Comparison of Seven Enzyme Immunoassay Systems for Measurement of Cytomegalovirus

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The relative sensitivities of seven different enzyme immunoassay (EIA) systems for the measurement of cytomegalovirus (CMV) were compared. Methods which used two separate antisera to CMV provided the greatest degree of sensitivity. Equivalent sensitivity was noted with the use of either enzyme-labeled antiglobulin or unlabeled staphylococcal protein A and rabbit enzyme-antienzyme complex to measure the second anti-CMV antibody bound to the solid phase. Single-antibody methods were less sensitive than the double-antibody methods but were more sensitive than an inhibition EIA. However, the sensitivity of the inhibition EIA was improved when CMV-antibody complexes were separated from unreacted antibody by means of precipitation with polyethylene glycol. Double-antibody EIA systems are preferable when antisera prepared in two different animal species are obtainable. However, a number of single-antibody EIA systems can be formulated for use in situations where only a single antiserum is available.

Enzyme immunoassays (EIA) have been attaining increasing usage for the measurement of bacterial, viral, fungal, and parasitic antigens (13, 14-16, 18). All EIA systems use an enzyme-substrate system to quantitate the reaction. However, there are a number of different ways in which enzyme-labeled immunoreactants can be used (15). Antigen detection systems have been developed that use single-antibody (12, 16), double-antibody (18, 19), and inhibition methods. Since these assay systems have been used with different antigens and antibodies, there are few studies available which compare the relative sensitivities of the different assay methods. We have devised several different methods for measuring a standard preparation of cytomegalovirus (CMV) and compared the relative sensitivities of the methods, using the same antigens and antisera.

MATERIALS AND METHODS

Goat anti-CMV serum was obtained from Microbiological Associates, Bethesda, Md. Human anti-CMV serum was obtained from a patient with systemic CMV infection. CMV complement-fixing antigen was obtained from Flow Laboratories, Springfield, Va. This preparation contained 16 complement-fixing units as determined by the method of Benyesh-Melnick et al. (3). Herpes simplex type 1 seed virus, 10^{3.3} 50% tissue culture infective doses per ml, was obtained from National Institute of Allergy and Infectious Diseases Reference Reagents. Epstein-Barr virus was obtained from James Robinson, Yale University Medical School.

Goat anti-human immunoglobulin G (IgG) and rabbit anti-goat IgG were purchased from Antibodies Inc.,

Davis, Calif. The globulin fractions of those antisera and the goat anti-CMV serum were prepared by precipitation with saturated ammonium sulfate, and these fractions were conjugated with alkaline phosphatase (Sigma type 7; Sigma Chemical Co., St. Louis, Mo.) by the method of Engvall and Perlmann (8), or with horseradish peroxidase (Sigma type 6) by the method of Avrameas et al. (1).

Rabbit peroxidase-antiperoxidase complex and peroxidase-labeled goat anti-human IgG were purchased from Cappel Laboratories, Downingtown, Pa. Staphylococcal protein A (SPA) was purchased from Pharmacia Fine Chemicals, Piscataway, N.J. A portion of the SPA was conjugated with alkaline phosphatase by the two-step method as described by Engvall (7). C1q, the subunit of the first component of complement which binds the Fc portion of some antigen-antibody complexes, was prepared from fresh human serum by the method of Yonemasu and Stroud (20).

Substrate for alkaline phosphatase was prepared by dissolving a 5-mg tablet of *p*-nitrophenyl phosphate (Sigma 104-105) in 5 ml of 1 M diethanolamine buffer (pH 9.8). Substrate for the peroxidase reactions was prepared by dissolving 20 mg of *o*-phenylenediamine (Sigma) into 50 ml of 0.01 M citrate buffer (pH 5.0), followed by the addition of 20 μ l of 30% H₂O₂. Both substrates were prepared immediately before use. The alkaline phosphatase reactions were stopped by the addition of 25 μ l of 3 M NaOH, and the peroxidase reactions were stopped by the addition of 25 μ l of 2 M H₂SO₄. The optical densities of the EIA reactions were measured in a microplate colorimeter (5).

