurogentec, Seraing, Belgium).

The TaqMan PCR mix contained Absolute QPCR mix (ABgene, Courtaboeuf, France), 15 pmol of forward and reverse primers, 10 pmol of the internal probe, and 5 µL of the template (equal to 500 ng of high–molecular-weight DNA) in a total volume of 50 µL. The amplification was performed in an ABI PRISM 5700 Sequence Detection System (PE Biosystem, Courtaboeuf, France) using the following sequence of steps: initial incubation for 2 minutes at 50 °C followed by 15 minutes at 95 °C to activate the DNA polymerase and then 45 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Each sample was amplified and quantified in duplicate. To normalize the viral load to the number of cells, we quantified in parallel in the same sample a 138-bp fragment of the human albumin gene (primers were A3: 5′ AAA CTC ATG GGA GCT GCT GGT T 3′ and A5: 5′ GCT GTC ATC TCT TGT GGG CTG T 3′ and internal probe was AlbS: FAM-CCT GTC ATG CCC ACA CAA ATC TCT CC-TAMRA) as described (31).

A previously generated standard curve was used to convert the fluorescence values from the albumin gene and HHV-8 amplification to viral copies per cell. The albumin gene standard was commercially provided (Novartis, Rueil Malmaison, France), and the HHV-8 standard was produced by cloning of the region of interest in the plasmid vector pCR2.1 (Invitrogen, Cergy-Pontoise, France). To determine the exact target number of PCR quantitative standards, we reproduced an analytical system distribution described by Wang et al. (32). Briefly, a DNA solution (containing about 100 000–300 000 copies of the target HHV-8 DNA per µL) was diluted to approximately one molecule per PCR, and 80 replicates of this solution were amplified by nested PCR. The real concentration of the diluted target was expressed in terms of signal-generating unit (SGU), the smallest volume element containing at least one PCR amplifiable template; the SGU value was determined in a Poisson distribution test using nested PCR positive and negative results. The SGU value allowed calculation of the real concentration of the undiluted HHV-8 standard.

**Terminal Repeat Analysis**

A description of the TR analysis has been reported previously (28). Briefly, during the HHV-8 lytic phase, viral genomes are replicated by a rolling-circle mechanism that produces linear genomes with varying numbers of direct tandem TRs at each terminus. Digestion of high–molecular-weight DNA with TaqI restriction enzyme cleaves the viral genome close to, but not within, the TR region and probing after Southern blotting with TR sequence generates a ladder of bands representing heterogeneous terminal fragments that vary in length by increments corresponding to a TR unit. When a latent infection is established, viral genomes are fused at the TR region, replicated accurately (with the same TR number), and maintained as multiple episomal copies by the cellular DNA polymerase. The same restriction and Southern blot analysis described above thus generates fragments corresponding to the fused TR region. Due to the variability in the number of TR units incorporated during circularization of the viral genome, independent infection events can be identified as differently sized TR-containing fragments with the presence of a unique fragment corresponding to a monoclonal population and heterogeneous profiles corresponding to oligoclonal populations.

The methods used for the HHV-8 TR analysis have been described in detail previously (28). Briefly, genomic high–molecular-weight DNA (10–30 µg) was digested at 65 °C with 4 U/µg of TaqI SX (New England Biolabs, Ipswich, MA) for 4 hours. Digested DNA fragments were separated on 1% agarose gel by pulsed-field gel electrophoresis (PFGE), transferred by capillarity onto a Biodyne B 0.45-µm nylon membrane (Pall Corporation, New York, NY), and hybridized with 50 ng of [32P]-labeled HHV-8 TR probe (803-bp pI7I fragment) (28,33).

**Results**

**Pathologic Characterization of the Kaposi Sarcoma Samples**

One hundred and thirty-nine biopsies were obtained from 98 patients presenting different forms of KS (Table 1). In 80 cases, only one KS biopsy was obtained for each patient, while in 18 patients with disseminated multicentric lesions, two or more KS biopsies were obtained. The origins of the 59 biopsies from these 18 patients we studied were as follows: 12 originated from six patients with epidemic-KS, nine came from four classical-KS patients, 23 were from four endemic-KS patients, and 15 were from four patients with iatrogenic-KS. Of the 11 lymph node biopsies, all but two originated from patients with epidemic-KS, as did the tonsil biopsy.

