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Sensitive Chemiluminescent Enzyme-Linked Immunosorbent Assay for Quantification of Human Immunoglobulin G and Detection of Herpes Simplex Virus

A. D. PRONOVOST,[1] A. BAUMGARTEN,[2]* AND G. D. HSIUNG[1]

Department of Laboratory Medicine, Yale University School of Medicine, New Haven, Connecticut 06510,[2] and Virology Laboratory, Veterans Administration Medical Center, West Haven, Connecticut 06516[1]

A chemiluminescent enzyme-linked immunosorbent assay (CELISA) was developed for detecting human immunoglobulin G and herpes simplex viral antigen. A comparison of CELISA with a conventional absorptiometric detection system showed that CELISA was 100 times more sensitive than absorptiometry for the measurement of human immunglobulin G. Similarly, CELISA detected as few as 40 plaque-forming units of herpes simplex virus in contrast to 2,500 plaque-forming units detected by absorptiometry. Of 18 specimens which were positive for herpes simplex virus type 1 by isolation in tissue culture, 15 (83%) were detected by CELISA within a few hours; in certain cases, several days were necessary for detection of virus by isolation techniques.

The enzyme-linked immunosorbent assay (ELISA) is widely applied in viral immunodiagnosis (1). The sensitivity of ELISA generally exceeds that of conventional assays, but is less than that of the radioimmunoasay (15). A limitation on the sensitivity of ELISA is the method used for the detection of the reaction product (13). Enzymatic activity is generally measured by absorptiometric methods.

The enzyme horseradish peroxidase has received wide use in ELISA because of its stability and ready availability (17). In addition to yielding colored reaction products, the enzyme catalyzes the oxidation of isoluminol by hydrogen peroxide. The latter reaction, which is accompanied by a measurable emission of light, is utilized for assays by chemiluminescence (CL) (4). Quantification of a variety of substances of biological and medical interest has indicated that the chemiluminescent ELISA (CELISA) has sensitivity comparable to that of the radioimmunoassay (16).

This report compares the sensitivity of CELISA and an absorptiometric detection system for the quantification of human immunoglobulin G (IgG) and detection of herpes simplex virus (HSV) antigen.

MATERIALS AND METHODS

Quantification of human IgG. A competitive ELISA for human IgG was standardized by absorptiometry to provide maximal sensitivity as described previously (6). Polystyrene (PS) balls (0.25-inch [ca. 0.64-cm] diameter) with a specular finish (Precision Plastic Ball Co., Inc., Chicago, Ill.) were precoated in duplicate for absorptiometry or in triplicate for CELISA with human IgG (Hyland Laboratories, Inc., Cosa Mesa, Calif.; 400 μl per ball at 10 μg/ml) in 0.1 M carbonate buffer, pH 9.6, for 18 h at 4°C and washed six times in 0.05% Tween 20-phosphate-buffered saline (TP). The assay was performed by mixing 200 μl of IgG, freshly diluted in phosphate-buffered saline (pH 7.2) containing 1% bovine serum albumin (fraction V), with 200 μl of peroxidase-conjugated goat anti-human IgG (Cappel Laboratories, Inc., Cochranville, Pa.) diluted 1:10,000 in the same buffer. The mixture was incubated for 30 min at 37°C, and then IgG-coated PS balls were added and incubated for 2 h at 37°C. The PS balls were then washed three times with TP and assayed for enzymatic activity by absorptiometry or CL.

Quantification of HSV. HSV type 1 (HSV-1; MacIntyre strain) was propagated in primary rabbit kidney or guinea pig embryo cells in Eagle minimal essential medium prepared in Hanks balanced salt solution (HMEM) (GIBCO Laboratories, Grand Island, N.Y.) containing 2% calf serum as previously described (10). When the virus-induced cytopathic effect was advanced, infected cells were frozen and thawed once, and virus infectivity titers were determined. Stock virus had an infectivity titer of 1.1×10^6 plaque-forming units per ml. The control antigen consisted of uninfected cells treated in a manner similar to virus-infected cells.

