Enzyme-Linked Immunosorbent Assay Method for Detection of Cytomegalovirus Strain-Specific Antibody Responses

Zdenek Novak,1* Shannon A. Ross,1 Raj Kumar Patro,2 Sunil Kumar Pati,2 Meera K. Reddy,1 Misty Purser,1 William J. Britt,1 and Suresh B. Boppana1

Department of Pediatrics, The University of Alabama at Birmingham, Birmingham, Alabama,1 and All India Institute of Medical Sciences, New Delhi, India2

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Reliable methods for the detection of cytomegalovirus (CMV) strain-specific serological responses are lacking. We describe a simple and reliable enzyme-linked immunosorbent assay method developed to detect antibodies against the polymorphic epitopes within the two envelope glycoproteins of CMV, glycoproteins II and B. This assay is useful for the detection of serologic responses to CMV strains and the identification of CMV reinfections.

Cytomegalovirus (CMV) is an important pathogen in immunocompromised hosts and a frequent cause of congenital infection. CMV isolated from clinical samples exhibits extensive genetic variation (7, 12, 13), and CMV reinfections have been demonstrated to occur in seropositive individuals. However, it is thought that these reinfections have little untoward consequences with respect to congenital infections. Recent studies documenting higher rates of congenital CMV infection in populations with nearly universal seroreactivity to CMV suggest that infection with new or different virus strains could be responsible for the intrauterine transmission of CMV in immune mothers (5, 17, 18). The frequency and consequences of infection with multiple CMV strains are unclear because of the lack of reliable methods for the accurate identification of CMV strain-specific antibody responses. By utilizing the defined heterogeneity within the antibody binding epitopes on envelope glycoprotein H (gH) and gB of the AD169 and Towne strains of CMV, an enzyme-linked immunosorbent assay (ELISA) method was developed to distinguish serological responses against infection with different CMV strains.

Serum samples from 96 CMV-seropositive women participating in an ongoing study and 51 seronegative individuals were tested for anti-CMV strain-specific antibodies. Informed consent was obtained from the study participants, and the study was conducted in accordance with the guidelines of the Institutional Review Board for Human Use of the University of Alabama at Birmingham.

Purified recombinant antigens based on polymorphic antibody binding sites defined on gH (antigen gpUL75) and gB (antigen gpUL55) were used as antigens (Fig. 1). The gH antigens were constructed as β-galactosidase fusion proteins containing the coding region for amino acids (aa) 15 to 142 of gpUL75 from the AD169 strain (the AP86 antigen) and aa 14 to 42 of the Towne strain (the TO86 antigen) of CMV (16). The recombinant peptides were expressed in Escherichia coli and were purified as described previously (8). gB antigens were prepared as six-His-tag-labeled peptides by cloning the coding region (aa 1 to 116) from strains AD169 (the AD55 antigen) and Towne (the TO55 antigen) (9) into expression vector pET21a (EMD, Gibbstown, NJ) by using the HindIII and BamHI endonuclease restriction sites. The peptides were expressed in E. coli Rosetta cells and were purified by using Talon Superflow metal affinity columns (Clontech, Mountain View, CA). A positive control antigen was constructed by cloning the antigen domain 1 (AD-1) region of the gene coding gB, which has been shown to be highly conserved among clinical isolates of CMV, as described previously (2), into each vector (AD-1). The reactivity against an empty vector expressing fusion protein alone or nonantigenic proteins of mouse origin was used as a negative control.

Strain-specific ELISA was performed on PolySorp microtiter plates (Nunc, Roskilde, Denmark). The wells of the plates were coated overnight with 50 μl of purified gH antigens (antigens AP86 and TO86) (1) or gB antigens (antigens AD55 and TO55) diluted in carbonate buffer and blocked with 3% goat serum in borate buffer (BB) for 2 h at 37°C. Serum samples diluted 1:100 in BB were added to the wells, and the plates were incubated at 37°C for 1 h. After the plates were washed three times with BB containing 0.05% Tween 20, goat anti-human immunoglobulin G (IgG) horseradish peroxidase (HRP)-conjugated antibody (Pierce, Rockford, IL) diluted 1:10,000 was added and the plates were incubated for 1 h at 37°C. The plates were developed by the addition of 50 μl of one-step Ultra TMB (3,3′,5,5′-tetramethylbenzidine) substrate (Pierce) for 10 min at room temperature (RT), and the reaction was stopped by the addition of 2 N sulfuric acid. The optical density (OD) values were determined with a spectrophotometer. A positive result was defined as an OD value more than three times the mean result obtained for each antigen with seronegative samples.

