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 indirect 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 Nunclo 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 10^3 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 and the number of positive cells

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 neutrali-
zation 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 neutral-
izing antibody was 1/45; and of the 23 patients with low
positive ELISA values for LA, the geometric mean neutral-
izing titer was 1/7. Among the 23 serum samples with low
positive ELISA values for LA, 5 were negative by neutrali-
zation 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 re-
ducible, 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 in-
fected 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 very low multiplicity of infection to avoid noninfectious
defective particles capable of binding the antibody (12). More-
over, we added to the immune complex of CMV and
antibody to CMV rabbit antibodies to human immunoglobu-
lins; 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 occur-
ing 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**

1. Gerna, G., P. M. Cereda, E. Cattaneo, G. Achilli, and M. G.
Revello. 1978. Immunoglobulin G to virus-specific early antigens
in congenital, primary and reactivated human cytomegalovirus

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**TABLE 1. Comparison between CMV neutralization titers and
titers by ELISA against CMV-induced IEA, EA, and LA in 92
serum samples**