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ORIGINAL ARTICLE

Kaposi's Sarcoma-Associated Herpesvirus Viremia is Associated with the Progression of Classic and Endemic Kaposi's Sarcoma

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In order to gain further insight on the role of Kaposi's sarcoma-associated herpesvirus (KSHV) in classic and endemic Kaposi's sarcoma (KS) pathogenesis, we aimed to determine (i) whether KSHV is detectable in peripheral blood mononuclear cells (PBMCs), (ii) which PBMCs subpopulation harbor the virus, (iii) which clinical, histologic, and immunologic parameters are associated with KSHV viremia in a population of classic and endemic KS. KSHV viremia and various immunologic parameters were screened on 81 patients. KSHV viremia was positive in 58% of the patients. KSHV was detected in B cells, T cells, and monocytes. CD34+ cells depleted in circulating endothelial cells (CECs) were never infected and 50% of the patients tested had CECs infected by KSHV. We observed a significant increase of IL-2 and IFN-γ production by CD4 T cells and an increase of IFN-γ production by CD8 T cells compared to control patients. KS progression ($P=0.001$) and KS staging ($P=0.03$) were significantly and independently associated with positive KSHV viremia. Our results show that there is no specific immunosuppression in classic or endemic KS. We showed that KSHV can be detected within CECs and that KSHV viremia could be an indicator of circulating mature or precursor spindle cells.

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INTRODUCTION

Kaposi's sarcoma (KS) is regarded as a multicentric neoplasm of lymphatic endothelium infected with Kaposi's sarcoma-associated herpesvirus (KSHV) (Chang *et al.*, 1994; Antman and Chang, 2000).

Three contexts for KS can be distinguished by clinico-epidemiologic features, all characterized by frequent cutaneous lesions: classic KS (CKS) affecting mostly men from Mediterranean countries with an indolent course; endemic KS (EKS) in people from sub-Saharan Africa with either indolent or locally aggressive cutaneous lesions; KS in immunosuppressed patients, either iatrogenically or with HIV infection, with progressive cutaneous disease and frequently serious visceral lesions (Antman and Chang, 2000).

KSHV infects most spindle cells of the KS lesions (Boshoff *et al.*, 1995). Spindle cells are endothelial cells expressing various specific lymphatic lineage proteins. Whether KSHV infects endothelial precursors and drives them to differentiate, or, equally, infects mature lymphatic and blood endothelial cells and induces gene expression similar those in to lymphatic cells is debated (Hong *et al.*, 2004; Wang *et al.*, 2004). The virus has been detected in all the major populations of peripheral blood mononuclear cells (PBMCs): B and T lymphocytes and monocytes (Ambroziak *et al.*, 1995; Harrington *et al.*, 1996; Henry *et al.*, 1999). Henry *et al.* (1999) also showed that KSHV may infect PBMC CD34 cells. We previously showed that KSHV virus levels in PBMCs correlated with both the degree of immunosuppression and KS progression, in epidemic and iatrogenic KS, suggesting KSHV reactivation parallels the decrease in immune surveillance (Pellet *et al.*, 2001, 2002). To our knowledge, immune suppression has not been unequivocally demonstrated in CKS or EKS, and apart from age and sex, the risk factors for KS among KSHV-positive, HIV-negative persons without overt immune suppression are unknown. Genetic cofactors such as variant genotypes of FcgammaRIIIA (Lehrnbecher *et al.*, 2000) or IL6 promoter polymorphism (Foster *et al.*, 2000) as demonstrated for HIV-related KS or a unknown recessive gene (Plancoulaine *et al.*, 2003) could also be involved in CKS or EKS.

