Chemiluminescent Immunoenzymatic Assay for Rapid Diagnosis of Viral Infections

ALLAN D. PRONOVOST,^{1,2} ALEXANDER BAUMGARTEN,^{1†*} AND WARREN A. ANDIMAN^{3,4}

Departments of Laboratory Medicine,¹ Pediatrics,³ and Epidemiology,⁴ Yale Medical School, New Haven, Connecticut 06510, and Virology Laboratory, Veterans Administration Medical Center, West Haven, Connecticut 06516²

Received 8 March 1982/Accepted 10 May 1982

Current methods of viral diagnosis have been criticized for slowness and insensitivity. However, immunoassay may provide the desired increase in the speed of diagnosis without sacrificing accuracy. This report describes the efficacy of the detection of viral antigen by means of an absorptiometric enzyme-linked immunoassay (ELISA) or by a chemiluminescent enzyme-linked immunoassay (CELISA). Human cytomegalovirus was detected in clinical specimens or culture fluid with comparable sensitivity by CELISA and by viral isolation but with 50 times lesser sensitivity by ELISA. Similarly, herpes simplex virus was detected in clinical specimens with markedly greater sensitivity by CELISA than by ELISA. These findings indicate that the detection of appropriate viruses by CELISA may be a practical alternative to their isolation in cell culture.

We previously reported that chemiluminescence (CL) enhances the sensitivity of an enzyme-linked immunoassay for the detection of herpes simplex virus antigen in clinical specimens (9) and for the quantification of serum proteins (8). The chemiluminescent enzyme-linked immunoassay (CELISA) was 64 times as sensitive as the absorptiometric (colorimetric) enzyme-linked immunoassay (ELISA) in the detection of enzyme-labeled immunoglobulin G. CELISA has also been used for the measurement of various drugs, metabolites, and hormones (1, 14).

CELISA effects quantification by the amount of light emitted during an enzymatically catalyzed reaction of an appropriate substrate (5). In this study the oxidation of isoluminol (isoaminophthalylhydrazide) by hydrogen peroxide to a dicarboxylic acid (aminophthalate) derivative was used. The oxidation can be catalyzed by various iron compounds but occurs at a much higher molar efficiency with the enzyme horseradish peroxidase. The emission of light results from the decay of the chemically excited newly formed aminophthalate (12, 13).

A rapid and sensitive method for the detection of human cytomegalovirus (CMV) is needed to diagnose congenital infection (7), infection in recipients of renal transplants (2), and interstitial pneumonia in bone marrow recipients (4). Isolation in cell culture not only may be slow and

tedious but may also yield a falsely negative result owing to lability of virus during transport and storage. Immunoelectron microscopy can detect CMV in urine (3) when the number of infectious particles is sufficiently high (typically over 10^4 per ml; M. Macris, P. Bailey, F. Lee, A. Visintine, J. Dannenbarger, A. Brann, and A. Nahmias, *Int. Conf. Hum. Herpesviruses*, Atlanta, Ga., abstr. no. G40, 1980), but the method is impractical for large numbers of specimens. ELISA is both rapid and convenient but lacks adequate sensitivity.

This report compares the efficacy of CELISA for the detection of CMV in clinical specimens and culture fluid with that of ELISA and of isolation in cell culture.

MATERIALS AND METHODS

Virus. The AD169 strain of human CMV was obtained from N. S. Swack of this laboratory and propagated in human embryonic fibroblast cells (Flow Laboratories, Inc., McLean, Va.) maintained in Eagle minimal essential medium supplemented with 2% fetal bovine serum. Stock virus was prepared by harvesting infected cultures 9 days after infection. The cultures were frozen and thawed three times and clarified by slow-speed centrifugation. The supernatant fluid containing the virus was dispensed in working volumes and frozen at -70°C . The stock suspension of virus had a titer of $10^{4.5}$ 50% tissue culture infectious dose units (TCID₅₀) per ml. Dilutions of the stock suspensions were made in virus transport medium (VTM; Eagle minimal essential medium with Hanks balanced salt solution, 10% bovine serum albumin [BSA], antibiotics) unless indicated otherwise.

