Three-Day Assay for Human Cytomegalovirus Applicable to Serum Neutralization Tests

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A fluorescing cell assay (FCA) technique utilizing the indirect fluorescent-antibody method to measure human cytomegalovirus (CMV) infected cells has been applied to the rapid determination of CMV-neutralizing antibody. Human sera with complement fixation titers to CMV of 1/32 or greater and fluorescein-conjugated rabbit anti-human globulin are the primary and secondary reagents in the fluorescent-antibody test. FCA measured in 3 days the same number of infectious units measured by plaque assay in 2 weeks. FCA and plaque assay yielded identical neutralizing antibody titers to CMV in 20 human sera.

The tedious methods available (2, 4-6) for assay of infectious human cytomegalovirus (CMV) have complicated neutralizing antibody tests for CMV. We describe in this report a fluorescing cell assay (FCA) which combines the quantitative accuracy of plaque counting with the convenience and specificity of immunofluorescence. The assay has been applied to measure CMV-neutralizing antibody in human sera.

MATERIALS AND METHODS

Cell cultures. Human fibroblast cell cultures were maintained on Eagle basal medium supplemented with 5% fetal calf serum, 100 units of penicillin per ml, and 100 μg of streptomycin per ml. Cells were grown in wells (16 mm in diameter) of Linbro-Disposotrays, on Lab-Tek 4-chambered slides, and 10.5- by 35-mm cover slips in Leighton tubes. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air.

Virus. The Davis strain of CMV (52nd through 55th passages) was used in all experiments. Stock virus was prepared by infecting monolayer cell cultures in 16-oz (475-ml) milk dilution bottles, from which the nutrient fluid had been removed, with approximately 5.0 × 10⁶ plaque-forming units (PFU). After 1 hr of adsorption, 10 ml of maintenance medium was added and incubation continued at 37°C. When 90% of the monolayer showed cytopathic effect, sorbitol from a 70% aqueous stock solution was added to the culture fluid to make a final concentration of 20%. The culture was frozen and thawed once. The resulting cell debris in medium was removed and clarified by centrifugation at 200 × g for 10 min. The virus-containing supernatant fluid, containing between 10⁴ and 10⁵ PFU/0.2 ml, was stored in 1.0-ml volumes at −70°C.

Plaque assay. Duplicate cultures prepared in Disposotrays or on cover slips were inoculated with 0.2 ml of virus suspension after removal of maintenance medium. The inoculum was aspirated after 1 hr of adsorption at 37°C in a 5% CO₂-air atmosphere, and the cultures were washed once with approximately 2 ml of Hanks balanced salt solution (HBSS) and overlayed with Eagle basal medium containing 2% methyl-cellulose. The methyl-cellulose-containing medium was replaced at 3- to 4-day intervals. Plaques were counted by microscope, without staining, 2 weeks after infection.

Fluorescing cell assay. Chamber slide or coverslip monolayer cultures were infected and treated as described for the plaque assay, but methyl-cellulose was omitted from the maintenance medium. Between 72 and 96 hr after infection, the cultures were air-dried, fixed in acetone for 10 min at room temperature, and stained immediately or stored at −20°C. Fixed monolayers were reacted for 60 min at 37°C with pooled human sera known to have complement-fixing (CF) titers to the Davis strain CMV of 1/32 or greater. Negative controls consisted of uninfected cell cultures treated in the same manner. The cultures were washed 3 times by dipping in approximately 50 ml of phosphate-buffered saline (0.15 M, pH 7.2) and reacted with fluorescein-conjugated rabbit anti-human globulin for 1 hr at 37°C. Three additional washes in phosphate-buffered saline removed excess conjugate. Fluorescing cells were counted at 100× with a Zeiss fluorescent microscope.

Sera. Human sera were obtained from Sherwin Kevy, Director of the Transfusion Service, Children's Hospital Medical Center, Boston, Mass. Twenty sera with CF titers of 1/4 to 1/256 to the Davis strain were tested for neutralizing antibody. The specimens were inactivated at 56°C for 30 min and sterilized by passage through a 0.4-μm polycarbonate filter.

