4. Other Data Relevant to an Evaluation of Carcinogenesis and its Mechanisms

4.1 Kaposi’s sarcoma

4.1.1 Cell biology

4.1.1.1 Origin of spindle cells

Spindle cells surrounding slit-like spaces are characteristic of advanced Kaposi’s sarcoma lesions. Endothelial cells (either vascular or lymphatic endothelium), cells from venous lymphatic junctions, fibroblasts, smooth-muscle cells, dermal dendrocytes and macrophages have all been proposed as possible progenitors of Kaposi’s sarcoma spindle cells (reviewed by Roth et al., 1992; Stürzl et al., 1992a; Browning et al., 1994; Kaaya et al., 1995). Like normal lymphatic endothelial cells, spindle cells stain with the monoclonal antibody EN-4, which detects both vascular and lymphatic endothelium, but lack reactivity with the monoclonal antibody Pal-E, which reacts with blood-vessel but not lymphatic endothelial cells (Rappersberger et al., 1990). Other markers for blood-vessel endothelium, such as OKM-5 and anti-factor VIII-related antigen (von Willebrand factor), stain Kaposi’s sarcoma endothelial or spindle cells, although varying results have been reported by different laboratories. Studies with Ulex europaeus agglutinin 1, another marker for endothelial cells, have also produced contradictory results (Nadji et al., 1981; Modlin et al., 1983; Little et al., 1986; Rappersberger et al., 1990; further references in Roth et al., 1992). Ultrastructural examination failed to show the presence of Weibel-Palade bodies, the storage vesicles for von Willebrand factor and therefore a characteristic feature of vascular endothelium, in spindle cells from Kaposi’s sarcoma lesions (Rappersberger et al., 1990). Staining with the monoclonal antibody BMA 120, which reacts with an antigen on endothelial cells, lends support to an endothelial origin of Kaposi’s sarcoma cells (Roth et al., 1988). Spindle cells and endothelia lining vascular spaces in Kaposi’s sarcoma lesions express leukocyte adhesion molecule 1 and thrombomodulin, which are markers of lymphokine-activated endothelial cells (Zhang et al., 1994). This observation further supports the notion that Kaposi’s sarcoma spindle cells are of endothelial origin and are activated by growth factors (see below).

The staining of spindle cells with antibodies to CD14, CD68 and factor XIIIa, observed by some laboratories, has been interpreted as reflecting a link between these spindle cells and cells of the monocyte/macrophage lineage, possibly dermal dendrocytes (Nickoloff & Griffiths, 1989; Rappersberger et al., 1990). These cells are distinct from Langerhans cells (Nickoloff & Griffiths, 1989). The staining of cultured Kaposi’s sarcoma spindle cells with an antibody to smooth-muscle α actin (Weich et al., 1991) and similar histochemical findings have been interpreted to suggest a relationship with smooth-muscle cells or myofibroblasts (reviewed by Roth et al., 1992). These discrepant results suggest either that cells of different lineages can adopt a spindle-like morphology or that these markers are common to different cells of mesenchymal origin and Kaposi’s sarcoma spindle cells derived from pluripotent mesenchymal progenitor cells. Currently,
CD34 (as detected by monoclonal antibody QBEND 10) is considered the best marker for Kaposi's sarcoma spindle cells (Russell Jones et al., 1995).

Cells expressing markers characteristic for vascular or lymphatic endothelium from Kaposi's sarcoma lesions have been cultured in a number of laboratories (Delli Bovi et al., 1986; Nakamura et al., 1988; Roth et al., 1988; Siegal et al., 1990; Corbeil et al., 1991; Herndier et al., 1994), but cultures expressing smooth-muscle α actin (Albini et al., 1988; Wittek et al., 1991) and mixed populations (Siegal et al., 1990; further references in Roth et al., 1992) have also been reported. The lineage of these cultured cells has been defined by staining for similar markers as in studies in situ, notably vimentin and cytokeratin (to discriminate mesenchymal and epithelial cells, respectively), endothelial markers such as von Willebrand factor, Pal-E, OKM-5, BMA 120 (specific for blood-vessel endothelium), EN-4 and Ulex europaeus agglutinin 1 lectin (which reacts with blood-vessel and lymphatic endothelium), CD14 and factor XIIIa (for the monocyte/macrophage lineage), smooth-muscle α actin (smooth muscle and myofibroblasts) and others (reviewed by Roth et al., 1992; Stürzl et al., 1992a; Kaaya et al., 1995). Spindle-shaped cells showing moderate expression of endothelial antigens have been cultured from peripheral blood of Kaposi's sarcoma patients (Browning et al., 1994).

4.1.1.2 Vascular lesions induced by Kaposi's sarcoma cell cultures in nude mice

The various cell cultures established from Kaposi's sarcoma lesions differ in their ability to induce angiogenic lesions in nude mice. [The Working Group noted that the similarity of some of these lesions to Kaposi's sarcoma is controversial.] A cell line expressing endothelial markers, established by Siegal et al. (1990) and studied by Herndier et al. (1994), induced Kaposi's sarcoma-like tumours of human origin in nude mice. This cell line expressed the endothelial markers factor VIII, EN-4 and Ulex europaeus agglutinin 1 lectin. In addition, it produced high levels of urokinase plasminogen activator and plasminogen activator inhibitor 1 (Herndier et al., 1994). Interestingly, plasminogen activator has been shown to be involved in the development of endothelial tumours in mice transgenic for the polyoma middle-T protein (Montesano et al., 1990). A second cell line capable of causing tumours of human origin in nude mice has also been described (Lunardi-Iskandar et al., 1995). The development of these cell lines suggests that a subpopulation of cells in Kaposi's sarcoma lesions may have progressed to a malignant phenotype; however, they do not contain KSHV/HHV8 DNA, and their relationship to spindle cells containing this virus (see section 4.5.1) is unclear.

