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Prevalence of Kaposi's sarcoma associated herpesvirus infection measured by antibodies to recombinant capsid protein and latent immunofluorescence antigen

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Summary

Background Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8, may be the infectious cause of KS. Its prevalence in the general population, on the basis of detection of the virus genome, is controversial. To investigate the seroprevalence, we measured antibodies to a recombinant capsid-related (lytic cycle) KSHV antigen and a latent antigen complex.

Methods We selected potentially immunoreactive capsid-related proteins of KSHV by expressing them as recombinant proteins and testing them in western blot assays. We used a truncated recombinant protein encoded by KSHV open reading frame 65 (orf 65) to develop a diagnostic enzyme-linked immunosorbent assay (ELISA) and tested sera from HIV-infected individuals with KS, HIV-uninfected patients with "classic" KS, other HIV risk groups, and blood donors. We also compared the antibody response to this capsid-related protein to the response to latent antigen(s) in an immunofluorescence assay.

Findings 77/92 (84%) sera from KS patients reacted with the KSHV orf 65 protein and 84/103 (81·5%) reacted with KSHV latent antigen(s). The dominant immunogenic region of orf 65 is within the carboxyterminal 80 aminoacids, a region with little sequence similarity to the related Epstein-Barr virus, suggesting that orf 65 is a KSHV specific antigen. Only three sera from patients with haemophilia (1/84) or from intravenous drug users (2/63) had KSHV specific antibodies in the orf 65 assay whereas none of these sera reacted with latent antigen. Antibodies to KSHV were also infrequently found in UK and US blood donors by either assay (UK, 3/174 with orf 65 and 4/150 with latent antigen; US, 6/117 with orf 65 and 0/117 with latent antigen). They were more common among HIV-infected gay men without KS (5/16 by orf 65 ELISA, 10/33 by IFA), HIV-uninfected STD clinic attenders (14/166 by IFA), and Ugandan HIV-uninfected controls (6/17 by orf 65 ELISA, 9/17 by IFA). Antibody reactivity to the orf 65 protein (ELISA) and to latent antigen(s) (IFA) was concordant in 89% of 462 sera tested but reactive blood donor sera were discordant in both assays. Four AIDS-KS sera were unreactive in both assays.

Interpretation The distribution of antibodies to both a capsid-related recombinant protein and latent antigen(s) of KSHV strongly supports the view that infection with this virus is largely confined to individuals with, or at increased risk for, KS. However, infection with KSHV does occur, rarely, in the general UK and US population and is more common in Uganda. Antibodies to latent antigen(s) or to orf 65 encoded capsid protein will not detect all cases of KSHV infection, and a combination of several antigens will probably be required for accurate screening and confirmatory assays.

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Introduction

Epidemiological evidence suggests that Kaposi's sarcoma (KS) in both HIV-uninfected and HIV-infected individuals is caused by a transmissible agent.[1,2] Among HIV-infected patients KS is much more common in homosexual men than it is in patients with haemophilia, transfusion recipients, and intravenous drug users (IVDU).[3] Transmission of the putative "KS agent" occurs independently of HIV[4] and shows a marked geographical variation,[1,5] as does the incidence of non-HIV KS (higher in central and eastern Africa than in the west or south, and within Europe commoner in some Mediterranean countries[1]).

KS-associated herpesvirus (KSHV),[6] otherwise known as human herpesvirus 8, is a γ_2-herpesvirus related to Epstein-Barr virus (EBV).[7] KSHV is consistently found in all forms of KS (AIDS-related,[6,8,9] African endemic,[10–12] classic,[8,12,13] and post-transplantation[8,12]). KSHV genomes are present in endothelial and spindle cells, the histological hallmarks of KS.[14] Detection of KSHV in peripheral blood correlates with, and in asymptomatic HIV-infected individuals predicts the development of, KS.[15,16] However, whether the distribution of KSHV matches that expected for the putative KS agent, remains controversial. Several groups[15,17] have not detected KSHV by polymerase chain amplification in the peripheral blood of healthy donors; others have reported it in 9% of peripheral blood mononuclear cells and lymphoid tissue from HIV-uninfected people.[18] Similarly, some groups,[19,20] but not others,[21,22] have reported a high prevalence in semen samples from healthy donors. Serological data from immunoblotting and immunofluorescence assays (IFA) using B-cell lymphoma cell lines infected with both KSHV and EBV,[7,23,24] or with KSHV alone,[25,26] are now emerging. Antibodies to latent KSHV antigen(s) were found in the vast majority of KS patients but not, or only rarely, in HIV-infected IVDU, patients with haemophilia, or healthy blood donors. Seroconversion to latent nuclear antigens can occur years before the onset of KS and is strongly predictive of subsequent disease.[24,25]

