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Evaluation of Immunoassays for Detection of Antibodies to Human Herpesvirus 7

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An enzyme immunoassay (EIA), an immunoblot assay (IB), and an indirect immunofluorescence assay were developed for detection of human herpesvirus 7 (HHV-7) antibodies in human serum. Cross-absorption studies with EIA or IFA using HHV-7 and human herpesvirus 6 (HHV-6) antigens indicated that most human sera contain cross-reactive HHV-6 and HHV-7 antibodies and that the degree of cross-reactivity varies between individual serum specimens. Inhibition of homologous antibody activity by absorption with heterologous virus ranged from 0 to 57% by EIA. However, for every sample tested, absorption with homologous virus removed more activity than did heterologous virus. An 89-kDa protein was identified as an HHV-7-specific serologic marker by IB. Activity to this protein was not removed by absorption with HHV-6 antigen. Of the three assays, the EIA was the most sensitive (94%), while the IB was the most specific (94%). Approximately 80% of specimens collected from German adults and children older than 2 years were positive for HHV-7 antibodies by these assays.

Human herpesvirus 6 variants A and B (HHV-6A and HHV-6B) are closely related to human herpesvirus 7 (HHV-7) on the basis of similar cell tropisms and growth characteristics (4, 5, 10, 18, 22), limited DNA cross-hybridization (4, 5, 10), and nucleotide and amino acid sequences (4, 7a). The following data suggest that HHV-6 and HHV-7 also share common antigens. (i) HHV-6 and HHV-7 can stimulate T-cell clones directed against the heterologous virus (26). (ii) Some monoclonal antibodies derived against HHV-6A or HHV-6B react weakly and variably with HHV-7-infected cells by immunofluorescence (3, 4, 9, 22, 23). (iii) Some monoclonal antibodies and hyperimmune mouse sera raised against HHV-7 react with HHV-6A- and HHV-6B-infected cells (9).

HHV-6B is the major cause of the childhood disease exanthem subitum (25), although primary HHV-7 infection has been associated with cases of exanthem subitum and febrile syndromes (2, 12, 17, 19, 20). HHV-6A has not been etiologically associated with any disease. Children seroconvert to HHV-7 independently of HHV-6 infection (20, 23), and the major immunogenic proteins of each virus differ in apparent size (9, 23). Thus, HHV-7 and HHV-6 are immunologically distinct. However, an exanthem subitum patient who seroconverted to HHV-6 also had a greater-than-fourfold rise in the titer of immunoglobulin G (IgG) to HHV-7 in serum (23). Other patients had smaller coincident rises. Tanaka et al. and Ueda et al. detected twofold or greater increases of HHV-6 IgG titer in sera from children with prior exposure to HHV-6 when these children had primary HHV-7 infection (19, 20). The increase in heterologous activity may be due to an anamnestic memory B-cell response to an antigen(s) common to both viruses (12), concurrent infection, or reactivation of one virus by the other (11). The degree to which cross-reactive antibody in human serum specimens contributes to reactivity in serologic assays must be evaluated to determine accurately the specificity of assays used for clinical diagnosis and seroepidemiology.

In this paper, we describe the development of an indirect immunofluorescence assay (IFA), an enzyme immunoassay (EIA), and an immunoblot assay (IB) for the detection of antibodies to HHV-7 in human serum. The ability of these assays to discriminate between HHV-7-specific antibody and cross-reactive HHV-6 antibodies in sera was examined by using a cross-absorption procedure. The sensitivity and specificity of these assays were then compared to evaluate their usefulness in seroepidemiologic studies.

MATERIALS AND METHODS

Cultivation of virus and cells. HHV-7 strain SB [HHV-7(SB)] (5) was propagated in human umbilical cord blood lymphocytes as previously described (5). HHV-6A strain U1102 [HHV-6A(U1102)] (8) and HHV-6B strain Z29 [HHV-6B(Z29)] (13), adapted for growth in the T-cell lines JJhan and Molt-3, respectively, were propagated in these cells in RPMI 1640 medium that contained 10% fetal calf serum and antibiotics. Human cytomegalovirus strain AD169 [HCMV(AD169)] was propagated in HLF cells grown in Eagle's minimal essential medium containing 10% fetal calf serum and antibiotics.