A few other Kaposi's sarcoma cell cultures, also of an endothelial phenotype, are angiogenic in vivo but induce the growth of 'Kaposi's sarcoma-like' vascular lesions of murine origin when inoculated into nude mice (Nakamura et al., 1988; Salahuddin et al., 1988). Spindle-shaped cells grown from the peripheral blood of Kaposi's sarcoma patients have also been reported to induce angiogenesis in nude mice (Browning et al., 1994). Although these cultures were not examined for KSHV/HHV8, similar cultures established more recently did contain viral DNA (Sirianni et al., 1997).
Most other cell cultures, established by several laboratories (Roth et al., 1988), were not angiogenic in nude mice (Delli Bovi et al., 1986; Albini et al., 1988; Roth et al., 1988; Wittek et al., 1991); furthermore, KSHV/HHV8 is either not present or is rapidly lost upon serial passage from such cultures (Ambroziak et al., 1995; Lebbé et al., 1995).

4.1.1.3 Growth factors involved in proliferation of spindle cells

The role of growth factors in the development of Kaposi’s sarcoma has been studied in several laboratories, with inconsistent findings, probably because of the use of different cell types. The role of growth factors in the development of Kaposi’s sarcoma in vivo and their interaction with KSHV/HHV8 are still unknown.

(a) Fibroblast growth factors

Basic fibroblast growth factor (FGF) is secreted by Kaposi’s sarcoma cultures expressing endothelial cell markers and may promote the growth of these cells in vitro (Ensoli et al., 1989). Other groups working with Kaposi’s sarcoma cultures of either endothelial phenotype (Corbeil et al., 1991) or mixed fibroblastoid/endothelial appearance (Werner et al., 1989) also found FGF-like activity in supernatants of these cultures which stimulated the growth of normal fibroblasts and endothelial cells.

Members of the FGF family, including basic FGF and endothelial cell growth factor, are known to stimulate the growth of normal endothelial cells, and cultured Kaposi’s sarcoma cells with endothelial characteristics have been shown to induce transient neo-angiogenesis in nude mice (Nakamura et al., 1988). The FGF family of cytokines may thus play a crucial role in the development of Kaposi’s sarcoma. Expression of basic FGF and FGF5 has been shown to occur in spindle cells of Kaposi’s sarcoma by in-situ hybridization (Xerri et al., 1991). Acidic FGF and FGF6 are also expressed in these lesions (Li et al., 1993), but the technique employed in this study (RT-PCR) did not permit identification of the cell type(s) that secrete(s) these two members of the FGF family. The importance of basic FGF in the development of experimental Kaposi’s sarcoma-like lesions is further supported by the report that basic FGF-specific antisense oligonucleotide can inhibit the angiogenic effect of cultured Kaposi’s sarcoma cells in nude mice (Ensoli et al., 1994a).

(b) Platelet-derived growth factor

Both normal endothelial cells (Ensoli et al., 1989; Roth et al., 1989) and short-term cultures of Kaposi’s sarcoma cells with endothelial characteristics (Ensoli et al., 1989) produce platelet-derived growth factor (PDGF). Cultures that produce this factor thus do not require exogenous PDGF to promote proliferation (Ensoli et al., 1989; Corbeil et al., 1991). Short-term cultures were also shown to express mRNA for the receptors for PDGF-A and PDGF-B (Roth et al., 1989; Werner et al., 1990). Kaposi’s sarcoma spindle cells express mRNA for the PDGF-β receptor in vivo, whereas mRNAs for PDGF-A and PDGF-B were expressed on some tumour cells located in the vicinity of slit-like spaces (Stürzl et al., 1992b). PDGFs may therefore play a role in the pathogenesis of Kaposi’s sarcoma.
(c) **Interleukin-1**

IL-1 has also been reported to be secreted by cultured Kaposi's sarcoma cells of the endothelial phenotype (Ensoli et al., 1989; Corbeil et al., 1991) and to have a potent stimulatory effect on these cells (Nakamura et al., 1988).

(d) **Interleukin-6**

Cultured Kaposi's sarcoma cells of an endothelial phenotype secrete and proliferate in response to IL-6 (Miles et al., 1990; Corbeil et al., 1991). The expression of receptors for IL-6 on cultured cells has also been reported (Miles et al., 1990), and Kaposi's sarcoma cells expressed IL-6 mRNA in vivo (Gillitzer & Berger, 1991). The former observation is of particular interest in the context of the IL-6 homologue encoded by KSHV/HHV8 (v-IL-6; see section 1.1); however, the v-IL-6 homologue is expressed only rarely in KSHV/HHV8-infected Kaposi's sarcoma spindle cells (Moore et al., 1996b), suggesting that it does not play a major role in the pathogenesis of this disease.

(e) **Tumour necrosis factor α**

Tumour necrosis factor (TNF) α has a potent stimulatory effect on some Kaposi's sarcoma cell cultures (Nakamura et al., 1988), but whether it is produced by cultures with endothelial characteristics is controversial (Ensoli et al., 1989; Corbeil et al., 1991). TNFα has been reported to be expressed by Kaposi's sarcoma cells in small amounts in vivo but was mainly found in epidermal cells adjacent to the tumour (Gillitzer & Berger, 1991), compatible with a possible paracrine role of this factor.

(f) **Miscellaneous growth factors**

Secretion of granulocyte–monocyte colony-stimulating factor and transforming growth factor β by Kaposi's sarcoma cell cultures with endothelial characteristics, but not by normal endothelial cells, has been reported (Ensoli et al., 1989). The latter also promotes the growth of cultured Kaposi's sarcoma cells. In lesions, mature transforming growth factor β1 is found mainly in macrophage-like cells and not in spindle cells (Williams et al., 1995). Hepatocyte growth factor (scatter factor) also promotes the growth of cultured Kaposi's sarcoma cells and may thus play a role in the pathogenesis of this disease (Naidu et al., 1994).

