Electrophoretic Analysis of Polypeptides Immune Precipitated from Cytomegalovirus-Infected Cell Extracts by Human Sera

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Serodiagnosis of cytomegalovirus (CMV) infection by complement fixation tests depends on showing a fourfold rise in antibody titer from acute- to convalescent-phase sera. Freeze-thaw and glycine-extracted, infected cell culture antigens used for these tests give markedly different titers in reactions with the same sera. In this study, we characterized the CMV-infected cell polypeptides contained in freeze-thaw and glycine-extracted antigens and identified the proteins precipitated by 23 pairs of human acute and convalescent sera. Our results were as follows. First, freeze-thaw and glycine-extracted antigens prepared from infected cells radiolabeled with [35S]methionine and subjected to electrophoresis in sodium dodecyl sulfate-polyacrylamide gels yielded similar patterns, and the bulk of the label was contained in late structural proteins and glycoproteins. Glycine-extracted preparations contained a greater proportion of soluble 66,000- and 50,000-molecular-weight proteins than did freeze-thaw antigens. Second, convalescent sera precipitated proteins migrating with apparent molecular weights of 150,000, 130,000, 110,000, 96,000, 74,000, 66,000, 50,000, 34,000, 32,000, and 25,000. Of these the 130,000-, 110,000-, 96,000-, 66,000-, 50,000-, and 25,000-molecular-weight proteins comigrated with glucosamine-labeled polypeptides. Both immunoglobulin G and M antibodies in human sera precipitated these proteins from CMV-infected cell preparations. Implications of the results for serodiagnosis of CMV infections are discussed.

Human cytomegalovirus (CMV) is an important human pathogen which produces a spectrum of clinical symptoms including congenital defects, infectious mononucleosis, postperfusion hepatitis, and interstitial pneumonia in transplant recipients (32). CMV has a linear double-stranded DNA genome, about 150 × 10^6 molecular weight (mw), with the capacity to code a large number of polypeptides (5, 26). About 30 polypeptides ranging in mw from 230,000 to 22,000 have been associated with purified CMV virions and the antigenically related dense bodies (7, 15, 21, 24). The architecture of CMV virions, similar to that of herpes simplex virus, is that of an icosahedral capsid, surrounded by an amorphous matrix which is enveloped in a bilaminar membrane (9, 12). Like herpes simplex virus, CMV acquires its envelope by budding from the membranes of infected cells containing viral glycoproteins. These glycoproteins specify the major immunological determinants of the virus. It has been shown that antisera prepared against purified virions and the glycoproteins extracted from them neutralize virus infectivity and react by immunofluorescence with CMV-infected cells (24).

For clinical diagnosis of CMV, virus can be isolated from infected newborns who excrete large amounts in urine. CMV-infected adults, however, seldom shed sufficient virus for isolation. In most cases, serological techniques which depend on showing a fourfold rise in antibody titer from the acute to convalescent sera are used for diagnosis (3). Measurement of complement-fixing (CF) antibody to CMV is routinely done with antigens prepared from infected cells by freezing and thawing (FT) or by extraction with glycine buffer (GE) (4, 13). Sera tested with both antigens give significantly higher titers with GE than with FT preparations, which suggests that the two preparations differ in the presence or amount of specific antigens (1, 4). We recently reported on the CF activity of density gradient fractions of FT and GE antigens analyzed by electron microscopy (4). In addition to a light peak (1.06 to 1.10 g/cm^3) of amorphous protein with CF activity, a more dense peak (1.18 to 1.22 g/cm^3) with nucleocapsids, dense bodies, and occasional enveloped virions was contained in GE antigen.

In this paper we report the electrophoretic characterization of viral polypeptides in FT and GE antigens prepared from cells labeled after infection with CMV. We also report identifica-
tation of the viral polypeptides immune precipitated by human convalescent sera from extracts of CMV-infected cells. The data show that FT and GE preparations contain different amounts of infected cell polypeptides with similar electrophoretic properties. They also show that CF antibody titers of acute and convalescent sera correlate with immune precipitation of polypeptides comigrating with viral glycoproteins, and that both immunoglobulin G (IgG) and IgM antibodies precipitate these polypeptides.

