Serotyping Herpes Simplex Virus Isolates by Enzyme-Linked Immunosorbent Assays

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An enzyme-linked immunosorbent assay (ELISA) using a double-antibody sandwich technique has been developed to serotype isolates of herpes simplex virus from clinical sources. The results obtained using this procedure were in agreement with those obtained with a standard neutralization test in typing stock cultures and 32 clinical isolates of herpes simplex virus. Clear differentiation between the two viral serotypes was obtained using rabbit immunoglobulin cross-absorbed with heterologous virus antigen. The ELISA procedure described appears to be a convenient and accurate substitute for the neutralization test in typing herpes simplex viruses. ELISA techniques require relatively small amounts of antigen and antibody and can be performed with very simple equipment.

Identification of herpes simplex virus (HSV) isolates in the diagnostic laboratory has been simplified by the development of fluorescent antibody reagents that are of considerable purity and specificity (2, 6). However, the typing of HSV isolates by immunofluorescence is a complex task requiring an interpretative evaluation of the results for determination of serotypes (7, 8). Thus, a procedure providing more clearly defined end points is desirable. Currently available biological and serological typing procedures have the disadvantage of being laborious; in some cases it takes several days to obtain results. Counterimmunoelectrophoresis has been developed as a typing procedure (4), but, despite the ease with which the test is performed, the procedure may require 24 h for development of the results.

The enzyme-linked immunosorbent assays (ELISA) described by Engval and Perlman (3) have been used in several studies for quantitative determination of antibodies to viral antigens (12, 13). Voller et al. (11) have noted the potential of ELISA methods for the detection and quantitative assay of viruses. The ELISA technique is sensitive, easily performed, and requires inexpensive equipment that is available in most diagnostic laboratories. The rapidity with which test results can be obtained suggests that the ELISA technique might be an attractive alternative to present methods of serotyping viruses.

In our laboratory, HSV isolates are identified by the type of cytopathic effect observed in Hep-2 cell cultures and by a positive immunofluorescent reaction. Therefore, we decided to de-

termine if these isolates could be serotyped more efficiently using the ELISA procedure.

MATERIALS AND METHODS

Immunoabsorbent preparation. A modification of techniques of Avrameas and Ternynck (1) and Jeansson (5) were employed for preparation of the immunoabsorbent. HSV MacIntyre type 1 ($10^{5.5}$ 50% tissue culture infectious doses per 0.1 ml) and MS type 2 ($10^{4.7}$ 50% tissue culture infectious doses per 0.1 ml) were inoculated in 10-ml amounts onto roller bottle cultures of BHK 21 cells grown in Eagle minimal essential medium containing 2% (vol/vol) fetal calf serum, 10% tryptose broth, and 1% glutamine. Cultures were allowed to progress to a 4+ cytopathic effect and cells were shaken from the plastic. Infected cells were collected by centrifugation at $1,800 \times g$. The supernatant fluid was drawn off, and 1 ml of pellet was suspended in saline to give a 5-ml total volume. One and one half milliliters of a 1% aqueous solution of glutaraldehyde (freshly diluted) and 0.5 ml of 0.1 M acetate buffer, pH 5.0, were added simultaneously. The suspension was stirred 3 h at room temperature. The suspension was then centrifuged 15 min at $1,800 \times g$. The pellet was homogenized with a tissue grinder in 0.2 M phosphate buffer (pH 7.2) and recentrifuged. Homogenization and centrifugation procedures were repeated three times. The immunoabsorbent was stored as a 10% suspension in phosphate-buffered saline (PBS) with 0.001% thimerosal.

Adsorption of immunoglobulins. One milliliter of anti-HSV 1 immunoglobulin (10 mg of protein per ml) or anti-HSV 2 immunoglobulin (25 mg of protein per ml) (Bio-Rad Laboratories, Richmond, Calif.) was mixed with 0.2 ml of packed heterologous immunoabsorbent. The mixture was stirred continuously for 1 h at 37°C and overnight at 4°C . The adsorption procedure was repeated three times. After each adsorption,

the immunoglobulin preparation was centrifuged at $48,000 \times g$ for 1 h in a Sorval RC-2B centrifuge.

Preparation of conjugate. Conjugate was prepared essentially by the method of Nakane and Kawaoi (9). Five milligrams of horseradish peroxidase type VI (Sigma Chemical Co., St. Louis, Mo.) was dissolved in 1.0 ml of freshly made 0.3 M sodium bicarbonate (pH 8.1). To this was added 0.1 ml of 1% dinitrofluorobenzene (Sigma Chemical Co.) in absolute ethanol. The above solution was gently mixed for 1 h at room temperature, followed by the addition of 1 ml of 0.01 M NaIO_4 (Sigma Chemical Co.) in distilled water, which was gently mixed for 30 min at room temperature. One milliliter of 0.16 M ethylene glycol in distilled water was added, and the solution was gently mixed for 1 h at room temperature. The solution was dialyzed against three 1-liter changes of 0.01 M sodium carbonate buffer, pH 9.5, at 4°C. Five milligrams of either anti-HSV type 1 or 2 immunoglobulin fraction suspended in 0.1 M NaCl with 15 mM NaN_3 was added to 3 ml of horseradish peroxidase-aldehyde solution and mixed for 3 h at room temperature. Five milligrams of NaBH_4 (Sigma Chemical Co.) was added, and the solution was held for 3 h at 4°C before dialysis against PBS at 4°C. The small amount of precipitate that formed was removed by centrifugation at $1800 \times g$ for 15 min. The supernatant fluid was the final conjugate preparation and did not require column purification as performed in the original method. One-milliliter portions were stored in vials at -20°C. These have been stable for 9 months.

