THE SEROPREVALENCE OF HUMAN HERPESVIRUS 8 INFECTION IN THE THAI POPULATION

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Abstract. The seroprevalence of human herpesvirus 8 (HHV-8) infection in the Thai population was investigated. Sera from 1,018 human immunodeficiency virus 1 (HIV-1)-negative and 436 HIV-1- positive individuals were tested for antibodies to latent and lytic HHV-8 antigens by indirect immunofluorescence assay (IFA) and an enzyme-linked immunosorbent assay (ELISA) using mixed recombinant orf HHV-8 proteins. The positive sera were further tested with recombinant HHV-8 protein expressed 293T cells by IFA. The seroprevalence of HHV-8 infection was determined by the concordant reactivity of sera among antibody testing assays. The results showed a low rate of HHV-8 seropositivity in both HIV-1- negative healthy individuals (0.6%) and HIV-1-infected patients (0.7%). These results are consistent with the fact that a small number of patients with AIDS-associated KS have been reported in Thailand and that HHV-8 is an uncommon pathogen in this country. Interestingly, we found that sera from the general population living in the north, but not other regions of Thailand, had antibodies to HHV-8.

INTRODUCTION

Human herpesvirus 8 (HHV-8), also known as Kaposi's sarcoma (KS)-associated herpesvirus (KSHV), is a lymphotropic oncogenic gammaherpesvirus which is closely related to *Herpesvirus saimiri* and the Epstein-Barr virus (EBV). It was first identified in the KS lesions of AIDS patients (Chang *et al*, 1994). HHV-8 DNA sequences have been demonstrated in all clinical forms of KS, including AIDS-related, classic Mediterranean, endemic African, and transplant-related KS (Dupin *et al*, 1995; Huang *et al*, 1995; Moore *et al*, 1995). The virus has been found in body-cavity-based lymphoma (BCBL), primary effusion lymphoma (PEL) (Cesarman *et al*, 1995; Nador *et al*, 1996), and multicentric Castleman's disease (Soulier *et al*, 1995), which are other AIDS-associated malignancies. The pathogenic importance of HHV-8, indicated by the presence of HHV-8 in the peripheral blood of HIV-positive patients, is that HHV-8 heralds the appearance of KS lesions; seropositivity for HHV-8 antibodies may also be predictive of future KS (Whitby *et al*, 1995; Gao *et al*, 1996b).

HHV-8 infection is uncommon in the general population of western countries, such as the United States and the UK, but commoner in some Mediterranean countries, including Italy and Greece (Lennette *et al*, 1996; Simpson *et al*, 1996; Whitby *et al*, 1998) and widespread in some parts of Africa (Lennette *et al*, 1996; Gao *et al*, 1996a). In non-endemic countries, sexual transmission appears to play an important role among high-risk groups (STD clinic patients; homosexual men) (Kedes *et al*, 1996; Lennette *et al*, 1996; Martin *et al*, 1998). It appears to be transmitted mainly during childhood in endemic countries (Mayama *et al*, 1998).

Several assays have been developed for the detection of HHV-8 antibodies to different target antigens. These include immunofluorescence antibody assays (IFA) for latent and lytic viral antigens from HHV-8 latently-infected B-

lymphoma cells (Kedes *et al*, 1996; Gao *et al*, 1996a; Lennette *et al*, 1996; Fujii *et al*, 1999; Huang *et al*, 2000), immunoblot assays with a variety of viral proteins (Gao *et al*, 1996b; Miller *et al*, 1996) and enzyme-linked immunosorbent assays (ELISA) for recombinant proteins (Simpson *et al*, 1996; Regamey *et al*, 1998), synthetic peptides (Davis *et al*, 1997; Pau *et al*, 1998; Raab *et al*, 1998), and whole viral lysates (Chatlynne *et al*, 1998). Recently, a new sensitive ELISA system, using potent antigenic proteins encoded by HHV-8 as antigens, was developed: this has been called *mixed-antigen* ELISA (Katano *et al*, 2000).

Few data are available on the epidemiology of HHV-8 infection in Asian countries; this scarcity of data extends to Thailand, an area with rare cases of KS, but a high prevalence of HIV infection. This study was conducted in order to determine the prevalence of HHV-8 antibodies in the Thai population using the standard IFA and a sensitive ELISA assay.