The antibody response to different antigen complexes of EBV varies in acutely or chronically infected individuals or in immunosuppressed patients with reactivated EBV infection.[27,28] Several potentially immunoreactive lytic-cycle proteins of KSHV, including the major capsid protein, are highly homologous to their EBV counterparts[7] and are thus unlikely to provide specific serological antigens. To investigate the prevalence of KSHV infection more comprehensively, we measured the antibody response to selected KSHV-encoded capsid-related (lytic cycle) proteins with low sequence similarity to the corresponding EBV proteins in addition to the antibody response to previously described latent antigen(s).

Patients and methods

Patient sera

We analysed 140 sera from HIV-infected gay men attending an HIV clinic, of whom 107 had KS and 33 had AIDS but no KS; 51 sera from HIV-infected Ugandan patients, of whom 17 had KS, and 17 HIV-uninfected controls;[25] 18 sera from Greek patients with "classic" KS and 26 sera from age and sex matched controls obtained from a case-control study in Athens; 110 sera from patients with haemophilia (84 Edinburgh, 26 London), of whom 54 (26 London, 28 Edinburgh) were infected with HIV; and 63 from intravenous drug users in Edinburgh, of whom 38
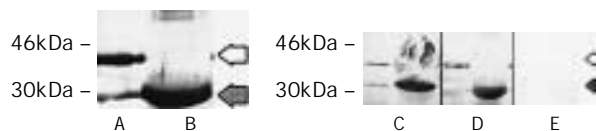

Figure: 1 **Western blot with recombinant KSHV antigens**
Reactivity of blood donor serum (A) and AIDS-KS serum (B) with recombinant orf 65.1 and orf 65.2 proteins panel. In each panel the left-hand lane is orf 65.1 (open arrow) and the right-hand lane is orf 65.2 (closed arrow). Orf 65.1 protein expressed less well and approximately 10-fold less protein was loaded, explaining weaker intensity of orf 65.1 band. Confirmation of ELISA-reactive sera by western blot on recombinant orf 65.1 and orf 65.2 proteins. (C) UK blood donor (ELISA+/IFA-/WB+); (D)=Greek control patient with epithelioma (ELISA+/IFA+/WB+); (E)=high titre EBV VCA serum.

were infected with HIV. We also studied 291 sera from blood donors (174 UK, 117 US), 166 sera from patients attending a sexually transmitted diseases clinic (see table 1), 15 HIV-infected women of African origin, and 24 HIV-uninfected children with rash or fever.