Substrate. A stock solution of 5-amino salicylic acid (Aldrich Chemical Co., San Leandro, Calif.) was prepared fresh daily by dissolving 80 mg of 5-amino salicylic acid in 100 ml of distilled water which had been heated to 70°C. The solution was cooled to room temperature. Immediately before use, the pH was adjusted to 6.0 with 1 N NaOH, and 1 volume of 0.05% hydrogen peroxide was mixed with 9 volumes of 5AS solution.

Viral cultures. Viral isolates of HSV types 1 and 2 were obtained from the Kansas Department of Health and Environment. Each isolate had previously been typed by a neutralization test (15) in that laboratory. Fifteen HSV type 1 and 17 HSV type 2 isolates were received and typed in this laboratory using the ELISA procedure. The samples were placed in tubes (16 by 125 mm) of Hep-2 cultures (Flow Laboratories, Inc., Rockville, Md., or Microbiological Associates, Inc., Bethesda, Md.) and allowed to progress to a 4+ cytopathic effect. Infected cells were removed by scraping with a rubber policeman and pelleted at $1,800 \times g$ in a GLC-2B Sorval centrifuge. Supernatant fluid was drawn off each tube, and the pelleted cells were suspended in 0.1 ml Eagle minimal essential medium (Microbiological Associates, Inc.). The suspension was frozen and thawed three times. Viral cultures of coxsackievirus type B-1, parainfluenza virus type 3, poliovirus type 1, and vaccinia virus, in addition to Hep-2 cell cultures (i) uninfected and (ii) infected with *Mycoplasma pneumoniae*, were treated in the same manner as the HSV cultures. These were used as negative antigen controls. Antigen preparations were diluted 1:10 in 0.01 M PBS containing 0.05% Tween 20 (Sigma Chemical Co.) and 0.5% bovine serum albumin.

ELISA protocol. Optimal dilutions of the antibody for coating the wells and for the enzyme-labeled conjugates were determined by block titrations. The commercial preparations of specific immunoglobulin against HSV types 1 and 2 were diluted 1:200 and 1:500, respectively, in 0.01 M sodium carbonate buffer (pH 9.5) (14). Two hundred microliters of one or the other immunoglobulin was placed into the wells of polyvinyl or polystyrene microtiter plates (Cooke 1-220-29 or 1-220-29S; Cooke Engineering Co. [Dynatech Corp.], Alexandria, Va.). These were incubated at 37°C overnight in a humid chamber. The coating procedure described above gave very satisfactory results, but later experiments have indicated that incubation at 56°C for 90 min provides adequate coating. The fluid was removed by vacuum, and the wells were filled with PBS containing 0.05% Tween 20, which was removed after 3 min. This procedure was repeated for a total of three washes. Two hundred microliters of antigen was placed into two wells that had been coated with anti-HSV type 1 immunoglobulin and two wells coated with anti-HSV type 2 immunoglobulin. These were incubated for 1 h at 37°C, followed by another wash procedure. Anti-HSV types 1 and 2 conjugates were diluted 1:200 and 1:300, respectively, in PBS with 0.05% Tween 20 and 1% bovine serum albumin. Two hundred microliters of anti-HSV type 1 conjugate was placed in each well that had been coated with specific anti-HSV type 1 immunoglobulin, and the same volume of anti-HSV type 2 conjugate was placed in the wells which had been coated with anti-HSV type 2 immunoglobulin. The plates were incubated for 1 h at 37°C, and the wash procedure was repeated. This was followed by the addition of 200 μl of freshly prepared substrate. The reaction was stopped after 30 min by the addition of 50 μl of 1 N NaOH. Reactions were compared in which the same antigen had been reacted with either specific anti-HSV type 1 immunoglobulin and anti-HSV type 1 conjugate or specific anti-HSV type 2 immunoglobulin and anti-HSV type 2 conjugate. A clear difference in color was detectable by viewing the trays against a white background. If one or both wells exhibited a colored reaction, the darkest reaction was recorded as positive. Wells containing light brown to colorless reactants were recorded as negative.

Spectrophotometry. The visual readings were recorded, and the tests were then read in a Beckman DU spectrophotometer, using a microcuvette, at 450 nm. The readings in absorbance were converted to percentage of transmittance for convenience.

