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Comparative Evaluation of Three Serological Methods for Detection of Human Herpesvirus 8-Specific Antibodies in Canadian Allogeneic Stem Cell Transplant Recipients

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Human herpesvirus 8 (HHV-8) has been associated with all types of Kaposi's sarcoma (KS), including post-transplantation KS. However, little is known regarding HHV-8 infections in hematopoietic stem cell transplant (SCT) recipients. In this study, we used a variety of serological assays, including in-house-developed enzyme immunoassays (EIAs) utilizing synthetic peptides corresponding to lytic viral antigens (ORFs 65 and K8.1) and a commercial EIA kit based on a whole virus lysate (Advanced Biotechnologies Inc.), as well as latent- and lytyc-antigen-based immunofluorescence assays (IFAs) to determine the seroprevalence of HHV-8 in 42 allogeneic SCT recipients from Canada. Using the two peptide-based EIA methods as for screening, HHV-8-specific antibodies were detected in five (12%) patients between days 21 and 91, although only one (2%) subject was positive for HHV-8-specific antibodies before transplantation. All positive results from these five patients were confirmed by at least one of the IFAs, with an additional patient showing seropositivity before transplantation. However, the commercial EIA was negative at all time points (days −7, 21, and 91) in those five patients. The episodes of seroconversion or reactivation were not associated with sustained viremia, since HHV-8 DNA was not detected by real-time PCR in the corresponding leukocytes and plasma of the seropositive patients. No clinical or laboratory abnormalities were clearly associated with HHV-8 seropositivity. This study confirms the utility of simple peptide-based EIA methods to assess the presence of HHV-8-specific antibodies in immunocompromised patients and emphasizes the need of conducting prospective studies to determine the source of HHV-8 infection in SCT recipients.

Human herpesvirus 8 (HHV-8), also known as Kaposi's sarcoma (KS)-associated herpesvirus, has been linked to all types of KS, including classic, endemic, epidemic (AIDS-related), and iatrogenic KS (8). HHV-8 has also been associated with two forms of lymphoproliferative disorders: body cavity-based lymphomas and multicentric Castleman's disease (12, 34). Several studies of HHV-8 infections in solid organ (liver, lung, heart, and kidney) transplant recipients have been reported previously (2, 3, 11, 16, 18, 22–24, 27; C. Frances, C. Mouquet, and V. Calvez, Letter to the editor, N. Engl. J. Med. **340:**1045-1046, 1999). The development of KS lesions in those patient populations has been shown to be highly correlated with immunosuppressive treatments and might result from HHV-8 transmission by the donors (27). Besides KS, HHV-8 infections in allograft recipients have been associated with cytopenias, splenomegaly, and marrow failure (23). Nevertheless, much remains to be known about HHV-8 infections in hematopoietic stem cell transplant (SCT) recipients, particularly in North America, where seroprevalence of this virus in the general population is very low (1). Because HHV-8 DNA has been detected in blood mononuclear cells (B cells and monocytes) (4, 32), viral transmission in the SCT population is plausible.

Many serologic tests have been developed for detection of HHV-8-specific antibodies (25, 31, 33). An enzyme-linked immunosorbent assay (ELISA) that uses sucrose-purified whole virus derived from the KS-1 cell line is now commercially available from Advanced Biotechnologies Inc. (ABI). It has been reported that this test is specific and sensitive when compared to results obtained from other assays and with the presence of KS (14). In one study, patients with a clinical (or histological) diagnosis of KS had antibodies in a proportion of 80 to 90%, whereas the seroprevalence in normal healthy individuals was 2 to 5% except in Central Africa, where the virus is endemic (1). Similar trends have been observed with two enzyme immunoassays (EIAs) using synthetic peptides from open reading frames (ORFs) 65 and K8.1 aimed at detecting lytic antigens (9). Although more tedious and more subjective than EIA tests, immunofluorescence assays (IFAs) are the most widely used tests for the detection of HHV-8-specific antibodies. Many cell lines latently infected by HHV-8 are commonly used as substrate cells for IFAs. HHV-8 lytic antigens can also be detected by IFAs following chemical induction of HHV-8-positive lymphoma cell lines with phorbol ester or sodium butyrate (19, 31, 33). However, there is imperfect correlation between all serological methods, and none can detect specific HHV-8-specific antibodies in all KS cases. An incomplete understanding of the viral proteins that may act as immunological targets and the wide geographic variations in the prevalence of HHV-8 infection may explain some of the discrepancies encountered in previous studies (17, 26).

