Enzyme-Linked Immunosorbent Assay for Determination of Antibodies Against Herpes Simplex Virus Types 1 and 2 in Human Sera

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A rapid and reproducible enzyme-linked immunosorbent assay (ELISA) is described for determining antibodies in human sera against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2). The sera were absorbed for 30 min with heterologous virus-infected-cell extracts to remove cross-reacting antibodies and then were applied to ELISA plates containing the target antigens, immunoaffinity-purified HSV-1 glycoproteins gC and gD and HSV-2 glycoproteins gD and gF. The absorbance index, defined as the ratio of A_{414} generated by a serum sample absorbed with a heterologous virus-infected-cell extract versus the A_{414} of a serum sample absorbed with an uninfected-cell extract, was used to determine the presence or absence of antibodies to HSV-1 and HSV-2. Results of the ELISA for detecting antibodies against HSV-2, when compared with results obtained for the same sera by the microneutralization test, showed an index of overall agreement of 91%. Results of the ELISA for detecting antibodies against HSV-1, when compared with microneutralization test results for sera negative for HSV-2 antibodies but positive for HSV antibodies by ELISA, showed an index of agreement of 99%.

Herpes simplex virus (HSV) type 1 (HSV-1) and type 2 (HSV-2) can be distinguished by their antigenic profiles (5) and by their genetic composition (14). Antibodies generated against HSV-1 or HSV-2 show extensive intertypic cross-reactivity (11), and an antigenically type-specific protein(s) has not been confirmed (29, 31, 33).

Numerous serological tests have been described for distinguishing antibodies to HSV-1 and HSV-2 in human sera (19). These include neutralization kinetics (21), microneutralization (MN) (16), indirect hemagglutination (7), immune lysis of infected cells (25), immunofluorescence (26), solid-phase radioimmunoassay (6), radioimmunoassay with purified viral proteins (12), immunoelectrophoresis (27), radioimmunoprecipitation-polyacrylamide-gel electrophoresis (10), and enzyme-linked immunosorbent assay (ELISA) (4, 28). In most seroepidemiological studies for determining antibodies to HSV-1 and HSV-2, the MN test, either as described by Pauls and Dowdle (16) or as modified by Rawls et al. (20), has been used.

We describe here an ELISA for determining the presence in human sera of antibodies directed against HSV-1 and HSV-2. In this assay, the target antigens are HSV-1 and HSV-2-induced glycoproteins purified with monoclonal antibodies, and infected-cell extracts are used for absorbing cross-reacting antibodies from test sera.

MATERIALS AND METHODS

Infected-cell extracts. Vero cells were grown in roller bottles (850 cm²) with Eagle minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin, and streptomycin. The cells were infected with either HSV-1 (strain 14012) or HSV-2 (strain 333) for 1 h at 37°C at a multiplicity of infection of 10 to 20. The inoculum was

removed, fresh medium containing 5% fetal bovine serum was added, and the cells were incubated for an additional 24 h and were harvested by sedimentation.

Packed infected cells (5 ml) harvested from 20 roller bottles were resuspended with 15 ml of extraction buffer (0.1 M Tris-hydrochloride [pH 8.0], 10% glycerol, 0.5% sodium deoxycholate, 0.5% Nonidet P-40, 0.2 mM phenylmethylsulfonyl chloride, 1% aprotinin [Sigma Chemical Co., St. Louis, Mo.]) by mixing for 1 h at 4°C on a rotator. The cells were disrupted by sonication and were clarified by centrifugation at 40,000 × *g* for 1 h at 4°C. The supernatant fluids (infected-cell extracts) were harvested and stored in aliquots at -70°C.

Purification of monoclonal antibodies. The following previously described monoclonal antibodies were employed: 4-S, which reacts with gD from HSV-1 and HSV-2 (24); 19-S, which reacts with HSV-1 gC (24); and 104-S, which reacts with HSV-2 gF and HSV-1 gC (33).

