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Avidity of Immunoglobulin G Directed against Human Cytomegalovirus during Primary and Secondary Infections in Immunocompetent and Immunocompromised Subjects

TIZIANA LAZZAROTTO, PATRIZIA SPEZZACATENA, PAOLA PRADELLI, DAVIDE A. ABATE, STEFANIA VARANI, AND MARIA PAOLA LANDINI*

Department of Clinical and Experimental Medicine, Division of Microbiology, University of Bologna, Bologna, Italy

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Diagnosis of primary human cytomegalovirus (HCMV) infection is accomplished exclusively by serologic testing. Among the possible methods, the determination of immunoglobulin G (IgG) avidity is one of the least explored. In this work, we used a commercially available kit to test anti-HCMV IgG avidity in 336 serum samples from pregnant women and transplant recipients undergoing virologically proven HCMV primary or nonprimary infections and from latently infected blood donors. Our results demonstrate that the anti-HCMV IgG avidity test differentiates primary from nonprimary HCMV infections in both pregnant women and solid organ transplant recipients. In fact, 88.6% of primary infections and no secondary infections showed low-avidity IgG to HCMV. In particular, low IgG avidity is a marker of primary infection for 18 to 20 weeks after onset of symptoms in both immunocompromised and immunocompetent subjects.

Human cytomegalovirus (HCMV) is a widespread human pathogen that is rarely pathogenic in healthy adults but is associated with several diseases in immunocompromised individuals, such as transplant recipients and people with AIDS. Furthermore, HCMV is the most common cause of congenital infection in humans. As infections are either asymptomatic or accompanied by symptoms that are not specific to HCMV, laboratory techniques are the sole means of diagnosing acute HCMV infection. Diagnosis of HCMV infection can be obtained by direct demonstration of the virus or virus components in pathological materials or indirectly through serologic testing (for a review, see reference 5). Primary infections have a greater clinical impact than recurrent infections or exogenous reinfections (nonprimary infections). In fact, intrauterine primary infections are second only to Down's syndrome as a known cause of mental retardation, while less severe complications are the result of nonprimary infections (for reviews, see references 4 and 10). This is also true for immunocompromised patients, in whom primary infections are often accompanied by symptoms while nonprimary infections are usually asymptomatic. Diagnosis of primary HCMV infection is accomplished exclusively by serological methods, i.e., demonstration of the appearance of antibodies to HCMV in a previously seronegative subject. HCMV-specific immunoglobulin M (IgM) is a sensitive indicator of an ongoing or recent infection. However, it is not a specific indicator of primary infection, as it is often produced during active nonprimary infections (1, 12, 20).

Another serological procedure that has been shown to be useful for identification of primary infections is the determination of IgG avidity, this term being indicative of low functional affinity of IgG class antibody. During the first weeks following primary infection, antibodies show low avidity. They progressively mature, acquiring higher avidity. Determination of IgG avidity for distinguishing primary from nonprimary infections has been established for several viruses, such as rubella virus (9), varicella-zoster virus (11), human herpesvirus type 6 (25), hepatitis C virus (26), and measles virus (22). Some data on the use of IgG avidity to identify a primary HCMV infection are also present in the literature. Of particular interest is the work of Boppana and Britt, in which examination of anti-HCMV antibody avidity revealed that the majority of HCMV-infected mothers who did not transmit the infection had high antibody avidity (3).

However, follow-up of patients was done only for transplant recipients and not for immunocompetent subjects such as pregnant women (17, 18, 24).

In this work, we used a commercially available kit to test anti-HCMV IgG avidity in 336 serum samples of which 240 were sequential serum samples from 42 pregnant women and 15 transplant recipients undergoing virologically proven HCMV primary or nonprimary infections and 96 were serum samples from long-term seropositive blood donors.

MATERIALS AND METHODS

Serum samples. (i) Blood donors. Ninety-six serum samples from 96 blood donors were used. Sixty-four serum samples were IgG and IgM negative for HCMV as judged by both conventional enzyme-linked immunosorbent assay (ELISA) and Western blotting (WB). Thirty-two serum samples were IgG positive and IgM negative as judged by both conventional ELISA and WB.

(ii) Pregnant women. Sixty-six sequential serum samples from 28 pregnant women undergoing primary HCMV infections and 44 serum samples from 14 pregnant women undergoing nonprimary HCMV infections were obtained from our diagnostic laboratory. Each woman was followed up for a minimum of 1 week to a maximum of 34 weeks.