In this study, we compared different serological methodologies to assess the prevalence of HHV-8-specific antibodies in Canadian SCT recipients after initial validation of the assays

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TABLE 1. HHV-8-specific antibodies in AIDS patients with biopsy-proven Kaposi's sarcoma by three serological methods[d]

Patient	IFA[a]		ABI-EIA		Peptide EIA (serological status[c])	
	Latent	Lytic	OD	OD/cutoff (serologic status[b])	ORF K8.1 OD	ORF 65 OD
A	1/200	1/100	0.206	0.980 (+/−)	0.200 (+)	0.380 (+)
B	1/100	1/200	0.232	1.100 (+)	0.376 (+)	0.900 (+)
C	−	1/100	0.646	3.070 (+)	0.720 (+)	1.700 (+)
D	1/100	1/100	1.383	6.570 (+)	2.070 (+)	1.060 (+)
E	1/100	1/100	0.875	4.160 (+)	2.340 (+)	1.970 (+)
F	−	−	0.057	0.270 (−)	0.170 (−)	0.150 (−)
G	1/50	1/100	0.839	3.950 (+)	1.890 (+)	0.420 (+)
H	1/50	−	0.127	0.600 (−)	0.604 (+)	0.190 (−)
I	−	1/100	0.196	0.930 (+/−)	0.720 (+)	0.240 (+)
J	−	−	0.194	0.920 (+/−)	0.760 (+)	0.460 (+)

[a] IFA values for both latent and lytic antigens correspond to the highest dilution at which membrane staining was observed by fluorescence microscopy. −, negative result.

[b] Serologic status was determined as described by the manufacturer's instructions (ABI) based on a cutoff value which is threefold higher than the mean of three negative samples. Absorbance/cutoff ratios were interpreted as follows: ≤0.75, negative sample; ≥1.00, positive sample; 0.76–0.99, equivocal (borderline [+/−]).

[c] Serologic status was determined by comparison with predetermined cutoff values, which were 0.194 and 0.220 for ORF K8.1 and ORF 65 EIAs, respectively. The mean absorbance of 20 healthy children's plasma samples plus 4 standard deviations was chosen as the cutoff value for the peptide EIAs.

[d] Note: each sample was tested in duplicate by the three serological methods.

using sera from AIDS-related KS patients and healthy children from the same country.

MATERIALS AND METHODS

Study population. Recipients of allogeneic blood or marrow SCT from a matched sibling donor were recruited in two hospitals of the province of Québec, Canada. A chemotherapy-based conditioning regimen with busulfan and cyclophosphamide was used in 80% of the patients, whereas others received high-dose cyclophosphamide and total body irradiation. Samples (plasma and leukocytes) used in this study were obtained from consecutive transplant patients enrolled in a preemptive ganciclovir protocol for treatment of cytomegalovirus viremia as described elsewhere (5). Ten AIDS patients with concurrent biopsy-proven KS (7) and ten healthy children <5 years old from the province of Québec were also included in this study as positive and negative controls, respectively.

In-house EIA assays. Detection of HHV-8-specific antibodies in plasma was first performed by two EIAs using synthetic peptides representing lytic viral antigens. The ORF K8.1 peptide contains 30 amino acids (aa) (aa 32 to 62) located in the N-terminal region of the gene product (35), and ORF 65 contains 14 aa (aa 157 to 170) derived from the C-terminal region of the protein (25). These peptides were synthesized with an automatic synthesizer, purified by reverse-phase liquid chromatography, and 500 ng of each peptide was used for EIA as previously described (25). The mean absorbance of 20 healthy children's plasma samples plus 4 standard deviations was chosen as a cutoff value for the EIAs.