MATERIALS AND METHODS

Subjects

A total of 1,454 serum (or plasma) samples were taken from 1,018 HIV-1-negative and 436 HIV-1-positive individuals during 1996-1999. The HIV-1-negative samples included: the sera of 117 children of 10 years of age or younger who had attended the hospital clinic with fever; the sera of 515 healthy individuals in four age groups from four regions of Thailand - these sera were obtained from the National Serum Reference Bank of the Department of Medical Sciences; the sera of 123 blood donors; the sera of 187 patients visiting a STD clinic; and the sera of 76 injecting drug users (IDU). The HIV-1- infected samples, obtained from hospitals in Bangkok, included: 2 sera from AIDS patients with KS (AIDS-KS); 434 sera from patients without KS, of whom 49 were homosexual men, 304 were heterosexual men, and 81 were IDU. All samples were stored at -20ºC until use.

Anti-latent and anti-lytic HHV-8 antibody assays

Indirect IFA for anti-latent and anti-lytic HHV-8 antibodies were carried out as described previously (Isarangkura Na Ayuthaya *et al*, 1998): BCBL-1 cells, an HHV-8 latently-infected human B cell line, (Renne *et al*, 1996), Ramos cells, and a normal human B cell line, (Klein *et al*, 1975) were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). BCBL-1 cells were treated with or without 12-0-tetradecanoyl phorbol-13 acetate (TPA) (20 ng/ml) for 2 days to produce lytic or latent HHV-8 antigens respectively. The cells were smeared onto slides that were subsequently fixed in acetone. The samples were diluted at 1:40 in serum diluent (1% bovine serum albumin in PBS) and were incubated on the lytic HHV-8 antigens-, latent HHV-8 antigens-, and Ramos cells-coated slides at 37ºC for 1 hour. The slides were washed in PBS with 0.1% Tween 20 (PBS-T) three times. The FITC conjugated goat anti-human IgG was then added and incubated for 40 minutes. After the final wash, the slide was examined by fluorescence microscopy. All positive samples at 1:40 dilution were determined for the end point titer, which was the reciprocal of the highest dilution giving positive fluorescence. Samples were considered true anti-latent or anti-lytic antibody-positive when Ramos cells were unstained. The plasma from AIDS-KS patients served as the positive control. The results were read in blind fashion and the positive samples were tested at least three times.

Procedure for orf K8.1 protein transient expression in 293T cells

The 293T cells that were maintained in Dulbecco modified Eagle medium (DMEM), supplemented with 10% FCS and antibiotics, were used for the transient-transfection of plasmid pcDNA3.1 carrying K8.1 protein encoded gene (pcDNA3.1-K8.1) constructed by the Department of Microbiology, Osaka Medical School, Japan. The cells were seeded

for 24 hours prior to transfection with the expression plasmid pcDNA3.1-K8.1 using SuperFect Transfection Reagent (QIAGEN, Hilden, Germany). The cells were harvested, spotted on glass slides, air-dried, and fixed with acetone at -20ºC. The slides were kept at -20ºC until used. Expression of K8.1 was then checked by IFA using anti-K8.1 monoclonal antibody (Mab) (Department of Microbiology, Osaka Medical School, Japan) and the FITC conjugated goat anti-mouse IgG (Dako, Copenhagen, Denmark). Specific immunofluorescence was observed by fluorescence microscopy.

Anti-K8.1 HHV-8 antibody test

IFA for anti-K8.1 HHV-8 antibody was performed among the samples positive for BCBL-1 cells. The 2-fold diluted samples were added to the slide for 1 hour at room temperature. The slides were washed prior to adding the FITC conjugated rabbit anti-human IgG (Dako, Copenhagen, Denmark) and further incubated at room temperature for 40 minutes. After being washed, the slides were observed for positive signals by fluorescence microscopy. The end point titer was determined in the reactive sample. The K8.1 Mab was applied as the positive control for each test.

