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Human Herpesvirus 6 DNA in Peripheral Blood Cells and Saliva from Immunocompetent Individuals

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Human herpesvirus 6 (HHV-6) genome equivalents were quantitated in peripheral blood mononuclear cells (PBMCs) and saliva from 20 healthy individuals by the polymerase chain reaction (PCR). Nineteen of 20 subjects (95%) harbored HHV-6 DNA: 18 (90%) had HHV-6 in their PBMCs and 18 had HHV-6 in their saliva. Quantitative PCR revealed HHV-6 DNA levels ranging from negative to 4,000 HHV-6 genome equivalents per 10^6 PBMCs and from negative to 200,000 HHV-6 genome equivalents per ml of saliva. Longitudinal saliva samples from 15 HHV-6-seropositive subjects revealed salivary HHV-6 DNA persistence in 13 subjects. HHV-6 antibodies were detected in 17 of 19 subjects, with titers ranging from 1:400 to 1:51,200 (geometric mean titer, 1:2,500). Antibody titers did not correlate with HHV-6 DNA levels in PBMCs or saliva (P = 0.27 and P = 0.44, respectively). One subject with persistent HHV-6 DNA lacked detectable HHV-6 antibodies. The high prevalence of HHV-6 DNA in PBMCs and saliva supports the concept that HHV-6 exists at these sites in normal individuals.

Human herpesvirus 6 (HHV-6) infection usually occurs before 2 years of age (41), resulting in seroprevalence rates ranging from 52% (33) to over 90% (27). Accumulating evidence suggests that HHV-6 may establish a life-long latent and/or persistent infection in normal people. Some investigators have suggested that HHV-6 persists in salivary secretions, although viral isolation rates from saliva have ranged from 0% (41) to 100% (20, 26). HHV-6 has also been isolated from the peripheral blood mononuclear cells (PBMCs) of immunosuppressed individuals (32), but rarely from the PBMCs of normal subjects (26). A study of healthy adults detected HHV-6 DNA in 63% of saliva samples and 49% of PBMC specimens by the polymerase chain reaction (PCR) (19). Furthermore, a recent report on HHV-7 suggested that previous reports of HHV-6 in saliva may actually represent misidentification of HHV-7 (38).

The correlation of HHV-6 antibody titers with HHV-6 infection has also been a source of controversy. Clinical studies have used HHV-6 antibody titers to identify subgroups of patients with "active" HHV-6 infection on the assumption that anti-HHV-6 immunoglobulin G (IgG) or IgM titers correlated with viral activity (2, 21, 22, 34). However, more detailed studies on other herpesviruses have shown variable antibody responses during reactivation of herpes simplex virus (11), cytomegalovirus (31, 37), and Epstein-Barr virus (30). In addition, anti-HHV-6 IgM can be detected long after primary infection without culture or PCR evidence for concurrent HHV-6 reactivation (16).

In the study described here, we quantified HHV-6 DNA in the PBMCs and saliva of healthy subjects using a highly sensitive and specific HHV-6 PCR method. The HHV-6 DNA concentrations were compared with HHV-6 antibody titers to better define the relationship between these parameters.

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MATERIALS AND METHODS

Subjects and specimens. Twenty healthy subjects (19 adults [8 males, 11 females] and one 9-year-old male) with a median age of 30 years (range, 9 to 55 years) were included in the present study. In addition, 10 umbilical cord blood specimens from normal newborn infants served as HHV-6-negative controls.

Each subject contributed blood and saliva, and 19 of 20 subjects donated a second saliva sample approximately 7 months after donating the first one (Table 1). Subjects 1, 2, and 5 also contributed additional blood and saliva specimens, as described below. Blood (30 ml) was drawn into a syringe coated with 300 U of preservative-free heparin. PBMCs and plasma were prepared by using Lymphocyte Separation Medium (Organon-Teknika, Durham, N.C.), according to the manufacturer's instructions. Purified PBMCs were washed two times with phosphate-buffered saline, and the cell pellet and plasma were stored separately at −20°C. Saliva was collected by asking the subjects to spit into a 100-ml sterile cup, and the saliva samples were stored at −20°C. Whole saliva (cells and fluid) was used for DNA extraction. For the first saliva collection, the specimen volume was not determined, and as a result, quantitation of HHV-6 DNA in these first specimens was not done. In the second saliva collection, 0.4 ml of saliva was used for the DNA extractions, providing the basis for HHV-6 DNA quantitation in saliva (HHV-6 genomes normalized per milliliter of saliva).

