Comparison of Specific Serological Assays for Diagnosing Human Herpesvirus 6 Infection after Liver Transplantation

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Cross-reactivity between human herpesvirus 6 (HHV-6) and human herpesvirus 7 (HHV-7) antibodies and the reliability of specific serological assays were analyzed for 12 patients with concurrent HHV-6 and HHV-7 antibody responses after transplantation with a liver from a living relative by using an immunofluorescence assay (IFA). A neutralizing antibody titer assay (NT) and an immunoblot assay (IB) designed to detect immunoglobulin M (IgM) antibody to the HHV-6 immunodominant 101-kDa protein were compared in the diagnosis of an active HHV-6 infection. A total of 9 of 12 patients demonstrated concurrent HHV-6 and HHV-7 antibody responses, including increased IgG titers and/or the presence of IgM by IFA, and were thus analyzed for cross-reactive antibody to heterologous virus. The average percentages of residual antibody to HHV-6 and HHV-7 after absorption with HHV-6 antigen were 32.6% (range, 6 to 50%) and 55.6% (range, 35 to 100%), respectively. All 12 patients were subsequently analyzed for HHV-6 antibody by using IB and NT. IB detected IgM antibody to the 101-kDa protein in 75% (9 of 12) of the recipients. A significant rise in the NT antibody titer was detected in the same nine samples. However, HHV-6 DNA was detected by PCR in only five of nine plasma samples collected from recipients with a specific serologic response against HHV-6.

Human herpesvirus 6 (HHV-6) (16) and human herpesvirus 7 (HHV-7) (12) are recently discovered members of the herpesvirus family. These two viruses are closely related based on similar cell tropism and growth characteristics, limited DNA cross-hybridization, and nucleotide and amino acid sequence homology (5). Moreover, primary infection with both viruses causes exanthem subitum (1, 3, 17, 19, 26), a common febrile disease of infancy. These viruses probably remain latent in the body throughout life and, like other human herpesviruses, reactivate during immunosuppressed states. In transplant patients, HHV-6 is associated with fever and skin rash (2, 28), interstitial pneumonitis (7, 9), encephalitis (11), and bone marrow graft suppression after bone marrow transplantation (10). Moreover, the virus has been associated with kidney transplant rejection (15) and several clinical features occurring after liver transplants (8, 22, 27). There are few reports describing HHV-7 activity post-organ or post-bone marrow transplant (5). However, these studies indicated that HHV-7 activity usually precedes that of human cytomegalovirus (HCMV) and may thus exacerbate disease associated with HCMV or serve as a marker of eminent HCMV disease.

Cross-reactivity between HHV-6 and HHV-7 antibodies has been demonstrated (6, 19, 20, 24), and an interaction between these viruses in vitro has been postulated (13). Although an indirect immunofluorescence assay (IFA) is commonly used to determine titers of antibody against these viruses, the inability of this assay to distinguish cross-reacting HHV-6 and HHV-7 antibodies is problematic. Another commonly used method to detect active virus infection is PCR analysis to detect viral DNA in peripheral blood mononuclear cells (PBMC) (21, 23). However, this method may detect the virus genomes in latently infected PBMC (false positive). False-negative results may also be obtained due to inappropriate sampling time, inhibitors present in the sample, or less sensitive assays. The false-positive or -negative results may confound understanding of the clinical symptoms associated with active HHV-6 infection. A specific serologic assay capable of discriminating between HHV-6 and HHV-7 cross-reacting antibodies would obviate the drawbacks of using PCR.

In this paper, we demonstrate that HHV-6 and HHV-7 cross-reactive antibody was present in plasma of patients who had concurrent HHV-6 and HHV-7 antibody responses after transplantation with a liver from a living relative by using a cross-absorption IFA. An immunoblot (IB) that specifically detects both immunoglobulin G (IgG) (26) and IgM (14) against the HHV-6 major immunogenic protein, which has a molecular mass of 101 kDa, was compared to the neutralization test (NT), which is generally considered to be a type-specific serological assay for most virus infections (1).

