SPECIAL REPORT

Studies on the Antibodies to Human Herpesvirus Type 6 Among Hungarian Patients with Asymptomatic HIV Infection*

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The occurrence and the possible role in promoting HIV infection by human herpesvirus type 6 (HHV-6) have not yet been revealed in Hungary. In different groups of patients, serum titre of IgM and IgG antibodies, as well as avidity of IgG were quantitated by indirect immunofluorescence and an enzyme-linked immunosorbent assay, using isolate U1102 of HHV-6 variant A as antigen. In 60% of HIV-seronegative adult controls, high avidity IgG antibodies were found in low titre suggesting childhood infection. In HIV-seronegative persons with high risk behaviour for HIV-infection, both IgM and low avidity IgG were frequently found in higher titre, representing either primary or frequent reinfections, or reactivation of latent HHV-6. In asymptomatic HIV-seropositive patients, high titre of high avidity IgG antibodies was predominant, proving virus infection in the near past. These results indicate the contribution of HHV-6 to immunosuppression prior to AIDS, predisposing the organism to HIV infection. (Pathology Oncology Research Vol 4, No 1, 56–61, 1998)

Key words: HHV-6, seroepidemiology, IgM, IgG avidity, asymptomatic HIV-1 infection

Introduction

Human herpesvirus type 6 (HHV-6) was isolated in 1986. It was originally designated human B lymphotropic virus (HBLV),[29] but was shown to replicate in cells other than B lymphocytes.[1] The virus is a typical β-herpesvirus, and so far two major variants (A and B) have been identified based on restriction enzyme maps[21] immunofluorescent assays using monoclonal antibodies (MAbs) and cell culture properties.[3,30] Group A contains isolates GS (the prototype) and U1102, while group B contains isolate Z29 and further isolates from exanthema subitum.[6,21] GS infects phenotypically immature CD4 positive T cells,[2,23] U1102 infects unstimulated peripheral blood lymphocytes (PBL), T cell lines as HSB-2 and JJHAN, but Z29, SF, KF and other B variants infect $CD4^+CD8^-$ T cells of mature phenotype.[21,35] The occurrence of the two variants in clinical syndromes differs. The B variant appears to be isolated more frequently, and has been recognised as the causative agent of exanthema subitum in infants.[32] Studies on the prevalence of HHV-6 antibody in children with no recorded history of exanthema subitum show that almost all children acquire HHV-6 antibody before the age of 1.[5] A higher prevalence of HHV-6 variant A has been found in Kaposi's sarcoma lesions,[4] chronic fatigue syndrome patients,[10] as well as in subjects with lymphoproliferative disorders as Hodgkin's and non-Hodgkin's lymphomas.[9] In immunodeficient patients, HHV-6 A is a co-factor in the progression of HIV infection and AIDS development,[19,24] due to prevalent tropism for $CD4^+$ cells,[31] induction of CD4 receptor expression on the surface of $CD4^-$ lymphocytes,[22] its direct transactivating effect on HIV replication,[11] and the stimulation of the release of HIV-1 activating cytokines.[26] Infection with one or both variants of HHV-6 results in seroprevalence in healthy adults from 50% to 90% worldwide.[9,19] The virus persists for life, sheding in the saliva,[19] but it is

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difficult to isolate from PBL.[33] Very rarely adolescents and young adults may experience a primary HHV-6 infection.[34] Longitudinal studies including a very limited number of patients revealed that HIV-1 and HHV-6 simultaneous gene expression studied by polymerase chain reaction (PCR);[27] significant rise of antibody titres to HHV-6;[15] as well as active and disseminated HHV-6 infection in the terminal stage of HIV-1 infection[16] all contribute to the dramatic loss of CD4 cells and fatal outcome of AIDS. The mode of infection and reactivation of latent HHV-6 in the process are still unknown. At the moment, serological methods are used to distinguish primary infections from recurrent ones. Well-timed collections of paired sera are often impossible, however therefore the simultaneous presence of IgM and IgG antibodies, as well as changes in the avidity of IgG antibodies in a single serum are suitable for the characterization and timing of the infection.[33,34]

In Hungary so far, no data have been obtained on the prevalence of HHV-6 variant A and its possible role in enhancing HIV-infection, *in vivo*. We studied its seroepidemiology and risk factor properties in patient' groups with or without HIV infection.

