Epidemiol. Infect. (2003), **131**, 1125–1129. © 2003 Cambridge University Press
DOI: 10.1017/S0950268803001146 Printed in the United Kingdom

Human herpesvirus-8 antibodies and DNA in HIV-1 infected patients in South Africa

L. ALAGIOZOGLOU[1], L. MORRIS[1], H. BREDELL[1], D. J. MARTIN[1] AND F. SITAS[2]*

[1] *AIDS Virus Research Unit, National Institute for Communicable Diseases and Department of Virology, University of the Witwatersrand, Johannesburg, South Africa*
[2] *Cancer Epidemiology Research Group and National Cancer Registry, National Health Laboratory Service, Department of Anatomical Pathology, University of the Witwatersrand, Johannesburg, South Africa*

(*Accepted 23 June 2003*)

SUMMARY

HIV-infected individuals with high levels of IgG antibodies against human herpesvirus-8 (HHV-8) are at increased risk of developing Kaposi's sarcoma. The aim of this study was to measure the association between HHV-8 viraemia and IgG antibody responses (by immunofluorescence) in a group of 201 HIV-infected individuals attending outpatient clinics, 91 in-patients with AIDS and 87 HIV-infected patients admitted with Kaposi's sarcoma. Compared to HIV-infected outpatients, the adjusted odds ratio in relation to Kaposi's sarcoma was 15·4 (95% CI 4·4–54·2) in those with viraemia, 25·1 (95% CI 6·6–95·6) in those with a positive immunofluorescent signal and ∞ (lower exact CI 33·6) in those with a high immunofluorescent signal (all P trend $<0{\cdot}001$). Among those without HHV-8 viraemia, 23% were IgG-positive, but only 5·5% had a high immunofluorescent signal. In those who were viraemic, 89·1% were IgG-positive, and 28·2% had a high immunofluorescent signal, suggesting viraemia is associated with high HHV-8 immunofluorescence IgG signal.

INTRODUCTION

Human herpesvirus-8 (HHV-8) is now recognized as the infectious agent responsible for causing Kaposi's sarcoma (KS) [1]. While the absolute risk of developing KS in HHV-8-infected individuals is low, it is much greater in those co-infected with HIV [2], or among those under post-transplant immunosuppressive therapy [3] suggesting that HHV-8 is under immunological control. The reservoir for HHV-8 appears to be the lymphoid organs, and reactivation is thought to be due in part to circulating HHV-8-infected spindle cells, which localize in tissues and participate in the formation of KS lesions [4, 5]. The incidence of KS is increasing in Africa in parallel to HIV infection [6, 7]. In South Africa, patients with KS had higher HHV-8 IgG antibody levels/titres compared to other cancer controls [2]. It was speculated that high antibody titres reflect, at least in part, increased HHV-8 viraemia, which in turn, causes the increased risk of developing KS. However, the direct association between HHV-8 antibody levels and viraemia has not been documented.

The aim of this study was to measure the association between HHV-8 viraemia and immunofluorescent IgG antibody response in a group of 379 individuals comprising 201 HIV-infected individuals attending outpatient clinics, 91 in-patients with AIDS and 87 HIV-infected patients admitted with KS.

* Author for correspondence: Dr F. Sitas, Cancer Research and Registers Division, The Cancer Council NSW, PO Box 572, Kings Cross, Sydney, NSW 2011, Australia.

METHODS

Patients

Ethical approval for this study was obtained from the University of the Witwatersrand Committee for Research on Human subjects. Peripheral blood was collected, consecutively, in EDTA from 379 anonymized, unlinked HIV-infected individuals from various hospitals in Johannesburg, South Africa and sent to the National Institute for Virology (now the National Institute for Communicable Diseases) for HIV and other diagnostic testing. Of these, 87 were recruited from another ongoing study and were confirmed as having KS based on clinical and histological examinations. The remainder of the specimens were from HIV-1 antibody-positive individuals (who had no clinical signs of KS), drawn from local outpatient clinics ($n=201$) and from those who had been hospitalized with AIDS-defining conditions ($n=91$). In this group, lymph node biopsies were available from eight patients who were undergoing routine diagnostic investigation for suspected tuberculosis.

