JOURNAL OF CLINICAL MICROBIOLOGY, June 1977, p. 629-634
Vol. 5, No. 6
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Solid-Phase Enzyme Immunoassay for Immunoglobulin M Antibodies to Cytomegalovirus

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Received for publication 13 Janaury 1977

A sensitive, solid-phase enzyme immunoassay for the detection of immunoglobulin M antibodies to cytomegalovirus is described. The results of the enzyme immunoassay correlated well with those obtained by an indirect immunofluorescence method. Horseradish peroxidase proved to be a more sensitive label than alkaline phosphatase. Nonspecific reactions, occurring with commercially available cytomegalovirus antigens, could be avoided by using a nuclear antigen prepared from sonically disrupted nuclei of cytomegalovirus-infected cells.

Overt disease caused by cytomegalovirus (CMV), such as CMV mononucleosis, hepatitis, and myocarditis and CMV-associated Guillain-Barré syndrome, can be reliably diagnosed by the presence of immunoglobulin M (IgM) titers to CMV, as we have shown using an indirect immunofluorescence method (10a, 11).

The immunofluorescence test is only feasible for well-equipped laboratories because it is based on the production of CMV-infected tissue culture slides. Furthermore, observer variation is considerable, and a standardized evaluation of the immunofluorescence test is difficult to achieve.

We therefore looked for a more simple but equally sensitive and reliable method for detecting the CMV-specific IgM antibodies, which avoids the handling of radioactive material. Recently Gerna et al. (5) described an assay for IgG antibodies to CMV, using CMV-infected tissue culture slides and immunoperoxidase-labeled anti-human antibodies. Also, Voller and Bidwell (15) reported an enzyme immunoassay for CMV IgG antibodies, using a commercially obtained antigen that was adsorbed to plastic plates. Encouraged by these reports, we attempted to develop a solid-phase enzyme immunoassay for IgM antibodies to CMV.

MATERIALS AND METHODS

Isolation of purified anti-μ antibodies. Anti-human μ-chain (anti-μ) antiserum of rabbit origin was obtained commercially (Dakopatts, Copenhagen, Denmark, or Hyland Laboratories, Los Angeles, Calif.). In indirect immunofluorescence tests both antisera were shown to react specifically, even with low concentrations of IgM antibodies to CMV. Cross-reactivity to CMV IgG antibodies was never observed (10a, 11).

To isolate the anti-μ antibody fraction, 1 ml of the anti-μ serum was mixed with 0.2 ml of human IgM paraprotein (5 mg/ml, stained with fluorescein isothiocyanate [FITC]). The FITC-stained IgM had been isolated by euglobulin precipitation followed by sedimentation in a sucrose gradient (1). The precipitate formed was washed in phosphate-buffered saline (PBS) and dissolved in 1 ml of glycine buffer, pH 2.6, during 5 min of incubation at 56°C. After clarification by centrifugation (1,000 $\times$ g, 5 min), 1 ml of the supernatant fluid was layered onto a sucrose-pH gradient (Fig. 1) prepared in a mixing chamber (1.8 ml of 10% sucrose in 0.1 M glycine buffer [pH 4] plus 0.15 M NaCl and 1.7 ml of 38% sucrose in 0.2 M tris(hydroxymethyl)aminomethane buffer [pH 8.5] plus 0.15 M NaCl). After ultracentrifugation for 5 h at 270,000 $\times$ g (SW60 Ti rotor, Beckman Instruments), the FITC-stained 19s IgM was found near the bottom of the tube (Fig. 1). The 7s material (i.e., the specific anti-μ antibody) present above the IgM band was removed by puncturing the tube and stored frozen in sucrose at −20°C.

Preparation of anti-μ antibody-enzyme conjugate. Two different conjugates were compared in this study. (i) Horseradish peroxidase-labeled anti-μ antibody (HRP-anti-μ) was prepared by the method of Avrameas and Ternynck (1). A 1-mg amount of lyophilized HRP (Boehringer, Mannheim, Germany) was dissolved in 0.2 ml of PBS containing 1.25% glutaraldehyde (25%, Serva, Heidelberg, Germany). After incubation overnight at 4°C, excess glutaraldehyde was removed by gel filtration (Sephadex G-25, Pharmacia, Uppsala, Sweden). To 1 mg of activated HRP, 0.5 mg of purified anti-μ antibody was added, and the mixture was concentrated to 0.1 ml by negative-pressure dialysis against PBS. After 2 h at room temperature, 5 ml of 5% bovine albumin in PBS was added. The conjugate was stored frozen in 0.5-ml amounts at −20°C until use.