Materials and Methods

Collection of patients' sera

Three groups of patients were chosen for our studies. Group I: Asymptomatic HIV-infected persons (age: 26 to 54 years, male/female ratio: 20/0), Group II: HIV-seronegative sexual partners of patients belonging to Group I (age: 18-48 years, male/female ratio: 6/4), and Group III: HIV-negative healthy persons, tested for other reasons (blood donors, employment, etc) (age: 12 to 76 years, male/female ratio: 1/9). Patients with CD4 counts >400 were included in the study and monitored regularly by flow cytometry (Becton Dickinson, Basel, Switzerland). Sera of patients were obtained from the tissue bank of the National Institute of Dermato-Venereology, Budapest, where Hungarian asymptomatic HIV-infected persons are under continuous care. The samples from the different patient' groups were chosen randomly and studied in a blind manner. HIV seropositivity was established by HIV ELISA and Western blot.[25] All sera were inactivated at 56°C for 30 min before studying HHV-6 seroepidemiology. Titres of IgM and IgG antibodies to HHV-6 variant A were quantitated by both an indirect immunofluorescent assay (IFA) and a cellular enzyme linked immunosorbent assay (ELISA).

Indirect immunofluorescent assay

JJHAN cells were maintained and grown in RPMI-1640 medium supplemented with 2 mM L-glutamine, 20 mM HEPES buffer, 100 U/ml of penicillin, 100 µg/ml of streptomycin (Sigma-Aldrich Ltd., Budapest, Hungary), and 10% heat inactivated fetal calf serum (FCS, Gibco Life Technologies Inc., Gaithersburg, MD). Strain U11O2 HHV-6 variant A was used throughout these studies. For infection, HHV-6 infected JJHAN cells exhibiting cytopathic effect (CPE) were pelleted 1:10 with the cells to be infected. The cells were resuspended at a concentration of 10^7 per ml. After 2h, the infected cells were diluted with fresh medium to a final concentration of 10^6 cells/ml. Mock infected cells were similarly treated, using uninfected JJHAN cells as inoculum.[26] Infected cells were incubated for 10 to 12 days, when characteristic CPE and positive fluorescence for intracellular virus antigens peaked (in around 38% of cells). Cells were washed in phosphate buffered saline (PBS, pH 7.4) three times, then 20,000 of either infected or uninfected cells in 20 µl PBS were mounted on multiwell glass slides (5 mm inner diameter, 12 circles per slide), and finally fixed in ice-cold 1:1 acetone-methanol for 10 min. Thereafter the preparations were washed with PBS and distilled water and stored at –20°C. Patients sera were diluted with PBS beginning 1:10 in twofold manner. Ten µl of diluted sera were incubated on fixed cells in a moist chamber at 37°C for 45 min. After three washes in PBS cells were incubated with prestandardized goat anti-human IgG or IgM, respectively, conjugated with fluoresceine isothiocyanate (Human Co., Gödöllő, Hungary). After three washes in PBS slides were counterstained with 1:20,000 dilution of Evans blue (Sigma) for 5 min at room temperature. Preparations were mounted in glycerol and examined under a Zeiss Fluoval microscope. A HHV-6 positive serum (gift from L. Ceccherini–Nelli, Pisa, Italy) in a dilution of 1:40, and a repeatedly negative serum (in a dilution of 1:10) of a 12 month old infant (gift from T. Tulassay, Budapest, Hungary) were used as controls. Serum dilutions were regarded positive if 5 or higher percent of cells showed fluorescence.[6]

Avidity of HHV-6 specific IgG antibodies

The IFA procedure described above was modified to include a stage in which low avidity antibody molecules were eluted with urea. Acetone-methanol fixed infected cells on multiwell glass slides were overlaid with twofold serial dilutions of the sera. Duplicate slides were made for each serum dilution and incubated as stated above. After this, one slide was washed with PBS three times, and the other was washed twice for 5 min with PBS containing 8 M urea to elute low avidity antibody and then once more for 5 min with PBS. The tests were then completed using FITC-conjugated IgG antibody as previously described. The sera where antibody titre was reduced eightfold (i.e. $\log_2$ reduction=3) or more in the presence of urea were considered to contain low avidity antibody

to HHV-6 and conversely, the sera where the titre showed fourfold reduction (i.e. $\log_2$ reduction=2) or less were designated as having high avidity antibody. Each determination was done in duplicate.[33,34]