ELISA for anti-HHV-8 antibodies

A recently developed mixed-antigen ELISA using open reading frames (orf) orf K8.1, orf 59, orf 65, orf 73N, and orf 73C as the source of HHV-8 antigens (Katano *et al*, 2000) was applied for the detection of anti-HHV8 antibodies. In brief, purified recombinant GST-HHV-8 fusion proteins (2 μg/ml, each protein) diluted in 100 mM carbonate buffer (pH9.0), were coated onto the 96-well microtiter plate at 50 μl/well and incubated overnight at 4ºC. After being washed with washing buffer [0.1M PBS (pH7.4); 0.02% Tween 20], the heat inactivated samples diluted at 1:100 in the dilution buffer containing 500 μg/ml Block Ace (Snowbrand Milk Products, Tokyo, Japan) in the lysate of *E. coli* producing recombinant GST protein were then allowed to react for 30 minutes at 37ºC with the recombinant proteins. After washing, mixed goat anti-human immunoglobulins (IgGs) conjugated with alkaline phosphatase were added and incubated 30 minutes at 37ºC. After washing, phosphate substrate tablets (5 mg/tablet; Sigma) were used as the substrate to develop the color for 30 minutes. The absorbance (Ab) of the wells was measured at a wavelength of 405 nm. Two serum samples from a Japanese AIDS-KS patient and a Japanese healthy donor were placed as positive and negative controls on every plate. The value of samples was calculated as follows: (sample Ab - negative control Ab) / (positive control Ab - negative control Ab). The samples are considered as positive when the value was greater than the cut-off value (0.1); this cut-off value was determined by the mean Ab plus 5 standard deviations (SD) for 43 normal serum samples.

RESULTS

In the preliminary experiment, antibodies to HHV-8 antigens including lytic, latent, orf K8.1 protein, and mixed recombinant HHV-8 proteins were measured in plasma samples of 2 AIDS-KS patients. The titers (IFA) or the sample values (ELISA) of anti-HHV-8 antibodies were moderately high and the presence of HHV-8 antibodies was as consistent and long-lasting as that observed in a case of AIDS-KS (Table 1).

The cytoplasmic staining pattern by IFA on TPA induced BCBL-1 cells is represented as anti-lytic antibodies (Isarangkura Na Ayuthaya *et al*, 1998). Of the HIV-1-infected patients without KS, 12% (6/49) of homosexual men, 16% (47/304) of heterosexual men, and 9% (7/81) of IDU had antibodies to lytic antigens (Table 2). There was no significant difference detected in seroprevalence among children aged 10 years or younger with fever and healthy HIV-1-negative individuals in different age groups (p= 0.13). This means that seroprevalence does not increase with age, suggesting that HHV-8 is not an endemic pathogen in the area studied. Seven percent 9/123 of blood donors,

Table 1
Reactivity of serum samples from AIDS-KS patients to lytic antigens, latent antigens, and orf K8.1 protein (determined by IFA) and to mixed-recombinant HHV-8 proteins (determined by ELISA).

Patient	collection date	Anti-HHV-8 antibody titer/sample value[a]			
		lytic	latent	orf K8.1	mixed HHV-8 proteins
KS 1					
	Oct,97	2,560	2,560	Not done	0.505
	Sep,98	2,560	2,560	1,280	0.472
	Dec,99	1,280	1,280	1,280	0.447
KS 2					
	May,99	640	640	320	0.219

[a]considered positive if greater than the cut-off value (0.1).

6% (11/187) of STD clinic attenders, and 8% (6/76) of IDU were reactive with HHV-8 IFA lytic antigens. The geometric mean titers (GMT) were 272 for HIV-1-infected patients without KS, 116 for HIV-1-seronegative healthy individuals, 80 for children aged under 10 years and blood donors, 91 for STD patients, and 90 for IDU.

The nuclear IFA-staining of latent antigens appeared clearly as in a previous study (Isarangkura Na Ayuthaya *et al*, 1998). In contrast to anti-lytic antibodies, antibodies to these nuclear latent antigens were presented in only 0.7% (3/434) of HIV-1-infected patients without KS (1 homosexual man and 2 heterosexual men) (Table 2). Seropositivity was also rare in HIV-1-negative individuals: 0.6 % (3/515) of the healthy adults aged under 50 years. No reactive serum was found among blood donors, STD clinic attenders, and IDU (Table 2). The GMT were 320 for HIV-1-infected patients and 253 for healthy individuals.