For purification of monoclonal antibody 104-S, ascites fluid (10 to 15 ml) was dialyzed extensively against 20 mM Tris-hydrochloride (pH 7.2) and was clarified by filtration through glass wool and centrifugation at 6,000 rpm for 10 min at 4°C. The fluid was then applied to a column (2.5 by 20 cm) of DEAE-Affi-Gel Blue (Bio-Rad Laboratories, Richmond, Calif.) (bed volume, 100 ml) at 4°C, and the column was washed with 300 ml of 20 mM Tris-hydrochloride (pH 7.2) and then with 300 ml of 25 mM NaCl-20 mM Tris-hydrochloride (pH 7.2). Immunoglobulin was eluted in ca. 60 ml of the 300 ml of 50 mM NaCl-20 mM Tris-hydrochloride (pH 7.2) added to the column. The remaining proteins were eluted with 300 ml of 1.4 M NaCl-Tris-hydrochloride (pH 7.2). Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (33) indicated that the immunoglobulin fraction was ca. 80 to 90% pure, with minor contamination with transferrin. The total immunoglobulin protein recovered, as measured by the Bio-Rad protein assay, was 3 mg.

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For monoclonal antibodies 4-S and 19-S, clarified ascites fluids (ca. 14 ml) were dialyzed extensively against 0.14 M sodium phosphate buffer (pH 8.0) and were mixed with protein A-Sepharose CL-4B beads (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) for 1 h at 4°C. The beads were washed four times with phosphate-buffered saline (PBS) (pH 8.0), loaded into a Bio-Rad polypropylene column, washed with PBS (pH 8.0), and then washed with 0.1 M sodium phosphate buffer (pH 7.2) (33). Immunoglobulins were eluted with 0.1 M sodium citrate buffer (pH 3.0) and dialyzed against 0.5 M NaCl-0.2 M sodium bicarbonate buffer (pH 8.5). The purity of the immunoglobulin preparations was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (33), and the protein yields were determined by the Bio-Rad protein assay.

Purification of viral antigens. gD and gC were purified from HSV-1-infected-cell extracts, and gD and gF were purified from HSV-2-infected-cell extracts by immunoaffinity chromatography (33). Monoclonal antibody 4-S was used for purification of HSV-1 gD and HSV-2 gD, monoclonal antibody 19-S was used for purification of HSV-1 gC, and monoclonal antibody 104-S was used for purification of HSV-2 gF.

Purified immunoglobulin was coupled to CNBr-activated Sepharose 4B beads (Pharmacia Fine Chemicals, Inc.) at 5 mg of protein per ml of gel, in accordance with the instructions of the manufacturer (33). The antibody-coupled beads were washed three times with extraction buffer, and 0.5-ml portions were mixed with 15 ml of infected-cell extract on a rotator at 4°C for 90 min. The beads were pelleted ($1,000 \times g$, 3 min) and washed three times with 10 ml of extraction buffer, and antigen was released by suspending the beads in 2 to 3 ml of 3 M NaSCN-0.2 M Tris-hydrochloride (pH 7.2). After 5 min, the beads were pelleted ($1,000 \times g$, 5 min, 4°C), and the antigen-containing supernatant fluid was dialyzed against PBS (pH 7.2). The purity of the preparation was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (33). Typical protein yields from 5-ml infected-cell packs were 0.3 mg each for HSV-1 gD and HSV-2 gD, 0.4 mg for HSV-1 gC, and 0.01 mg for HSV-2 gF.

Human sera. A battery of human sera that have been characterized by MN (20) for antibodies to HSV-1 and HSV-2 was kindly furnished by W. Rawls (McMaster University, Ontario, Canada). Additional human sera, used in previous studies (12), were kindly furnished by K. Hsu (Columbia University).

ELISA. Purified antigens were diluted 25- to 50-fold in 0.05 M sodium bicarbonate-carbonate coating buffer (pH 9.6) and were adsorbed to wells (100 μ l per well) of either U- or flat-bottomed Immulon II microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.). For detecting antibodies to HSV-2, a mixture consisting of HSV-2 gD (0.2 μ g) and HSV-2 gF (0.05 μ g) was adsorbed to each well. For detecting antibodies to HSV-1, a mixture consisting of HSV-1 gD (0.2 μ g) and HSV-1 gC (0.2 μ g) was adsorbed to each well. Adsorption was carried out overnight at 4°C. The wells were washed one time with distilled water and four times with PBS (pH 7.2) containing 0.05% Tween-20 (PBS-T). The protein binding sites were then blocked by adding 200 μ l of 5% bovine serum albumin (Miles Laboratories, Inc., Elkhart, Ind.) in PBS (pH 7.2) (BSA-PBS) to each well and incubating for 1 h at 37°C in a humidified box.

