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Solid-Phase Enzyme Immunoassay for Determination of Antibodies to Cytomegalovirus

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A solid-phase enzyme immunoassay for the determination of immunoglobulin G (IgG) and IgM antibodies to cytomegalovirus is described. The enzyme immunoassay gave reliable and consistent results which were in concordance with those obtained by the complement fixation test and the indirect immunofluorescence test. Antibodies to herpes simplex and varicella-zoster viruses did not interfere in the enzyme immunoassay for cytomegalovirus IgM antibodies. In a few patients with IgM antibodies to Epstein-Barr virus, cytomegalovirus IgM antibodies were also detected. False-positive cytomegalovirus IgM antibody results were observed in sera containing both the rheumatoid factor and cytomegalovirus IgG antibodies. This rheumatoid factor interference was overcome by the absorption of sera with polymerized human gamma globulin. The absorption did not affect true cytomegalovirus IgM antibody titers. Also described is a simple enzyme immunoassay that makes possible a more sensitive detection of the rheumatoid factor than the latex agglutination test.

Cytomegalovirus (CMV) has been recognized as a causative agent of viral mental retardation in congenitally infected newborns, as well as of postnatally acquired syndromes, including hepatitis, pneumonitis, and cytomegalic mononucleosis (13). CMV infections are common among immunosuppressed individuals, e.g., after renal transplantations and in recipients of blood transfusions (5). Sensitive and rapid methods for the detection of specific immunoglobulin G (IgG) and IgM antibodies to CMV would thus offer valuable information to help diagnose infection by this virus. Detection of IgM antibodies in serum would reveal a recent or current CMV infection, whereas that of IgG antibodies would speak of an earlier exposure to CMV (16).

Among the serological tests for the determination of CMV antibodies, complement fixation (CF) (4), indirect immunofluorescence (IIF) (9, 16, 24), and immunoperoxidase techniques (7) have been commonly used. Although promising for the assay of IgG and IgM antibodies to CMV, radioimmunoassays (6, 12) have been bypassed by the more elegant and hazard-free enzyme immunoassays (EIA) (1, 2, 3, 23, 28). The precise determination of specific IgM antibodies by IIF, radioimmunoassay, and EIA methods presents some difficulties owing to the presence of antinuclear antibodies and the IgM class rheumatoid factor (RF) in patients having high titers of specific IgG antibodies (10, 20, 23, 26, 27). False-positive IgM antibody results have been recognized and eliminated by several methods in assays for antibodies against different viruses. Absorption of the RF with aggregated human gamma globulin or with IgG-coated latex particles has been used in assays for antibodies to rubella virus (20), Epstein-Barr virus (EBV) (10), measles virus (26), and CMV (12). Absorption of IgG antibodies with staphylococcal protein A has been used in an EIA for rubella antibodies (17), despite the variable affinities of this reagent for the different IgG subgroups (15) and the fact that part of the IgM also will be absorbed (18). Alternatively, IgM fractions have been separated before assays by sucrose gradient centrifugation (4, 22) or by gel filtration (21). However, despite the realization of false-positive IgM results in indirect immunoassays for antibodies to CMV (12, 22, 23), more definitive experimental evidence on the participation of the RF and on its elimination is needed.

In this paper we describe an EIA for the determination of IgG and IgM antibodies to CMV in human sera. A simple EIA for the estimation of RF activity in sera, as well as a method for the elimination of the RF interference in the assay for CMV IgM antibodies by preabsorption of sera with polymerized human gamma globulin (PGG), are also included.

MATERIALS AND METHODS

Preparation of antigens. Confluent monolayers of human embryonic fibroblasts in roller cultures were

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infected with CMV, strain AD-169, and grown in double-strength Eagle minimum essential medium supplemented with heat-inactivated 10% calf serum at 35 to 37°C. When a 100% cythopathic effect was observed, the cells were scraped off, washed with K_2HPO_4 buffer (pH 7.2)–0.15 M NaCl (PBS), and extracted with 0.1 M glycine-NaOH–0.1 M NaCl buffer (11, 14). Control antigen was prepared in the same way but from uninfected fibroblasts. The same antigens were used for the CF and EIA tests. Nuclear antigens from CMV-infected and uninfected fibroblasts were prepared by the method of Schmitz et al. (23), but the sucrose gradient centrifugation was replaced by Ficoll-400 (Pharmacia, Uppsala, Sweden) centrifugation at 2,500 × *g* for 15 min (H. Schmitz, personal communication). Herpes simplex virus (HSV) and varicella-zoster virus (VZV) CF antigens were prepared by the glycine extraction method from virus grown in rabbit kidney cells (RK-13) or human embryonic fibroblasts, respectively.