Histopathologic study of these clinically diagnosed KS lesions (Fig. 1) confirmed the diagnosis in all cases with typical features.
(neoangiogenesis with slit-like vascular spaces, infiltration by inflammatory cells, and proliferation of spindle cells) and/or specific LANA immunolabeling (Fig. 2). However, no infiltration by spindle cells was detected in 19 of the skin samples (most of which were from patients with epidemic-KS), and these were thus classified as “−.” These 19 biopsies corresponded mainly to early plaque-stage lesions with proliferation of irregular dilated endothelial lined spaces in the superficial dermis (Figs. 1 and 2). Histologic analysis indicated that 60 of the 127 skin lesions were highly infiltrated by spindle cells (++) in addition, 19 skin biopsies had only a few spindle-shaped cells and thus were considered as “+/−,” and 29 had scattered groups or few fascicles of typical spindle cells (+, Fig. 2). Furthermore, nine of the 11 lymph node biopsies and the tonsil biopsy were found to be highly infiltrated (+++) (Table 2).

**Human Herpesvirus 8 Clonality Pattern**

HHV-8 clonality analysis using PFGE was performed on the 62 of 139 samples (52 skin biopsies, nine lymph node biopsies, and one tonsil biopsy) for which sufficient (10–30 µg) high–molecular-weight DNA was available (Table 2). Samples in which there were scattered groups or few fascicles of typical spindle cells (+) or massive infiltration by large fascicles of spindle cells (++) constituted the great majority (61/62) of the studied samples (Table 2), and most of these corresponded to advanced nodular skin or lymph node lesions. Furthermore, 70% of these 62 samples had a high viral load (>5 copies/cell), 27% had an intermediate viral load (1–5 copies/cell), and only 3% had a low viral load (<1 copy/cell) (Table 2).

A clonal pattern (defined as the presence of at least one visible sharp TR band) was observed for 59 samples (49 skin, nine lymph node, and one tonsil). In the three other samples for which sufficient DNA was obtained (KS33, KS34, KS44), no band was seen after hybridization with the HHV-8 TR-specific probe (negative samples in Table 2). In these three cases, we cannot eliminate the possibility that there are multiple small clones of HHV-8–infected spindle cells in the lesions, none of them detectable due to the small quantity of DNA that each contributed to the sample and the low sensitivity of the Southern blot technique used.

Among the 59 samples from which we obtained a clonal pattern, 11 skin biopsies were classified as monoclonal (because a unique clear, strong band was observed) and 48 biopsies (38 skin biopsies, nine lymph nodes, and one tonsil) were classified as oligoclonal due to the presence of more than one sharp band (Figs 3 and 4 and data not shown). In some oligoclonal tumors,
Fig. 2. Four levels of spindle cell infiltration in skin biopsies from patients with Kaposi sarcoma (KS). (−) = no evidence of typical spindle cells; (+/−) = only very few spindle-shaped cells observed; (+) = scattered groups of few fascicles of typical spindle cells; and (++) = massive infiltration by large fascicles of spindle cells. Paraffin-embedded sections were cut at 5 µm thickness and A) stained with hematoxylin–eosin (HE) or B) incubated with an antibody against the latent nuclear antigen (LANA) coded by the open reading frame 73 of human herpesvirus 8, which is nearly always expressed in spindle cells present in KS lesions. The number of samples studied for each level of spindle cell infiltration is indicated.

Discussion

Despite recent insights into KS pathogenesis, the question of whether or not KS is a reactive process (i.e., nonneoplastic tumor cell proliferation mediated by different factors including cytokines and lymphokines) or a true malignant proliferation of spindle cells remains unclear (9,12,34,35). The aggressiveness and rapid evolution of some endemic-KS tumors, as suggested by the lymph node involvement in children and the observation of epidemic-KS with disseminated and visceral lesions, suggest that KS is a malignant process (1). However, the chronic and slow evolution of classic-KS lesions, the partial reversibility of iatrogenic-KS lesions after diminution of immunosuppressive regimens, and complete epidemic-KS regression after anti-retroviral therapy seem more consistent with reactive proliferation (36–39).