A double-antibody sandwich ELISA for HSV-1 was standardized by means of an absorptiometric detection system for maximal sensitivity and then compared with the CELISA. Before the assay, PS balls were coated in duplicate for absorptiometry or in triplicate for CELISA with rabbit anti-HSV-1 (MacIntyre strain) immunoglobulin (DAKO, Denmark) at a 1:20 dilution (400 μl per ball) in carbonate buffer, as described above for IgG. After absorption, the PS balls were washed six times with TP and then incubated in

400 μl of appropriate dilutions of HSV-1 stock or normal uninfected cell control antigen in HMEM containing 1% bovine serum albumen for 30 min at 37°C. PS balls were subsequently washed three times with TP and incubated in 400 μl of human serum (obtained from a patient from whom HSV-1 was isolated; complement-fixation titer, 1:256) diluted 1:160 in 1% bovine serum albumin-phosphate-buffered saline for 30 min at 37°C. After three washes with TP, 400 μl of peroxidase-conjugated rabbit anti-human IgG (Cappel Laboratories, Inc., Cochranville, Pa.) diluted 1:160 in 1% bovine serum albumin-phosphate-buffered saline was added, and the balls were incubated again for 30 min at 37°C. The PS balls were then washed three times with TP, and the enzymatic activity was assayed by absorptiometry or CL. The absorbance or CL obtained with normal, uninfected cell control antigen was subtracted from the value of each virus-containing sample.

Absorptiometric detection system. Horseradish peroxidase was measured by adding 400 μl of fresh reaction mixture (0.5 mg of *o*-phenylenediamine per ml, 0.006% hydrogen peroxide in 0.1 M sodium citrate buffer, pH 5.0) to PS balls, which were then incubated at room temperature for 30 min in the dark (7). The reaction was stopped by the addition of 200 μl of 2 M sulfuric acid, and absorbance was read at 493 nm. The reaction mixture with stop solution added at 0 min served as a blank.

CELISA detection system. CELISA measured the oxidation of isoluminol by hydrogen peroxide at pH 10.5 (12). The assay was performed at room temperature in the dark by successive addition of the following reagents to a vial which contained 1.25 ml of distilled water and the enzyme bound to the PS balls: 300 μl of 1 mM ethylenediaminetetraacetic acid; 30 μl of isoluminol (0.1 mM in 1.0 M glycine-NaOH buffer, pH 9.0); and 300 μl of 1.0 M glycine-NaOH, pH 10.5. Immediately upon addition of 1 ml of 0.3 mM hydrogen peroxide, the emitted light was quantified in a liquid scintillation counter with the coincidence circuitry disconnected (11). Data were recorded as counts per minute $\times 10^3$ above the background, which was ascertained from a control that contained all of the reagents except the enzyme. To facilitate comparison of CL and absorbance, data were normalized and expressed as percentages of the maximal response.

Detection of HSV in clinical samples. Clinical specimens, including lesion swabs, throat swabs, and cerebrospinal fluids, which had been submitted for HSV isolation and stored at −70°C for up to 9 months in virus transport medium containing HMEM, 10% bovine serum albumin, and antibiotics, were thawed and examined by CELISA and absorptiometric ELISA. All specimens had been previously assayed for HSV by isolation in cell culture, and virus type was determined by cell culture selection (10). Virus isolations and infectivity titers were performed in primary rabbit kidney cell cultures. Specimens which exceeded background luminescence (virus transport medium alone) by 10% (≥10,000) of the maximum obtained in the titration of HSV-1 antigen (see Fig. 2) were considered to be positive by CELISA.

RESULTS

Quantification of human IgG. Figure 1 illustrates the dose-response curves obtained for human IgG by a competitive assay method for CELISA or absorptiometric ELISA. Absorbance values ranged from 1.00 without inhibition to 0.2 at full inhibition. CELISA values ranged from 0 to 200,000 above background. The amount of detected IgG ranged from 0.05 to 2.5 μg/ml (threshold sensitivity, 10 ng per tube) by CELISA in contrast to 1 to 500 μg/ml by absorptiom-

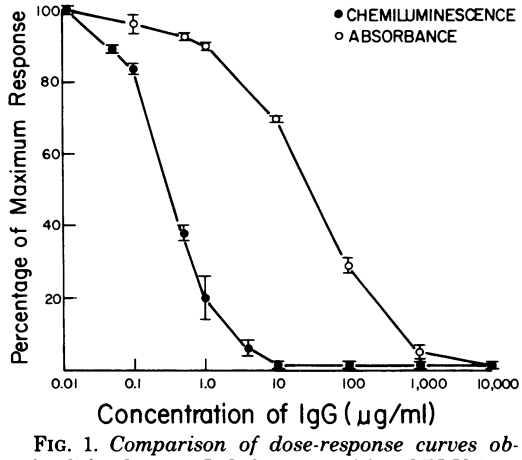

FIG. 1. *Comparison of dose-response curves obtained for human IgG by competitive CELISA or absorptiometric ELISA. The brackets represent ±1 standard deviation of six CL or four absorptiometric determinations. Concentration of IgG are expressed logarithmically: number on abscissa is in $\log_{10}$ micrograms per milliliter.*

