Detection of Immunoglobulin M and G Antibodies Against Cytomegalovirus Early and Late Antigens by Enzyme-Linked Immunosorbent Assay

JAAP M. MIDDELDORP,^{1*} JOKE JONGSMA,¹ ANITA TER HAAR,¹ JURJEN SCHIRM,² AND T. HAUW THE¹

Department of Clinical Immunology, University Hospital,¹ and Department of Virology, Public Health Laboratory,² Groningen, The Netherlands

Received 13 December 1983/Accepted 2 April 1984

A sensitive and reproducible enzyme-linked immunosorbent assay (ELISA) is described for the detection of immunoglobulin M and antibodies with specificity for human cytomegalovirus (CMV) early (CMV-EA) and late (CMV-LA) antigens. The emphasis is on the production of high-quality CMV antigens, CMV-EA and CMV-LA separately, and conditions for their application in the ELISA. The induction of CMV-EA and -LA in infected cell extracts was studied in detail by using human sera with defined antibody specificity for CMV-EA and CMV-LA. This resulted in the development of a simple whole cell extraction procedure that provided a high yield of CMV antigens with reproducible antigen quality. The antigens were specific for the detection of anti-CMV antibodies. The influence of autoantibodies on the determination of CMV-specific antibodies was investigated. Parallel analysis of 322 human sera by indirect immunofluorescence and ELISA showed a high correlation between both assays ($r = 0.9674$ for CMV-EA and 0.9362 for CMV-LA). Antibody titers determined by ELISA were equal to (for CMV-EA) or slightly higher (for CMV-LA) than those determined by immunofluorescence but significantly higher (20- to 5,120-fold) than those determined by complement fixation. From 191 sera positive by ELISA (titer ≥ 40) 4 (2.1%) were negative by immunofluorescence (titer < 40), and from 61 ELISA-positive sera 12 (19.6%) were negative (titer < 8) when tested by complement fixation. Consequently, ELISA for CMV may prove to be more reliable for the selection of CMV-seronegative blood donors than these other methods. The use of high-quality antigens allows more economic handling of large-scale serum determinations. Possibilities for further automation are discussed.

Serological screening by means of indirect immunofluorescence (IF) for the detection of antibodies against human cytomegalovirus (CMV) has proven to be very sensitive and specific in comparison to other techniques (13).

However, IF is laborious and requires well-trained personnel for interpretation of the results. Moreover, the presence of antinuclear antibodies (ANA) in a patient's serum may give misleading results. The IF test allows the detection of immunoglobulin M (IgM) and IgG antibodies against CMV early (CMV-EA) and late (CMV-LA) nuclear antigens separately (19, 28, 32, 33). Thus far, anti-CMV-EA antibodies could only be detected by means of IF (9, 10, 23, 28, 33).

The presence of high levels of anti-CMV-EA antibodies may be a reflection of active virus replication in the host as indicated in previous studies (10, 23, 28, 32) and in studies on vaccination with attenuated CMV (9).

The presence of CMV-specific IgM in a patient's serum may reflect a recent or ongoing active CMV infection and probably forms the best parameter for the early diagnosis of acute CMV infection (11, 24, 27). This has been shown for congenital CMV infection (11, 12), posttransplantation CMV infection (24, 31), and CMV infection in previously healthy individuals (19, 24). Both primary and secondary CMV infection may be associated with the presence of virus-specific IgM (24, 31), although in some instances IgM is not detectable during reinfection with CMV (12, 24, 31). The measurement of IgM, however, is complicated by false-positive reactions due to the presence of rheumatoid factor (RF), thus requiring its removal before testing for specific IgM (34). Recently, a number of new serological techniques have been described for the detection of antibodies against

CMV, either based upon the principles of a radioimmunoassay (11, 24, 36) or an enzyme-linked immunosorbent assay (ELISA) (4, 7, 18, 20, 26, 27, 30, 35, 36, 39), which appear to be both rapid and sensitive. This would justify their application for routine serology (2, 3, 13).

However, the detection of anti-CMV-EA antibodies by these techniques has not been described as yet, and comparisons of radioimmunoassay or ELISA data with those of a sensitive serological test such as IF are scarce (13, 18, 40). In most instances, comparisons have been made with relatively insensitive, although still widely used, tests such as complement fixation (CF) or indirect hemagglutination inhibition (3, 5, 7, 26, 28). A major advantage of ELISAs and radioimmunoassays compared with IF is the possibility to quantify the results by using automated equipment for reading the results and a table top computer for data processing (8, 17, 25).

Such an approach for routine serology requires standardization of all procedures and ingredients, including the antigens used (2). This aspect has been relatively neglected in previous reports on ELISA for CMV (4, 7, 18, 30, 36, 39, 40).

In this paper we describe conditions for isolation of CMV-EA and CMV-LA which are suitable for application in the ELISA. A high content of CMV-specific proteins in the antigen preparations provides the basis for a reliable and reproducible technique for the specific detection of antibodies against CMV-EA and CMV-LA with a sensitivity equal to or better than that of IF. In addition, the technique can possibly be used with automated data processing.

MATERIALS AND METHODS

Tissue culture. Human embryonic lung fibroblasts (FF; passage 10 to 25) grown in Dulbecco modified Eagle medium

* Corresponding author.

(Flow, Irvine, Scotland) containing 2 mM glutamine, 0.3% bicarbonate, and antibiotics and supplemented with 10% fetal calf serum (GIBCO Europe, Hoofddorp, The Netherlands) were free of mycoplasma contamination as was routinely tested by DNA staining (6). Infectious cell-free human CMV strain AD-169, plaque purified three times, was obtained from the supernatant fluid of FF cultures inoculated at 0.01 PFU per cell in 830-cm² roller bottles (50 ml of Dulbecco modified Eagle medium plus 3% fetal calf serum per bottle, changed weekly; Corning, Avon, France) 4 days after 100% cytopathic effect (CPE). Cells and debris were removed by centrifugation at $1,200 \times g$ for 10 min, and small volumes were frozen directly in liquid nitrogen and stored at -80°C . Infectivity was usually 1×10^7 to 4×10^7 PFU/ml.