All specimens were processed in a pre-PCR room by using reagents dedicated for pre-PCR work. All operators wore isolation gowns, surgical hats, shoe covers, surgical masks, and latex gloves while in the room. Disposable cotton-plugged serologic pipettes or disposable positive-displacement pipette tips (Microman; Gilson Medical Electronics, Sarcelles, France) were used for all liquid transfers.

DNA from thawed PBMCs representing 7.5 ml of blood or from 0.4 ml of saliva was prepared by (i) overnight digestion at 50°C with 0.1 mg of proteinase K per ml, 0.5% sodium dodecyl sulfate, 25 mM EDTA, 100 mM NaCl, and 10 mM

TABLE 1. HHV-6 DNA levels and serologic titers from 20 healthy individuals

Subject no.	Sex[a]	Age (yr)	PBMCs			Saliva			HHV-6 serology[f]
			HHV-6/μl of DNA[a]	Beta-globin/μl of DNA[b]	HHV-6/10^6 cells[c]	First specimen	Second specimen (HHV-6/ml)[d]	Interval (mo)[e]	
1	F	23	Negative	500,000	0	Negative	Negative	7	Negative
2	F	51	Negative	1,000,000	0	Positive	200,000	7	Negative
3	M	26	10	5,000,000	4	Positive	5,000	7	800
4	F	25	0.5	100,000	10	Positive	5,000	7	1,600
5	M	37	0.5	100,000	10	Positive	2,500	7	400
6	F	30	5	500,000	20	Positive	25,000	8	1,600
7	M	28	0.4	10,000	80	Positive	20,000	7	1,600
8	F	33	50	1,000,000	100	Positive	25,000	7	3,200
9	F	21	5	50,000	200	Positive	50,000	6	51,200
10	F	29	250	2,500,000	200	Positive	200,000	7	1,600
11	F	49	10	500,000	80	Positive	10,000	8	12,800
12	M	38	10	100,000	200	Positive	500	5	6,400
13	M	23	10	50,000	400	Positive	2,500	8	800
14	M	38	10	50,000	400	Positive	Inhibited	7	3,200
15	F	30	100	100,000	2,000	Negative	Negative	8	6,400
16	M	9	50	100,000	1,000	Positive	200,000	7	3,200
17	M	35	50	100,000	1,000	Positive	Inhibited	7	800
18	M	39	50	100,000	1,000	Negative	2,500	7	NE[g]
19	F	34	50	50,000	2,000	Positive	Not done	NA[h]	1,600
20	F	28	10	5,000	4,000	Positive	25,000	7	3,200

[a] F, female; M, male.
[b] Number of HHV-6 or cellular DNA targets per microliter of purified DNA.
[c] Number of HHV-6 genomes per 10^6 PBMCs.
[d] Number of HHV-6 genomes per milliliter of saliva.
[e] Months between saliva collections.
[f] Reciprocal of highest reactive dilution.
[g] NE, not evaluable; a high nonspecific enzyme immunoassay reactivity obscured the potential HHV-6-specific signal.
[h] NA, not available.

Tris (pH 8.0), (ii) extraction with equal volumes of phenol, phenol-chloroform, and chloroform, and (iii) precipitation with sodium acetate (0.25 M), glycogen (100 μg/ml), and 2 volumes of ethanol. The DNA was resuspended in 200 μl of 10 mM Tris (pH 8.0). Each amplification reaction contained 10 μl of the resuspended DNA.

PCR. Two HHV-6 primer pairs (primers 101R and 5R) and one beta-globin primer pair were used (Table 2). Plasmid pZ101 (6.8-kb insert; kindly provided by Phil Pellet) was cloned from HHV-6 strain Z-29. The plasmid insert is homologous to the cytomegalovirus region containing genes UL-1 to UL-9. The 101R primers were derived from plasmid pZ101, which was partially sequenced in our laboratory by using Sequenase (United States Biochemical Corp., Cleveland, Ohio) according to the manufacturer's instructions. The 134-bp sequence of the PZ101R PCR product (primers and probe underlined) is GTATCCCGAC GGCAGAGGTT GCGGTTTGGC CTTCGGTACA GTGAAAGAGT GCTC GATTCC AGAATCTCAA AGAATATATT TAAAGAAC TG GAAAGAAGCA TCCGAATTTT TCCCCCCTGA CAA CGGCTGT TTAA. The 5R primers (19) were derived from the pHD5α subclone of HHV-6 strain U-1102 (Table 2). The 223-bp product represents part of the 13R open reading frame, which has not been associated with a specific function (25). The 5R probe was designed in our laboratory by using the published HHV-6 sequence (25). The human beta-globin gene primers (12) and probe (14) amplify a highly conserved 294-bp product (Table 2).

