Rapid Quantitation of Cytomegalovirus and Assay of Neutralizing Antibody by Using Monoclonal Antibody to the Major Immediate-Early Viral Protein

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An overnight assay, based on staining cytomegalovirus-infected cells with monoclonal antibody to the 72,000-molecular-weight major immediate-early viral protein, was compared with a conventional 14-day plaque assay for quantitation of cell-free stocks of cytomegalovirus laboratory strain AD-169 and 20 other clinical strains. Viral titers were quantitatively similar when determined by either method, but centrifugation of monolayers during inoculation enhanced viral infectivity an average of 4.1-fold. When used for scoring neutralizing antibody assays, monoclonal antibody staining yielded titers within one dilution of 14-day plaque-reduction assays in 54 of 56 titrations. Of 21 cytomegalovirus strains, 2 were not recognized by the monoclonal antibody used. Assay with monoclonal antibody offers a rapid and accurate alternative to plaque assays for quantitation or neutralization of cytomegalovirus.

MATERIALS AND METHODS

Virus stocks and sera. CMV strain AD-169 was obtained from the American Type Culture Collection. Twenty other CMV strains were from clinical isolates which had been passed up to 20 times in vitro. DNA extracts from these viral isolates were examined by restriction enzyme digest analysis (4) to confirm that each strain was distinct from the others. Human foreskin fibroblasts at passage 12 to 18 were used for viral propagation. Culture medium was Eagle minimal essential medium containing 10% calf serum. Cell-free stocks were prepared from cultures showing 100% cytopathic effect by dislodging cells from monolayers, sonicating for 30 s with a microtip, and centrifuging at 6,000 × g. The supernatant containing virus was frozen at −70°C after the addition of 5% dimethyl sulfoxide. Immune sera were collected from CMV-seropositive transplant recipients representing a broad range of antibody titers by enzyme-linked immunosorbent assay (ELISA).

Viral quantitation. Fibroblast monolayers were prepared in 24-well tissue culture cluster plates (Corning Glass Works) by adding suspensions of approximately 6 × 10⁴ cells per well and were used 1 day after cells had become confluent (about 4 days after seeding). Monolayers that were not at this stage of growth were less satisfactory. Plates used to compare techniques (absorption, scoring for plaques or infected cells) were seeded with fibroblasts in parallel from the same flask of cells and inoculated with virus simultaneously.

Culture medium was removed from wells and replaced with viral inocula consisting of 0.2-ml samples of serial twofold dilutions of cell-free virus stock. Virus stocks were always used immediately upon thawing and were never reused or refrozen. Virus absorption was by the traditional method of 1 h of incubation at 37°C in a CO₂ incubator or by spinning the plate at 500 × g at room temperature for 20 min (Sorvall RT6000 centrifuge, plate-spinning bucket). Centrifugation for longer than this adversely affected the integrity of the fibroblast monolayer.

After virus absorption, plaque titration was completed by traditional techniques (26). The inoculum was removed and...
replaced with a 1-ml overlay of Eagle minimal essential medium containing 0.6% agar and 10% calf serum. After 1 week another 1 ml of the same overlay was added. At the end of the 2-week period (10 days for strain AD-169), the overlay was removed, the monolayer was fixed in methanol, viral plaques were stained with 0.03% methylene blue for 20 min, and the number of plaques per well was counted under an inverted microscope. Quantitation was based on plaque counts in the dilution giving about 200 plaques per well.

Rapid scoring of virus titrations was performed by using mouse monoclonal antibody L-14 (fast protein liquid chromatography purified; Johnson & Johnson Biotechnology Center, La Jolla, Calif.) which is specific for the 72K major IE protein of CMV (21). Plates to be evaluated were incubated for 20 h at 37°C after virus absorption in regular culture medium (no agar). Monolayers were then washed once with sucrose-phosphate (SP) buffer (16 mM sodium phosphate, 2 mM potassium phosphate, 2% sucrose, pH 7.8) and fixed in methanol. After monolayers were washed twice in SP buffer, they were “blocked” for 20 min in 0.5 ml of 5% nonfat dry milk to reduce nonspecific binding of antibody and then washed once with SP buffer. Antibody L-14 (4 μg/ml in SP buffer) was added (0.1 ml per well). The plate was incubated at 37°C for 15 min with continuous rocking to distribute the reagent evenly. After two washes with SP buffer, bound monoclonal antibody was visualized by sequential incubation with biotinylated antimouse antibody (1:100 dilution) and an avidin-peroxidase complex (Vectastain ABC mouse kit; Vector Labs, Burlingame, Calif.), according to directions provided with the commercial kit. Aminoethylcarbazole substrate (200 μg/ml in 50 mM sodium acetate buffer, pH 5.5) was used as the peroxidase substrate. Cells positive for CMV revealed dark red nuclear staining 2 to 10 min after substrate addition. Quantitation was based on counts of stained nuclei in the dilution giving about 200 positive cells per well.

