Detection by Polymerase Chain Reaction Amplification of Human Herpesvirus 6 DNA in Peripheral Blood of Patients with Exanthem Subitum

KAZUHIRO KONDO,[1] YASUHIKO HAYAKAWA,[1] HIROYUKI MORI,[1] SHIGEO SATO,[1] TOSHIO KONDO,[2] KAZUO TAKAHASHI,[3] YOICHI MINAMISHIMA,[4] MICHIAKI TAKAHASHI,[1] AND KOICHI YAMANISHI[1]*

Department of Virology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565,[1] Kondo Clinic, Toyonaka, Osaka,[2] Department of Virology, Kagoshima University School of Medicine, Kagoshima,[3] and Department of Microbiology, Miyazaki Medical School, Miyazaki,[4] Japan

The polymerase chain reaction was used to detect human herpesvirus 6 (HHV-6) DNA in peripheral blood mononuclear cells from patients with exanthem subitum. Amplified products were detected by agarose gel electrophoresis and dot blot hybridization with a cloned DNA probe. No cross-hybridization with DNAs of five other human herpesviruses was observed in this system, and all HHV-6 strains gave positive reactions when the primer pairs were used. Immunoglobulin M antibody appeared about 5 days after the onset of disease, reaching a maximum after about 2 to 3 weeks and then decreasing to less than 1:10 1 month after the onset of disease in almost all patients. Mononuclear cells from seven patients with exanthem subitum all gave positive reactions in this system, and viral DNA was found in samples even during the convalescent phase of the disease. We conclude that this test, using polymerase chain reaction amplification, which takes only 1 to 2 days, is useful for the diagnosis of HHV-6 infection.

A novel human herpesvirus, now named human herpesvirus 6 (HHV-6), has been isolated from patients with lymphocytic disorders independently by several groups of investigators (2, 5, 10, 18, 21). HHV-6 is an enveloped virus (diameter, about 200 nm) with an electron-dense icosahedral core composed of 162 capsomers containing a large double-stranded DNA genome (5, 7, 18). This virus was initially named human B lymphotropic virus, because it infected B lymphocytes (18). However, later it was found mainly to infect and replicate in lymphocytes of the T-cell lineage (1, 11, 12, 20). Preliminary characterization of HHV-6 indicated that it was antigenically and genetically distinct from other human herpesviruses (cytomegalovirus [CMV], herpes simplex virus types 1 and 2 [HSV-1 and HSV-2, respectively], varicella-zoster virus [VZV], and Epstein-Barr virus [EBV] [7, 10, 18]). However, Efstathiou and colleagues (6) have recently reported that the HHV-6 genome shows sequence homology to that of human CMV. Most children have antibodies against this virus by the time they are 2 years old (15), but it is not known whether HHV-6 causes disease in humans. We recently discovered that HHV-6 is a causal agent of exanthem subitum (22). An immunofluorescence test is usually used for the diagnosis of HHV-6 infection in serum, but this method takes at least 1 week, because serum should be collected during the convalescent phase of the disease.

A novel technique, the polymerase chain reaction (PCR), was recently developed for the amplification of DNA or RNA in vitro, and this technique can be used for many purposes, such as analysis of inherited disorders and the detection of somatic diseases (14, 16, 17). One of the most important applications of this method, which gives results in a short time, has been for the detection of infectious agents that are present in small numbers in clinical samples (4, 19). Recently, Buchbinder and associates (3) reported that PCR

could be used to detect HHV-6 DNA in peripheral blood cells of patients with the acquired immune deficiency syndrome (AIDS) and various lymphoproliferative disorders. We report here the use of PCR for detecting HHV-6 in peripheral blood mononuclear cells of patients with exanthem subitum.