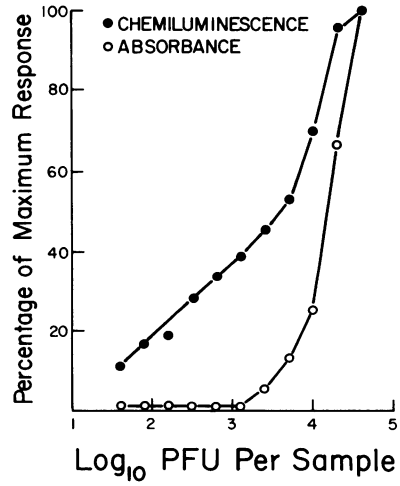

FIG. 2. *Comparison of sensitivity of CL and absorptiometry for detection of HSV-1 by double-antibody ELISA. Each point represents the mean of triplicate CL or duplicate absorptiometric determinations.*

etry (assuming lower and upper positive cutoff limits of 10 and 90% of the maximal response, respectively). At the midpoint of the dose-response curve (50% of the maximal response), the difference in detection represented a 100-fold increase in sensitivity of CELISA above that obtained by absorptiometry. The intra- and interassay coefficients of variation did not exceed 10% at maximum.

Quantification of HSV-1. Figure 2 compares the sensitivity of a double-antibody sandwich method for CELISA and absorptiometric ELISA in the detection of HSV-1. The mean variance exhibited in the dose-response curve was ±4.0% of maximum for CELISA and ±0.9% of maximum for absorptiometry. Variation in results obtained for the lowest detected amount of HSV antigen was ±0.9% by either method. However, owing to the greater variation noted at other points of the curve, a 10% increase above background was arbitrarily selected as the minimum at which antigen was considered to have been detected. At that level, the amount of HSV-1 detected by CELISA was ≥40 plaque-forming units per test sample versus ≥2,500 plaque-forming units per test sample detected by absorptiometry, representing a 64-fold-greater sensitivity of the former method.

Clinical specimens. Table 1 illustrates the effectiveness of CELISA for the detection of HSV in clinical specimens from which HSV was isolated initially. Background CL was determined as the number of counts emitted by virus transport medium alone. All specimens known to be negative for virus isolation in cell culture yielded counts which did not exceed the background by more than 0.2% of the maximal response obtained in the titration of HSV-1 antigen (Fig. 2). Specimens considered to be positive were those which exceeded the background by at least 10%. Of the 11 clinical specimens which yielded isolates of HSV-1 in cell culture within 1 to 2 days (≥2.25 $\log_{10}$ 50% tissue culture infectious doses per 0.2 ml), all were positive by CELISA and absorptiometric ELISA. These specimens were also positive for virus upon reisolation although the infectivity titers were reduced. By contrast, of seven specimens which took longer than 2 days to exhibit a cytopathic effect (≥2.25 $\log_{10}$ 50% tissue culture infectious doses per 0.2 ml), or which exhibited a cytopathic effect only upon repeated culture, four were identified as positive by CELISA, but only one was identified as positive by absorptiometric ELISA. It is noteworthy that virus was recovered from only three of the seven specimens upon reisolation. The overall yield of samples positive by CELISA was 15 of 18 (83%) versus 12 of 18 (67%) by absorptiometric ELISA.

Of six specimens containing HSV-2, all of which showed a cytopathic effect within 1 to 2 days in cell culture (≥2.25 $\log_{10}$ 50% tissue culture infectious doses per 0.2 ml), two (33%) were positive by CELISA using HSV-1 antiserum-coated PS balls. Sixteen clinical specimens, including those from three facial lesions, three genital lesions, four throat swabs, and six cerebrospinal fluid samples showed no infectivity by virus isolation in the initial tests and were negative by CELISA and absorptiometric ELISA.

These results indicate a specificity of 100% but a sensitivity of 83% for CELISA, applied to

TABLE 1. *Comparison of virus isolation in cell culture and assay by absorptiometric ELISA and CELISA for the detection of HSV in clinical specimens*

Virus	Source of specimen	No. tested	Infectivity titer[a]	No. of viruses isolated	No. positive		No. of viruses reisolated
					Absorptiometric ELISA[b]	CELISA[c]	
HSV-1	Lip lesion	6	2.25–5.50	6	6 (3.5+)	6	6
	Genital lesion	3	2.25–5.50	3	3 (2+)	3	3
	Throat swab	2	2.25–4.50	2	2 (1.5+)	2	2
		3	1.00–2.25	3	1 (1.5+)	3	3
		4	<1.00	4	0	1	0
HSV-2	Arm lesion	1	4.50	1	ND[d]	0	1
	Genital lesion	5	2.25–5.50	5	ND	2	5

[a] $\log_{10}$ 50% tissue culture infectious doses per 0.2 ml.