Amplification reactions were set up in 0.65-ml microcentrifuge tubes containing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.4), 1 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus), 200 μM (each) dGTP, dATP, dTTP,

TABLE 2. Oligonucleotides used for DNA amplification

Amplimer	Oligonucleotide	Function	Sequence (5'→3')
5R (223 bp)	H6-6	Primer	AAGCTTGCACAATGCCAAAAAACAG
	H6-7	Primer	CTCGAGTATGCCGAGACCCCTAATC
	5R Probe	Probe	CATTTCCTCACTGTCAAAGAAACGACGGCG
101R (134 bp)	101(R)A	Primer	GTATCCCGACGGCAGAGGTT
	101(R)C	Primer	TTAAACAGCCGTTGTCAGGG
	101(R)B	Probe	GAGATTCTGGAATCGAGCAC
Beta-globin (294 bp)	β-1	Primer[a]	GGCCTGGGCATAAAAGTCA
	β-2	Primer	AATAGACCAATAGGCAGAG
	β-probe	Probe	GCAGACTTCTCCTCAGGAG

[a] The previously published sequence (12) differs at position 3.

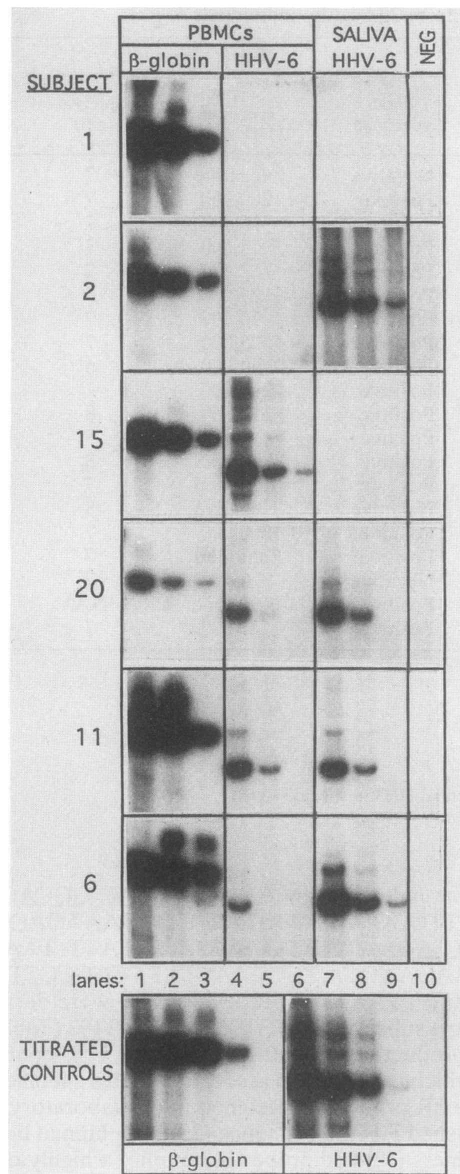

FIG. 1. Representative autoradiograms showing PCR products from titrations of purified specimen DNA. Lanes 1 to 3, 10^{-3}, 10^{-4}, and 10^{-5} dilutions, respectively, of PBMC DNA amplified with beta-globin primers; lanes 4 to 6, undiluted and 10^{-1} and 10^{-2} dilutions of the PBMC DNAs, respectively, amplified with 5R HHV-6 primers; lanes 7 to 9, undiluted and 10^{-1} and 10^{-2} dilutions of salivary DNA amplified with 5R HHV-6 primers; lane 10, HHV-6 amplification of a coprocessed negative control. Titrated controls show 10,000, 1,000, 100, 10, and 0 genomes of human DNA (lanes 1 to 5, respectively) and HHV-6 DNA (lanes 6 to 10, respectively).

and dCTP (Pharmacia), 0.83 μM (2.5×10^{13} molecules) (each) high-pressure liquid chromatography-purified primer (Midland Certified Reagents, Midland, Tex.), and 10 μl of purified DNA in a final volume of 50 μl. HHV-6 and beta-globin amplifications were performed with various dilutions of the purified DNA in order to achieve quantitative determinations in the linear range of the assay (Fig. 1). Other dilutions were amplified in successive runs if these did not adequately define the number of genomes. Thermal cycling was done in a programmable forced-air oven (Biosycler; Bios Corporation, New Haven, Conn.) with the following temperature settings: 94°C for 6 min; 30 cycles of 55°C for 1 min, 72°C for 1 min, and 94°C for 1 min; and a final cycle of 72°C for 10 min.

