Microbiol. Immunol., **46**(3), 177−180, 2002

Detection of Herpesvirus DNA in the Serum of Immunocompetent Children

Shinya Hara*[,1], Hiroshi Kimura[1], Yo Hoshino[1], Naoko Tanaka[1], Kazuo Nishikawa[2], Masaru Ihira[3], Tetsushi Yoshikawa[4], and Tsuneo Morishima[5]

[1]*Department of Pediatrics,* [5]*Department of Health Science, Nagoya University School of Medicine, Nagoya, Aichi 461−8673, Japan,* [4]*Laboratory of Virology, Research Institute for Disease Mechanism and Control, Nagoya University School of Medicine, Nagoya, Aichi 466−8550, Japan,* [2]*Department of Pediatrics, Ekisaikai General Hospital, Nagoya, Aichi 454−8052, Japan, and* [3]*Department of Pediatrics, Fujita Health University School of Medicine, Toyoake, Aichi 470−1192, Japan*

Received November 13, 2001. Accepted December 25, 2001

***Abstract*: The DNA of herpesviruses such as Epstein-Barr virus (EBV), cytomegalovirus (CMV), human herpesvirus-6 (HHV6), and human herpesvirus-7 (HHV7) has been detected in the serum of patients with primary infection or with immunosuppression. However, it is unknown how frequently herpesvirus DNA can be detected in the serum of immunocompetent children, or whether the detection of herpesvirus DNA indicates an active infection or virus-related diseases. Using a real-time polymerase chain reaction assay, attempts were made to detect herpesvirus DNA in the serum of 176 ambulatory children who visited a hospital for various reasons. EBV was detected in 4 (2.2%), HHV6 in 4 (2.2%), and HHV7 in 2 (1.1%) of 176 children, but CMV was not detected. Of the 10 positive patients, only 4 were considered, by virtue of clinical and serological characteristics, to have primary infections. The other 4 positive patients had other infections, such as mycoplasma and salmonella. Although herpesvirus DNA could be detected in the serum of immunocompetent children, there was not always a relationship between clinical manifestations and the detection of virus DNA. When herpesvirus DNA is detected in the serum, a careful interpretation is necessary to diagnose a primary infection or a virus-associated disease.**

***Key words*: Herpesviruses, Serum, Real-time polymerase chain reaction assay**

Presently, 8 herpesviruses are known to be associated with human disease. They are classified into three subfamilies: herpes simplex virus type 1, herpes simplex virus type 2 and varicella-zoster virus are α herpesviruses. Cytomegalovirus (CMV), human herpesvirus-6 (HHV6), and human herpesvirus-7 (HHV7) are β herpesviruses. Epstein-Barr virus (EBV) and human herpesvirus-8 are γ herpesviruses. Primary infection with these viruses occurs mostly in childhood, and latent infection persists in the host for life. It has been reported that some herpesvirus can be detected in the serum of patients who either have an active virus infection or are affected by an immunosuppressive condition, such as AIDS or the sequelae of organ or stem cell transplantation (2, 3, 5, 7). Specifically, latent β and γ herpesviruses in blood cells are detectable in blood from patients with these conditions (15, 18). It has also been reported that the quantification of these viral DNA is useful in the diagnosis of virus-associated diseases and in monitoring virus activity (2, 9, 14, 20). However, it is unknown how frequently herpesvirus DNA can be detected in the serum of immunocompetent children or whether the detection of herpesvirus DNA indicates an active infection or virus-related disease. In this study, sera were collected from immunocompetent children, and a real-time polymerase chain reaction (PCR) was used to detect EBV, CMV, HHV6, and HHV7. The relative importance of viral detection in these children is discussed, along with clinical and serological data.