Human serum samples were diluted 1:5 with BSA-PBS (final serum dilution tested was 1:10). For assaying antibodies to HSV-2, one serum sample was mixed with an equal volume (100 μ l) of HSV-1-infected-cell extract diluted 1:10

with BSA-PBS, and a duplicate sample was mixed with an equal volume of uninfected-Vero cell extract diluted 1:10 with BSA-PBS. For assaying antibodies to HSV-1, one serum sample was mixed with an equal volume (100 μ l) of HSV-2-infected-cell extract diluted 1:10 with BSA-PBS, and a duplicate sample was mixed with an equal volume of uninfected-Vero cell extract diluted 1:10 with BSA-PBS. Absorptions were carried out for 30 min at room temperature.

Absorbed serum samples (uncentrifuged) were added to microtiter plate wells (50 μ l per well), and the plates were incubated for 90 min at 37°C in a humidified box. Each well was washed one time with distilled water and four times with PBS-T. To each well was then added 50 μ l of peroxidase-conjugated goat anti-human immunoglobulin (Kirkegard and Perry Laboratories, Inc., Gaithersburg, Md.) diluted 1:200 in 0.5% bovine serum albumin in PBS-T. The plates were then incubated for 1 h at 37°C in a humidified box. The wells were washed five times as above, and 100 μ l of a color-producing substrate solution [150 μ g of 2,2'-azino-di-(3-ethyl-benzylthiazoline-sulfonate) (Sigma Chemical Co.) per ml, 0.05 M sodium citrate buffer (pH 4.0), 0.03% hydrogen peroxide] was added to each well to detect bound peroxidase activity. The plates were incubated in the dark at room temperature for 30 min, and A_{414} was measured with a Titertek Multiskan spectrophotometer (Flow Laboratories, Inc., McLean, Va.).

The presence of antibodies against HSV in a 1:10 dilution of human serum was determined by measuring its absorbance index, which is the ratio of A_{414} generated by a serum absorbed with an infected-cell extract versus A_{414} generated by a serum absorbed with an uninfected-cell extract. For determining antibodies to HSV-2, the absorbance index for each serum was analyzed to determine a best-fit with MN results furnished by W. E. Rawls for the same sample. Based on this analysis, a best-fit for copositivity and conegativity (3, 8) between the screening ELISA and the reference MN test was observed at an absorbance index of 0.4. A serum was classified as antibody negative if its A_{414} was less than 0.5 after absorption with an uninfected-cell extract.

For determining antibodies to HSV-1, the data were analyzed for sera which scored negative for HSV-2 antibodies (ratio, <0.4) but generated an absorbance of 0.5 or greater against HSV-2 antigens when absorbed with an uninfected-cell extract. An absorbance of 0.5 or greater against HSV antigens (type 1 or 2) by a serum absorbed with an uninfected-cell extract indicated the presence of antibodies to HSV. Based on this analysis, sera were classified as HSV-1 antibody positive when the absorbance index at 414 nm was 0.5 or greater against HSV-1 target antigens.

To summarize, the presence of antibodies to HSV-1 and HSV-2 was determined by ELISA as follows. Serum was considered positive for antibodies to HSV-2 when the absorbance index, a/b , was ≥ 0.4 and b was ≥ 0.5 ; serum was considered negative for antibodies to HSV-2 when a/b was <0.4 or when b was <0.5 . a was defined as A_{414} generated by a serum absorbed with an HSV-1-infected-cell extract and reacted with a mixture of HSV-2 gD and gF, and b was defined as A_{414} generated by a serum absorbed with an uninfected-cell extract and reacted with a mixture of HSV-2 gD and gF. Serum was considered positive for antibodies to HSV-1 when the absorbance index, c/d , was ≥ 0.5 and d was ≥ 0.5 ; serum was considered negative for antibodies to HSV-1 when c/d was <0.5 or when d was <0.5 . c was defined as A_{414} generated by a serum absorbed with an HSV-2-infected-cell extract and reacted with a mixture of

HSV-1 gD and gC, and *d* was defined as A_{414} generated by a serum absorbed with an uninfected-cell extract and reacted with a mixture of HSV-1 gD and gC.