PCR amplification of HHV-8 DNA

DNA lysates from peripheral blood mononuclear cells (PBMC) or from isolated lymph node cells were screened for HHV-8 DNA by nested PCR using primers in two different regions. The KS330BAM$_{233}$ primers which amplify a 330 bp were used as described [8]. An 804-bp fragment in the ORF75 gene was amplified as described in an earlier study [9]. A sample from a KS patient who was previously 330BAM$_{233}$ PCR-positive was used as a positive control and water was used as a negative control in all PCR reactions. Samples were considered HHV-8 DNA-positive if they amplified in either gene region.

Detecting antibodies to HHV-8 latent antigens

Plasma specimens were screened for antibodies to the latency-associated nuclear antigen (LANA) [10, 11] using an immunofluorescence assay (IFA). BCP-1 cells were cultured in RPMI 1640 growth medium containing 100 IU/ml penicillin and 100 μg/ml streptomycin with 20% foetal calf serum (FCS) and 1% L-glutamine. The method was similar to that of Simpson et al. [12] except that cells were permeabilized with 0·5% Triton X-100. Approximately 10 000 cells were placed in a 5 mm well of a 12-well HTC super-cured slide (Erie Scientific Co., Portsmouth, NH, USA) and allowed to dry and stored at -70 °C until use. Plasma samples were diluted 1 in 100 in 3% FCS and incubated in the wells in a humidified chamber for 30 min at room temperature. Slides were washed five times in 1% FCS. Mouse anti-human IgG-FITC conjugate (Zymed Labs Inc., CA, USA) was diluted at 1 in 40 with Evans Blue counter stain and incubated on the slides in humidity for 20 min at room temperature. Cells were washed as before and analysed using a Microflex HFX-II fluorescence microscope (Nikon, Japan) at 400× magnification within 48 h of preparation. Three known positive plasmas with high, intermediate and low levels of anti-HHV-8 antibodies and one negative plasma sample were included on each slide as controls. Specimens were scored as positive when punctate fluorescent spots were visible in the nucleus indicating the presence of latent nuclear antigens. Positive samples were scored as low, intermediate, or high depending on their fluorescence intensity. Homogeneous cytoplasmic fluorescence was recorded as negative.

Statistical analysis

The risk [odds ratio (OR), and 95% confidence intervals (CI)] of the association between HHV-8 and KS was estimated using logistic regression analysis. ORs were adjusted for age, sex, current residence and race group. Associations between the two different assays were estimated using χ^2 statistics.

RESULTS

The HIV-infected outpatients were largely asymptomatic with an average age of 36 years and a median CD4 count of 280 cells/μl while the in-patients with AIDS were mostly admitted with tuberculosis or meningitis and were on average 34 years old with a median CD4 count of 201 cells/μl. Patients with KS had an average age of 36 years. No CD4 counts were available for this group. Table 1 shows that amplification of HHV-8 gene sequences in PBMC (viraemia) was detected in 3% of HIV outpatients, 5·5% of in-patients with AIDS and in 50·6% of patients with KS (P trend $<0{\cdot}0001$). Of the 55 PCR-positive samples, 46 were positive for both gene regions, 5 were positive in ORF26 only and 4 were positive in ORF75 only. Compared to the HIV outpatients, the adjusted OR in relation to KS was 15·4 (95% CI 4·4–54·2). In the 281 patients where there was sufficient blood for measuring immunofluorescence.

Table 1. *Relationship between HHV-8 infection and disease condition*

	HHV-8 PCR assay			HHV-8 LANA immunofluorescence assay				HHV-8 LANA immunofluorescence assay			
				Any positive (+, ++, +++)				Moderate/high intensity (++, +++)			
Condition	No./total	OR	(95% CI)	No./total	OR	(95% CI)	PCR+/LANA+	No./total	OR	(95% CI)	PCR+/LANA+
HIV (outpatients)	6/201 (3·0%)	1		16/129 (12·4%)	1		2/16	0/129 (0%)	1		0/0
AIDS (in-patients)	5/91 (5·5%)	0·4	(0·1–2·1)	17/72 (23·6%)	4·6	(0·8–24·7)	3/17	4/72 (5·6%)			0/4
KS (in-patients)	44/87 (50·6%)	15·4	(4·4–54·2)	62/80 (77·5%)	25·1	(6·6–95·5)	36/62	22/80 (27·5%)	13·9*	(2·5–78·9)	13/22
Total	55/379 (14·5%)	*P* trend <0·001		95/281 (33·8%)	*P* trend <0·001		41/95	26/281 (9·3%)	*P* trend <0·001		13/26

