Arch Virol (1994) 134: 357–368

Archives of Virology

Low-incidence latent infection with variant B or roseola type human herpesvirus 6 in leukocytes of healthy adults

J. Rajčáni[1,*], R. Yanagihara[1], M. S. Godec[1], J. W. Nagle[1], M. Kudelova[2], and D. M. Asher[1]

[1] Laboratory of Central Nervous System Studies and Center for Neurogenetics, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland, U.S.A.

[2] Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovak Republic

Accepted August 31, 1993

Summary. Nested primer-based polymerase chain reaction was employed to determine the frequency of latent infection with human herpesvirus 6 (HHV-6) among healthy adults from Bratislava, Slovak Republic. A 592-bp region, upstream from the gene encoding the putative large tegument protein of HHV-6, was amplified from DNA extracted from peripheral blood mononuclear cells (PBMC) of only one of 29 seropositive adults, suggesting that as few as 1 in 10^5 PBMC may be infected with the virus. Direct sequencing of the 592-bp fragment indicated that the virus harbored by the seropositive Slovak subject (designated B38) differed by only 3 nucleotides from an HHV-6 variant B strain (R-147) isolated from an American infant with a roseola-like illness and by 32 bases from the variant A strain GS isolated from a patient with lymphadenopathy (5.4% sequence divergence). None of these strains had a deoxyadenosine at base position 1251, when compared to the published sequence of strain GS clone pZVH14. Although this discrepancy did not affect the large tegument protein gene, it altered the predicted amino acid sequences of two putative proteins coded by open-reading frames 1 and 2 (ORF 1 and ORF 2) located upstream from this gene.

Introduction

Human herpesvirus 6 (HHV-6), originally isolated from cultures of peripheral blood mononuclear cells (PBMC) derived from patients with lymphoproliferative disorders [30] and acquired immunodeficiency syndrome (AIDS) [22, 27, 38], is now known to be the etiological agent of roseola infantum or

* Permanent address: Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovak Republic.

exanthem subitum [39] and possibly an infectious mononucleosis-like syndrome [3, 19, 26, 34]. HHV-6 infection is usually acquired during infancy and childhood, and infection is widespread in populations from widely separated geographical regions [5, 6, 20, 27]. Although HHV-6 was initially believed to be B-cell lymphotropic, accumulated data indicate that mononuclear cells which support HHV-6 replication display markers characteristic of T cells, namely CD2, CD4, CD5, CD7, and CD8 [23, 35], but not markers of B cells, such as CD21 or CD20 [9].

Like other human herpesviruses, HHV-6 can establish latent infection with nonintegrated viral DNA persisting in a covert, nonproductive form. Kondo and colleagues [18] demonstrated that adherent monocytes harbor HHV-6 for more than a month without expression of detectable antigen or virus replication. Similarly, HHV-6 genomic sequences were detected in monocytes by polymerase chain reaction (PCR), without detectable viral antigen, during the convalescent phase of roseola [16, 17]. PCR has also been employed to demonstrate HHV-6 DNA in throat washings and leukocytes of a small proportion of healthy adults [15] and patients infected with human immunodeficiency virus (HIV) [31] and in lymph nodes of some patients with Hodgkin's and non-Hodgkin's lymphomas [11, 37]. Here, we report the enzymatic amplification and direct sequencing of a 592-base pair (bp) region of HHV-6 in DNA extracted from PBMC of a healthy adult from the Slovak Republic. Our analysis also indicates a possible error in the originally reported sequence of HHV-6 strain GS. This finding alters the predicted amino acid sequences of the two proteins encoded upstream to the tegument gene and markedly increases the predicted size of the protein encoded by open-reading frame (ORF 1).

Materials and methods

Blood specimens and DNA extraction

Heparinized blood (5-10 ml) was obtained with informed consent from 45 healthy adults (age range, 20-60 years) in Bratislava, Slovak Republic. Plasma was saved and PBMC were separated by Ficoll-gradient centrifugation. DNA was extracted from uncultured PBMC by the saturated phenol-chloroform method following proteinase K digestion. From suspensions of HHV-6 infected and uninfected HSB-2 cells (10^7 cells/ml), DNA was extracted into PCR buffer, as described by Higuchi [10].