MATERIALS AND METHODS

Cells and viruses. Umbilical cord blood mononuclear cells were cultured at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum, L-glutamine, penicillin, kanamycin, 0.1 U of recombinant human interleukin-2 (kindly supplied by Takeda Chemical Industries) per ml, and 5 μg of phytohemagglutinin (Honen Oil Co.) per ml. Umbilical cord blood lymphocytes were infected with HHV-6 (Hashimoto strain), which was isolated in our laboratory from a patient with exanthem subitum, and were cultured as described previously (22). When cytopathic effects were observed, approximately 1 to 2 weeks after infection, the cells were collected by centrifugation and infected cells were disrupted by sonication. The supernatant that was obtained by centrifugation (K-80H; Kubota) at 3,000 rpm at 4°C was stored at −80°C until use. The Kawaguchi strain of VZV, the AD 169 strain of human CMV, and the Seibert and UW268 strains of HSV-1 and HSV-2, respectively, were used for these studies. Human embryonic fibroblast cells, which were passaged 10 to 15 times, were cultured in a mixture of Eagle minimal essential medium and medium 199 containing 10% fetal bovine serum for growth, and 3% fetal bovine serum for maintenance. Human embryonic fibroblast cells were infected with each virus and cultured for certain periods of time. When infected cells showed a cytopathic effect, they were harvested and their DNAs were extracted as described below. A cell line producing EBV (B95-8 cell line) was also propagated, and its DNA was extracted in the same fashion. The titer of HHV-6 was determined on MT-4 cells (13) and

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* Corresponding author.

expressed as the 50% tissue culture infectious dose (TCID$_{50}$).

Collection of specimens from patients and purification of DNA from mononuclear cells. Blood samples (approximately 1 ml) from patients with exanthem subitum were collected during the acute and convalescent phases of the disease in heparinized tubes, and mononuclear cells were separated by Ficoll-Paque (Pharmacia LKB) gradient centrifugation. The cells were incubated for 6 h at 37°C in 500 μl of NET buffer (150 mM NaCl, 15 mM Tris hydrochloride, 1 mM EDTA) with 0.1% sodium dodecyl sulfate–1.0 mg of proteinase K (Boehringer Mannheim Biochemicals) per ml. Then, the mixture was extracted five times with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). The DNA was precipitated by adding ethanol, washed with 80% ethanol, and suspended in distilled water. The DNA concentration was determined by measuring the A_{260}/A_{280} ratio. Samples of about 100 ng of DNA were used for the PCR.

Oligomer synthesis. The sequences used as primers were parts of the SalI fragment (approximately 6 kilobase pairs) derived from the Hashimoto strain with the sequences (5' to 3') 5'-GTGTTTCCATTGTACTGAAACCGGT-3' and 5'-TAAACATCAATGCGTTGCATACAGT-3'. These oligomers were synthesized in a DNA synthesizer (Applied Biosystems). The PCR amplification products were 776 base pairs in length.

PCR. DNA was amplified in a total volume of 100 μl of a reaction mixture consisting of 50 mM KCl; 10 mM Tris hydrochloride (pH 8.3); 1.5 mM MgCl$_2$; 0.01% (wt/vol) gelatin; 200 μM each dATP, dGTP, dTTP, and dCTP; and 2.5 U of Taq polymerase. The sample was first denatured at 94°C for 10 min and then subjected to 30 amplification cycles. Annealing was performed at 62°C for 2 min, extension was performed at 72°C for 5 min, and denaturation was performed at 90°C for 1 min. The primers were used at a concentration of 1.0 μM each. The amplification reaction was carried out in a DNA thermal cycler (Perkin-Elmer/Cetus). The primers of λ phage DNA (Perkin-Elmer/Cetus) and β-globin (16) were also used as positive controls in samples in which the amplification of viral DNA was not observed. The conditions of PCR were the same as those described above, except that the annealing temperature was 55°C.

Detection of amplified product. The amplified product was detected by direct gel analysis and a dot blot hybridization assay by using cloned DNA probe (part of the SalI fragment of DNA described above).

For direct gel analysis, 10 μl of the reaction mixture was subjected to electrophoresis on a 1.5% agarose gel, and DNA was located by UV fluorescence after staining with ethidium bromide. Molecular weight markers were included in each gel.