Western blot assays were performed with a subset of 12 samples. Appropriate antigens were run on a 12.5% sodium dodecyl sulfate-polyacrylamide gel and then blotted onto a polyvinylidene difluoride membrane (Immobilon P; Millipore,
Billerica, MA), according to the manufacturer’s recommendations. The membranes were blocked for 2 h in 3% goat serum in SuperBlock buffer (Pierce, Rockford, IL) and 0.05% Tween 20 at RT. Human sera were diluted 1:5,000 in blocking buffer and applied to the membrane, and the membrane was shaken at RT for 2 h. The membranes were washed four times in wash buffer (BB with 0.05% Tween 20), and goat anti-human IgG HRP-conjugated antibody (Pierce) diluted 1:100,000 in blocking buffer was added. After incubation at RT for 2 h, the membranes were washed four times in wash buffer and soaked into the substrate Luminol West Femto (Pierce) for 10 min. The membranes were placed on X-ray film, and images were developed and acquired by using a VersaDoc imaging system (Bio-Rad, Hercules, CA).

Of the 96 baseline serum samples from CMV-seropositive women participating in an ongoing study testing for strain-specific antibodies, 58 (60%) samples were positive for at least one of the four antigens and 18 samples were positive for two or more antigens. The OD values (mean ± standard deviation) for each antigen for the group of 51 CMV-seronegative individuals and the samples that were considered positive and negative from the 96 CMV IgG antibody-positive women are shown in the Table 1. Forty-five percent (43/96) of the samples had reactivity to the AP86 antigen, with a mean OD value of 1.240 ± 0.498, whereas the OD values were 0.291 ± 0.134 and 0.196 ± 0.052 for the negative samples and the CMV IgG antibody-negative control serum samples, respectively. Fifteen percent (14/96) of the study samples were positive for the TO86 antigen, with an average OD reading of 1.069 ± 0.317. Responses against the AD55 and TO55 antigens were seen in 0.196 (53) of the samples, with corresponding OD values of 0.196 ± 0.052 for the strain-specific antibody-positive samples and 0.143 ± 0.078 ± 0.278 (Table 1).

To verify the reproducibility of the assay results, two positive control samples with known reactivity to the gH and gB antigens were tested on 10 different occasions and consistently yielded similar results. Serum sample 1 was reactive against AP86 (OD = 1.442 ± 0.569) and TO55 (OD = 0.809 ± 0.438), while serum sample 2 contained antibodies against the TO86 antigen (OD = 1.132 ± 0.485). The OD values of CMV IgG antibody-positive samples against the positive (AD-1) and the negative control antigens were 1.21 ± 0.45 and 0.28 ± 0.17, respectively.

The strain specificities of the antibody responses were confirmed with a subset of 12 samples by Western blot assay. Figure 2 demonstrates the recognition of the antigens by four serum samples. The sizes of the reactive bands by the Western blot assay were similar to the predicted sizes of the recombinant peptides (16).