Negative controls, consisting of uninfected human embryonic fibroblast lysates, were added to each test. A dilution of CMV was considered to be positive if the mean absorbance at that dilution differed by at least 2 standard deviations from the mean of the negative controls.

Enzyme assays. Optimal dilutions of all reagents were determined by checkerboard titration. For each assay, the dilutions chosen were those which allowed for detection of minimal quantities of antigen. All reactants were added in 100- μ l volumes unless otherwise stated. The assay systems are summarized in Table 1.

(i) Single antibody: sandwich assay.

- (a) Goat anti-CMV serum was diluted in 0.06 M carbonate buffer (pH 9.6) and added in the wells of a polyvinyl microtiter plate (Dynatech 220-24). The plate was incubated for 14 h at 4°C.
- (b) The plate was washed five times with phosphate-buffered saline (pH 7.4) containing 0.5% Tween-20 (PBS-T).
- (c) CMV antigen, diluted fourfold with PBS-T, was added to duplicate wells. The plate was incubated for 2 h at 37°C.
- (d) The plate was washed five times with PBS-T.
- (e) Alkaline phosphatase-labeled goat anti-CMV, diluted in PBS-T containing 1% fetal calf serum and 1% normal goat serum (PBST-S), was added to the wells and incubated for 2 h at 37°C.
- (f) The plate was washed five times with PBS-T.
- (g) Substrate was added. The plate was incubated for 30 min at 37°C, and the results were recorded as described above.

(ii) Single antibody: C1q method.

- (a) C1q was diluted to a concentration of 2 μ g/ml in carbonate buffer and added to the wells of a polyvinyl microtiter plate. The plate was stored at 4°C until use in step (c) below.
- (b) Fourfold dilutions of CMV antigen were added to the wells of a hard microtiter plate (Dynatech 223-24). An equal volume of goat anti-CMV diluted in PBS-T was also added. The plate was incubated for 2 h at 37°C.
- (c) The contents of this plate were transferred to the wells of a plate coated with C1q as described in step (a) above after it had been washed five times with PBS-T. The plate was incubated for 2 h at 37°C.
- (d) The plate was washed five times with PBS-T.
- (e) Alkaline phosphatase-labeled anti-goat IgG was added to the wells and incubated for 1 h at 37°C.
- (f) The plate was washed five times with PBS-T.
- (g) Substrate was added. The plate was incubated for 30 min at 37°C; the results were measured and interpreted as described above.

(iii) Double antibody: antiglobulin method.

- (a) The plate was prepared according to steps (a) through (d) of the single-antibody sandwich method.
- (b) Unlabeled human anti-CMV, diluted in

TABLE 1. Outline of EIA methods"

Component	Single antibody			Double antibody		Inhibition	
	Sandwich	Human C1q	Labeled antiglobulin	Labeled SPA	SPA-PAP complex	Without complex separation	Complex separation with PEG precipitation
Reactant bound to solid phase	Goat anti-CMV	Human C1q	Goat anti-CMV	Goat anti-CMV	Goat anti-CMV	CMV antigen	CMV antigen
Antibody added to well (liquid phase)	Enzyme-labeled goat anti-CMV	Human Anti-CMV	Human anti-CMV	Human anti-CMV	Human anti-CMV	Human anti-CMV	Human anti-CMV
Other reactants (in order of addition)	Substrate	Enzyme-labeled anti-human IgG	Enzyme-labeled anti-human IgG	Enzyme-labeled SPA	Unlabeled SPA	Anti-human IgG	Anti-human IgG
		Substrate	Substrate	Substrate	Rabbit PAP	Substrate	Substrate

" Abbreviations: CMV, Cytomegalovirus; SPA, staphylococcal protein A; PAP, peroxidase-antiperoxidase complex; PEG, polyethylene glycol.

PBS-T, was added and incubated for 1 h at 37°C.