Preparation of CMV-EA and CMV-LA. CMV early- and late-infected cells for IF were prepared as described previously (33). Antigens for ELISA were prepared by alkaline-glycine extraction by the method of Kettering et al. (16) with some modifications. CMV-infected FF monolayers (usually 1×10^7 to 2×10^7 cells per Roux bottle) were washed twice with phosphate-buffered saline, (PBS; pH 7.2), and the cells were dislodged in PBS containing 5 mM EDTA. After centrifugation at $700 \times g$ for 10 min, the cell pellet was suspended by repeated pipetting in ice-cold 0.1 M glycine-NaOH buffer (pH 9.75) containing 0.9% NaCl (glycine buffer; 1 ml per 10^7 cells), shaken for 20 min at 0 to 4°C , and finally homogenized completely by four 30-s bursts of an MSE sonifier at maximal power setting with a small sonication tip (0°C). Cell debris was removed by centrifugation at $700 \times g$ for 10 min, and the supernatant fluid was collected and stored at -80°C (GE antigens).

For the preparation of CMV-EA (EA-GE), cells were infected at 5 PFU per cell and cultured for 48 h at 37°C in Dulbecco modified Eagle medium plus 3% fetal calf serum in the presence of 0.5 mM phosphonoformate (PFA) to completely block viral DNA synthesis and induction of CMV-LA (37). CMV-LA (LA-GE) was prepared from cells infected at 2 PFU cell and harvested 10 days after inoculation. Control antigen (FF-GE) was prepared from uninfected FF cultures. Modifications of these procedures used to study optimal antigen induction are described below. Total protein content of GE antigen preparations was measured by the method of Lowry et al. (21), with bovine serum albumin (BSA) in 0.1 M glycine buffer as a standard. ELISA reactivity of GE antigens was determined by block titration of serial antigen dilutions made in 0.1 M carbonate buffer (pH 9.6; see below). Antigen dilutions allowing optimal discrimination between positive and negative reference sera were selected, and GE antigens were stored in a $50\times$ concentrated form in glycine buffer at -80°C . Before use, the antigens were diluted in carbonate buffer. FF-GE was always used at the same protein concentration as the CMV-GE antigens. The quality of GE antigens (antigenicity), which was defined as the amount of protein producing optimal ELISA reactivity with a standard reference serum (see below), was reproducible among different antigen preparations.

ELISA procedure. Several parameters affecting ELISA reactivity of human sera were investigated, which resulted in an optimal procedure as outlined below which was used throughout this study.

For coating, individual wells of 96-well polystyrene flat-bottomed microelisa plates (M 129 A; Dynatech Laboratories, Inc., Alexandria, Va.) received 0.1 ml of GE antigen (diluted 1:50 in carbonate buffer, pH 9.6), and the plates were sealed (Titertek plate sealing tape) and left at 4°C for 40 h. Immediately before use, the coating solution was aspirat-

ed, the wells were washed three times (with a 5-min incubation in the first wash) with 0.3 ml of PBS containing 0.05% Tween 20 (PBS-T) per well, and subsequently the plates were dried by beating onto a paper towel. Serum samples to be tested for CMV antibody were diluted in PBS-T containing 4% BSA and additional 0.15 M NaCl (0.3 M final concentration) to minimize nonspecific binding. Of each serum dilution (fourfold dilutions starting from 1:40 up to 1:40,960), 0.1 ml was added in duplicate and plates were covered and incubated at 37°C for 45 min. Thereafter, the plates were washed again as described above and shaken dry. Affinity-purified horseradish peroxidase-labeled sheep anti-human antibodies (horseradish peroxidase conjugates: anti-IgG H+L, anti-IgG γ -chain, and anti-IgM μ -chain; l'Institut Pasteur, Paris, France were diluted 1:1,000 in PBS-T containing 2% BSA, and 0.1 ml was added to each well. The plates were covered, incubated, and subsequently washed as above. Finally, 0.1 ml of fresh substrate solution (*ortho*-phenylenediamine dihydrochloride, 0.2 mg/ml of 50 mM phosphate buffer, at a final pH of 5.6, containing 0.006% H_2O_2) was added. The reaction was allowed to proceed at 20 to 22°C in the dark for 30 min and was stopped by adding 0.1 ml of 1 M H_2SO_4 .

Endpoint determination and controls. The color intensity of the wells as measured at 492 nm with the Titertek Multiskan. As a control in each assay and for standardization among different assays, each ELISA plate received a CMV antibody-positive and a CMV antibody-negative reference pool serum (see below) together with six patient sera in identical dilutions. All sera were tested on control antigen (FF-GE)-coated plates to detect non-CMV specific reactions directed against normal human fibroblast antigens. The antibody titer of individual sera was determined by endpoint titration by using fourfold dilution steps starting from 1:40. Sera giving optical density at 492 nm (OD_{492}) values below that of the CMV-negative reference pool sera at the same dilution were considered negative. Sera giving OD_{492} values above that of the reference pool serum on control antigen were considered to contain autoantibodies. The endpoint of positive sera was calculated by subtracting the value for the control reaction (OD_{492} on FF-GE) from that of the reaction on CMV antigen, either EA-GE or LA-GE, at the same dilution. The last dilution giving a difference of at least 0.100 was taken as the endpoint titer. The cutoff point of 0.100 OD_{492} units represented about twice the mean observed difference between the value for the reaction of the CMV antibody-negative reference serum on CMV antigen and that of the control antigen at a 1:40 dilution. At further dilutions, this difference was always smaller. For comparative studies (Fig. 1 through 6), data are expressed for the reaction with the anti-IgG H+L horseradish peroxidase conjugate, whereas for patient studies IgM and IgG were measured specifically.

Other serological tests. CF and IF assays for the detection of CMV antibodies were performed as described previously (19, 33). Rheumatoid factor (RF) was measured by latex agglutination (38), IgM-RF was measured by ELISA (unpublished data), and anti-nuclear antibodies (ANA) were determined by IF with 50% (vol/vol) acetic acid-ethanol-fixed human fibroblasts (14).