Serum samples. Four panels of serum samples were used. (i) One hundred umbilical cord blood samples were collected from consecutive deliveries at a private suburban hospital in Louisville, Ky. Most of the obstetrical patients from this hospital have private insurance (94%), they have a mean age of 28.5 years (range, 18 to 42 years), and 48% were HCMV seropositive (14). (ii) Fifteen serum samples well characterized as to their antibody activity to several herpesviruses were obtained from healthy adults. (iii) Two hundred seventy-eight serum samples from children and adolescents, ages 1 month to 20 years (median age, 5 years), residing in northern Germany, were collected. Information on the sex of 250 of these individuals was available: 100 females and 150 males. (iv) One hundred eighty serum samples from residents of Munich who ranged in age from 6 to 94 years (median age, 44 years) were collected. All samples were collected with permission from either the individual or a parent.

EIA. (i) Antigen preparation. Infected cells were harvested at the peak of

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cytopathic activity (approximately 5 to 7 days after infection), washed in phosphate-buffered saline (PBS), pH 7.4, and frozen overnight at −70°C at a concentration of 2×10^6 cells per ml in PBS containing 1% bovine serum albumin. Thawed cells were disrupted by using a cup horn sonicator (Heat Systems W 375; Farmingdale, N.Y.) at a setting of 4, 50% duty cycle, for two 10-s bursts. Uninfected-cell counterparts were prepared in the same manner.

(ii) Absorption procedure. Virus-infected cells were pelleted and suspended in PBS at a concentration of 1.5×10^6 cells per 50 μl, frozen at −70°C, thawed, and then sonicated for two 10-s bursts. Serum specimens were diluted 1:10 with absorbing antigen and incubated at 37°C for 1 h. The samples were centrifuged at $8,740 \times g$ for 2 min, and the supernatant was tested by EIA.

(iii) Assay procedure. Microplates were coated with infected and uninfected control antigens in alternating columns. The plates were air dried, fixed with 10% buffered neutral formalin (Fisher Scientific, Norcross, Ga.) for 10 min at room temperature, washed twice with PBS–0.5% Tween 20, and then stored in sealed plastic bags at −70°C until use. The optimal antigen concentration for each batch of cells (usually 10^5 cells per well) was determined on the basis of the activity of known HHV-7 and HHV-6 positive and negative control sera. Absorbed and unabsorbed samples were diluted to a final concentration of 1:150 in diluent containing 1% BSA, 0.05% Tween 20, and 1% (vol/vol) of an HLF cell extract (6). Diluted serum was added to two wells containing viral antigen and two wells containing control antigen; the plates were then incubated for 1 h by floating them in a 37°C water bath. The plates were then washed three times with PBS containing 0.05% Tween 20 and incubated with alkaline phosphatase-conjugated goat anti-human IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) for 1 h in a 37°C water bath. The plates were washed again and *p*-nitrophenyl-phosphate substrate was added, and after color development the reaction was stopped with 3 N NaOH and the plates were read at 405 nm with a microplate reader (Vmax; Molecular Devices, Palo Alto, Calif.). The net absorbance value for each sample was determined by subtracting the mean of the uninfected control antigen optical density (OD) value from the mean of the viral antigen OD value. Results from duplicate wells with a greater-than-30% difference in OD values were rejected. To control for lot-to-lot antigen variability, the final EIA value was determined by a calibration procedure that used positive and negative control serum samples on each plate and standard linear regression analysis (21). The percentage of activity remaining for each sample after absorption was calculated by using the final EIA values obtained in the presence and in the absence of an absorption step and is hereafter referred to as the percentage of residual antibody.

IFA. Virus-infected cells were washed once in PBS, aliquoted onto 12-well teflon-coated slides at a concentration of approximately 10^4 cells per well, air dried, and then fixed in acetone for 5 min at −20°C. Slides were incubated with sera diluted 1:11 at 37°C in a moist chamber for 1 h. After two washes in PBS for 5 min each, slides were incubated with fluorescein isothiocyanate-conjugated goat anti-human IgG (Kallestad, Austin, Tex.) as described above. The slides were again washed twice in PBS, air dried, and examined with a fluorescent microscope (Dialux 20-EB equipped with a 75-W xenon bulb and an epifluorescence filter system consisting of an excitation filter of 450 to 490 nm and a barrier filter of 515 nm).