Immunoassay. The techniques of ELISA and CELISA used for CMV were similar to those de-

† Address reprint requests to: A. Baumgarten, Clinical Immunology Laboratory, Yale-New Haven Hospital, New Haven, CT 06504.

FIG. 1. Comparison of the results of an infectivity assay with those of CELISA and ELISA for the detection of human CMV in culture fluid. The intensity of the response in ELISA and CELISA to each viral concentration has been represented on the ordinate as a percentage of the maximal response of which the immunoassay was capable (see text); brackets indicate one standard deviation from the mean. The TCID₅₀ of each suspension of CMV (represented along the abscissa and illustrated by reference to the guide along the upper border) varied inversely with the dilution of the suspension that contained the viral antigen (represented along the lower border).

scribed previously for herpes simplex virus (9). A double-antibody-sandwich ELISA for CMV was standardized by conventional absorptiometric methods (15). Polystyrene spheres, 6.4 mm in diameter, with a specular finish (Precision Plastic Ball Co., Chicago, Ill.) were used for the assay. The polystyrene spheres were processed by successive 30-min incubations at 37°C in 300 μ l of fluid, followed by three washes with 0.05% Tween 20 in sodium phosphate (0.02 M)-buffered saline (TP), pH 7.2, unless stated otherwise. The initial incubation was for 18 h at 4°C in goat anti-AD169 strain CMV antiserum (Microbiological Associates, Bethesda, Md.) diluted 1:50 in 0.1 M sodium carbonate buffer, pH 9.6, followed by six washes with TP. The second incubation was with an appropriate dilution of the CMV stock virus suspension, a sample of the original or absorbed clinical material, cell culture supernatant, or a control lysate of uninfected human embryonic fibroblasts in Eagle minimal essential medium containing 1% BSA. The third incubation was with human serum (obtained from a patient who had previously had a systemic CMV infection) that had a complement fixation titer of 1:256 and was diluted 1:200 in 1% BSA-phosphate-buffered saline, pH 7.2. The last incubation was with horseradish

peroxidase-conjugated goat anti-human immunoglobulin G (Cappel Laboratories, Downingtown, Pa.) diluted 1:3,200 in BSA-phosphate-buffered saline. The enzymatic activity was assayed in duplicate by absorptiometry but in triplicate by CL.

For absorptiometry, *o*-phenylenediamine was used as the indicator, with readings made at 493 nm (8). With CELISA, the light emitted during the peroxidase-catalyzed oxidation of isoluminol by hydrogen peroxide at pH 10.5 was measured as described previously (8, 9, 11). The emission was recorded as counts per minute in a scintillation counter with coincidence circuitry disconnected (10). The data for each method were normalized by subtracting the result obtained for VTM (72,100 cpm by CL) from that for each assay and expressing the difference as a percentage of the maximal difference, i.e., that given by the stock suspension of CMV diluted 1:25 in VTM (129,000 cpm by CL). The normalization was made to facilitate a comparison of the results.

Clinical specimens. Clinical specimens (23 urine specimens and 1 postmortem liver tissue specimen) which had been submitted for CMV isolation and stored at 4 or -70°C for up to 4 years were examined by CELISA and ELISA. All specimens had been previously assayed for CMV by isolation in cell culture. Virus reisolations and infectivity titers were performed in human embryonic fibroblast cell cultures. Negative controls consisted of pooled urine from apparently healthy individuals. A clinical specimen was considered to be positive for CMV if the mean absorbance or counts per minute exceeded that for VTM by at least 10% ($\geq 85,000$ cpm by CL). The specificity of the positive response exhibited by clinical specimens in the ELISA test was ascertained by preincubation (30 min at 37°C) of representative urine samples with an equal volume of goat or human CMV antiserum diluted 1:50, followed by a subsequent addition of the neutralized sample to antibody-coated polystyrene spheres as described above.