4.1.1.4 **Role of HIV-1 Tat in promoting Kaposi's sarcoma lesions**

AIDS-associated Kaposi's sarcoma is clinically more aggressive than classic or endemic Kaposi's sarcoma, suggesting that HIV is a cofactor in the progression of this tumour. Experimental evidence suggests that the Tat protein of HIV-1 (see IARC, 1996) can enhance the growth of cultured 'endothelial' Kaposi's sarcoma cells (Ensoli et al., 1990); the effect of Tat on other cell cultures was inconsistent (Roth et al., 1992). Several cytokines, including TNF, IL-1 and interferon γ, can render normal endothelial and smooth-muscle cells susceptible to the growth-promoting effect of Tat (Barillari et al., 1992), possibly by increasing the expression of integrin receptors which interact with Tat (Barillari et al., 1993; Ensoli et al., 1994a). Injection of Tat into nude mice
(Ensoli et al., 1994b) or immunocompetent C57Bl mice (after incorporation into Matrigel; Albini et al., 1994) induces angiogenesis, and this effect is potentiated by basic FGF (Ensoli et al., 1994a,b) and heparin (Albini et al., 1994, 1996b). Tat- and heparin-induced neoangiogenesis can be inhibited by the matrix metalloproteinase inhibitor TIMP-2 (Albini et al., 1994), and Tat and basic FGF synergize to increase the expression of collagenase IV in nude mice (Ensoli et al., 1994b). In addition, one group reported the emergence of Kaposi's sarcoma-like lesions in mice transgenic for HIV-1 tat (Vogel et al., 1988); however, other lines of transgenic mice carrying the complete HIV-1 genome failed to develop similar lesions (Leonard et al., 1988). Transgenic mice carrying the early region of BK virus included in a long terminal repeat-tat construct also develop 'Kaposi's sarcoma-like' lesions, in addition to other malignancies (Coralini et al., 1993), and extracellular Tat released by tumour cell lines derived from these animals protects them from apoptosis under conditions of serum starvation (Campioni et al., 1995). Tat can be released from HIV-infected cells and can act on HIV-uninfected cells. The growth promoting effect of extracellular Tat on cultured Kaposi's sarcoma cells and endothelial cells (Ensoli et al., 1990; Barillari et al., 1992) suggests that infection of cells not directly involved in the Kaposi's sarcoma lesion may be sufficient to trigger the sequence of events leading to the development of this tumour. In keeping with this interpretation, in tat-transgenic mice which did develop Kaposi's sarcoma-like lesions, expression of tat was not found in spindle cells but in neighbouring keratinocytes (Vogel et al., 1988).

HIV-1 Tat has been reported to be detectable by histochemical techniques in Kaposi's sarcoma lesions in AIDS patients, probably originating from a few HIV-1-infected mononuclear cells (Ensoli et al., 1994b). Thus, the angiogenic properties of Tat, alone or in concert with other growth factors, has been documented in a variety of experimental systems; however, the molecular basis for its angiogenic properties is still controversial. Tat has been reported to bind to \( \alpha_\beta \) and \( \alpha_\beta \) integrins via an RGD sequence element in a manner similar to, and replaceable by, their physiological ligands fibronectin and vitronectin (Barillari et al., 1993; Ensoli et al., 1994b). Baboons infected with HIV-2, however, also developed Kaposi's sarcoma-like lesions, although HIV-2 Tat lacks an RGD domain (Barnett et al., 1994). Tat binds with high affinity to the Flk-1/KDR receptor for vascular endothelial cell growth factor (Albini et al., 1996c), and this interaction promotes angiogenesis. A basic heparin binding-like domain in tat, rather than the RGD domain, is thought to be involved in this interaction (Albini et al., 1996b).

### 4.1.1.5 Clonality of Kaposi's sarcoma lesions

Individual Kaposi's sarcoma nodules in female patients have been shown, by studying X-inactivation markers, to contain monoclonal or oligoclonal cell populations (Rabkin et al., 1995), and different nodules from the same patient have the same monoclonal origin (Rabkin et al., 1997). Southern blotting of Kaposi's sarcoma DNA with KSHV/HHV8 terminal-repeat probes showed one or a few bands, which is consistent with a monoclonal or oligoclonal expansion of virus-infected cells. Whether different lesions from the same patient have the same KSHV/HHV8 clonality pattern has not yet been
addressed by terminal-repeat hybridization, and it is not known whether this assay is sufficient to determine monoclonality (Russo et al., 1996). Short-term cultures of biopsy samples from Kaposi’s sarcomas have been found to contain chromosomal rearrangements, but no consistent pattern has emerged (Delli Bovi et al., 1986). Two tumorigenic cell lines derived from Kaposi’s sarcoma lesions have been reported to have a 3(p14) translocation in common (Popescu et al., 1996); however, as neither is infected with KSHV/HHV8, the relationship of these findings to Kaposi’s sarcoma spindle cells latently infected with the virus (see section 4.1.4) is unclear.

4.1.2 Role of KSHV/HHV8 in development of Kaposi’s sarcoma

The consistent detection of KSHV/HHV8 in Kaposi’s sarcoma biopsy samples and the epidemiological data discussed in Section 1 strongly support a causative role of KSHV/HHV8 in the pathogenesis of Kaposi’s sarcoma. Although its precise role is not yet understood, the presently available data are compatible with the notion that KSHV/HHV8 exerts a direct transforming effect on endothelial cells, the likely precursors of Kaposi’s sarcoma spindle cells.