Materials and Methods

Patients and samples. The patients enrolled in this study were 176 ambulatory children (96 boys and 80 girls, mean age of 4.9 years old, range from 0−15 years),

**Address correspondence to* Dr. Shinya Hara, Department of Pediatrics, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466−8550, Japan. Fax: 052−744−2974. E-mail: shinyah@med.nagoya-u.ac.jp

Abbreviations: CMV, cytomegalovirus; EBNA, EB nuclear antigen; EBV, Epstein-Barr virus; ELISA, enzyme-linked immunosorbent assay; HHV6, human herpesvirus-6; HHV7, human herpesvirus-7; PCR, polymerase chain reaction; VCA, viral capsid antigen.

who visited Nagoya Ekisaikai General Hospital. They consisted of patients with a variety of health complaints, including upper respiratory tract infection, pneumoniae, bronchitis, bronchial asthma, gastroenteritis, fever of unknown origin, and treated epilepsy. None had any underlying immunodeficiency or was receiving any immunosuppressive therapy. Blood specimens were drawn for clinical purposes. Informed consent was obtained from the parents of all children enrolled in this study.

Real-time PCR assay. Viral DNA was detected by the use of a real-time quantitative PCR assay. DNA was extracted from 200 µl of serum with a QIAamp Blood Kit (Qiagen, Hilden, Germany), eluted in 50 µl of distilled water, and stored at −20 C until use. Five microliters of solution were used for the real-time PCR assay. The primers and probe conditions for EBV, CMV, and HHV6 were as described in previous papers (8, 18, 19). The PCR primers and probe for HHV7 were derived from a DNA sequence of the U37 gene. The sequence data were obtained from GenBank. The upstream primer was 5′-CGGAAGTCACTGGAGTAATGACAA-3′, and the downstream primer was 5′-CCAATCCTTCC-GAAACCGAT-3′. A fluorogenic probe (5′-CTCGCA-GATTGCTTGTTGGCCATG-3′) was located between the primers. The PCR reaction was performed with a TaqMan PCR kit and a Model 7700 Sequence Detector (PE Applied Biosystems, Foster, U.S.A.), as previously described (8). Real-time fluorescence measurements were made and a threshold cycle value for each sample was calculated by determining the point at which the fluorescence exceeded a threshold limit (10 times the standard deviation of the baseline) (6). A standard graph was constructed from the threshold cycle values obtained from serially diluted positive control plasmid (8, 18, 19). The threshold cycle values from clinical samples were plotted on the standard curve, and the copy number was automatically calculated by the use of a Sequence Detector v1.6 (PE Applied Biosystems), a software package for data analysis. Samples were defined as negative when the threshold cycle values exceeded 50 cycles. The copy number of each virus DNA was expressed as copies per ml. The detection limits of each virus DNA were approximately 50−100 copies/ml. To exclude contamination from our laboratory, adequate care was taken in the experimental design (11). For positive cases, DNA was extracted from the original samples, and the PCR was repeated to confirm the results.

Antibodies to 4 viruses. Antibodies to EBV and CMV were tested by enzyme-linked immunosorbent assay (ELISA). The DiaSorin VCA IgG, VCA IgM, and EBNA IgG ELISA kits (DiaSorin, Stillwater, U.S.A.) were used to measure EBV-specific antibodies, such as anti-viral capsid antigen (VCA) IgG, IgM, and anti-EB nuclear antigen (EBNA) antibody. Specific anti-CMV IgG antibody was determined by using Enzygnost Anti-CMV/IgG (Behring Diagnostics GmbH, Deerfield, U.S.A.). Antibodies to HHV6 and HHV7 were tested by an indirect immunofluorescence assay (IFA), as previously described (17, 21).

Results

Frequency of Viral DNA Detection

We found EBV-DNA in 4 (2.2%) of the 176 sera, HHV6-DNA in 4 (2.2%), and HHV7-DNA in 2 (1.1%). No CMV-DNA was detected. All samples were assessed for antibodies to EBV and CMV. Ninety-six samples were positive for anti-CMV IgG antibody, which indicated previous or recent infection by CMV, and 81 samples were negative, suggesting that these children had not been infected. A total of 116 samples were positive for anti-VCA IgG antibody, indicating that these patients were previously or recently infected with EBV. EBV-DNA was detected in 4 of these 116 samples (3.4%), and all 4 were positive for anti-VCA IgG antibody. EBV-DNA was not detected in samples that were negative for anti-VCA IgG antibody.