The PCR products were detected by liquid hybridization at 14°C below the melting temperature (13). Seven microliters of products from the 50-μl reaction mixture were mixed to final concentrations of 50 mM Tris (pH 8.0), 1.2 M NaCl, 100 μM deoxynucleoside triphosphates, 60% formamide (40% formamide used for beta-globin hybridization), and 10^6 cpm of ^{32}P-labeled probe per reaction in a total volume of 25 μl. The mixtures were hybridized by heating to 97°C for 5 min and cooled to room temperature over 15 min. Ten microliters of each hybridized reaction mixture was electrophoresed in a 6% acrylamide (19:1 acrylamide:bisacrylamide; Bio-Rad) gel (Hoefer Scientific Mighty Small II) with TBE (0.089 M Tris base, 0.089 M boric acid, 2 mM EDTA). The gels were dried and autoradiographed with Kodak X-Omat XR-5 film at room temperature for 1 to 18 h. We were able to routinely detect 10 HHV-6 genomes or 10 human genome equivalents per reaction.

Negative controls were derived by matching each specimen with an aliquot of uninfected HSB-2 cells (ATCC CCRF-HSB-2) processed in parallel from proteinase K digestion through autoradiography. In addition, each run included up to three controls consisting of PCR reagents without added specimen DNA. Contamination of stock ingredients did not occur during the present study, and none of the negative controls were PCR positive. An additional 10 negative controls consisted of purified DNA samples from 10 cord blood specimens.

Every sample was checked for endogenous inhibition by performing a separate PCR amplification that contained an aliquot of the sample and 100 copies of purified HHV-6 DNA. Negative PCR results were accepted only when the corresponding internal positive control for that sample was amplifiable. PCR inhibition occurred in 10 of 50 saliva specimens and 4 of 26 PBMC specimens. The inhibition was corrected in most cases by repurifying and reamplifying by the methods described above using an aliquot of the original specimen; two saliva specimens remained inhibited (Table 1).

All positive HHV-6 PCR results were confirmed with both HHV-6 primer sets, which amplify different regions of the viral genome. Conversely, all negative HHV-6 PCR results were negative with both primer sets.

Both of the HHV-6 primer sets, 101R and 5R, were characterized with DNA from 20 HHV-6 isolates propagated in vitro: 10 isolates from PBMCs of Japanese children with roseola (a gift from Koichi Yamanishi) (24, 40), 4 isolates from South Africans infected with human immunodeficiency virus or human T-cell leukemia virus type 1 (a gift from M. L. Becker) (6), 1 isolate (HHV-6_{SF}) from a human immunodeficiency virus-infected adult (a gift from Evelyne Lennette) (26), 1 isolate (U-1102) from a human immunodeficiency virus-infected Ugandan (15) and 1 isolate (ANZ) from a Nigerian with multiple immune deficiencies (gifts from Ian Teo); 1 isolate (SIE) from an African with AIDS (a gift from Helene Collandre) (3), 1 isolate from the saliva of an Australian with AIDS (a gift from G. B. Harnett) (29), and 1 isolate (Z-29) from an African with AIDS (a gift from Phil Pellet) (8). The HHV-6 primer sets reacted with all of the cultured HHV-6 isolates.

Amplification of other herpesvirus DNAs, including 2 herpes simplex virus strains, 10 cytomegalovirus strains, 1

Epstein-Barr virus strain, 1 varicella-zoster virus strain, and 2 HHV-7 strains, by our HHV-6 PCR method did not result in detectable products. Furthermore, HHV-7 amplification with HHV-7-specific primers demonstrated HHV-7 DNA in both HHV-7 samples (data not shown). The HHV-7 DNA from strains RK (18) and JI (7) were kindly provided by Niza Frenkel and Zwi Berneman, respectively.

The intensities of the autoradiographic signals from the PCR products corresponded to the number of HHV-6 and human genomes in each sample (1). Standard HHV-6 DNA dilution curves (purified DNA from lysates of HHV-6 strain U-1102) and human DNA dilution curves (purified human placental DNA) consisting of 10, 100, 1,000, and 10,000 preamplification copies were included in every run (Fig. 1). Serial 10-fold dilutions of each specimen were independently amplified to generate a titration curve as described above. The signal intensity from each dilution representing less than 1,000 template molecules was compared with the standard curve from each dilution run by visual inspection. For quantitation, HHV-6 was amplified with the 5R primer set only.