Viruses and cells

Uninfected HSB-2 cells and HSB-2 cells infected with the GS strain of HHV-6 variant A were provided by the AIDS Research and Reference Reagent Program, Rockville, MD, U.S.A. Uninfected J-Jhan cells and J-Jhan cells infected with the R-147 strain of HHV-6, isolated from a 5-month old female infant with a roseola-like illness characterized by high fever (40.5 °C), malaise, bulging anterior fontanelle, diarrhea and rhinorrhea but without rash, were kindly provided by Dr. C. B. Hall, Dr. M. T. Caserta and Ms. K. McIntyre of the Department of Pediatrics, University of Rochester, School of Medicine, Rochester, NY, U.S.A. Cells were maintained in culture as previously described [39].

Indirect immunofluorescent antibody test

For fluorescent antibody tests, 5 μl aliquots of a suspension containing approximately 10^7 cells/ml in PBS with 1% bovine serum albumin were spotted onto wells of 10-well microscopic slides (Cel-line Associates, Newfield, NJ, U.S.A). Smears were air dried, fixed in cold acetone for 10 min and blocked with normal goat serum (diluted 1:20 in PBS) for 20 min at 37 °C. Each plasma, diluted 1:20, was tested on HHV-6-infected and uninfected HSB-2 cells by the indirect fluorescent antibody technique, using fluorescein isothiocyanate-labeled goat anti-human IgG (Cappel-Organon Teknika Corp., Durham, NC, U.S.A.). To assure accurate end-point readings, most specimens were also tested using commercially available HHV-6-infected cell preparations (Universal Biotechnologies, Rockville, MD, U.S.A.); each dilution of plasma was tested in parallel on virus-positive and negative HSB-2 cells, which had been checked for the presence or absence of HHV-6 DNA by PCR; each plasma was tested at least twice, initially at dilutions of 1:20 and 1:40, and positive samples were tested at serial two-fold dilutions from 1:40 to 1:160; at each staining the end-point was read using a standard antibody-positive serum (titer, 1:160; Universal Biotechnologies). Anti- HHV-6 antibody titers were expressed as the reciprocal of the highest dilution of plasma giving virus-specific fluorescence.

Gene amplification

Oligonucleotide primers for PCR, synthesized using an Applied Biosytems model 391 DNA synthesizer (Foster City, CA, U.S.A.), were derived from a *Hin*d III fragment of the HHV-6 clone pZVH14 [12], which encodes the putative large tegument protein [14]. Primer P1 (bases 814-833, 5′-ACCGCGCACGTGTTTTTTAT-3′) and the reverse primer P2 (bases 1546-1527, 5′-TAGAATGCTTACGAGAAGGT-3′) flanked a 732-bp fragment, while primers for nested PCR, P3 (bases 884-904, 5′-CATACTAGAACAACAGAGAG-5′) and P4 (bases 1476-1457, 5′-ATTTTAAAGATTACGGACTT-3′), flanked a 592-bp portion, with an *Alu* I cleavage site at bases 1151–1154. Primers were used at a final concentration of 1 μM in a reaction mixture of 100 μl containing 2 μg of test DNA and 2.5 units of *Thermus aquaticus* DNA polymerase (Perkin-Elmer/Cetus, Norwalk, CT, U.S.A.) in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, and 0.2 mM each dNTP. Using a DNA thermal cycler (Perkin-Elmer/Cetus), the mixture was initially denatured at 94 °C for 7 min, and then subjected to 40 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 3 min, with a final extension of 7 min at 72 °C before storing at 4 °C [7].

The sensitivity of nested PCR for detection of HHV-6 DNA was also evaluated in mink lung cells [33], co-cultivated in the presence of polybrene (20 μg/ml) with HHV-6-infected HSB-2 cells in a proportion of 1:1 and propagated for 7 passages over 24 days.