Expression of recombinant KSHV proteins

KSHV open reading frames (orf) 52 and 65 are homologous, respectively, to the EBV genes BLRF2 and BFRF3 which, as recombinant proteins, react with more than 80% of sera from EBV-infected individuals. However, the aminoacid identity between these KSHV and EBV homologues is only 37% for orf 52 vs BLRF2 and 26% for orf 65 vs BFRF3, low enough to permit detection of KSHV specific antibodies. Orf 52 and orf 65 (or segments) were fused to the carboxyterminal end of mouse dihydrofolate reductase, and placed under the control of a T7 promoter in the expression vector pQE 42 (Quiagen, Hilden, Germany). This vector also provides a histidine tag at the aminoterminal end of the fusion protein and allows purification of recombinant proteins by affinity chromatography on Ni-NTA resin (Qiagen). To generate construct orf 65·1 (aminoacids 2–170 of orf 65), we used primers GAG AGA GAT CTG TTC CAA CTT TAA GGT GAG AGA C and TCT GCA TGC CGG TTG TCC AAT CGT TGC CTA. These primers generate a 589 bp fragment which was cloned using the BglII and SphI sites. Construct orf 65·2 (aminoacids 86–170) was assembled using primers AGA GAG ATC TGT GCT GAC CGA GTT TCC GCG GCG and TCT GCA TGC CGG TTG TCC AAT CGT TGC CTA. The carboxyterminal half of orf 65 (aminoacids 86–170) and the equivalent region of EBV BFRF3 share only 18 evenly distributed aminoacids (21% identity), which makes antibody cross-reactivity unlikely, and, like the latter,[29,30] contains the most important serological epitopes (figure 1). Both recombinant orf 65 proteins reacted well with the same number of sera from KS patients but not with 10 sera with high
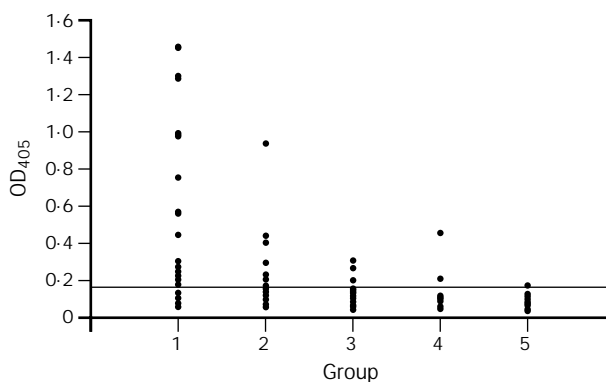

Figure 2: **Representative results obtained with orf 65 ELISA**
Results of 21 serum samples from each of the following patient groups, tested by ELISA at 1 in 100 dilution, are shown: (1) AIDS-associated KS; (2) AIDS without KS; (3) UK blood donors; (4) intravenous drug users; (5) patients with haemophilia. Horizontal line represents "cut-off" of 0·16 (see text).
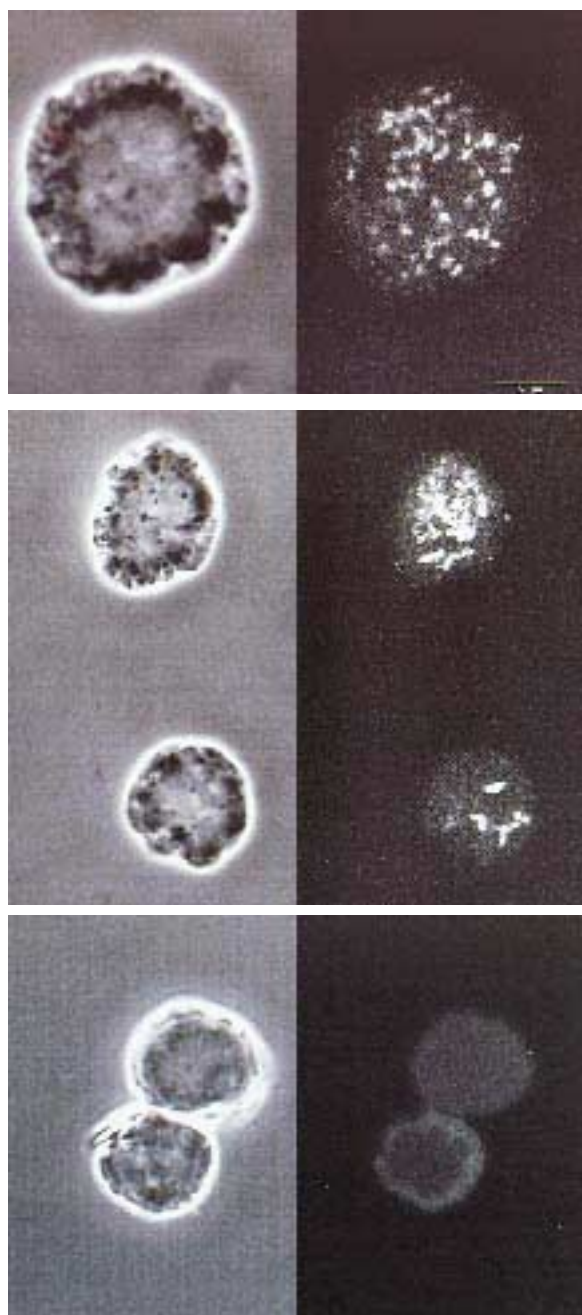
Group	Proportion (and %) testing positive by		
	Orf 65 ELISA	Latent antigen IFA	PCR
AIDS-KS			
UK/US	46/57 (81)	84/103 (81·5)	48/82 (59)
Uganda	14/17 (82)	. .	. .
Classic Greek KS	17/18 (94)	17/18 (94)	9/15 (60)
HIV-infected, no KS			
Homosexual men	5/16 (31)	10/33 (30)	4/28 (14)
Female STD clinic attenders	. .	3/15 (20)	2/15 (13)
Haemophilia			
HIV infected	0/28*	0/26†	0/26†
HIV uninfected	1/56*	. .	. .
Intravenous drug users			
HIV infected	2/38	0/38	. .
HIV uninfected	0/25	0/25	. .
HIV-uninfected STD clinic attenders			
Homosexual men	. .	8/65 (12)	. .
Heterosexual men	. .	4/75 (5)	. .
Heterosexual women	. .	2/26 (8)	. .
Children with rash and fever	. .	0/24	. .
Blood donors			
UK	3/174 (1·7)‡	4/150 (3)	. .
USA	6/117 (5)‡	0/117	. .
Greek age/sex matched controls	3/26 (12)§	3/26 (12)	0/15
Ugandan controls			
HIV-infected	16/34 (47)	18/34 (53)	. .
HIV-uninfected	6/17 (350	9/17 (53)	. .