Serum specimens. The study material comprised the following serum groups: (i) sera from patients with verified or suspected CMV infection: (ii) sera from patients with EBV, HSV, or VZV infections; (iii) sera from patients with detectable RF activity; (iv) sera from healthy persons with high CMV antibody titers; and (v) nonselected sera from healthy blood donors. In each assay, a positive control serum (CF titer, 240; IIF IgM antibody titer, 160) and a negative control serum (CF titer, <10; IIF IgM antibody titer, <10) were included. These control sera were stored in lyophilized form.

Coating of the solid phase with antigen. The correct dilutions of the CMV and control antigens were determined by checkerboard titration against CMV-positive and -negative control sera. A 1:75 dilution of the CF antigen was found to be optimal. Portions (75 μl) of antigen solutions in 0.05 M sodium carbonate buffer (pH 9.6) with 10 μM sodium ethylmercurithiosalicylate (Merthiolate) were filled in the wells of flat-bottom microtiter plates (Falcon 3040; Becton, Dickinson & Co., Oxnard, Calif.) and let stand with the tops covered at room temperature overnight (16 to 18 h). The excess antigen was then aspirated off, and the plates were washed twice with PBS containing 0.1% Tween-20 (PBST) for about 5 min. The plates were gently tapped dry and stored at 4°C until use.

EIA conjugates. Horseradish peroxidase-conjugated pig antihuman IgG and antihuman IgM from Orion Diagnostica (Helsinki, Finland) were used. These were routinely diluted 1:200 with PBST containing 5% porcine serum (GIBCO Diagnostics, Glasgow, Scotland). The conjugates were tested for cross-reactivity toward other immunoglobulin classes and toward viral and control antigens. Only negligible nonspecific reactivity was observed.

EIA procedure. Test and control sera were diluted in the antigen-coated wells with PBST containing 5% porcine serum as the diluent in fourfold steps from 1:10 to 1:10,240. The plates were incubated at 37°C for 2 h, followed by two washes with PBST as described above. Thereafter, all the wells except the beginning 10-fold dilution rows, which later served as blanks, received 75 μl of peroxidase-conjugated anti-human IgG or anti-human IgM. Incubations with conjugates were carried out at 37°C for 90 min, followed by two washes with PBST as before and a final wash with PBS. The peroxidase activity bound to the wells was assayed by adding to each well 75 μl of freshly prepared substrate solution (1,2-phenylenediamine [1 mg/ml] with 0.03% hydrogen peroxide in 0.05 M citric acid–K_2HPO_4 buffer [pH 5.5]). After about 5 min, when significant yellow coloration occurred in the wells, 150 μl of 4 M H_2SO_4 was added to all the wells to stop the reaction. The color intensities were quantitated by reading the plates with a multichannel automatic photometer (Titertek Multiskan; Flow Laboratories, Inc., Rockville, Md.) with the vertical optical path at 450 nm. The first dilution rows that did not receive the enzyme conjugates served as blanks for respective sera. Serum dilutions showing absorbance values of 2.1 times those of the respective negative controls were considered positive for antibodies to CMV.

CF tests. Antibody titers to CMV, HSV, and VZV antigens were measured by the standard CF test applied to a microtechnique (25).

IIF assay. IgG antibodies to EBV and IgM antibodies to CMV, EBV, HSV, and VZV were determined by IIF tests with infected cells fixed on immunofluorescence slides as antigens (14). For the IgG antibody assay, whole sera were used, and for the IgM antibody assay, the sera were fractioned by column chromatography on agarose, and the IgM-containing fractions were collected for testing (21).

Determination and removal of the RF. The presence of the RF was checked by the classical latex agglutination (LA) test (Hyland Laboratories, Inc., Costa Mesa, Calif.), according to the instructions given by the manufacturer, and by a modified EIA. In the EIA procedure, purified human IgG (protein content, 18 mg/ml; Cappel Laboratories, Downingtown, Pa.) was diluted 1:200 in PBS, and 100-μl amounts were pipetted into the wells of microtiter plates. After overnight incubation at room temperature, the excess antigen was aspirated off, and the IgG-coated wells were then fixed with 0.5% glutaraldehyde reagent (12). The EIA was then done as described for CMV antibodies, employing peroxidase-conjugated antihuman IgM to detect the RF molecules bound to the IgG on the plate.