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Abbreviations: CEC, circulating endothelial cell; CKS, classic Kaposi's sarcoma; EKS, endemic Kaposi's sarcoma; KS, Kaposi's sarcoma; KSHV, Kaposi's sarcoma-associated herpesvirus; PBMC, peripheral blood mononuclear cell

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In order to explore the pathogenic role of KSHV in CKS and EKS, we aimed to see whether: (1) KSHV was detectable in PBMCs from actual cases, and whether or not KSHV viral load in PBMCs was similar in the two settings compared with immunosuppressed patients; (2) circulating endothelial cells (CECs) harboured the virus; (3) which clinical, histologic, and immunologic features were associated with positive KSHV viremia in such specific KS patients.

RESULTS

Eighty-one patients, whose characteristics are given in Table 1, were included. The median age at enrollment was 67 years, whereas the median time from first symptoms of KS was 6.7 years. Most patients had CKS whereas 11/81 (14%) had EKS. Forty-nine patients (60%) had limited disease with fewer than 10 lesions. Lymphedema was present in 32 (40%) whereas painful disabling lesions were recorded in 17 (21%). KS was progressive in 32 (40%) but only 25 (31%) required systemic treatment (interferon $n=20$, bleomycin $n=2$, vinblastin $n=2$, thalidomide $n=1$) within 6 months after enrollment.

KSHV viremia occurred in 47 (58%) patients (median value of KSHV viremia restricted to the 47 patients was 3.48 log (2.01–4.76)).

PBMCs sorting was performed for eight patients with positive KSHV viral load in total PBMCs. As shown in Table 2, three of eight (37.5%) had KSHV-infected B cells, two of seven (28.5%) had infected monocytes, and only one of eight (12.5%) had infected T cells. None had CEC-depleted CD34 + cells infected by KSHV and 50% (4/8) of the patients tested had CECs infected by KSHV. All had progressive or stable KS (five progressive disease and three stable disease). Although KSHV was detectable in total PBMCs in the eight patients, KSHV remained undetectable in either subpopulations in two patients. This result is probably related to the cell loss during washes and to the low total viral load (respectively 2.48 and 2.95 log). We tried to quantify the number of separated CECs using the Malassez count; the quantification of rosetted CECs was performed in triplicate, and CECs were identified when bearing more than 10 beads and a cell size in the range of 30–50 μm as described by George *et al.* (1992, 1993). We found less than 1 CEC/ml of peripheral blood among PBMCs of normal subjects ($n=5$) but 100 to 10,000 CEC/ml in the KS patients tested ($n=8$).

Among immunologic parameters studied in our KS cohort, we observed a significant increase of IL-2 and IFN-γ production by CD4 T cells as well as an increase of IFN-γ production by CD8 T cells compared to control patients matched for age (Table 3). Increase of IL-2 production by CD8 T cells was not significant. Moreover, the number of lymphocytes, percentage of total CD3, CD3-HLA-DR, CD3-CD25, and CD3-CD38 lymphocytes as well as percent of total CD4, CD4-CD28, CD4-CD45RA, and CD4-CD45RO lymphocytes were not significantly different between KS patients and controls. No significant difference was also

Table 1. Main characteristics of patients

	Mean value (SD)/or number (%)
Sex ratio	4.4 (66 H, 15 F)
Age at inclusion	67 (14)
Time from first symptoms of KS (years)	6.7 (7.7)
Type of KS	
Endemic KS	11 (14)
Classic KS	70 (86)
Previous systemic therapy	15 (19)
Current systemic therapy	6 (7)
Associated malignancy	7 (9)
Diabetes	11 (14)
Decision of systemic therapy within 6 months	25 (31)
Histologic form	
Lymphangiomatosis	14 (20)
Patch	12 (17)
Plaque	11 (16)
Nodular	32 (46)
Number of lesions (n)	
$n<0$	5 (6)
$n=1$	13 (16)
$1<n\leqslant 10$	31 (38)
$10<n\leqslant 50$	26 (32)
$50<n\leqslant 100$	4 (5)
$n>100$	2 (2)
Painful lesions	17 (21)
Lymphedema	32 (40)
KSHV-positive viremia	47 (58)

F; female; H, M=male; KS, Kaposi's sarcoma; KSHV, Kaposi's sarcoma-associated herpesvirus; SD, standard deviation.

