Detection of Kaposi sarcoma associated herpesvirus in peripheral blood of HIV-infected individuals and progression to Kaposi's sarcoma


Summary
Kaposi sarcoma-associated herpesvirus (KSHV) is consistently found in biopsy samples from patients with AIDS-related and "classical" Kaposi's sarcoma (KS). Although highly suggestive of a causal role of KSHV in the pathogenesis of KS, this observation does not exclude the possibility that KSHV, like other herpesviruses, is widely distributed and is a mere "passenger" in these lesions.

We report that KSHV was detectable in peripheral blood mononuclear cells of 24/46 (52%) of KS patients, but in none of 134 blood donors or 26 HIV-uninfected hospital controls. KSHV detection increased with immunosuppression, as shown by a correlation with a reduced number of CD4-positive T-cells. Moreover, KSHV detection in peripheral blood cells of HIV-infected individuals without KS predicted the subsequent appearance of KS lesions. 143 patients who did not have KS at the time of their first (or only) blood sample were followed up for a median of 30 months. Of the 11 who had been KS-positive 6 developed KS compared with only 12 out of 132 who were KSHV negative.

These findings are compatible with a causative role of KSHV in KS. KSHV was rarely detected in sputum and throat swabs of HIV-infected patients, providing a potential explanation for the apparently limited spread of this virus.

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Introduction
Kaposi's sarcoma (KS) is a common AIDS-defining illness but also occurs rarely in elderly individuals of Mediterranean and eastern European origin and more frequently in immunosuppressed organ transplant recipients. 1-3 Epidemiological features, in particular the high incidence of KS in HIV-infected homosexual men but virtual absence in HIV-infected patients with haemophilia, suggest the involvement of a sexually transmitted agent. 1,4,5 After several infectious agents, 2 including herpesvirus-like particles 6 had been suggested as possible candidates, DNA sequences of a new herpesvirus, provisionally termed KS-associated herpesvirus (KSHV), were recently identified in the vast majority of AIDS-associated KS lesions. 7 We and others have shown that KSHV is also found in most cases of HIV-negative KS, including those of Mediterranean and African origin, as well as in post-transplant KS cases. 8,9 The presence of KSHV in all forms of KS (but not in other angiogenic lesions 5) supports a causative role for this virus in the development of KS. However, it is still possible that KSHV, like many other herpesviruses, could have a wider distribution than predicted for the putative "KS agent". It could also represent a "passenger" in KS tissue, perhaps because its replication is favoured in either endothelial cells or spindle cells found in these lesions. We therefore investigated whether detection of KSHV in peripheral blood correlates with the presence of KS in HIV-infected individuals and whether the detection of KSHV in HIV-infected individuals without KS would predict the subsequent development of KS lesions. We also investigated its presence in sputum/throat swabs, serum/plasma and faecal material to identify possible routes of transmission.

Patients, materials, methods

Patients and controls
We studied 189 HIV-infected individuals (173 male, 16 female) with a median age of 34 (range 22–64), of whom 70 had AIDS at the time of sampling. Of the 70 with AIDS 46 had KS and 24 did not. Of the remainder, 98 were symptom-free (CDC groups 10 II or IJ), 16 had non-AIDS CDC group IV disease, and for 5 patients the CDC stage was not known. 145 individuals were homosexual, 7 were bisexual, 4 possibly homosexual or bisexual, 21 acquired HIV by a different route, and for 12 individuals the route of transmission was unknown. 77 of the 98 patients with symptom-free HIV infection were enrolled in the Middlesex and University College Hospital, London, cohorts of the MRC/INSERM Concorde study and the analysis was done on additional samples taken together with routine blood samples. Patients with symptomatic disease were selected because they were inpatients or outpatients at the time of the study and consented to an extra blood sample being taken in addition to those needed for routine investigations.

Studies reported here were approved by the Clinical Investigation Panel, Middlesex Hospital.