RESULTS

Figure 1 illustrates the sensitivity of isolation in cell culture, CELISA, and ELISA for the titration of infectious CMV or CMV antigen in culture fluid. CELISA detected as little as $10^{0.3}$ TCID₅₀ of CMV per assay in contrast to $10^{2.0}$ TCID₅₀ detected by ELISA. This represents a 50-fold increase, assuming a lower positive cut-off limit of 10% above the background value observed with uninfected cell control lysates. Measurement of CMV in 1% BSA-Eagle minimal essential medium or pooled normal urine (instead of culture fluid) gave comparable results. The mean variance within and between runs did not exceed 8% by either method. Undiluted normal urine or VTM did not interfere with the assay. No cross-reactivity was observed with $\geq 10^{4.5}$ TCID₅₀ of herpes simplex virus or varicella-zoster virus.

Table 1 summarizes the results of assays performed on clinical specimens. It is noteworthy that all negative specimens of urine gave lower yields by CL (60,000 to 62,000 cpm) than

TABLE 1. Comparison of the results of viral isolation in cell culture with those of CELISA and ELISA for the detection of human CMV in clinical specimens

Specimen no. ^a	Conditions of storage	Clinical data ^b	Virus isolation in cell culture ^c		ELISA ^d	CELISA ^d
			Original isolation	Reisolation ^e		
1-14	-70°C, 3 yr		-	-	-	-
15	-70°C, 1 yr	Pneumonia, hepatosplenomegaly (5.5 mo) ^f	+	+ (<0.25)	-	+
16	-70°C, 4 yr	Cytomegalic inclusion disease	+	+ (1.5)	-	+
17a	-70°C, 4 yr	Agammaglobulinemia; mononucleosis syndrome with pneumonia, splenomegaly, and atypical lymphocytosis (12 yr) ^f	+	+ (1.0)	-	+
17b	4°C, 4 yr		+	-	-	+
18a	-70°C, 4 yr	Shock, hepatitis, coma, seizures, hearing loss (4 mo) ^f	+	+ (0.5)	-	+
18b	4°C, 4 yr		+	-	-	+
19a	-70°C, 3 yr	Cytomegalic inclusion disease ^f	+	-	-	+
19b	4°C, 3 yr		+	-	-	+
19b ^g			+	-	-	-
20	-70°C, 4 mo	Pneumonitis, hepatosplenomegaly (1 mo)	+	+ (1.0)	-	+
20 ^g			+	-	-	-
21	-70°C, 1 yr	Guillain-Barre syndrome, hepatomegaly, serum anti-CMV antibody titer of 1:128	-	-	-	+
21 ^g			-	-	-	-

^a All specimens were urine samples except for no. 16 which was a homogenate of liver tissue obtained at autopsy; a and b indicate either separate specimens from the same patient or a divided specimen stored at different temperatures.

^b Age of patient given in parentheses.

^c -, Negative for virus; +, positive for virus.

^d The positive control included in each assay contained $10^{2.0}$ TCID₅₀ of CMV per 300 μ l of normal urine. Specimens giving a response $\geq 10\%$ above the background (normal urine) were considered positive by either method.

^e Number in parentheses indicates the infectivity titer upon reisolation ($\log_{10}$ TCID₅₀ per 0.2 ml).

^f Indicates patients in whom CMV was also isolated from throat swabs.

^g Antibody was added. The specificity of the positive response was confirmed by a loss of the reactivity of the specimen after its preincubation with a specific antiserum before assay (see text).

did the VTM which was used as a blank (72,100 cpm). Five of six urine specimens as well as a liver tissue homogenate that were originally positive for CMV by isolation in cell culture and had been stored at -70°C were also positive upon reisolation. Three urine specimens stored at 4°C for over 3 years were no longer infectious. All of the specimens were negative by ELISA in two separate determinations. By contrast, all of the specimens that had been initially positive by isolation were also found to be positive by CELISA. Neither ELISA nor CELISA yielded false-positive results.