EDTA-treated peripheral blood was collected from patients who received a liver transplant donated by a parent at Kyoto University Hospital, at the time of transplantation and bi-
weekly after transplantation for 2 months. Plasma was separated from whole blood by density gradient centrifugation (Ficoll-Paque; Amersham Pharmacia Biotech). All specimens were stored at $-70^\circ$C. Samples from twelve recipients (seven male and five female) demonstrated concurrent HHV-6 and HHV-7 IgG and/or IgM antibody responses by IFA and were further analyzed by NT, IB, and PCR as described below. NT antibody titers were measured in paired plasma samples that spanned the same time points as when significant increases in both HHV-6 and HHV-7 IgG titer were detected or over a period when IgM against both viruses was detected by IFA. Samples were tested by IB at the same time point as the posttransplant samples used for determining NT titer. None of the patients were HHV-6 IgM positive prior to transplantation. The median age of the recipients was 12 years old (ranging from 7 months to 52 years old) at the time of transplantation. All guardians of these patients consented to participation in this study.

Titers of antibody to HHV-6 and HHV-7 were measured by IFA as described previously (29). The representative strain of HHV-6 variant B (FG-I), isolated from PBMC obtained from an exanthem subitum patient, was used as the standard antigen. HHV-7 strain Sato, isolated from saliva obtained from a healthy adult, was used for making HHV-7 antigen. For IgM determination, plasma samples were pretreated with RF-Absorbant (Hoechst-Behring, Kanata, Ontario, Canada) to absorb interfering IgG and rheumatoid factor. The antibody titer was defined as the reciprocal of the serum dilution showing specific fluorescence. A significant increase in HHV-6 or HHV-7 IgG antibody titer was considered evidence of active HHV-6 or HHV-7 infection.

Details of the procedure for absorption were described elsewhere (6). Briefly, HHV-6 (FG-1)-infected cells were pelleted and suspended in phosphate-buffered saline, frozen at $-70^\circ$C, thawed, and then sonicated. Serum samples were diluted 1:10 with absorbing antigen and then incubated at $37^\circ$C for 1 h. The samples were centrifuged, and the supernatant was twofold serially diluted and then tested by IFA. The percentage of residual antibody was calculated by using the IFA titer obtained in the presence and in the absence of an absorption step.

HHV-6-specific antibody titers were determined by NT (1). Serial twofold plasma dilutions prepared on disposable plastic 96-well trays were mixed with an equal volume (100 µl) of HHV-6 preparation containing $10^5$ 50% tissue culture infectious doses of HHV-6. After 1 h of incubation at $37^\circ$C, cord blood mononuclear cells were added to each well, and incubation was continued for 7 days. The antibody titer was determined as the reciprocal of the highest dilution of serum that completely prevented large-cell formation. A significant increase in the NT titer was defined as a $\geq 4$-fold increase.

IB for detecting HHV-6 IgM was performed as previously described (14). Briefly, proteins from virions that were pelleted from an HHV-6 variant B (Z29)-infected cell supernatant were separated on a sodium dodecyl sulfate--9% polyacrylamide gel by electrophoresis and then transferred to nitrocellulose. Serum samples were treated with IgG absorbent (Gullsorb; Gull Labs, Salt Lake City, Utah) according to the manufacturer’s instructions and then reacted with the nitrocellulose. After treatment with alkaline phosphatase goat anti-human IgM, immunoreactive bands were visualized by using a commercial alkaline phosphatase kit according to the manufacturer’s instructions (Immune-Blot assay kit; Bio-Rad, Hercules, Calif.).