ABI tests. The HHV-8-specific immunoglobulin G antibody ELISA (Advanced Biotechnologies Inc., Columbia, Md.) test was performed following manufacturer's instructions. This assay is made from a whole virus extract, derived from sucrose gradient purified HHV-8 virions isolated from the KS-1 cell line.

Latent and lytic IFAs. Latent and lytic HHV-8-specific antibodies were detected by IFA using noninduced and induced body-cavity-based lymphoma-1 cells, respectively (17). For induction, 10^6 body-cavity-based lymphoma-1 cells (kindly provided by Benoît Barbeau, Centre de Recherche en Infectiologie, Québec City, Canada) were treated with 12-*O*-tetradecanoylphorbol 13-acetate at a final concentration of 20 ng/ml for 96 h in RPMI medium (Life Technologies, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS). Cells were then resuspended in 1 ml of phosphate-buffered saline (PBS), and 10 μl was spotted onto 10-well slides, air dried, and fixed with cold acetone. All slides were previously blocked with PBS supplemented with 5% FBS for 30 min at 37°C. Each plasma sample was assayed by testing three serial twofold dilutions (1/50, 1/100, and 1/200) during a 1-h incubation step at 37°C. After three washes with PBS, 10 μl of rabbit-anti-human immunoglobulin G-fluorescein isothiocyanate conjugate (Caltag Laboratories, San Francisco, Calif.) diluted 1:100 in sample diluent (PBS supplemented with 3% FBS) was added to each well before proceeding to another 1-h incubation step as described elsewhere (30). Following washes, all slides were examined by fluorescence microscopy by a single blind researcher and those exhibiting punctuate membrane staining at a dilution of 1:50 or more were considered positive. For both lytic and latent IFAs, two sets of negative controls were performed, one consisting of serial dilutions of plasma from young immunocompetent Canadian children and another one without serum. All samples were tested in duplicate.

Quantitative real-time PCR. HHV-8 DNA was quantified in 200 μl of plasma or in 10^4 polymorphonuclear leukocytes (PMNLs) using real-time PCR in a LightCycler instrument (Roche Diagnostics, Laval, Quebec, Canada) as previously described (6, 7). Two sets of adjacent fluorogenic probes, including one for the HHV-8 target (ORF 26) and one for an internal control. were designed to quantify HHV-8 amplicons and verify the presence of PCR-inhibitory substances (6).

RESULTS

The validation of the three serological methods was first done using two groups of patients. Among the 10 AIDS patients with biopsy-proven KS, 9 were positive by at least one of the in-house EIAs based on synthetic peptides (ORF K8.1 or 65) compared to 8 that were positive by at least one of two IFAs and five by the ABI test (Table 1). Three other patients had equivocal results as determined by the ABI test. A single AIDS patient with KS (patient F) was seronegative by all three methods. There was a good correlation between peptide-based EIA and IFAs except for one patient (patient J) who was seronegative by IFA but positive by the two peptide-based EIAs. Discrepant serological results with the two EIA tests were observed for one patient (patient H). However, the negative sample by EIA for ORF 65 had an optical density (OD) value very close to the assay's cutoff. Plasma samples obtained from 10 healthy children were negative by all three serological methods (data not shown).

Due to superior sensitivity, peptide-based EIA tests were first used to evaluate the presence of HHV-8-specific antibodies in plasma of 42 consecutive allogeneic SCT recipients collected on day 21 and 77 to 91 posttransplantation. As shown in Table 2, plasma from 5 (12%) patients tested positive between day 21 and 91 by the two ELISA tests (ORF K8.1 and 65), while the remaining 37 patients tested negative by the two peptide-based EIAs. Pretransplant plasma samples from the five seropositive patients were also tested using the same serological method. Only one of the five patients (patient 2) had HHV-8-specific antibodies on day −7 (Table 2).

