Elucidation of the Cross-Reactive Immunoglobulin M Response to Human Herpesviruses 6 and 7 on the Basis of Neutralizing Antibodies

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Human herpesvirus 6 (HHV-6) (5, 9) and HHV-7 (3) have been discovered recently and are T-lymphotropic viruses that have been classified as betaherpesviruses. Clinically, HHV-6 and HHV-7 are the common etiologic agent of exanthem subitum (ES) (4, 10, 14, 13). Serologic studies have shown that the seroprevalence of HHV-6 and HHV-7 infections is very high throughout the world and that almost all people are exposed first to HHV-6 and second to HHV-7 in childhood (4, 6). HHV-6 and HHV-7 are closely related in terms of genome organization and sequence, and the cross-reactive antibody responses between HHV-6 and HHV-7 have been reported by using immunofluorescent techniques (6). The neutralizing (NT) antibody response is thought to be important in preventing infection with these viruses. Therefore, we established a dot blot method for detecting NT antibody responses to each virus (12). Recently, we have shown that NT antibody responses to HHV-6 and HHV-7 are specific to each virus and do not cross-react in healthy individuals (17). We took advantage of this and used NT antibody response to estimate the time of seroconversion to each virus and examined the pattern of humoral immune response, especially the immunoglobulin M (IgM) response, against each virus antigen in the natural course of infection with HHV-6 and HHV-7. In children who experienced HHV-6 infection first, followed by HHV-7 infection, the IgM response at the first HHV-6 infection was directed only against HHV-6, while no IgM response was directed against HHV-7 at the second HHV-7 infection. In contrast, in children who experienced HHV-7 infection first, followed by HHV-6 infection, the IgM response at the first HHV-7 infection was directed not only against HHV-7 but also against HHV-6. These data suggest that cross-reactive responses to heterologous viruses should be taken into consideration when making a diagnosis based on IgM antibody.

Human herpesvirus 6 (HHV-6) (5, 9) and HHV-7 (3) have been discovered recently and are T-lymphotropic viruses that have been classified as betaherpesviruses. Clinically, HHV-6 and HHV-7 are the common etiologic agent of exanthem subitum (ES) (4, 10, 14, 13). Serologic studies have shown that the seroprevalence of HHV-6 and HHV-7 infections is very high throughout the world and that almost all people are exposed first to HHV-6 and second to HHV-7 in childhood (4, 6). HHV-6 and HHV-7 are closely related in terms of genome organization and sequence, and the cross-reactive antibody responses between HHV-6 and HHV-7 have been reported by using immunofluorescent techniques (6). The neutralizing (NT) antibody response is thought to be important in preventing infection with these viruses. Therefore, we established a dot blot method for detecting NT antibody responses to each virus (12). Recently, we have shown that NT antibody responses to HHV-6 and HHV-7 are specific to each virus and do not cross-react in healthy individuals (17). We took advantage of this and used NT antibody response to estimate the time of seroconversion to each virus and examined the pattern of humoral immune response against each virus antigen in the natural course of infection with HHV-6 and HHV-7. In this report, we thought it important to determine the degree of immunological cross-reactivity between HHV-6 and HHV-7 based on the NT antibodies. Therefore, we established an enzyme-linked immunosorbent assay (ELISA) method for detecting the immunoglobulin G (IgG) and IgM antibodies, in addition to the IF and the NT antibodies. We collected 138 serum samples sequentially from 36 pediatric patients (up to 5 years old) diagnosed with exanthem subitum (ES) or without ES and subjected them to each assay to detect these four different antibodies. We plotted the data obtained from each assay on a chart for each child and sorted these charts into two groups: children who experienced HHV-6 infection first, followed by HHV-7 infection, and children who experienced HHV-7 infection first, followed by HHV-6 infection. We then analyzed these chart patterns based on the categories of the immunological cross-reactivity between antigens and antibodies in heterologous viruses like HHV-6 and HHV-7.

Here, we describe the following. (i) The NT antibody response is specific among the humoral immune responses between HHV-6 and HHV-7. (ii) The IgM antibody response is not applicable to the detection of primary infection with these viruses.

