

## Reactivity to p52 and CM2 Recombinant Proteins in Primary Human Cytomegalovirus Infection with a Microparticle Agglutination Assay

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**We evaluated the reactivities of sera against p52 and CM2 recombinant antigens of human cytomegalovirus (HCMV), coated on microparticles, for the differentiation of primary HCMV infection from an established infection. Two different test formats of the CMV Multiplex Copalis assay were evaluated. The 214 serum samples tested were immunoglobulin M (IgM) positive or equivocal by our reference assay. Reactivities against p52 and CM2 antigens were tested for sera from 37 patients with a well-documented seroconversion within the preceding 3 months (119 serum specimens), 31 patients known to have had a seroconversion at least 8 months earlier (31 serum specimens), and 57 patients without a documented seroconversion (64 serum specimens). The assay had a sensitivity for the detection of a primary infection of 70 or 86% by the first test format and a sensitivity of 88 or 94% by the second test format, according to the criteria used to indicate a primary infection by this test. A good correlation of the results of the assay with our in-house avidity index was found. The specificity of the assay warrants further evaluation. With IgM-positive sera, the assay was not sufficiently specific to make a distinction between a primary infection and an established infection.**

Human cytomegalovirus (HCMV) causes morbidity in immunocompromised patients and the fetus in case of pregnancy (2, 3, 10, 12, 21). The distinction between a primary infection, reactivation, or convalescence is not easy by serological assays alone, because immunoglobulin M (IgM) antibodies can be present in sera over a long period and can reappear during reactivation (1, 13, 14). Besides classical IgM detection through indirect or capture assays, an alternative could be to detect antibodies against some antigenic targets. It has been shown that reactivity against the recombinant p52 (ppUL44) and CM2 (a recombinant protein of ppUL44 and pUL57) antigens is associated with primary infection (4, 5, 6, 18, 20). Reactivity against the p52 and CM2 antigens increases during primary infection. A few months after onset, reactivity against p52 sharply falls, while reactivity against CM2 can be detected for several more months in immunocompetent patients (13, 20). We evaluated the CMV Multiplex Copalis assay, which is an automated qualitative test that uses coupled light scattering technology to discriminate between recent or past infection. It allows the simultaneous detection of antibody reactivity against p52, CM2, and whole-virion protein (VP).

### MATERIALS AND METHODS

**HCMV IgM serology.** All sera were tested for the presence of HCMV IgM by an indirect enzyme immunoassay (EIA; Enzygnost CMV-IgM; Behring AG, Marburg, Germany). The procedures and interpretation of the results (positive, equivocal, or negative) were as recommended by the manufacturer. This included an absorption of IgG and rheumatoid factor before testing.

**Patients.** In this evaluation 214 serum specimens obtained from 125 patients were tested. All the sera were positive or equivocal for HCMV IgM, as tested by our reference EIA (Enzygnost CMV-IgM; Behring AG).

The samples were classified into three groups, according to the serological

follow-up of the patients. The first group consisted of 119 serum samples from 37 patients (15 pregnant women and 22 transplant patients) with a well-documented seroconversion for HCMV positivity within the preceding 3 months (seroconversion group). The second group was composed of 31 serum samples from 31 patients who were known to be HCMV infected for at least 8 months (established infection group). The third group of samples was used only for comparison of the avidity indices (AIs) of the IgG antibodies (see below). The third group included 64 serum samples from 56 pregnant women and 1 transplantation patient who presented with a positive or equivocal HCMV IgM serology but no documented seroconversion (unknown seroconversion group).

**Copalis assay serology.** In the CMV Multiplex Copalis assay (DiaSorin, Saluggia, Italy) polystyrene microparticles of three different sizes are coated with three different antigens. The antigens are recombinant proteins (p52 and CM2) or a viral particle. The IgM antibodies present in the sample are essentially bound to p52 and CM2 antigens, while the VP antigen binds to IgG antibodies. Binding of antibodies results in cross-linking of the microparticles. After incubation, the aggregated microparticles are recognized by counting them as they flow through a laser beam.