Further evidence supporting the designation of KS as a reactive process derives from the many differences between KS spindle cells and standard tumor cells (40). KS spindle cells are typically diploid, even in advanced lesions, and lack the genetic instability usually observed in tumor cells. In the majority of KS tumors, no

tumor with such a degree of spindle cell infiltration. Six samples were informative as to clonality (Table 2). All exhibited different oligoclonal patterns with a large number of bands that varied greatly in size and intensity (Fig. 4). Finally, in the case of the KS107 biopsy, a lytic profile—a ladder of bands representing heterogeneous terminal fragments whose molecular weights were less than 6 kb and varied by increments that corresponded to the molecular weight of a TR unit—was observed (Fig. 4).

A 65-year-old man (ID85) developed iatrogenic-KS lesions in 1995, 12 years after a renal transplant. Five skin biopsies (from different lesions on the left leg) were obtained: three in 1999 and two in 2002. The four found to have a high viral load used for clonality testing (Table 2). The three lesions obtained in 1999 exhibited different oligoclonal patterns, but each was characterized by a strong band of similar size, accompanied by a second fainter band that differed in size between the samples. The fourth sample, obtained in 2002, exhibited faint bands of variable size (Fig. 4).

Clonal Pattern of Multicentric Lesions

To study the clonal pattern of multicentric KS lesions (Fig. 1, E), we obtained multiple biopsy samples (n = 59) from 18 patients with disseminated skin lesions. These lesions came from different skin sites and in some cases had been biopsied years apart (Table 2 and data not shown). Sufficient high–molecular-weight DNA for PFGE and determination of the clonal pattern was obtained in 26 samples originating from six different patients (Table 2). The clinical and virologic results for four patients for whom HHV-8 clonality patterns are presented in Fig. 4 are as follows.

In a 30-year-old woman with AIDS (ID11), two skin biopsies (one from leg [KS12] and the other from the back [KS13]) were studied. Both lesions were highly infiltrated by spindle cells and had a very high viral load (Table 2) and exhibited an oligoclonal pattern with no evidence of a common clone (Fig. 4).

An 82-year-old man (ID73) experienced endemic-KS for 10 years. Seven skin biopsies (mainly nodular from the either the foot or the leg) were studied. Six were highly infiltrated by spindle cells and exhibited a high HHV-8 load; in one, fewer spindle cells were observed despite a high viral load. Five of his samples were studied for clonality (Table 2); two were monoclonal, and the others each exhibited a different oligoclonal pattern (Fig. 4).

The third patient was a 62-year-old man (ID78) who developed endemic-KS lesions on the left foot about 15 years ago. He was treated by radiotherapy, but multiple new lesions appeared subsequently including knee lesions in 2004 (Fig. 1, E). Seven skin biopsies from nodular lesions from the knee area were performed (four in 2004 and two in 2005 and one from the hand in 2005). All lesions were highly infiltrated by spindle cells, and, in most of the cases, they exhibited viral loads slightly lower than expected for a

one predominant band was associated with a few bands of lower intensity (e.g., ID38/KS43 and ID51/KS57 in Fig. 3). In the majority of the oligoclonal patterns, five bands or fewer could be clearly identified with just a few cases showing 10 or more clear bands (e.g., ID78/KS102 in Fig. 3). The oligoclonal patterns consisted of DNA fragments that ranged in size from 2 to 60 kb (Figs 3 and 4 and data not shown).