MATERIALS AND METHODS

Cell culture, media, and virus. Human fetal diploid lung (HFDL) cells were grown in fortified Eagle minimal essential medium containing twice the standard concentration of vitamins and amino acids and supplemented with 10% fetal bovine serum. AD169 strain of human CMV was used throughout the study.

Human sera. Acute and convalescent sera used in this study were submitted to the California Department of Health Services for serological diagnosis of CMV. Sera used for immune precipitation tests with IgM antibody were checked for rheumatoid factor, IgM antibody reactive with IgG by the slide test with Hyland Laboratories latex globulin reagent.

CF tests. CF tests were done by standard methods described previously (16).

Preparation of immune sera to CMV. Procedures used to prepare hamster immune sera to gradient purified CMV virions and dense bodies were previously reported (8).

Radiolabeling of proteins synthesized by infected cells. Confluent monolayers of HFDL cells were infected with 5 to 10 PFU per cell at 37°C before radiolabeling for various intervals as described below. Radiochemicals were purchased from New England Nuclear Corp., Cambridge, Mass. For labeling polypeptides, the cultures were replenished with minimal essential medium containing 1/10 the normal amount of methionine and supplemented with 5 μCi of [35S]methionine (specific activity, 1,200 mCi/mmoll per ml. To radiolabel the glycoproteins, complete media with 0.1 μCi of [14C]glucosamine (45 to 60 mCi/mmoll per ml was added. At the end of the labeling period, the cells were rinsed with ice-cold phosphate-buffered saline to terminate incorporation.

Preparation of samples for electrophoresis. The labeled cells were denatured and solubilized by heating for 2 to 3 min at 80°C in the presence of 2% sodium dodecyl sulfate (SDS), β-mercaptoethanol, and 0.05 M Tris-hydrochloride (pH 7.0).

Polyacrylamide gel electrophoresis. The polyacrylamide gel electrophoresis was done in a discontinuous buffer system containing 0.1% SDS. The stacker and separation gels contained 3 and 9% acrylamide, respectively, cross-linked with N,N-diallyltartardiamide (Bio-Rad Laboratories, Richmond, Calif.). Gels were fixed in methanol and acetic acid and prepared for fluorography with EnHance (New England Nuclear). The proteins used for calibration of molecular weights were myosin, β-galactosidase, phosphorylase B, bovine serum albumin, ovalbumin, and trypsinogen.

Preparation of radiolabeled antigens. HFDL cells grown in 32-oz. (ca. 960-ml) bottles were infected and radiolabeled with [35S]methionine (5 μCi/ml) in media with 1/10 the normal amount of methionine from 96 to 120 h postinfection. At the end of the radiolabeling period, the cells were washed with cold phosphate-buffered saline, dislodged from the glass, and centrifuged. To prepare FT antigen, 1.4 ml of phosphate-buffered saline was added to the cell pellet, and the cells were frozen and thawed three times. For the preparation of GE antigen, the infected cells were resuspended in 1.4 ml of glycine buffer (pH 9.5). To extract with nonionic detergents, infected cells were sonicated in 1.4 ml of phosphate-buffered saline containing 1% Nonidet P-40 and 1% sodium deoxycholate (Sigma Chemical Co., St. Louis, Mo.). Radiolabeled FT, GE, and nonionic detergent-extracted, infected cell extracts were centrifuged at 24,000 rpm in an SW27.1 rotor for 1 h at 4°C to remove insoluble proteins before use in immune precipitation tests.

Immune precipitation tests. Radiolabeled antigen (100 μl) was mixed with immune sera or human acute and convalescent sera (100 μl) and incubated at 37°C for 2 hr. To collect the immune precipitates, 5 mg of protein A-Sepharose (Sigma) was added, and the mixtures were incubated for 1 h with gentle agitation. To remove unreacted antigen, the beads were washed five times with phosphate-buffered saline, glycine buffer, or nonionic detergent containing buffer, as prescribed by the antigen preparation used, and centrifuged in an Eppendorf microfuge. To separate IgG and IgM, the immune precipitates were collected on anti-IgG or anti-IgM Sepharose beads (Bio-Rad).