- (c) The plate was washed five times with PBS-T.
 - (d) Alkaline phosphatase- or horseradish peroxidase-labeled goat anti-human IgG, diluted in PBST-S, was added and incubated for 1 h at 37°C.
 - (e) The plate was washed five times with PBST-S.
 - (f) The appropriate substrate was added. The plate was incubated for 30 min at 37°C, and the results were determined as described above.
- (iv) Double antibody: enzyme-labeled SPA method.**
- (a) The plate was processed according to steps (a) through (c) of the double-antibody antiglobulin method.
 - (b) Alkaline phosphatase-labeled SPA was added and incubated for 1 h at 37°C.
 - (c) The plate was washed five times with PBS-T.
 - (d) Substrate was added, and the results were determined as described above.
- (v) Double antibody: SPA-peroxidase-antiperoxidase method.**
- (a) The plate was processed according to steps (a) through (c) of the double-antibody antiglobulin method.
 - (b) SPA, diluted to 1 µg/ml in PBST-S, was added to the wells and incubated for 1 h at 37°C.
 - (c) The plate was washed five times with PBS-T.
 - (d) Rabbit peroxidase-antiperoxidase complex, diluted in PBST-S, was added and incubated for 30 min at 37°C.
 - (e) The plate was washed five times with PBS-T.
 - (f) Peroxidase substrate was added, and the plate was incubated for 30 min at 37°C; the results were determined as described above.
- (vi) Inhibition method.**
- (a) A hard plastic microtiter plate (Dynatech 223-24) was coated with CMV antigen diluted in carbonate buffer. The plate was stored at 4°C until use in step (c) below.
 - (b) Fourfold dilutions of CMV antigen (diluted in PBS-T) were added to the wells of a blank hard plastic microtiter plate (Dynatech 223-24). Human anti-CMV was also added to the wells. The plate was incubated for 2 h at 37°C.
 - (c) The contents of the uncoated plate were transferred to the antigen-coated plate prepared in step (a) after the plate had been first washed five times with PBS-T. The plate was incubated for 30 min at 37°C.
 - (d) The plate was washed five times with PBS-T.
 - (e) Enzyme-labeled anti-human IgG was added and incubated for 1 h at 37°C.

(f) The plate was washed five times with PBS-T.

(g) Substrate was added. The plate was incubated for 30 min at 37°C and quantitated as described above. A dilution was considered to be positive for CMV if it yielded an optical density value which was 2 standard deviations less than the mean of the negative controls.

(vii) Inhibition assay with antigen-antibody complex separation.

- (a) Fourfold dilutions of CMV antigen (diluted in PBS-T) were added to Eppendorf microcentrifuge tubes. An equal volume of human anti-CMV serum was added, and the tubes were incubated at 37°C for 2 h.
- (b) Polyethylene glycol 9000 (Sigma) was added to the tubes to give a final concentration of 4%. The tubes were incubated for 30 min at 4°C and centrifuged at approximately 10,000 rpm for 10 min.
- (c) The supernatant fluids were transferred to a plate coated with CMV antigen and processed according to steps (c) through (g) of the inhibition method.

RESULTS

The results of the seven different assays for fourfold dilutions of a standard preparation of CMV are depicted in Table 2. The double-antibody methods proved to be the most sensitive, with both the labeled antiglobulin methods and the method using SPA and rabbit peroxidase-antiperoxidase conjugates detecting 1:6,400 dilutions of the standard preparations. The double-antibody method using enzyme-labeled SPA was less sensitive, allowing for the detection of 1:1,600 dilutions.

In the case of the single-antibody assays, the sandwich method, in which the goat anti-CMV was directly labeled with enzyme, was the most sensitive, allowing for the detection of 1:1,600 dilutions of the test antigen. On the other hand, the C1q method allowed for the detection of a 1:400 dilution of the antigen. The inhibition method, which did not involve separation of the antigen-antibody complexes, was the least sensitive, allowing for the detection of only a 1:100 dilution of antigen. However, the sensitivity of the inhibition assay was increased fourfold when the antigen-antibody complexes were precipitated with polyethylene glycol before transfer to the antigen-coated plate.