Table 2. KSHV viral load in peripheral blood mononuclear cells after magnetic cell sorting (patients with positive KSHV viremia)

	KSHV viral load (copies/microgramm of DNA)					
Patient	CD19+ cells	CD2+ cells	CD14+ cells	CD146+ cells	CD146− CD34+ cells	KS staging
1	<10	<10	<10	458	<10	PD
2	45	11	75	244	<10	PD
3	688	<10	<10	276	<10	SD
4	<10	<10	2,260	<10	<10	SD
5	<10	<10	<10	280	<10	PD
6	<10	<10	<10	<10	<10	PD
7	<10	<10	<10	<10	<10	SD
8	397	<10	ND	<10	ND	PD

KS, Kaposi's sarcoma; KSHV, Kaposi's sarcoma-associated herpesvirus; ND, not done; PD, progressive disease; SD, stable disease.

observed in percent of CD8 lymphocytes, of CD19 lymphocytes, and in percent of NKT and NK cells.

Using univariate analysis, detection of KSHV by PCR in peripheral blood was significantly associated with EKS versus CKS ($P=0.02$), KS progression ($P=0.001$), decision of systemic therapy ($P=0.03$), KS staging ($P=0.03$), lymphedema ($P=0.01$), whereas it did not depend on the histologic type, length of KS evolution, the presence of painful KS lesions (Table 4). None of the immunologic parameters studied was significantly associated with KSHV viremia.

When all significant parameters were considered jointly by multivariate analysis, only KS progression ($P=0.001$) and KS staging (as evaluate by the number of lesions) ($P=0.03$) remained significantly and independently associated with positive KSHV viremia (Table 4).

Table 3. Significant differences in intracellular cytokines expressed as percent of T CD4 or T CD8 positive lymphocytes

	Kaposi		Controls	
Intracellular cytokines	***N***	**Mean (SD)**	**Mean (SD)**	***P*-value**
CD4 IFN-γ	29	32.7 (18.3)	15.3 (9.8)	0.012
CD4 IL-2	29	60.2 (15.1)	21.6 (12.2)	<0.001
CD8 IFN-γ	29	68.8 (21.6)	44 (16.3)	0.018

Median values and standard deviation for the KS patients and 10 controls matched for age.
KS, Kaposi's sarcoma; KSHV, Kaposi's sarcoma-associated herpesvirus; *N*, number; SD, standard deviation.

DISCUSSION

The demographic, clinical, and histologic features of the 81 CKS and EKS patients reported in this study is in accordance with previous reports. Like Friedman-Birnbaum *et al.* (1990), we found a high prevalence of diabetes (15% in our series, all type II with a median age of onset of 61 years; 16% in theirs), whereas the rate of accompanying malignancies seems lower than previously reported (9% – four prostate adenocarcinoma, two T-cell lymphomas, and one polycythemia vera, compared to 19% for Brenner *et al.*, 2002). Up to 31% of patients had an extension or progression of KS requiring systemic therapy compared to 19% in the series of Brenner *et al.* (2002). This difference may be related to differences in patient population.

The detection rate of KSHV virus in PBMCs (58%) is similar to our previously published observations (Lebbe *et al.*, 1997; Cattani *et al.*, 1998; Boneschi *et al.*, 2001). KSHV viral load in PBMCs appears lower in CKS or EKS (3.48 log) versus HIV or transplanted KS where median values were, respectively, 4.13 and 4.60 log in previously published data (Pellet *et al.*, 2001, 2002). This could be related to an increased viral reactivation due to immunosuppression or a higher tumor burden in epidemic or post-transplant KS. The latter hypothesis is supported by the fact that KSHV viremia was significantly associated with disease progression and tumor burden,