(iii) Transplant recipients. Seventy-four sequential serum samples from seven transplant recipients (three kidney and four heart transplants) undergoing primary HCMV infections and 56 serum samples from eight transplant recipients (six heart and two kidney transplants) undergoing nonprimary HCMV infection were obtained from our diagnostic laboratory. Each patient was followed up for a minimum of 1 week to a maximum of 34 weeks after transplantation.

Diagnosis of active HCMV infection. (i) Pregnant women. In pregnant women, the presence of an active HCMV infection was documented by one or more of the following parameters: virus isolation from urine, saliva, or blood and/or demonstration of seroconversion for HCMV-specific antibody. Virus isolation was carried out by the shell vial procedure (7). Inoculated human embryo fibroblasts were fixed 24 to 48 h after inoculation and stained in an indirect

* Corresponding author. Mailing address: Department of Clinical and Experimental Medicine, Division of Microbiology, St. Orsola General Hospital, Via Massarenti no. 9, 40138 Bologna, Italy. Phone: 39.51.341652. Fax: 39.51.341632. E-mail: TEL1811@IPERBOLE.BOLOGNA.IT.

immunofluorescence assay using a monoclonal antibody reacting with the HCMV-IE1/IE2 gene product (E13 from Argene, Varilhes, France). Seroconversion was documented by a commercially available enzyme immunoassay (EIA) (Enzygnost Anti-HCMV/IgG EIA Alpha Method; Behring AG, Marburg, Germany).

(ii) Immunocompromised patients. For immunocompromised patients, antigenemia and/or PCR on polymorphonuclear leukocytes (PMNL) was performed. The presence of HCMV pp65 (ppUL83) in PMNL (antigenemia) of immunocompromised patients was determined as originally described by van der Biji et al. (23) and modified by Revello et al. (21), by using an HCMV pp65-specific pool of two monoclonal antibodies (1C3 and AYM-1 from Argene) in indirect immunofluorescence tests. The presence of the HCMV genome in PMNL of immunocompromised patients was detected by PCR. Aliquots of 1.5×10^5 PMNL were used, and the PCR was carried out as previously described (15). The HCMV-specific primers from the fourth exon of the HCMV immediate-early gene (the *Eco*RI J fragment of the AD169 strain), corresponding to nucleotides 1767 to 1786 and nucleotides 1894 to 1913 were used. A third oligonucleotide, consisting of nucleotides 1807 to 1847, which was complementary to the antisense DNA strand in the region between the binding sites of the other oligonucleotides was used for hybridization (15). The amplification reaction was carried out in a DNA thermal cycler (Perkin-Elmer/Cetus, Norwalk, Conn.) for 35 cycles. Each sample was initially subjected to analysis with oligonucleotide primers GH26 and GH27 (Perkin-Elmer/Cetus) (20 pmol of each), with a conserved region of the HLA-DQa locus, to determine the capacity of DNA to be amplified. The sensitivity (one viral genome) and specificity of the PCR were determined as previously described (15).

Determination of the time of primary HCMV infection. For pregnant women, the time of infection is difficult to determine. Among the HCMV-seronegative pregnant women we followed up, five had symptoms that could have been related to HCMV infection in an immunocompetent subject (fever and/or an increase in hepatic transaminases). We also took serum samples from these women weekly from the time when they showed symptoms onwards. Therefore, we could calculate the average time span between the appearance of symptoms (in one case, an antigenemia-positive result was obtained by coincidence) and seroconversion, which was 3.4 weeks (range, 2 to 5 weeks). Therefore, in cases in which a time for the appearance of symptoms was recorded that was considered the starting time and in cases in which no indication of the time of symptom onset was available we deducted 3.4 weeks from the date of seroconversion (for either IgG or IgM) and the resulting date was considered the starting time.

In transplant recipients, we assumed that the initial infection took place at the time of transplantation because all of the seronegative recipients we followed up received an organ from a seropositive donor and developed an acute infection during the first 2 months afterward. However, to compare the results with those obtained with pregnant women, we arbitrarily considered as the starting time the date of the onset of symptoms, which preceded seroconversion (for either IgG or IgM) by an average of 6.1 weeks (range, 5 to 8 weeks).