Neutralization assay. Neutralization titers of immune sera were determined by incubating serial twofold dilutions of heat-inactivated serum (56°C for 30 min, 0.2 ml of each dilution) with 0.2 ml of virus stock dilution, each containing approximately 800 PFU (20). Virus dilutions were made in serum-free Eagle minimal essential medium with guinea pig complement (sterile filtered; GIBCO) added to make a concentration of 10%. Serum dilutions were made in Eagle minimal essential medium containing 100 μg of amikacin per ml. Serum-virus mixtures were incubated at 37°C for 1 h, and 0.2 ml of each mixture was then used as a viral inoculum for quantitation of infectivity as described above. Neutralization titers were obtained either at 10 to 14 days by the traditional plaque reduction method (17, 20) or at 1 day when plates were scored with monoclonal antibody. Neutralization endpoints were those serum dilutions giving a 60% reduction in counts of plaques or infected nuclei compared with counts for a no-serum control (20). To improve reproducibility, we required that control counts be in the range of 100 to 600.

ELISA. Immunoglobulin G anti-CMV antibody titers were determined with an ELISA as described previously (8), except that strain AD-169 antigen of our own preparation was used (5). Serum dilutions were started at 1:1,000, and serial twofold dilutions were tested out to the endpoint. Titers were determined in parallel with neutralization titers on some sera.

RESULTS

Viral quantitation. Technical factors affected the apparent titer of individual CMV stocks. Fibroblast monolayers not at the specified stage of growth tended to give lower virus yields. Centrifugation during absorption increased plaque counts greatly. Overall, with 23 CMV stocks representing 19 distinct strains, we found that the viral titer (PFU per milliliter) as determined by the traditional plaque assay was enhanced an average of 4.1-fold (range, 1.8- to 8.9-fold) by absorption with centrifugation for 20 min at room temperature compared with absorption in an incubator for 1 h. A similar effect was noted when titers were determined by monoclonal antibody staining. Thus, the enhancement of infectivity observed with centrifugation is independent of the scoring method used. The degree of enhancement (1.8- to 8.9-fold) did not appear to be dependent on the viral strain; other factors such as state of growth of fibroblast monolayers and in vitro adaptation of CMV strains were probably more important. All subsequent experiments described below were performed with centrifuge-assisted absorption.

Traditional plaque assays were used to determine the titers of 31 CMV stocks (19 strains) in comparison with titers in an overnight assay by monoclonal antibody staining of parallel monolayers inoculated similarly. Figure 1 shows the correlation of the infectivity titers as determined by the two methods. Neither scoring method was consistently more sensitive in detecting infectious CMV. This was seen throughout the range of viral titers, ranging from 10^2 to 10^7 PFU/ml. Most clinical strain stocks had titers in the range of 10^4 PFU/ml, whereas laboratory strain AD-169 was readily propagated to a titer of 10^7 PFU/ml.

Each assay method was occasionally problematic. As is widely known, some clinical strain stocks do not plaque well on the traditional assay; plaques were sometimes too small or poorly formed, at least at 14 days postinoculation. Further propagation of the strain was then required to obtain stocks which formed better plaques. Serial propagation was not necessary for antibody staining. However, among the 21 CMV strains studied, 2 were stained weakly or not at all by antibody L-14, despite attempts to stain at various intervals from 0.5 to 7 days postinoculation with stocks and inocula

![FIG. 1. Comparison of viral titers of 31 CMV stocks by plaque assay and monoclonal antibody staining. The stock with the titer of 10^7 PFU/ml was strain AD-169; all others were clinical strains.](http://jcm.asm.org/Downloaded from September 20, 2017 by guest)
giving moderately high plaque counts by the traditional assay. These strains were verified as CMV by DNA hybridization (4). The other strains were stained satisfactorily at 16 to 20 h, and staining was not improved on further holding.