All sera were tested twice by the CMV Multiplex Copalis assay (see below), once before and once after adaptation of the test, unless the serum sample was exhausted. The sera were tested by following the order of the laboratory number. Because most serum samples had been stored frozen for various periods of time, 100  $\mu$ l of the serum was centrifuged at 8,000  $\times$  g for 10 min before testing. After centrifugation, 50  $\mu$ l was transferred into the specimen cup (which was separated from the test cup). Up to 24 serum samples could be tested in one test cycle, within 1 h. The antigen-coated microparticles are supplied as dried reagents and are solubilized with buffer, which is automatically added by the instrument to the test cups. The serum sample is then added by the instrument and is incubated at room temperature for 10 min, followed by reading of the test.

In the second test format anti-IgM antibodies were added in lyophilized form. After binding of HCMV IgM by p52 and CM2 antigens, the anti-IgM antibodies improve cross-linking of the microparticles. This should increase the level of IgM detection by the assay. CM2 and p52 antigens mostly bind to IgM antibodies and not to IgG antibodies.

Quality control and the specificity of the assay were ensured with internal reference particles. These particles are not coated with antigen and will aggregate if unspecific interactions are present. They can be measured because their sizes are different from those of the coated particles. According to the manufacturer, an acute primary infection is characterized by the presence of antibodies against p52 and CM2 in the sample, while an established infection is characterized by binding of the antibodies only to the VP antigen. A convalescent-phase primary infection is characterized by binding of the CM2 antigen, while the binding of the p52 antigen disappears within weeks after the beginning of the infection.

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TABLE 1. Reactivities against p52 and CM2 antigens in sera from patients with proven seroconversion with first and second test formats

| Test format <sup>a</sup> | No. of serum specimens <sup>b</sup> :                |                             |                                                           |                                                    | Total           |
|--------------------------|------------------------------------------------------|-----------------------------|-----------------------------------------------------------|----------------------------------------------------|-----------------|
|                          | p52+, CM2+<br>(early primary infection) <sup>c</sup> | p52+, CM2- (?) <sup>c</sup> | p52-, CM2+<br>(convalescent-phase infection) <sup>c</sup> | p52-, CM2-<br>(established infection) <sup>c</sup> |                 |
| First                    | 25                                                   | 1                           | 6                                                         | 5                                                  | 37 <sup>d</sup> |
| Second                   | 30                                                   | 0                           | 2                                                         | 2                                                  | 34 <sup>d</sup> |

<sup>a</sup> For explanation of test formats, see the text.<sup>b</sup> +, positive; -, negative; ?, equivocal.<sup>c</sup> Interpretation according to the manufacturer is given in parentheses.<sup>d</sup> Only the first IgM-positive serum sample of each patient is counted in the total.

**HCMV IgG antibody avidity.** One serum sample from each of 15 patients in the seroconversion group, 2 patients in the established infection group, and 57 patients in the unknown seroconversion group were tested by the urea denaturation procedure as described previously (1). The 74 serum samples were from 66 pregnant women and 8 transplant patients.

Briefly, the sera were added to wells coated with HCMV antigen (Enzygnost CMV-IgG; Behring AG). Each serum sample was tested in duplicate. After incubation, the first well for each serum sample was rinsed with 8 M urea and was soaked for 5 min to remove low-avidity antibodies. The second well (reference well) for each serum sample was rinsed as recommended by the manufacturer. The optical density after urea denaturation is expressed as a percentage of the optical density of the reference well. This percentage is the AI.

## RESULTS

Results for the seroconversion group are given according to the possible combinations of p52 and CM2 reactivity (Table 1). Only the first sample that was positive or equivocal for IgM antibodies by the Behring EIA was considered. Two serum samples were equivocal for IgM antibodies and 35 serum samples were positive for IgM antibodies.