The recombinant proteins of orf K8.1, orf 59, orf 65, and orf 73, representing immunoreactive lytic- and latent-cycle proteins of HHV-8, are mixed as HHV-8 antigens in this ELISA (Katano *et al*, 2000). Antibodies to mixed HHV-8 antigens were detected in 0.7 % (3/434) of HIV-1-infected patients without KS, (one homosexual man and two heterosexual men). Reactive sera were rare in the HIV-1-negative group: 0.6% (3/515) of healthy individuals

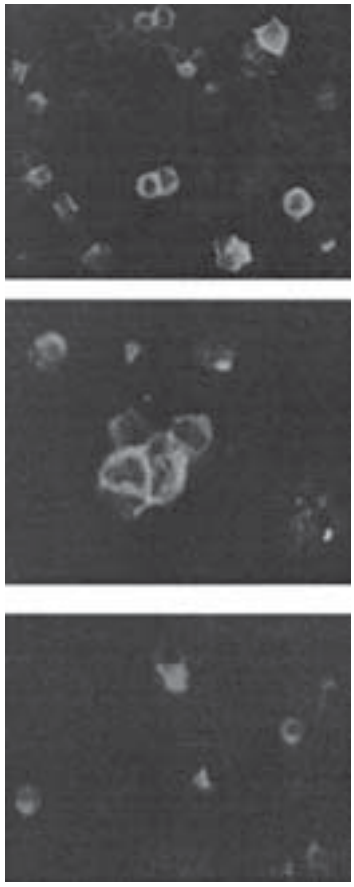

Fig 1–Immunofluorescence assay of orf K8.1 protein expressed 293T cells using 1:80 dilution of serum samples positive for IFA on BCBL-1 cells; sera from HIV-1 negative (A); sera from HIV-1-positive individuals (B); control monoclonal antibody to orf K8.1 protein (C).

Table 2
Seroprevalence of antibodies to HHV-8 lytic, latent, mixed-recombinant proteins and orf K8.1 among HIV-1 infected and uninfected individuals without KS.

Groups	No. tested	No.(%) of sera with HHV-8 antibodies			
		BCBL-IFA		Mixed-recombinant HHV-8 protein-ELISA	Orf K8.1-IFA
		lytic	latent		
HIV-1(+)					
Homosexuals	49	6 (12)	1 (2)	1 (2)	0
Heterosexuals	304	47 (16)	2 (0.6)	2 (0.6)	1 (0.3)
Injecting drug users	81	7 (9)	0	0	0
HIV-1 (-)					
By risks, Bangkok					
Blood doners	123	9 (7)	0	0	0
STD patients	187	11 (6)	0	0	0
Injecting drug users	76	6 (8)	0	0	0
By ages (yrs)					
<10	117	5 (4)	0	0	0
10-14	110	6 (5)	1 (0.9)	1 (0.9)	0
15-20	116	9 (8)	1 (0.9)	1 (0.9)	1 (0.9)
21-50	172	16 (9)	1 (0.6)	1 (0.6)	0
>50	117	15 (13)	0	0	0
By geographical regions					
Central	99	5 (5)	0	0	0
North	137	20 (15)	3 (2.2)	3 (2.2)	1 (0.7)
Northeast	137	9 (7)	0	0	0
South	142	12 (8)	0	0	0

The samples analyzed by age groups from 10 years to >50 years were those used to categorize by region.

between 10 and 50 years of age, all of whom were from the north. No reactive samples were found in the risk groups from Bangkok (Table 2).

Fig 1 shows the cytoplasmic staining pattern that represents a positive IFA reaction with orf K8.1 protein expressed 293T cells. Antibodies to orf K8.1 were confirmed in one (0.2%) of 434 HIV-1-infected patients – a heterosexual man – and one of the 515 (0.2%) healthy HIV-1-negative individuals living in the northern region. Both had high levels of anti-lytic and anti-latent antibody titers: greater than 640.