Sera. The sera used in this study were obtained from normal healthy donors that were seropositive ($n = 38$) or seronegative ($n = 41$) for CMV, or from patients with CMV mononucleosis or hepatitis ($n = 15$), patients who were renal or liver allograft recipients ($n = 132$), and patients who had undergone open heart surgery ($n = 18$). Furthermore, multi-

ple sera from patients who had a primary ($n = 10$) or secondary ($n = 20$) CMV infection after renal transplantation were analysed retrospectively.

Diagnosis of CMV infection was based upon clinical parameters (heterophyl antibody-negative mononucleosis; hepatitis B surface antigen-negative hepatitis; or allograft dysfunction associated with [spiking] fever, leukopenia or thrombopenia or both, myalgia, arthralgia, and occasionally with gastrointestinal infection[s] and pneumonitis) and was confirmed within 1 to 2 weeks by serology (IF or ELISA or both) by a fourfold rise in virus-specific IgG, in most cases (86%) accompanied by a positive IgM against CMV-LA; by virus isolation from urine or throat or both in 8 of 15 mononucleosis-hepatitis patients and in 19 of 30 allograft recipients; and in some cases by histopathology. Viral cultures were performed at the Public Health Laboratory, Groningen. Specificity control sera with antibodies against Epstein-Barr virus, herpes simplex virus, or varicella zoster virus were kindly donated by F. P. Schröder (Public Health Laboratory, Groningen) and A. J. Scheffer (Department of Medical Microbiology, University Hospital, Groningen). Sera containing IgM RF were a gift from M. H. van Rijswijk, and sera positive for ANA were a gift from P. C. Limburg (both at University Hospital, Groningen).

Reference pool sera. For standardization of the CMV ELISA test three different reference serum pools were made, each consisting of 10 individual sera negative for RF and ANA.

Pool 1 was CMV antibody negative (titer <40) and consisted of sera from healthy donors, each CMV seronegative as tested by IF and ELISA. Pool 2 was CMV IgG antibody positive and consisted of sera from healthy seropositive donors and had a titer of 2,560 for CMV-EA and 10,240 for CMV-LA in both IF and ELISA. Pool 3 was CMV IgM and IgG antibody positive and consisted of sera from CMV mononucleosis patients obtained during the acute or convalescent phase of infection (for IgM, CMV-EA titer = 640, CMV-LA titer = 40,960; for IgG, CMV-EA titer = 640,

CMV-LA titer = 2,560). The three serum pools were stored in 0.1-ml volumes at -80°C .

RESULTS

CMV-EA and CMV-LA. In searching for optimal conditions for the preparation of CMV-EA and CMV-LA that were suitable for use in the ELISA, alkaline glycine extraction followed by extensive sonication was found to be superior as compared with other extraction methods. In these studies we noticed a great variation in the antigen content per microgram of protein (antigenicity) among different preparations, even though isolation was performed by identical protocols (results not shown). To obtain a more reproducible quality of CMV-GE antigens, their induction was studied in detail (Fig. 1 and 2). Subconfluent FF monolayers were inoculated with different concentrations of virus, and CMV antigen was extracted (see above) at different times after infection. For each extract, 0.01 mg of protein per ml of carbonate buffer was used for coating ELISA plates.

The induction of late antigens (Fig. 1) was studied by using an anti-CMV-LA-positive serum without detectable antibodies against CMV-EA (measured by IF, CMV-EA titer <40 , CMV-LA titer = 10,240). When infected at 2 PFU per cell, early cell rounding was apparent within 12 to 24 h postinfection but significant CMV-LA reactivity was not detectable until 2 days after inoculation. At 5 to 6 days postinfection, late CPE was seen in about 100% of the cells, nuclear inclusions were apparent, and newly produced virus was detectable in the supernatant fluid. At this time there still was a strong increase in antigenicity, reaching a maximum level about 3 days later. Although the production of infectious virus decreased, nuclear inclusions remained very large and the antigenicity of the extracts remained constant over the next few days. Subsequently, the advancing CPE caused cells to detach from the growth surface, leading to a decrease in the net yield per Roux bottle. Similar results were found with LA-GE antigens extracted from cells infect-

FIG. 1. Induction of CMV-LA. Subconfluent FF monolayers were inoculated with CMV strain AD169 at 0.1 (●) or 2 PFU per cell (○), and antigens were extracted on different days after 100% CPE (●) or inoculation (○) as described in the text. The production of infectious virus in the supernatant fluid was studied in parallel in cultures inoculated at 2 PFU per cell (*). Each point is the mean of two independent experiments. ELISA reactivity (OD_{492}) of the extracts (all tested at $1 \mu\text{g}$ of protein per well) was determined under standard conditions (see the text) by using a serum (at a dilution of 1:40) without antibodies against CMV-EA as described in the text. CMV antibody-negative sera ($n = 5$) and the CMV negative reference serum all reacted within the shaded area.

FIG. 2. Induction of CMV-EA. Subconfluent FF monolayers were inoculated at 0.5 (*), 1.0 (▲), 2.0 (●), or 5.0 (■) PFU per cell with CMV strain AD169 and cultured in the presence of 0.5 mM PFA to block viral DNA synthesis and induction of CMV-LA. EA was extracted as described in the text at different times after inoculation. ELISA reactivity of the extracts (all tested at 1 μ g of protein per well) was determined under standard conditions by using the CMV IgG-positive reference serum (pool 2) at a 1:40 dilution. CMV antibody-negative sera ($n = 5$) and the CMV-negative reference serum all reacted within the shaded area.

ed at 0.1 PFU per cell. At the point of 100% CPE, the relative amount of CMV-specific antigens in the extracts still increased strongly and reached a maximal value 3 to 4 days later, which was similar to the extracts from cells infected at high multiplicity infection. Isolation of LA-GE at 8 to 10 days after infection at 2 PFU per cell or 3 to 4 days after 100% CPE with a multiplicity of infection of 0.1 PFU per cell gave very reproducible results.