Southern blot hybridization

Enzymatically amplified HHV-6 DNA were size fractionated by electrophoresis on 1.5% agarose (NU-Sieve, Seakem GTG, Rockland, ME, U.S.A.) prepared in 0.5 × TBE buffer (45 mM Tris-HCl pH 8.0, 45 mM boric acid, 1 mM EDTA) and were transferred to nylon membranes (Nytran, Schleicher and Schuell, Keene, NH, U.S.A.), after alkaline denaturation and neutralization, for Southern analysis using a 30-mer synthetic oligonucleotide probe (bases 1119–1148, 5′-CAACTCTACGGCACACCATATTACAACCCT-3′), designed to anneal to the amplified product just upstream of the *Alu* I site (SP13 in Fig. 1). The oligonucleotide probe, end-labeled by direct phosphorylation of the free 5′-terminal OH-groups by T4 polynucleotide kinase in the presence of $^{32}P(\gamma)$-ATP (specific activity of probe, 5.4×10^5 dpm/ml), was purified on Nensorb-20 columns. Membranes were hybridized

overnight, washed under high- stringency conditions [41] and exposed to Kodak X-Omat AR film for 6 to 18 h at −80 °C.

Direct sequencing of PCR products

Sequencing of PCR products, purified using ultrafiltration membranes (Centricon 100, Amicon Corp., Beverly, MA, U.S.A.), were performed in both directions at least twice using six overlapping primers by the *Taq* DyeDeoxy-Terminator Cycle sequencing kit (Applied Biosytems Inc., Foster City, CA, U.S.A.) on an automated sequencer (model 370A-1, Applied Biosystems Inc.) [24, 36]. The sequencing strategy and primers for sequencing reactions are shown in Fig. 1. SP11 (bases 884–914, 5′-CATACTAGAACAACAGAGAGCCATCT-TGAT-3′) and SP14 (bases 1476–1447, 5′-ATTTTAAAGGATTACGGACTTTATTAC-GGTG-3′) flanked the ends of the 592-bp subfragment, while SP3 (bases 1075–1092, 5′-GGTAACGTATTTTTCCCT-3′) and SP4 (bases 1277–1259, 5′-CCATAGGTATAACG-TAGA-3′) started approximately 200 bases apart; finally, SP12 (bases 1184–1156, 5′-CTCT-GATAGACCTCTTTGCAGATATCCCT-3′) and SP13 (bases 1119–1148, 5′-CAACTCTA-CGGCACACCATATTACAACCCT-3′) started on each side of the *Alu* I site.

Sequence analysis

Nucleotide sequences were assembled using the DNAStar Seqman program, and the consensus sequence for each PCR fragment was compared to the GenBank database of the HHV-6 strain GS sequence of clone pZVH14 (Entrez: Sequences, National Center for Biotechnology Information, Bethesda, MD, U.S.A.) using the DNAStar Align Software (DNAStar Inc., Madison, WI, U.S.A.). Multiple alignment was facilitated with the SeqApp program.

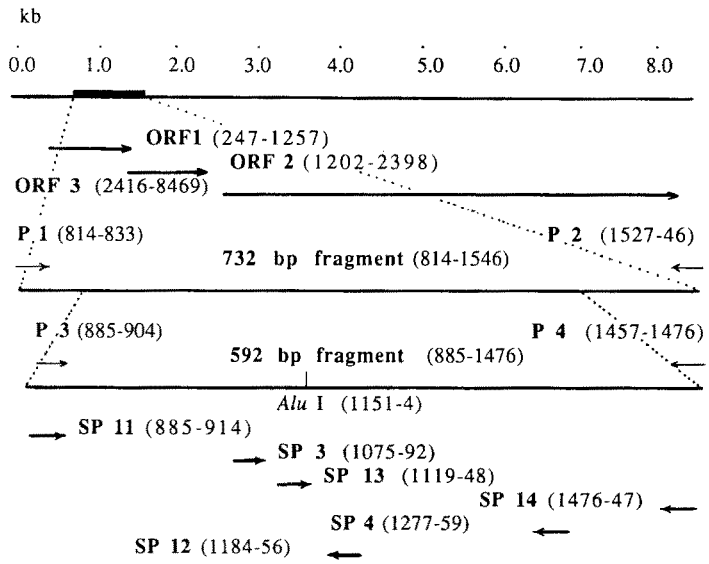


Fig. 1. The 8717-bp *Hind* III H fragment from clone pZVH14 [14] contains 7 open-reading frames (ORFs), of which 5 are transcribed downstream. The large tegument protein is encoded by ORF 3, while the amplified region (reported here) starts in ORF 1 (base 814) and ends in ORF 2 (base 1546). The putative proteins encoded by ORFs 1 and 2 have not been identified. SP12 was ^{32}p-end-labeled for Southern hybridization