The Characteristics of the Cases in Which Viral DNA Was Detected

Table 1 shows the clinical and virological character-

Table 1. Characteristics of 4 cases positive for EBV-DNA

				Antibody			Viral load		
				Anti-VCA		Anti-EBNA	EBV-DNA		
No.	Age	Sex	Symptoms	IgG	IgM		(Copies/ml)	Diagnosis	Date of sampling[a)]
1	2 yr	M	fever, cough	+	−	+	3.0×10^2	Mycoplasma pneumoniae	6
2	2 yr	M	fever, cough	+	−	+	2.6×10^2	Viral bronchitis	8
3	2 yr	M	fever, cough	+	+	+	5.5×10^2	Unknown hepatitis	13
4	2 yr	F	CLN swelling	+	+	−	3.3×10^3	Primary EBV infection	1

M: male, F: female, CLN: cervical lymph node.

[a)] The first day of symptoms was assigned as day 0.

Table 2. Characteristics of 6 cases positive for HHV6/7-DNA

No.	Age	Sex	Symptoms	Viral load		Antibody				Diagnosis	Date of sampling[a)]
				HHV6-DNA (Copies/ml)	HHV7-DNA (Copies/ml)	Anti-HHV6 IgG	Anti-HHV6 IgM	Anti-HHV7 IgG	Anti-HHV7 IgM		
5	9 mo	M	fever, rash	1.7×10^3	undetected	<8	<8	<8	n.d.	Exanthem subitum	2
6	2 mo	F	fever	1.0×10^4	undetected	<8	<8	16	n.d.	Unknown fever	15
7	5 mo	M	fever	1.2×10^4	undetected	<8	<8	<8	n.d.	Unknown fever	3
8	2 yr	M	vomiting, diarrhea	1.2×10^4	undetected	64	<8	<8	n.d.	Salmonella infection	2
9	11 mo	M	fever, rash	undetected	1.1×10^3	<8	n.d.	<8	<8	Exanthem subitum	5
10	3 yr	M	fever, cough	undetected	2.7×10^2	64	n.d.	64	8	Mycoplasma pneumoniae	4

M: male, F: female, n.d.: not done.

[a)] The first day of symptoms was assigned as day 0.

istics of the 4 cases in which EBV-DNA was detected. The clinical symptoms in cases 1 and 2 did not seem to be due to EBV. They were diagnosed as mycoplasma pneumoniae and viral bronchitis, respectively, both of which are unrelated to EBV. Case 3 had mild liver dysfunction of unknown etiology. The detection of both anti-VCA IgG antibody and anti-EBNA antibody indicated that this patient had been infected with EBV previously, but he also had anti-VCA IgM antibody, suggesting that he might have been experiencing a reactivation of EBV. Any relationship of his infection to other hepatitis viruses, such as hepatitis A, B, and C, or to CMV was discounted, because of negative results with virus-specific antigens or antibodies. Based on clinical and serological characteristics, case 4 was diagnosed as a primary EBV infection, there were 10 times more EBV-DNA copies than found in the other 3 cases.

Table 2 shows the clinical and virological characteristics of the 4 cases with HHV6-DNA and the 2 cases with HHV7-DNA. Cases 5 and 9 were clinical cases of exanthem subitum. In cases 6 and 7, fever was the only clinical finding, and no skin rash was found after the fever subsided. In each case, neither antibody titers to HHV6 nor the patient's age could be used to rule out the possibility of primary HHV6 infection. Case 8 was a salmonella infection, and case 10 was a mycoplasma infection.

Discussion

Using a real-time PCR assay, we detected EBV-DNA in serum from 4 immunocompetent children. The detection rate of EBV-DNA was about 3.4% in samples from EBV-seropositive children. Two previous studies, though small, detected EBV-DNA in the serum of 7−10% of healthy adults (5, 12). Our results for immunocompetent children are in general accord with these previous findings. Of 4 cases with EBV-DNA, one was diagnosed as a primary EBV infection, but 2 had no serological or clinical findings suggestive of active EBV infection. We confirmed that the presence of EBV-DNA in their serum was not due to contamination or technical problems. The final case (case 3) had mild hepatitis of unknown origin. Serological examinations indicated that this patient had been previously infected with EBV, but a positive anti-VCA IgM antibody suggested that he might be experiencing a reactivation of EBV. It is unclear whether the liver dysfunction was associated with EBV in this patient.