RESULTS

Preparation of radiolabeled FT and GE antigens. Three series of experiments were done to characterize the infected cell polypeptides and glycoproteins in FT and GE antigens.

The first series of experiments was designed to determine the optimal time interval for radiolabeling the late polypeptides before the decline of protein synthesis in the infected cells. These experiments were based on procedures used in serological tests in which CMV antigens, prepared from cells showing advanced cytopathic effect, were harvested 96 to 120 h postinfection. Cells were radiolabeled with [35S]methionine for 24-h intervals from the time of infection, disrupted in SDS and β-mercaptoethanol, and subjected to electrophoresis in SDS-polyacrylamide gels (Fig. 1). The electrophoretic profiles showed that the late viral proteins were synthesized in large amounts from 4 to 5 days postinfection, at which time host protein synthesis began to decline and viral polypeptides were clearly detected. Two prominent polypeptide bands synthesized at this time were the 150,000-mw capsid protein and the 66,000-mw matrix protein. Polypeptides with apparent mw of 220,000, 165,000, 130,000, 115,000, 110,000, 96,000, 88,000, 74,000, 50,000, 45,000, 34,000, 32,000, 27,000, and 25,000 were also synthesized late in infection.
In the second series of experiments, infected cells were labeled with $[^{14}C]$glucosamine at different times to identify late polypeptides which were glycosylated (Fig. 2). Eight electrophoretically distinct glycoproteins with apparent mw of 130,000, 110,000, 96,000, 66,000, 50,000, 48,000, 46,000, and 25,000 were synthesized in large quantities from 4 to 5 days postinfection.

In the third series of experiments, the polypeptides in FT and GE antigens were characterized by electrophoretic mobility, and the structural polypeptides were identified by immune precipitation tests with antisera to purified virions and dense bodies. Infected cells labeled with $[^{35}S]$methionine from 4 to 5 days postinfection were prepared by FT, GE, or extraction with nonionic detergents as described above. The extracts were centrifuged at 2,000 and 24,000 rpm, disrupted in SDS, and subjected to electrophoresis in polyacrylamide gels. Polypeptide profiles of the antigen preparations are shown in Fig. 3. It was found that GE-infected cell extracts or cell extracts prepared with nonionic detergents contained greater amounts of late proteins than did FT cell extracts. A large number of the proteins were insoluble since the electrophoretic profiles of infected cell extracts subjected to high-speed centrifugation gave less intense autoradiographic images than did low-speed-centrifuged extracts. GE and FT preparations yielded similar polypeptide profiles, except that the GE preparation was enriched in the 66,000- and 50,000-mw polypeptides.

To identify the structural proteins in infected cell extracts, immune precipitation experiments were done with hamster immune sera produced against gradient-purified virions and dense bodies. Immune precipitates of radiolabeled FT and GE antigens from high-speed-centrifuged extracts contained polypeptides with apparent mw of 150,000, 110,000, 96,000, 74,000, 66,000, 50,000, 48,000, and 32,000 (Fig. 3). With the exception of the 74,000-mw polypeptide these bands were precipitated from both antigens and in greater quantity from GE antigen. The electrophoretic profiles of $[^{35}S]$methionine- and

**FIG. 1.** Electrophoretic profiles of polypeptides radiolabeled with $[^{35}S]$methionine at different times after infection of cells with CMV.

**FIG. 2.** Electrophoretic profiles of polypeptides radiolabeled with $[^{14}C]$glucosamine at different times after infection of cells with CMV.
[14C]glucosamine-labeled infected cell lysates were compared to identify the glycosylated polypeptides (Fig. 3). Polypeptide bands corresponding to apparent mw of 110,000, 96,000, 66,000, 50,000, 48,000, and 32,000 comigrated with bands produced by lysates of [14C]glucosamine-labeled, infected cells.

Additional immune precipitation experiments with antigens clarified only by low-speed centrifugation had a high background of proteins non-specifically bound to the precipitates (data not shown). As a result, all immune precipitations with human sera described hereafter were done with high-speed-centrifuged FT, GE, and non-ionic detergent-extracted antigens.