MATERIALS AND METHODS

Serum samples. One hundred and thirty-eight serum samples were sequentially obtained from 36 pediatric patients (up to 5 years old) diagnosed with ES or without ES under informed consent from the mothers (Shingu Municipal Hospital, Shingu, Japan).

Host cells. Fresh cord blood mononuclear cells (CBMCs) were obtained under agreement with informed consent and prepared by centrifugation through a Ficoll-Conray gradient from heparinized blood and cultured for 3 days in RPMI 1640 medium containing 10% heat inactivated fetal bovine serum, recombinant human interleukin-2 (0.1 U/ml; Gibco BRL Life Technology Inc., Grand Island, N.Y.) and phytohemagglutinin (5 g/µl; Sigma Chemical Co., St. Louis, Mo.) at 37°C in a 5% CO2 incubator. After 3 days, the CBMCs were infected with the viruses and cultured for different experiments in RPMI 1640 medium containing the above-listed reagents.

Preparation of virus stocks. The Z29 strain of HHV-6B and the SB strain of HHV-7 used throughout this study were prepared by Yoshida et al. (16). The cells infected with each virus were cocultivated with uninfected cells at a ratio of 1:5 for 7 days. Virus stocks were prepared by centrifugation of culture fluids at 2,000 × g for 10 min and stored at −80°C. Titration of virus stocks was performed by an end point dilution method using a dot blot assay (15, 16). In brief,
25 μl of CBMCs (adjusted to 5 × 10⁶ cells/ml) was divided into each well of a 96-well U-bottom microtiter plate. The CBMCs were then infected with 25 μl of virus preparation (in 10-fold dilution series) and incubated for 7 days at 37°C in a 5% CO₂ incubator. After incubation, the supernatant medium was removed and the cells were washed with phosphate-buffered saline (PBS) (pH 7.4). A dot blot assay was performed to detect viral antigens as described below. After treatment with Lumi-Phos 530, the membrane was exposed to Fuji RX-U film. The 50% cell culture infectious dose was calculated according to the method of Reed-Muench (8).

MAbs. Monoclonal antibodies (MAb) to HHV-6 and HHV-7 were established in our laboratory (12) and characterized by radioimmunoprecipitation. For monitoring HHV-6 replication, the MAb TK-2, which recognizes a 135-kDa late polypeptide, was used. The MAb IK-3 reacts to both variants of HHV-6. For HHV-7, the MAb IK-3, which recognizes a 125-kDa corresponding polypeptide, was used. The MAb IK-3 is specific to HHV-7.

**IF antibody assay.** An indirect IF antibody assay, described elsewhere (15), was performed to determine either the anti-HHV-6 or -7 antibody titer of human serum samples. In brief, HHV-6- or -7-infected CBMCs were mounted on a 14-well slide, dried, and kept at −20°C for 30 min and washed in PBS for 15 min three times. Fluorescein isothiocyanate-conjugated goat anti-human IgG F(ab')₂ antibody (Cappel, West Chester, Pa.) was diluted 1:100 in PBS and added to each well of the slide. The slides were incubated at 37°C for 30 min and washed in PBS for 15 min three times. Fluorescein isothiocyanate-conjugated goat anti-human IgG F(ab')₂ antibody (Cappel, West Chester, Pa.) was diluted 1:100 in PBS and added to each well of the slide. After a 30-min incubation at 37°C, the slides were washed three times in PBS for 15 min and examined with a fluorescence microscope. The end point of the positive fluorescence was determined visually by low magnification, and the IF antibody titer was calculated as the reciprocal of the serum dilution.

**NT antibody assay.** A dot blot method described elsewhere (12) was performed for determining the titers of NT antibody to HHV-6 and -7. In brief, 25-μl aliquots of serial twofold serum dilutions or medium without serum (control) were prepared in each well of a 96-well U-bottom microtiter plate and mixed with equal volumes of the strains of HHV-6 or HHV-7, containing 2 × 10⁷ 50% tissue culture infective doses per ml. After the microtiter plate was incubated for 1 h at 37°C in a 5% CO₂ incubator, 50 μl of CBMCs (2.5 × 10⁶ cells/ml) was added to each well and centrifuged at 800 × g for 1 h (7). For removing the added sera, 100 μl of medium was added to each well of the plate and centrifuged at 800 × g for 5 min, and about 180 μl of the supernatant was aspirated. Two hundred microliters of medium was added to each well of the plate, and the microtiter plate was incubated for 7 days at 37°C in a 5% CO₂ incubator.