KSHV/HHV8 establishes a persistent infection in most Kaposi’s sarcoma spindle cells, which involves the expression of at least three viral genes. All spindle cells, as well as the atypical endothelial cells of early lesions, express abundant mRNA for ORF K12, which encodes a putative small hydrophobic protein of unknown function (Zhong et al., 1996; Stürzl et al., 1997; Staskus et al., 1997). Kaposi’s sarcoma spindle cells also express the high-molecular-mass latent nuclear antigen (LNA) encoded by ORF 73, as shown by immunohistochemistry with affinity-purified antibodies to this protein (Rainbow et al., 1997). Expression of mRNA for the ORF 72-encoded D-type cyclin homologue has been demonstrated by in-situ hybridization (Stürzl et al., 1997); however, as the mRNA encoding ORF 73 extends through the ORF 72/v-cyclin gene, this result requires confirmation by immunohistochemical studies with specific antibodies. The ORF K13 gene is also contained within the mRNAs encoding ORF 72/v-cyclin and ORF 73/LNA (Rainbow et al., 1997) and may therefore be expressed in Kaposi’s sarcoma spindle cells, but no immunohistochemical studies on the protein it expresses have yet been reported (see section 4.4.5). The KSHV/HHV8 homologue to IL-6 (ORF K2) is expressed during latency in KSHV/HHV8-infected haematopoietic cells but is not generally expressed in Kaposi’s sarcoma lesions (Moore et al., 1996b).

A subpopulation (approximately 10%) of Kaposi’s sarcoma spindle cells also expresses a polyadenylated nuclear T1.1 RNA (Staskus et al., 1997), which is abundant in primary effusion lymphoma cell lines induced into lytic replication (Renne et al., 1996a; Zhong et al., 1996). Expression of T1.1 may therefore be indicative of lytic replication within Kaposi’s sarcoma lesions, suggesting that a subpopulation of spindle cells can produce KSHV/HHV8 virions. Intracellular herpesvirus-like particles or intranuclear inclusions characteristic of herpesviruses can be found in Kaposi’s sarcoma tissues, indicating that the tumour is a source of productive infection (Walter et al., 1984). Expression of ORF 74, encoding a functional chemokine receptor (Arvanitakis et al., 1997), can
be found by RT-PCR, but it is not clear whether this gene is expressed during the latent or the lytic viral expression programme (Cesarman et al., 1996a).

As discussed in section 1.1.6, v-cyclin can phosphorylate Rb and histone H1 and therefore dysregulate the cell cycle, but it has not so far been shown to have transforming properties of its own. The functions of ORF 73/LNA and K12 are unknown; however, the expression of a limited set of genes in persistently infected cells is a familiar pattern with other (both oncogenic and non-oncogenic) herpesviruses and suggests that the role of KSHV/HHV8 in the pathogenesis of Kaposi’s sarcoma is likely to be a direct one.

4.2 Primary effusion lymphomas

Primary effusion lymphomas have a unique constellation of features that distinguishes them from all other known lymphoproliferations: they are predominantly confined to ‘body cavities’ as an effusion; cells with cytomorphological features bridging those of large-cell immunoblastic and anaplastic large-cell lymphoma suggest that they represent mature B cells; a nil phenotype, although some primary effusion lymphomas with B- and/or T-cell markers have been described; clonal immunoglobulin gene rearrangements, further implying a B-cell phenotype; uniform lack of c-myc rearrangements and the presence of KSHV/HHV8 with or without EBV (see section 2.2.1). In at least one primary effusion lymphoma cell line tested, KSHV/HHV8 is clonal by terminal-repeat analysis (Russo et al., 1996).

Most cases of primary effusion lymphoma have been described in severely immunocomprised individuals with HIV infection, in keeping with the notion that lack of immunosurveillance favours primary effusion lymphoma cell proliferation. Why these lymphomas persist predominantly as effusions, without lymph node or other lymphoid tissue involvement, is unclear, although a lack of ‘homing markers’ has been suggested (Karcher & Alkan, 1995).

Multiple, complex chromosomal abnormalities have been described in cases of primary effusion lymphoma; however, apart from a consistent absence of c-myc rearrangement, none of the described abnormalities is present in all cases (Ansar et al., 1996).

In view of the frequent co-infection with EBV and KSHV in the cells of such tumours, these two viruses may act together to induce neoplastic transformation and/or the peculiar phenotypic features of these lymphomas. The presence of activation markers (similar in EBV-positive and EBV-negative KSHV/HHV8-positive cases) suggests that cell activation is secondary to viral infection (Ansari et al., 1996; Nador et al., 1996). Of note, all KSHV/HHV8-positive primary effusion lymphomas lack c-myc rearrangements. The few cases of ‘effusion-based lymphomas’ described that have c-myc rearrangements do not have a nil surface antigen phenotype, display different cytomorphological features and do not contain KSHV/HHV8 (see Table 8).

4.3 Multicentric Castleman’s disease

Multicentric Castleman’s disease is a polyclonal lymphoproliferation characterized by prominent vascularity in lymphoid tissue and associated systemic symptoms. There is a
strong association between multicentric Castleman’s disease and Kaposi’s sarcoma in patients with AIDS (see section 2.2.2).