ORs were adjusted for age (18–54, 55+, missing); sex (M, F, unknown); race group (Black, other, missing); and place of residence (Johannesburg, other, missing).
* The OR could not be calculated (=∞) using standard methods given a baseline of 0%. Instead, 'exact' methods were used; relative to the baseline category of 0%, the Cornfield lower CI was 12·4 among AIDS patients and 33·6 among those with KS.

The HHV-8 antibody prevalence was 12·4% in the outpatient group, 23·6% in the in-patients with AIDS and 77·5% in those with KS (*P* trend <0·0001). Moderate to high HHV-8 immunofluorescence was found in none of the outpatients, 5·6% in the in-patients with AIDS and in 27·5% of the KS patients (*P* trend <0·0001).

Table 2 shows an analysis of those patients where both PCR and LANA assays were performed ($n=281$). Of the 46 patients who had viraemia (i.e. HHV-8 DNA detected in PMBC), 41 (89·1%) also had a positive LANA result with 13 (28·2%) having moderate to high antibody immunofluorescence scores. Among the 235 patients who had no viraemia, the HHV-8 antibody prevalence was lower (23·0%) and an even lower proportion (5·5%) had moderate to high antibody immunofluorescence scores. There was a strong association between HHV-8 viraemia and HHV-8 antibody prevalence ($\chi^2=75$, 1 D.F., $P<0{\cdot}0001$) and between viraemia and moderate to high immunofluorescence ($\chi^2=24$, 1 D.F., $P<0{\cdot}0001$). In patients without KS seven were viraemic, but none of these had high/moderate immunofluorescence scores.

Out of eight patients admitted with suspected tuberculosis, one lymph node biopsy was found, on histological examination, to have KS spindle cells but no visible skin lesions suggesting an early, preclinical KS diagnosis. This patient presented with splenomegaly, hepatomegaly and generalized lymphadenopathy. PCR analysis using the KS330BAM$_{233}$ primers revealed that this patient harboured HHV-8 DNA in both PBMC and lymph nodes (Fig. 1). The other seven were KS-negative clinically and on histological examination of the lymph nodes. Two patients were also found to be HHV-8 DNA-positive in the lymph nodes, but were negative in the blood. All other patients were HHV-8-negative in both lymph node and blood samples.

DISCUSSION

A significantly higher proportion of individuals with KS had anti-HHV-8 antibodies and HHV-8 DNA in their blood (78 and 51% respectively) compared to HIV-infected outpatients (12 and 3%) and patients with AIDS (24 and 5·5%) confirming the association between KS and HHV-8. Patients who were viraemic (i.e. had HHV-8 DNA in their blood) were significantly more likely to be HHV-8 antibody-positive than those who had no detectable virus in their blood

Table 2. *HHV-8 infection: association between HHV-8 PCR and HHV-8 LANA assays*

HHV-8 PCR status	*n*	HHV-8 LANA assay Total	HHV-8 LANA assay ++, +++
Negative	235	54 (23·0%)	13 (5·5%)
Positive	46	41 (89·1%)	13 (28·2%)
Total	281	95 (33·8%) $\chi^2=75$; $P<0{\cdot}0001$	26 (9·3%) $\chi^2=24$; $P<0{\cdot}0001$

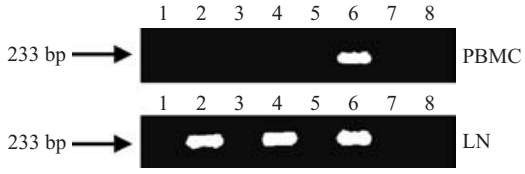

Fig. 1. HHV-8 DNA PCR on matched PBMC and lymph nodes (LN) from 8 HIV-1-infected individuals. KS330BAM$_{233}$ PCR amplified HHV-8 in both LN and blood from a patient with histologically confirmed KS in the LN (lane 6). HHV-8 DNA was detected in the LNs from two other patients (lanes 2–4) but not in their blood. None of these patients had visible KS lesions at the time of the investigation.