HCMV serology. (i) Conventional EIA. The evaluation of anti-HCMV IgG was carried out with a commercial kit (Enzygnost Anti-HCMV/IgG EIA Alpha Method, Behring AG). Plates were read on a microEIA automatic reader (Behring AG). The evaluation of anti-HCMV IgM was performed with the Enzygnost Anti-HCMV/IgM kit (Behring AG). The kits were used and the results were interpreted as suggested by the manufacturer.

(ii) Avidity test. Determination of IgG avidity was carried out by using a kit that has recently been made available (Cytomegalovirus IgG Avidity EIA WELL, produced by RADIM, Rome, Italy) and is based on the difference between the absorbance values due to antibody binding in the absence and in the presence of 4.5 M urea. The test was run and the results were interpreted as suggested by the manufacturer. In particular, an avidity index (AI) (absorbance reading after urea wash/absorbance reading without urea wash $\times$ 100) of $<35\%$ was considered low, $>45\%$ was considered high, and between 35 and 45% was considered moderate.

RESULTS

AI and IgM in blood donors. A group of 32 serum samples from HCMV-seropositive patients and a group of 64 serum samples from HCMV-seronegative blood donors were used in this part of the study. Of the 32 HCMV-positive serum samples, 31 were shown to have a high AI of IgG to HCMV by the avidity test. Furthermore, of the 64 HCMV-negative serum samples, 62 were judged negative by the avidity test. The data are shown in Table 1 and indicate that 1 seropositive and 2 seronegative serum samples reacted with the antigen present in the avidity test that we used, showing the presence of low-avidity IgG. The serological status of the donors was previously determined by ELISA and conventional WB. However, to verify the real seronegativity of the two sera that were judged IgG negative and gave a low AI, the samples were retested by the WB technique that we recently developed (16) by using both viral structural proteins and recombinant proteins. The two sera also gave a negative result with this test (data not shown). No IgM positivity was observed in this population.

TABLE 1. Detection of low-avidity IgG and positivity for IgM in different groups of subjects

Group and infection status	No. of persons (no. of serum samples)	No. with low AI	No. IgM positive by ELISA	No. with low AI and/or IgM positivity
Blood donors				
Uninfected seropositive	32 (32)	1	0	0
Uninfected seronegative	64 (64)	2	0	0
Transplant recipients				
Primary	7 (74)	7	6	7
Nonprimary	8 (56)	0	6	6
Pregnant women				
Primary	28 (66)	24	24	27[a]
Nonprimary	14 (44)	0	12	12[b]

[a] The sample that was IgM negative and showed a high AI was from a woman whose first serum sample was obtained approximately 28 weeks after primary infection.

[b] The two samples that were IgM negative and showed high AIs were from two pregnant women whose first serum samples were obtained approximately 7 months after infection. Nevertheless, they were IgM positive by the new WB method.

AI and IgM in transplant recipients and pregnant women undergoing primary or nonprimary HCMV infection. Seven of seven transplant recipients with an ongoing primary HCMV infection developed HCMV-specific IgG in 4.2 to 14.4 weeks (mean, 7.3 weeks) after transplantation. As shown in Table 1, all of the patients showed IgG with a low AI and six patients also showed the appearance of HCMV-specific IgM.

Among eight transplant recipients with a nonprimary HCMV infection (no attempt was made to differentiate between endogenous reactivation and exogenous reinfection), none showed low-avidity IgG while six produced IgM to HCMV.

As shown in Table 1, of 28 pregnant women undergoing a primary HCMV infection, 24 showed low-avidity IgG, 2 gave a medium AI, and 2 gave a high AI. The two sera that gave a high AI were obtained 28 and 34 weeks after primary infection.

Among four women whose sera did not reveal the presence of low-avidity IgG, three (two with medium AIs and one with a high AI) showed the presence of HCMV-specific IgM. Twenty-four pregnant women also showed the presence of HCMV-specific IgM. Among the four women who were IgM negative, three had a low AI.

Among 14 women who underwent a nonprimary HCMV infection during pregnancy, none showed a low AI while 12 produced HCMV-specific IgM. The two serum samples found to be IgM negative by ELISA and WB were from two pregnant women whose first serum sample was obtained 6 to 7 months after infection. These sera were retested by the new WB and found to be slightly positive for p52.