Results

Serosurvey of HHV-6 infection and HHV-6 antibody titration

HHV-6 antigen-specific fluorescence was clearly visible in the cytoplasm as well as in the nuclei of virus-infected HSB-2 cells. The proportion of the HHV-6 antigen-containing cells in the producer HSB-2 and J-Jhan cell lines slightly exceeded 10%. Sera from 29 of 45 healthy adult individuals from Bratislava contained IgG antibodies against HHV-6, as determined by the indirect immunofluorescent antibody technique, giving an overall seroprevalence of 64%. In the majority of sera, the HHV-6 antibody titers were 20 (17 cases) or 40 (6 cases), and the antibody titers exceeded 80 in 6 individuals. The geometric mean titer was 37. Antibody titers were identical using our slide preparations and the commercially available HHV-6 smears.

Sensitivity of HHV-6 PCR

PCR was performed on serial five-fold dilutions of DNA extracted from HSB-2 cells infected with HHV-6 strain GS (approximately 10% of HSB-2 cells exhibiting virus-specific fluorescence designated as "high-producer" cultures). Distinct amplification of HHV-6 DNA, visualized by ethidium bromide staining, was obtained at a template dilution of $1:5^6$, which corresponded to approximately 16 HHV-6-infected cells (640 pg of total DNA). The identity of amplified HHV-6 DNA was verified by Southern hybridization, which detected the complementary sequence at a DNA dilution of $1:5^7$ (approximately 3–4 infected cells, or 128 pg of total DNA). By nested PCR, using primers P3 and P4, the predicted 592-bp HHV-6 fragment was detected at the threshold dilution of $1:5^8$, corresponding to 26 pg of total DNA, equivalent to the amount of HHV-6 DNA produced in less than a single HHV-6 antigen-bearing cell (Fig. 2). Thus, nested PCR increased the sensitivity of detecting HHV-6 DNA in PBMC by at least 100 fold.

The presence of HHV-6 DNA was also examined in a "low-producer" HHV-6-infected HSB-2 cell culture, which showed $<0.01\%$ positively stained cells. HHV-6 genomic sequences were detected by direct PCR at a DNA dilution of $1:5^2$, and, by nested PCR, the 592-bp fragment was visualized at a DNA dilution of $1:5^3$. Thus, the amount of the HHV-6 DNA in this "low-producer" HSB-2 cell culture was at least 625-times lower than that in the "high-producer" HSB-2 culture. This estimate was consistent with the nearly 1 000-fold decrease in the number of HHV-6 antigen-bearing cells.

The high sensitivity of nested PCR to detect HHV-6 DNA was also demonstrated in mink lung cells, co-cultured with "high-producer" HHV-6-infected HSB-2 cells (Fig. 2). No amplification was found by direct PCR, but by nested PCR and subsequent confirmation by Southern hybridization, HHV-6 sequences were detected in DNA extracted from the HHV-6/mink lung co-cultures.

