

Rises in Antibody to Human Herpesvirus 6 Detected by Enzyme Immunoassay in Transplant Recipients with Primary Cytomegalovirus Infection

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Immunoglobulin G to human herpesvirus 6 (HHV-6) and cytomegalovirus (CMV) in sera from solid organ recipients was measured by an enzyme-linked immunoassay (ELISA) before and after transplant. The HHV-6 ELISA was developed from glycine extracts of HHV-6-infected and uninfected HSB-2 cells. At a serum dilution of 1:500, 80 (91%) of 88 recipients were seropositive for HHV-6 before transplant, while only 14 (16%) were seropositive for CMV. Posttransplant HHV-6 serologic rises were observed in 38 (43%) recipients; rises in 25 of these recipients were associated with primary CMV infection. Titration of sera revealed much higher HHV-6 titer rises among those with primary CMV infection than among those with CMV reactivation or with no CMV infection. Elevated HHV-6 antibody titers persisted for up to 2 years after primary CMV infection. No correlation was noted between CMV and HHV-6 antibody titers in individual serum samples.

Human herpesvirus 6 (HHV-6) was first described in 1986 as a virus infecting B lymphocytes of six patients with lymphoproliferative disorders (22). The virus is now known to infect T cells *in vitro* (19), and primary human infection may cause the syndrome of roseola infantum (3, 25). The reported HHV-6 seroprevalence has ranged from an early report of 2% (4 of 220) (22) to as high as 98% (41 of 42) (15), with recent studies tending to confirm a high seroprevalence in all populations surveyed (4, 5, 15, 16, 18, 21, 23). Most serologic surveys have been done by indirect immunofluorescence with infected cultured cells. Interpretation of these assays is complicated by variations in techniques and reagents and an element of subjectivity in reading results. Enzyme-linked immunosorbent assays (ELISAs) and immunoblot (Western blot) assays for HHV-6 have been reported (1, 23), but published experience so far is limited. A few reported cases of HHV-6 seroconversion or serologic rise have been found to occur in conjunction with cytomegalovirus (CMV) seroconversion and to be accompanied by CMV-like syndromes, thus raising questions about cross-reactivity (12, 14, 17, 20). In addition to this concern is the observation of a limited DNA homology between CMV and HHV-6 (10).

During a past investigation of the serology and virology of CMV infection in organ transplant recipients, we collected serum specimens which were subsequently characterized in terms of the CMV antibody and the CMV infection status of the respective recipients. Using some of these sera and antigens extracted from HHV-6-infected cells, we developed and tested a new HHV-6 ELISA to determine (i) the proportion of recipients with HHV-6 antibody pretransplant, (ii) changes in HHV-6 antibody levels posttransplant in those with various categories of CMV infection, and (iii) whether there is a correlation between HHV-6 and CMV antibody titers which might suggest cross-reactivity. The results are reported here.

MATERIALS AND METHODS

Viral stocks and cells. HHV-6-infected cell supernatant, permissive HSB-2 cells (bearing T-cell markers), and a subgenomic molecular clone (pZVH14) of viral DNA were obtained from Ablashi and colleagues (2, 13) of the National Cancer Institute (Bethesda, Md.). HHV-6 was passaged in HSB-2 cells to obtain cultures containing a large proportion of enlarged refractile cells with multiple large inclusion bodies. The presence of HHV-6 was confirmed by probing dot blots of DNA extracts of these cells with biotinylated pZVH14. Uninfected HSB-2 cells gave no signal under the same conditions.

Sera. Sera were selected from past organ (kidney, heart, or liver) recipients at the Oregon Health Sciences University to represent those who were CMV seronegative and seropositive and those who were undergoing primary CMV infection. Primary CMV infection was defined as CMV seroconversion (negative to positive by the ELISA described below) associated with CMV shedding (viruria, viremia, or both). Selection of recipients for testing was not random, in that CMV-seronegative recipients, and especially those known to have undergone primary CMV infection, were overrepresented in relation to our total recipient population (which was about 50% CMV seropositive). However, other than the availability of sera, no further selection criteria were applied. For each recipient, pre- and posttransplant sera were tested for CMV and HHV-6 antibody by ELISA, as described below. Sera from several recipients known to have acquired transfusion-associated primary CMV infection prior to transplant were also tested. The sera have been stored for up to 6 years at -80°C . CMV culture data were available on most recipients.

CMV ELISA. Assay for immunoglobulin G (IgG) antibody to CMV was performed with a locally developed ELISA, as described previously (8). At specified antigen (1:300) and serum (1:1,000) dilutions, a specific absorbance (SA) value of 0.1 was the criterion used to determine CMV seropositivity. The accuracy of this assay has been compared with those of commercial CMV antibody assays; it is our reference assay for donor and recipient CMV serologic screening.