Nested PCR was carried out for amplification of HHV-6 DNA using two primer sets as described previously (18). Three hundred microliters of plasma was incubated overnight in lysis buffer (10 mM Tris HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 0.4% sodium deoxycholate, 1 mg of proteinase K per ml) at 65°C. DNA was extracted by phenol-chloroform treatment followed by ethanol precipitation. Five hundred nanograms of DNA (quantified spectrophotometrically) was used for the PCR. PCR resulted in the amplification of a 751-bp DNA fragment encoding a putative large tegument protein gene. HHV-7 DNA was amplified according to procedures reported elsewhere (4). The primers amplified a DNA fragment of 124 bp. Reaction mixtures consisted of 50 µM KCl, 10 mM Tris (pH 8.3), 1.5 mM MgCl$_2$, a 200 µM concentration of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), a 0.5 mM concentration of each primer, and 2.5 U of Taq DNA polymerase (AmpliTaq Gold; Perkin-Elmer, Norwalk, Conn.). A Perkin-Elmer Cetus model 9600 DNA thermocycler was programmed as follows: 10 min at 95°C, 30 cycles of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C; and 10 min at 72°C. Samples were held at 4°C until the products could be analyzed. Aliquots of Tris-EDTA buffer were processed as a negative control, and appropriate precautions were taken during sample preparation and performance of the PCR to avoid cross contamination.

A total of 9 of the 12 plasma samples (patients 1, 2, 6, 7, 8, 9, 10, 11, and 12) which demonstrated concurrent HHV-6 and HHV-7 antibody responses by IFA (Table 1) had sufficient volumes to be examined for cross-reacting HHV-6 and HHV-7 antibodies by cross absorption. These nine samples were absorbed with HHV-6 antigen, and the percentage of residual antibody to HHV-6 and HHV-7 was calculated (Fig. 1). Residual antibody values which were greater than 100% were plotted as 100% residual activity, indicating that no cross-reactive antibodies were detected. The average percentages of residual antibody to HHV-6 and HHV-7 after absorption with HHV-6 antigen were 32.6% (range, 6 to 50%) and 55.6% (range, 35 to 100%), respectively. These results demonstrate the presence of HHV-6 and HHV-7 cross-reactive antibodies.

Results of NT were then compared to results obtained using IB. The plasma samples obtained from patients 1 through 9 demonstrated HHV-6-specific IgM antibody reactivity with the 101-kDa protein. A significant rise in the NT antibody titer was also demonstrated in the same nine samples (Table 1). These results indicate that active HHV-6 infection occurred in these nine recipients during the observation period. However, HHV-6 DNA was not detected in plasma obtained from four of the nine recipients (patients 6 to 9). Although a significant increase in HHV-6 IgG antibody titers or positive HHV-6 IgM antibody was observed in three recipients by IFA (patients 10, 11, and 12), an HHV-6-specific antibody response was not demonstrated in these patients by either NT or IB.

HHV-6 antigen absorbed an average of 70% of the HHV-6 antibody activity and 50% of the HHV-7 antibody activity, indicating cross-reactivity between HHV-6 and HHV-7 anti-
bodies. The quantity of residual antibody titers varied in each individual as previously described (6). Although IFA is a commonly used method for detecting HHV-6 and HHV-7 infection, it appears that this assay cannot discriminate between cross-reacting antibodies in all cases and thus may be inadequate for the diagnosis of specific virus infection.

Yamamoto et al. (25) reported that a 101-kDa HHV-6 virion protein was highly immunoreactive in IB and was a useful specific serological marker of infection. There is no cross-reactivity between the HHV-6 101-kDa protein and its HHV-7 counterpart, the 89-kDa protein (6). Thus, the results of IB are considered to reveal genuine active HHV-6 infection in the recipients. Since the results of NT were identical to those of IB, NT is also a useful serologic assay for the diagnosis of active HHV-6 infection in organ transplant recipients. Since the results of NT were identical to those of IB, NT is also a useful serologic assay for the diagnosis of active HHV-6 infection until an optimal sampling schedule is determined. In spite of the presence of the HHV-6 specific serological responses during the observation period, plasma PCR was negative at all time points for four recipients (patients 6, 7, 8, and 9). Possible explanations for the discrepancy include the following. (i) Polyclonal antibody response may cause an increase in HHV-6 antibody titers without reactivation of the virus. (ii) The concentration of viral DNA in these samples may have been below the detection threshold of the assay. (iii) There may have been inhibitors present in the plasma. (iv) The peak plasma DNA levels may not have been present during the chosen sample collection intervals. It is possible that collecting samples weekly rather than biweekly may help increase the sensitivity of PCR. An appropriate sampling schedule needs to be optimized for PCR, as results can be obtained in 1 day. A specific serologic assay should be combined with PCR analysis to determine active HHV-6 infection until an optimal sampling schedule is determined.