HHV-8 serological status of the five peptide-based-EIA-

TABLE 2. Serological and molecular analysis of five allogeneic SCT recipients with HHV-8-specific antibodies in plasma by peptide EIAs[d]

Patient no. and transplantation day	IFA[a]		ABI-EIA		Peptide EIAs OD (serologic status[c])		Real-time PCR	
	Latent	Lytic	OD	OD/cutoff (serologic status[b])	ORF K8.1	ORF 65	PMNL	Plasma
1								
−7	1/50	1/50	0.070	0.330 (−)	0.110 (−)	0.097 (−)	ND[e]	ND
21	−	−	0.050	0.237 (−)	0.240 (+)	0.230 (+)		
91	1/200	1/100	0.071	0.336 (−)	0.330 (+)	0.250 (+)		
2								
−7	1/50	1/50	0.056	0.265 (−)	0.692 (+)	0.372 (+)	ND	ND
21	1/100	1/100	0.052	0.246 (−)	1.000 (+)	0.360 (+)	ND	
91	−	1/50	0.072	0.341 (−)	1.200 (+)	0.400 (+)		
3								
−7	−	−	0.092	0.436 (−)	0.077 (−)	0.076 (−)	ND	ND
21	1/50	1/100	0.056	0.265 (−)	0.230 (+)	0.288 (+)	ND	
77	1/50	−	0.077	0.365 (−)	0.250 (+)	0.250 (+)	ND	
4								
−7	−	−	0.076	0.360 (−)	0.062 (−)	0.099 (−)	ND	ND
21	1/100	1/50	0.051	0.242 (−)	0.270 (+)	0.230 (+)		
91	−	−	0.058	0.275 (−)	0.320 (+)	0.220 (+)		
5								
−7	−	−	0.051	0.242 (−)	0.070 (−)	0.058 (−)	ND	ND
21	1/100	1/100	0.053	0.251 (−)	0.450 (+)	0.230 (+)		
91	1/50	1/50	0.058	0.275 (−)	0.440 (+)	0.310 (+)		

[a] IFA values for both latent and lytic antigens correspond to the highest dilution at which membrane staining was observed by fluorescence microscopy.
[b] Serologic status was determined as described by the manufacturer's instructions (ABI) based on a cutoff value which is threefold higher than the mean of three negative samples. Absorbance/cutoff ratios were interpreted as follows: ≤0.75, negative sample; ≥1.00, positive sample; 0.76–0.99, equivocal (borderline).
[c] Serologic status was determined by comparison with predetermined cutoff values, which were 0.194 and 0.220 for ORF K8.1 and ORF 65 EIAs, respectively. The mean absorbance of 20 healthy children's plasma samples plus 4 standard deviations was chosen as the cutoff value for the peptide EIAs.
[d] Note: each sample was tested in duplicate by the three serological methods and by quantitative real-time PCR. −, negative result; +, positive result.
[e] ND, not done.

positive patients was further investigated by the use of two additional serological methods. The results obtained with the IFAs were in moderate agreement with those of the peptide-based EIAs. Indeed, four of the five plasma samples from day 21 were positive for the lytic antigen as well as three from day 91. With the latent antigen, four of the five plasma samples were positive on day 21, as well as three obtained on day 77 to 91 (Table 2). Two of the five pretransplant sera (including one positive specimen by the peptide-based EIA test) were found positive for the presence of latent and lytic HHV-8-specific antibodies on day −7 (Table 2). In contrast, all plasma samples (collected on day −7, 21, and 77 to 91) from the five patients with positive peptide-based EIA results tested negative with the ABI test (Table 2). Overall, we observed that 5 of 42 (12%) of our patients had HHV-8-specific antibodies after transplantation using the two peptide-based EIAs (ORF K8.1 and 65) as screening assays and the IFAs as confirmation tests. One of these five patients had HHV-8-specific antibodies measured before transplantation by the two types of serological assays with a second one being positive by the IFAs only at the lowest dilution (1/50).