Immune precipitation of CMV antigens with human sera. For this series of experiments, 23 paired sera from CMV-infected patients were selected for immune precipitation tests. CF titers of acute and convalescent sera with GE and FT antigens are summarized in Table 1. The sera were divided into three groups based on reactivity as follows: (i) sera with rising titer to FT and GE antigens, (ii) sera with rising titer to FT antigen and stationary titer to GE antigen, and (iii) sera with stationary titer to FT and GE antigens. Additional CF tests were done with selected sera to determine whether the CF antibody titer found with GE antigen prepared by low-speed centrifugation would be affected by high-speed centrifugation of antigen prepared for immune precipitation tests (Table 2). An overall decrease in CF antibody titer was found in tests with GE antigen subjected to centrifugation at 24,000 rpm; however, all sera which showed a rise in CF titer with low-speed-centrifuged antigen continued to do so with high-speed-centrifuged antigen extracts.

Two series of immune precipitation tests with radiolabeled FT and GE preparations were done with representative sera from each group to identify the antigenic polypeptides. Sera were reacted with radiolabeled FT and GE antigens, and identical amounts of solubilized, SDS-denatured immune precipitates were applied to adjacent sample slots of an SDS-polyacrylamide gel slab to compare the polypeptide profiles.

Results of the first series of immune precipitation tests with [35S]methionine-labeled FT antigen are shown in Fig. 4. The weak intensity of polypeptide bands precipitated with acute serum St from group 1 correlated with the low CF antibody titer. In comparable tests, convalescent serum St precipitated appreciably more protein from FT antigen than found with the acute serum; both CF and immune precipitation tests showed a substantial rise in CMV antibody concentration. Immune precipitates obtained with paired sera Fa from group 2 showed less difference in intensity of electrophoretic profiles of acute and convalescent sera, in agreement with a smaller rise and lower CF antibody titer. Polypeptide profiles of group 3 paired sera with stationary CF titers were similar in intensity and polypeptide content (serum R). All CF-positive sera precipitated large amounts of polypeptides with apparent mw of 66,000, 50,000, 34,000 and 32,000. Paired sera St and R precipitated large
TABLE 1. Antibody titers of acute (a) and convalescent (c) sera in CF tests with FT and GE antigens prepared from CMV-infected cells

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* CF titers with CMV-infected cell antigens centrifuged at 2,000 rpm.

amounts of the 150,000-mw capsid protein. A 74,000-mw band was also precipitated; however, patient sera Fa failed to precipitate large quantities of this polypeptide. Trace amounts of the 130,000-, 110,000-, 96,000-, and 25,000-mw polypeptides were precipitated from FT antigen by all the convalescent sera.

In parallel immune precipitation tests, sera from the same patients were reacted with [35S]methionine-labeled GE antigen (Fig. 5). The 66,000- and 50,000-mw polypeptides were the predominant bands contained in all immune precipitates with acute and convalescent sera, with the exception of the acute serum from patient ST which precipitated only trace amounts of the polypeptides. The intensity of the electrophoretic profiles reflected the antibody titer of the sera in CF tests (Table 1). Convalescent sera from patient ST contained a greater amount of precipitating antibody than the acute sera, in agreement with the higher CF titer. In contrast, the stationary CF titer of patient sera Fa and R with GE antigen was reflected in the similar intensity of electrophoretic patterns of polypeptides precipitated by acute and convalescent sera. For each of the paired sera, comparison of CF titers and intensity of precipitated polypeptide bands showed that convalescent sera were more reactive with GE than with FT antigen. Moreover, greater amounts of the 66,000- and 50,000-mw polypeptides were precipitated from GE infected cell antigen.

Results of the second series of immune precipitation experiments with patient sera are shown in Fig. 6 and 7. Electrophoretic profiles of precipitates from reactions of radiolabeled FT
antigen and four paired sera representing group 1 (Pa and Yo), group 2 (Ce), and group 3 (G) are shown in Fig. 6. Rising CF titers with FT antigen for group 1 sera correlated with the increased intensity of radiolabeled polypeptide patterns obtained with convalescent sera in immune precipitation tests. Although group 2 and 3 paired sera gave low titers in CF tests with FT antigen, the intensity of the polypeptides precipitated showed that more antibody was present than indicated by CF tests. Immune precipitates obtained in reactions with radiolabeled GE antigen were similar in pattern to those with FT antigen, but of greater intensity (Fig. 7). Polypeptides of 150,000, 130,000, 66,000, 50,000, 34,000, 32,000, and 25,000 mw were the most prominent bands precipitated. Variable amounts of polypeptides with apparent mw of 110,000, 96,000, 74,000, and 40,000 were also precipitated.