Laboratory methods
The number of CD4-positive T-cells in peripheral blood ("CD4 count") was measured by flow cytometry in a single laboratory. Samples from 134 healthy blood donors and 26 HIV-negative oncology patients undergoing chemotherapy or radiotherapy were also tested.

0·5 mL heparinised peripheral blood were mixed with "glycigel" 7 and 10–20 mL of heparinised blood used to separate peripheral blood mononuclear cells on Ficoll-Hypaque. DNA was extracted by standard methods. To extract DNA from sputum samples, 100 jtg/mL of sputum were mixed with 100 µL extraction buffer containing 10 mmol/L "tris" (pH 8·3), 2·5 mmol/L MgCl2, 0·5% NP40, and 0·5% Tween 20, 50 µg/mL protease K and incubated for 60 min at 56°C. After heat inactivation of protease K at 95°C for 5 min 5 µL of

Virology Laboratory, Institute of Cancer Research (D Whitby BSc, C Boshoff BSc, T Hatzioannou MSc, Prof R A Weiss MD, Prof T F Schulz MD); and Division of Virology (M R Howard BSc, N S Brink MSc, Prof R S Tedder MSc and Academic Department of Genito-Urinary Medicine (M Tenant-Flowers MSc, A Copas MSc, F E A Suggett RN, D M Aldam RN, A S Denton MSc, R F Miller MSc, Prof I V D Wellar FRCP), University College London Medical School, London, UK

Correspondence to: Prof T F Schulz, Institute of Cancer Research, 237 Fulham Road, London SW3 6BJ, UK

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this extract were used in a polymerase chain reaction. Throat swabs were placed into viral transport medium, cellular debris pelleted by centrifugation and the supernatant boiled for 10 min. Testing for KSHV by PCR was carried out on 1 µL and 10 µL boiled supernatant. Plasma and serum were diluted with an equal volume of water, boiled for 10 min, centrifuged at 15 000 rpm, and 1 µL as well as 10 µL of supernatant analysed by PCR. Fecal material was suspended in water to give a 10% slurry, shaken vigorously and pelleted at 1000 g for 10 min. 500 µL of supernatant were pelleted at 100 000 rpm for 10 min, the pellet resuspended in 20 µL of water, boiled, and 5 µL used for PCR.

Amplification by nested PCR to detect KSHV DNA was carried out independently on blinded samples in two of the participating institutions (Virology Laboratory, ICR, and Division of Virology, UCLMS): one group used previously described conditions9 and the other used published outer primers10 and the oligonucleotides GTG CTC GAA TCC AAC GGA TT and ATG ACA CAT TGG TGG TAT AT as inner primers. Sensitivity, as determined by end-point dilution of a positive sample, was equivalent in both methods. 3 samples gave discrepant results in the two laboratories. These did not affect the analyses carried out on the results presented here. The quality of extracted DNA was tested by amplifying the endogenous retroviral genome ERV-3,11 and all sputum and stool samples were tested for the presence of inhibitory factor(s) by mixing them with an end-point dilution of a known positive sample. PCR conditions for the detection of cytomegalovirus (CMV) and Epstein-Barr Virus (EBV) were as described.11

Statistical analysis

For associations between the detection of KSHV and other variables we used the chi-square test (association with KS), Fisher's exact test (when table entry numbers were low), or Mann-Whitney (association with CD4 numbers) as indicated in the text. To control for the effect of CD4 count on the association between KS and the detection of KSHV, logistic regression was used. CD4 counts (cells/µL) were grouped as 0–50, 51–100, 101–200, 201 or more. The fitted odds ratio and confidence interval were then calculated for having KS if KSHV was detected compared with KSHV not detected. A Kaplan-Meier plot was used to represent progression to KS of initially asymptomatic individuals in whom KSHV could or could not be detected and the significance of this was tested by log-rank test.