A band of 776 base pairs was seen when samples were amplified. For dot blot analysis, 10 μl of the reaction mixture was mixed with 190 μl of 5× SSPE (0.75 M NaCl, 50 mM NaH$_2$PO [pH 7.4], and 5 mM disodium EDTA) and was then denatured at 95°C for 5 min. The DNA was promptly applied to a nylon filter membrane (Hybond-N$^+$; Amersham Corp.) by vacuum filtration; the filter was then denatured in a 1.5 M NaCl–0.5 N NaOH solution and neutralized with 1.5 M NaCl–0.5 M Tris hydrochloride (pH 7.2), and the DNA was fixed by treatment with 0.4 N NaOH for 20 min (alkali fixation). The DNA samples were then hybridized with a homologous ^{32}P-labeled cloned probe (2.0 × 10^6 cpm/ml) by using a rapid hybridization system (Multiprime; Amersham) for 1 h. Then, the filter was washed twice with 2× SSPE–

0.1% sodium dodecyl sulfate for 10 min each time at room temperature, once with 1× SSPE–0.1% sodium dodecyl sulfate for 15 min at 65°C, and then twice in 0.1× SSPE–0.1% sodium dodecyl sulfate for 20 min each time at 65°C. Bound probe was detected by autoradiography at −70°C for 8 h with intensifying screens. Specific amplification was confirmed by Southern blotting, when the dot blot signal was weak.

Antibody detection. The Hashimoto strain was inoculated onto HPB-ALL cells, which were derived from an acute lymphatic leukemia cell line and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. When some cells showed a cytopathic effect with the characteristic balloonlike syncytia, the cells were mounted onto spotted glass slides and fixed in acetone at −20°C for 10 min. Uninfected cells were also prepared on spotted glass slides in the same way. In the indirect immunofluorescence test, approximately 30 to 50% of the infected cells stained with antibody-positive human serum, whereas uninfected cells were not stained. Therefore, these HHV-6 infected HPB-ALL cells were used as standard cells for titration of antibody in serum, as described previously (20).

Serially diluted sera were mounted onto spotted slides and incubated for 1 h at 37°C, and the slides were then washed twice with phosphate-buffered saline. For the detection of immunoglobolin M (IgM), antibody in serum was treated with Streptococcus pyogenes as described elsewhere (8), and the supernatant that was obtained by centrifugation (13N rotor; Tomy) at 5,000 rpm was used for the test. Fluorescein isothiocyanate-conjugated antibody to human IgG or IgM (Cappel Laboratories) was used in the indirect immunofluorescence antibody test. The slides were treated with fluorescein isothiocyanate-conjugated goat serum and incubated for another 30 min at 37°C. They were then washed with phosphate-buffered saline and observed with a fluorescence microscope after the addition of buffered glycerin. Titers of antibodies are expressed as the highest serum dilution yielding detectable immunofluorescence.

RESULTS

Amplification of different HHV-6 strains. The ability of our primers to detect eight different clinical isolates (six samples isolated from patients with exanthem subitum in Japan, one sample isolated from a patient in Japan who had a renal transplant, and one sample isolated from a patient with AIDS in the United States) was evaluated. Samples of 0.05 to 0.2 μg of DNA from infected cells were used as templates in the PCR. All strains gave a positive band on direct gel electrophoresis (Fig. 1, lanes 1 through 8).