*From Edinburgh. †From London. ‡Also reactive on western blot on recombinant orf 65.1 and orf 65.2 (figure 1). 11 blood donor sera were initially reactive in the orf 65.2 ELISA. §Also reactive on western blot.

Table 1: **Antibodies to KSHV orf 65 protein and latent immunofluorescence antigen in different HIV risk groups and controls**

Figure 3: **Immunofluorescence for latent antigen**
Upper and middle: two positive sera at 1 in 150 dilution showing punctate nuclear immunofluorescence. Lower=negative serum at 1 in 20 dilution to show diffuse background fluorescence. Left-hand panels=phase contrast; right-hand panels=immunofluorescence. Scale line in upper panel is 5 μm; middle and lower panels are at roughly half that magnification.

EBV viral capsid antigen titre (figure 1). Near complete orf 52 (aminoacids 4–131), expressed as for orf 65, showed no reactivity with sera from KS patients and was therefore not used any further.

ELISA with orf 65.2 protein
ELISA plates (Immulon 4, Dynatech) were coated with 100 μL purified orf 65·2 protein (about 5 μg/mL) in 0·1 mol/L NaHCO₃, pH 8·5 for 16 h at room temperature. A conventional ELISA protocol was used with phosphate-buffered saline containing 0·1% Tween 20 (PBS-T) for washes, 5% dried skimmed milk in PBS-T (blocking buffer) to saturate plates and to dilute patient sera (1 in 100), and an alkaline phosphatase conjugated affinity-purified goat anti-human IgG (Seralab), diluted 1 in 1000 in blocking buffer containing 1% goat serum, followed by 0·1 mg/mL of nitrophenyl phosphate (Sigma) in glycine buffer (0·1 mol/L glycine, 1 mmol/L MgCl₂, 1 mmol/L ZnCl, pH 10·4) as substrate. The colorimetric reaction was stopped after 1 hour at 37°C with 50 μL 3 mol/L NaOH and read spectrophotometrically at 405 nm.

For the blinded testing of sera we used the mean of optical density values at 405 nm (OD_{405}) of 10 blood donor sera plus five standard deviations as the "cut-off" value to define reactive sera; we also included 2 sera which gave reactivities close to the cut-off, 2 "low positive", and 1 "high positive" sera, to ensure inter-assay comparability.