To compensate for variation in the sample size and recovery of DNA after purification, the estimate of HHV-6 genomes in PBMCs was expressed as a ratio of the number of HHV-6 genomes per million cells (the number of HHV-6 genomes per 10^6 PBMCs). Although the amplifications contained different absolute amounts of cellular DNA, they were normalized to the number of HHV-6 genomes per 10^6 PBMCs for ease of comparison among subjects. PCR results for saliva are expressed as the number of HHV-6 genomes per milliliter of saliva because we found that salivary cellular DNA levels did not correlate with HHV-6 genome levels (unpublished data).

HHV-6 serology. An enzyme immunoassay was used to detect HHV-6 plasma antibodies in a microtiter system with immobilized detergent extracts of HHV-6-infected (strain U-1102) or mock-infected HSB-2 cells (9). Bound antibodies were detected with horseradish peroxidase-conjugated goat anti-human IgG (gamma chain; Boehringer Mannheim Biochemicals, Indianapolis, Ind.), and ABTS substrate (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was added for 20 min. The endpoint titer was taken as the reciprocal of the highest dilution giving an absolute absorbance value of greater than 0.5 and a relative absorbance value at least twofold greater against the viral antigen than against the mock-infected cell antigen. Specimens that did not react at the lowest dilution (1:100) were considered negative for HHV-6 antibodies (5).

Statistics. Correlation coefficients were calculated with general linear regression by using Systat statistical software (36), version 5.0, on a Macintosh IIcx personal computer. Calculations for HHV-6 quantitative PCR data and serologic data were performed on $\log_{10}$ values.

RESULTS

HHV-6 DNA in PBMCs. Eighteen of the 20 subjects had HHV-6 DNA in their PBMCs, as evidenced by reactivity with both HHV-6 primer sets (Table 1; Fig. 1). HHV-6 DNA levels ranged from 0 to 4,000 HHV-6 genomes per 10^6 PBMCs, with a median of 200 HHV-6 genomes per 10^6 PBMCs. The levels of HHV-6 genomes per microliter of purified DNA (Table 1) were derived by adding 10 μl of purified DNA to each amplification reaction. Therefore, 0.5 HHV-6 genomes per μl of DNA (e.g., subject 4, Table 1) was detected as 5 HHV-6 genomes in 10 μl of undiluted, purified DNA. The absolute levels of cellular DNA varied among specimens because of differences in the recoveries of PBMCs and purified DNAs. The ratio of HHV-6 copies per 10^6 cellular genomes normalized these variations, enabling comparisons among specimens. Equivalent numbers of PBMCs from the 10 umbilical cord blood specimens yielded negative HHV-6 PCR results.

HHV-6 DNA in saliva. Concentrations of salivary HHV-6 DNA ranged from 0 to 200,000 HHV-6 genomes per ml of saliva (median, 5,000 genomes per ml of saliva; Fig. 1 and Table 1). Seventeen of the 20 subjects provided two saliva specimens separated by a median of 7 months (range, 5 to 8 months). Saliva pairs were not available for the remaining three subjects because the second specimen from two subjects consistently inhibited amplification and one subject was not available for the second saliva collection (Table 1). Of the 17 saliva pairs that were successfully evaluated by HHV-6 PCR, 14 (82%) contained HHV-6 DNA in both specimens, suggesting the continuous presence of salivary HHV-6 in those subjects. One subject (subject 18) had intermittent salivary HHV-6 and two others (subjects 15 and 1) were consistently negative for HHV-6 in saliva by PCR. Subject 1 was probably not infected with HHV-6, as evidenced by negative HHV-6 serology and negative HHV-6 PCR of PBMCs. Although subject 15 lacked detectable salivary HHV-6 DNA, her HHV-6 serology (1:6,400) and HHV-6 PCR of her PBMCs (2,000 HHV-6 genomes per 10^6 cells) clearly demonstrated HHV-6 infection.