The induction of CMV-EA (Fig. 2) was studied as described above except that we used the reference serum pool 2 for the detection of EA reactivity in the extracts. Before extraction, a small sample of the cells was prepared for IF to analyze the type of nuclear staining (33). No late antigens (condensed nuclear staining) were found at any time with PFA at 0.5 mM to block viral DNA synthesis (not shown). EA reactivity was first detectable at a significant level at about 3 h postinfection with a multiplicity of infection of 5 PFU per cell and increased strongly during the first 18 to 36 h postinfection depending on the inoculum used. It reached a maximum level around 24 h postinfection. Thereafter, EA reactivity decreased slightly. Immediately after infection, antigenicity was already greater than the control antigen (uninfected cell extract) level due to the presence of inoculum proteins attached to the cell monolayer. This was confirmed when cultures were treated with cycloheximide (0.05 mg/ml) before, during, and after infection to completely block the induction of new antigens. In extracts from these cycloheximide-treated cultures, no additional CMV-specific antigens were induced above the inoculum level (not shown). The level of EA reactivity was dependent on the amount of input virus used and was maximal at 5 PFU per cell. Also, higher levels of EA reactivity were obtained when FF cultures were inoculated at the subconfluent stage as compared to cultures inoculated 2 days after reaching confluency. Preparation of EA-GE from cells infected at 5 PFU/cell for 48 h in the presence of 0.5 mM PFA provided a reproducible source of CMV-EA.

In the kinetic studies described above (both for EA-GE and for LA-GE), no specific increase of reactivity of CMV

GE antigens was noticed with the CMV-negative reference serum. However, the reactivity of CMV-negative sera on CMV GE antigens always was slightly higher than that on FF-GE coated at the same protein concentration. Preincubation of CMV GE antigen-coated plates with different animal sera could not inhibit this reactivity.

Specificity of the GE antigens. The EA and LA specificity of the GE antigens was analyzed in a number of tests. As mentioned above, we did not find LA antigens (as nuclear inclusions detected by IF on fixed cells) in cultures treated with 0.5 mM PFA after infection. Furthermore, we checked the EA-GE for LA reactivity in the ELISA by using a set of sera shown to be anti-EA negative and anti-LA positive by IF (Table 1). Although all sera were positive on LA-GE (sera 1 and 4 were strongly positive), no reaction on EA-GE was detectable with EA-negative sera (sera 1 through 3), and low reactivity was found with sera slightly positive for CMV-EA

TABLE 1. Determination of the EA or LA specificity of the glycine extracts

Serum ^a	IF titer		OD ₄₉₂ at a 1:40 dilution (ELISA titer) ^b		
	EA	LA	FF-GE	EA-GE	LA-GE
1	<40	40,960	0.206	0.231 (<40)	1.865 (40,960)
2	<40	640	0.237	0.248 (<40)	0.961 (2,560)
3	<40	1,280	0.183	0.191 (<40)	1.036 (2,560)
4	40	10,240	0.214	0.563 (160)	1.572 (10,240)
5	40	160	0.167	0.459 (40)	0.696 (640)
6	10,240	10,240	0.213	1.416 (10,240)	1.535 (10,240)
7	2,560	10,240	0.224	1.163 (2,560)	1.624 (40,960)
8	640	10,240	0.169	0.837 (1,280)	1.493 (10,240)
9	160	10,240	0.192	0.552 (320)	1.568 (20,480)
10	2,560	10,240	0.201	1.263 (2,560)	1.624 (10,240)
11	40	10,240	0.315	0.563 (80)	1.702 (40,960)
12	640	160	0.350	0.933 (1,280)	0.828 (640)

^a Sera 1 through 4 and serum 9 were taken from patients early after onset of acute primary CMV infection. Sera 10 through 12 were used in adsorption experiments: 10, unadsorbed reference pool serum 2; 11, serum 10 adsorbed with EA-GE; 12, serum 10 adsorbed with LA-GE.

^b Mean value of duplicate dilutions.

by IF (sera 4 and 5). In addition, absorption of reference pool serum 2 with EA-GE (0.5 mg of protein per 0.5 ml serum) at room temperature for 1 h and absorption overnight at 4°C strongly reduced the reaction of that serum on EA-GE but not on LA-GE (sera 10 and 11, before and after absorption, respectively).

Sera with different titers against CMV-EA but comparable titers against CMV-LA (by IF) were tested by LA-GE (sera 6 through 9) to determine the influence of anti-EA antibodies on LA reactivity (LA specificity of LA-GE). Results showed that, irrespective of the amount of anti-EA antibodies, the reactions on LA-GE were comparable. Selective absorption of pool serum 2 with LA-GE reduced the reactivity on LA-GE strongly but also reduced the EA reactivity of that serum to some extent (sera 10 and 12, respectively).

The virus specificity of the extracts was analyzed with CMV antibody-negative sera which were positive for Epstein-Barr virus, herpes simplex virus, or varicella zoster virus and by a set of sera from patients with an acute infection due to one of these viruses. No positive reactions were detectable in any of these control sera, indicating that the CMV GE antigens, both EA-GE and LA-GE, were specific for the detection of CMV antibodies (data not shown).

Detection of nonspecific reactions. The influence of nonspecific factors such as RF or ANA on the detection of CMV-specific antibodies was studied (Table 2). ANA, which in most cases completely prevented the determination of CMV antibody titer by IF, were always detected on control antigen (FF-GE). Moreover, changes in CMV-specific antibody levels in sera containing ANA were easily identified by ELISA by an increase on EA-GE or LA-GE and a stable reaction on control antigen (sera 1 and 2; Table 2). In

addition, antibodies against cytoskeletal and other intracellular components of normal fibroblasts also reacted on FF-GE antigen (not shown). RF is known to cause false-positive IgM determinations. To study the effect of RF in our system, we selected a number of sera containing IgM RF, CMV antibodies, or both and analyzed them separately or as mixtures (Table 2, sera 3 through 7). In total, we tested four mixtures of CMV IgG-negative, IgM RF-positive sera with CMV IgG-positive, IgM RF-negative sera for CMV IgM reactivity. We found no false-positive IgM for either CMV-EA or CMV-LA. However, after reducing the NaCl and BSA concentrations of the serum dilution buffer twofold, a higher level of nonspecific binding was observed.