For removal of the RF, the sera were absorbed with heat-aggregated human gamma globulin (protein content, 160 mg/ml; Swiss Serum Institute, Berne, Switzerland) which was polymerized with glutaraldehyde (PGG) (19). In a typical run, 20 μl of serum was mixed with 180 μl of the appropriately diluted PGG (1 volume of PGG plus 1 volume of PBS) and incubated at 37°C for 1 h. For the unabsorbed control, 20 μl of serum was mixed with 180 μl of PBS and incubated as above. After incubation, the mixtures were centrifuged at 3,000 × *g* for 20 min, and the supernatants were assayed for the RF and for CMV IgM antibody by the EIA.

PAS absorbtion. IgG antibodies were absorbed from sera with protein A-Sepharose (PAS, Pharmacia, Uppsala, Sweden) with 20% (vol/vol) suspensions in PBS as described by Leinikki et al. (17). The absorbed sera were then assayed for CMV IgG and IgM antibodies.

RESULTS

Selection of the type of CMV antigen for the EIA. The glycine extract CF antigen and the nuclear antigen, with respective control antigens, were compared in the EIA with a known CMV antibody-positive serum and a negative control serum. With both antigen types, the nonspecific binding, as measured by the reactivity of the positive serum with control antigens and of the negative serum with both viral and control antigens, was sufficiently low. A constant finding was that negative sera also showed a stronger reactivity with viral than with control antigens. This seems to indicate that in the cells, CMV infection induces changes which nonspecifically increase the binding of antibodies to the cellular material. Both in IgG and IgM antibody tests, however, with optimal dilution of the CF, antigen-positive serum produced absorbance values three to four times as high as those with optimal dilution of the nuclear antigen. Because the nonspecific binding to both antigen types was almost equal, with the CF antigen, better positive/negative differences and better assay sensitivity were observed. Therefore, in all subsequent assays, the CF antigen and the respective control antigen were used.

Specificity of EIA, CF, and IIF tests for CMV antibodies. Preliminary experiments were performed with 63 sera of known CF and IIF antibody titers and without detectable RF activity. All sera showed negligible reactions with the uninfected control antigen in EIA tests. All known negative sera with CF titers <10 against CMV had EIA titers of ≦40, the arbitrarily selected initial dilution in our tests. Nonspecific reactions with the viral antigen were not observed. The comparisons of EIA IgG antibody titers with CF titers and those of EIA IgM antibody titers with IIF IgM antibody titers are depicted in Fig. 1 and 2, respectively.

As an additional control, sera from 50 healthy blood donors from the Cantonal Hospital of St. Gallen were tested for CMV IgM antibodies by the EIA. The profiles of these sera together with those of known CMV IgM antibody-positive and -negative control sera are shown in Fig. 3. All the blood donor sera were negative for IgM antibodies to CMV. Sera with absorbance values matching those of the negative controls were pooled, filtered sterile, and lyophilized to be used as negative controls in subsequent tests. Approximately 60% of these blood donor sera had IgG antibodies to CMV when measured by the EIA, the geometric mean titer of the positive sera being 640.

The specificity of the EIA for IgM antibodies to CMV was further studied by testing under identical conditions 33 sera with IgM antibodies to other herpesviruses, namely, EBV, HSV, and VZV (Table 1). These sera were tested both unabsorbed and after absorption with PAS or with PGG to eliminate the possible interference caused by specific IgG antibodies or by the RF, respectively. All of these sera had IgG antibodies to CMV which were effectively removed by absorption with PAS. Whereas none of the HSV and VZV IgM antibody-positive sera showed positivity in the CMV IgM antibody test, three of the EBV IgM antibody-positive sera had IgM antibodies to CMV. In two cases, the titers remained even after absorption with PAS and with PGG, thus indicating a true CMV IgM antibody response.

