Rapid Neutralization Assay for Human Cytomegalovirus Antibody

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Human cytomegalovirus induces the appearance of immediate early antigens in infected cells 1 h after infection. This provided the basis for the development of a rapid neutralization assay for cytomegalovirus antibody which was able to yield results within a single day. Indirect immunofluorescence to visualize immediate early antigen-positive cells was applied to the rapid determination of cytomegalovirus-neutralizing antibody. The neutralization titers obtained with this assay on 92 serum samples were in accordance with the immune status as determined by enzyme-linked immunosorbent assay to cytomegalovirus-induced immediate early, early, and late antigens.

In recent years, several neutralization assays for cytomegalovirus (CMV) antibody based on plaque assays or immunofluorescence staining of infected cells at the end of replication cycle have been described (8, 10, 11, 14). However, neutralization tests for CMV antibody have been considered technically demanding and time-consuming because of the long replication cycle of CMV.

It is known that in cells infected with CMV several antigens appear at varying intervals after infection and that immediate early antigens (IEA) can be detected in the nucleus of infected cells by 1 h after infection with the indirect immunofluorescence (IIF) technique (2).

This prompted us to develop a rapid and sensitive neutralization test for CMV antibody, in which the immunofluorescence technique for visualizing IEA-positive cells was applied to the neutralization assay.

In our study, the presence of neutralizing antibodies in the samples examined was compared with the presence of antibodies to CMV-induced late antigens (LA), early antigens (EA), and IEA as determined with an enzyme-linked immunosorbent assay (ELISA). The determination of antibodies against these various antigens permits a serological diagnosis of CMV recent or past infection, since the presence of antibodies to IEA and EA and high ELISA optical density values for late antigens may be associated with an active CMV infection (1, 4, 6, 13).

MATERIALS AND METHODS

Cell cultures. Human embryo fibroblast cell cultures (Flow Laboratories, Inc., McLean, Va.) were maintained in Eagle minimum essential medium supplemented with 10% fetal bovine serum and antibiotics. Cells were grown on cover slips in wells (16 mm in diameter) on Nunclon plates (Nunc).

Virus. The Towne strain of CMV was used in all of the experiments. CMV was propagated and harvested as previously described (5). In brief, stock virus was prepared by infecting cells at a low multiplicity, and released virus was harvested from the extracellular fluid at 3 to 4 days after 100% cytopathic effect. Cell debris was removed by centrifugation, and the virus-containing supernatant fluid, with a titer ranging between 10^5 to 10^6 PFU/0.2 ml, was stored at −70°C in volumes of 1 ml for no longer than 2 months. During this period, no decrease in infectivity titer of the virus preparation was noticeable.

Human sera. Ninety-two serum samples from renal transplant recipients and normal blood donors were tested.

ELISA. Serum samples were tested by an ELISA for antibodies to CMV-induced LA with commercially available kits (M. A. Bioproducts). The sera were also tested for antibodies against CMV-induced IEA and EA with an ELISA previously described (3) by using as antigens purified IEA and EA obtained from nuclei of CMV-infected cultures at 1 and 24 h after infection, respectively.

Neutralization test. Serial twofold serum dilutions in phosphate-buffered saline (PBS) (0.15 M; pH 7.4) were mixed with equal volumes (0.2 ml) of virus suspension containing 300 PFU/0.2 ml. In negative controls, PBS was used instead of human serum in the same volume. Negative and positive reference sera (G.P. 401 [LA negative, EA negative, IEA negative]; F.G. 182 [LA titer of >1/320, EA titer of >1/320, IEA titer of >1/320]) were included in every test.

The mixtures were incubated for 1 h at 4°C, 0.4 ml of rabbit anti-human immunoglobulin G (Dako) at a dilution of 1/20 was added, and the mixtures were incubated for another 1 h at 4°C. The reaction mixtures were adsorbed on cells at 37°C for 60 min and then removed, and growth medium was replaced. Two cover slip cultures were inoculated for each serum dilution. At 3 h after infection, cells were fixed with acetone and processed for IIF to detect IEA-positive cells (15). In brief, cells were incubated with a reference serum (A.P. 184 [IEA titer of 1/320]), diluted 1/40 for 45 min at 37°C, washed three times in PBS incubated with a 1/20 dilution of fluorescein isothiocyanate-conjugated rabbit immunoglobulins to human immunoglobulin G (Dako) for 45 min at 37°C, washed three times with PBS, and mounted in glycerol-PBS (1:1).