(ii) To obtain an alkaline phosphatase anti-μ conjugate (Pase-anti-μ), the method of Engvall and Perlmann was employed (4). Briefly, to 1.5 mg of sedimented Pase (calf intestine, Boehringer, Mann-

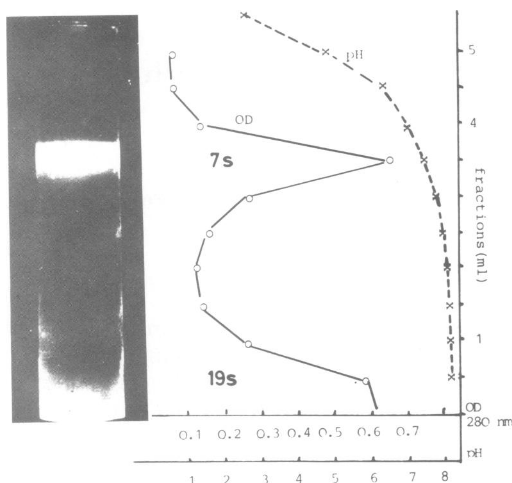

FIG. 1. *Isolation of anti-μ antibodies by ultracentrifugation in a sucrose gradient. Both antigen and antibody had been FITC stained prior to precipitation. Thus, the FITC-stained anti-μ antibodies in the 7s region and the FITC-stained IgM in the 19s region can be visualized. After fractionation, pH and optical density (OD) were measured in each fraction of 0.5 ml.*

heim, Germany), 0.5 mg of anti-μ antibody was added, and the mixture was dialyzed against PBS overnight and then concentrated to 0.3 ml. Coupling was carried out in 0.25% glutaraldehyde (final concentration) for 2 h at room temperature. To stop the reaction, the conjugate was diluted in 2 ml of PBS, and, after dialysis against PBS, 3 ml of 5% bovine albumin in PBS was added. The final product was stored in 0.5-ml quantities at −20°C.

Antigens. Two commercially obtained antigens and two antigens prepared in this laboratory were used in this study. A glycine buffer-extracted complement-fixing antigen was purchased from Behringwerke, Marburg, Germany. Another complement-fixing antigen was obtained from Flow Laboratories, Glasgow, United Kingdom. Both antigens were used at a dilution of 1:10 in RSB buffer [10 mM tris(hydroxymethyl)aminomethane (pH 7.5)-1.5 mM $MgCl_2$-10 mM KCl].

Also, an antigen prepared from the nuclei of CMV-infected cells (nuclear antigen) was employed. The nuclei were isolated from CMV-infected, nonconfluent fibroblast cells in which the cytopathogenic changes had been developing for at least 5 days (11). The cells from a Roux bottle were suspended in 10 ml of 0.4% KCl in distilled water by vigorous shaking, sedimented by centrifugation, resuspended in 5 ml of 0.4% KCl-1% Nonidet P-40 (nonionogenic detergent, Shell, Hamburg, Germany), and then homogenized in a tight-fitting Dounce homogenizer (Braun, Melsungen, Germany) with 60 strokes. The homogenate was layered onto a sucrose cushion (0.25 M sucrose in 0.4% KCl) and centrifuged at 700 × *g* for 5 min. To the sediment, 5 ml of RSB containing 0.5% bovine albumin was added. After sonic treatment (Branson sonifier, Danbury, Conn.) for 30s (2 intervals; output control 4, microtip) in an ice bath, the disrupted material was sedimented at 1,000 × *g* for 5 min. The supernatant fluid, which could be stored lyophilized, was used as antigen.

A nucleocapsid antigen was obtained by further purification of the nuclear antigen by ultracentrifugation through a sucrose cushion (35% in RSB) as has been described by McCombs (8).

A control antigen from noninfected cells was prepared in a manner identical to that described for the nuclear antigen. The nuclear, nucleocapsid, and control antigens were used in the enzyme immunoassay at dilutions of 1:2 in RSB.

Fixation of the antigens. Twenty-microliter volumes of the various antigens were evenly distributed in the holes of glass plates (Assistent, Hamburg, Germany) (Fig. 2). The antigens were air-dried and fixed in acetone at −20°C for 5 min, and the plates were incubated in 20% heat-inactivated bovine serum in PBS for 10 min at room temperature and again washed in PBS. Excess PBS was removed by placing the glass plates upside down on filter paper.