RESULTS

Block titrations using specific immunoglobulins, positive antigen controls, and enzyme-labeled conjugates indicated that polyvinyl plates were superior to polystyrene plates for the attachment of immunoglobulins. Specificity of the reactions was essentially the same in both plates, but intensity of the reaction was decreased in the polystyrene plates. Therefore, polyvinyl plates were used for the determinations recorded in this section.

Fifteen HSV type 1 isolates typed by the ELISA technique on three separate occasions showed complete agreement with results obtained by neutralization tests performed in the Kansas Department of Health and Environment Laboratories. Complete agreement as to type was also demonstrated for the 17 HSV type 2 isolates. Figure 1 is a photograph of an ELISA plate showing reactions of four isolates and two ATCC strains of HSV. The negative controls, as listed in Table 1, were all nonreactive (i.e., colorless). MacIntyre HSV type 1, ATCC strain, clearly showed a dark positive reaction in the anti-HSV type 1 system and only a slight reaction in the anti-HSV type 2 system. Just the opposite sites of reaction were seen with the HSV type 2 ATCC strain MS. The HSV isolates pictured showed a darker reaction in their homologous antibody system. Spectrophotometric readings of the reactions demonstrated a definite difference, with positive reactions having a transmittance $\leq 17\%$. Negative reactions all had readings of $\geq 28\%$ transmittance. These results are summarized in Table 2. Although the anti-HSV type 2 system did not give the intensity of reaction observed in the anti-HSV type 1 system, a detectable difference still made the plates readable.

DISCUSSION

ELISA techniques have been applied to antibody detection and used for the detection of viral antigens. The present study used a double-antibody sandwich version of the ELISA method to distinguish between HSV types 1 and 2 isolated from clinical specimens.

HSV types 1 and 2 were clearly differentiated by the rapid ELISA performed in microtiter plates. The HSV type 2 strain ATCC MS had a much darker reaction in the anti-HSV 1 anti-

body system than was encountered in testing the clinical isolates. Roizman et al. (10) have stated that strains of HSV form a continuously varying spectrum of antigenic variants. Wolontis and Jeanssen (16) have theorized that this conclusion was based largely on studies of highly

TABLE 1. Reactions of HSV cultures and control antigens when tested by an ELISA serotyping procedure

Antigen tested	No. tested	ELISA results using:	
		HSV type 1 anti-body	HSV type 2 anti-body
HSV isolates:			
Type 1 ATCC VR-539	1	+	-
Type 2 ATCC VR-540	1	-	+
Type 1 isolates (clinical)	15	+	-
Type 2 isolates (clinical)	17	-	+
Negative controls:			
Coxsackievirus type B1	1	-	-
Parainfluenzavirus type 3	1	-	-
Poliovirus type 1	1	-	-
Vaccinia virus	1	-	-
<i>Mycoplasma pneumoniae</i>	1	-	-
Hep-2 cells	1	-	-
Reagent controls ^a	4	-	-

^a Reagent controls were ELISA tests with the following reagents omitted: antigen, antibody coated on wells, conjugated immunoglobulin, and substrate.

TABLE 2. Summary of spectrophotometric readings of ELISA reactions in typing HSV

Reactions	Transmittance (%)	
	HSV type 1 ab	HSV type 2 ab
Positive	≤ 5.9	≤ 16.5
Negative	≥ 30.9	≥ 28.8
Negative controls	≥ 45.7	≥ 45.7

FIG. 1. Demonstration of ELISA reactions in serotyping HSV. Wells in row 1 are ELISA reactions for detecting HSV type 1 antigen. Wells in row 2 are ELISA reactions for detecting HSV type 2 antigen. Antigens tested: A, negative control wells in which antigen was omitted from procedure; B, Hep-2 cell antigen; C, ATCC strain HSV type 1 MacIntyre; D, ATCC strain HSV type 2 MS; E and F, HSV type 2, antigen preparations of clinical isolates; G and H, HSV type 1, antigen preparations of clinical isolates.

passed viral strains. The reactions we observed with the MS strain may be indicative of this phenomenon.

HSV comprise a large proportion of the viruses isolated in a diagnostic laboratory. A rapid method for typing these viruses using readily obtained reagents is desirable. The use of highly diluted antisera, the preparation of antigen from cell cultures of clinical specimens, and the ability to provide results within 3 h make the ELISA procedure very functional for a clinical virology laboratory. Conventional methodology may require the use of additional cell cultures and comparatively low dilutions of antisera, thus increasing the cost of identifying serotypes. All materials used in this study were from commercial sources; therefore, this procedure should be readily available to any laboratory.

We have not performed stability studies on immunoglobulin-sensitized plates. Those laboratories testing many isolates may find storage of sensitized plates to be desirable. However, this should not be attempted without performing stability studies. Positive and negative controls for each serotype should be included in any diagnostic use of the ELISA procedure for serotyping.

The potential for virus typing using the ELISA method is obvious, its greatest attributes being its specificity, speed, and economy. Problems, however, may arise in obtaining high-titered antisera with good specificity; therefore, each lot of antiserum should be checked carefully before it is accepted for diagnostic procedures.

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