**Neutralizing antibody assay.** Neutralizing antibody titers of 21 sera against one or more of 10 CMV stocks (10 distinct strains) were determined by traditional plaque reduction assay in comparison with a rapid (overnight) assay based on staining with L-14 IE antibody. Each of the stocks was tested against 2 to 14 sera. Endpoints obtained with either technique were within 1 dilution in 54 of 56 titrations, and a majority of titers were identical with both assays (Fig. 2). However, when the titers differed, there was a tendency for the rapid assay to give a slightly higher titer. We speculate that this may be due to partial inhibition of the inoculum by diluted neutralizing antibody, causing a delay in the appearance of IE antigen, which would affect the overnight assay but not the 2-week reading. Nevertheless, this effect appears to be a minor one.

**Neutralization versus ELISA titers.** Figure 3 shows the correlation of neutralization titers (determined by overnight assay) and ELISA titers on the same sera. All titrations were performed against laboratory CMV AD-169. Although there is a rough correlation between endpoints obtained with the two methods, there are instances of considerable disparity (two or more dilutions), such as two sera with the same ELISA titer but with neutralizing titers of <8 and 128.

**DISCUSSION**

We show here that assay of various strains of CMV by plaque titration can be replaced by staining of infected-cell nuclei for the CMV major IE antigen, with comparable results in terms of viral quantitation and neutralizing titer endpoints. The close similarity of endpoints suggests that infected cells consistently display IE antigen at 16 to 24 h postinoculation and that these cells become foci of cytopathology over the ensuing weeks. Neither scoring method appears inherently more sensitive than the other for detection of CMV.

The overnight availability of results with monoclonal antibody staining is a major advantage over traditional methods, whether used for rapid detection, viral quantitation, or neutralizing antibody assays. In the past, the need for time-consuming plaque-reduction neutralization assays has inhibited the study of neutralizing antibody in various patient populations. Newer, rapid assays such as those described here and elsewhere (11, 28), should facilitate these studies, but each new method must be calibrated against established neutralization assays, tested for applicability to a variety of CMV strains, and based on the use of reproducible reagents.

Interest has grown in assaying CMV neutralizing antibody because of an increased understanding of this phenomenon on a molecular level (3, 7, 18, 19) and because of its potential importance in protection against CMV disease, as demonstrated by controlled trials showing the protective effect of passively infused CMV immune globulin (15, 23, 27). Neutralization assays have been reported to show strain-dependent quantitative differences (2, 25). Whether this has clinical significance in terms of susceptibility to reinfection or symptomatic disease is a matter for further study. Our results showing some discordance of binding (ELISA) titers with neutralizing titers are consistent with other published data (13, 22). Thus, during development of CMV immune globulins and vaccines, it will probably be necessary to assay a variety of immune sera for neutralizing activity against strains of CMV.

A number of technical factors need to be controlled for optimal results when quantitating CMV. It is clear that centrifugation markedly enhances apparent infectivity of viral inocula, independent of the method used to visualize infected cells. Each scoring method has potential pitfalls. Problems with traditional plaque assays are maintaining the integrity of monolayers for 2 weeks under semisolid medium and the variable plaquing properties of different clinical CMV stocks. Problems with use of monoclonal antibody staining include the limited period of maximal expression of
IE antigen, reagent expense, and possible failure to detect certain strains of CMV due to variation in the corresponding IE antigen. In this study, 2 of 21 strains were not detected by the L-14 antibody we used. This has important implications for clinical diagnostic laboratories seeking to replace conventional CMV cultures with rapid assays with monoclonal antibodies; the performance characteristics of each monoclonal antibody must be similarly determined. Based on limited experience with other CMV IE monoclonal antibodies, we expect that differences will be found in optimum working concentrations, in the expected degree of background staining of uninfected nuclei (which was very low for L-14), and in the range of CMV strains detected.

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LITERATURE CITED