Optimization of the ELISA procedure. To increase long-term reproducibility of the assay, to allow comparison among different tests without the need for retesting of sera, and to examine the possibility of using this system with automatic data processing, we analyzed several conditions related to the use of CMV GE antigens. First, we determined the antigen concentration that would give optimal discrimination between positive and negative reactions and a near linear slope over a range of serum dilutions. A coating of 1.0 to 2.0 µg of protein per well gave the best results both for EA-GE and LA-GE (Fig. 3). For the control reaction, FF-GE was always used at the same protein concentration as the CMV antigens.

Second, we determined the time needed for optimal coating of the wells with an antigen concentration of 0.5 µg per well (Fig. 4). With reference serum 2 we found an optimal reaction after coating for 40 h at 4°C both for EA-GE and LA-GE. When higher protein concentrations were used, the coating time could be reduced to 16 h. Prolonged storage at 4°C had no effect on LA reactivity, but the level of the reaction on EA-GE slightly decreased with time. Control reactions remained stable. After coating, the plates could be stored at -80°C for more than 6 months and at -20°C for at least 3 months without loss of antigenicity. GE antigens, in a 50× concentrated form, were always stored at -80°C since storage at higher temperatures or storage at 4°C after freeze-drying increased nonspecific binding and reduced specific reactions. However, these effects only became important after prolonged storage (>3 months). Finally, we selected conditions for washing, incubation, and the final color reaction that would give the most reproducible results. Optimal conditions as outlined above, with freshly prepared reagents, were essential for a reproducible test with little day-to-day variation.

Detection of IgM and IgG against CMV-EA and CMV-LA. By using heavy chain-specific horseradish peroxidase conjugates we were able to measure IgM and IgG on EA-GE and LA-GE with high sensitivity. In retrospective studies we found a significant rise in antibody titer against CMV-LA nearly at the onset of clinical illness in a group of renal transplant patients, which was 1 or several weeks before a rise in CF titer became detectable (J. M. Middeldorp, unpublished data). In a study on the development of humoral immune response to CMV in different groups of patients with acute CMV infection we found CMV-LA-specific IgM in 18 of 18 (100%) cases of primary CMV infection and in 17 of 20 (85%) cases of secondary CMV infections. IgM against CMV-EA was present less frequently and at reduced titers as compared with IgM against CMV-LA. The development of IgM or IgG responses against CMV-EA lagged behind those against CMV-LA. No IgM antibodies against CMV-EA or CMV-LA were detectable in sera from 25 healthy seropositive donors selected from laboratory personnel, although IgG against CMV-LA was present up to

TABLE 2. Influence of RF and ANA on the determination of anti-CMV-LA antibodies by ELISA

Serum ^a	Antigen	IgG (OD ₄₉₂) at a dilution of: ^b			IgM (OD ₄₉₂) at a dilution of: ^b		
		1:40	1:160	1:640	1:40	1:160	1:640
-	FF-GE	0.203	0.125	0.050	0.248	0.117	0.036
+		0.230	0.146	0.055	0.248	0.132	0.051
1		0.673	0.370	0.129	0.241	0.121	0.027
2		0.659	0.418	0.130	0.269	0.146	0.048
3		0.195	0.104	0.050	0.264	0.117	0.054
4		0.116	0.039	0.004	0.090	0.038	0.025
5		0.165	0.070	0.009	0.215	0.093	0.060
6		0.193	0.094	0.041	0.201	0.099	0.031
7		0.200	0.102	0.036	0.226	0.117	0.027
-	LA-GE	0.226	0.173	0.086	0.274	0.143	0.030
+		1.012	0.616	0.347	1.423	1.110	0.771
1		0.703	0.383	0.157	0.258	0.143	0.061
2		1.165	0.738	0.354	0.796	0.358	0.186
3		0.990	0.650	0.380	0.687	0.370	0.271
4		1.281	1.000	0.655	0.110	0.054	0.034
5		1.170	0.880	0.522	0.370	0.276	0.181
6		1.634	1.261	0.839	0.241	0.139	0.065
7		1.386	0.987	0.792	0.301	0.178	0.099

^a A number of sera containing ANA on RF were tested by ELISA against control (FF-GE) and LA-GE antigens at dilutions of 1:40, 1:160, and 1:640. Sera: -, reference serum 1 (CMV negative); +, reference serum 3 (IgM and IgG positive); 1, ANA-positive serum before CMV infection (CMV seronegative); 2, ANA-positive serum from the same patient as serum 1 after onset of primary CMV infection; 3, CMV IgG- and IgM-positive serum, negative for RF; 4, IgM RF-positive serum (also CMV IgG positive); 5, 1:1 mixture of sera 3 and 4; 6, highly CMV IgG-positive serum (CMV IgM negative), negative for RF; 7, 1:1 mixture of sera 4 and 6.

^b Mean of duplicate determinations.

FIG. 3. Effect of antigen (protein) concentration on the detection of antibodies against CMV-EA and CMV-LA. Individual wells of 96-well ELISA plates were coated with 0.25 ($\square$), 0.5 ($\blacksquare$), 1.0 ($\circ$), or 2.0 ($\bullet$) μ g of protein in 0.1 ml of coating buffer for 40 h at 4°C. A high-titered serum (—) (EA titer, 10,240; LA titer, 40,960; by IF and ELISA), a low-titered serum (---) (EA titer, 160; LA titer, 640; by IF and ELISA), and the CMV antibody-negative reference serum (shaded area) were analyzed under standard ELISA conditions. Control plates (FF-GE) were coated and processed identically. Each point represents the mean of two independent measurements.

titers of 40,960. In 8 of 10 renal allograft recipients with primary CMV infection, the virus was repeatedly isolated as compared with 11 of 20 patients with secondary CMV infection. There was no relation between the level of antibodies (IgM or IgG) against CMV-EA and positive or negative virus isolation (Fisher exact test, $P > 0.3$). Chronic CMV excretors had levels of IgM and IgG against CMV-EA comparable to those of non- or short-term excretors. In both renal allograft recipients and patients with community-acquired CMV infection, IgG against CMV-EA remained detectable for years after convalescence and after cessation of virus excretion. In addition, IgG against CMV-EA was detectable, occasionally in substantial titers (up to 10,240), in healthy CMV-LA seropositive donors who did not have a history of symptomatic CMV infection. No data on virus excretion in this group were available, however.