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Titers determined from the ELISA values were obtained by serial twofold dilution of sera beginning at 1:1,000; the highest dilution which qualified as seropositive (SA, >0.1) was recorded as the endpoint titer.

HHV-6 ELISA. By using methods similar to those used for the CMV ELISA (7, 8), ELISA antigen was made from glycine extracts of HHV-6-infected and uninfected cells. Briefly, 30×10^6 infected or uninfected cells were collected by centrifugation at $1,200 \times g$, suspended in 4 ml of glycine buffer (pH 9.5), chilled on ice, sonicated for 30 s, and then centrifuged at $5,000 \times g$ for 20 min. The supernatant was frozen at -80°C in aliquots and used as the assay antigen. The optimal working antigen dilution (typically 1:40) was determined by titration with strongly reactive sera as well as with weak or nonreactive sera, and the final dilution was selected to give the strongest possible positive signals while minimizing reactivity with uninfected cell antigen.

Polystyrene, 96-well ELISA plates (Corning Glass Works, Corning, N.Y.) were coated with 50 μl of working dilutions (in phosphate-buffered saline [PBS], pH 7.4) of infected and uninfected cell antigen per well in alternating columns, as previously described for the CMV ELISA (8). Coated plates were stored at 4°C for up to 2 weeks. Prior to use, plates were washed three times with PBS containing 0.05% Tween 20, emptied, loaded with 0.2 ml of PBS-10% calf serum per well, and incubated at 37°C for 1 h. Plates were then emptied and loaded with 50 μl of test sera per well. Each serum specimen was diluted in PBS-10% calf serum and tested in duplicate against both infected and uninfected cell antigens. A 1:500 serum dilution was adopted for screening purposes. Sera of interest were titrated in serial twofold dilutions. Plates containing the diluted test sera were incubated for 2 h at 37°C , washed three times with PBS containing 0.05% Tween 20, and emptied; and 50 μl of peroxidase-conjugated goat anti-human IgG antibody (1:2,500 dilution; Tago, Burlingame, Calif.) was added to each well. After another 2 h at 37°C , plates were washed five times with PBS containing 0.05% Tween 20 and five times with deionized water. Wells were then loaded with 100 μl each of a substrate solution of 0.5 mg of 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma Chemical Co., St. Louis, Mo.) per ml and 0.03% hydrogen peroxide in 50 mM sodium citrate buffer (pH 4.5). After 15 min, the reaction was stopped with 100 μl of 1% electrophoresis-grade sodium dodecyl sulfate per well. The A_{405} was read on an automated ELISA reader (Titertek MCC/340; Flow Laboratories, Inc., McLean, Va.). SA was calculated by subtracting the average absorbance in wells containing uninfected cell antigen from the average absorbance in wells containing infected cell antigen. Control sera (positive and negative pools) were included with each run. An SA of >0.1 was considered positive; in titrations, the highest dilution giving this SA was read as the endpoint.

RESULTS

Single-dilution ELISA. Sera tested at a single 1:500 dilution gave a wide range of signals, with SA readings ranging from 0.0, presumably representing HHV-6-seronegative sera, to 1.5 (Fig. 1). There was no clear cutoff value to distinguish seropositive from seronegative sera, but based on experience with CMV ELISAs, we chose an SA value of 0.1 (about twice the absorbance of a control well without serum). By using this criterion, 80 (91%) of 88 recipients were HHV-6 seropositive at the time of transplant; 14 (16%) were CMV seropositive by ELISA.

The intensity of single-dilution SAs was used to estimate

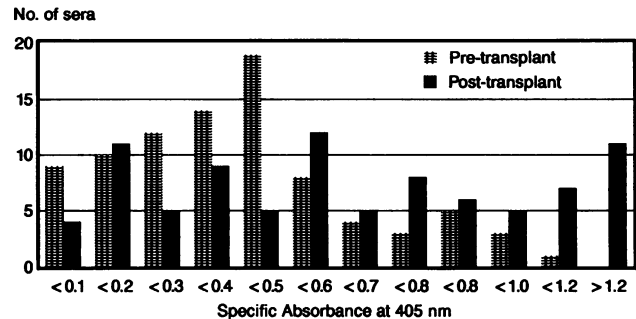


FIG. 1. Distribution of HHV-6 SAs of 176 serum specimens tested by ELISA at a dilution of 1:500. Pre- and posttransplant sera are compared.

the proportion of those recipients with posttransplant serologic rises to HHV-6 antibody. An antibody rise was considered to have occurred with a twofold increase in the SA, or an absolute increase of 0.4 units. By these criteria, over 40% of recipients had an antibody rise to HHV-6 in the period from 6 to 40 weeks posttransplant (Table 1). HHV-6 antibody rises were strongly associated with CMV seroconversion; 75% of those undergoing CMV seroconversion (negative to positive) had a concomitant HHV-6 antibody rise (usually from a positive to a higher positive). This was more than twice the incidence of HHV-6 antibody rises seen in groups with no CMV infection (who remained CMV seronegative) or who were CMV seropositive. In the latter category, 12 of 14 were known to have active CMV infection (viral shedding) posttransplant.

