Confirmation of Human Cytomegalovirus by Reverse Passive Hemagglutination with Monoclonal Antibodies Reactive to the Major Glycosylated Peptide (GP-66)

KWANG SOO KIM,* VICTOR SAPIENZA, AND CHENG-MO J. CHEN

Department of Virology, Institute for Basic Research in Developmental Disabilities, New York State Office of Mental Retardation and Developmental Disabilities, Staten Island, New York 10314

Received 3 March 1986/Accepted 23 May 1986

Sheep erythrocytes coated with three monoclonal antibodies, each reactive to a different epitope of the 66-kilodalton cytomegalovirus (CMV) matrix protein, were used in a reverse passive hemagglutination test with CMV-infected cell lysate to identify and confirm the CMV. The test is specific only for CMV, since 5 laboratory strains of CMV (AD169, Davis, Espilat, C-87, and Towne) and 10 clinical isolates reacted well, but uninfected MRC-5 cell lysate, lysates of herpes simplex virus types 1 and 2, varicella-zoster virus, and adenoviruses did not react. The reactive CMV lysate was confirmed by the pretreatment of CMV lysate with the three monoclonal antibodies followed by the addition of antibody-coated erythrocytes. The reverse passive hemagglutination test and the confirmatory blocking test are performed at the same time, requiring 2 h to complete. Since V-bottom microtiter 96-well plates and a 25-μl pipette can be used to perform the test, it is ideal for CMV confirmation, especially when the equipment to read the fluorescent-antibody test or enzyme-linked immunosorbent assay is not available.

Human cytomegalovirus (CMV) has been implicated in various disease syndromes in humans, some of which have serious consequences, particularly for newborns (1–7, 14, 16–18). Rapid diagnosis of CMV infection is important in the treatment and management of these patients. Because of the lack of a simple method that does not require expensive instruments, a small laboratory relies on the recognition of a specific type of viral cytopathic effect which develops slowly (days to weeks) in suitable human embryonic fibroblast cells inoculated with clinical specimens. Recently, Shuster et al. (15) reported an elegant and sensitive way to detect CMV, but many small laboratories are not able to conduct sedimentation inoculation followed by the indirect immunofluorescent-antibody test with monoclonal antibody reactive to CMV early protein. We reported the successful production of murine monoclonal antibodies to a CMV major glycosylated protein with a molecular weight of 66,000 (GP-66) (13). GP-66 represents 35% of the total protein in purified CMV and dense bodies (12). To develop a simpler and less expensive method, the cell agglutination method first reported by Juji and Yokochi (10), which has sensitivity close to that of the enzyme immunoassay or the radioimmunoassay, was adopted for the detection of human CMV. We used a mixture of three GP-66-reactive monoclonal antibodies, each reactive to a different epitope, to develop a method using agglutination of anti-CMV-coated sheep erythrocytes as an indicator of the presence of CMV antigen. This method, if made available in the form of a prepared test kit, would be a simple, reliable, and inexpensive alternative for small laboratories over the indirect fluorescent-antibody assay and the enzyme immunoassay, since expensive equipment is not needed to read the test results. Furthermore, a simple confirmation of the positive results by specific antigen blocking is accomplished at the same time. In this report, we present an evaluation of the sensitivity, specificity, and stability of the developed test.

* Corresponding author.
TABLE 1. Specificity of the assay against various laboratory strains and clinical isolates

<table>
<thead>
<tr>
<th>CMV strain or isolate</th>
<th>Reciprocal of HA titer</th>
<th>Reciprocal of CF titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Towne</td>
<td>8</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Espilat</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>AD169</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>C87</td>
<td>128</td>
<td>16</td>
</tr>
<tr>
<td>Davis</td>
<td>32</td>
<td>2</td>
</tr>
</tbody>
</table>