According to the manufacturer's recommendations, acute HCMV infection is characterized by reactivity against both p52 and CM2. By following these criteria, the sensitivity of the assay is 68% for the first test format and 88% for the second test format. We found p52 reactivity alone for the first serum sample from a patient in the seroconversion group with the first test format. If this is taken into account, the sensitivity of the first test format becomes 70%. If the isolated reactivity against CM2 (convalescent-phase acute infection) is also considered a criterion for acute infection, the sensitivity rises to 86% for the first test format and 94% for the second test format. An increased reactivity was generally found with the second test format, meaning that individual antigen reactivities in the first test format were also found in the second test format but with additional reactivities.

Sera from patients in the established infection group were evaluated in the same way as sera from patients in the seroconversion group (Table 2). We found p52 reactivity only in the serum of a patient in this group by the first test format. By the second test format sera from two patients in the established infection group showed reactivity only against p52. The sera of two patients in the unknown seroconversion group also had

this pattern. If reactivity against both p52 and CM2 must be negative, then the rate of false-positive results for this selected patient groups for acute or recent HCMV infection is 58% for the first test format and 81% for the second test format. If reactivity against CM2 is accepted for an established infection, then the rates are 35% for the first test format and 61% for the second test format.

In the seroconversion group 17 transplant patients were monitored several times. At least one sample from all patients showed p52 and CM2 reactivities with the second test format. Nevertheless, sera from two transplant patients showed a delayed reactivity for p52 and CM2, and serum from one transplant patient showed a delayed reactivity only against p52, whereas IgM antibodies were detected by our reference EIA. These were the same patients whose sera showed delayed reactivities with the first test format. An exact estimation of the delay could not be calculated because blood samples were collected at irregular times. Reactivity against p52 disappeared faster than reactivity against CM2 for one patient with the first test format and for seven patients with the second test format (data not shown). An exact calculation of the timing of disappearance of the two parameters was not possible because of incomplete follow-up of the patients. Antibodies against VP were present in almost every serum sample tested with the first test format (99%), whereas their presence diminished in samples tested with the second test format (87%).

We also compared the CMV Multiplex Copalis test result with the AI. For this we considered all samples for which an AI was available for the seroconversion and established infection groups and all serum samples from the unknown seroconversion group. Of 15 serum samples from the seroconversion group, 11 had AIs of less than 50% and 4 had AIs between 51 and 58%. The two serum samples from the established infection group tested for AI had values of more than 90%. The AIs for 57 patients in the unknown seroconversion group ranged between 13 and 100%. The results of the AIs for all the sera were divided into three different groups according to their values (Table 3). A comparison of individual combinations of test results with the AIs is provided. An AI of less than 50% indicates a primary infection within the preceding 3 months

TABLE 2. Reactivities against p52 and CM2 antigens in a group with established infection with first and second test formats

| Test format <sup>a</sup> | No. of serum specimens <sup>b</sup> :                |                             |                                                           |                                                    | Total |
|--------------------------|------------------------------------------------------|-----------------------------|-----------------------------------------------------------|----------------------------------------------------|-------|
|                          | p52+, CM2+<br>(early primary infection) <sup>c</sup> | p52+, CM2- (?) <sup>c</sup> | p52-, CM2+<br>(convalescent-phase infection) <sup>c</sup> | p52-, CM2-<br>(established infection) <sup>c</sup> |       |
| First                    | 10                                                   | 1                           | 7                                                         | 13                                                 | 31    |
| Second                   | 17                                                   | 2                           | 6                                                         | 6                                                  | 31    |

<sup>a</sup> For explanation of test formats, see the text.<sup>b</sup> +, positive; -, negative; ?, equivocal.<sup>c</sup> Interpretation according to the manufacturer is given in parentheses.