Results

None of the blood samples from 134 healthy blood donors and 26 HIV-negative cancer patients contained detectable KSHV (table 1). By contrast we detected KSHV in 24 out of 46 patients with KS (52%) but only in 11 out of 143 of those without KS (8%, table 1). Thus, 24/35 (69%) samples with detectable KSHV, but only 22/154 (14%) KSHV-negative samples, came from patients with KS (χ², p<0-0005).

For comparison we looked for Epstein-Barr virus (EBV)—which is also a γ-herpesvirus—in 73 samples from patients with and without KS and in 50 blood donors. In this selected group of 73 patients there was no association between the detection of EBV and the presence of KS but the relation between KS and KSHV persisted as in the main group (Fisher's exact; p=0-0005). The detection rate for EBV in this subset of HIV-positive patients with low CD4 counts was much higher (61%) than it was for healthy blood donors (12%, table 2), reflecting the immunosuppression in this group of HIV-infected patients which contained a higher proportion of individuals with low CD4 counts than the total group (median CD4 count 60/µL [range 0–790] vs 280/µL [range 0–1720]).

Because KS is more common in immunosuppressed transplant recipients12 we looked for a correlation between KSHV and CD4-positive peripheral blood lymphocytes. The median CD4 count in samples with detectable KSHV was 120/µL (range 0–680) compared with 300/µL (range 0–1720) in samples in which KSHV could not be detected (Mann-Whitney test, p=0-007), suggesting that KSHV, like EBV and cytomegalovirus, is indeed under immunological control. Because of this finding, and since KS is correlated with a low CD4 count,11 we re-examined the relation between the detection of KSHV in blood and KS after controlling for CD4 count by logistic regression. The fitted odds ratio for having KS if KSHV is detected rather than not detected was 23 (95% CI 8–66). This result indicates that, among these patients, having detectable KSHV in peripheral blood confers a greatly increased risk of KS, irrespective of CD4 count.

We further investigated whether HIV-positive individuals without KS, but with detectable KSHV in their blood, are at increased risk of developing KS. We followed up the 143 patients who did not have KS at the time of their only or first sample for a median of 30 months (range 0–70) from that sample date. During this follow-up 6 out of 11 individuals (55%) who initially had KSHV in their blood progressed to KS in contrast to only 12 out of 132 patients initially negative for KSHV (9%). In terms of CDC group, 15 of the 98 symptom-free patients developed KS, as did 3 of 40 patients in CDC group IV but none of the 5 patients whose CDC group was unknown. Follow-up of the symptom-free group was longer (median 52 months, range 0–70) than for those with CDC group IV disease (median 2 months, range 0–48). To account for both time to progression to KS and loss to follow-up, KS-free survival in KSHV-positive and KSHV-negative individuals was calculated and represented in a Kaplan-Meier plot. Figure 1 shows that patients with detectable KSHV progressed much more rapidly to KS than those in whom KSHV could not be detected (log-rank test; p<0-0005). Both groups were, however, comparable with respect to CD4 counts: the median CD4 count in the group of patients with detectable KSHV was 60 (range 0–790) versus 280 (range 0–1720).

Table 1: Detection of KSHV in blood of patients and controls

<table>
<thead>
<tr>
<th>Group</th>
<th>KSHV Detected</th>
<th>KSHV Not detected</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-infected patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>With KS</td>
<td>24 (52%)</td>
<td>17</td>
<td>41</td>
</tr>
<tr>
<td>Without KS*</td>
<td>11 (8%)</td>
<td>132</td>
<td>143</td>
</tr>
<tr>
<td>Proportion with KS if KSHV detected/not detected</td>
<td>69%†</td>
<td>14%†</td>
<td></td>
</tr>
<tr>
<td>Oncology patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>26</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Blood donors</td>
<td>0</td>
<td>134</td>
<td>134</td>
</tr>
</tbody>
</table>

*Without KS when tested for KSHV at first (if several samples were available) or only sample date. tp<0-0005 (Fisher's exact).