Comparison of EIA and LA tests for the assay of the RF. A total of 55 sera tested by the Immunology Department of this Institute by the LA method were subjected to the EIA as described above. The two tests showed a good correlation (Fig. 4); however, the EIA was more sensitive. A total of 10 sera negative in the LA

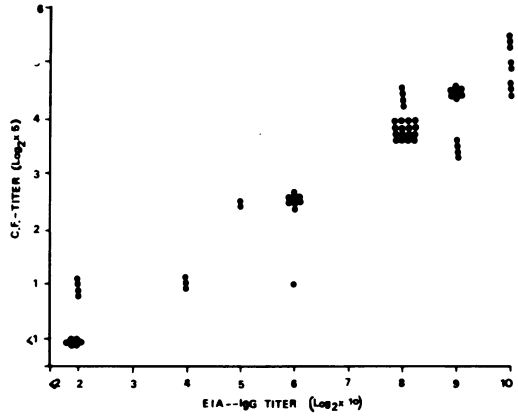

FIG. 1. *Comparison of CMV IgG antibody titers obtained by the EIA and by the CF test.*

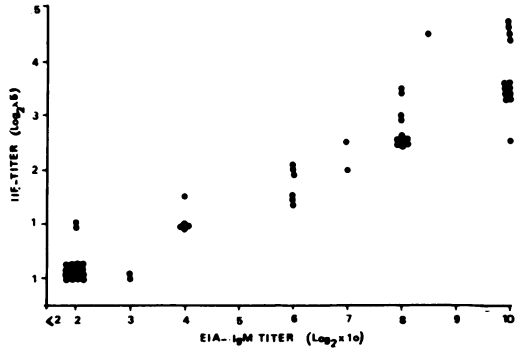

FIG. 2. *Comparison of CMV IgM antibody titers obtained by the EIA and by the IIF test.*

test were low positives in the RF-EIA. With sera positive by both methods, the RF-EIA produced titers approximately 8 to 16 times as high as those produced by the LA test.

Interference of the RF in the EIA for IgM antibodies to CMV. Sera from different patient groups were tested for the RF and for CMV IgM antibodies before and after absorption with PGG to remove the RF (Table 2). False-positive CMV IgM antibody results were consistently observed in sera of patients with high RF titers and occasionally in sera of patients with low RF titers. These false-positive results were effectively eliminated by the removal of the RF with PGG absorption. RF titers were also observed in patients with acute CMV infections and IgM antibodies to CMV. In these cases, the PGG absorption removed the RF activity without affecting the true CMV IgM antibody titers (patient group 4, Table 2). The interference of the RF in the IgM antibody assay was subsequently verified experimentally by mixing RF-positive, CMV antibody-negative sera with RF-negative, CMV IgG antibody-positive but IgM antibody-negative sera and showing that these serum mixtures without true CMV IgM antibodies produced positive results in the CMV IgM antibody assay.

DISCUSSION

Several research workers studying antibodies to CMV have successfully employed CF anti-

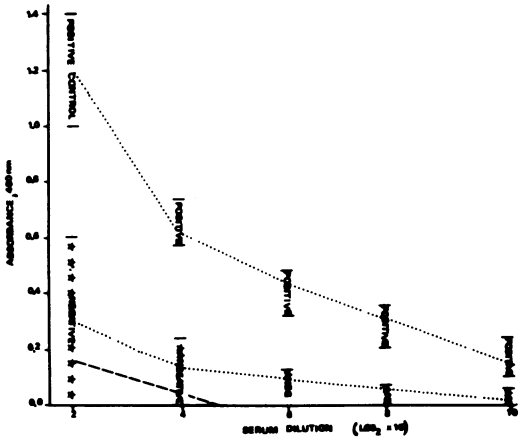

FIG. 3. *CMV EIA IgM antibody results for sera from 50 healthy blood donors (☆) and for known positive and negative control sera. The bars blocking the symbols denote the limits of scatter among the sera tested. The mean absorbance values obtained with all serum groups in titration against the control antigen are shown by the broken line.*