Immunofluorescence assay (IFA) for antibodies to latent KSHV antigens
This was done on a body-cavity related B-cell lymphoma cell line (primary effusion lymphoma) latently infected with KSHV (BCP-1).[25] In brief, BCP-1 cells were fixed in 4% paraformaldehyde, rendered permeable with 0·2% Triton X100, incubated with human sera (diluted 1 in 150) for 30 min at room temperature, washed three times in PBS/3% bovine serum albumin, incubated with rabbit anti-human IgG-fluorescein isothiocyanate conjugate (DAKO) for 30 min, washed again, and examined by fluorescence microscopy. Micrographs (see figure 3) were taken with a BioRad MRC600 confocal imaging system in conjunction with a Nikon fluorescence Optiphot×60 plan-apo. In preliminary experiments a serum dilution of 1 in 150 had been shown to be the best cut-off point to avoid the homogeneous non-specific cytoplasmic background staining without affecting the nuclear punctate staining (figure 3). Selected negative sera were examined at higher serum concentrations (1 in 20, 1 in 50) without obtaining a higher rate of positive results. Antibodies to KSHV were titrated for 10 seropositive patients, with a median titre of 1625 (range 200–50 000). The IFA results for US blood

donors, Ugandan KS patients, and Ugandan HIV-infected and uninfected controls used here for comparison with ELISA results (table 1) have been published previously.[25]

PCR for KSHV

We extracted peripheral blood mononuclear cell DNA from 151 KS or HIV infected KSHV patients from samples taken at the same time as the sera. The DNA samples were tested by nested PCR.[8,15]

Results

Antibodies to KSHV orf 65

In preliminary experiments most sera from KS patients reacted with both recombinant proteins, the complete orf 65 protein (orf 65.1) and the aminoacid 86–170 sequence (orf 65.2) (figure 1). We used orf 65.2 as an antigen in a diagnostic ELISA. Figure 2 shows examples of ELISA results.

The ELISA detected antibodies to orf 65 in 80·7% UK or US AIDS-KS sera, 82·4% Ugandan AIDS-KS sera, and 94·4% of HIV-negative classic KS sera (table 1). Antibodies to orf 65 were found in 31·3% of homosexual men with AIDS but no KS. 18/18 ELISA-reactive sera from individuals with AIDS-KS or AIDS in the absence of KS were also reactive by western blot on recombinant orf 65.1 and 65.2 antigen (figure 1).

Reactive sera were rare in other risk groups for HIV transmission: 1·2% of patients with haemophilia (no reactive serum among HIV-infected patients with haemophilia) and 5·3% of intravenous drug users with HIV infection had antibodies to KSHV orf 65 (table 1). Among UK blood donors, 11/169 sera were reactive by ELISA; of these, 3 were also reactive with recombinant orf 65.1 and orf 65.2 on western blots (figure 1), suggesting a prevalence of KSHV infection in UK blood donors of at least 1·6% (table 1). Among US blood donors, we found 6/117 (5·0%) orf 65 reactive by ELISA and western blot sera. 3 of 26 Greek control sera were reactive by ELISA and all three were also positive by western blot and/or immunofluorescence. Infection with KSHV seems to be more widespread in Uganda among HIV-infected and HIV-uninfected individuals (47·1% and 35·3% orf 65 reactive sera, respectively).

We tested, blinded, two to four sequential samples from 14 KS patients and obtained concordant results, except in one individual whose last (of three) sample was non-reactive.