To assess the effect of the use of different enzymes, the relative sensitivities of assays using alkaline phosphatase- and peroxidase-labeled antiglobulins were compared in the single-antibody human C1q, the double-antibody-labeled immunoglobulin, and the inhibition assays for each assay system. The concentrations of CMV which could be detected were identical regard-

TABLE 2. Comparison of sensitivity of EIA methods for detection of CMV^a

CMV dilution	Single antibody, alk phos		Double antibody				Inhibition, alk phos	
	Sandwich	Human C1q ^b	Labeled antiglobulin		Labeled SPA alk phos	SPA-PAP complex perox	Without complex separation ^b	PEG complex separation ^b
			Alk phos	Perox				
1:100	0.27	0.18	0.51	0.49	0.24	0.55	0.22	0.08
1:400	0.24	0.13	0.48	0.44	0.20	0.43	0.34	0.20
1:1,600	0.20	0.10	0.34	0.31	0.16	0.41	0.38	0.36
1:6,400	0.11	0.09	0.22	0.22	0.14	0.33	0.37	0.34
1:25,600	0.12	0.07	0.17	0.15	0.14	0.28	0.39	0.39
Neg ctr	0.11 ± 0.03	0.09 ± 0.02	0.14 ± 0.03	0.15 ± 0.03	0.12 ± 0.02	0.27 ± 0.03	0.34 ± 0.04	0.37 ± 0.04

^a Underlined values are 2 standard deviations greater or less than the mean of the negative controls. Results are the means of triplicate values expressed as optical density units (5). Abbreviations: CMV, cytomegalovirus; SPA, staphylococcal protein A; PAP, rabbit peroxidase-anti-peroxidase complex; PEG, polyethylene glycol; Neg ctr, mean ± 2 deviations of uninfected tissue culture lysates; alk phos, alkaline phosphatase; perox, horseradish peroxidase.

^b These assays were also performed with peroxidase-labeled reagents. In each case the endpoints were identical to the results with alkaline phosphatase (data not shown).

less of the enzyme used. Suitable reagents were not available for such a comparison in the other assay systems.

Each assay was also tested for activity with herpes simplex type 1 and Epstein-Barr virus. No assay system showed any reactivity with these non-CMV herpesviruses.

DISCUSSION

Enzyme immunoassays offer a number of advantages, including sensitivity, convenience, and low cost (14, 15, 17). However, there is a great deal of variation in how EIAs are performed. We therefore set up seven different EIA systems, using available antisera directed against CMV, and compared the relative sensitivities of the systems in testing fourfold dilutions of a standard antigen preparation.

In general, the most sensitive assays were the ones which used two separate antisera to CMV. These results thus correlated with previous studies in which double-antibody systems proved to be more sensitive than single-antibody systems for detecting cholera toxin (18), human rotavirus (17), *Haemophilus influenzae* type b (J. M. Pepple, E. R. Moxon, and R. H. Yolken, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 19th, Boston, Mass., Abstr. no. 754, 1979), and meningococcus (J. E. Sippel and A. Voller, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, C119, p. 330). In addition to the labeled-antiglobulin method, which was used in the previously reported studies, we found that we could attain equal sensitivity by using SPA and rabbit peroxidase-antiperoxidase complex. This method makes use of the fact that SPA will not significantly interact with goat serum, which was used to coat the plate, but will react with the Fc portion of the human anti-CMV serum and the rabbit peroxidase-antiperoxidase complex (4). Although this method requires an extra incuba-

tion step, it has the advantage that a specific enzyme-labeled antiglobulin does not have to be prepared. The use of enzyme-labeled SPA as the antibody detector led to an assay which had fourfold less sensitivity, presumably because of the lower specific activity of the SPA enzyme conjugate as compared with enzyme-antibody complexes. Our results thus correlated with those of Engvall (7), who found that SPA-alkaline phosphatase conjugates were not as effective as alkaline phosphatase-anti-human IgG conjugates in measuring antibody by EIA. It should be noted that the SPA methods are only applicable in double-antibody systems in which the antibody used to coat the plate is prepared in an animal, such as a goat or horse, which does not interact with SPA (4, 10).