Table 4. Parameters associated with positive KSHV viremia

	Crude OR (95% CI)	*P*-value	Adjusted OR (95% CI)	*P*-value
KS progression	9.8 (3.4–28.0)	<0.001	14.2 (2.7–73.9)	0.0017
Time from first symptoms of KS	1.5 (0.6–3.7)	0.05		
Endemic KS versus classic KS	8.9 (1.08–73.5)	0.02	6.3 (0.6–71.1)	0.14
Age at enrolment (less than 60 vs superior or equal to 60 years)	0.9 (0.3–2.3)	0.8		
Age at first symptoms of KS (less than 60 vs superior or equal to 60 years)	0.4 (0.2–1.1)	0.07	0.7 (0.2–2.3)	0.56
Decision of systemic therapy	3.3 (1.5–9.2)	0.03	0.4 (0.06–2.3)	0.28
Previous systemic therapy	0.8 (0.3–2.5)	0.7		
Current systemic therapy	0.7 (0.1–3.7)	0.7		
Histologic form (S vs A, I, CM)	0.9 (0.3–2.3)	0.8		
Number of lesions (more than 1 vs equal or less than 1)	4.4 (1.6–12.0)	0.03	6.6 (1.3–35.1)	0.03
Painful lesions	3.0 (0.9–10.1)	0.07	1.4 (0.3–7.6)	0.7
Lymphedema	3.5 (1.3–9.5)	0.01	1.3 (0.3–6.4)	0.7
Intracellular cytokines				
CD4 IFN-γ	1.0 (0.99–1.01)	0.3		
CD8 IFN-γ	1.0 (0.99–1.01)	0.5		
CD4 IL-2	1.0 (0.99–1.01)	0.3		
CD8 IL-2	1.0 (0.99–1.01)	0.7		

95% CI, 95% confidence interval; A, angiomatous; CM, cellular mixt; I, inflammatory; KS, Kaposi's sarcoma; KSHV, Kaposi's sarcoma-associated herpesvirus; OR, odds ratio; S, sarcomatous.

whereas no immunologic parameter was apparently involved. Although the association between KSHV viremia and KS progression has already been demonstrated in HIV-infected patients by us (Pellet *et al.*, 2001) and by others (Marcelin *et al.*, 2004), it has never been demonstrated in non-HIV-infected KS patients. The low number of patients (11) included in Marcelin *et al.*'s study (2004) probably explain why these authors failed to demonstrate a relationship between HIV-negative KS extension and viral load in PBMCs.

As already demonstrated by others using a similar methodology (Ambroziak *et al.*, 1995; Harrington *et al.*, 1996; Henry *et al.*, 1999), our results show that KSHV can infect B lymphocytes and monocytes. In only one patient KSHV sequences were found in the T lymphocytes at a very low viral load (just above the limit of the test sensitivity). It is therefore very difficult to assume that T cells are infected with KSHV from these results. Anti-S-endo-1 is a pan-endothelial monoclonal antibody, and S-endo-1 (CD146) is ubiquitously and highly expressed in the majority of human endothelial cells of different types of vessels, including lymphatic vasculature (George *et al.*, 1991; Bardin *et al.*, 1996). The anti-S-endo-1 antibody recognizes mature endothelial cells and endothelial cell progenitors obtained in culture (Ingram *et al.*, 2004). Therefore, it could recognize mature endothelial cells and young cells from circulating progenitors in the blood. Our results suggest that CD34+ cells infected by KSHV are CECs. Solovey *et al.* (1997) found a mean of 2.6 CEC/ml of peripheral blood in normal subjects, but a mean of 22.8 CEC/ml in patients with acute painful crisis of sickle cell anemia. George *et al.* (1991, 1992, 1993), whose methodology we used, found less than 1 CEC/ml in normal subjects but a mean of 162 CEC/ml (ranging from 0 to 1600) in Mediterranean spotted fever subjects before treatment. Our CEC counts accord with those in the literature with regard to healthy subjects but the number of CECs in Kaposi subjects seems elevated compared to CECs count in other clinical situations. As the CD146+ population is a minority among PBMCs and because of cell loss during washings, we believe that we have probably underestimated the CD146+ population. Although we did not succeed in visualizing KSHV-infected CECs by FACS analysis and could not exclude contaminating cells in the CD146+ population, the 100- to 100,000-fold difference between the numbers of CD146+ cells separated from normal and KS individuals could be related to KS pathogenesis. Indeed, KS spindle cells are thought to be derived from either KSHV-infected endothelial precursors, or from mature lymphatic and blood endothelial cells, with KSHV having induced lymphatic gene expression (Hong *et al.*, 2004; Wang *et al.*, 2004). Therefore, two non-exclusive hypotheses can be drawn from the presence of KSHV in the CECs: circulating CD146 cells could be mature spindle cells that separate from the KS lesions and migrate toward the peripheral blood, or KSHV-infected CECs could be spindle cell precursors migrating from the peripheral blood toward tissues to colonize target tissues and then develop into KS lesions. The latter hypothesis could be paralleled with Barozzi *et al.* (2003) who detected the presence of the donor neoplastic KSHV-CD34-infected cells in the KS lesions from five of eight kidney recipients.