Specificity of PCR. The specificity of the PCR and the primers for HHV-6 were evaluated by using five other human herpesviruses, HSV-1, HSV-2, VZV, CMV, and EBV. DNAs from cells infected with these viruses were extracted and purified as described above, and samples of 0.01 to 1 μg of DNA were used as templates in the PCR. No amplification was detected by direct gel electrophoresis (Fig. 1) or Southern blot hybridization (data not shown). Because samples might have been contaminated with inhibitors, DNA samples of approximately 1 μg containing 1 ng of phage DNA were used as templates, and the PCR was performed in accordance with the instructions provided with the GeneAmp DNA amplification kit (Perkin-Elmer/Cetus). A positive band was observed on direct gel electrophoresis (Fig. 1, lane A). Next, β-globin DNA in all samples except EBV were also amplified by using primers, as described by
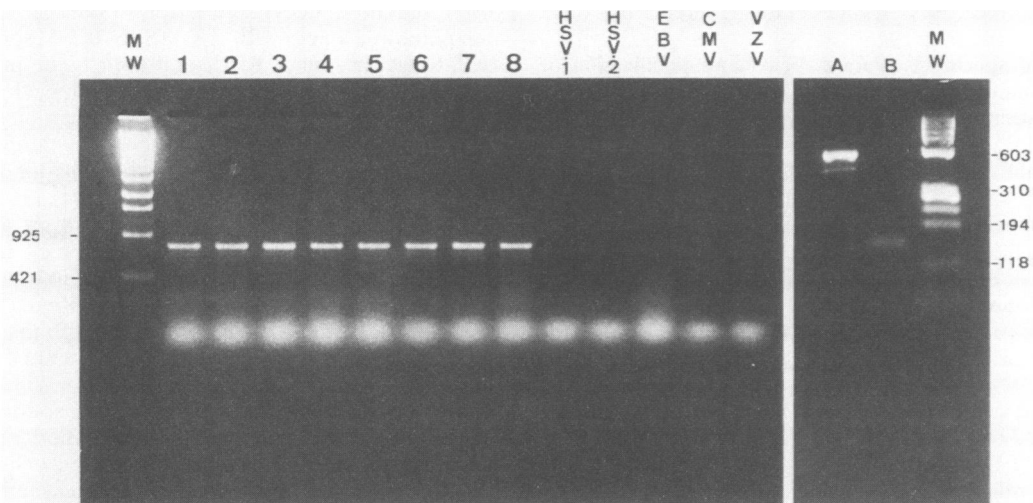
FIG. 1. Specificity of PCR. Virus strains in lanes 1 through 6 were isolated from patients with exanthem subitum in Japan. The virus in lane 7 was isolated from a patient with AIDS in the United States. The virus in lane 8 was isolated from a patient who underwent a renal transplant. λ phage DNA and its primers and β-globin primers were used in the reaction as positive controls. Lanes A and B, Positive patterns when a sample of HSV-1-infected cells with λ phage DNA was used and when λ phage and β-globin were used as the primers, respectively. MW, Molecular weight.

Saiki et al. (16). A band with a molecular weight of 150 was observed (Fig. 1, lane B).

Sensitivity of PCR. For determination of the sensitivity of PCR in this system, a viral sample with an infectivity titer of $10^{5.0}$ $TCID_{50}$/ml was diluted to 10^{-9} and assayed by PCR. A sample of 10 μl of virus equivalent to 10^{-1} $TCID_{50}$ was detectable by direct gel analysis (Fig. 2), while a sample equivalent to 10^{-4} $TCID_{50}$ could be detected by dot blot hybridization (Fig. 3).

Detection of HHV-6 DNA in peripheral blood mononuclear cells of patients with exanthem subitum. Blood samples were collected from eight patients diagnosed clinically as having exanthem subitum, and HHV-6 DNA in their mononuclear cells was examined by PCR. Mononuclear cells from umbilical cord blood were used as controls. Seven samples were obtained from different patients during the acute phase (four samples during the febrile period and three samples during
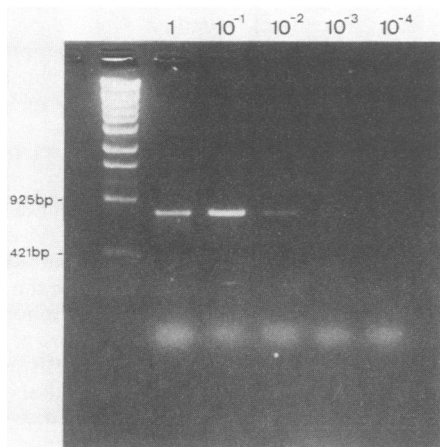
the exanthem period) and one sample was obtained 1 week after disappearance of the rash. In addition, seven samples were obtained from the patients during the convalescent phase (approximately 1 month after recovery from exanthem subitum). The DNA was extracted from mononuclear cells of these samples. On direct gel electrophoresis, two specimens (specimens 2 and 7) gave a positive band, but the other specimens did not (data not shown). By dot blot analysis, HHV-6 DNA was detected in seven of eight samples (Fig. 4), but is was not detected in sample 8 (Fig. 4). When β-globin primers were used in this sample as a positive control as described above, a positive band was detected by direct gel electrophoresis (data not shown). The amount of DNA appeared to decrease during the convalescent phase of the disease. No HHV-6 DNA was detected in samples from umbilical cord blood cells or blood cells from healthy adults (age, 25 to 47 years) with antibody against HHV-6 by the immunofluorescence test (data not shown).