Distribution of AI in different groups of sera. The distribution of avidity of IgG to HCMV in 57 patients with primary ($n = 35$) or nonprimary ($n = 22$) HCMV infection is shown in Fig. 1. It can immediately be seen that the AI obtained with most of the sera ($n = 31$) from primary infections fall in the first part of the graph (0 to 35% AI). These sera have a mean AI of 16.0% (range, 3.5 to 34.0%). Two serum samples of the same group gave a medium AI (mean AI, 39.3%), and two

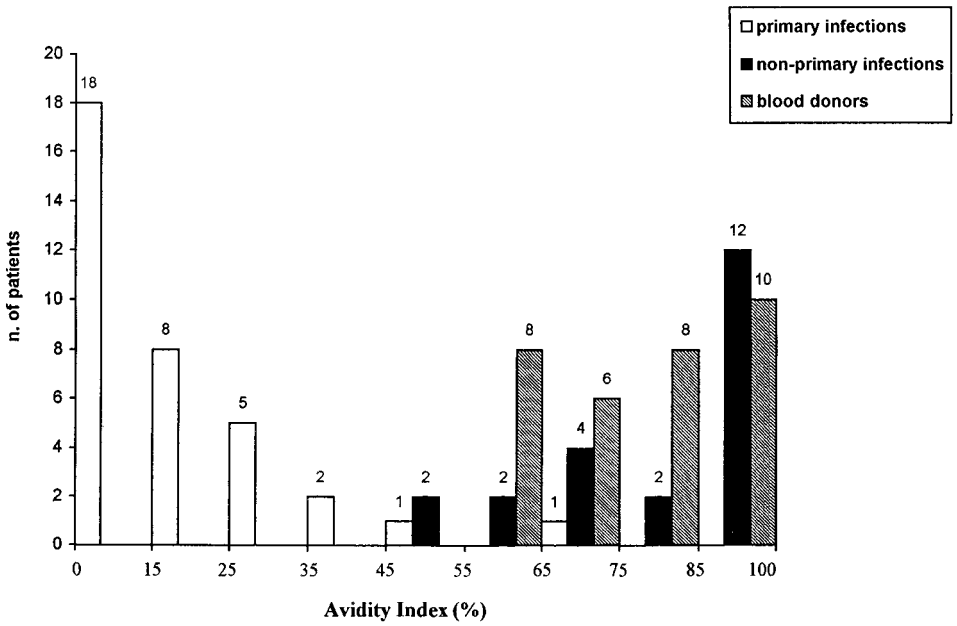

FIG. 1. Distribution of avidity of IgG antibody to HCMV in 57 patients with primary (open) or nonprimary (striped) HCMV infection and 32 long-term seropositive blood donors. Bars represent the numbers of patients whose AIs fall within the range shown on the abscissa.

gave a high AI (mean AI, 60.4%). These last serum samples were obtained from two pregnant woman 28 and 34 weeks after primary infection.

In contrast, all of the data from the 22 patients with nonprimary infections fell into the second part of the graph. Their sera had a mean AI of 81.5% (range, 53.6 to 100%).

The difference between the mean AI obtained with sera from primary infections and that obtained with sera from nonprimary infections is extremely significant ($P < 0.0001$ obtained by the Student t test).

Figure 1 also shows the distribution of the AIs obtained with serum samples from 32 long-term seropositive blood donors. As expected, the AIs obtained with these sera overlap those obtained with sera from patients undergoing nonprimary infections. The mean AI was 79.53% (range, 59.3 to 100%).

Temporal change in AI after primary infection in transplant recipients and pregnant women. As shown in Fig. 2, transplant recipients undergoing primary HCMV infections showed low IgG avidity (AI below 35%) for approximately 17 weeks after the beginning of HCMV-specific symptomatology. This period of time was followed by a period of progressive maturation of antibodies. Antibodies appear to have reached full maturation (AI of >45%) approximately 25 weeks after symptoms appeared. It was interesting that the two serum samples giving an AI of >45% at less than 17 weeks were from the same patient. This patient had a much shorter (7-day) viremia phase than the other patients (mean, 126 days; range, 30 to 240 days).

Furthermore, after 17 weeks, three samples gave AIs of less than 35%. Two of them were from the same patient, who had the longest viremia phase observed in our study (240 days). Correlation analysis of the joint distribution of the two series of values (weeks after infection and AI) gave a correlation coefficient of 0.8726, indicating a very strong association between the two characters.

As shown in Fig. 3, pregnant women undergoing a primary HCMV infection showed low IgG avidity (AIs below 35%) for approximately 17 weeks after infection. Unfortunately, only a few sera obtained from women at times later than 16 weeks after infection could be tested and these showed moderate to high AIs. Correlation analysis of the joint distribution of the two series of values (weeks after infection and AI) gave a correlation coefficient of 0.875, indicating a strong association between the two characters.