[b] Number (and mean intensity) of positive clinical specimens. Intensities are recorded as 3 to 4+ (strongly positive), 1 to 2+ (moderately positive), and 0 to 1+ (negative). The results represent averages of duplicate assays.

[c] Clinical specimens in virus transport medium giving a response 10% above background (virus transport medium alone) were considered positive by CELISA (see text).

[d] ND, Not determined.

the detection of HSV-1 viral antigen, in comparison with tissue culture isolation methods.

DISCUSSION

Luminometry has been applied clinically in immunoassays and specific competitive binding assays for the quantification of a variety of medically important substances (16), although it has not yet been applied to the detection of viral antigen. The sensitivity of CELISA is comparable to that of the radioimmunoassay (4) and other nonisotopic techniques, such as fluorescence (18). The results of this study demonstrated that the application of CL to ELISA increased the sensitivity for detection of antigen 50- to 100-fold above that obtained by absorptiometry. A comparable increase in sensitivity was obtained with IgG and HSV-1, although the methods of assay of the two antigens differed in detail.

Compared with the radioimmunoassay, CELISA offers the added advantages of greater stability of reagents and safety in handling. In comparison with absorptiometric enzyme immunoassays using peroxidase, luminescent reagents pose no toxic or mutagenic risk (15) and provide a means for very sensitive detection of the enzyme label. The use of other oxidation systems may allow for further increases in the sensitivity of CELISA (12). Luminometers and scintillation counters are readily available for luminescence quantification, and luminescence measurements have been automated in various cases (4). The manual method of CL used here required fresh reagents and approximately 3 min per sample. It was therefore more tedious than absorptiometric ELISA.

The applicability of CELISA to the quantification of IgG suggests that the method could be used in a highly sensitive assay for antibody. The results obtained with the presented method for measuring human IgG indicated a greater sensitivity than those reported by Hersch et al. (5a).

Miranda et al. (9) developed a solid-phase absorptiometric ELISA for HSV-1 which required the incubation of test samples in a specially designed filtration apparatus. A solid-phase radioimmunoassay for HSV required the growth of tissue culture cells on the bottom of reaction vials (2). Mills et al. (8) serotyped HSV isolates by double-antibody ELISA, but the limits of sensitivity for the method were not established. In the present study the use of commercially available rabbit anti-HSV immunoglobulin and peroxidase-conjugated rabbit anti-immunoglobulin without the need for the individual conjugation of viral antisera and specially designed equipment was an important feature. The rabbit anti-HSV immunoglobulin preparation had been previously shown to be specific for HSV by immunofluorescence and did not cross-react with other herpesviruses (M. J. August, J. J. Nordlund, and G. D. Hsiung, 4th Int. Congr. Virol., The Hague, Netherlands, 1978, p. 137). The human serum had a high complement-fixation antibody titer to HSV but no detected antibody to varicella-zoster virus or cytomegalovirus (complement-fixation titer, <1:4). It is possible that the human serum could be replaced by a second antiserum to HSV commercially made in another animal species.

The percentage of clinical specimens positive for virus isolation in cell cultures which proved also positive by absorptiometric ELISA and CELISA was comparable to that reported with previously described immunoassays (2, 8, 9). The proportion of clinical specimens positive by CELISA was probably related to the number of infectious and noninfectious virus particles present in the specimens. The inability to detect some HSV-2 isolates in this study was probably caused by the high dilution of rabbit anti-HSV-1 immunoglobulin used to coat the PS balls. Since the HSV-1 antiserum had a neutralizing antibody titer of 1:160 to HSV-1 and 1:40 to HSV-2, it is possible that a higher concentration of capture antibody would allow detection of a higher number of HSV-2 isolates. The differentiation of HSV-1 and HSV-2 viral antigen by absorptiometric ELISA (8, 9) and radioimmunoassay (2) has been reported, as has the differentiation of HSV-1- and HSV-2-specific antibodies in sera (3, 5, 14).

CELISA seems to be applicable to the detection of other viruses. It appears that the technique is well suited to rapid detection of viral antigen (or antibody) in body fluids (cerebrospinal fluid, urine), although it is not yet as sensitive as cell culture isolation when a low concentration of viral antigen is present. Nevertheless, further development of the technique may bridge the remaining gap in sensitivity.

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