HHV-6 specific ELISA

JJHAN cells were cultured and infected as described above. At the time of maximal CPE and viability close to 100%, cells were washed three times in PBS, then adjusted to $2x10^6$ cells in 25 ml PBS (a quantity enough to prepare 5x96 samples). Polystyrene 96 well tissue culture plates (Greiner, Frickenhausen, Germany) were treated with 100 µl poly-L-Lysine (PLL, MW: 70,000 to 150,000, Sigma), 100 µg/ml concentration at 37°C for 30 min. After removal of PLL, plates were dried and subsequently washed with 0.25% Tween-20 in PBS. Plates were dried again at 50°C for 10 min and stored at +4°C in the presence of anhydrous $CaCl_2$. Next, 50 µl of JJHAN cell suspension per well was introduced and subsequently plates were centrifuged at 2000 rpm for 5 min. Without removing cells, the wells were treated with 50 µl of 0.025% glutaraldehyde at 22°C for 15 min. After discarding supernatants, wells were washed with 200 µl of 0.25% Tween-20 three times and finally dried. Wells were blocked with 100 µl of freshly prepared 1% solution of bovine serum albumine (BSA) at 37°C for 1h. Supernatants were discarded, the plates were dried and stored as mentioned. Immediately before use, each well was filled in with 100 µl of 0.25% Tween-20 solution. To quantitate both IgM and IgG, parallel serial dilutions of patients' sera in the Tween-20 solution were prepared in twofold manner at the beginning in a dilution of 1:400. The plates were incubated in a moist chamber at 37°C for 60 min, then the residual serum was removed using Tween-20 three times, finally plates were dried. Peroxidase-conjugated anti-human IgG and IgM (Sigma), respectively, were diluted to 1:15,000 using 1% FCS solution. Their 100 µl volumes in wells were incubated at 37°C for 60 minutes. The microwells were washed with Tween-20 three times. Subsequently 100 µl of freshly prepared ortho-phenyl-diamine (OPD) substrate in phosphate-citrate buffer (pH 5.0) was introduced in each well at 22°C in the dark for 15 minutes. After stopping reaction with 100 µl of 12.5% sulfuric acid, colour intensity was read in parallel at 492 and 620 nm in a microplate reader (Life Sciences International Ltd, Budapest, Hungary). Standard deviation of the absorbance using concentrated sera of seronegative individuals from other studies[15,26,27] did not exceed 150% of the mean, therefore samples with absorbance values greater than this empirical cut-off value were considered positive for antibody titre.

Results

Ninety percent of control persons had no detectable HHV-6 A specific IgM in their sera, except for one positive case (a 19 year old female), who might have had a fresh infection in her adolescence *(Table 1)*. By both IFA and ELISA, 40% of controls had no detectable anti-HHV-6 A IgG antibodies at all *(Table 2)*. The mean level of IgG was found to be moderate *(Table 3)*. The avidity of anti-HHV-6 antibodies found in their sera was high, also suggesting primary infection and/or reinfections a long time before the collection of specimens *(Figure 1)*.

In the group of HIV-seronegative persons with high risk for HIV-infection, the anti-HHV-6 antibody pattern was completely different from that of the first group. Using IFA, although 40% of the probands proved to be negative for HHV-6 specific IgM, but the more sensitive ELISA detected IgM antibodies in all persons without exception

***Table 1.* Distribution of IgM antibody-titres to HHV-6 „A" in the sera of different patients' groups (in percentage of patients)**

Group	*IFA*								*ELISA*							
	Negative	*20[a]*	*40*	*80*	*160*	*320*	*640*	*1280*	*Negative*	*100[a]*	*200*	*400*	*800*	*1600*	*3200*	*6400*
HIV-seropositive patients n=20	70	0	20	5	5	0	0	0	50	35	5	10	0	0	0	0
High-risk group n=10	40	0	10	20	30	0	0	0	0	90	0	0	10	0	0	0
Control persons n=10	90	10	0	0	0	0	0	0	90	10	0	0	0	0	0	0

[a]Reciprocal values of serum dilutions

Table 2. **Distribution of IgG antibody-titres to HHV-6 „A" in the sera of different patients' groups (in percentage of patients)**

Group	IFA								ELISA							
	Negative	20[a]	40	80	160	320	640	1280	Negative	100[a]	200	400	800	1600	3200	6400
HIV-seropositive patients n=20	0	0	10	10	25	40	10	5	0	15	0	10	10	15	30	15
High-risk group n=10	40	0	0	0	0	20	10	30	0	0	0	0	30	40	30	0
Control persons n=10	40	10	10	20	20	0	0	0	40	10	10	0	20	20	0	0