To monitor the virus growth in each well, a dot blot antigen detection assay was performed as described previously (12, 15). In brief, after 7 days of incubation the microtiter plate was centrifuged at 500 × g for 10 min. The supernatant medium was removed, and the cells were washed with PBS (pH 7.4). One hundred and eighty microliters of lysis buffer (20 mM Tris, 0.5 M NaCl, and 0.5% Nonidet P-40, pH 7.5) was added to each well. The membrane was washed and incubated with anti-mouse IgG alkaline phosphatase conjugate (Sigma Chemical Co.). Finally, the membrane was treated with Lumi-Phos 530 (Boehringer Mannheim Biochemical, Indianapolis, Ind.) through a dot blot apparatus. The nylon membrane was washed, treated with blocking solution, and incubated with one of the MAb described below overnight at 4°C. The membrane was washed and incubated with anti-mouse IgG alkaline phosphatase conjugate (Sigma Chemical Co.). The membrane was treated with Lumi-Phos 530 (Boehringer Mannheim Corporation) and exposed to Fuji RX-U film.

**Antigen preparation for ELISA method.** The CBMCs infected with HHV-6 (Z9 strain) or HHV-7 (SB strain) were cultured for 5 to 7 days and harvested in 15-ml conical tubes when the numbers of IF-positive cells had reached almost 80%. The infected cells were pelleted down by centrifugation at 500 × g for 10 min and washed with PBS (including Mg²⁺ and Ca²⁺) ([PBS(-)]) (pH 7.4) followed by centrifugation. The lysis buffer (20 mM Tris, 0.5 M NaCl, and 0.5% Nonidet P-40, pH 7.5) was added to each pellet at four times the pellet volume. The uninfected CBMCs were cultured for 6 days and prepared for control antigen in the same manner as described above.

**ELISA.** The wells of a microplate (Immunoplate II; Nunc, Aarhus, Denmark) were coated with 50 μl of appropriate dilutions in PBS(-) of each virus or control antigen overnight at 4°C. After coating, the wells were washed and blocked for 30 min at room temperature by adding 200 μl of the blocking solution [PBS(-) containing 5% skim milk]. After discarding the blocking solu-
tion, 50 µl of serum diluted (1:100 for IgG and 1:25 for IgM) in the blocking solution was added to the wells of the plate and incubated at 37°C for 2 h. The plate was washed five times with 200 µl of the blocking solution. Then, 50 µl of alkaline phosphatase-conjugated goat anti-human IgG (γ-chain specific) F(ab’)2 fragment (Sigma Chemical Company) or alkaline phosphatase-conjugated goat anti-human IgM (μ-chain specific) F(ab’)2 fragment (Sigma Chemical Company) diluted in the blocking solution (1:1,000) was added to each well of the plate. After 2 h of incubation at 37°C, the plate was washed five times with 200 µl of the blocking solution and twice with PBS(+) . Seventy-five microliters of substrate, p-nitrophenylphosphate in diethanolamine buffer (alkaline phosphatase substrate kit [Bio-Rad]), was added to each well of the plate and incubated at 37°C for 1 h. The reaction was then stopped by adding 75 µl of 0.4 M NaOH, and the absorbance of each well at 405 nm was read with a microplate reader (model 550; Bio-Rad). The results (net absorbance) were expressed as the absorbance reading of the each viral-antigen-coated well minus the absorbency reading for the wells coated with control antigen.