Neutralization test. Serial twofold serum dilutions in HBSS were mixed with equal volumes of...
virus suspension containing 200 PFU/0.2 ml. Controls contained only HBSS in a volume equal to that of the virus suspension. Each serum was tested in the presence and absence of complement. Pooled guinea pig serum served as the complement source and was incorporated into test and control mixtures to a final concentration of 5%. The reaction mixtures were incubated at 37 °C for 30 min and assayed by the plaque and fluorescing cell methods. The highest serum dilution neutralizing 80% of the virus was judged to be the antibody end point.

RESULTS

Optimum time to fix infected cultures for FCA. Three culture chambers on each of five Lab-Tek slides were infected with 200 PFU per chamber, with the fourth culture chamber serving as a negative control. One slide from this group was fixed every 24 hr for 5 consecutive days, and an average fluorescing cell count per chamber was determined for each day. The number of infected cells resulting from the initial inoculum peaked between 48 and 96 hr (Fig. 1). The sharp increase after 96 hr reflected a second cycle of virus replication. Infected cell cultures for FCA, therefore, were fixed for staining between 72 and 96 hr after infection.

Dose-response curve. Fluorescing cell counts varied directly with the virus dilution, demonstrating that cell infection is initiated by a single infectious unit (Fig. 2).

Serial twofold virus dilutions assayed by fluorescing cell counts and plaque-forming units. Stock virus was diluted in HBSS. Cell cultures were prepared on cover slips in Leighton tubes to provide equivalent adsorption areas for each assay. Before inoculation, each cover slip was removed and transferred to a tissue culture dish, 35 mm in diameter, where 0.2 ml of virus dilution was placed onto the monolayer. Four cover-slip cultures were inoculated per dilution. Two were used for FCA and two for plaque assay. Depending on the type of assay, duplicate cultures were maintained either with periodical changes of the methyl-cellulose medium for 2 weeks, or else for 72 to 96 hr on maintenance medium without change. The infectious counts obtained by both methods were comparable (Table 1).

Comparison of neutralization test titers using fluorescing cell and plaque assays. Twenty sera were tested individually in groups of five for neutralizing antibody content with each dilution tested by the fluorescing cell and plaque assay techniques in the presence and absence of complement. Identical neutraliza-

![Graph](image)

**Fig. 2. Dose-response curve for Davis strain CMV by FCA.**

| TABLE 1. Comparison of plaque and fluorescing cell assays |
|---------------------------------|
| Virus dilution | Fluorescing cell counts* | Plaque-forming units* |
|----------------|-------------------------|-----------------------|
| 1:2            | TNTC                    | TNTC                  |
| 1:4            | 463                     | 218                   |
| 1:8            | 226                     | 120                   |
| 1:16           | 130                     | 67                    |
| 1:32           | 73                      | 32                    |
| 1:64           | 39                      | 32                    |

*Average count of two infected cover-slip cultures.
*Too numerous to count.
tion titers were obtained by both methods. In both systems, the addition of complement enhanced virus neutralization from 4- to 16-fold, thereby confirming previous reports (1, 3).

Reproducibility of the FCA in determining serum-neutralizing antibody titers to CMV. Triplicate examinations of 10 sera using the FCA and incorporating complement into the test mixture resulted in identical results in all but 3 of 30 tests (Table 2).

**DISCUSSION**

Studies of CMV-neutralizing antibody have been neglected in comparison to those of CF antibody because there has been no convenient procedure for assay of infectious virus. Desirable improvements in methods to detect neutralizing antibody would include speed, accuracy, and use of available reagents. The fluorescing cell and plaque assays are herein shown to be equivalent for the detection of infectious virus, but the former is completed in 3 days. FCA requires human sera reactive by immunofluorescence with CMV-infected cells, and the laboratory capability of performing indirect fluorescent-antibody tests. Both of these are available in most diagnostic and research laboratories.

Since the FCA utilizes antiserum of undefined specificity, it cannot be used to specifically distinguish cells infected with CMV from cells infected with other herpesviruses. There is, also, no assurance that sera positive for CMV by CF will be reactive in immunofluorescence. It is essential to use pooled sera which have been shown by prior testing to be suitable for the detection of CMV-infected cells by indirect immunofluorescence.

Consideration should also be given to the CMV strain used in the test. Other CMV strains at different passage levels might possess characteristics not compatible with the test procedure as described in this report for Davis strain, passages 52 through 55.

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