(89 *vs.* 23% and 28 *vs.* 6% respectively in those with high immunofluorescence signals). This indicates that a higher HHV-8 IgG immunofluorescence signal is a good marker of viraemia, possibly due to higher levels of replicating virus and antigen stimulating B cells. A similar order of magnitude of risk between KS in relation to HHV-8 immunofluorescence was found in a previous South African study [2], although in this current study there were too few patients with high immunofluorescence signals to make proper comparisons. There was a good correlation between the PCR results obtained from the two non-overlapping nested primer sets, which verified the presence of HHV-8 DNA in these patients. Many of these fragments were also sequenced and shown to cluster phylogenetically with HHV-8 [9].

Our finding that 51% of the patients with KS had HHV-8 DNA in the circulation is similar to previous studies from the United States (57%) [13], United Kingdom (52%) [14] and Europe (45%) [15]. HHV-8 is a latent virus appearing in the blood when immunological control is compromised such as during HIV infection [14]. Detection of HHV-8 DNA in the blood may therefore be associated with dissemination of the virus when CD4 counts decline. In this study, there was very little difference in viraemia between HIV-positive patients and those admitted with AIDS (3 and 5·5%) probably because the difference in CD4 counts was too small (280 *vs.* 201 cells/μl) to make any proper inferences about this mechanism. However, the incidence of KS has declined significantly in places where highly active anti-retroviral therapy has been offered to HIV-positive individuals [16], probably because of an improvement in CD4 counts in these populations.

Studies done elsewhere on patients with advanced AIDS but without KS have found 7% to be HHV-8 DNA-positive in blood [17]. This compares well to the 5·5% found in this study. Although the numbers are small, HHV-8 DNA was detected more frequently in the lymph nodes (3/8) compared to blood (1/8) concurring with the view [4, 5] that the lymph node may be a latent site for HHV-8 infection. Thus, the prevalence of HHV-8 in the lymph node may be higher than suggested by just analysing blood.

In 5 of the 46 cases where HHV-8 viraemia was detected in the absence of an antibody response, this may reflect cases of recent acute infection prior to seroconversion. However, the strong association between HHV-8 viraemia and immunofluorescence (the latter being a strong risk factor associated with KS), suggests that increased HHV-8 viral loads may be an important mechanism of KS development. The data are, however, cross sectional but in prospective studies the presence of HHV-8 in the blood is highly predictive of KS with 50% of positive individuals developing KS within 3–5 years [13, 14].

HHV-8 seroprevalence of 34·6% was reported in rural KwaZulu–Natal province [18] and 32% in Johannesburg [2]. In the latter study (where the immunofluorescence assays were performed by the Wohl Virion Centre, London, UK), the prevalence of HHV-8 was similar between HIV-negative and HIV-positive persons, and among black blood donors the seroprevalence was reported to be 20%. Our finding of 16% seroprevalence among KS-negative HIV-infected individuals could reflect age differences

between the different studies and/or differences in the assays used to detect HHV-8 antibodies. In a separate study comparing immunofluorescence results in approximately 400 patients (Stein et al., unpublished data), the overall scores tested by one of us (L.A.) at the National Centre for Communicable Diseases in Johannesburg were, on average, slightly lower than readings measured by the Wohl Virion Centre, which may account for the lower seroprevalence found in this study, but nevertheless agreement between the two readers was good ($\kappa = 0{\cdot}6$). In a former study of 3344 patients immunofluorescence scores were highly correlated with antibody titres [2]. Nevertheless, the results from this study, and those of Sitas [2] and Wilkinson [18], still indicate a high background prevalence of HHV-8 in South Africa. In the presence of an accelerating HIV epidemic, KS is likely to become a leading cause of cancer and an important HIV-related condition in South Africa.

ACKNOWLEDGEMENTS

We thank Drs R. Sher, D. Spencer, J. Murray, P. Sonnenberg, E. Silber and D. Brittain, for providing clinical samples and Dr C. Boshoff (Wohl Virion Center, Cancer Research Campaign, University of London, UK) for providing the BCP-1 cell line, and Mr H. Carrara, Dr R. Pacella-Norman and Ms S. Nyoka for data and sample preparation.