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<td>93</td>
<td>Skin</td>
<td>Classic</td>
<td>6.66</td>
<td>++</td>
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<td>M</td>
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<td>2.41</td>
<td>++</td>
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</tr>
<tr>
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<td>KS72</td>
<td>M</td>
<td>59</td>
<td>Skin</td>
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<td>++</td>
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<td>KS75</td>
<td>M</td>
<td>60</td>
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<td>4.63</td>
<td>++</td>
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</tr>
<tr>
<td>ID72</td>
<td>KS83</td>
<td>M</td>
<td>69</td>
<td>Skin</td>
<td>Endemic</td>
<td>0.44</td>
<td>++</td>
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</tr>
<tr>
<td>ID73</td>
<td>KS88</td>
<td>M</td>
<td>83</td>
<td>Skin</td>
<td>Endemic</td>
<td>13.42</td>
<td>++</td>
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</tr>
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<td>ID74</td>
<td>KS99</td>
<td>M</td>
<td>83</td>
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<td>Endemic</td>
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<td>++</td>
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</tr>
<tr>
<td>ID77</td>
<td>KS99</td>
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<td>60</td>
<td>Skin</td>
<td>Endemic</td>
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<td>+/-</td>
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<tr>
<td>ID78</td>
<td>KS109</td>
<td>M</td>
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<td>Endemic</td>
<td>9.89</td>
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<td>ID85</td>
<td>KS124</td>
<td>M</td>
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<td>Iatrogenic</td>
<td>10.88</td>
<td>++</td>
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<tr>
<td>ID86</td>
<td>KS125</td>
<td>M</td>
<td>65</td>
<td>Skin</td>
<td>Iatrogenic</td>
<td>16.59</td>
<td>++</td>
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<tr>
<td>ID87</td>
<td>KS126</td>
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<td>Iatrogenic</td>
<td>14.16</td>
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<td>KS130</td>
<td>F</td>
<td>53</td>
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<td>KS133</td>
<td>M</td>
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<td>29.59</td>
<td>++</td>
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<td>+</td>
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<td>31</td>
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<td>1.23</td>
<td>++</td>
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<td>Epidemic</td>
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<td>++</td>
<td>Oligo</td>
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<td>KS137</td>
<td>M</td>
<td>62</td>
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<td>++</td>
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<td>M</td>
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<td>Tonsil</td>
<td>Epidemic</td>
<td>17.10</td>
<td>++</td>
<td>Oligo</td>
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</table>

* KS = Kaposi sarcoma; ID = identification; HHV-8 = human herpesvirus 8; F = female; M = male; oligo = oligoclonal; mono = monoclonal; neg = negative results.
† +/− = very few spindle-shaped tumor cells; + = some scattered groups or few fascicles of typical spindle cells; ++ = massively infiltrated (well-formed aggregates and large fascicles of spindle cells).
characteristic chromosomal abnormalities were found. Moreover, in vitro, spindle cells display few features commonly associated with transformed cells (40). Finally, in contrast to the monoclonal origin of conventional tumors, some of the data obtained on KS suggest that the lesions are of oligoclonal origin (22).

The first attempts to determine whether KS has a monoclonal or polyclonal origin were based on the pattern of X chromosome inactivation, as evidenced by the methylation status of the androgen receptor gene (19–24). A sensitive PCR-based assay was performed on microdissected KS tumor lesions. All the lesions were from female patients, and all of them contained a large proportion of spindle cells. In the first study, the monoclonal nature of the epidemic-KS lesions was demonstrated in two of the three patients studied (23). Subsequently, the same authors provided strong evidence for the monoclonal origin of multicentric KS lesions by studying 32 different tumors from eight HIV-infected women with disseminated nodular skin lesions (24). However, these findings soon elicited controversy (20,21). Indeed, another group studying skin KS lesions from seven women with either classic or epidemic-KS found a polyclonal pattern of inactivation and concluded that KS is characterized by polyclonal cellular proliferation (19). Finally, Gill et al. (22) found evidence for the multiclonality of multicentric KS lesions in studying 24 biopsies from 12 women, 10 of whom had been diagnosed with epidemic-KS.

The history of conflicting results might be explained in part by the fact that the assay to determine the methylation status of the androgen receptor gene can be biased by infiltration of the lesion with nontumor cells; that is, using this technique, a tumor can be scored as polyclonal if, as is often the case in KS lesions, it contains a sufficient number of nontumor cells. Furthermore, the authors of these papers studied tumors at different stages (advanced tumor as well as initial plaque process), so tumors would have different levels of infiltration by spindle cells (19). Finally, reliance on an assay of the methylation status of the androgen receptor gene limits KS studies because it can only be applied to women, even though, in western countries, classic-KS, endemic-KS, and epidemic-KS occur mainly in men. It should also be noted that in the two largest studies (22,24), a substantial proportion (36% and 57%, respectively) of the biopsies were not informative using this approach.