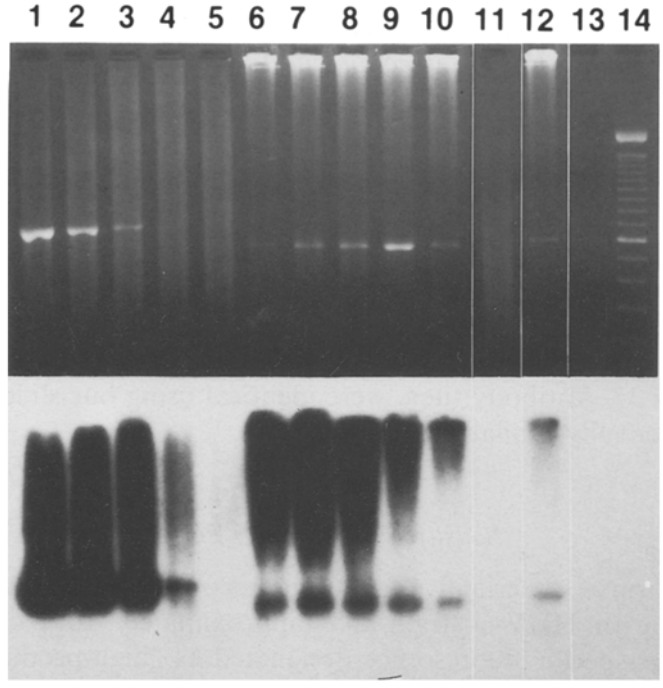
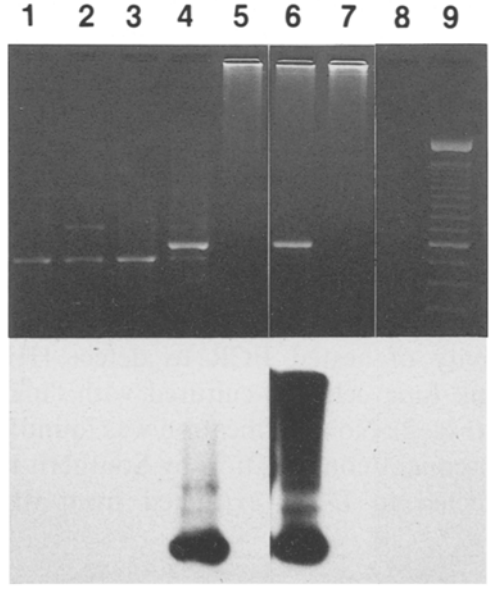


Fig. 2. Sensitivity of direct and nested PCR (top) and Southern blot hybridization (bottom). Direct PCR (*1–5*) and nested PCR (*6–10*) of five-fold serial dilutions of DNA extracted from the "high-producer" HHV-6 (strain GS)-infected HSB-2 cells. PCR in *4*, conducted on 128 pg of total DNA (dilution, 5^7) and HHV-6 produced by about 3 virus antigen-producing cells, was positive by Southern hybridization but showed a barely visible band by ethidium bromide staining. Nested PCR showed a clear-cut band at a dilution of 5^8, corresponding to 26 pg of total DNA and the amount of HHV-6 produced by 0.5 infected cell (10). Direct (*11*) and nested PCR (*12*) of DNA from mink lung cells co-cultivated with HHV-6-infected HSB-2 cells and passaged 7 times over 24 days. *13* Water control; *14* 1 kb DNA ladder



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          885                                                                954
GS-Publ   CATACTAGAA CAACAGAGAG CCATCTTGAT CATCGAACGA AATGATTATT CCACACTCTG GAAGCAAATA
 GS-Seq   .......... .......... .......... .......... .......... .......... ..........
    B38   .......... .......... ....T..... .......... .....A.... .......... ...A......
  R-147   ....T..... .......... ....T..... .......... .....C.... .......... ...A......

          955                                                               1024
GS-Publ   TCTGATCACA TCGACTGTCT CTTCAACGTC TCTCTCTCCG AATCCTTTTT TAAAGAATAT ACCAAAGGAG
 GS-Seq   .......... .......... .......... .......... .......... .......... ..........
    B38   .......... .......... .......A.. .......... .......... .......... ..........
  R-147   .......... .......... .......A.. .......... .......... .......... ..........

          1025                                                              1094
GS-Publ   GAAACGAAGA TCAAAAACAG TTTCTATACA AAAACCTATT CGAAAAATGG GGTAACGTAT TTTTCCCTTT
 GS-Seq   .......... .......... .......... .......... .......... .......... ..........
    B38   .......... A..T...... .......... .......... .......... ...G....C. ..........
  R-147   .......... A..T...... .......... .......... .......... ...G....C. ..........

          1095                                                              1164
GS-Publ   CACGTACTCG GTATCCACCT CAAACAACTC TACGGCACAC CATATTACAA CCCTTGAGCT AAGGGATATC
 GS-Seq   .......... .......... .......... .......... .......... .......... ..........
    B38   .......... ...A...... ....A..... C......... .......... .......... .....CC...
  R-147   .......... ...A...... ....A..... C......... .......... .......... .....CC...