IB. IBs were performed as previously described for HHV-6 (24). Briefly, proteins from filtered, pelleted, HHV-7(SB)-infected cell supernatant were separated on a sodium dodecyl sulfate–9% polyacrylamide gel by electrophoresis and then transferred to nitrocellulose. The nitrocellulose strips were treated with serum diluted 1:11 in 5% skim milk, and immunoreactive bands were visualized by using a commercial alkaline phosphatase kit according to the manufacturer's instructions (Immune-Blot assay kit; Bio-Rad).

RESULTS

The final OD values for 100 cord blood samples tested against HHV-7 were normally distributed over a wide range of values with no evidence of bimodality (Fig. 1); a clear cutoff value was thus not obtainable. The mean value from 10 tests using an adult serum identified as HHV-7 negative by IFA and IB was 0.020 (range, 0.009 to 0.034). Final OD values below 0.05 (mean × 2.5) were therefore considered to be negative. In support of 0.05 being an appropriate cutoff value, final OD values were all below 0.05 in a panel of sera collected from children 6 to 8 months old which were negative by IB and IFA and which had a less than 10% reduction in activity after HHV-7 absorption. These children are the appropriate age to be HHV-7 seronegative (7, 23).

The efficiency of the HHV-6 and HHV-7 absorbing antigens, described in Materials and Methods, was evaluated by EIA. HHV-6–HHV-7 dual-positive sera were absorbed with 0.25×, 0.5×, 1×, 2×, and 3× concentrations of homologous and heterologous absorbing antigens, and the percentage of residual

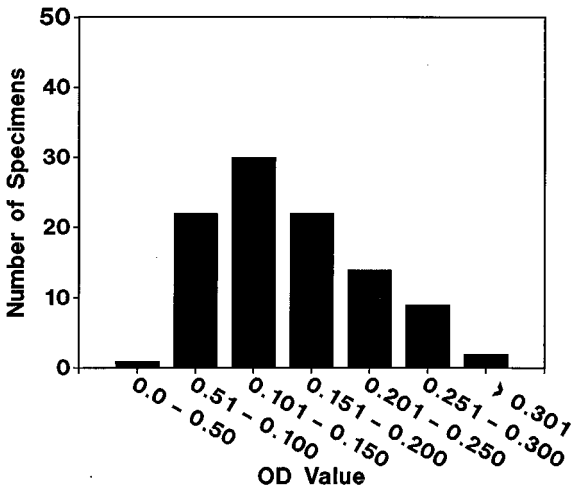

FIG. 1. Distribution of OD values obtained from 100 samples of cord blood plasma. Plasma samples were evaluated for reactivity to HHV-7 by EIA without absorption. The numbers of samples with final OD values falling within the indicated ranges are shown.

antibody activity was calculated. The percentage of residual antibody remained fairly constant at all absorbing-antigen concentrations (data not shown). The percentage of residual activity using the 1× concentration of absorbing antigen was 18% when the activity was absorbed with HHV-7 and tested against the HHV-7 EIA, and the percentage of residual antibody plateaued at 10 to 12% for the highest antigen concentration tested; therefore, 1× was used as the final concentration for all absorbing antigens to conserve material.

Antigenic cross-reactivity between HHV-6B and HCMV using monoclonal antibodies has been reported previously (1, 24); therefore, we performed cross-absorption studies with HHV-7 and HCMV to assess specificity. The HCMV EIA was performed as previously described (21). Three serum samples positive for both HCMV and HHV-7 were absorbed with homologous and heterologous antigens and then tested by EIA. The average percentages of residual antibody activity to HHV-7 and HCMV after absorption with heterologous antigen were 92 and 91%, respectively. Homologous antigen left less than 30% of activity in both cases (data not shown). On the basis of these results, we conclude that the potential for HCMV cross-reacting antibody in serum does not confound the results obtained for HHV-7 with our EIA.