An example of progressive antibody maturation during an acute primary infection in an HCMV-seronegative heart transplant recipient is shown in Fig. 4.

DISCUSSION

In some developed countries, the percentage of women of childbearing age who do not have IgG to HCMV has always been higher than 50% (6). The low immunity level of this population puts them at particular risk of primary infection

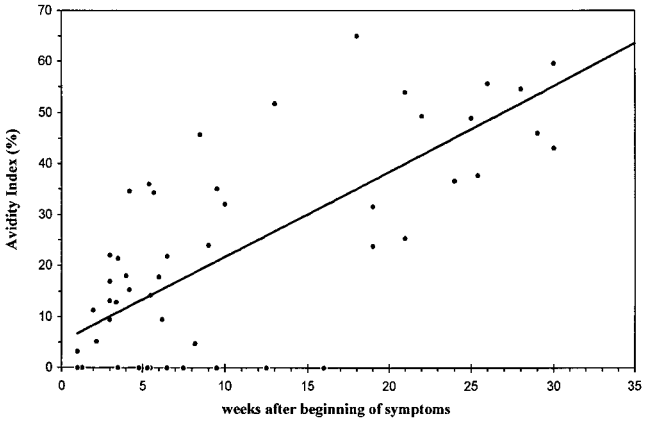

FIG. 2. Cograduation graph showing the correlation between the AI and the number of weeks after primary HCMV infection in transplant recipients. Correlation analysis of the joint distribution of the two series of values gave a correlation coefficient of 0.875, indicating a strong association between the two parameters.

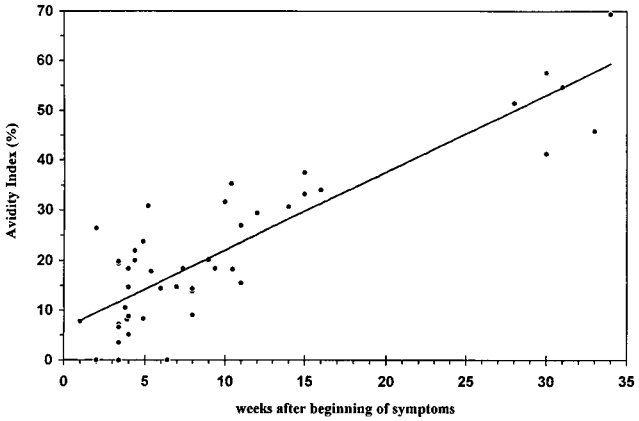

FIG. 3. Cograduation graph showing the correlation between the AI and the number of weeks after primary HCMV infection in pregnant women. Correlation analysis of the joint distribution of the two series of values gave a correlation coefficient of 0.8726, indicating a strong association between the two parameters.

during pregnancy. Furthermore, in other developed areas, such as northern Italy, the percentage of HCMV positivity, which was higher than 70% a decade ago, is decreasing (16a, 21a). Therefore, we can forecast an increase in the number of primary HCMV infections during pregnancy even in countries where they have always been considered rare.

The diagnosis of a primary HCMV infection can only be achieved by serologic testing, and there are four main methods: detection of seroconversion (for a review, see reference 10), detection of HCMV-specific IgM (20), detection of a particular profile of reactivity to HCMV proteins (for a review, see reference 12), and detection of low IgG avidity (2). Detection of seroconversion is uncommon in the absence of a specific follow-up. Many data indicate that IgM also appears during nonprimary infection (8). Furthermore, a well-standardized commercial kit for detecting a particular antibody profile is not available (14). For these reasons, we examined the avidity of IgG to HCMV during primary and nonprimary infections in both immunocompetent and immunocompromised subjects by using a kit that has recently been made available.

This study is the first work in which the maturation of antibodies after HCMV infection was correlated with virological and serological parameters in both pregnant women and transplant recipients. Our results demonstrate that the anti-HCMV IgG avidity test differentiates primary from nonprimary HCMV infections in both pregnant women and solid organ transplant recipients. In fact, 88.6% of primary infections and 0% of secondary infections showed low-avidity IgG to HCMV.

In two transplant recipients, we found four serum samples giving a few anomalous results (a high AI among a series of samples showing low avidity). The clinical records showed that both patients had been treated with anti-HCMV immunoglobulins which were likely to be of high avidity. This is in agreement with Lutz et al. (18), who tested the AI of a batch of anti-HCMV hyperimmune gammaglobulins and found an AI of 95%.