It is well known that both HHV6 and HHV7 cause exanthem subitum. Of 6 cases in which HHV6 or HHV7 was detected, 2 (cases 5 and 9) were clinically diagnosed exanthem subitum. Their blood samples may have been negative for IgG and IgM antibodies to HHV6 or HHV7 because they were taken during a febrile period. It has been reported that specific antibodies for HHV6 and HHV7 are not detected during febrile periods and that IgM antibodies appear 3 to 5 days after the onset of exanthem subitum (10, 16). Cases 6 and 7, which presented with fever but no rash, might also have had a primary HHV6 infection because primary infection of HHV6 does not necessarily elicit a rash (13). Case 6 had neither IgG nor IgM antibodies to HHV6, fifteen days after the onset of symptoms. Since this case was a very young infant (2 months), the appearance of virus-specific antibodies might have been delayed because of the immaturity of the immune system. The other two cases (cases 8 and 10) had bacterial infections. The existence of IgG antibodies in the acute phase suggests that they had been infected in the past. It is possible that a subclinical reactivation of HHV6 or HHV7 had occurred and that cell-free viral DNA was detected in the serum of these patients. It has been reported that neither HHV6 nor HHV7 is detected in the serum of healthy adults (4, 14).

As we have shown in this study, herpesvirus DNA can be detected in the serum of immunocompetent children, at low frequency. Although some cases were thought to have a primary infection, there were others in whisch we could find no relationship between clinical manifestations and the detection of viral DNA. Therefore a careful

diagnosis is required subsequent to the detection of herpesvirus DNA. It is unclear why virus DNA was detected in the cases without virus-specific symptoms. A reactivation might occur in tandem with other infections. In this study, 4 of 10 cases co-occurred with other infections, such as mycoplasma and salmonella. There is a report that *Salmonella typhimurium* activates human immunodeficiency virus type 1 in chronically infected promonocytic cells by inducing tumor necrosis factor-alpha production (1). Through such a mechanism, bacterial infection might cause the reactivation of herpesviruses.

We thank Dr. K. Watanabe, professor of the Department of Pediatrics, Nagoya University Graduate School of Medicine, for his critical reading of the manuscript and valuable suggestions. This work was supported by a grant from the Japan Society for the Promotion of Science (JSPS-RFTF97L00703).