Table 2: Detection of KSHV and EBV in blood of 73 selected patients and 50 controls

<table>
<thead>
<tr>
<th>Group</th>
<th>KSHV Detected</th>
<th>KSHV Not detected</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-infected patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>With KS</td>
<td>23 (58%)</td>
<td>17</td>
<td>40</td>
</tr>
<tr>
<td>Without KS*</td>
<td>2 (6%)</td>
<td>31</td>
<td>33</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>48</td>
<td>73</td>
</tr>
<tr>
<td>Proportion with KS if KSHV detected/not detected</td>
<td>92%†</td>
<td>5%‡</td>
<td></td>
</tr>
<tr>
<td>Blood donors</td>
<td>0</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

*Without KS when tested for KSHV at first (if several samples were available) or only sample date. tp<0-0005 (Fisher's exact).
median CD4 count in KSHV-positive and KSHV-negative patients was 450 and 380/μL, respectively (no association by Mann-Whitney test). The proportion of individuals with AIDS (but without KS) at the time of testing for KSHV was also similar in both groups: of the KSHV-positive patients, 2/11 (18%) had AIDS at sample date compared with 22/132 (17%) of KSHV-negative patients. Although limited by the small number of KSHV-positive individuals, these findings suggest that 50% of HIV-infected patients progress to KS within 3-5 years of KSHV being detected in their peripheral blood.

We looked for KSHV in plasma, sputum, and stool samples to identify possible routes of transmission for this virus. KSHV was only rarely detected in sputum or throat swabs (table 3) but 10 out of the 27 KS patients whose sputum was tested had KSHV in their blood. The single patient with KSHV-positive sputum did not have KSHV in blood. None of the sputum samples contained PCR inhibitors. The single positive throat swab came from a patient with pulmonary KS. All 21 KS patients from whom throat swabs were taken had detectable KSHV in their peripheral blood. CMV was detected in 60% of sputum and 70% of throat swab samples in the same DNA extracts as for KSHV (table 3). It seems that KSHV, in contrast to other herpesviruses, is present only rarely in saliva.

KSHV was detected more often in the serum than in the plasma of KS patients (table 3), and all positives in plasma and/or serum were in patients whose whole blood was positive for KSHV too.

Because oral/anal contact has been suggested as a risk factor for KS, 18 stool samples of patients with KS were tested for KSHV but none were positive, suggesting that cell-free KSHV is unlikely to be shed by this route.

### Table 3: Detection of KSHV in sputum, faeces, plasma, and serum of HIV-infected patients

<table>
<thead>
<tr>
<th>Sample</th>
<th>KS</th>
<th>KSHV positive</th>
<th>CMV positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum</td>
<td>No</td>
<td>0/24</td>
<td>5/24 (21%)</td>
</tr>
<tr>
<td>Throat swabs</td>
<td>No</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Faeces</td>
<td>Yes</td>
<td>1/27</td>
<td>16/27 (59%)</td>
</tr>
<tr>
<td>Plasma</td>
<td>No</td>
<td>0/22</td>
<td>ND</td>
</tr>
<tr>
<td>Serum</td>
<td>No</td>
<td>0/18</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>8/51 (16%)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>11/24 (46%)</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND=not done.

### Discussion
On epidemiological evidence, the putative "KS agent" would be expected to be confined to individuals at high risk for KS in western countries but be more prevalent in central Africa. Several groups have reported that KSHV is found in almost all biopsy specimens from patients with KS, irrespective of whether they are HIV-infected or not, but not in a wide variety of non-lymphoid tumours in patients from non-KS endemic areas. However, KSHV could still, by analogy to some other herpesviruses, be widely distributed and present as a mere "passenger" in KS lesions, particularly if its replication were favoured in this proliferative tissue. Also KSHV has been detected in peripheral blood of patients with AIDS-associated KS.