Clinical isolates

1 128 8
2 128 8
3 128 8
4 64 2
5 64 2
6 64 <2
7 64 <2
8 32 <2
9 64 <2
10 32 <2

The method described by Kim et al. (13) was used for obtaining hybrid cell lines which yielded the three monoclonal antibodies reactive to CMV GP-66. The three monoclonal antibodies, 4D11, 7D2, and 322A2, were determined to be reactive to GP-66 by immunoblotting and to be reactive to different epitopes on GP-66 by a competitive binding inhibition assay. Immunoglobulins were purified from ascites fluid with the Affi-Gel Protein A MAPS system from Bio-Rad Laboratories, Richmond, Calif. The mixture of the three purified monoclonal antibodies was prepared so that the same concentration of each monoclonal antibody was present. All three monoclonal antibodies were of the immunoglobulin G1 subclass, as determined by Ouchterlony double diffusion tests with goat anti-mouse immunoglobulin G.

The method described by Ikram and Prince and by Hollinger et al. (8, 9) was used with some modifications for glutaraldehyde treatment and sensitization of the erythrocytes. Sheep erythrocytes in Alsever solution were washed five times with phosphate-buffered saline (PBS) (0.15 M [pH 7.2]), and a 10% suspension was prepared. A 3% glutaraldehyde solution (1 volume) (pH 7.2) in PBS was mixed gently with 1 volume of the 10% cell suspension for 18 h at room temperature. The mixture was washed five times with PBS, filtered through eight layers of gauze, and stored as a 10% suspension.

Glutaraldehyde-treated erythrocytes were washed once with 0.1 M acetate buffer at various pH levels and resuspended to a concentration of 10%. In a siliconized tube, 1 ml of 10% glutaraldehyde-treated erythrocytes was added to 1 ml of the three monoclonal antibodies, and the volume was adjusted to 10 ml with acetate buffer. The mixture was incubated at room temperature. The cell suspension was shaken on a rotary mixer for various times. The cells were washed five times with PBS and resuspended to 0.2% in 0.15 M PBS. The mixture of the three monoclonal antibodies contained 1 mg of each monoclonal antibody per 3 ml. V-bottom microtiter plates (Dynatech Industries, Inc., McLean, Va.) were used. Serial twofold dilutions of CMV-infected lysate (25 μl) in 0.15 M PBS (pH 7.2) containing 0.1% gelatin were prepared. To each well, 25 μl of coated erythrocytes was added, and the plates were sealed with Dynatech plate sealer and incubated at room temperature for 2 h.

Confirmation of the presence of CMV antigen in the RPHA test was performed by specific blocking of the antigen as follows. Serial twofold dilutions (1:2 through 1:128) of normal uninfected MRC-5 lysate (25 μl) were prepared in row 1. Serial twofold dilutions (1:2 through 1:128) of CMV-infected lysate (25 μl) were prepared in rows 2 and 3. To row 2, 25 μl of monoclonal antibody mixture (0.8 μg/ml) was added, and to row 3, only 25 μl of PBS containing 0.1% gelatin was added. The wells were left at room temperature for 15 min. The 25 μl of antibody-coated erythrocytes was added, mixed, and incubated at room temperature for 2 h.

Antibody-coated erythrocytes were made 3% with goat serum and 4% with either lactose, mannitol, or sucrose, quick-frozen in liquid nitrogen, and lyophilized overnight. The sensitized erythrocytes were suspended in PBS and used.

To obtain the maximal absorption of the antibodies by the erythrocytes, several different pHs (3.0, 4.0, 4.6, and 5.0) of acetate buffer were used to coat the antibodies to the erythrocytes. At pHs 3.0 and 4.0, the control antigen gave a nonspecific reaction; therefore, at these pHs we were unable to determine the hemagglutination (HA) titer of the CMV CF antigen. However, at pHs 4.6 and 5.0, the control CF antigen showed an HA titer of <1:2, whereas the CMV CF antigen showed titers of 1:128 and 1:32, respectively. Therefore, the optimal combination of sensitivity with a nonreactive control pattern (HA titer of <1:2) was obtained with pH 4.6 acetate buffer.