The seroepidemiologic study of CMV strain diversity has been hampered thus far by the lack of simple and reliable methods that can be used to accurately identify infection with multiple strains of CMV. In this study, we report the findings of an ELISA method that was used to identify the presence of strain-specific antibodies in sera from 96 seropositive women against the polymorphic epitopes on CMV gH and gB from the prototypic laboratory strains of CMV, strains AD169 and Towne. Using this method, we could demonstrate the presence of strain-specific antibodies against the antigenic determinants

| Antigen | CMV IgG antibody-positive samplesa (n = 96) | CMV IgG antibody negative (n = 51) | Positive control sera b |
|---------|---------------------------------|---------------------------------|------------------------|
|         | Strain-specific antibody positive | Strain-specific antibody negative | Serum sample 1 | Serum sample 2 |
| AP86    | 1.240 ± 0.498 (43)               | 0.291 ± 0.134 (53)              | 0.196 ± 0.052         | 1.464 ± 0.578 | 0.458 ± 0.143 |
| TO86    | 1.069 ± 0.317 (14)               | 0.301 ± 0.158 (82)              | 0.238 ± 0.066         | 0.443 ± 0.119 | 1.155 ± 0.495 |
| AD55    | 0.681 ± 0.103 (8)                | 0.238 ± 0.120 (88)              | 0.198 ± 0.068         | 0.348 ± 0.115 | 0.368 ± 0.125 |
| TO55    | 0.708 ± 0.094 (19)               | 0.170 ± 0.094 (77)              | 0.132 ± 0.032         | 0.803 ± 0.434 | 0.191 ± 0.053 |

a Fifty-eight of 96 samples were positive for at least one antigen.

b Serum sample 1 had known reactivity with AP86 and TO55, and serum sample 2 had known reactivity with TO86.

c Numbers in parentheses represent numbers of samples.
on envelope glycoproteins gH and gB. The reliability of this assay for the identification of CMV strain-specific antibodies was documented by comparing the serological reactivities to the antigens tested between CMV-seropositive and -seronegative individuals. As can be seen in Table 1, the mean OD values for each antigen were similar between the group of CMV IgG antibody-negative individuals and the group of CMV IgG antibody-positive women who were categorized as negative for antibodies against specific gH or gB antigenic determinants. The reproducibility of the assay was demonstrated by repeated testing of two serum specimens reactive with three of the four antigens (antigens AP86, TO86, and TO55) tested. The strain-specific serological responses were confirmed in a Western blot assay with a subset of 12 serum specimens (Fig. 2).

Clinical strains of CMV exhibit extensive genetic polymorphisms in their envelope glycoproteins (10), and no two clinical isolates have been documented to be identical (7), even when they are examined by restriction fragment length polymorphism analysis (11). Studies with populations with increased exposure to CMV, such as sexually transmitted disease clinic attendees (4) and human immunodeficiency virus-infected individuals, have shown that infection with new CMV strains occurs frequently (3, 14, 15). However, the impact of infection with multiple CMV strains and/or reinfection with new virus strains with respect to the severity of CMV disease among immunocompromised hosts and intrauterine transmission of CMV are unclear. In a recent study, we documented the occurrence of infection with new strains of CMV in seropositive women between pregnancies and identified an association between reinfections and intrauterine transmission and severe fetal infection (1). Reinfection with different CMV virus strains in organ donors has been associated with an increased incidence of transplant rejection and CMV disease, as shown by a more recent study of renal transplant recipients (6).

The CMV strain-specific ELISA method described in this report could be a useful tool for determination of the CMV strain diversity in populations and, therefore, could provide a better understanding of the implications of infection with multiple CMV strains. In addition, the ability to identify the appearance of new antibody specificities over time will make it possible to document CMV reinfections in seroinmune individuals and allow the study of the factors associated with reinfections and the impact of reinfections in different populations. One of the limitations of this assay is that not all CMV-specific IgG-positive individuals can be identified by use of the four antigens used. Specimens from more than a third of the seropositive individuals (38/96) did not contain antibodies against any of the four antigenic determinants tested, suggesting the presence of additional polymorphic epitopes on glycoproteins gH and gB as well other envelope glycoproteins of CMV, such as gN. Identification of these additional epitopes could further extend the sensitivity of our assay for the detection of infection with multiple CMV strains and to determine the rates of reinfection with new virus strains in seroinmune individuals. In addition, with a clearer understanding of the frequency of CMV reinfections in seroinmune individuals and the CMV strain diversity in different populations, one could begin to address the role of the strain-specific antibody response in protective immune responses against CMV.

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We have no commercial interest to report.

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