Discussion

We (unpublished) and others have found KSHV in peripheral blood CD19-positive B-cells but it may infect other cell lineages. Our group has found KSHV in the flat endothelial cells lining the vascular spaces of KS lesions, as well as in KS spindle cells, but not in normal vascular endothelium adjacent to KS lesions. A cell population with some of the properties of KS spindle cells has been shown to circulate in peripheral blood. Interpretation of our findings therefore is that the higher detection rate of KSHV in peripheral blood of AIDS patients with KS reflects either an increased number of B-cells infected by KSHV originating from endothelial cells of KS lesions, or circulating KSHV-infected spindle or endothelial cells. In this scenario KSHV could simply be a passenger virus replicating in KS lesions but our observation that detection of KSHV predicts progression to KS is difficult to reconcile with such a passenger status. Most patients progressing to KS after KSHV had been detected in their blood had high CD4 counts (median 450/μL) when the virus was detected. It is unlikely that they would have had clinically undetectable KS in other tissues at that stage, although we cannot formally exclude the possibility. We therefore feel that the lack of detectable KSHV in blood samples of healthy donors and patients with other neoplasms and the detection of this virus before progression to KS support a causative role for KSHV.
KSHV in KS. The delayed progression to overt KS (50% KS-free survival was at 3-5 years [figure 1]) suggests that events and co-factors other than immunosuppression are necessary to promote the development of KS lesions. Individual KS nodules have been shown to be of monoclonal origin, suggesting a situation similar to that in nasopharyngeal carcinoma (an EBV-associated epithelial tumour) and some EBV-associated B-cell lymphomas, where infection with EBV precedes transformation into a monoclonal tumour.

AIDS-associated KS is more severe than Mediterranean and endemic African KS. This clinical observation, together with the growth-promoting effect of HIV-tat on KS spindle cell cultures in vitro, suggests that infection with HIV accelerates progression towards KS through both immunosuppression and tat activity. KSHV may infect rare endothelial cells and promote their transformation into spindle cells with the help of cofactors. Alternatively, other factors, such as HIV-tat, are responsible for the activation of individual endothelial cells which then form the target for KSHV. However, HIV is rarely detectable in KS lesions and tat levels will presumably be low.

Since the frequency of KS is increased in HIV-negative transplant recipients, the putative KS agent had been expected to establish a persistent infection, controlled by the immune system. We have shown here that KSHV is easier to detect in individuals with low CD4 counts, suggesting that its replication, or the numbers of KSHV-infected cells, are indeed under immunological control. In this respect KSHV resembles its close relative EBV. Although KS occurs occasionally in individuals who contracted HIV through blood transfusion, it is very rare in haemophiliacs, suggesting that the KS agent is cell-associated or that is inactivated during factor VIII purification. We could detect KSHV DNA in a few plasma samples and more often in serum. Since KSHV DNA positive serum or plasma came from patients with KSHV DNA in their whole blood, we cannot exclude the possibility that KSHV DNA found in serum had leaked from cells during the clotting process and that the detection of KSHV DNA in serum or plasma may not reflect the presence of infectious virus. In contrast to cytomegalovirus KSHV was only rarely found in sputum samples or throat swabs (table 3), and this may explain the apparently limited spread of this virus. KSHV DNA was not detected in faecal samples after removal of cellular material but it is still possible that cells of the intestinal tract harbour infectious KSHV, as suggested by the risk of oral-anal contact for contracting KS.

Our findings are compatible with a causative role of KSHV in the development of KS with a high rate of detection in patients with this disease. Furthermore, the presence of KSHV in blood indicates a high likelihood of subsequent development of KS. In contrast, KSHV is rarely found in non-KS HIV-infected patients and not in blood donors. However, a recent report of detection of KSHV in squamous cell skin carcinomas of HIV-negative immunosuppressed transplant recipients points to the need for a serological assay to determine its prevalence in the general population.

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References