One disadvantage of the double-antibody methods is that they require antibody prepared in two separate animal species. Therefore they are not applicable for some antigens, such as hepatitis A virus or Norwalk virus, for which antibody can only be practicably produced in a single animal species (11). Thus, these antigens must be assayed by a single-antibody system. Of the single-antibody systems tested, the sandwich method, in which the same antibody used to coat the plate was labeled with enzyme, proved to be the most sensitive. Although this method has been successfully used to detect a number of viral antigens, it has the disadvantage of requiring a separate labeled antibody for each antigen to be tested. We therefore devised an alternate system in which C1q, the component of human complement which binds to the Fc portion of antigen-antibody complexes, is purified from human serum and bound to the solid phase. This C1q binds to antigen-antibody complexes formed by the subsequent addition of test antigen and specific antibody (20). Although this method proved to be less sensitive than the

sandwich method, it has the advantage of allowing for the use of labeled antiglobulin to detect a number of different antigens. In addition, it is possible that a system consisting of C1q on the solid phase and pooled antisera in the liquid phase would allow for detection of a number of different antigens in the same well. In addition, the use of C1q which has been further purified by affinity chromatography might lead to a more sensitive C1q EIA (11).

Another form of EIA which uses a single antiserum is an inhibition assay. In inhibition EIAs, the antiserum is incubated with the antigen to be measured and transferred to a solid phase containing a fixed amount of antigen. Antibody not bound by the initial antigen-antibody reaction will bind to the antigen on the solid phase. Therefore antigen in the test specimen will result in a decreased optical density as compared with the negative control. This system has been used to measure *Candida albicans* (14) and alpha-fetoprotein (2). However, we found that the simple inhibition test could only detect a 1:100 dilution of CMV antigen and was therefore less sensitive than either the single-antibody or double-antibody methods. We postulated that one reason for this decreased sensitivity might be the fact that the CMV antibody has a relatively low affinity for antigen and that the antigen-antibody complexes might dissociate after being added to the solid-phase antigen. We therefore attempted to increase the sensitivity of the inhibition assay by removing the complexes by means of precipitation with polyethylene glycol by the method of Creighton et al. (6). In fact, such separation resulted in a fourfold increase in sensitivity. Immune complex separation by polyethylene glycol thus provided for a simple means of increasing the sensitivity of inhibition assays. It is possible that the use of antibody with higher affinity might result in inhibition EIAs of increased sensitivity.

Both alkaline phosphatase- and peroxidase-labeled reagents were available for testing in four of the seven assay systems. In each case, the results were independent of the enzyme used. This suggests that the differences noted in the sensitivities of the systems were largely due to the nature of the antigen-antibody interactions rather than the kinetic properties of the enzyme-substrate systems.

If the antigen-antibody system for CMV is typical of the systems for other antigens, it can be concluded that the double-antibody method, using either enzyme-labeled antiglobulin or SPA and peroxidase-antiperoxidase complex, will provide the greatest sensitivity. This increased sensitivity is probably due to the fact that a

single molecule of antibody can react with a number of antiglobulin or SPA molecules, thereby resulting in a magnification of the initial antigen-antibody reaction (9, 17). However, in cases where antibody from two different animal species is not available, the single-antibody sandwich will provide a useful alternative. C1q binding, although it proved to be less sensitive than the sandwich method, could be the most useful assay if labeled antibody is not available or if multiple antigens are to be tested. In addition, the inhibition method, augmented by the separation of immune complexes by polyethylene glycol precipitation or other methods, might be used if satisfactory solid-phase antigen can be obtained.

Further experiments on the measurement of CMV in body fluids should be undertaken to determine the relative sensitivity and specificity of the methods for the measurement of CMV clinical specimens. In addition, experiments on antigens other than CMV should be undertaken to determine whether the results of these experiments can be generally applied to the measurement of other antigens in tissue culture and body fluids.

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