References

1) Andreana, A., Gollapudi, S., Kim, C.H., and Gupta, S. 1994. *Salmonella typhimurium* activates human immunodeficiency virus type 1 in chronically infected promonocytic cells by inducing tumor necrosis factor-alpha production. Biochem. Biophys. Res. Commun. **201**: 16–23.
2) Brytting, M., Xu, W., Wahren, B., and Sundqvist, V.A. 1992. Cytomegalovirus DNA detection in sera from patients with active cytomegalovirus infections. J. Clin. Microbiol. **30**: 1937–1941.
3) Clark, D.A., Kidd, I.M., Collingham, K.E., Tarlow, M., Ayeni, T., Riordan, A., Griffiths, P.D., Emery, V.C., and Pillay, D. 1997. Diagnosis of primary human herpesvirus 6 and 7 infections in febrile infants by polymerase chain reaction. Arch. Dis. Child. **77**: 42–45.
4) Drago, F., Ranieri, E., Malaguti, F., Battifoglio, M.L., Losi, E., and Rebora, A. 1997. Human herpesvirus 7 in patients with pityriasis rosea. Electron microscopy investigations and polymerase chain reaction in mononuclear cells, plasma and skin. Dermatology **195**: 374–378.
5) Gan, Y.J., Sullivan, J.L., and Sixbey, J.W. 1994. Detection of cell-free Epstein-Barr virus DNA in serum during acute infectious mononucleosis. J. Infect. Dis. **170**: 436–439.
6) Heid, C.A., Stevens, J., Livak, K.J., and Williams, P.M. 1996. Real time quantitative PCR. Genome Res. **6**: 986–994.
7) Huang, L.M., Kuo, P.F., Lee, C.Y., Chen, J.Y., Liu, M.Y., and Yang, C.S. 1992. Detection of human herpesvirus-6 DNA by polymerase chain reaction in serum or plasma. J. Med. Virol. **38**: 7–10.
8) Kimura, H., Morita, M., Yabuta, Y., Kuzushima, K., Kato, K., Kojima, S., Matsuyama, T., and Morishima, T. 1999. Quantitative analysis of Epstein-Barr virus load by using a real-time PCR assay. J. Clin. Microbiol. **37**: 132–136.
9) Kimura, H., Nishikawa, K., Hoshino, Y., Sofue, A., Nishiyama, Y., and Morishima, T. 2000. Monitoring of cell-free viral DNA in primary Epstein-Barr virus infection. Med. Microbiol. Immunol. (Berl.) **188**: 197–202.
10) Kondo, K., Hayakawa, Y., Mori, H. Sato, S., Kondo, T., Takahashi, K., Minamishima, Y., Takahashi M., and Yamanishi, K. 1990. Detection by polymerase chain reaction amplification of human herpesvirus 6 DNA in peripheral blood of patients with exanthem subitum. J. Clin. Microbiol. **28**: 970–974.
11) Kwok, S., and Higuchi, R. 1989. Avoiding false positives with PCR. Nature **339**: 237–238.
12) Lo, Y.M., Chan, L.Y., Lo, K.W., Leung, S.F., Zhang, J., Chan, A.T., Lee, J.C., Hjelm, N.M., Johnson, P.J., and Huang, D.P. 1999. Quantitative analysis of cell-free Epstein-Barr virus DNA in plasma of patients with nasopharyngeal carcinoma. Cancer Res. **59**: 1188–1191.
13) Pruksananonda, P., Hall, C.B., Insel, R.A., McIntyre, K., Pellett, P.E., Long, C.E., Schnabel, K.C., Pincus, P.H., Stamey, F.R., Dambaugh, T.R., et al. 1992. Primary human herpesvirus 6 infection in young children. N. Engl. J. Med. **326**: 1445–1450.
14) Secchiero, P., Carrigan, D.R., Asano, Y., Benedetti, L., Crowley, R.W., Komaroff, A.L., Gallo, R.C., and Lusso, P. 1995. Detection of human herpesvirus 6 in plasma of children with primary infection and immunosuppressed patients by polymerase chain reaction. J. Infect. Dis. **171**: 273–280.
15) Spector, S.A., Merrill, R., Wolf, D., and Dankner, W.M. 1992. Detection of human cytomegalovirus in plasma of AIDS patients during acute visceral disease by DNA amplification. J. Clin. Microbiol. **30**: 2359–2365.
16) Suga, S., Yoshikawa, T., Asano, Y., Nakashima, T., Yazaki, T., Fukuda, M., Kojima, S., Matsuyama, T., Ono, Y., and Oshima, S. 1992. IgM neutralizing antibody responses to human herpesvirus-6 in patients with exanthem subitum or organ transplantation. Microbiol. Immunol. **36**: 495–506.
17) Suga, S., Yoshikawa, T., Asano, Y., Yazaki, T., and Hirata, S. 1989. Human herpesvirus-6 infection (exanthem subitum) without rash. Pediatrics **83**: 1003–1006.
18) Tanaka, N., Kimura, H., Hoshino, Y., Kato, K., Yoshikawa, T., Asano, Y., Horibe, K., Kojima, S., and Morishima, T. 2000. Monitoring four herpesviruses in unrelated cord blood transplantation. Bone Marrow Transplant. **26**: 1193–1197.
19) Tanaka, N., Kimura, H., Iida, K., Saito, Y., Tsuge, I., Yoshimi, A., Matsuyama, T., and Morishima, T. 2000. Quantitative analysis of cytomegalovirus load using a real-time PCR assay. J. Med. Virol. **60**: 455–462.
20) Yamamoto, M., Kimura, H., Hironaka, T., Hirai, K., Hasegawa, S., Kuzushima, K., Shibata, M., and Morishima, T. 1995. Detection and quantification of virus DNA in plasma of patients with Epstein-Barr virus-associated diseases. J. Clin. Microbiol. **33**: 1765–1768.
21) Yoshikawa, T., Asano, Y., Kobayashi, I., Nakashima, T., Yazaki, T., Suga, S., Ozaki, T., Wyatt, L.S., and Frenkel, N. 1993. Seroepidemiology of human herpesvirus 7 in healthy children and adults in Japan. J. Med. Virol. **41**: 319–323.