[a]Reciprocal values of serum dilutions

belonging to this group, without *(Table 1)*. Prevalence of IgG antibodies was similar *(Table 2)*. Using either IFA or ELISA, the mean titres of both antibodies to HHV-6 A were detected in higher concentration than the corresponding values of the control subjects *(Table 3)*. The most striking changes in the avidity of anti-HHV-6 IgG antibodies were found in this group: the majority of the risk persons had low avidity, relatively fresh IgG molecules in their sera *(Figure 1)*. This finding, plus the frequent detectability and higher titre of IgM antibodies strongly suggest recent reinfections or reactivation of latently carried HHV-6 in their organism.

In the group of HIV-seropositive homosexual men, prevalence of anti-HHV-6 IgM antibodies fell to 30 to 50% depending on the sensitivity of the method used *(Table 1)*. The mean level became moderate as compared to that in the high risk group, but still exceeded that of the control

Table 3. **Mean antibody-titres to HHV-6 „A" in seropositive subjects**

Group	IgM		IgG	
	IFA	ELISA	IFA	ELISA
HIV-seropositive patients n=20	83[a]	170	308[a]	2935
High-risk group n=10	113	170	853	1840
Control persons n=10	20	100	90	850

[a]Reciprocal values of serum dilutions

values *(Table 3)*. Without exception asymptomatic HIV-seropositive patients showed high level IgG antibodies *(Tables 2 and 3)*. In the majority of these patients, the avidity of their anti-HHV-6 IgG molecules changed to high again *(Figure 1)*. These latter results, together with the observed decreasing frequency and mean level of IgM antibodies suggest possible reinfection or endogenous reactivation by HHV-6 long before the specimen collection.

In all three groups the results obtained by either IFA or ELISA show the same trend. The cellular ELISA established in our laboratory seems to be approx. 10 times more sensitive than IFA, especially if detecting IgG antibodies *(Tables 1 to 3)*. There seems to be another interesting observation in the high risk group. Although IFA detected the most significant increase in the level of both IgM and IgG antibodies, the results of ELISA were not completely parallel to this finding. In this patient group, among the reacting anti-HHV-6 IgG molecules, the ratio of low avidity molecules was found to be frequent, and the extensive washing procedures during assay might have removed them from the polystyrole plate. This observation might be utilised to distinguish between low and high avidity IgG molecules in the ELISA method, similarly to their quantitation in IFA. Experiments are in progress to reveal this possibility.

Discussion

Serological evidence suggests that HHV-6 A is prevalent in Hungary. Our other studies (not shown here) indicate that infection occurs before 18 months of age. By the end of that period the same ratio of seroprevalence is established as found in healthy adults. Seronegatives might experience a mononucleosis-like infection or mild afebrile lymphadenopathy in the adulthood,[17] as one of the subjects

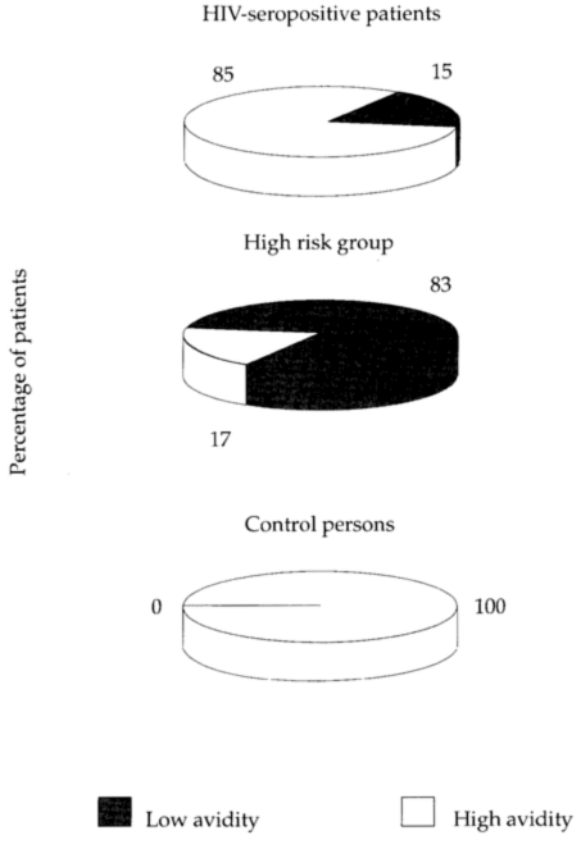

Figure 1. Reappearance of low avidity IgG antibodies to HHV-6 A in subjects with high risk for HIV-infection as compared to HIV-infected patients and normal persons.