Comparison of ELISA with IF and CF. A total of 322 sera were analyzed in parallel by IF and ELISA (Fig. 5). Linear regression showed that the correlation between the two assays was high both for the detection of anti-CMV-EA ($r = 0.9674$) and for anti-CMV-LA antibodies ($r = 0.9362$). CMV-negative sera and sera with titers exceeding 40,960 were excluded from this calculation. A total of 187 sera were positive in both tests. Four sera of 191 (2.1%) were positive for CMV-EA and CMV-LA by ELISA and negative by IF.

Sixty-one sera that were positive by ELISA on LA-GE were tested by CF (Fig. 6). The ELISA titers for LA were 20 to 5,120 times higher than the CF titers. In addition 12 of 61 (19.6%) sera that were positive by ELISA at titers ranging from 160 to 10,240 were negative (titer < 8) by CF. Moreover, ELISA screening frequently detected a significant rise (at least fourfold) in CMV antibody titer several weeks

FIG. 4. Coating and stability of glycine-extracted antigens. Glycine extracts were prepared as described in the text, and 0.5 μ g of protein in 0.1 ml of coating buffer was added to the wells at different days before testing and left at 4°C. All plates were processed on the same day by using the CMV IgG-positive reference serum at a 1:160 dilution under standard ELISA conditions.

FIG. 5. Comparison of CMV-EA and CMV-LA antibody titers obtained by ELISA and IF. A total of 322 sera were analyzed for CMV-EA and CMV-LA antibody by ELISA and IF. Linear regression analysis (—) included all sera ($n = 187$) that were positive (titer $\leq 40,960$) in both tests (EA correlation, 0.9674; LA correlation, 0.9362).

before CF, hence allowing a very early diagnosis of acute CMV infection.

DISCUSSION

In recent years, a number of papers have described the development of ELISA technology for the detection of antibodies against CMV in human sera (4, 7, 18, 20, 26, 27, 30, 35, 36, 39, 40). However, despite the fact that antigen quality is a major determinant in the specificity and reproducibility of the technique (2, 15), very little attention has been paid to the production of CMV antigens suitable for use in the ELISA. Most authors use commercially available CF antigens (4, 7, 27, 36, 39, 40) or produce their own antigen by freeze-thawing (3, 5, 20, 26), glycine extraction (18, 27), or other procedures (24, 26, 27, 30, 35).

The procedure described in this paper employs a simple whole cell extraction method for the preparation of both CMV-EA and CMV-LA. Alkaline glycine extraction on ice followed by extensive sonication was found to be superior to other techniques (16; J. M. Middeldorp, unpublished data).

By using human sera with well-defined specificity for CMV-EA and CMV-LA as defined by IF, we were able to study the induction of EA- and LA-reactive proteins after infection of fibroblasts in vitro as separate entities (Fig. 1 and 2). For CMV-LA (Fig. 1) we found that after the first point of 100% CPE, when most authors prepare CMV antigen (3, 5, 18, 27, 30, 35), there was still a strong increase in LA reactivity per total amount of extracted protein. Isolation of LA-GE at later stages after infection (see also references 20 and 26) not only increased the amount of virus-specific protein, which was in agreement with molecular studies by Stinski (29), but it also increased the reproducibility among different preparations, since the ratio of viral to cellular proteins appears to reach a maximum (and thereafter constant) level late after infection (Fig. 1). In cells treated with 0.5 mM PFA after infection, EA-reactive proteins were maximally present at 24 to 48 h postinfection and decreased thereafter (37). Experiments shown in Table 2 indicated that EA-GE did not contain LA-reactive proteins, since sera with

high titers against CMV-LA, as measured by both IF and ELISA, were negative by ELISA on EA-GE (Table 1, sera 1 through 3). This was in agreement with the finding that CMV-infected cells treated with 0.5 mM PFA showed no fluorescent inclusions (late antigen staining) (37). Adsorption experiments further demonstrated the EA and LA specificity of EA-GE and LA-GE, respectively. The amount of protein needed to produce optimal ELISA reactivity (0.5 to 1.0 μg of protein per well for LA-GE and 1.0 to 2.0 μg of protein per well for EA-GE) was comparable to that needed for purified virion antigen (0.5 μg per well [26]) or purified nuclear antigen (0.4 μg per well [35] or 1 μg per well [30]). Consequently, the protocol described here provides a simple and economical method for the production of CMV antigens suitable for use in ELISA.

The ratio of CMV-specific proteins to non-CMV antigenic proteins in a mixture composed of multiple proteins may strongly influence the binding of ELISA plates of the relevant antigens (15). This applies to all CMV ELISA techniques described thus far. The use of a high multiplicity of infection to achieve simultaneous antigen induction in all cells greatly increases the quantity of CMV antigenic proteins relative to the total amount of normal cell proteins (29), which may explain the high antigenicity of the CMV-GE extracts as discussed above.

The reproducible production of these glycine extracts, CMV-EA and CMV-LA, respectively, has contributed significantly to the establishment of a sensitive technique which has proven to be a reliable tool for the selection of CMV-negative blood donors in autologous bone marrow transplantation (L. F. Verdonk, J. M. Middeldorp, H. A. Kreeft, T. H. The, A. Hekker, and G. C. de Gast, submitted for publication). This technique has also proven to be a valuable tool for the early diagnosis of CMV infection in a number of different patient groups (J. M. Middeldorp, A. M. Tegzess, J. Jongsma, H. W. Roenhorst, and T. H. The, in S. Plotkin, ed., *Pathogenesis and Prevention of Human Cytomegalovirus Disease*, in press; Verdonk et al., submitted for publication). The ELISA test is equal in sensitivity to the IF test

FIG. 6. Comparison of CMV antibody titers obtained by ELISA on LA-GE and CF. A total of 61 sera positive by ELISA on LA-GE were also tested by CF. ELISA titers were 20 to 5,120 times higher than CF titers, with a low correlation between both assays ($r = 0.4990$).

that has been applied for many years in our laboratory (Fig. 5) (13, 32, 33) and is considerably more sensitive than the widely used CF assay (Fig. 6) and also superior to indirect hemagglutination inhibition (data not shown).