The presence of active HHV-8 replication was assessed in the five seropositive patients by performing real-time PCR on PMNLs and plasma. No viral DNA was detected by real-time PCR (lower limit of detection = 50 copies) in either plasma or PMNLs at different time points (day 21 and days 77 to 91) posttransplantation (Table 2).

A passive transfer of HHV-8-specific antibodies due to administration of gamma globulins was also investigated in the five seropositive patients. With the exception of one patient (patient 5) who had received serial administrations of γ-globulins from day 1 to day 98, passive transfer of HHV-8-specific antibodies was unlikely to be involved in the seropositivity of the other two patients who had received them because they were either seropositive before the first administration (patient 2) or remained positive for more than 2 months after the last dose (patient 4). No laboratory or clinical abnormalities (fever of unknown etiology, persistent cytopenia, hepatitis, encephalitis, and pneumonitis) were observed in the five HHV-8-infected patients. Only one of the five seropositive patient (patient 5) was born outside Canada. This patient from Romania also received a transplant from the same country. Sera from SCT donors were unavailable for testing.

DISCUSSION

Serological assays could constitute useful monitoring tools for HHV-8 infection in transplant populations at risk of KS. However, routine use of serological assays has been hampered by many factors, including the absence of a "gold standard" and few detailed epidemiological studies from various countries. In this study, we report a comparison of three serological approaches for the detection of HHV-8-specific antibodies in a cohort of 42 allogeneic SCT recipients from Canada. In addi-

tion, the assays were validated in two well-defined populations consisting of 10 AIDS patients with biopsy-proven KS and 10 healthy children below the age of 5 years.

Our study emphasizes the variable performance of different serological approaches for the diagnosis of HHV-8 infection. Our results show that the peptide-based EIAs for lytic antigens were most sensitive and convenient for detecting HHV-8-specific antibodies in plasma samples from human immunodeficiency virus (HIV)-infected patients and SCT recipients. The additional testing of samples by IFAs added little to EIA methods for both patient populations except for one pretransplant specimen (patient 1, Table 2). However, additional investigation is warranted considering that EIA-negative samples from SCT recipients were not tested by IFA in our study. Also, assessment of seroconversion was clearer with EIAs since some of the patients (patients 2, 3, and 4 in Table 2) appeared to serorevert at some point after transplantation when tested by IFA. The performance of both ORF 65 and ORF K8.1 peptide-based EIAs used in this study has been previously evaluated in different populations including HIV-infected patients with and without biopsy-proven KS as well as healthy blood donors (25, 35). The specificity of the ORF 65 peptide has also been demonstrated in a competition study with an Epstein-Barr virus peptide analogue (25). Although the OD values of the peptide-based EIAs obtained in the five seropositive SCT recipients were lower than those of HIV-seropositive patients with KS (Tables 1 and 2), most results were confirmed by both latent and lytic IFAs. Therefore, our serological data are unlikely to represent false-positive results. However, all plasma samples from the five SCT recipients with serological evidences of HHV-8 infection tested negative by the ABI test: such discrepancy is probably due to lower sensitivity of the latter method. In fact, Spira et al. (35) have demonstrated that even if the ABI test was specific, this serological method could miss some true-positive results. The same authors recommended a combination of peptide-based EIAs (ORFs K8.1 and 65) for screening HHV-8-specific antibodies in order to reach a sensitivity of approximately 93% in HIV-infected patients with current and subsequent KS.