          1165                                                              1234
GS-Publ   TGCAAAGAGG TCTATCAGAG CGATTCCCCG GACGCATATG AATCTTTACT TCCCTACAGC ACTCATCCAT
 GS-Seq   .......... .......... .......... .......... .......... .......... ..........
    B38   .......... .......... .......... ..G.....C. ....G..... .......... .........G
  R-147   .......... .......... .......... ..G.....C. ....G..... .......... .........G

          1235                                                              1304
GS-Publ   CTTTCAAAAC ATTATTACGT TAAAATCTAC GTTATACCTA TGGTTTCACA CATTACCAAT CTTACCTTTG
 GS-Seq   .......... ......*... .......... .......... .......... .......... ..........
    B38   ....T..... ......*.A. .......... .......... ....CA..T. .......... ..........
  R-147   ....T..... ......*.A. .......... .......... ....CA..T. .......... ..........

          1305                                                              1374
GS-Publ   ATAAATTACA ATCTGATTGC AGACTCTTAA CTCTAATCCA CGCCTGCAAA CTCTTACTCC CCTCGCAACA
 GS-Seq   .......... .......... .......... .......... .......... .......... ..........
    B38   .......... ........A. ......A... ....G..... ......T... .......... ..........
  R-147   .......... ........A. ......A... ....G..... ......T... ...C...... ..........

          1375                                                              1444
GS-Publ   TCTACTACTA CACTACATGG CGTGGCTATA TGCCTTCTCC ATAAACGTGG ACCATATAGA TCTAGGCACT
 GS-Seq   .......... .......... .......... .......... .......... .......... ..........
    B38   .......... .......... T......... C..T..T... ........A. ....C..... ..........
  R-147   .......... .......... T......... C..T..T... ........A. ....C..... ..........

          1445                          1476
GS-Publ   TTCACCGTAA TAAAGTCCGT AATCTTTAAA AT
 GS-Seq   .......... .......... .......... ..
    B38   .......... .......... .......... ..
  R-147   .......... .......... .......... ..
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Fig. 4. Nucleotide sequences of enzymatically amplified DNA from HHV-6 strain GS, strain B38 and strain R-147 aligned and compared to the published sequence of HHV-6 strain GS (clone pZVH14) [14]. The sequences of HHV-6 strain GS, B38 and R-147 differed from the published sequence of HHV-6 strain GS (clone pZVH14) by one base (a missing deoxyadenosine at position 1251, indicated by asterisks). Strains B38 and R-147 differed from strain GS by 32 and 34 nucleotides, respectively. Nucleotide numbers are according to Josephs et al. [14]

Fig. 3. Nested PCR for HHV-6 genomic sequences in DNA from five representative seropositive adults from Bratislava (*1–5*). Sample B38 in *4* was positive by PCR as confirmed by Southern hybridization. Nested PCR for DNA from HHV-6 strain GS-infected (*6*) and uninfected (*7*) HSB-2 cells. *8* and *9* are water control and 1 kb DNA ladder, respectively

Detection and sequencing of HHV-6 DNA in PBMC

By direct PCR using primers P1 and P2, HHV-6 genomic sequences were not detected in leukocyte DNA from the 29 seropositive individuals from Bratislava. After re-amplification using the internal (nested) primers P3 and P4, one sample, B38, from a 48 year-old healthy woman with an anti-HHV-6 antibody titer of 40, revealed a 592-bp band, confirmed as HHV-6 by Southern hybridization (Fig. 3). Thus, the demonstrated latency rate of HHV-6 infection in leukocytes of these healthy adults was only 3.5% (1/29) by PCR.

Nucleotide sequences of the PCR-amplified 592-bp fragments for each of the HHV-6 strains studied (B38, R-147 and GS) were aligned and compared to the published sequence of HHV-6 variant A strain GS clone pZVH14. Our sequence of strain GS was identical to the published sequence except for a missing deoxyadenosine at base position 1251. This deoxyadenosine was also absent in HHV-6 strains B38 and R-147 (Fig. 4). Because of the sequencing strategy, the region in question was sequenced repeatedly with primers SP13, SP3, SP14 and SP4. The absence of deoxyadenosine at position 1251 was verified by analysis of more than 20 independent sequencing reactions.