Regarding immunologic analysis, our results show that KS patients do not have lymphocyte counts significantly lower than age-matched controls, which contrasts with the findings of Touloumi *et al.* (1999). Similarly, KS patients did not significantly differ from controls in respect of total T-cell count, CD4 and CD8 T-cell subpopulation, or in B cells, whenever percentages or total values are considered. This agrees with published findings, except for those of Touloumi *et al.* (1999) where CD4 T lymphocytes were fewer in KS than in sex-and age-matched controls. Notably we found an increase in IFN-γ-expressing T cell, in the CD8, but also and to a higher extent in the CD4-positive population as well as an increase in IL-2 CD4 T cells among KS patients. Our findings can be compared to those of Sirianni *et al.* (1998), although our methodology using intracellular cytokine flow cytometry may reflect a more relevant physiological impact and allows discrimination between CD4 and CD8 T cells. Sirianni *et al.* (1998) assessed cytokine production of PBMCs isolated from KS patients after 72 hours of *in vitro* stimulation by phyto-hemagglutinin. They showed a preferential IFN-γ expression both in CKS and AIDS KS. Moreover, they showed high level of IFN-γ production by KS infiltrating CD8 T cells. Whereas positive KSHV viremia was not associated with IFN-γ-producing T cells in KS patients, nor with any other immunologic marker, Fiorelli *et al.* (1998) have demonstrated a relationship between IFN-γ and HLADR+ T cells infiltrating tissues and the presence of KSHV in the same specimen. Physiological immune responses to herpesvirus involve both T CD8+ and T CD4+ cells (Amyes *et al.*, 2003; Gamadia *et al.*, 2003). Although IL-2 and IFN-γ intracellular staining was performed after non-specific stimulation, we can suppose that detected cells include KSHV-specific cells that may contribute to the control of KSHV infection. Recently, cytomegalovirus-specific IFN-γ-producing CD4 effector cells have been described indispensable in protection against cytomegalovirus disease (van Leeuwen *et al.*, 2004). However, from our data, the detection of IFN-γ-producing CD8 or CD4 cells was not associated with negative KSHV viremia. Moreover, IL-2/IFN-γ T CD4+ cells have been described to be associated with antigen persistence and low antigen levels in chronic HSV, EBV, and cytomegalovirus infections (Harari *et al.*, 2005). Those secreting IL-2 and IFN-γ T CD4+ cells found to be significantly associated with KS could be a marker of KSHV chronic infection.

As neither Sirianni *et al.* (1998) nor us measured KSHV-specific IFN-γ production, we cannot exclude involvement of T cytokine production driven by other unknown stimuli. IL-2 and IFN-γ are Th1 cytokines with an established role in defense against viral and other pathogens and in the induction of immune-mediated inflammatory response. The strong association between Th1 profile and KS disease could suggest that Th1 pattern may contribute to the development of KS disease.