Alternatively, 20-μl volumes of antigen were distributed in the wells of flat-bottom polystyrene microtiter plates (Cooke Engineering, Alexandria, Va.) with a vibrator, dried, fixed in ethanol at −20°C, and treated with PBS as described.

Test procedure. 50-microliter amounts of the human serum specimens, used in doubling dilutions beginning at 1:64, were incubated in antigen-coated wells for 1.5 h at 37°C. The plates were then washed in PBS-0.05% Tween 20 (Serva, Heidelberg, Germany) for 10 min at room temperature. Then 50 μl of HRP-anti-μ or Pase-anti-μ diluted 1:160 or 1:20, respectively, was added and incubated for 1.5 h at 20°C.

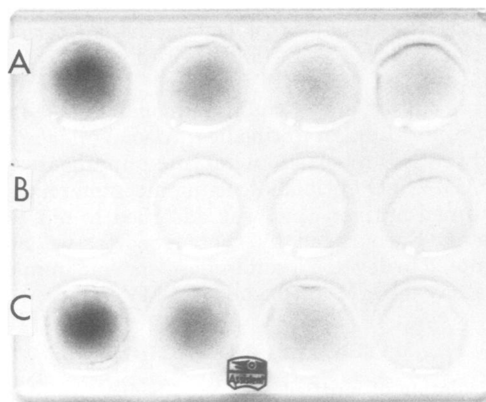

FIG. 2. *Enzyme immunoassay carried out on glass plates (slightly reduced size). Plates were photographed 15 min after the substrate had been added. Sera were diluted 1:64, 1:128, 1:256, and 1:512. (A) Positive control serum; (B) negative control serum (containing 5 mg of IgM per ml); (C) serum of a patient with anti-nuclear IgM antibodies.*

After a final washing in PBS-Tween 20 for another 10 min, the plates were placed on a white sheet of paper and 50 μl of the appropriate substrate was added to each well (*o*-phenylendiamine, 1 mg/ml, in 0.1 M phosphate buffer [pH 6] containing 0.03% H_2O_2 [16] or alternatively *p*-nitrophenylphosphate, 1 mg/ml, in 0.1 M carbonate buffer [pH 9.8] [4]; all reagents were from E. Merck AG, Darmstadt, Germany). The observation of a clear yellow coloration after 15 min at room temperature was judged to be positive, whereas negative wells remained completely colorless for at least 0.5 h.

Alternatively, the substrate (20 μl) was diluted 1:10 in 0.1 N HCl, and the optical density was measured at 400 nm.

FA tests. The human serum specimens were tested for CMV IgM antibodies by using the double indirect technique (IgM fluorescent-antibody [FA] test) (7, 11). After incubation of the human sera on the acetone-fixed cover slip cultures, anti-μ antiserum of rabbit origin (diluted 1:60) was applied and finally the cultures were stained with FITC anti-rabbit globulin (of sheep origin).

To confirm that low CMV IgM titers were specific, the IgM fractions of those sera with IgM antibody levels of 1:≤64 in the IgM FA test were isolated by ultracentrifugation (13). These IgM fractions were then tested in an anticomplement immunofluorescence test (11, 12), using human serum without CMV antibodies as a source of complement at a dilution of 1:100 and FITC anti-C_3 globulin conjugate at a dilution of 1:150.

The IgG antibodies to CMV in human sera were tested by an immunofluorescence technique, using FITC anti-γ-chain globulin of rabbits, diluted 1:60, as described previously (11).

Human serum specimens. A total of 103 sera of 44 patients with acute CMV infections diagnosed by successful virus isolation and positive IgM antibody response in FA tests were included in this study to control the results of the enzyme immunoassay. Another 127 serum specimens with CMV IgG antibody levels of 1:128 to 1:4,096, but without CMV IgM antibodies, served as negative controls.

Radial immunodiffusion tests. The IgM concentration in all human serum specimens was measured by radial immunodiffusion in Tri-Partigen IgM plates (Behring, Marburg, Germany). The concentration of the anti-μ sera used was also determined by radial immunodiffusion against purified IgM (10 μg/ml in 1% agarose in PBS). The plates were read after a diffusion time of 72 h.

RESULTS

Specificity and potency of the conjugates. After ultracentrifugation of the dissolved anti-μ-IgM immunocomplexes, about 50% of the anti-μ antibody could be isolated from the gradient in a highly purified form.