Immunoregulatory abnormalities probably contribute to these lymphoproliferations. It was hypothesized that a virus acts as a cofactor, perhaps as a stimulus for cytokine production (Peterson & Frizzera, 1993). The association between multicentric Castleman’s disease and the presence of IL-6 is notable. Thus, IL-6 is present at high levels in biopsy samples from patients with this disease, and PBMC from patients with multicentric disease secrete high levels of IL-6 (Yoshizaki et al., 1989; Burger et al., 1994). IL-6 has prominent actions on cells of the immune system, including stimulation of immunoglobulin synthesis by activated B cells and differentiation of cytotoxic T cells (Kikutani et al., 1985). It also commits myeloid progenitors to differentiate into granulocytes and macrophages. IL-6 is synthesized in response to a number of stimuli, which include viruses and other cytokines such as IL-1 and TNF (Kishimoto, 1989). IL-6 also acts as an auto- and paracrine growth factor for multiple myeloma cells (Kawano et al., 1988). Retroviral-mediated transfer of IL-6 into haematopoietic cells of mice results in a syndrome resembling multicentric Castleman’s disease (Brandt et al., 1990a,b). These mice develop anaemia, polyclonal hypergammaglobulinaemia, splenomegaly and peripheral lymphadenopathy, further supporting the hypothesis that dysregulated synthesis of IL-6 has a causative role in multicentric Castleman’s disease.

KSHV/HHV8 is nearly always present in HIV-positive individuals with multicentric Castleman’s disease; in immunocompetent hosts, the virus is present in about 40% of cases (see Table 10). The presence of HHV6 in two of five cases of multicentric Castleman’s disease and EBV in two of five cases has also been reported (Barozzi et al., 1996), but the significance of this finding is unknown.

4.4 Viral genes with cellular growth promoting or oncogenic potential

As discussed in Section 1 (Table 1), the KSHV/HHV8 genome contains multiple non-conserved viral genes, some of which are strikingly similar to human genes involved in cellular growth control. The specific viral genes discussed in this section are those with sequence similarity to known human oncogenes and/or are expressed during viral latency.

4.4.1 Open reading frame K1

The first gene at the left end of the KSHV/HHV8 genome (Figure 2), ORF K1, encodes a putative 289-amino acid, type I transmembrane protein, featuring a signal peptide, an extracellular domain with multiple cysteine residues, a typical transmembrane anchor domain and a short intracellular domain containing several potential tyrosine phosphorylation motifs (Russo et al., 1996; Neipel et al., 1997a). No experimental data are available to indicate that the K1 protein is phosphorylated, and neither its function nor its expression pattern in Kaposi’s sarcoma-associated tumours has been described. The same genomic region is also highly variable between different strains of Herpesvirus saimiri (Jung et al., 1991; Jung & Desrosiers, 1991; Biesinger et al., 1995).
A comparison of deposited genomic sequences indicates that it may also be highly variable (Russo et al., 1996; Lagunoff & Ganem, 1997; Neipel et al., 1997a).

4.4.2 Growth factor homologues

Three KSHV/HHV8 genes have sequence similarity to members of the C-C-chemokine family (Russo et al., 1996; Neipel et al., 1997a; Nicholas et al., 1997a,b). Two of these, encoded by ORF 6 and 4 (v-MIP-I and v-MIP-II) are expressed at low levels in latently infected primary effusion lymphoma cells, and that expression increases during the lytic cycle of viral replication (Moore et al., 1996b; Nicholas et al., 1997a,b). v-MIP-I can interact with the C-CR5 co-receptor to inhibit the entry of some primary HIV strains (Moore et al., 1996b).

The viral IL-6 homologue (v-IL-6), encoded by ORF K2, is 25% identical at the amino-acid level to human IL-6. Several groups have identified this protein (Moore et al., 1996b; Russo et al., 1996; Neipel et al., 1997b; Nicholas et al., 1997a,b) and shown that it supports the growth of the IL-6-dependent mouse myeloma cell line, B9 (Moore et al., 1996b; Nicholas et al., 1997b). It is expressed in latently infected lymphoma cell lines and primary effusion lymphoma tissue, but not generally in Kaposi’s sarcoma tissue. Only a few scattered CD20+ B cells expressing v-IL-6 were found in one of eight Kaposi’s sarcoma specimens examined by immunohistochemistry (Moore et al., 1996b). v-IL-6 activates the same Janus kinase-signal transducer and activator of transcription (JAK/STAT) pathway in HepG2 hepatoma cells as human IL-6; however, human IL-6 and v-IL-6 differ in their cellular IL-6 receptor interaction. Whereas human IL-6 requires both IL-6Rα and gp130 receptor protein for signal activation, v-IL-6 requires only gp130 (Molden et al., 1997). Murine B9 plasmacytoid cell proliferation in response to v-IL-6 is inhibited by antibody to murine IL-6Rα, suggesting possible differences between human and mouse systems (Nicholas et al., 1997b).

4.4.3 bcl-2 homologue

ORF 16 of KSHV/HHV8 encodes a homologue of the cellular anti-apoptotic protein bcl-2, with which it shares 16% sequence homology (Russo et al., 1996; Cheng et al., 1997; Neipel et al., 1997a; Nicholas et al., 1997a; Sarid et al., 1997). The BH1 and BH2 domains, which are conserved in the bcl-2 family of proteins, are also found in the KSHV/HHV8 homologue. Functional studies indicate that bcl-2 prevents Bax-mediated toxicity or apoptosis in yeast, Sindbis virus-infected cells and transfected fibroblasts (Cheng et al., 1997; Sarid et al., 1997). There is contradictory evidence for the ability of v-bcl-2 to heterodimerize with human Bcl-2 or Bax: Sarid et al. (1997) found evidence of v-bcl-2–human Bcl-2 interactions in a two-hybrid yeast system, but Cheng et al. (1997) found no specific interaction between the KSHV/HHV8 protein and other bcl-2-like proteins by co-immunoprecipitation. [This contradiction could be due to differences in the sensitivities or specificities of the assays used or to differences in the recombinant bcl-2 constructs.] Both studies demonstrate, however, that the v-bcl-2 has functional anti-apoptotic activity.
v-bcl-2 Transcripts can be induced in primary effusion lymphoma cell lines by 12-O-tetradecanoylphorbol 13-acetate and can be detected at low levels in Kaposi's sarcoma lesions and primary effusion lymphoma cell lines (Cheng et al., 1997; Sarid et al., 1997), similar to the proposed function of the EBV bcl-2 homologue BHRF1 (Roizman, 1993). [The function of v-bcl-2 of KSHV/HHV8 may be to prolong the survival of lytically infected cells.] Since lytic virus replication is thought to be incompatible with survival of the cell (Roizman, 1993), expression of BHRF1 may not play a direct role in EBV-mediated transformation.