To determine the range of levels of HHV-6 and HHV-7 cross-reacting antibodies in human sera, 10 samples with activity to both HHV-6 variants and HHV-7 were evaluated with absorption assays and EIA. The samples were absorbed with heterologous antigen, and the percentage of residual antibody was compared with that remaining after absorption with homologous antigen. Residual antibody values greater than 100% were plotted as 100% residual activity, indicating no heterologous absorption. HHV-7 absorption left from 53 to 82% of HHV-6A activity (mean, 60%) (Fig. 2A) and from 58 to 100% of HHV-6B activity (mean, 79%) (Fig. 2B). The percentage of residual activity after HHV-6A absorption was 46 to 100% of HHV-7 EIA activity (mean, 84%) (Fig. 2C), whereas HHV-6B absorption left from 66 to 100% of HHV-7 EIA activity (mean, 83%) (Fig. 2D). In comparison, the percentage of residual antibody ranges and means after homologous absorptions were as follows: HHV-6A, 14 to 40% (mean, 28%); HHV-6B, 7 to 43% (mean, 23%); HHV-7, 15 to 47% (mean, 30%). In all

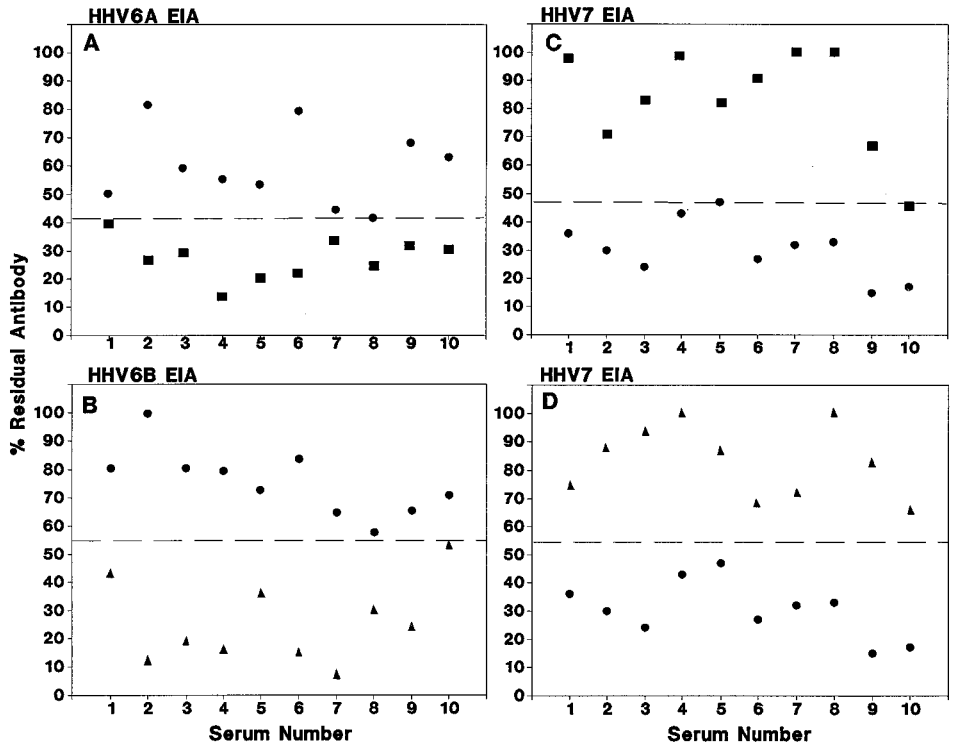

FIG. 2. Range of HHV-6- and HHV-7-cross-reactive antibody found in dual-positive sera. Ten sera reactive with both HHV-6 and HHV-7 were absorbed with HHV-6A (squares), HHV-6B (triangles), and HHV-7 (circles). The percentages of residual antibody activity to HHV-6A (A), HHV-6B (B), and HHV-7 (C and D) were determined by EIA. The dashed line is placed somewhat arbitrarily to delineate the difference in absorption efficiency between homologous virus and heterologous virus.

cases, the inhibition produced by absorption with homologous virus exceeded that produced by heterologous virus.