Three concentrations of antibody (0.25, 0.5, and 1 mg/ml) were used to coat the erythrocytes for 60 and 120 min. A concentration of 0.25 mg/ml gave an HA titer of 1:64 with CMV CF antigens, whereas concentrations of 0.50 and 1.00 mg/ml both gave HA titers of 1:128. Maximum titers were obtained at 60 min; no further increase was achieved with an additional 60-min incubation. Control CF antigen had an HA titer of <1:2 at all antibody concentrations. Therefore, the optimal conditions for the sensitization of the erythrocytes used were as follows. Samples (1-ml) of 10% (vol/vol) glutaraldehyde-treated cells were washed once with 0.1 M sodium acetate (pH 4.6). The sedimented cells were suspended in 1 ml of a mixture of the three monoclonal antibodies (each 1 mg/3 ml). The erythrocyte suspension was put on a rotary shaker at room temperature for 1 h. The cells were then washed and processed as described above.

The antibody-sensitized erythrocytes were suspended at various concentrations (0.1, 0.2, and 0.5%) and were added to twofold serial dilutions of CMV antigen prepared in Dynatech V-bottom microtiter plates. The best pattern of agglutination and ease of reading occurred with an erythrocyte concentration of 0.2%. The tests were readily readable in 2 h at room temperature. After 18 h at room temperature, cell patterns were occasionally more distinct, but the titers remained the same. HA titers for all these erythrocyte concentrations were the same.

To determine how soon p.i. the CMV RPHA test system would detect the synthesis of GP-66, a time course experiment was done. Monolayers of MRC-5 were infected at a multiplicity of infection of 3 PFU with CMV strain AD169. After 2 h of virus absorption at 37°C, cells were washed three times with growth medium, and then growth medium was added at different times (0, 24, 48, 72, 96, and 144 h). The amount of antigen in the infected cell lysate was tested by using the sensitized erythrocytes. GP-66 was detected in the infected cell lysates from 24 h p.i., when the HA titer was 1:2, as opposed to <1:2 at time zero. The HA titer steadily increased with time to a maximum HA titer of 1:128 at 144 h p.i. An increase in the CF titer of the same lysates was

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detected only at 72 h p.i., and the CF titer reached a maximum of 1:4 at 144 p.i. MRC-5 control antigen gave negative results (HA titer, <1:2), as did infected lysates of HSV-1 (72 h p.i.), HSV-2 (72 h p.i.), adenovirus (120 h p.i.), and commercial CF antigen of varicella-zoster, which had CF titers of 1:16, 1:8, 1:16, and 1:8, respectively.

The effect of storage conditions on the stability of the coated erythrocytes as determined by the HA titer was studied. Antibody-coated erythrocytes (0.2%) were stored at 4°C. In addition, a 2% suspension was lyophilized under various conditions and stored at 4°C. The sensitized erythrocytes at 4°C were stable for at least the 15 days tested (Table 1). When a 2% erythrocyte suspension in PBS was lyophilized and suspended in either deionized water or PBS to 0.2% erythrocytes, nonspecific agglutination occurred. When a 2% erythrocyte suspension was lyophilized in the presence of 3% goat serum, 4% sucrose, or 4% sucrose plus 3% goat serum and reconstituted in deionized water or PBS, nonspecific agglutination did not occur, and the cells were very stable, i.e., the HA titer remained unchanged for the 15 days tested at 4°C.

The specificity of the assay was evaluated by testing various laboratory strains of CMV and several clinical CMV isolates which were confirmed by an indirect fluorescent-antibody test with GP-66-specific monoclonal antibody. All the laboratory CMV strains and clinical isolates showed a positive HA pattern after 2 h at room temperature. The relative CMV antigen titer varied from strain to strain and from one isolate to another (Table 2). This was a reflection of the different degree of cytopathic effect and the amount of CMV antigen accumulated at the time of harvest and not strain or isolate variations (11). This was evidenced by the amount of CF antigen present in the samples. In previous experiments, we showed that the antigen titer detected by monoclonal antibodies to GP-66 was always directly proportional to infectivity for all the CMV strains we tested (data not shown). We also showed that 1 U of human CMV CF antigen is equivalent to approximately 10^7 PFU (K. S. Kim, C. Juneja, V. J. Sapienza, and K. Wisniewski. Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, S49, p. 243). The results presented in Table 2 clearly show that sensitized erythrocytes gave a titer much higher than the titer obtained by the CF test in 2 h at room temperature.