For the IgG antibody, the absorbance readings (mean ± standard deviation [SD]) for HHV-6 and HHV-7 obtained from about 20 pediatric negative reference serum samples were 0.052 ± 0.067 and 0.027 ± 0.034, respectively. Studied samples with net absorbance readings of ≥3 SDs above the mean absorbance of 20 negative reference serum samples were considered reactive for anti-HHV-6 or HHV-7 antibody. The cutoff values for HHV-6 and -7 were determined with absorbance readings of 0.26 and 0.13, respectively. For the IgM antibody, the absorbance readings (mean ± SD) for HHV-6 and HHV-7 were 0.049 ± 0.030 and 0.033 ± 0.027, respectively, and the cutoff values for HHV-6 and -7 were determined with absorbance readings of 0.14 and 0.12, respectively.

RESULTS

Prevalence of NT antibodies against HHV-6 and HHV-7 in childhood. First, to assess the titers of NT antibody against each virus, we examined by a dot blot method 138 serum samples which were sequentially obtained from 36 pediatric patients (up to 5 years old) diagnosed with ES or without ES. We analyzed the time of seroconversion to each virus in the natural course of infection with HHV-6 and HHV-7, and the results are shown in Fig. 1. The titers of NT antibody against HHV-6 increased from about 6 months after birth and reached a peak value up to 1.5 years old, and none were negative after 6 months. This means the time of seroconversion to HHV-6 mainly occurs from the age of about 6 months to 2 years. In contrast, the titers of NT antibody against HHV-7 increased from the age of about 1 year but were still negative in many samples up to the age of about 2 years. This means the times of seroconversion to HHV-7 occur later and that patients remain negative for HHV-7 longer than they do for HHV-6. It is interesting that the titers of NT antibody against HHV-6 are higher than those against HHV-7 in the primary infection of childhood, because we have reported that the titers of NT antibody against HHV-7 are higher than those against HHV-6 for individuals of all ages (17).

Immunological cross-reactivity between antigen and antibody in heterologous viruses like HHV-6 and HHV-7. First, we subjected all the samples obtained sequentially to assessments of IF, ELISA (IgG), and ELISA (IgM) antibodies. We plotted these data, including NT antibody titers, on a chart for each child, as shown in Fig. 3 to 6. We then used the NT antibody to estimate the time of seroconversion to each virus and analyzed the cross-reactivity of NT, IF, IgG, and IgM antibody...
responses to each virus in the natural course of infection with HHV-6 and HHV-7. The categories of the cross-reactivity between antigen and antibody in heterologous viruses like HHV-6 and HHV-7 are summarized in Fig. 2A. Category I shows the cross-reaction that the antibodies obtained by one virus infection are cross-reacting to another antigen or not, and vice versa. Category II shows the cross-reaction obtained when one virus infection occurs first and is followed by the other virus infection and whether the levels of antibody titers against the former virus change or not at the time of seroconversion to the latter virus, and vice versa. We demonstrated the cross-reactivity of humoral antibody responses between HHV-6 and HHV-7 based on these categories.

(i) Immunological cross-reactivity in children who experienced HHV-6 infection first followed by HHV-7 infection. First, we analyzed the cases of children who experienced HHV-6 infection first in which the time of seroconversion to HHV-6 was clear but HHV-7 infection was still negative (Fig. 3). All of these cases belonged to category I. The NT, IF, and IgG antibody responses clearly showed the time of seroconversion to HHV-6, and the titers of NT and IgG antibodies against comparable HHV-7 antigen did not change at the time of seroconversion to HHV-6. These means that the NT and IgG antibody responses to HHV-6 do not cross-react with HHV-7. The IgM antibody response was clearly detected in all cases just before the time of seroconversion to HHV-6.