After enzyme labeling of the purified anti-μ antibody, both conjugates could be used at a 1:10 dilution in the indirect IgM FA assay (11), where they revealed the same high μ-chain specificity as the unlabeled antibody.

Table 1 (row 1 and columns a, b, c, e, f, and g) shows that both conjugates were able to detect even traces of IgM, which adsorbed nonspecifically to the surface of the untreated glass plates during incubation. On the other hand, concentrated serum preparations freed from the IgM antibody by ultracentrifugation (column d) adsorbed to the plates or, when dried and fixed in acetone, were always negative.

The nonspecific adsorption to the glass plates could be greatly reduced by preincubating the plates with 20% bovine serum (Table 1).

The potency of the enzyme-anti-μ conjugates

TABLE 1. *IgM antibody titers to CMV obtained in the enzyme immunoassay as related to the human sera or immunoglobulin fractions (columns a to g) and antigens (rows 2 to 7) used.*[a]

Antigen	IgM antibody titer						
	(a) Serum, CMV IgM +++	(b) Serum, CMV IgM negative, CMV IgG +++	(c) Purified IgM (5 mg/ml) CMV IgM negative	(d) IgG + IgA (40 mg/ml)	(e) Anti-nuclear IgM +++, CMV IgM negative	(f) Rheumatoid factor +++, CMV IgM negative, CMV IgG +++	(g) Serum with bacterial contamination, CMV IgM negative
1. None	*1,024*	*512*	*2,048*	Negative	*512*	*512*	*512*
2. 20% BS	Negative	Negative	Negative	Negative	Negative	Negative	*512*
3. Flow Laboratories (+BS)	512	*128*	*256*	Negative	*128*	*128*	*512*
4. Behring (+BS)	1,024	*128*	*256*	Negative	*128*	*256*	*512*
5. Nucleocapsids (+BS)	512	Negative	*128*	Negative	*512*	*256*	*512*
6. Nuclear (+BS)	2,048	Negative	Negative	Negative	*512*	*256*	*512*
7. Control (+BS)	Negative	Negative	Negative	Negative	*1,024*	Negative	*512*

[a] The antigens were incubated in 20% bovine serum (BS) to reduce adsorption of human IgM. Italics indicate nonspecific reactions.

was tested in a chessboard titration on glass plates containing dilutions of nonspecifically adsorbed IgM paraprotein. Both conjugates detected IgM up to a dilution of 1:2,048, but to give identical optical densities with the appropriate substrates, the HRP conjugate could be used at an eightfold-higher dilution (1:160) as compared with the Pase conjugate (1:20). When the enzyme immunoassay was carried out in microtiter plates instead of glass plates, both enzyme conjugates had to be used at a higher concentration (1:80 and 1:10, respectively).

Specific and nonspecific reactions. Table 1 summarizes the specific and nonspecific reactions obtained with the various antigens and sera used (rows 3 to 7). There was considerable nonspecific binding of IgM to crude CMV antigens, and preincubation with 20% bovine serum did not completely inhibit this nonspecific IgM adsorption (Table 1, rows 3 and 4). However, with antigens freed from the cytoplasmatic material (rows 5 to 7), the nonspecific adsorption was greatly reduced. The best results, i.e., highest specific titers and lowest nonspecific reactions, were obtained with the nuclear antigen. Therefore, all further enzyme immunoassays were carried out with the nuclear antigen (row 6). Even with the nuclear antigen, false-positive reactions may occur if the human sera contain anti-nuclear IgM antibodies or rheumatoid factors or are contaminated with bacteria (Table 1, columns e, f, and g). As can be seen from row g in Table 1, misinterpretations due to antinuclear antibodies or bacterial contamination of the human sera can be avoided if all positive sera are additionally tested with a control antigen prepared from noninfected fibroblast nuclei.

Correlation between the titers obtained in the CMV IgM FA test and the enzyme immunoassay. Table 2 shows that when the nuclear antigen and HRP anti-μ (diluted 1:160) were used in the enzyme immunoassay, the correlation between the IgM antibody levels to CMV in the IgM FA test and the enzyme immunoassay was highly positive ($r = 0.94$).