In nude mice, IFN-γ has been shown to induce endothelial cells to acquire phenotypical and functional characteristics of KS spindle cells and to induce angiogenic lesions (Fiorelli

et al., 1998). IFN-γ is able to induce KSHV reactivation *in vitro* (Chang *et al.*, 2000). Worsening of KS has been reported after treatment with IFN-γ. Therefore, our data do not provide any evidence of immunosuppression in CKS or EKS. We did not assess KSHV-specific responses and therefore cannot exclude a KSHV-specific immune defect that would allow the virus to escape immune cell control. We show physiological T-cell response to a herpesvirus infection. A pathogenic role of IFN-γ in KSHV reactivation and KS progression can therefore be suggested.

In conclusion, we have provided evidence that, whatever the clinical context of KS, KSHV viremia is associated with tumor burden and KS progression. In CKS and EKS populations, KSHV viral load is less than that in immunosuppressed patients. We have shown that KSHV can be detected within CECs, and that KSHV viremia could be an indicator of circulating mature or precursor spindle cells. The variation in KSHV viral load is clearly insufficient to permit the monitoring patients with KSHV viremia. When no specific immunosuppression is evident, the numerous circulating activated IFN-γ-producing T cells suggest that both classic and EKS patients have a close to normal immunologic parameters to viral infection.

MATERIALS AND METHODS

KSHV viremia and various immunologic parameters were measured in 81 patients suffering from non-HIV-related KS followed in our institution from January 1994 to January 2002. Those with histologically confirmed KS and with at least a 6 months follow-up were included in this cross-sectional study.

The study was approved by the medical ethical committee of Saint-Louis Hospital. The study was conducted according to the Declaration of Helsinki Principles. Patients gave their written informed consent.

Main clinical measures at enrollment

The following parameters were recorded: age at enrollment and at KS diagnosis, histologic type, that is, patch, plaque, nodular, and lymphangiomatosis (Chor and Santa Cruz, 1992), and KS extension classified from 0 to 5 as follows: no lesion, 1 lesion, 1–10 lesions, 10–50 lesions, 50–100 lesions, and >100 lesions. The interval time from KS diagnosis was also recorded along with the presence of lymphedema, painful lesions, past or current history of specific treatment for KS. The disease progression was defined as proposed by Krown *et al.* (1989). Patients were classified as having endemic KS when they came from sub-Saharan Africa, or as having CKS when they came from Mediterranean countries.

Control subjects

For immunologic parameters study, control patients undergoing surgery, without KS, KSHV sero-negative, and matched for age were selected.

DNA extraction and KSHV quantitative viral load

PBMCs were obtained from blood samples after separation with Ficoll. DNA was extracted using QIAamp blood DNA extraction kit (Qiagen, Hilden, Germany) and was amplified using real-time quantitative PCR performed on Light Cycler 1.3 (Roche, Neuilly Sur Seine, France) in the presence of an internal probe within orf 26 as described by Kennedy *et al.* (1997). PCR conditions and primers sequences have been previously described (Pellet *et al.*, 2001). Dilutions of known amounts (10–10^7 copies) were used for the standard curve. This method allowed us to quantify from 10 to 10^7 copies of this fragment.

Flow cytometry analysis

Lymphocyte immunophenotyping was performed on fresh whole blood samples by direct 4- 3- or 2-color immunofluorescence.