TABLE 3. Reactivities against p52 and CM2 antigens for sera previously tested for AI

| Test format <sup>a</sup> | AI (%) | No. of serum specimens <sup>b</sup>                  |                             |                                                           |                                                    | Total |
|--------------------------|--------|------------------------------------------------------|-----------------------------|-----------------------------------------------------------|----------------------------------------------------|-------|
|                          |        | p52+, CM2+<br>(early primary infection) <sup>c</sup> | p52+, CM2- (?) <sup>c</sup> | p52-, CM2+<br>(convalescent-phase infection) <sup>c</sup> | p52-, CM2-<br>(established infection) <sup>c</sup> |       |
| First                    | <50    | 20                                                   | 0                           | 4                                                         | 2                                                  | 26    |
|                          | 50-64  | 6                                                    | 0                           | 2                                                         | 1                                                  | 9     |
|                          | ≥65    | 5                                                    | 0                           | 9                                                         | 25                                                 | 39    |
| Second                   | <50    | 20                                                   | 0                           | 3                                                         | 0                                                  | 23    |
|                          | 50-64  | 7                                                    | 0                           | 1                                                         | 0                                                  | 8     |
|                          | ≥65    | 12                                                   | 2                           | 16                                                        | 9                                                  | 39    |

<sup>a</sup> For explanation of test formats, see the text.<sup>b</sup> +, positive; -, negative; ?, equivocal.<sup>c</sup> Interpretation according to the manufacturer is given in parentheses.

(1). Two of the 26 serum samples with low AIs were equivocal for HCMV IgM with our reference assay. If p52 reactivity with or without CM2 reactivity is chosen as reflecting an infection less than 3 months old, then the sensitivity of the assay is 77% for the first test format and 87% for the second test format. If the p52 or CM2 reactivity is chosen as an infection less than 3 months old, the sensitivity of the assay rises to 92% for the first test format and 100% for the second test format.

An AI of more than 64% is seen for patients with infections that are more than 3 months old. By our reference assay 33 of the 39 serum specimens were equivocal for HCMV IgM. If CM2 reactivity or no reactivity at all was chosen as an old infection (more than 3 months), then the assay showed false-positive rates of 13% for the first test format and 36% for the second test format for these selected samples. If CM2 reactivity must be negative for old infections, then the false-positive rates rise to 36% for the first test format and 77% for the second test format.

An AI equal to or greater than 50% but smaller than 65% is difficult to interpret.

## DISCUSSION

Recombinant proteins, instead of naturally occurring HCMV antigens, are increasingly used to distinguish a primary infection from an established infection (4, 5, 9, 13, 18, 20). Several attempts have been made to make the best possible antigen combination to ensure a sensitive and specific detection of primary infection (7, 8, 11, 17, 19). The present assay detects separately reactivity against p52 and CM2 recombinant proteins and a viral particle (VP). The p52 recombinant protein is the full ppUL44 protein sequence of HCMV, expressed in *Escherichia coli*. The CM2 antigen is a combination of two C-terminal sequence portions of ppUL44 and the central sequence of pUL57 (20). IgM antibodies are highly reactive against ppUL44 (p52) and pUL57 (p130) antigens (5, 7, 8, 9, 19, 20). p52 and CM2 reactivities are considered early markers of acute HCMV infection (6, 8, 9, 19). Furthermore, the p52 antibody titer rapidly declines 2 to 3 months after the onset of the infection in immunocompetent patients (13), while CM2 reactivity can be detected several months later (18, 20). The antigens are each coated on microparticles of different sizes. The IgM antibodies present in the sample mainly bind to p52 and CM2, while IgG antibodies mainly react with the total viral particle. This results in cross-linking of the microparticles. In the second test format, anti-IgM antibodies were added to improve the cross-linking between the microparticles, once the HCMV IgM antibodies in the sample were bound to the p52 antigen or the CM2 antigen. The microparticles are counted as

they flow through a laser beam. In this evaluation we could not determine the exact time of disappearance of p52 and CM2 reactivities because most patients were not monitored long enough at regular intervals. We found, however, that p52 could be detected several months later in immunocompromised transplant patients, which correlates with the findings of another study (13). According to the information from the manufacturer, reactivity against the p52 antigen alone should not be found. After the evaluation of the second test format we found four serum samples that showed isolated p52 reactivity. A change in interpretation should be validated with more tests.