in our control group had, with presenting IgM antibodies. These data correspond to previous findings,[9,18,34] although reported prevalences of antibodies vary between 20 and 80%.[17,21] Most of the serology to date have relied on IFA, using infected cell cultures, but the specificity and sensitivity of this method always vary.[13] We used cell cultures prepared in our laboratory, because commercially available IFA preparations do not differentiate between antibodies to HHV-6 variants A and B, although the reactivities of human sera from healthy adults might show the existence of antigenic differences of virus variants, especially with the use of radioimmunoprecipitation.[6] Using commercial IFA kits, approximately 75% of pregnant women, and 90% children had antibodies to HHV-6 variant B (G. Berencsi, personal communication). Such differences in the antibody prevalence to HHV-6 variants A and B are well established.[18] It has proved very difficult to develop solid phase enzyme- or radioimmunoassays especially for IgM class antibodies, due to difficulties in producing good antigen preparations for these types of tests.[8] Our ELISA system proved to be specific and sensitive. Sera of healthy children and adults, that of patients with different types of lymphomas and multiple sclerosis have been studied recently with this ELISA system using both JJHAN cells infected with HHV-6 variant A and MOLT-3 cells infected with variant B. Results clearly demonstrate prevalence of high titre antibodies to variant B with low titre or absence of antibodies to variant A. Experiments are in progress using monoclonal antibodies (gift of PE Pellett, CDC, Atlanta, GA) in a competitive ELISA to quantitate parallel presence of antibodies to both HHV-6 variants. Furthermore, variations of our HHV-6 ELISA – under development – might be suitable to detect both low and high avidity IgG molecules separately, beside IgM antibodies.

In the high risk group, frequent detection of IgM antibodies might reflect either primary infections in 40% of subjects free of virus since their childhood, or recurrent infections in virus carrier persons. It is well established that specific IgM can be detected in recurrent infections with other herpesviruses (e.g. human cytomegalovirus [HCMV][28]). Furthermore, high risk persons – due to promiscuous behaviour – are infected with several types of microorganisms, among them HCMV and Epstein-Barr virus (EBV). Both are known to enhance production of anti-HHV-6 antibodies in recurrent HHV-6 infections. This phenomenon is not a cross-reaction between different herpesvirus antibodies.[20] Extremely frequent prevalence of low-avidity IgG antibody molecules in the sera of high risk persons also supports reinfection or reactivation of HHV-6 within the previous 5 months, because this is the period in which antibodies mature to high avidity from the initial response.[33] Beside saliva,[19] HHV-6 has already been recovered from the cervix and vaginal secretions.[18] High risk persons for HIV-1 infection are, therefore, at high risk for HHV-6 A infection, too.[19]

HHV-6 antibody titres in the specimens of HIV-infected patients were established 6 to 24 months after HIV-seropositivity had been diagnosed. Possible changes in their life style – e.g. giving up promiscuity or practicing safer sex – diminish the risk for reinfection by HHV-6 and other viruses. HHV-6 is known to transactivate several other types of viruses, e.g. HIV-1, papillomavirus, EBV, CMV,[7,14,18,20,26] from which EBV and CMV enhance the production of anti-HHV-6 antibodies.[33,34] Breaking this vicious circle stops the release of fresh IgG antibodies with low avidity, consequently the majority of IgG molecules detected in this group matured to high avidity. In one case, anti-HHV-6 antibody profile was established few weeks after diagnosing HIV seropositivity and both, both IgM and low avidity IgG were found in the serum of this patient.

Our recent results demonstrate that HHV-6 A contributes to the immunosuppression prior to HIV infection and, consequently, it predisposes the organism to HIV infection. In the asymptomatic stage of disease, the coincidence of periodical expressions by HHV-6 and HIV might occur,[27] resulting in severe immunosuppression. In the course of AIDS, no data on the anti-HHV-6 antibody

profile have been revealed as yet, therefore, longitudinal studies have been started in this group of patients to establish the role of HHV-6 A during all stages of AIDS.

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