The specimens that were originally positive by viral isolation in cell culture were not only low in infectivity titer upon reisolation ($\leq 10^{1.5}$ TCID₅₀/0.2 ml) but also low in total reactive antigen content, because no specimen yielded a greater response by CL than that exhibited by a positive control which contained only $10^{2.0}$ TCID₅₀ of CMV per sample.

It is noteworthy that one urine specimen (no. 21) which had been negative in the initial cell culture as well as upon attempted reisolation after storage at -70°C was positive only by CELISA.

TABLE 2. Comparison of the results of viral isolation in cell culture and assay by ELISA and CELISA for the detection of virus in clinical specimens

Virus tested	No. positive/no. tested (%)			
	Isolation in cell culture		Assay of stored material	
	Original isolation	Reisolation	ELISA	CELISA
HSV-1 ^a	17/33 (52)	14/33 (42)	12/33 (36)	15/33 (45)
CMV ^b	6/21 (29)	5/21 (24)	0/21 (0)	7/21 (33)

^a HSV-1, Herpes simplex virus type 1. Adapted from Pronovost et al. (9). The specimens included lip lesions, genital lesions, throat swabs, and cerebrospinal fluid.

^b Values do not include repeat specimens held under various conditions of storage (see Table 1).

The specificity of the positive response exhibited by CELISA with respect to the viral antigen was tested as follows. Representative cell culture-positive and -negative urine specimens held at -70°C , and a culture-positive isolate held at 4°C for 4 years were preincubated with CMV antiserum and then reexamined in the assay. Samples to which the antiserum was added were found to be negative both by CELISA and in cell culture.

The effect of serum protein in urine was tested by mixing 1 part of a normal human serum (without complement-fixing antibody to CMV) with 24 parts of urine. Normal urine specimens yielded no positive results despite the addition of serum. Urine specimens that contained CMV reacted as strongly after the addition of the serum as before. This result indicates that serum proteins in urine are unlikely to interfere with the outcome of CELISA.

DISCUSSION

The results of this study indicate that CELISA is a rapid (3 to 4 h), specific, and sensitive method for the detection of very low concentrations of CMV antigen in stored clinical specimens. Unlike isolation in cell culture, CELISA does not require infectious virions, which means that clinical samples could even be inactivated before assay. Preliminary observations indicate that storage at 4°C or delay in transportation does not affect the ability of the assay to detect viral antigen.

A double-antibody-sandwich method was employed in this study because it provided the greatest degree of sensitivity (15). The assay described can be used with readily obtainable reagents and can be adapted to rapid screening of numerous specimens. The human serum that was used as a detector antibody in the assay could be replaced by a second CMV antiserum prepared in another animal species. Human serum with a high antibody titer to CMV but with a low antibody titer to varicella-zoster virus or

herpes simplex virus gave the best results. It is noteworthy that the addition of a normal human serum diluted 1:25 with urine did not interfere with the results obtained for either positive or negative assays. Urine treated in this manner contained approximately 0.4 g of immunoglobulin per liter. This concentration is unlikely to be exceeded in most specimens of urine; therefore, even marked gammaglobulinuria will probably not interfere with the assay.

A comparison of the sensitivity of CELISA with that of ELISA for the detection of human herpes simplex virus type 1 had previously shown that the former method detected as few as 40 PFU per sample, whereas the latter method required 2,500 PFU for detection (9). Furthermore, CELISA detected the viral antigen in 88% of the clinical specimens that had been found to contain the virus during original isolation, but it yielded no false-positive results. Table 2 compares the results of CELISA, ELISA, and viral isolation for the detection of herpes simplex virus type 1 and CMV in clinical specimens.

Although CL is rather more tedious to perform than absorptiometry, the two methods require a similar amount of manipulation. However, the latter method requires long reaction periods and the use of a stopping reagent which may affect the development of color (6). Detection of enzyme activity by CL (11) is better suited to automation because indicator enzyme activity is measured during an initial period when the reaction is linear with respect to antibody concentration. The use of luminogenic substrates with higher quantum efficiencies and peak light intensities may permit a further increase in sensitivity (11).