As shown in Table 2, 5%, 15%, 7% and 8% of healthy HIV-1-negative individuals from the central, the north, the northeast, and the south respectively had antibodies to lytic antigens. However, only 3 sera collected from the north were reactive to latent antigens (2.2%), and mixed-antigen ELISA (2.2%). One of the sera showed specific antibody to orf K8.1 (0.7%) by IFA.

Table 3 summarizes the seroprevalence of HHV-8 of 1,454 individuals based on serological assays. With the agreement of sera reactivity from all the assays as the criterion, sera from 2 AIDS-KS were reactive, whereas the prevalence of HHV-8 antibody was extremely low in HIV-1-infected patients without KS (0.2%) and similarly low in healthy HIV-1-negative adults aged between 10 and 50 years (0.2%). With regard to the concordance among the 3 assays, with the exception of the confirmation test for anti-orf K8.1 antibody, the seroprevalence rates were 0.6% (3/515) in HIV-1-negative healthy adults and 0.7% (3/434) in

HIV-1-infected patients without KS. A higher seroprevalence of HHV-8 was found in all groups by IFA for anti-lytic antibodies: approximately 10% of HIV-1-positive patients and approximately 5-10% of HIV-1-negative individuals.

DISCUSSION

No current assay consistently detects anti-HHV-8 antibodies in all patients with KS and no single assay is sufficiently sensitive and specific to detect HHV-8 in asymptomatic infection (Rabkin *et al*, 1998). In this study, we attempted to evaluate the prevalence of HHV-8 antibodies in the population of Thailand, a country with few cases of KS but a high prevalence of HIV-1 infection; our subjects included HIV-1-negative and HIV-1-positive individuals; we used a standard IFA method, using HHV-8 antigens from a HHV-8 latently-infected B lymphoma cell line, and a second generation assay containing the major immunogenic antigens, including latent and lytic proteins orf 73, orf 59, orf 65, and orf K8.1. We found that samples of two AIDS-KS patients had 100% correlation between seropositivity by all assays and the presence of the HHV-8 genome by DNA polymerase chain amplification on KS tissues and peripheral blood mononuclear cells (data not shown). Therefore, Thais with AIDS-KS have been infected with HHV-8.

From our data, all sera positive for latent antigens by IFA were reactive to mixed HHV-8 proteins by the ELISA system; however, shown in only a few sera positive for lytic antigens by IFA; moreover, the sera positive for anti-lytic antibodies and anti-K8.1 antibody would have antibodies to mixed-HHV-8 antigens. Since the lytic and latent HHV-8 proteins encoded by orf K8.1, orf 59, orf 65, and orf 73 were included in this ELISA, our findings suggest that HHV-8 proteins encoded by orf K 8.1 and orf 73 may be a useful tool for HHV-8 seroepidemiological or pathological studies, as claimed in other studies (Gao *et al*, 1996b; Kedes *et al*, 1997; Raab

Table 3
Seroprevalence of HHV-8 in study populations based on serologic assays.

Assay					No. of positive by the assay results							
					HIV (+)		HIV (-)					
IFA lytic	IFA latent	ELISA mixed-antigens	IFA orf K8.1	No. of assays positive	KS + (%)	KS - (%)	<10 yr (%)	10-50 yr (%)	BD[a] (%)	STD[b] (%)	IDU[c] (%)
+	+	+	+	4	2 (100)	1 (0.2)	0	1 (0.2)	0	0	0
+	+	+	-	3	0	2 (0.5)	0	2 (0.4)	0	0	0
+	-	-	-	1	0	57 (13)	5 (4)	43 (8)	9 (7)	11 (6)	6 (8)
-	-	-	-	0	0	374 (86)	112 (96)	469 (91)	114 (91)	176 (94)	70 (92)
Total					2	434	117	515	123	187	76

[a]blood donors.
[b]sexual transmitted diseases clinic attenders.
[c]injecting drug users.

et al, 1998). In addition, we found that when using an anti-lytic assay, cross-reaction with unknown antigens can not be excluded, and may lead to an overestimation of the prevalence of HHV-8 antibodies (Dupin *et al*, 1998).