Second, we analyzed the cases of children who experienced HHV-6 infection first in which the time of seroconversion to both viruses was clear (Fig. 4). All of these cases belonged to both category I and category II, which are comparable to the times just before and after the seroconversion to HHV-7 in these charts. In category I for all of these cases, the NT and the IgG antibody responses were also not cross-reactive as shown in Fig. 3. However, the IF antibody response to HHV-6 exhibited a marked cross-reaction with the HHV-7 antigen in the case of patient 4. In category II, namely, when the HHV-6 infection occurred first and was followed by the HHV-7 infection, the titers of NT antibody against the HHV-6 antigen did not change at the time of seroconversion to HHV-7, except in the case of patient 32. In the case of patient...
FIG. 4. HHV-6 infection followed by HHV-7 infection. Seroconversion against HHV-6 is positive and that against HHV-7 is also positive. Symbols: *, cross-reaction; shaded ellipse encircled with a dashed line, IgM positive samples. OD, optical density.
it was difficult to direct the NT antibody response to the HHV-6 antigen to either the reactivation of HHV-6 or the cross-reaction with HHV-6 antigen, because the change in titers of the NT antibody were small and likely encompassed errors. The titers of the IF and IgG antibodies changed in almost all cases. These findings mean that the NT antibody response to HHV-6 is not cross-reactive to the HHV-7 antigen but that the IF and IgG antibody responses to HHV-6 are more cross-reactive to the HHV-7 antigen. The IgM antibody responses to HHV-6 were detected in two cases, patients 32 and 37, just before the time of seroconversion to HHV-6. In contrast, it was noticeable that the IgM antibody response to HHV-7 did not detect anything at the time of seroconversion to HHV-7.

Third, we analyzed the cases of children who experienced HHV-6 infection first in which the time of seroconversion to HHV-6 was not clear but that to HHV-7 was clear (Fig. 5). All of these cases belonged to category II. As with the results described above, the titers of NT antibody against the HHV-6 antigen did not change at the time of seroconversion to HHV-7. The titers of the IF and the IgG antibodies changed markedly in all cases. It was also interesting that none of the IgM antibody responses to HHV-7 were detected at the time of seroconversion to HHV-7.

These findings suggested that the NT antibody response to HHV-6 is specific and does not cross-react with the HHV-7 antigen, but the IF and IgG antibody responses to HHV-6 cross-react with the HHV-7 antigen. Also, the IgM antibody response to HHV-6 is applicable in identifying primary infection with HHV-6, but that to HHV-7 is not useful in identifying primary infection with HHV-7 in the natural course of infection with HHV-6 first and HHV-7 second.

In all of the cases in Fig. 3, 4, and 5, it was generally observed that the titers of IgG antibody against HHV-7 were considerably lower than those against HHV-6.

(ii) Immunological cross-reactivity in children who experienced HHV-7 infection first followed by HHV-6 infection.

In the natural course of infection with HHV-6 and HHV-7, HHV-6 infection occurs first and is followed by HHV-7 infection generally. Therefore, it is difficult to collect many serum samples from patients who experienced HHV-7 infection first. We collected three samples from patients diagnosed with ES for which the time of seroconversion to first HHV-7 infection was clear. These three samples belonged to category I or cat-
Category II, which are comparable to the times just before or after the seroconversion to HHV-6 in these charts (Fig. 6). The periods before the seroconversion to the second infection with HHV-6 belonged to category I in all cases. In these periods, none of the NT, IF, and IgG antibody titers against HHV-6 antigen changed at the time of seroconversion to HHV-7. These facts suggested that none of these antibody responses to HHV-7 are cross-reactive to the HHV-6 antigen. In category II, namely, when the HHV-7 infection occurs first and is followed by HHV-6 infection (patients 11 and 20), the titers of NT antibody against the HHV-7 antigen did not change at the time of seroconversion to HHV-6. These findings revealed that none of these antibody responses to HHV-7 are cross-reactive to the HHV-6 antigen. In category II, when the HHV-7 infection occurs first and is followed by HHV-6 infection (patients 11 and 20), the titers of NT antibody against the HHV-7 antigen did not change at the time of seroconversion to HHV-6. These findings revealed that none of these antibody responses to HHV-7 are cross-reactive to the HHV-6 antigen. Category II, which are comparable to the times just before or after the seroconversion to HHV-6 in these charts (Fig. 6). The periods before the seroconversion to the second infection with HHV-6 belonged to category I in all cases. In these periods, none of the NT, IF, and IgG antibody titers against HHV-6 antigen changed at the time of seroconversion to HHV-7.