HHV-6 ELISA titers. In order to provide more precise information about the magnitude of the HHV-6 serologic rises, titers of pre- and posttransplant sera from 15 patients in each of the groups (no CMV infection, primary CMV, or CMV reactivation and reinfection with documented viral shedding) were determined to obtain an endpoint dilution. Results are shown in Fig. 2. Pretransplant titers were similar in all groups. However, in the posttransplant period, almost all of those with primary CMV infection showed a rise in HHV-6 antibody to $>1:8,000$, whereas only one of those in the other two groups (including all of those identified as having titer rises [Table 1]) had a posttransplant HHV-6 antibody titer of $>1:8,000$. HHV-6 titers in some of those with primary CMV infection reached 1:512,000.

We also had an opportunity to examine serum specimens from three renal transplant candidates before and after they acquired primary CMV infection from pretransplant blood transfusions. These patients were not on immunosuppressive regimens. HHV-6 antibody titers for these three patients at 3 to 4 months posttransfusion (with documented CMV seroconversion and shedding) were 1:8,000, 1:64,000,

TABLE 1. Posttransplant HHV-6 antibody rise stratified by CMV status

CMV status	No.	No. (%) with HHV-6 antibody rise ^a
CMV seroconversion	32	24 (75)
Primary CMV infection ^b	3	1
CMV seronegative, uninfected	39	10 (26)
CMV seropositive	14	3 (21)
Total	88	38 (43)

^a Rise defined as an SA increase of twofold, or >0.4 , posttransplant.

^b Posttransplant serum obtained prior to CMV seroconversion.

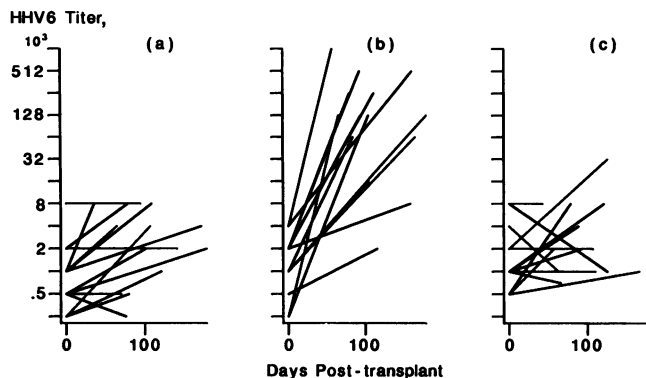


FIG. 2. Pre- and posttransplant HHV-6 ELISA titers (connected by straight lines) observed in 15 recipients each in those with no CMV infection (CMV seronegative) (a), primary CMV infection (b), and CMV reactivation and reinfection with viral shedding (c).

and 1:128,000. Thus, primary CMV infection unassociated with posttransplant immunosuppression also appears to be capable of inducing high anti-HHV-6 antibody.

Time course of HHV-6 antibody rise. The duration of the HHV-6 antibody rise associated with primary CMV infection was examined in three recipients for which long-term follow-up sera were available. HHV-6 antibody titers were 1:64,000 at 1,018 days, 1:512,000 at 685 days, and 1:32,000 at 856 days. These titers were unchanged, increased by one dilution, and decreased by four dilutions, respectively, when compared with values obtained from the same recipients within several months of transplant. Figure 3 shows the evolution of HHV-6 titers in two of these individuals over time. Titers rose rapidly to high levels by the time of onset of CMV shedding and were relatively sustained over the follow-up period.

Comparison of CMV and HHV-6 titers. Antibody titers to HHV-6 and CMV in 18 serum specimens with high single-dilution absorbances or measured titers of either antibody were compared (Fig. 4). The highest CMV titers tended to be seen in those with secondary CMV infection (reactivation and reinfection), whereas the highest HHV-6 antibody titers tended to be seen in those with primary CMV infection. In individual serum specimens, CMV and HHV-6 titers differed greatly.

DISCUSSION

Using an HHV-6 ELISA developed from glycine-extracted infected and uninfected cell antigens, we found a

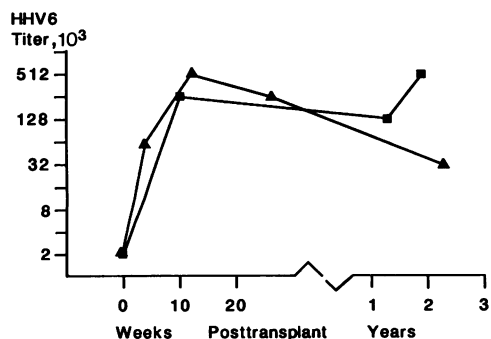


FIG. 3. Evolution of HHV-6 titers by ELISA during and after primary CMV infection in two recipients. Titers for each recipient are connected by straight lines. Both recipients shed CMV and had CMV seroconversion (4 and 10 weeks posttransplant).