Previous studies (10, 23, 33) have suggested that determination of anti-CMV-EA antibodies could be of diagnostic value, either indicating current or very recent infection (33) or reflecting active virus replication or excretion or both (10, 23). Our present data failed to show a distinct relationship between the level of anti-CMV-EA antibodies and virus excretion. Additionally, antibodies against CMV-EA were frequently detectable in normal CMV-seropositive individuals (9, 28).

Consequently, the detection of anti-CMV-EA antibodies may not provide additional information on the activity of CMV in the human host (9, 28). However, the persisting presence of anti-CMV (both against CMV-EA and CMV-LA) antibodies after primary infection is suggestive of continued (subclinical) virus replication, probably reflecting the delicate balance between CMV and host immunological defense. Retrospectively (Middeldorp et al., in press), parallel determination of IgG and IgM against CMV-LA proved the most sensitive and reliable test for the detection of active CMV infection in different patient groups.

IgM-RF did not produce false-positive reactions (4, 18, 24, 27, 31, 34, 39) in our ELISA protocol; however, the number of RF-positive sera studied by us was small. Non-CMV-specific reactions were observed (Table 2) due to the presence of ANA (14) or autoantibodies against other fibroblast constituents (1, 14). These nonspecific reactions, only occasionally mentioned by others (4, 27), are frequently temporarily detectable during the course of symptomatic CMV infections (1, 2) and require further investigation. In the studies mentioned above (with CMV strain AD-169), no

serological cross-reactions with other human herpesviruses were observed.

The growing demand for CMV serology in clinical (acquired immune deficiency syndrome or prodromes) and nonclinical (donor selection) situations calls for further standardization and simplification of the assay, including the possibility using the assay with automatic data processing (8, 17, 22, 25). This requires a reproducible quality of antigens and reagents and a standardized working protocol (2, 15, 22). The ELISA technique described here fulfills these requirements. A set of reference sera has been constructed which gave very reproducible results over many tests in the past 2 years.

Negative reference sera were selected by a sensitive test (IF) which is necessary for the reliable identification of weakly positive reactions (17).

Although for this study we used a standard dilution protocol with six serial dilutions measured in duplicate, further simplification can be achieved by means of measurements at a single serum dilution (2, 3, 7, 35) or better by means of logarithmic transformation of optical density values at different dilutions and expression of the data relative to a standard (8, 25, 35). However, before this can be done in an ELISA system that uses complex antigens, more variables need to be assessed such as the slope of dilution curves in different sera (e.g., acute-phase versus convalescent-phase sera), which may be influenced by different antibody specificities and affinities in individual sera.

ACKNOWLEDGMENTS

This study was supported by grant 13-40-45 from the Foundation for Medical Research with financial aid from the Netherlands Organization for the advancement of Pure Research.

LITERATURE CITED

1. Andersen, P., and H. K. Andersen. 1978. Immunoglobulin levels and specific viral antibodies in relation to smooth-muscle antibodies in cytomegalovirus infection. *J. Clin. Lab. Immunol.* 1:233-236.
2. Blomberg, J., I. Nilsson, and M. Andersson. 1983. Viral antibody screening system that uses a standardized single dilution immunoglobulin G enzyme immunoassay with multiple antigens. *J. Clin. Microbiol.* 17:1081-1091.
3. Booth, J. C., G. Hannington, T. M. F. Baker, H. Stern, H. Kangro, P. D. Griffith, and R. B. Heath. 1982. Comparison of enzyme-linked immunosorbent assay, radioimmunoassay, complement fixation anti-complement immunofluorescence and passive haemagglutination techniques for detecting cytomegalovirus IgG antibody. *J. Clin. Pathol.* 35:1345-1348.
4. Cappel, R., F. de Kuyper, and J. de Broekelaar. 1978. Rapid detection of IgG and IgM antibodies for cytomegalovirus by the enzyme linked immunosorbent assay (ELISA). *Arch. Virol.* 58:253-258.
5. Castellano, G. A., G. T. Hazzard, D. L. Madden, and J. L. Sever. 1977. Comparison of the enzyme-linked immunosorbent assay and the indirect haemagglutination test for detection of antibody to cytomegalovirus. *J. Infect. Dis.* 136:5337-5340.
6. Chen, T. R. 1977. In situ detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain. *Exp. Cell. Res.* 104:255-262.
7. Chia, W. K., and L. Spence. 1979. Quantitative determination of cytomegalovirus IgG antibody by enzyme-linked immunosorbent assay (ELISA). *J. Can. Microbiol.* 25:1082-1086.
8. Fey, H. 1981. Use of a computer to evaluate sigmoidal curves in serology by a new procedure. *J. Immunol. Methods* 47:109-112.
9. Friedman, A. D., T. Furukawa, and S. A. Plotkin. 1982. Detection of antibody to cytomegalovirus early antigen in vaccinated, normal volunteers and renal transplant candidates. *J. Infect. Dis.* 146:255-259.