Moreover, the results obtained showed that in both populations there is progressive acquisition of antibody avidity within the first 6 months after primary infection. In fact, for both populations 6 months seems to be long enough to guarantee the full maturation of antibodies. This result is in disagreement with the data of Lutz et al. (17), who found that in solid organ transplant recipients approximately a year is needed for IgG to mature to high avidity, in contrast to the 3 to 6 months expected for immunocompetent subjects. The discrepancy could be explained by the difference in the determination of the starting time. Lutz and coworkers correctly assumed that the infection took place at the time of transplantation because all of the seronegative recipients they followed up received an organ from a seropositive donor and developed an acute infection during the first 2 months afterward. Even though this approach is unquestionable, it does not allow a correct comparison with the maturation time in immunocompetent subjects in whom the exact date of infection cannot be determined. As we could determine the time of symptom onset in some immunocompetent subjects, we decided to use the time of symptom onset as a starting point also for transplant recipients. Under these conditions, the time spans between symptom onset and the presence of completely mature IgG (AI higher than 45%) were very similar in the two populations (24 and 25 weeks in pregnant women and transplant recipients, respectively).

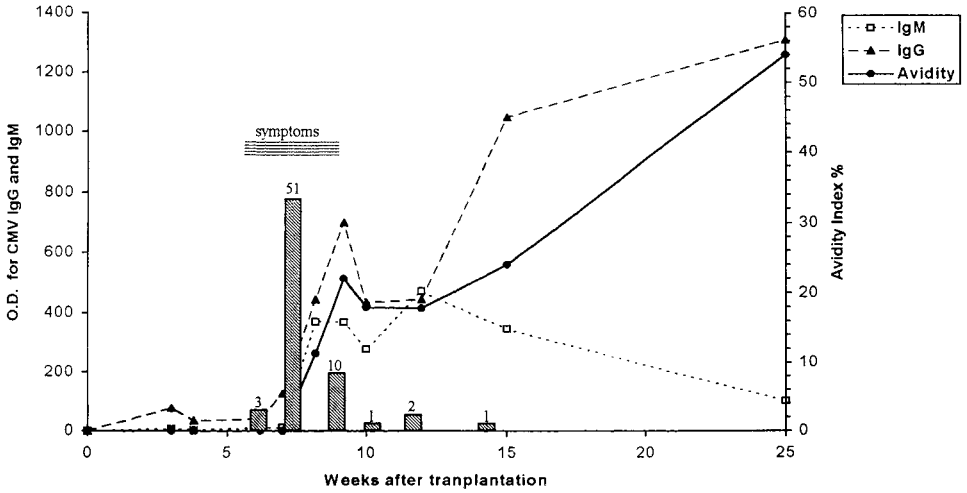

FIG. 4. Comparison of anti-HCMV IgG avidity with other serological and virological markers during an acute primary infection in an HCMV-seronegative heart transplant recipient who received an organ from a seropositive donor. The serological tests performed were ELISAs for IgG and IgM and the avidity test. Antigenemia results are also reported. The values above the bars are numbers of pp65-positive PMNL among 2×10^5 PMNL. O.D., optical density.

In the transplant recipient population, we observed a longer antibody maturation time than in pregnant women. In particular, one patient who had a very quick acquisition of IgG avidity had the shortest viremia phase, while another patient showing some delay in antibody maturation had the longest viremia phase observed in our study. The possibility that the AI could have a prognostic value is under evaluation.

The determination of IgG avidity was carried out in parallel with the determination of the presence of IgM. HCMV-specific IgM was detected in 85.7% of primary infections and 81.2% of nonprimary infections. No IgM positivity was observed in sera from blood donors. These results confirm other data present in the literature (13, 19) on the high incidence of IgM during viral reinfection or reactivation.

In conclusion, the IgG avidity test described here can differentiate primary from recurrent or latent infections. In particular, a low IgG avidity (AI, 0 to 35%) is a marker of primary infection for 18 to 20 weeks after symptom onset in both immunocompromised and immunocompetent subjects. In our opinion, this test could be used in every case of a positive IgM result to determine whether the IgM is the result of a recent primary infection. In the case of a low AI, an approximation of the date of infection can be attempted. In the case of high avidity, the infection is either nonprimary or took place more than 18 to 20 weeks earlier.

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