4.4.4 Viral interferon regulatory factor

**ORF K9** encodes the homologue of v-IRF (Moore et al., 1996b; Russo et al., 1996). This gene has low but significant homology to the IRF family of proteins responsible for interferon signal transduction. Two members of this pathway, IRF-1 and IRF-2, are antagonistic to each other in their effector functions (Taniguchi et al., 1995a). IRF-1 positively regulates interferon signaling by binding to specific enhancer elements, called interferon-stimulated response elements, in the promoter regions of interferon-inducible genes. Activated transcription of interferon-stimulated genes results in phenotypic changes characteristic for the interferon-induced antiviral state, which include (but are not limited to) induction of CDKI p21, which can lead to shut-down of the cell cycle through inhibition of Rb (Taniguchi et al., 1995a), and up-regulation of the major histocompatibility complex antigen (Taniguchi et al., 1995b). IRF-2 has oncogenic activities in NIH 3T3 cells which can be reversed by IRF-1 overexpression (Harada et al., 1993). Like KSHV/HHV8-encoded cytokine homologues, v-IRF is expressed in latently infected primary effusion lymphoma cell lines, and its expression is markedly enhanced after lytic cycle induction (Moore et al., 1996b).

4.4.5 Viral proteins that inhibit fas-associated death domain protein interleukin-1β converting enzyme (FLICE)

**ORF K13** encodes a small protein similar to the ‘death effector’ domains found in **ORF 71**-encoded proteins of three other rhadinoviruses, HVS, equine herpesvirus 2 and BHV-4 (Thome et al., 1997); however, there is no significant, overall sequence similarity between KSHV/HHV8 K13 and the **ORF 71** of other rhadinoviruses (Russo et al., 1996). The protein of equine herpesvirus 2 encoded by **ORF 71** and a similar protein of molluscipoxvirus have been shown to act as dominant negative inhibitors of apoptotic signals by interfering with the interaction of fas-associated death domain protein (FADD) and tumour necrosis factor receptor-associated death domain protein (TRADD) and the assembly of the interleukin-1β-converting enzyme (ICE)-like protease complex and are therefore known as v-FLIPs (Bertin et al., 1997; Thome et al., 1997). No information on the function of the v-FLIP of KSHV/HHV8 is available, but conservation of the critical sequence elements suggests that it may also be able to inhibit this apoptotic pathway. Although the HVS v-FLIP may be expressed primarily during the lytic cycle of replication and not in tightly latent HVS-transformed human T-cells, the KSHV/HHV8 v-FLIP may be expressed in Kaposi’s sarcoma spindle cells and primary effusion lym-
phoma cells, as its reading frame is included in mRNAs that encode the neighbouring ORF 72 (v-cyclin) and ORF 73 (LNA) proteins which are expressed in both Kaposi’s sarcoma spindle and primary effusion lymphoma cells (Rainbow et al., 1997). No direct evidence for its expression in endothelial tumour (spindle) or B-lymphoma cells is available.

4.4.6 Viral cyclin

ORF 72 of KSHV/HHV8, like that of HVS, encodes a protein that has about 30% amino-acid identity with human cellular cyclin D2 (Cesaran et al., 1996a; Russo et al., 1996; Li et al., 1997). Cellular D-typed cyclins are implicated in the pathogenesis of several human malignancies (Sherr, 1995, 1996). v-Cyclin contains a region of high sequence similarity to the cyclin box domain of cellular cyclins, responsible for interactions with cyclin-dependent kinases (Chang et al., 1996a; Godden-Kent et al., 1997; Li et al., 1997). The KSHV/HHV8 v-cyclin associates predominantly with cyclin-dependent kinase 6 and more weakly with kinase 4 (Godden-Kent et al., 1997; Li et al., 1997), and the cyclin-dependent kinase 6–v-cyclin complex can phosphorylate the physiological target of D-type cyclins, the retinoblastoma protein Rb (Chang et al., 1996a; Godden-Kent et al., 1997; Li et al., 1997). Two-dimensional electrophoresis indicates that KSHV/HHV8 v-cyclin induces phosphorylation of Rb at authentic sites (Chang et al., 1996a). The activation of cyclin-dependent kinase 6 activity induced by the v-cyclins of both HVS and KSHV/HHV8 is much more pronounced than that by human cyclin D2: the two have a broader specificity and can also phosphorylate histone H1 (Godden-Kent et al., 1997; Li et al., 1997). The activity of KSHV/HHV8 v-cyclin in vivo was demonstrated in the osteosarcoma cell line SAOS-2, which has homozygous deletions of both Rb alleles. When wild-type Rb is transfected into SAOS-2 cells, they stop replicating and take on a senescent phenotype characterized by an enlarged cytoplasm. Co-transfection of KSHV/HHV8 v-cyclin with Rb prevents entry of SAOS-2 into senescence, and the cells continue to proliferate (Chang et al., 1996b).

KSHV/HHV8 v-cyclin is expressed in latently infected primary effusion lymphoma cell lines, in uncultured primary effusion lymphomas and in Kaposi’s sarcoma tissue (Cesaran et al., 1996a; Godden-Kent et al., 1997). It is expressed in persistently infected cells in concert with K13/v-FLIP and ORF 73 (Rainbow et al., 1997).