Grant sponsors: Poliomyelitis Research Foundation and the Medical Research Council of South Africa. The Cancer Epidemiology Research Group is sponsored by the Medical Research Council, the Cancer Association of South Africa, University of the Witwatersrand and the National Health Laboratory Service. The National Cancer Registry is also sponsored by the Department of Health.

REFERENCES

1. Moore PS, Chang Y. Kaposi's sarcoma (KS), KS-associated herpesvirus, and the criteria for causality in the age of molecular biology. Am J Epidemiol 1998; **147**: 217–221.
2. Sitas F, Carrara H, Beral V, et al. Antibodies against human herpesvirus 8 in black South African patients with cancer. N Engl J Med 1999; **340**: 1863–1871.
3. Sheil A. Patterns of malignancies folowing renal transplantation. Transplant Proc 1999; **31**: 1263–1265.
4. Sirianni MC, Uccini S, Angeloni A, Faggioni A, Cottoni F, Ensoli B. Circulating spindle cells: correlation with human herpesvirus-8 (HHV-8) infection and Kaposi's sarcoma. Lancet 1997; **349**: 255.
5. Bigoni B, Dolcetti R, de Lellis L, et al. Human herpesvirus 8 is present in the lymphoid system of healthy persons and can reactivate in the course of AIDS. J Infect Dis 1996; **173**: 542–549.
6. Chokunonga E, Levy L, Bassett M, et al. AIDS and cancer in Africa: the evolving epidemic. AIDS 1999; **13**: 2583–2588.
7. Wabinga HR, Parkin DM, Wabwire-Mangen F, Mugerwa JW. Cancer in Kampala, Uganda, in 1989–91: changes in incidence in the era of AIDS. Int J Cancer 1993; **54**: 26–36.
8. Chang Y, Cesarman E, Pessin MS, Lee F, Culpepper J, Knowles DM, Moore PS. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science 1994; **265**: 1865–1869.
9. Alagiozoglou L, Sitas F, Morris L. Phylogenetic analysis of human herpesvirus-8 in South Africa and identification of a novel subgroup. J Gen Virol 2000; **81**: 2029–2038.
10. Gao SJ, Kingsley L, Hoover DR, et al. Seroconversion to antibodies against Kaposi's sarcoma-associated herpesvirus-related latent nuclear antigens before the development of Kaposi's sarcoma. N Engl J Med 1996; **335**: 233–241.
11. Kedes DH, Operskalski E, Busch M, Kohn R, Flood J, Ganem D. The seroepidemiology of human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus): distribution of infection in KS risk groups and evidence for sexual transmission. Nat Med 1996; **2**: 918–924.
12. Simpson GR, Schulz TF, Whitby D, et al. Prevalence of Kaposi's sarcoma associated herpesvirus infection measured by antibodies to recombinant capsid protein and latent immunofluorescence antigen. Lancet 1996; **348**: 1133–1138.
13. Moore PS, Kingsley LA, Holmberg SD, et al. Kaposi's sarcoma-associated herpesvirus infection prior to onset of Kaposi's sarcoma. AIDS 1996; **10**: 175–180.
14. Whitby D, Howard MR, Tenant-Flowers M, et al. Detection of Kaposi sarcoma associated herpesvirus in peripheral blood of HIV-infected individuals and progression to Kaposi's sarcoma. Lancet 1995; **346**: 799–802.
15. Quinlivan EB, Ye D, Wehbie RS, Stewart PW, Flepp M, Vernazza PL. Kaposi's sarcoma patients in the Swiss HIV Cohort Study with and without detectable human herpesvirus 8 in peripheral blood mononuclear cells. AIDS 1997; **11**: 1893–1895.
16. International Collaboration on HIV and Cancer. Highly active antiretroviral therapy and incidence of cancer in human immunodeficiency virus-infected adults. J Natl Cancer Inst 2000; **92**: 1823–1830.
17. Boshoff C, Schulz TF, Kennedy MM, et al. Kaposi's sarcoma-associated herpesvirus infects endothelial and spindle cells. Nat Med 1995; **1**: 1274–1278.
18. Wilkinson D, Sheldon J, Gilks CF, Schultz TF. Prevalence of infection with human herpesvirus 8/Kaposi's sarcoma herpesvirus in rural South Africa. S Afr Med J 1999; **89**: 554–557.