RESULTS

ELISA. Serum samples were tested by ELISA for antibodies to CMV-induced LA, IEA, and EA. Of the 92 serum samples examined, 35 showed high ELISA optical density values (≥1.15) for antibodies to LA and also had positive ELISA values for antibodies to IEA and EA; 6 sera had high positive values for LA but were negative for IEA and EA; 13 showed mid-range positive values (0.46 to 1.14) for LA and were negative for IEA and EA; 23 had low positive values (0.25 to 0.45) for LA and were negative for IEA and EA; and 15 samples were negative for all three antigens.

Neutralization assay. A dose-response curve of stock virus was determined. Stock virus was inoculated at various dilutions on cell cultures, and the number of positive cells
showing IEA by IIF was determined (Fig. 1). Each point on the graph represents the average number of IEA-positive cells counted on three different cover slips. The number of IEA-positive cells was seen to vary directly with the virus dilution, indicating that cell infection (i.e., the appearance of IEA) was initiated by a single infectious unit. In neutralization assays, the presence of neutralizing antibody was based on the absence of immunofluorescence staining of IEA in cells inoculated with sensitized serum-virus mixtures. The highest serum dilution which gave negative IIF staining for CMV IEA was considered the neutralization titer of the serum sample.

The neutralizing antibody titers of the 92 sera examined are shown in Table 1 and compared with ELISA values. Of the 35 patients showing high ELISA values for antibodies to LA and positive values for antibodies to IEA and EA, the geometric mean neutralizing titer was 1/250; of the 6 serum samples with high ELISA values for LA but negative values for IEA and EA, the geometric mean neutralizing titer was 1/79; in the group of 13 patients with ELISA mid-range positive values for LA, the geometric mean titer of neutralizing antibody was 1/45; and of the 23 patients with low positive ELISA values for LA, the geometric mean neutralizing titer was 1/7. Among the 23 serum samples with low positive ELISA values for LA, 5 were negative by neutralization assay. The 15 samples that were negative for all three antigens by ELISA also proved negative for neutralizing antibody.

The neutralizing titers determined were found to be reproducible, since the test performed onto two different cell cultures gave the same results. Moreover, different runs and different batches of stock virus (used always at 300 PFU/0.2 ml) were checked with positive and negative reference sera and gave similar results.

**DISCUSSION**

In the present study, we describe a rapid neutralization assay for CMV antibody, based on the use of IIF to visualize CMV-induced IEA which are present in the nuclei of infected cells by 1 h after infection. With this method it was possible to perform a neutralization assay for CMV antibody in a single day. In our study, we optimized the sensitivity of the assay by using a stock of CMV that had been propagated at a low multiplicity of infection to avoid noninfectious defective particles capable of binding the antibody (12). Moreover, we added to the immune complex of CMV and antibody to CMV rabbit antibodies to human immunoglobulins; this has been shown to increase the sensitivity of the assay more efficiently than does complement (9). In our study, the Towne strain of CMV was used, as it was demonstrated to cross-react with 14 other naturally occurring CMV strains when analyzed with a panel of monoclonal antibodies to surface membrane proteins (7).

The neutralizing titers obtained with this assay were in accordance with the antibody profiles determined by ELISA to CMV-induced LA, IEA, and EA. High neutralization titers were associated with high ELISA values for antibodies to LA in the presence of antibodies to IEA and EA, and mid-range positive and low positive ELISA values for LA in the absence of antibodies to IEA and EA corresponded to proportionally lower neutralization titers. The neutralization assay we describe fulfills the criteria of a rapid, sensitive, and reproducible assay for the detection of neutralizing antibody to CMV, which gives results comparable to those obtained by ELISA and could be used in routine diagnostic laboratories.

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**LITERATURE CITED**