In view of the discordant results of previous studies, we decided to use the HHV-8 TR as a marker to study KS clonality, thus adopting a strategy used previously with EBV and its associated tumors (26,27). Using a small series that consisted of mostly...
epidemic-KS, we showed that some KS lesions were monoclonal tumors (28). This led us to conduct the current study in which we determined the viral clonality status of a larger series of KS samples originating from the four KS forms and from patients of both sexes. In addition, we investigated whether multicentric KS lesions arose from independent clones or corresponded to metastatic lesions originating from the same clonal proliferation.

The main limitations of this study were due to the technique that we used to analyze the viral clonality. The method requires a substantial amount of DNA of high molecular weight that cannot be obtained from all KS biopsy samples. Typically only highly spindle cell–infiltrated lesions are informative. Moreover, fixed biopsies cannot be used for such a technique providing, generally, fragmented nucleic acid.

To ensure that we had a large series of informative tumors for such a technique (i.e., samples with high infiltration by spindle cells and sufficient quantity and quality of high–molecular-weight DNA for PFGE), we first estimated the level of spindle cell infiltration. Histological analysis indicated that half of the samples (70/139) were highly infiltrated by spindle cells (+). Furthermore, sufficient DNA for study of clonality was available in 62 of the 139 samples, and these informative samples originated mostly from highly infiltrated tumors with high HHV-8 viral load.

We then determined the number of TR in the fused termini of HHV-8 episomes in these well-characterized KS samples to assess the viral clonality of these spindle cell proliferations in vivo. Our results indicated the following. 1) Advanced KS lesions could exhibit either an oligoclonal (in 82% of lesions) or a monoclonal (in 18% of the lesions) pattern of HHV-8 episomes (Figs 3 and 4). Among the oligoclonal patterns, the sizes and intensities of the bands were quite variable and frequently a few (one to three) major bands were present along with fainter ones. 2) HHV-8 oligoclonal patterns were found in all four clinico-epidemiologic KS forms, in skin lesions, as well as in lymph node or tonsil tumor lesions and both in women and men.

These data indicated that most of the advanced KS lesions were oligoclonal expansions of HHV-8–infected cells, with several different HHV-8–infected clones coexisting at the same time within a single clinical KS lesion. However, true monoclonal expansions of HHV-8–infected spindle cells, although rare, were present in KS lesions. These findings suggest that, at least in some tumors, the virus was present before monoclonal expansion of spindle cells and therefore they support an etiologic role of HHV-8 infection in spindle cell proliferation in vivo. Herpesvirus-induced multigenotypic proliferation recalls EBV postransplant lymphoproliferative diseases, which can be polyclonal, oligoclonal, or monoclonal in origin (41–44).

Based on the analysis of 26 informative samples from six patients with disseminated skin tumors, it is clear that KS lesions occurring at different anatomic sites in a given patient (and even if sampled years apart) are generally found to harbor HHV-8 episomes of different sizes, with some lesions being monoclonal and others oligoclonal (Table 2 and Fig. 4). Different episomes at different lesions strongly suggest that disseminated lesions represent multiple distinct primary expansions of HHV-8–infected spindle cells originating from different infectious events rather than metastatic proliferations.

In conclusion, Gill et al. (22) were the first to suggest the multiclonality of multicentric KS in a series of epidemic-KS lesions. However, their data were in disagreement with those obtained by Rabkin et al. (24), who argued for the monoclonality of multicentric KS lesions using the same experimental procedure. Our study was based on a very different assay and including three of the four clinico-epidemiologic forms (epidemic-KS, iatrogenic-KS, and endemic-KS), thus providing evidence that multifocal KS lesions arise from independent clones. These findings of multiclonality in multicentric KS are similar to the situation found in several postransplant lymphoproliferative diseases, and also in some other EBV-associated lymphomas occurring in AIDS patients, where multifocal tumors with different clones are found at different tissue sites (41–43).

References


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