Table: 2. Confirmatory blocking test against various strains of CMV

<table>
<thead>
<tr>
<th>CMV strain or isolate</th>
<th>Reciprocal of HA titer before and after specific antigen blocking</th>
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<tbody>
<tr>
<td></td>
<td>Before</td>
</tr>
<tr>
<td>C-87</td>
<td>128</td>
</tr>
<tr>
<td>Towne</td>
<td>32</td>
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<td>Davis</td>
<td>16</td>
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<td>Espilat</td>
<td>32</td>
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<tr>
<td>AD169</td>
<td>8</td>
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<tr>
<td>Clinical isolates</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>16</td>
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<tr>
<td>2</td>
<td>8</td>
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<td>3</td>
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</table>

The performance of the confirmatory blocking test is shown in Table 2. Endpoint titers for laboratory strains and the 10 clinical isolates were reduced to <1:2 when dilutions of CMV CF antigen preparations were tested on the sensitized erythrocytes in anti-CMV-containing diluent. The blocking test for only three clinical isolates is shown in Table 2.

The primary focus of this study was to investigate the use of the RPHA for the detection of CMV antigen for a possible application to CMV confirmation in clinical virology. To achieve this objective, three monoclonal antibodies against GP-66, a major glycosylated peptide of CMV, were used for sensitization of sheep erythrocytes, and the sensitized erythrocytes were used for the detection of CMV in infected lysates. These three monoclonal antibodies are reactive to different epitopes on GP-66 and are also reactive to GP-66 even after sodium dodecyl sulfate treatment, as shown by immunoblotting (13). These antibodies are reactive, therefore, to very stable epitopes on the CMV virion, which makes them very suitable for use in detecting clinical laboratory isolates. In addition, we showed that antibody-coated erythrocytes are stable for at least 15 days both as a suspension and when lyophilized with stabilizers, which suggests that they are suitable for making prepared test kits.

In our previous study (13), a highly effective double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) for the detection of antigen was devised that used GP-66-reactive monoclonal antibodies. We used 4D11 as the capture antibody and 7B4, which reacts with a different epitope on GP-66, as the detector antibody. This ELISA detected as little as 0.00490 U of CF antigen per ml or 0.000245 U of CMV CF antigen (13). In contrast, the newly developed CMV RPHA can only detect 0.03 to 0.06 U of CMV CF antigen per ml, which is equivalent to 3 x 10^5 to 6 x 10^5 PFU per ml. Therefore, the CMV RPHA is 6- to 12-fold less sensitive than the CMV ELISA method we reported. However, the test is 16- to 64-fold more sensitive than the CF test. The specificity, stability, simplicity, rapidity, and relative ease with which the test can be performed make the CMV RPHA an important and excellent alternative method for the detection and confirmation of CMV in infected cell lysates. So far, all the CMV antigen preparations from laboratory strains and the clinical isolates tested have given unequivocal positive results. Also, a simple confirmatory blocking test against CMV-infected cell lysates can be conveniently performed at the same time. The assay system is now ready to test to see how well it can perform in the confirmation of clinical CMV isolation and to compare it to a method such as sedimentation inoculation followed by the fluorescent-antibody test (15). Finally, the newly developed CMV RPHA using sheep erythrocytes and CMV GP-66-specific monoclonal antibodies would be a valuable CMV confirmation test if made available in the form of a prepared test kit, since the test can be completed in approximately 2 h, it is against a stable epitope on the CMV virion, and the results can be read by eye without the need for expensive equipment, such as a fluorescence microscope or ELISA reader. These advantages would make this test especially attractive to a small clinical laboratory with a limited budget.

We thank Dina Esposito for her technical assistance and Adele Monaco for processing the manuscript.

LITERATURE CITED