Six sera; 3 from HIV-1-negative individuals and 3 from HIV-1-positive patients without KS, who had anti-lytic and anti-latent HHV-8 antibodies by IFA, were also reactive in the mixed-antigen ELISA. However, two sera, one of each group, were positive for anti-orf K8.1 antibody. The other two samples of each group, which showed the negative results for anti-K8.1 antibody, had low antibody titers (<320) to both latent and lytic HHV-8 antigens: this may result in anti-orf K8.1 antibody being undetected. Therefore, antibodies to orf K8.1 protein are not likely to be appropriate serological markers for HHV-8 infection in areas without endemic KS, like Thailand, in contrast to those countries with a high risk of KS, as suggested in other studies (Raab *et al*, 1998; Li *et al*, 1999).

Interestingly, HHV-8 infections, confirmed by all of the assays of 3 sera of healthy HIV-1-negative patients and by one serum sample from an HIV-1 positive patient without KS, were derived from the northern region: this may suggest that people living in the north of Thailand are at greater risk of developing KS. Whether the people of this region are susceptible to this virus remains to be shown; a larger study would be needed. If a large study suggests a higher rate of KS in this region, the difference in cofactors, such as the environment or HLA types, among Thais might play a role in the rate of KS development in HHV-8 infected individuals.

There were two reports on the prevalence of HHV-8 antibodies in Thais. In our previous study, using IFA to detect anti-lytic and anti-latent antibodies (Isarangkura Na Ayuthaya *et al*, 1998), we found that the rates of seroprevalence of HHV-8 among HIV-1-negative individuals, HIV-1 carriers, and HIV-1 positive patients with skin diseases were 0% (0/19), 7.4% (2/27), and 25 % (11/44) respectively. Another group reported that 4% (3/75) of healthy HIV-negative donors and 11.2% (22/196) of HIV- infected donors had HHV-8 antibodies, determined by whole viral lysate as the source of antigens in ELISA (Ablashi *et al*, 1999); the samples in this study were collected at a single vaccine trial center and the sample sizes in both the studies cited above were small compared with that used in this study. Our data showed lower seroprevalence, in both HIV-1-negative and HIV-1-positive groups, than that shown by Ablashi *et al* (1999): this discrepancy may be the result of different cohorts and different assays.

Four studies on seroepidemiology in Asia have been published recently. Ablashi *et al* (1999) reported the low seroprevalence of HHV-8 in some Asian countries (*eg* Malaysia, India, and Sri Lanka): 3.8-4.4% in the general population and 2.4 % among HIV-1-positive-individuals; the method was whole-virus lysate ELISA. In Taiwan, Huang *et al* (2000) demonstrated that about 4% of sera from children of less than 10 years of age and about 19% of healthy adults were positive for HHV-8 antibodies by IFA to both latent and lytic antigens. The other two reports from Japan found that the seroprevalence of HHV-8 infection among healthy Japanese blood donors was 0.2% using IFA to detect latent antigens (Fujii *et al*, 1999) and 1.4% using mixed-antigen ELISA (Katano *et al*, 2000). Within their limits of detection the techniques used in this study gave seropositive rates for anti-HHV-8 antibodies among HIV-1-negative healthy individuals and HIV-1-positive patients of 0.6% (3/515) and 0.7% (3/434) respectively. The prevalence of HHV-8 infection is extremely low in Asian countries; these results suggest that HHV-8 is not widespread in this region compared with the western world, where 5-10% of the general population and 20-70% of homosexual HIV-infected patients are seropositive for HHV-8 (Cathomas, 2000).

Although the prevalence of HHV-8 antibodies in the general population and in HIV-1-infected individuals in this country can not yet be determined, we estimate that it is likely

to be low because the results from our data were derived from an expanded sample; serum samples from HIV-1-negative subjects, sampling from the 4 main regions of Thailand, and the use of a second generation assay system to confirm the positivity of sera might also add validity to our study. Between 1984, the year of the first report of a HIV-1 infected case in Thailand, and 1996, only 0.19% (41/21,897) of AIDS-KS patients were reported (Office of the Permanent Secretary for Public Health, 1996); the finding of 1% HHV-8 seroprevalence in either the general population or HIV-1-infected patients agrees with this report. Follow-up studies of HHV-8 seropositive individuals will provide more information on the role of this virus in the pathogenesis of KS and other diseases.

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