IB analysis revealed an immunodominant HHV-7-reactive protein of approximately 89 kDa (Fig. 3). Reactivity to the 89-kDa protein was not affected by preabsorption of the sera with uninfected-cell antigen or HHV-6B antigen but was completely removed after absorption with HHV-7. No reactivity was detected when the same sera were tested against uninfected-cell antigen. Reactivity with the 101-kDa immunodominant HHV-6B protein (101K) (24) was unaffected by absorption with HHV-7 and completely removed by absorption with HHV-6. Genetic analysis of the HHV-6A homolog of the 101K gene, p100, showed that these genes are virtually identical, and polyclonal rabbit sera raised against recombinant 101K reacted with HHV-6A and HHV-6B by IB (15, 16). Therefore, HHV-6B was used as the antigen source.

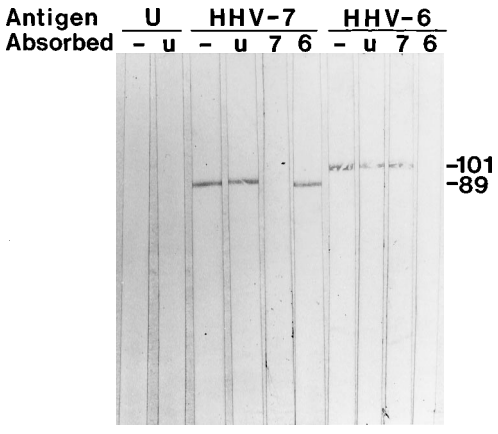

FIG. 3. Specificity of the 89-kDa HHV-7 immunoreactive protein. Nitrocellulose strips containing proteins from HHV-6B and HHV-7 infected-cell supernatants were reacted with a dual-positive serum without absorption (−) and after absorption with uninfected-cell antigen (u), HHV-7 antigen (7), and HHV-6 antigen (6) as described in Materials and Methods. U, strips with uninfected-cell proteins.

These results indicate that the major immunoreactive proteins of HHV-6 and HHV-7 are of distinct molecular weights, in agreement with the findings of Wyatt et al. (23), and are non-cross-reactive. Serum samples reacted in immunoblots with a protein of 89 kDa present in virions purified from infected-cell supernatants by equilibrium centrifugation in a sucrose gradient, indicating that this protein is most likely a virion component (data not shown). Reactivity with the 89-kDa protein was thus considered to be HHV-7 specific, and sera reactive to this protein were scored positive. The 278 serum samples collected in northern Germany were screened for antibody to HHV-7 by IB. Ninety percent of the IB-positive sera reacted with the 89-kDa band. No significant difference in seropositive rates was found between males and females in this group. Sera obtained from 200 individuals residing in Munich were tested for reactivity to the 89-kDa protein by IB. Seventy-eight percent of this population was HHV-7 seropositive (data not shown).

IB, EIA, and IFA were each performed on a set of 174 of the serum samples collected in northern Germany (Fig. 4). The basic results of the three assays agree. Approximately 65% of the sera of infants (ages, 1 to 2 months) reacted with HHV-7, most likely because of the presence of maternal antibody. The

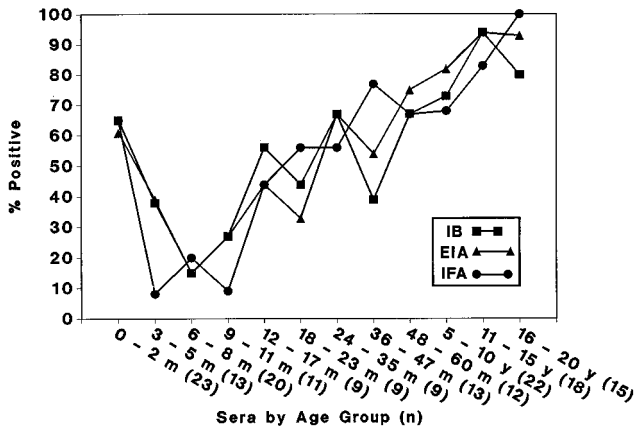

FIG. 4. Comparison of EIA, IB, and IFA for detection of HHV-7-specific antibody in human sera. A subset of 174 serum samples from the group of 276 samples collected from children and adolescents was evaluated for HHV-7 antibody by EIA, IB, and IFA. Sera were diluted 1:150 for the EIA and 1:11 for both the IFA and IB. Antigen absorption was performed for the EIA and IFA as described in the text.

percentage of positive specimens decreased to approximately 15% for infants up to 9 months of age and then steadily increased for older children. Approximately 80% of the samples from subjects up to 20 years of age were HHV-7 seropositive. The results obtained with the IFA show greater differences in some age groups with the IB and EIA; however, we have no reason to believe these differences are relevant. IFA nonspecific reactivity was often difficult to distinguish from true activity, even after absorption with uninfected-cell antigen or heterologous antigen (data not shown). Approximately 10% of samples scored as positive to HHV-7 in the IFA had activity removed after absorption with either HHV-6 or uninfected-cell antigen.