CD3, CD4 and CD8 T cells, activated CD25, HLA-DR and CD38 T cells, competent CD28 CD4 and CD8 T cells, memory CD4RO and CD45RA CD4 T cells as well as NK CD3-CD16 + CD56 + and B CD19 cells were quantified using standardized procedures (Fournier *et al.*, 2001). The following monoclonal antibodies from Becton Dickinson (San Jose, CA) were used: CD45-FITC or -PerCP, CD14-PE, CD3-FITC or -PerCP, CD4-FITC, -PE or -APC, CD8-PE, CD2-FITC, CD16 + CD56-PE, CD19-PE, CD28-PE, CD25-PE, HLA-DR-PE, CD38-PE, CD45RA-FITC, CD45RO-PE. Appropriate isotype-matched controls were carried out on each sample. Five thousand gated lymphocytes were analyzed with a FACScalibur analyzer (Becton Dickinson). Lymphocyte gate purity was $\geqslant 98\%$.

Intracellular cytokines

Intracellular IFN-γ and IL-2 were detected by flow cytometry on activated lysed whole blood with a scatter gate on the CD3 + T population (Sewell *et al.*, 1997).

Fresh whole blood sodium heparinate samples were activated in the presence of phorbol 12-myristate 13 acetate (25 ng/ml, Sigma, St Louis, MO), and calcium ionophore (ionomycin 1 μg/ml, Sigma) for 4 hours at 37°C – 5% CO_2 in the presence of brefeldine A (BFA 10 μl/ml, Sigma). Double-color surface staining was then performed with FITC- or PE-conjugated anti-CD4 or anti-CD8 and PerPC-anti-CD3 monoclonal antibodies (Becton Dickinson). Cells were then permeabilized with permeabilizing solution (Becton Dickinson) and stained with FITC-anti-IFN-γ or phycoerythrin-anti-IL-2 (Becton Dickinson). Isotypic controls, permeabilizing control (Bcl-2-FITC, 25 μ/ml, Dako, Glostrup, Denmark), and activation control (CD69-PE, Becton Dickinson) were performed at the same time. 5,000 lymphocytes were initially gated by FSC/SSC and secondary set on CD3.

Results were expressed as % of the CD4 and CD8 T-cell populations.

Cell separations

PBMC samples of eight patients suffering from CKS with positive KSHV viremia were available for cell separation. Forty million PBMCs were obtained from lithium heparinate-treated blood specimens after Ficoll separation.

The majority (B and T lymphocytes, monocytes) and minority (CD146 and CD34 cells) PBMC subpopulations were isolated using immunomagnetic Dynabeads (Dynal, Osb, Norway). Immunomagnetic separations were conducted following the manufacturer's protocol. CECs were depleted first using immunomagnetic Dynabeads coated in our laboratory with mouse anti-human CD146 (S-endo-1) MoAb (Biocytex, Marseille, France) as a second layer, as described by George *et al.* (1992). CD146-CD34 + cells, monocytes, B lymphocytes, and then T lymphocytes were then separated using standardized coated Dynabeads CD34, CD14, CD19, and

CD2, respectively. Dynabeads depletion was assessed to be complete in these experiments. Specificity of the anti-S-endo-1 antibody was tested using human microvascular endothelial cells and human umbilical vein endothelial cells as positive controls and BC-3 cells (KSHV-infected pleural effusion lymphoma cell line) as negative control. After selection with anti-S-endo-1 coated Dynabeads of sequential dilution of human umbilical vein endothelial cells cells in PBMCs from a healthy patient, about 80% of the cells remained, which means that 20% were lost during washes. Using microscopy, rosetted human umbilical vein endothelial cells appeared large, with at least 10 beads on their surface. We could observe less than 1% of contaminating cells that were small and with fewer than three beads on the surface.

Statistical analysis

Patients with positive KSHV viremia ($n=47$) and those without ($n=34$) were compared for several clinical and histologic variables, using the χ^2. All significant variables at 0.2 level were included in a multiple logistic regression model. The results are presented with odds ratios and 95% confidence intervals.

Flow cytometry data and expression of intracellular cytokines were compared by analysis of variance. *P*-values were corrected for multiple tests with the Hochberg–Benjamini method (Hochberg and Benjamini, 1990). All the statistical analyses were performed using SAS 8.2 software.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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