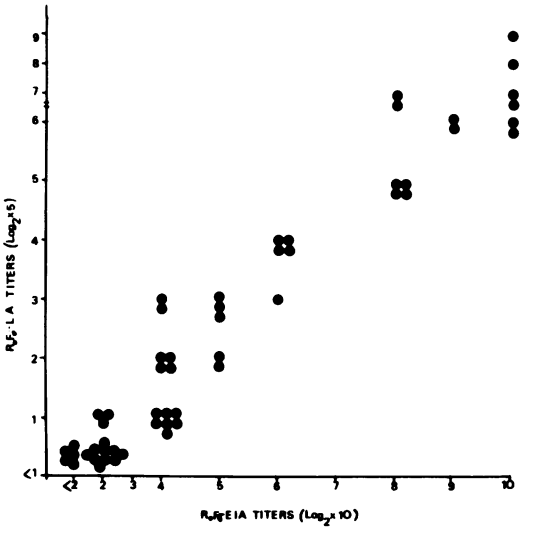

FIG. 4. *Comparison of RF titers obtained by the EIA and by the LA test.*

TABLE 1. *Effect of IgM antibodies against EBV, HSV, and VZV on the EIA for IgM antibodies to CMV*

Serum group	Total no. tested	EIA for antibodies to CMV				
		Absorption of sera[a]	IgG antibody result		IgM antibody result	
			No.	Titer	No. positive	Titer
EBV IgG+, IgM+	25	None	25	160–2,560	3	640–2,560
		PAS	25	<40	2	320–1,280
		PGG	25	160–2,560	3	320–2,560
HSV IgG+, IgM+	5	None	5	160–640	0	
		PAS	5	<40	0	
		PGG	5	160–640	0	
VZV IgG+, IgM+	3	None	3	160–640	0	
		PAS	3	<40	0	
		PGG	3	160–640	0	

[a] Serum specimens were absorbed with PAS to remove IgG antibodies and with PGG to remove the RF.

TABLE 2. *Interference of the RF in the EIA for IgM antibodies to CMV*[a]

Patient group	No. of sera tested	RF titer by the EIA		CMV IgM titer by the EIA		No. of CMV IgM-positive sera after absorption
		Before absorption	After absorption	Before absorption	After absorption	
1. Healthy persons with CMV IgG antibodies	103	<40	<40	<40	<40	0
2. Rheumatoid arthritis patients with CMV IgG antibodies	14	160–10,240	<40	≦40–640	<40	0
3. Pregnant women with CMV IgG antibodies	5	640–10,240	<40	640–2,560	≦40–1,280	1
4. Patients with CMV mononucleosis	14	160–10,240	<40	160–2,560	160–2,560	14

[a] Serum specimens from four different patient groups were assayed for the RF and for CMV IgM antibodies before and after removal of the RF by absorption with PGG.

gens, either glycine extract or the freeze-thaw type, for the determination of CMV antibodies by the radioimmunoassay RIA or by the EIA (1, 2, 3, 12). Our EIA results are in agreement with those cited above. We could not repeat the observation of Schmitz et al. (23), who found in the EIA high numbers of nonspecific reactions when the CF antigen was used and therefore stressed the importance of using the nuclear antigen in the EIA. The applicability of CF antigens in the EIA is a major advantage as these are commercially available, and the test thus becomes adoptable by laboratories without tissue culture facilities.

Data presented here indicate that the EIA for CMV antibodies is both sensitive and specific. However, it is becoming apparent that in some patients with EBV-caused infectious mononucleosis, IgM antibodies to CMV may also be detected. This observation was originally made by Hanshaw et al. (8) with an IIF test. A possible explanation for this phenomenon is the manifestation of common antigenic determinants between these two related herpesviruses (8).

The RF, by forming complexes with specific IgG antibodies, is known to be capable of causing false-positive IgM antibody results in indirect immunoassays (12, 20, 23). This problem becomes pronounced in assays for CMV IgM antibodies as CMV infection itself seems to induce the formation of the RF (12, 29). A procedure for the elimination of the RF interference is therefore required in indirect immunoassays for CMV IgM antibodies. Serum fractionation methods (21, 22), although generally reliable, are too laborious for large-scale routine use. The absorption with PAS (17) is apparently useful, but as often up to 20% of the IgM antibodies will be absorbed (18), this may lead to a decrease in the assay sensitivity. We therefore preferred the absorption with PGG, which effectively removed the RF without affecting the specific IgG and IgM antibody titers.

The present EIA methodology seems to be readily adaptable for diagnostic purposes. The use of a standardized microtiter system with automatic or semiautomatic pipetting and dilution devices and with the reading of the absorbance values directly on the plate with an automatic multichannel photometer makes it possible to apply the test also to large-scale testing of routine serum specimens.

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