The next series of immune precipitation experiments was designed to characterize the polypeptides precipitated from infected cell lysates.

**FIG. 4.** Electrophoretically separated $^{35}$S-methionine labeled polypeptides immune precipitated from CMV-infected cell FT antigen by human acute (a) and convalescent (c) sera. Control serum was negative for CF antibody to CMV.

**FIG. 5.** Electrophoretically separated $^{35}$S-methionine-labeled polypeptides immune precipitated from GE infected cell antigen by human acute (a) and convalescent (c) sera. Control serum was negative for CF antibody to CMV.
FIG. 6. Electrophoretically separated, [35S]methionine-labeled polypeptides immune precipitated from CMV-infected cell FT antigen by human acute (a) and convalescent (c) sera.

enriched for viral glycoproteins. Nonionic detergent extracts of CMV-infected cells were reacted with representative sera from each group. The electrophoretic profiles of [35S]methionine-labeled immune precipitates were similar to those of FT and GE antigens, but more closely resembled profiles obtained with GE antigen (Fig. 8). Precipitated in large quantities were the 66,000-, 50,000-, 34,000-, and 32,000-mw polypeptides. In addition, variable amounts of the 130,000-, 96,000-, and 25,000-mw polypeptides and trace amounts of the 150,000-, 76,000-, 74,000-, 45,000-, and 40,000-mw polypeptides were precipitated.

**Immune precipitation of CMV antigens with IgG and IgM.** Immune precipitation tests were done to determine whether IgG and IgM antibody reacted by immunoprecipitation with extracts of CMV-infected cells. For these experiments, radiolabeled GE antigen was first reacted with whole sera, and then the immune precipitates were removed with anti-IgG or anti-IgM cross-linked to Sepharose beads as described above. Immunoglobulins from acute group 1 sera Yo and St did not precipitate any detectable CMV proteins (Fig. 9). Trace amounts of the 66,000-mw polypeptide was detected with group 1 acute Pa sera (Fig. 10). Electrophoretic profiles of polypeptides precipitated by IgG and IgM from the convalescent sera correlated with CF antibody titers and were similar to patterns described for previous immune precipitation tests. Paired sera B from group 2 gave similar profiles for both immunoglobulins, precipitating

FIG. 7. Electrophoretically separated, [35S]methionine-labeled polypeptides immune precipitated from GE infected cell antigen by human acute (a) and convalescent (c) sera.
FIG. 8. Electrophoretically separated, [35S]methionine-labeled polypeptides immune precipitated by human acute (a) and convalescent (c) sera from CMV-infected cells treated with nonionic detergents.

large amounts of the 66,000- and 50,000-mw polypeptides. More of the 150,000-mw capsid protein was detected in immune precipitates with IgM (Fig. 10). Acute serum Ce of group 2 precipitated large amounts of the 66,000- and 50,000-mw proteins with IgM and IgG; however, the convalescent serum (taken 6 weeks later) showed a declining immune response (Fig. 9).

DISCUSSION

Characterization of FT and GE antigens. In this study we characterized CMV-infected cell polypeptides in FT and GE antigens by their electrophoretic mobility in SDS-polyacrylamide gels. Antigens prepared from cells radiolabeled from 4 to 5 days postinfection contained primarily structural polypeptides that were synthesized late in the virus replication cycle. Of these, eight polypeptides with apparent mw of 130,000, 110,000, 96,000, 66,000, 50,000, 48,000, 46,000, and 25,000 were contained in both antigens. Insomuch as these bands comigrated with glycoprotein bands, they are probably the major glycoproteins in CMV-infected cells and we shall refer to them as such.