4.4.7 Latency-associated nuclear antigen

ORF 73 of KSHV/HHV8 encodes a large nuclear protein which is identical (Rainbow et al., 1997) to the previously described high-molecular-mass (224–236 kDa) LNA (Gao et al., 1996b) and a component of the LANA, defined by immunofluorescence (Gao et al., 1996a; Kedes et al., 1996). Its size varies between different virus isolates, due to varying lengths of the moi repeat region within this ORF (Russo et al., 1996; Rainbow et al., 1997). The moi region is translated as a long acidic repeat. ORF 73/LNA also encodes a leucine zipper region in the carboxy terminus of the protein (Russo et al., 1996). Its apparent molecular mass (by sodium dodecyl sulfate polyacrylamide gel electrophoresis) is much higher than that predicted from its amino-acid sequence, pro-
bably because of the highly charged nature of the moi repeat region and possibly also as a result of post-translational modification. Post-translational modification may also explain the doublet nature of LNA on western blots (Gao et al., 1996b; Rainbow et al., 1997).

The function of ORF 73/LNA is not yet known. It is a latent protein, expressed in latently infected primary effusion lymphoma cell lines and Kaposi's sarcoma spindle cells (Gao et al., 1996a,b; Rainbow et al., 1997) and associated with subnuclear domains (Gao et al., 1996a,b; Kedes et al., 1996; Rainbow et al., 1997), the identity of which remains to be resolved. There is no evidence that ORF 73/LNA has transforming properties. [It is conceivable that, in analogy with trans-activating proteins of other herpesviruses, it is primarily involved in controlling transcription of other viral genes but could also affect the expression of cellular genes.]

4.4.8 G Protein-coupled receptor homologue

ORF 74 of both KSHV/HHV8 and HVS encodes a homologue of a G protein-coupled receptor with seven membrane-spanning domains (Cesarman et al., 1996a; Arvanitakis et al., 1997). This receptor has highest sequence homology to IL-8 chemokine receptors and is also related to the EBI-1 cellular protein induced by EBV infection (Birkenbach et al., 1993). Studies of transient expression of viral G-protein-coupled receptor suggest that it is constitutively active and does not require ligand binding or is activated by a ligand commonly expressed into cell culture medium. The receptor is unusual in that it can bind chemokines belonging to both the C-X-C and C-C families. Transfection of the KSHV/HHV8 G protein-coupled receptor enhances proliferation of rat kidney fibroblasts (NRK-49F), suggesting that it may contribute to tumour-cell proliferation (Arvanitakis et al., 1997). Its increased expression in primary effusion lymphoma cell lines treated with 12-O-tetradecanoylphorbol 13-acetate suggests, however, that it is expressed primarily during lytic replication. While its expression in Kaposi's sarcoma tissue has been documented by RT-PCR (Cesarman et al., 1996b), it is not clear whether it is expressed in persistently infected Kaposi's sarcoma spindle cells or in the few cells in these lesions that are undergoing lytic viral replication.

4.5 Summary of potential roles of KSHV/HHV8 in tumorigenesis

4.5.1 Kaposi's sarcoma

KSHV/HHV8 establishes a persistent infection in most Kaposi's sarcoma spindle cells, characterized by a restricted pattern of gene expression (Zhong et al., 1996). All spindle cells and the atypical endothelial cells of early Kaposi's sarcoma lesions express abundant mRNA for ORF K12, which encodes a small hydrophobic protein of unknown function (Zhong et al., 1996; Stürtzl et al., 1997; Staskus et al., 1997). Kaposi's sarcoma spindle cells also express the high-molecular-mass LANA encoded by ORF 73, as shown by immunohistochemistry with affinity-purified antibodies to this protein. Expression of mRNA for the ORF 72-encoded D-type-cyclin homologue has been demonstrated, but as the mRNA encoded ORF 73/LANA extends through the ORF 72/v-cyclin gene, this result
should be confirmed by immunohistochemical studies with v-cyclin. The \textit{K13/v-FLIP} gene is also contained within the mRNAs encoding \textit{ORF 72/v-cyclin} and \textit{ORF 73/LANA} (Rainbow \textit{et al.}, 1997) and may therefore also be expressed in Kaposi’s sarcoma spindle cells, but no immunohistochemical studies on this protein have been reported. The KSHV/HHV8 homologue of IL-6 (\textit{ORF K2}) is expressed during latency in KSHV/HHV8-infected haematopoietic cells but is not generally expressed in Kaposi’s sarcoma lesions (Moore \textit{et al.}, 1996b). Expression of \textit{ORF 74}, which encodes a functional chemokine receptor (Arvanitakis \textit{et al.}, 1997), can be found by RT-PCR (Cesarman \textit{et al.}, 1996c). KSHV/HHV8 virions have been seen in a subpopulation of Kaposi’s sarcoma spindle cells and infiltrating haematopoietic cells (Orenstein \textit{et al.}, 1997).

These findings indicate that all or nearly all endothelial tumour (spindle) cells in Kaposi’s sarcoma lesions are infected with KSHV/HHV8 (Boshoff \textit{et al.}, 1995b). There is not yet enough evidence to conclude that the gene expression programme of KSHV/HHV8 (i.e. lytic and latent gene expression programmes) is similar to that of other herpesviruses. A set of viral genes that is expressed in primary effusion lymphoma cells (\textit{ORF K12, ORF K13, ORF 72, ORF 73}) is not up-regulated by chemical treatment and could therefore represent latent genes that are also expressed in Kaposi’s sarcoma spindle cells. Since a minority of these cells appears to undergo full lytic replication, it is too early to conclude whether KSHV/HHV8 establishes a latent infection in most spindle cells; however, it is also unclear whether a latent expression pattern is required for the expansion of spindle cells. The presently available evidence is insufficient to conclude whether a limited lytic expression programme is compatible with virus-mediated cellular proliferation.