Since there is no standard assay to compare the results of these assays for sensitivity and specificity calculations, each sample was scored negative or positive on the basis of agreement between two or more tests, and this value was then used as the consensus standard. The sensitivity and specificity of the EIA, IB, and IFA were then calculated by comparing the results of each test from the 174 serum specimens with the consensus standard. The IB was the most specific assay for HHV-7 antibody detection (94%), with a sensitivity of 90%, while the EIA was the most sensitive (94%), with a specificity of 90%. The IFA had a sensitivity of 89% and a specificity of 92%. The three assays were in agreement for 75% of the samples, the EIA and IB were in agreement for 85%, the EIA and IFA were in agreement for 83% and the IB and IFA were in agreement for 82%.

DISCUSSION

HHV-6 (either variant) and HHV-7 commonly absorb 20 to 40% of the antibody activity to the reciprocal virus in human serum samples, thus directly demonstrating cross-reactivity. The amount of cross-reacting antibody varies considerably between individuals; however, the clear separation of absorption activities when homologous versus heterologous antigen is used (Fig. 2) and the lack of cross-reactivity between the major IB-reactive proteins indicates that this cross-reactivity is partial and type-specific responses can be detected. Thus, these viruses can be immunologically distinguished, as previously described (5, 18, 23).

The reported seroprevalence of HHV-7 in healthy adults ranges from 60 to 92% using methods that were not evaluated for the effects of cross-reactivity between HHV-7 and HHV-6 (4, 7, 23, 27). In preliminary experiments similar to those described in this paper, we detected HHV-7-reactive antibody in 96% of 158 serum samples obtained from cord blood and healthy adults in the absence of an absorption step and found that 92% of 36 HHV-7-reactive specimens remained positive for HHV-7 after the absorption step (data not shown). These results extrapolate to a seroprevalence of 88%, which represents a reduction of 8%, due to the absorption step. The differences between the rates obtained in the presence and in the absence of an absorption step indicate the need for an absorption step for accurate estimates of seroprevalence.

Ninety percent of the sera tested were reactive to an HHV-7 virion protein of approximately 89 kDa by IB. In a similar study with HHV-6, Yamamoto et al. (24) found that more than 90% of sera were reactive to a 101-kDa virion protein. We found no evidence for antigenic cross-reactivity between these proteins; thus, these proteins appear to be specific serologic markers of HHV-6 and HHV-7 infection and will be valuable tools in discriminating between HHV-7 and HHV-6 activity.

Since the results of this study indicated that the IB was the most specific assay for detection of HHV-7 antibody and our IB methods do not require an absorption step, the IB may be the most convenient assay to implement and score by using reactivity to the 89-kDa HHV-7 virion protein as a positive indicator. Sera that are negative or nonreactive to the 89-kDa protein can then be tested by EIA, which is slightly more sensitive than the IB but requires an absorption step for specificity. Although the overall IFA results were in close agreement with the EIA and IB results, individual samples were in disagreement slightly more frequently with this test. In addition to the reader subjectivity inherent in interpreting the results of an IFA, nonspecific reactivity varies with different cord blood lymphocyte cultures used for antigen production, and this may contribute to the difficulty in distinguishing nonspecific activity from true activity. We therefore believe that the IFA is a less robust assay. A cell line-adapted isolate of HHV-7 might decrease the nonspecific activity seen with the IFA and help increase the specificity of the EIA. The development of such a reagent is in progress. The cross-absorption EIA and the IB will be most useful in evaluating the age-specific prevalence of HHV-7 in various populations and in studying the specific HHV-7 serologic response in various disease states.

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