Our results show that HHV-8 seropositivity among Canadian SCT recipients is approximately 12% when a sequential and conservative serological approach is used based on screening all plasma samples by a peptide-based EIA test for lytic antigen followed by confirmation of positive results by IFA. Importantly, most of our seropositive patients lacked HHV-8-specific antibodies before transplantation. The discrepancy between our results and the low seroprevalence observed in North America (<5%) could be due to many factors such as higher transfusion rates in the SCT population and donors originating from countries where HHV-8 seroprevalence is higher. However, only one of our seropositive patients had a donor from a country where HHV-8 is more prevalent. Unfortunately, plasma samples from donors were unavailable for serological analysis. Consequently, we cannot definitively distinguish HHV-8 transmission by donor cells or transfusion from viral reactivation. Only a few studies have addressed the question of HHV-8 seropositivity and/or KS development in allogeneic bone marrow transplant (BMT) recipients (20, 21, 29). Our seropositivity rate is slightly lower than that reported in a study by Rosenzwajg et al. (29) in which 18% of French BMT patients had HHV-8-specific antibodies after transplantation versus 10% before transplantation. These authors have suggested that acquisition of HHV-8 could be attributed to blood transfusions instead of graft transmission since they found no relationship between posttransplantation seroconversion and the donor's serological status. Although this hypothesis is possible, their serological approach was based on a latent IFA only. Spira et al. (35) have reported that the IFA for latent antigen was only moderately sensitive for detecting HHV-8-specific antibodies. Thus, the main mode of HHV-8 transmission in SCT recipients in countries with a low HHV-8 seroprevalence rate remains unclear. Other studies have demonstrated that KS occurred rarely after bone marrow transplantation (21); only one case of KS, possibly transmitted by the donor, has been reported in a French cohort (20). In contrast, several studies of HHV-8 infection and KS have been reported after renal transplantation (10, 11, 16, 22, 27; Frances et al., letter). In two studies from different geographic areas (Switzerland and United States), HHV-8 seroprevalence increased from 5 to 6% before to 16 to 18% within the year after renal transplantation (22, 27). Notably, KS is clearly more prevalent in the renal transplant population, approaching 4% of transplants (10, 11), indicating that the pathogenesis of HHV-8 infection in the two transplant populations is different, which may be related in part to the shorter period of immunosuppression in SCT recipients.

Despite serological evidences of HHV-8 infections, none of the five SCT recipients in our study had detectable viremia. Previous studies have shown that the detection of HHV-8 antibodies for lytic proteins was generally more sensitive than the detection of HHV-8 DNA in peripheral blood mononuclear cells for the assessment of HHV-8 infections in HIV-infected patients with KS (13, 33). By contrast, some authors (15, 28) have found HHV-8 DNA by PCR in bone marrow stromal cells obtained from patients with multiple myeloma in whom HHV-8-specific antibodies had not been detected. Many reasons might explain the discrepancy between the seropositivity of our patients and the absence of viral DNA in their blood, including short and transient viremic episodes, viral replication at sites other than peripheral blood, lack of sensitivity of the quantitative PCR method, and suboptimal cell sampling with the use of PMNLs instead of peripheral blood mononuclear cells in our study. In addition, the possibility of a passive transfer of antibodies was examined closely for patients who received gamma globulins after transplantation. However, this hypothesis could be verified for only one of our five HHV-8-seropositive SCT recipients. Remarkably, the detection of lytic HHV-8-specific antibodies in the posttransplantation period was not associated with any adverse clinical and laboratory outcomes such as pneumonitis, hepatitis, encephalitis, fever of unknown etiology, and severe persistent cytopenia in our patients.

In summary, our study confirms the variable performance of the different serological HHV-8 tests and emphasizes the importance of adequate evaluation of the assays using well-defined populations before performing HHV-8 epidemiological studies in special groups such as transplant recipients. Our results also confirm the reliability of simple EIA tests based on synthetic peptides for lytic antigens compared to the well-characterized but more cumbersome IFAs. Using such meth-

odologies, we were able to report a relatively high prevalence (12%) of HHV-8-specific antibodies in our Canadian allogeneic SCT population. The exact mode(s) of acquisition of HHV-8 in that population and the clinical consequences attributable to this infection require additional prospective studies.

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