ACKNOWLEDGMENTS

We thank Grace Tucker for supplying us with the clinical isolates.

This study was supported by Public Health Service research training grant T-32-AD 07018 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Gorus, F., and E. Schram. 1979. Applications of bio- and chemiluminescence in the clinical laboratory. *Clin. Chem.* (Winston-Salem, N.C.) **25**:512-519.
2. Ho, M., S. Surwansirikul, J. M. Dowling, L. A. Youngblood, and T. A. Armstrong. 1975. The transplanted kidney as a source of cytomegalovirus infection. *N. Engl. J. Med.* **293**:1109-1112.
3. McIntosh, K., K. Wilfert, M. Chernesky, S. Plotkin, and M. J. Mattheis. 1980. Summary of a workshop on new and useful techniques in rapid viral diagnosis. *J. Infect. Dis.* **182**:793-802.
4. Meyers, J. D., H. C. Spencer, Jr., J. C. Watts, M. B. Gregg, J. A. Stewart, R. H. Troupin, and E. D. Thomas. 1975. Cytomegalovirus pneumonia after human marrow transplantation. *Ann. Intern. Med.* **82**:181-188.
5. Nilsson, R. 1964. On the mechanism of peroxidase catalyzed oxidations studied by means of chemiluminescence measurements. *Acta Chem. Scand.* **18**:389-401.
6. Pesce, A. J., D. J. Ford, and M. A. Gaizutus. 1978. Qualitative and quantitative aspects of immunoassays. *Scand. J. Immunol.* **8**(Suppl. 7):1-6.
7. Plotkin, S. A., and I. Furukawa. 1978. Herpesviridae: cytomegalovirus, p. 93-112. *In* G. D. Hsiung and R. H. Green (ed.), CRC handbook series in clinical laboratory science, section H, virology and rickettsiology, vol. 1, part 2. CRC Press, Inc., Boca Raton, Fla.
8. Pronovost, A. D., and A. Baumgarten. 1981. Comparison of chemiluminescence and absorptiometry in enzyme immunoassays for protein quantification. *Experientia* **38**:304-305.
9. Pronovost, A. D., A. Baumgarten, and G. D. Hsiung. 1980. Sensitive chemiluminescent enzyme-linked immunosorbent assay for quantification of human immunoglobulin G and detection of herpes simplex virus. *J. Clin. Microbiol.* **13**:97-101.
10. Schram, E., F. Demuylder, J. Derycker, and H. Roosens. 1976. On the use of liquid scintillation spectrometers for chemiluminescence assays in biochemistry. p. 243-254. *In* A. A. Moujaim, C. Ediss, and L. Wiebe (ed.), Liquid scintillation science and technology. Academic Press, Inc., New York.
11. Schroeder, H. R., and F. M. Yeager. 1978. Chemiluminescence yields and detection limits of some isoluminol derivatives in various oxidation systems. *Anal. Chem.* **50**:1114-1119.
12. Seitz, W. R., and M. P. Neary. 1974. Chemiluminescence and bioluminescence in chemical analysis. *Anal. Chem.* **46**:189A-202A.
13. White, E. H., and R. B. Brundrett. 1973. The chemiluminescence of acyl hydrazides, p. 231-241. *In* M. J. Cormier, D. M. Hercules, and J. Lee (ed.), Chemiluminescence and bioluminescence. Plenum Publishing Corp., New York.
14. Whitehead, T. P., L. J. Kricka, T. J. N. Carter, and G. H. G. Thorpe. 1979. Analytical luminescence: its potential in the clinical laboratory. *Clin. Chem.* (Winston-Salem, N.C.) **25**:1531-1546.
15. Yolken, R. H., and P. J. Stopa. 1980. Comparison of seven enzyme immunoassay systems for measurement of cytomegalovirus. *J. Clin. Microbiol.* **11**:546-551.