NT, IB, and PCR did not detect HHV-6 activity in three of the recipients who demonstrated either a significant increase in HHV-6 IgG titers (patients 11 and 12) or the presence of HHV-6 IgM (patient 10) by IFA (Table 1). Since HHV-7 DNA was detected in patients 10 and 11, and since both patients showed the presence of HHV-7 plasma IgM by IFA, it is likely that active HHV-7 infection occurred in these two recipients. Since these patients were 10 and 26 years of age and their HHV-7 IgG titers at week 0 were 128 and 256, respectively, the HHV-7 activity is likely the result of reactivation. The rises in HHV-6 IgG titer in these two patients is probably due to HHV-7 cross-reacting antibody, as absorption with HHV-6 antigen removed approximately 50 and 80%, respectively, of the IFA-detectable HHV-7 IgG activity from these plasma samples (Fig. 1). To clarify the HHV-7 activity in patient 12, further virological examinations are necessary. Thus, a specific sero-

![FIG. 1. Range of HHV-6 and HHV-7 cross-reactive antibody found in plasma obtained from the recipients with concurrent antibody responses against both viruses as detected by IFA. Plasma samples were absorbed with HHV-6 variant B. Residual antibody to HHV-6 and HHV-7 was determined as described in the text. Mean residual titers of antibody against HHV-6 and HHV-7 are shown.](http://cvi.asm.org/)

### TABLE 1. Results of serological assays and plasma PCR for both viruses

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>DNA detection</th>
<th>IgG titer increase</th>
<th>IgM titer increase</th>
<th>HHV-6 NT titer</th>
<th>Result of IB</th>
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<tbody>
<tr>
<td></td>
<td>Pretransplant</td>
<td>Posttransplant</td>
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* Time points, in weeks, after transplant. NC, no change in IgG titer. IgM titers of ≤1:8 are considered positive. Neg, IgM titer of less than 1:8 at all time points. Boldface type indicates the time periods of concurrent HHV-6 and HHV-7 antibody responses.

** NT antibody titers shown were measured using paired plasma samples that spanned the same time points as when significant increases in HHV-6 and HHV-7 IgG titers were detected, or over a period when HHV-6 and HHV-7 IgM were detected by IFA. Numbers in parentheses are time points in weeks after transplant.

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In spite of the presence of the HHV-6 specific serological responses during the observation period, plasma PCR was negative at all time points for four recipients (patients 6, 7, 8, and 9). Possible explanations for the discrepancy include the following. (i) Polyclonal antibody response may cause an increase in HHV-6 antibody titers without reactivation of the virus. (ii) The concentration of viral DNA in these samples may have been below the detection threshold of the assay. (iii) There may have been inhibitors present in the plasma. (iv) The peak plasma DNA levels may not have been present during the chosen sample collection intervals. It is possible that collecting samples weekly rather than biweekly may help increase the sensitivity of PCR. An appropriate sampling schedule needs to be optimized for PCR, as results can be obtained in 1 day. A specific serologic assay should be combined with PCR analysis to determine active HHV-6 infection until an optimal sampling schedule is determined.

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logical assay for determining HHV-7 infection is also important. Based on the results of this study and others, we believe that there is a need for the continued development of rapid serologic assays to specifically detect active HHV-6 or HHV-7 infection in the transplant patient population.

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REFERENCES