Antibodies to latent KSHV antigen(s)

Figure 3 shows the nuclear staining pattern representing a positive reaction with KSHV nuclear antigen(s).[25,26] 81·5% of AIDS-KS patients, 94% of classic KS patients, but only 30% of HIV-infected homosexual men without KS, had antibodies to this nuclear antigen (table 1). In contrast, none of 26 HIV-infected patients with haemophilia had antibodies to this latent antigen. There was no significant difference detected in seroprevalence between HIV-uninfected STD clinic attenders who were homosexual men (12%), heterosexual men (5%, 2-tailed Fisher's exact test p=0·25) or heterosexual women (8%, p=0·71), suggesting that the mode of KSHV transmission is not confined to homosexual practices. 4/150 (2·7%) blood donor sera were reactive with KSHV IFA nuclear antigen and none of 24 children who had sera taken when they presented with fever and rash.[31] Among individuals,

whose sera were tested by IFA, we found similarly low CD4 counts in AIDS-KS patients (range 0–400, median 80), HIV-infected patients with haemophilia (0–440, 150), and HIV-infected, non-African women (10–340, 90), suggesting a comparable degree of immunosuppression despite very different seropositivity rates for KSHV.

Concordance of ELISA, immunofluorescence, and PCR results

Table 1 shows that antibodies to both orf 65 and the latent immunofluorescence antigen have the same distribution among KS patients, different risk groups for HIV transmission, and blood donors, supporting the conclusion[15,16,24–26] that infection with KSHV is largely confined to people with, or at risk of, KS, and that it is uncommon in the general UK and US population. Furthermore, table 2 shows that 89·2% of 462 sera tested by both ELISA and immunofluorescence gave a concordant result. 78·1% of 105 immunofluorescence-positive sera were also positive by ELISA and 75·2% of 109 ELISA positive sera were also reactive in immunofluorescence. Among the 14 AIDS-related KS patients from whom sequential sera were available, we observed three individuals who consistently lacked antibodies to the orf 65-encoded protein despite being reactive by immunofluorescence on one or more occasions, suggesting that the lack of orf 65 reactivity can be a stable phenomenon. 4 patients with AIDS-related KS were negative in both assays. The 9 orf 65 reactive (ELISA and western blot) blood donor sera were negative by immunofluorescence and four immunofluorescence positive blood donor sera did not react with orf 65. Among the Ugandan sera, the concordance rate between the two assays was lower at 69·6%; 68% of immunofluorescence-reactive samples were ELISA reactive, and 78% of ELISA-reactive samples were also positive by immunofluorescence. Thus both assays suggest a high rate of KSHV infection among HIV-

Reactivity to KSHV orf 65 (ELISA/WB)	Reactivity to KSHV latent antigen (IFA)					
	KS*		HIV infection no KS†		UK/US blood donors‡ and Greek controls	
	+	−	+	−	+	−
+	66	9	14	8	2	9
−	9	5	9	19	5	307

These correlations in 2×2 tables were significant by kappa test: for KS κ=0·24 (p=0·01, one tailed); for HIV infection, no KS κ=0·31 (p=0·01), and for blood donors and controls κ=0·20 (0=0·001).
*Includes UK/US and Ugandan AIDS KS cases.
†Includes HIV-infected gay men with AIDS, but without KS, and HIV-infected Ugandans without KS.
‡Includes western blot confirmed blood donor results (see text).

Table 2: **Comparison of antibody reactivity to orf 65 protein and to latent antigen(s)**

Detection by PCR	Serological reactivity					
	Orf 65		Latent IF antigen		Orf 65/latent antigen combined*	
	+	−	+	−	+	−
+	25	6	26	6	32	3
−	18	5	17	5	17	2

*Reactivity with either orf 65 or latent antigen.
‡Includes western blot reactive blood donor results (see text).

Table 3: **Comparison of PCR detection of KSHV and antibody reactivity to orf 65 and latent antigen(s)**

infected and HIV-uninfected Ugandans.

Tables 1 and 3 show that KSHV genomes were detected in peripheral blood monocyte DNA from KS patients less frequently than antibodies to either KSHV antigen in serum. Although both ELISA and immunofluorescence are thus more sensitive indicators of KSHV infection than PCR, not all PCR positive samples were seropositive. The concordance between genome detection by PCR and serology was very similar for both serological assays 30/54 [56%] for immunofluorescence, 31/54 [57%] for ELISA.