DISCUSSION

The FA test for IgM antibodies to CMV, although of great value in diagnosing acute infections, has not become a routine diagnostic method in many laboratories. This is probably due to time-consuming work that is needed to prepare hundreds of cover slip cultures. The enzyme immunoassay for IgM antibodies to CMV now offers some technical advantages. Although as sensitive as the immunofluorescence method, it does not require cover slip tissue cultures or a fluorescence microscope. Moreover, the enzyme immunoassay is more economical to perform than the immunofluorescence method. The nuclear antigen prepared from one Roux bottle of CMV-infected cells is sufficient for testing about 400 serum dilutions. The purified anti-μ antibody could be used 100 times more dilute when coupled to HRP as compared with FITC-labeled anti-μ antibody for the FA test. Therefore, only small amounts of antibody are required for enzyme labeling. As compared with affinity chromatography, the ultracentrifugation method described here is especially suitable for preparing small amounts of purified anti-μ antibody. The specific antibody can be isolated as a sharp band from the gradient, and no antibody is lost due to nonspecific adsorption to a gel matrix, to which the antigens have to be coupled for affinity chromatography.

The time-consuming isolation of the specific anti-μ antibodies was undertaken not only for the reduction of background staining, but also for economic reasons, because the enzymes, especially Pase, are very expensive.

Only a few disadvantages of the enzyme immunoassay are noteworthy. Since microscopic examination of the antigen is not carried out,

TABLE 2. *Correlation between the IgM antibody levels in 230 sera obtained by immunofluorescence and the enzyme immunoassay*[a]

Titer of enzyme immunoassay	$r = 0.94$[b] at immunofluorescence titer of:							
	32	32	64	128	256	512	1,024	2,048
2,048							3	9
1,024						2	11	
512						10	5	
256					18	1		
128			4	6	2			
64			4	8				
<64	127	11	9					

[a] Total of 103 sera were positive and 127 were negative in the IgM FA test.

[b] Confidence limits of r, 0.91 to 0.96.

bacterial contamination cannot be ruled out, nor can different antigenic structures such as nuclear inclusions, nuclei, or cytoplasm of the cells be distinguished. Therefore, human sera with antinuclear antibodies of the IgM class and sera that are contaminated with bacteria may give rise to false-positive reactions. The bacteria, presumably coated with IgM while growing in the serum specimens, adhere to the plates during the test procedure. These false-positive reactions can be easily recognized by using a control antigen prepared from noninfected fibroblast nuclei. Sera with IgM antibodies to CMV containing no antinuclear antibodies or bacterial contaminants should not react with the control antigen, whereas sera with antinuclear antibodies of the IgM class or with bacterial contaminations consistently do so.

Other pitfalls of the enzyme immunoassay are the same as those of the IgM FA test. Occasionally nonspecific reactions are caused by rheumatoid factors in the serum specimens. Therefore, as in the IgM FA test, all positive sera have to be checked for the presence of rheumatoid factors (14). This can be done quickly with the commercially available latex agglutination test. If positive, the sera must be absorbed with IgG-coated latex particles before retesting in the enzyme immunoassay. However, it should be stressed that sera producing false-positive reactions due to rheumatoid factor have rarely been found in our testing (in about 5% of all sera with CMV IgM antibody titers of 1:>64) and that bacterial contamination can be simply avoided if NaN_3 is added to the serum specimens.

The greatest single difficulty in setting up the enzyme immunoassay was associated with the purity of the antigens used. The commercially available antigens were prepared from whole cells and, in contrast to the nuclear antigen, contained cytoplasmatic components, which can bind human IgM nonspecifically. Only antigens prepared from isolated nuclei of infected cells had the desired degree of specificity.

With sera from adults containing IgM concentrations of >4 mg/ml and neonates (IgM > 1 mg/ml), dilutions of less than 1:64 or 1:16, respectively, could not be used due to high background reactivity. This background reactivity was also found when the nuclear antigen was bound to plastic plates during prolonged incubation at an alkaline pH (3, 4, 9, 15). Furthermore, under these conditions most of the antigen washed off the plastic plates during the test procedure, possibly because of its corpuscular structure.

It is possible to standardize the method, if desired, by measurement of the optical density of the substrate. Although this is not needed for the interpretation of our results, it might be useful for a more automated reading procedure (10).

We have shown (10a, 11) that IgM antibody levels to CMV of 1:≥128 indicate a recent CMV infection. When the enzyme immunoassay is used, such antibodies can be detected without any special technical equipment. All of the reagents required can be stored for long periods and can be used at high dilutions with corresponding economic advantages. Therefore, we hope that this technique will help to simplify the diagnosis of acute CMV infections.

ACKNOWLEDGMENTS

We would like to thank G. Lips for helpful assistance.

This work was supported by grants from the Deutsche Forschungsgemeinschaft (Schm 4/2).

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