HHV-6 seroprevalence. HHV-6 antibody titers ranged from <1:100 (negative) to 1:51,200 (Table 1). A comparison of HHV-6 antibody levels versus the HHV-6 DNA levels in PBMCs or versus the HHV-6 DNA levels in saliva did not reveal convincing associations (P = 0.27 and P = 0.44, respectively). The 17 seropositive subjects had a geometric mean HHV-6 antibody titer of 1:2,500. The serology for subject 18 was not evaluable because his plasma produced excessive reactivity in control wells coated with an extract of uninfected cells. Resampling of the two seronegative individuals after 7 months confirmed continued seronegativity in those subjects.

DISCUSSION

All of the 17 HHV-6-seropositive individuals in the present study had HHV-6 DNA in their PBMCs, and none of the 10 umbilical cord blood PBMCs were HHV-6 DNA positive. Other investigators have used PCR to detect HHV-6 DNA in PBMCs from healthy people at prevalences ranging from 49% (19) to 88% (23). Differences in apparent HHV-6 DNA prevalences among studies may be related to the low levels of HHV-6 in normal PBMCs. All of our seropositive subjects had demonstrable HHV-6 DNA when we used more than 25,000 PBMCs per reaction mixture. As with the subjects in a study by Jarrett et al. (23), progressively fewer specimens were PCR positive as fewer PBMCs were used for amplification. Quantitative determinations of HHV-6 DNA in our healthy subjects ranged over 3 orders of magnitude, from 1 HHV-6 genome per 250,000 PBMCs to 1 HHV-6 genome per 250 PBMCs, with a median of 1 HHV-6 genome per 5,000 cells. These data do not address how many HHV-6 genomes may be present in each infected PBMC. Even though PBMC cultures from healthy adults rarely yield HHV-6 (26), our PCR data suggest that HHV-6 genomes are usually present in the PBMCs of healthy, HHV-6-seropositive individuals.

The concentrations of HHV-6 genomes in PBMC and

saliva specimens reported here agree with previous semiquantitative HHV-6 PCR studies of specimens from these sites (19, 23). Considering that HHV-6 PCR is 10 to 10,000 times more sensitive than culture when examining the peripheral blood of patients with roseola (24), it is not surprising that normal individuals with lower levels of HHV-6 DNA in PBMCs are typically culture negative. HHV-6 latency in PBMCs may also contribute to culture negativity.

The 85% prevalence of salivary HHV-6 DNA in the present study reproduces and extends previous reports on the prevalence of HHV-6 in saliva by other investigators (17, 20, 23, 26, 28). HHV-6 DNA was present in the saliva of 16 of 17 HHV-6-seropositive subjects, and the persistence of salivary HHV-6 DNA was demonstrated in 14 HHV-6-seropositive subjects by measuring two independent saliva samples separated by a median of 7 months. This 82% prevalence of HHV-6 DNA in adult saliva provides one explanation for the high frequency of HHV-6 acquisition during early childhood (4).

Our results differ from those of Wyatt and Frenkel (38), who reported a low frequency of HHV-6 isolation but a high frequency of HHV-7 isolation from saliva. They speculated that HHV-6 may have overgrown HHV-7 isolates in previous studies. Our HHV-6 PCR results on uncultivated saliva eliminate the possibility of cross-contamination during culture. Also, our stringent procedures for preventing DNA contamination and the extensive use of negative controls in the present study make it very unlikely that the positive HHV-6 PCR results can be explained by DNA contamination. Wyatt and Frenkel (38) also proposed that HHV-7 may have been misidentified as HHV-6; our HHV-6 PCR specificity showed no cross-reaction with HHV-7 DNA.

Our finding that an HHV-6-seronegative individual harbors HHV-6 DNA was reproduced three times with different serum and saliva specimens collected over a 1-year period. The presence of salivary HHV-6 in a seronegative individual has also been reported by Jarrett et al. (23), and Stewart and Sanderlin (35) discussed the problem of false-negative serologies from HHV-6-infected individuals. This paradoxical seronegativity may occur because the serologic tests are not sensitive enough to detect very low levels of specific antibodies or because some individuals may have antibodies that react poorly with the HHV-6 strain used in the assay (10, 39). Of interest, the HHV-6-seronegative subjects in the present study were cytomegalovirus seropositive, indicating that they were capable of mounting a humoral immune response to at least one member of the herpesvirus family.

In summary, our data suggest that healthy adults commonly have HHV-6 DNA in their PBMCs and saliva. These findings confirm the permanent nature of HHV-6 infection in normal hosts, a characteristic common to all herpesviruses, and suggest that PBMCs and the oropharynx make up two reservoirs of HHV-6 infection. The particularly high levels of viral DNA in saliva from normal individuals support the concept that HHV-6 transmission occurs via an oral route.

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