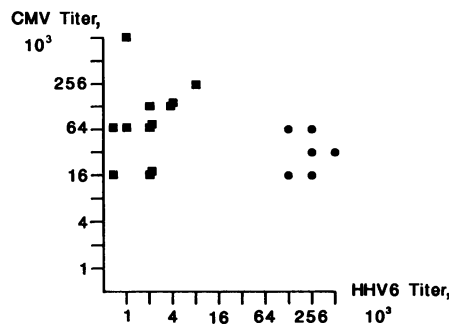


FIG. 4. Comparison of CMV and HHV-6 titers by ELISA in individual serum specimens. Each point represents one serum specimen. Sera were obtained from those with CMV reactivation and reinfection (■; secondary CMV) or those with recent primary CMV infection (●).

high seroprevalence of HHV-6 infection and observed that HHV-6 antibody rises associated with primary CMV infection occurred after transfusion or transplantation. Very high anti-HHV-6 titers were reached. These very high titers were not seen in sera from those uninfected with CMV or those undergoing CMV reactivation or reinfection and having high titers of CMV antibody.

The high base-line seroprevalence of HHV-6 infection is consistent with recent serologic surveys done by immunofluorescence assays or ELISA (15, 21, 23), although there appears to be no consensus regarding a precise criterion for seropositivity, and most normal adult populations appear to have a relatively low titer of HHV-6 antibody (21), similar to our pretransplant samples (Fig. 1). Variable sensitivity and interpretation of immunofluorescence assays is probably responsible for the wide divergence in seroprevalence reported to date. Whether the absence of HHV-6 antibody detectable by a particular assay implies susceptibility to primary infection can only be established by further serologic and virologic studies. Of eight recipients classified in this study as HHV-6 seronegative at the time of transplant, six became seropositive posttransplant (two with primary CMV infection).

During primary CMV infection, HHV-6 antibody levels rise rapidly and usually to very high levels. We generally observed HHV-6 antibody rises rather than seroconversions. Possible explanations for this phenomenon might include (i) cross-reactivity between CMV and HHV-6, (ii) increased reactivity to non-CMV antigens because of polyclonal B-cell activation during primary CMV infection analogous to that seen in Epstein-Barr virus mononucleosis, and (iii) a true increase in HHV-6 viral activity (reactivation) as a result of CMV effects on leukocytes (stimulation, suppression) or of viral interactions like those that have been postulated to occur between herpesviruses and human immunodeficiency virus (11).

In the absence of data from viral isolation studies (which are in progress), assessment of the relative likelihood of these possibilities must be somewhat tentative. Because of reports of HHV-6 antibody rises associated with primary CMV infection (12, 14, 17, 20), there has already been considerable interest in the issue of cross-reactivity between CMV and HHV-6. So far, direct evidence for cross-reactivity has not been reported. Absorption of sera with CMV- or HHV-6-infected cells removes antibody to the homologous virus, but not to the other (6, 23). Limited homology

among herpesvirus genomes is increasingly reported (9) and does not necessarily imply serologic cross-reactivity.

The data presented here, especially for individual serum samples that showed extreme differences in antibody titers to HHV-6 versus CMV, favor the conclusion that there is no broad cross-reactivity between the viruses. It is still possible that there is some limited component of the CMV antigen for which high antibody levels are produced only during the primary infection; this component may cross-react with HHV-6. However, because of the magnitude of CMV and HHV-6 titer differences, this antigen component would have to have been poorly represented in our present CMV ELISA antigen and well represented in the HHV-6 antigen. There is also little precedent for an extreme difference in reactivity to specific CMV proteins in primary versus secondary infections. The possibility of an HHV-6 antibody rise caused by polyclonal B-cell activation during primary CMV infection is also reduced by the height and apparent durability of the antibody response in the few long-term follow-up studies we have done.

Viral isolation studies in transplant recipients would be an important means of resolving some of the uncertainties encountered in performing HHV-6 serologic studies. The technical complexities and unknown sensitivities of the isolation procedures that have been described to date are an obstacle which could be overcome by newer rapid diagnostic techniques such as DNA amplification. However, DNA amplification must still be validated by comparing it with viral isolation. Meanwhile, a case report of HHV-6 isolation in a liver transplant recipient (24) suggests that, as with other herpesviruses, HHV-6 infection may complicate the post-transplant period.

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