Discussion

We describe here the first recombinant KSHV protein which is recognised by most sera from KS patients, and we show the ELISA based on this protein to be useful for the serological diagnosis of infection with KSHV. Orf 65 encodes a protein which, according to its positional homology to EBV BFRF3, is probably a capsid protein[30] and therefore expressed during the lytic cycle of viral replication. Its predicted molecular weight (about 18 kD) is not identical with a previously described 40 kD lytic-cycle KSHV protein, recognised by about 67% of patient sera, or the 27 kD and 60 kD lytic-cycle proteins which react with only a small proportion of KS sera.[23] Its immunogenic determinants lie in the carboxyterminal half, which shares only 21% of its aminoacids with the corresponding (also immunogenic) EBV-BFRF3 segment.[29,30] Of 10 sera with high titre EBV VCA antibodies none reacted with both recombinant orf 65 proteins. The carboxyterminal region (86–170) is therefore a promising candidate for a specific serological antigen.

Latently infected body-cavity related B-cell lymphoma cell lines express at least one latent antigen which has been reported to react (by immunofluorescence) with about 90% of sera from AIDS-related KS patients[25,26] and whose identity is presently unknown. A high molecular weight immunoreactive doublet antigen which is localised to the nucleus has also been reported but it is unknown whether this is the antigen being assayed in the BCP-1 IFA assay.[24,25]

With an ELISA based on recombinant orf 65.2 protein, we have found 80% or more of KS sera to be reactive, while sera from patients with haemophilia and intravenous drug users, who have a low risk of developing KS, even after infection with HIV,[1] were seldom ELISA reactive. We obtained very similar results by measuring antibodies to a latent KSHV immunofluorescence antigen, as described recently.[24–26] Thus antibodies to both a latent and a capsid protein with low homology to EBV are uncommon in individuals at low risk for KS. Taken together with the recently reported small number of IFA-reactive US blood donor sera,[26] the serological results reported here support our earlier conclusion, reached on the basis of KSHV detection by PCR,[15,16] that infection with KSHV is not common in the general UK and US population.

In EBV infection, antibodies to viral capsid antigen and nuclear (latent) antigen persist,[27] so antibody responses to both capsid and latent proteins of KSHV are probably due to infection with, rather than reactivation of, KSHV.[24,25] We also found that HIV-positive women and patients with haemophilia had low CD4 counts, comparable to those of AIDS-KS patients. It is therefore unlikely that the higher frequency of KSHV antibodies in AIDS-KS patients compared to HIV-infected women or patients with haemophilia is due to virus reactivation following immunosuppression.

The sensitivities of both the orf 65 ELISA and IFA were comparable and a similar number of sera was "missed" by either assay alone. This suggests that a combination of lytic and latent recombinant antigens will be required for an ELISA with the sensitivity and specificity needed for reliable testing of sera from patients. While the concordance between the two assays was high for KS sera, all orf 65 reactive blood donor sera were immunoflourescence negative, and vice versa. It is therefore difficult at the moment to estimate the prevalence of KSHV infection in UK/US blood donor sera accurately. However, with a combined sensitivity of both assays of 94% (84/89 KS sera detected; see table 2) we would estimate the prevalence of KSHV infection in UK blood donors to be at most about 5% (1·7%+2·7%, see table 2). A recent study,[32] using an immunofluorescence assay on undefined structural (lytic cycle) antigens, found a higher prevalence of KSHV antibodies among US blood donors. However, the possibility of cross-reactivity with homologous EBV structural proteins[7] remains to be investigated further.

Infection with KSHV may be more common among patients attending a sexually transmitted disease clinic than among blood donors. The epidemiology of KSHV may therefore be more similar to that of herpes simplex virus type 2[33,34] than to that of EBV or other human herpesviruses. In this respect, KSHV fulfils the criteria required for a transmissible co-factor involved in KS pathogenesis.[1,3] Our study found that both HIV positive gay men and African women had higher KSHV seroprevalences than HIV positive haemophilia patients which is similar to the trend for AIDS-KS among these different HIV risk groups. Our ELISA results also confirm that KSHV infection is more common in Uganda than in the UK or US[25] but its exact prevalence in different geographical areas remains to be established, most likely by a combination of tests measuring antibodies to both lytic and latent antigens, as illustrated here.

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