The overall sensitivity of the assay for the detection of primary infection is 86% for the first test format and 94% for the second test format. We tested sera from only a small group of patients by the Copalis assay, and only the first IgM-positive serum from each patient during seroconversion was tested. For the first serum from one patient for which no p52 or CM2 reactivity was found, the serum was also tested for HCMV IgM antibodies by alternative assays (AxSYM and ETI-Cytok IgM) and was found to be negative. Sera from two further patients in the seroconversion group, both of whom were transplant recipients, showed no p52 reactivity by the first test format of the assay. Antibody production against p52 has been reported not to be suppressed or delayed in transplant patients (12, 13, 14, 16, 17). The addition of anti-IgM antibodies in the second test format improved the sensitivity of detection of p52 and CM2 reactivities. The delayed reactivity for p52 alone for one patient shows that the addition of CM2 will increase the sensitivity of the assay. The earlier reactivity with CM2 for this patient could possibly be due to a better reactivity against the midsequence of the pUL57 protein of the CM2 antigen. As shown in previous studies, isolated p52 reactivity is an insufficient early marker of primary infection (5, 18, 20). The addition of recombinant CM2 increases the sensitivity of the assay (20). It is known that the central part of pUL57 is a major reactive protein during acute HCMV infection (9, 19, 20). Even though the sensitivity of the assay with the second test format seems to be higher for p52 reactivity, this reactivity also disappears earlier in the seroconversion group. This is in accordance with previous studies (13, 20), but in our study we regularly monitored only transplant patients, who may have a prolonged synthesis of IgM (12). The results of the present assay are classified as positive or negative. The introduction of a gray zone could be indicative of the need for further testing of some samples.

The purpose of this evaluation was to make a distinction between an acute and an established reactivating HCMV infection in IgM-positive patients. After screening by IgM-specific assays the large number of false-positive results by this



assay is due to the selected study population, with all patients being HCMV IgM positive or equivocal. We did not test random patients, which would be needed to establish the true specificity of the test. Many patients in the established infection group showed p52 and CM2 reactivities. We know that they had had a primary HCMV infection at least 8 months earlier. The meaning of the detection of IgM in these patients may differ: persistent IgM after resolved primary infection, reactivation, or reinfection (10, 11). This may well influence the results. Reactivity to VP was lower with the second test format (87 versus 99% with the first test format). This may be due to the fact that the second format enhances IgM reactivity, while IgG mainly reacts with the VP antigen. The added anti-IgM could hinder the cross-linking through binding with the few IgM molecules present on the particles.

We also compared the assay with our AI method (1). The determination of the AI of IgG allowed us to differentiate a primary infection from an older infection independently from the p52 and CM2 reactivity. A good correlation between AIs of less than 50% (infection less than 3 months old) and p52 and CM2 reactivities was found, especially for the second test format of the assay. This shows that reactivity against p52 and CM2 antigens can be used to detect a primary infection. These results correspond to the sensitivity of the p52-CM2 assay that we found for the seroconversion group. On the other hand, reactivity against p52 and CM2 was often found in patients with AIs above 64% (an infection more than 3 months old). This means that the p52 and CM2 reactivities of a serum sample are not sufficient to differentiate between a primary and an established infection. Our evaluation with selected sera (IgM positive) does not allow us to define the true specificity with random serum samples. As shown by others, p52 and CM2 reactivities can be detected up to several months after primary infection and also probably during secondary infections (8, 11, 13, 17, 20).

The test is not suited as a confirmatory assay for IgM-positive samples to distinguish primary from recurrent infection. On the practical side, the assay is very easy to handle and the time to the result is 12 min for 1 serum sample or 1 h for up to 24 serum samples. In conclusion the CMV Multiplex Copalis assay appears to have a good sensitivity for the detection of primary HCMV infection if the second test format is used. It may have a future as a screening test. Grouping of p52 and CM2 might increase the sensitivity but may lower the specificity. This specificity should be further tested with random serum samples.

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