Antibody response to HHV-6. Seven paired serum samples (acute- and convalescent-phases samples 1 through 6 and 8) and one serum specimen obtained from a patient in the acute phase of the disease (sample from patient 7) were tested for antibody to HHV-6 by the immunofluorescence technique (Table 1). No antibody was detected (a titer of less than 1:10) in sera obtained from patients in the febrile and exanthem phases of disease (samples from patients 1 through 5, 7, and 8). The antibody titer of the sample from patient 6 was 1:160, because the sample was obtained 1 week after disappearance of the rash. The antibody titers of six paired serum samples showed that the antibody titer became positive in the con-

FIG. 2. Analysis of virus DNA by PCR and direct gel electrophoresis. The diluted samples of virus (equivalent to 1 to 10^{-4} $TCID_{50}$s) were amplified by PCR, and 10-μl volumes of the reaction mixtures were subjected to electrophoresis. bp, Base pairs.
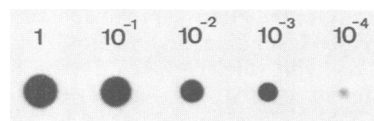

FIG. 3. Analysis of DNA by dot blot hybridization. The diluted samples were amplified by PCR. Dot blot analysis was performed as described in the text.
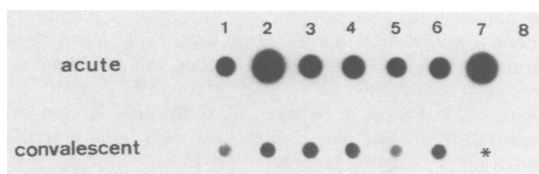
FIG. 4. Detection of HHV-6 DNA in peripheral blood mononuclear cells of patients with exanthem subitum. Blood samples were collected from patients during the febrile phase (patients 1, 2, 7, and 8) or the exanthem phase (patients 3, 4, and 5). A sample was obtained from patient 6 1 week after the disappearance of a rash. *, No sample was collected.

TABLE 1. Antibody response to HHV-6

Patient no.[a]	Antibody titer by immunofluorescence for samples obtained from patients in the:	
	Acute phase	Convalescent phase
1	<10	1,280
2	<10	2,560
3	<10	1,280
4	<10	1,280
5	<10	2,560
6	160[b]	2,560[b]
7	<10	ND[c]
8	<10	<10

[a] Patients 1, 2, and 7 were in the febrile and convalescent phases; patients 3, 4, and 5 were in the exanthem and convalescent phases.

[b] Samples were obtained 12 days and 1 month after the onset of the illness for patients in the acute and convalescent phases, respectively.

[c] ND, Not determined.

valescent phase, but the antibody titer of the serum from patient 8 was still <1:10 during the convalescent phase (Table 1). Twenty-six serum samples, including those used for PCR, obtained from patients with exanthem subitum during the acute and convalescent phases were assayed for IgM antibody. As shown in Fig. 5, IgM antibodies were not detected in the sera during the acute phase, which lasted until day 4 of onset of disease, but it was detected on day 7 and persisted for 3 weeks, but it was not detectable in most sera 1 month after the onset of disease.

DISCUSSION

In this study we found that PCR could be used for the detection of HHV-6. The sequence of HHV-6 DNA is not yet available, so we used primers corresponding to the partial sequence of a cloned DNA fragment. There are reports that there is no cross-reaction at the DNA level between HHV-6 DNA and the DNAs of five other human herpesviruses (7, 10), although cross-hybridization of a HHV-6 DNA fragment and a CMV DNA fragment has been observed (6). As shown in Fig. 1, the primers used in our experiment were specific for HHV-6, showing no detectable amplification of five other human herpesviruses. PCR amplified DNA sequences of six other HHV-6 strains isolated from patients with exanthem subitum in different regions of Japan, from a patient who had a renal transplant, and from a patient with AIDS in the United States. Our rate of virus