The overall sequence similarity between the HHV-6 strain B38 from Bratislava and the HHV-6 strain GS was 94.6% (32 substitutions in 592 nucleotides). The HHV-6 strain R-147, from an American infant with a roseola-like illness, also diverged by 34 nucleotides (5.7%) from the variant A GS strain. By contrast, the HHV-6 strain B38 differed from strain R-147 by only 3 (0.5%) of 592 nucleotides. Nucleotide changes in strains B38 and R-147 were primarily transitions (nearly 70%), with the majority being deoxycytosine to deoxythymidine or vice versa (Fig. 4).

Discussion

Previously reported serological studies indicate moderately to extremely high prevalences of anti-HHV-6 antibodies, ranging from 52% to 95%, in healthy adult populations from widely separated geographical regions [5, 25, 40]. Some of the variations between studies can be attributed to the different serological techniques employed (indirect immunofluorescence, anticomplement immunofluorescence, ELISA), as well as to the ages of the study subjects. An age-related decrease, from 100% among children aged 2-3 years to approximately 70% among adults, has been reported [6]. Our own data from 45 adults are in accord with serological surveys conducted on larger population samples. Moreover, the anti-HHV-6 antibody titers among individuals from Bratislava were similar to those reported for most populations, except those from Ghana [20] and Melanesia (J. Rajčáni and R. Yanagihara, unpubl. obs.), where much higher titers have been recorded.

HHV-6 DNA can be detected by PCR in PBMC of nearly all patients during the acute phase of roseola [17] and the more common acute illness sometimes called "roseola sine eruptione", which is the syndrome most typical of HHV-6 infection in North American infants [28]. This high rate of detection decreases

during convalescence [16]. HHV-6 genomic sequences have been detected in PBMC and throat washings of only 9% and 3% of seropositive healthy adult individuals, respectively [15, 31]. Similarly, HHV-6 DNA was found by PCR in saliva from only 2 of 25 (8%) infected people [29]. The sensitivity of PCR for detecting HHV-6 DNA in these studies was as low as 10 genomes. In the present study, despite the high sensitivity of nested PCR, the incidence of HHV-6 carriage in leukocytes of healthy adults was low. Our data further indicate that as few as 1 in 10^5 PBMC may be infected with HHV-6.

The genomes of HHV-6 strain GS [30] and an isolate from a patient with AIDS from Zaire (termed strain Z29) [21] exhibit restriction fragment-length polymorphism [13]. Based on these differences, as well as on growth properties (strain GS grows in HSB-2 cells, strain Z29 in J-Jhan or Molt-3 cells) and antigenic domains, HHV- 6 isolates have been classified into two groups: variant A (prototype strain GS) and variant B (prototype strain Z29) [1, 2]. Similarly, by restriction endonuclease analysis and cross- hybridization with cloned DNA fragments, Aubin et al. [4] found that HHV-6 strains can be placed into at least two groups based on specific point mutations. Like strain Z29, viral isolates from patients with roseola show a variant B profile [8]; additional minor differences in their electrophoretic patterns suggest two subtypes within variant B. On the other hand, the U1102 strain, isolated from a patient with lymphadenopathy and AIDS in Uganda, closely resembles the GS strain, and thus, it can be classified as variant A [32]. Our observations are consistent with these findings. The B38 strain amplified from leukocytes of a healthy adult from Bratislava and the R-147 strain isolated from an American patient with a roseola-like illness, both variant B isolates, differed by only 0.5% (3 of 592 nucleotides), while these strains differed from the variant A GS strain by more than 5% (32 to 34 base substitutions).

Quite unexpectedly, neither the HHV-6 variant A strain GS nor the two variant B strains (R-147 and B38) that we sequenced had a deoxyadenosine at base position 1251, when compared to the published sequence of strain GS clone pZVH14 [14]. This result was repeatedly confirmed by sequencing different templates of the GS strain, suggesting a possible error in the originally reported sequence of HHV-6 strain GS. Although this discrepancy did not affect the large tegument protein gene, it altered the predicted amino acid sequences of two putative proteins coded by ORF 1 and ORF 2 located upstream from this gene. Thus, rather than ending at base 1257, ORF 1 extends to base 1991 and encodes a much larger protein of approximately 40 kilodaltons. The functional significance of this protein needs clarification.

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Authors' address: Dr. R. Yanagihara, National Institutes of Health, Bldg. 36, Rm. 5B21, Bethesda, MD 20892, U.S.A.

Received May 10, 1993