Comparison of the electrophoretic profiles of FT, GE, and nonionic detergent-extracted antigens showed that they contained different amounts of polypeptides with similar electrophoretic properties. Glycine buffer more efficiently extracted the bulk of the late proteins from CMV-infected cells, particularly the 66,000- and 50,000-mw glycoproteins. In fact, the polypeptide profile of GE antigen, comparable to that of nonionic detergent-extracted antigen, was enriched for the highly insoluble viral

FIG. 9. Electrophoretically separated, [35S]methionine-labeled polypeptides immune precipitated from CMV-infected cell GE antigen by IgM and IgG from human acute (a) and convalescent (c) sera.
glycoproteins extracted from infected cell membranes.

**Infected cell polypeptides reactive with antibody in human acute and convalescent sera.** Human acute and convalescent sera were used to immune precipitate the antigens in radiolabeled FT and GE preparations. Immune precipitation tests showed that all of the CMV glycoproteins, particularly the 66,000- and 50,000-mw bands, were immune precipitated by human sera with CF antibody titer. It has been well established that herpes simplex virus glycoproteins are highly immunogenic and are the major antigens precipitated by human convalescent sera (6, 17, 23). Our results show that CMV convalescent sera with large amounts of CF antibody also precipitate the viral glycoproteins. Data from this study agreed with and extended previous observations that soluble GE antigen from AD169 and Davis strains contained two immunogenic proteins with mw of 150,000 and 67,000 to 85,000 (14, 29). It has been shown that immune serum produced against purified CMV virions neutralizes virus infectivity (24), reacts with glycoproteins in CMV-infected cell membranes (27), and has CF activity (8). Our results showed that all sera with CF antibody to CMV precipitated the major glycoproteins from extracts of infected cells, and it is likely that a large proportion of the antibodies were neutralizing.

It is of interest to note that some variation in the polypeptide profiles of immune precipitates with different sera were found. This may reflect differences in antibody reactive with particular polypeptides or antigenic variation among CMV strains. Purified CMV virions have similar structural proteins (10) and are serologically related (31). They have been shown to differ also in restriction enzyme cleavage sites of their DNAs (11), fixation of complement-dependent antibody (31), and kinetics of neutralization (30). Preliminary analysis of the infected cell polypeptides of a small number of CMV isolates showed that one strain failed to produce large amounts of the 66,000-mw glycoprotein synthesized by four isolates of limited passage (data not shown). Since this glycoprotein is a major antigen in extracts of AD169-infected cells, sera from patients infected with strains which fail to produce the normal amount of this protein might give artificially low CF titers in tests employing the standard antigen preparations.

**Immunoglobulin classes reactive with CMV antigens.** Immune precipitation tests with GE antigen showed that CMV glycoproteins were precipitated by IgG and IgM antibodies. These results are supported by previous studies which showed that IgM antibodies react with the membrane antigens on CMV-infected cells (28) and with nucleo antigens late in infection (20). It has recently been reported that CMV-specific IgM antibody possesses CF activity (2, 3). Our results showed that IgM antibody immune precipitated soluble CMV glycoproteins and suggest that IgM contributes to the antibody titer in CF tests with GE antigen. Although the sera in this study were negative for rheumatoid factor, we cannot rule out potential interference of rheumatoid factor in highly sensitive immune precipitation tests with radiolabeled antigens. Serological studies have shown that IgM antibody is present in both primary and reactivated CMV infections and may be diagnostic for intrauterine CMV infections (19, 22). Considering the serious se-

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**FIG. 10.** Electrophoretically separated, [35S]methionine-labeled polypeptides immune precipitated from CMV-infected cell GE antigen by IgM and IgG from human acute (a) and convalescent (c) sera.
quelea of such infections, further effort should be directed to development of more specific tests for detection of IgM antibody to CMV.

In this study we showed that the viral glycoproteins are the major antigens in extracts of CMV-infected cells. Results of serological tests with human sera suggest that different strains may specify glycoproteins with heterologous immunologic determinants. We recently reported the serological analysis of herpes simplex virus type 1 and 2 strains by using monoclonal antibodies to dissect the antigenic domains on the viral glycoproteins (18). We found that strains of each serotype differ with respect to particular antigenic sites and that strains can be grouped on this basis. Similar serological studies of the antigenic determinants on glycoproteins specified by different CMV strains are currently in progress.

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LITERATURE CITED