isolation from patients with clinically diagnosed exanthem subitum during the acute phase of infection was approximately 67% (data not shown). As shown in Fig. 3, PCR detected HHV-6 DNA equivalent to 10^{-1} $TCID_{50}$ by gel electrophoresis and equivalent to 10^{-4} $TCID_{50}$ by dot blot analysis. By this assay, no HHV-6 DNA was detected in mononuclear cells of umbilical cord blood (data not shown). From these data, PCR was concluded to be sufficiently sensitive for the diagnosis of HHV-6 infection. As shown in Fig. 4, in all patients with exanthem subitum in which HHV-6 infection was detected by the antibody test, HHV-6 DNA was detectable, even when the samples were collected during the convalescent phase of the disease. However, the amount of viral DNA in the blood was less during the convalescent phase than during the acute phase. These data suggest that during the convalescent phase, the viral DNA is present in the peripheral blood cells, although antibody was detected at high levels and we were unable to isolate the virus from patients during the convalescent phase. These observations suggest that the virus may be in a latent stage in the cells during the convalescent phase.

HHV-6 is supposed to be the main causative agent of exanthem subitum, but other viruses, such as coxsackievirus
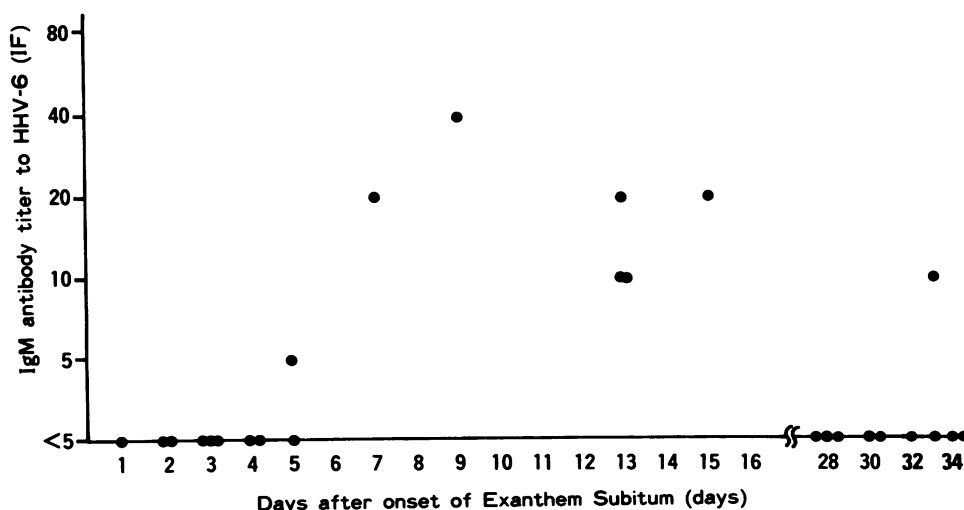

FIG. 5. IgM antibody titers in patients with exanthem subitum. Twenty-six serum samples were collected from 20 patients, and IgM antibody titers were tested as described in the text. IF, Immunofluorescence.

and echovirus, have been reported to cause symptoms very similar to those of exanthem subitum (9). Therefore, it is important to distinguish this disease. Patient 8 was diagnosed as having exanthem subitum because febrile and exanthem phases were recognized, but no DNA was detected in blood samples from the patient and no increase in antibody against HHV-6 was detected by the immunofluorescence test. Therefore, this patient may have been infected with another virus.

IgM antibody was first detected in patients on day 5, and it persisted for 2 weeks (Fig. 5). On the other hand, IgG antibody was first detected on day 7 of disease and reached a maximum in 2 to 3 weeks (22). These data on the antibody response suggest that HHV-6 causes a typical acute viral infection.

The PCR assay is sensitive and specific and takes only 1 to 2 days to perform. The application of PCR for the detection of HHV-6 should be very useful, particularly when a drug for the treatment of HHV-6 infection has been developed, such as acyclovir and ganciclovir, which are available for the treatment of HSV, VZV, and CMV infections.

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