\section*{4.5.2 Primary effusion lymphoma}

Ninety percent of the described cases of primary effusion lymphoma contain both KSHV/HHV8 and EBV (see Section 1), and the cells contain multiple episomal copies of KSHV/HHV8 (Cesarman \textit{et al.}, 1995a,b; Nador \textit{et al.}, 1996). KSHV/HHV8 gene expression in primary effusion lymphoma has been studied mainly in derived cell lines, and the results may not fully reflect the gene expression pattern \textit{in vivo}. It is, however, largely similar to that in Kaposi’s sarcoma lesions (Zhong \textit{et al.}, 1996; see section 4.5.1). In addition, of the genes investigated, v-bcl-2, v-MIP-I, v-MIP-II, v-IRF, v-IL-6 and v-GCR are expressed at low levels in primary effusion lymphoma cell lines, and their expression can be up-regulated by treatment with phorbol esters and/or sodium butyrate. The untranslated \textit{T1.1} RNA (see Section 1) is strongly expressed in unstimulated primary effusion lymphoma cell lines but can be further up-regulated by chemical treatment. Unlike Kaposi’s sarcoma, primary effusion lymphoma cells strongly express v-IL-6 protein, as shown by immunohistochemistry of fresh primary effusion lymphoma cells (Moore \textit{et al.}, 1996b).

In cell lines concomitantly infected with EBV and KSHV/HHV8, the EBV is monoclonal and its expression is limited to EBNA-1 and (in one case) LMP-2 (Moore \textit{et al.}, 1996a; Nador \textit{et al.}, 1996).
Despite attempts by several groups, there is currently no evidence that KSHV/HHV8 can transform or immortalize lymphocytes in vitro.

Given the low level of expression of several KSHV/HHV8 genes shown to dysregulate the cell cycle and/or prevent apoptosis, it is at least conceivable, although unproven, that these viral genes play an important role in the development of primary effusion lymphoma.

4.5.3 Multicentric Castleman's disease

Unlike Kaposi's sarcoma and primary effusion lymphoma, multicentric Castleman's disease is not universally associated with KSHV/HHV8 infection, although infection with this virus is very common in HIV-associated cases (see Section 2). Multicentric Castleman's disease is a polyclonal disorder and, like Kaposi's sarcoma, characterized by prominent angiogenesis. No published data are available on KSHV/HHV8 gene expression in these lesions. Given the polyclonal nature of the disease and the inconsistent association with KSHV/HHV8, it is likely that multicentric Castleman's disease has a multifactorial etiology and that the role of KSHV/HHV8 is indirect, conceivably mediated by virus-encoded cytokines.

4.6 Antiviral agents

Specific antiviral therapy has been evaluated in only a few studies and a case series (Morfeldt & Torssander, 1994; Jones et al., 1995; Glesby et al., 1996; Mocroft et al., 1996). All of the currently available antiviral drugs that have been evaluated belong to the class of DNA polymerase inhibitors, which are active against lytic but not latent herpesviral infection. Morfeldt and Torssander (1994) described clinical regression of AIDS-associated Kaposi's sarcoma lesions in three of five patients after administration of high doses of phosphonoformic acid (foscarnet), but no untreated control patients were available for comparison. Jones et al. (1995) examined follow-up data on 20,228 HIV-positive persons and AIDS patients enrolled in a study of adult diseases, of whom 1033 (5%) developed Kaposi's sarcoma. Proportional hazards analysis was used to demonstrate that the risk of patients receiving foscarnet for developing Kaposi's sarcoma after a median of 14 months' follow-up was 30% of that of patients who did not receive herpesviral therapy, after taking into account CD4 count, age, sex, race, route of exposure to HIV, other opportunistic illnesses and antiretroviral therapy (p = 0.001). Similar analysis for gancyclovir (odds ratio, 1.0; p = 0.8) and acyclovir (odds ratio, 1.4; p < 0.001) showed either no effect or an increased risk for disease with therapy. A similar analysis of 935 participants in a multicentre study of AIDS showed a non-significant preventive effect against Kaposi's sarcoma for foscarnet (RR, 0.40; 95% CI, 0.05–3.10; p = 0.38) and gancyclovir (RR, 0.56; 95% CI, 0.22–1.44; p = 0.23) but not for acyclovir (Glesby et al., 1996). Mocroft et al. (1996) followed 3688 patients with HIV infection or AIDS for a median of 4.2 years, of whom 598 (16%) developed Kaposi's sarcoma. After adjustment for sex, route of exposure to HIV, age, antiretroviral treatment, prophylaxis for Pneumocystis carinii pneumonia, opportunistic infections and CD4 count, use of foscarnet (relative hazard, 0.38; 95% CI, 0.15–0.95; p = 0.038) and
gancyclovir (relative hazard, 0.39; 95% CI, 0.19–0.84; \( p = 0.015 \)) but not acyclovir (relative hazard, 1.1; 95% CI, 0.88–1.4; \( p = 0.40 \)) were associated with a decreased risk of developing Kaposi’s sarcoma.

Foscarnet has some direct antiretroviral activity (Sandstrom et al., 1985) and may therefore have activity against Kaposi’s sarcoma, but other opportunistic tumours and infections (aside from susceptibility to herpesviral infections) are not known to respond to foscarnet therapy.

Kedes and Ganem (1997) demonstrated that gancyclovir, foscarnet and cidofovir at pharmacological concentrations inhibit virion induction \textit{in vitro} in the primary effusion lymphoma cell line BCBL-1, whereas acyclovir does not. An anti-retroviral protease inhibitor and ritonavir analog, A 77003, did not act on KSHV/HHV8 replication.