These facts suggested that none of these antibody responses to HHV-7 are cross-reactive to the HHV-6 antigen. In category II, namely, when the HHV-7 infection occurs first and is followed by HHV-6 infection (patients 11 and 20), the titers of NT antibody against the HHV-7 antigen did not change at the time of seroconversion to HHV-6. These findings revealed that none of these antibody responses to HHV-7 are cross-reactive to the HHV-6 antigen. In category II, when the HHV-7 infection occurs first and is followed by HHV-6 infection (patients 11 and 20), the titers of NT antibody against the HHV-7 antigen did not change at the time of seroconversion to HHV-6. These findings revealed that none of these antibody responses to HHV-7 are cross-reactive to the HHV-6 antigen.

These data suggest that the IgM antibody response to HHV-7 does not identify the primary infection of HHV-7 because of the IgM antibody cross-reaction with HHV-6, even when the HHV-7 infection occurs first, which is rare.

Cross-reactivity of IgM antibody. The IgM antibody responses between HHV-6 and HHV-7 are summarized in Fig. 7. The IgM response at the first HHV-6 infection was directed only to HHV-6, while no IgM response was detected against HHV-7 at the second HHV-6 infection. In contrast, in children who experienced HHV-7 infection first followed by HHV-6 infection, the IgM response at the first HHV-7 infection was directed not only against HHV-7 but also against HHV-6. These data suggest that the cross-reactive response between HHV-6 and HHV-7 should be taken into consideration in diagnoses based on the IgM antibody.

We also summarized the cross-reaction with the NT antibody, IF antibody, and ELISA(IgG) antibody responses between HHV-6 and HHV-7 in Fig. 2B.
**DISCUSSION**

It is well-known that the immune response is cross-reactive between HHV-6 and HHV-7 based on the antibody titers determined by IF or ELISA methods (6). Serologic studies have also shown that almost all individuals are exposed first to HHV-6 and second to HHV-7 in childhood, acquire the NT antibodies against these viruses, and keep them at high levels for 2 or 3 decades after primary infection (6). Recently, we have reported that the immunological cross-reactivity between HHV-6 and HHV-7 is not exhibited in the NT antibodies detected by a dot blot method (17).

In this report, we intended to further investigate more precisely the degree of the immunological cross-reaction between HHV-6 and HHV-7. We confirmed again in this report that the NT antibody response is specific and useful in determining the time of seroconversion. We categorized the cross-reaction between antigen and antibody in heterologous viruses such as HHV-6 and HHV-7. According to these categories, we analyzed the immune response patterns between HHV-6 and HHV-7 in each child. Category I means the antibodies obtained by HHV-6 infection cross-react with HHV-7 antigen or not and vice versa. Category II means the HHV-6 infection occurs first and is followed by HHV-7 infection, and the titers of antibody HHV-6 antigen change or not at the time of seroconversion to HHV-7 and vice versa. In category I, the NT and IgG antibody responses were not cross-reactive between HHV-6 and HHV-7, but the IF antibody response was more cross-reactive. In category II, the NT antibody response is only specific to each virus independent of the course of infection with HHV-6 and HHV-7. The cross-reaction between HHV-6 and HHV-7 was observed in the IF and IgG antibody responses depending on the course of infection with each virus resulting when HHV-6 infection occurs first and is followed by HHV-7 infection. These findings suggested that the humoral immune response to HHV-6 is more cross-reactive than that to HHV-7. We are interested in the immunological cross-reactivity between HHV-6 and HHV-7 in that HHV-6 infection generally occurs first in the natural course of infection in childhood and acquires the cross-reactive antibodies against HHV-7; nevertheless, the NT antibody response, which has an important role in preventing the infection, is not cross-reactive. These findings suggest that the NT epitopes of these two viruses are apparently distinct.

Generally, the IgM antibody response is thought to be useful in diagnosing the primary infection and the reactivation of infection with several viruses. This is the first report on the cross-reactivity of IgM antibody response between HHV-6 and HHV-7. These findings suggested that the IgM antibody response is rather useless in the identification of primary infection with HHV-6 and HHV-7. We should consider these disadvantages when the IgM antibody responses to HHV-6 and HHV-7 are used for the diagnosis of the primary infection. Next, we would like to investigate the cross-reactivity of the IgM antibody response in cases of the reactivation of HHV-6 associated with drug induced hypersensitivity syndrome (1, 2, 11).

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