10. 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 infection. *Infect. Immun.* 22:833-841.
11. Griffiths, P. D., S. Stagno, R. F. Pass, R. J. Smith, and C. A. Alford. 1982. Congenital cytomegalovirus infection: diagnostic and prognostic significance of the detection of specific immunoglobulin M antibodies in cord serum. *Pediatrics* 69:544-549.
12. Griffiths, P. D., S. Stagno, R. F. Pass, R. J. Smith, and C. A. Alford. 1982. Infection with cytomegalovirus during pregnancy: specific IgM antibodies as a marker of recent primary infection. *J. Infect. Dis.* 145:647-653.
13. Horodniceau, F., and S. Michelson. 1980. Assessment of human cytomegalovirus antibody detection techniques. *Arch. Virol.* 64:287-301.
14. Kallenberg, C. G. M., J. van der Meulen, G. W. Pastoor, J. A. M. Snijder, T. E. W. Feltkamp, and T. H. The. 1983. Human fibroblasts, a convenient nuclear substrate for the detection of antinuclear antibodies including anticentromere antibodies. *Scand. J. Rheumatol.* 12:193-200.
15. Kenney, G. E., and C. L. Dunsmoor. 1983. Principles, problems, and strategies in the use of antigenic mixtures for the enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* 17:655-665.
16. Kettering, J. D., N. J. Schmidt, and E. H. Lennette. 1977. Improved glycine-extracted complement-fixing antigen for human cytomegalovirus. *J. Clin. Microbiol.* 6:647-649.
17. Kramer, S. M., N. P. Jewell, and N. E. Cremer. 1983. Discriminant analysis of data in enzyme immunoassay. *J. Immunol. Methods* 60:243-255.
18. Krishna, R. V., O. H. Meurman, T. Ziegler, and H. H. Krech. 1980. Solid-phase enzyme immunoassay for detection of antibodies to cytomegalovirus. *J. Clin. Microbiol.* 12:46-51.
19. Langenhuisen, M. M. A. C., T. H. The, H. O. Nieweg, and J. G. Kapsenberg. 1970. Demonstration of IgM cytomegalovirus-antibodies as an aid to early diagnosis in adults. *Clin. Exp. Immunol.* 6:387-393.
20. Levy, E., and I. Sarov. 1980. Determination of IgA antibodies to human cytomegalovirus by enzyme-linked immunosorbent assay (ELISA). *J. Med. Virol.* 6:249-257.
21. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
22. Malvano, R., A. Boniolo, M. Davis, and M. Zannino. 1982. ELISA for antibody measurement: aspects related to data expression. *J. Immunol. Methods* 48:51-60.
23. Musiani, M., M. P. Landini, C. Feletti, and M. LaPlaca. 1978. Serum antibodies against cytomegalovirus-induced early and immediate early antigens and presence of viruria in renal transplant recipients. *Microbiology* 1:101-105.
24. Rasmussen, L., D. Kelsall, R. Nelson, D. Winston, J. Preiksaitis, and F. C. Merigan. 1982. Virus-specific IgG and IgM antibodies in normal and immunocompromised subjects infected with cytomegalovirus. *J. Infect. Dis.* 145:191-199.
25. Ritchie, D. G., J. M. Nickerson, and G. M. Fuller. 1981. Two simple programs for the analysis of data from enzyme-linked immunosorbent (ELISA) assays on a programmable desk-top calculator. *Anal. Biochem.* 110:281-290.
26. Sarov, I., P. Anderson, and H. K. Andersen. 1980. Enzyme linked immunosorbent assay (ELISA) for determination of IgG antibodies to human cytomegalovirus. *Acta Pathol. Microbiol. Scand. Sect. B* 88:1-9.
27. Schmitz, H., H. W. Doerr, D. Kampa, and A. Vogt. 1977. Solid-phase enzyme immunoassay for immunoglobulin M antibodies to cytomegalovirus. *J. Clin. Microbiol.* 5:629-634.
28. Stagno, S., D. W. Reynolds, A. Tsiantos, D. A. Fucillo, W. Long, and C. A. Alford. 1975. Comparative serial virologic and serologic studies in symptomatic and subclinical congenitally and natively acquired cytomegalovirus infections. *J. Infect. Dis.* 132:568-577.
29. Stinski, M. F. 1977. Synthesis of proteins and glycoproteins in cells infected with human cytomegalovirus. *J. Virol.* 23:751-767.
30. Sundquist, V., and B. Wahren. 1981. An interchangeable ELISA for cytomegalovirus antigen and antibody. *J. Virol. Methods* 2:301-312.
31. Sutherland, S., and J. D. Briggs. 1983. The detection of antibodies to cytomegalovirus in the sera of renal transplant patients by an IgM antibody capture assay. *J. Med. Virol.* 11:147-159.
32. The, T. H., H. K. Andersen, E. S. Spencer, and G. Klein. 1977. Antibodies against cytomegalovirus-induced early antigens (CMV-EA) in immunosuppressed renal-allograft recipients. *Clin. Exp. Immunol.* 28:502-505.
33. The, T. H., G. Klein, and M. M. A. C. Langenhuisen. 1974. Antibody reactions to virus-specific early antigens (EA) in patients with cytomegalovirus (CMV) infection. *Clin. Exp. Immunol.* 16:1-12.
34. Torfasen, E. G., and H. Diderholm. 1982. False RIA IgM to herpes simplex virus and cytomegalovirus: factors causing them, and their absorption by protein A-sepharose/IgG-protein A-sepharose. *J. Med. Virol.* 10:157-170.
35. van Loon, A. M., J. T. M. van der Logt, and J. van der Veen. 1981. Enzyme-linked immunosorbent assay for measurement of antibody against cytomegalovirus and rubella virus at a single serum dilution. *J. Clin. Pathol.* 34:665-669.
36. Voller, A., and D. E. Bidwell. 1976. Enzyme-immunoassays for antibodies in measles, cytomegalovirus infections and after rubella vaccination. *Br. J. Exp. Pathol.* 57:243-247.
37. Wahren, B., and B. Oberg. 1979. Inhibition of cytomegalovirus late antigens by phosphonoformate. *Intervirology* 12:335-339.
38. Waller, M. V., and J. H. Vaughan. 1956. Use of anti-Rh sera for demonstrating agglutination activating factor in rheumatoid arthritis. *Proc. Soc. Exp. Biol. Med.* 92:198-200.
39. Yolken, R. H., and F. J. Leister. 1981. Enzyme immunoassays for measurement of cytomegalovirus immunoglobulin M antibody. *J. Clin. Microbiol.* 14:427-432.
40. Ziegelmaier, R., F. Behrens, and G. Enders. 1981. Class-specific determination of antibodies against cytomegalovirus (CMV) and rubella virus by ELISA. *J. Biol. Stand.* 9:23-33.