Statistical methods. The correlation between the ELISA and MN test results was performed with both the Pearson and the nonparametric Spearman methods. Similar results were obtained by the two methods. The tabular comparison of the ELISA and MN test results involves terms and calculations described by Buck and Gart (3).

RESULTS

Preliminary studies were carried out to delineate some of the variables associated with the ELISA. Antigen mixtures, HSV-1 gC and gD and HSV-2 gD and gF, were selected on the basis of tests in which mixtures and individual antigens were compared. Since all HSV antigens were not tested, however, it is possible that other antigen combinations would also prove effective. Preliminary studies to determine the efficiency of the absorption procedure were carried out with rabbit antisera immunized with either HSV-1 or HSV-2. Both infected-cell extracts and purified antigens (0.5 μ g) proved equally effective for absorption. The former were chosen based on cost and time required for preparation. Finally, all sera were tested at a dilution of 1:10 and 1:100. The results were essentially the same at both dilutions, although in a few cases, antibody activity was equivocal at the 1:100 dilution. The 1:10 dilution was chosen so as to maximize sensitivity.

A battery of 157 human sera was tested by ELISA, and the absorbance index was used to determine the presence or absence of antibodies to HSV-1 and HSV-2 (Table 1). A serum was classified as antibody negative if its A_{414} was less than 0.5 after absorption with uninfected cells. For determining antibodies to HSV-2, the absorbance index was compared for best-fit with MN test results for the same serum. An index of 0.4 or greater indicated the presence of antibody to HSV-2. When this criterion was applied to a battery of 276 sera, an index of overall agreement (3, 8) of ca. 91% was observed between the screening ELISA and the reference MN test (Table 2). The degree of copositivity, defined as the probability that the screening ELISA would be called positive, given that the reference MN diagnosis was positive (3), was 0.87; the degree of conegativity,

TABLE 1. Results of ELISA of human serum for antibodies against HSV-1 and HSV-2

Antibody content of serum sample (n) ^a	Results ^b			
	Test for antibody against HSV-2		Test for antibody against HSV-1	
	<i>b</i>	<i>a/b</i>	<i>d</i>	<i>c/d</i>
+/+ (37)	1.48 $\pm$ 0.26	0.66 $\pm$ 0.19	1.43 $\pm$ 0.23	0.86 $\pm$ 0.10
+/- (84)	1.29 $\pm$ 0.35	0.20 $\pm$ 0.09	1.39 $\pm$ 0.30	0.84 $\pm$ 0.11
-/+ (5)	1.57 $\pm$ 0.16	0.86 $\pm$ 0.05	1.14 $\pm$ 0.22	0.36 $\pm$ 0.06
-/- (31)	0.11 $\pm$ 0.11	NS	0.10 $\pm$ 0.07	NS

^a +/+, Antibodies to both HSV-1 and HSV-2; +/-, antibody to HSV-1 only; -/+, antibody to HSV-2 only; -/-, antibody to neither HSV-1 nor HSV-2.

^b Expressed as mean $\pm$ standard deviation. *a*, A_{414} generated by a serum absorbed with an HSV-1-infected-cell extract and reacted with a mixture of HSV-2 gD and gF; *b*, A_{414} generated by a serum absorbed with an uninfected-cell extract and reacted with a mixture of HSV-2 gD and gF; *c*, A_{414} generated by a serum absorbed with an HSV-2-infected-cell extract and reacted with a mixture of HSV-1 gC and gD; *d*, A_{414} generated by a serum absorbed with an uninfected-cell extract and reacted with a mixture of HSV-1 gC and gD; NS, not significant.

TABLE 2. Overall agreement between MN and ELISA for determining antibodies against HSV-2^a

MN result	No. of sera with the following ELISA result:	
	Positive	Negative
Positive	111	16
Negative	10	139

^a ELISA results were determined as described in the text. MN results were furnished by W. Rawls and were determined by the II/I neutralization index (20). Overall agreement was 0.91.

similarly defined, was 0.93. The correlation between the ELISA and the MN test also was evident when the values obtained in the two tests were analyzed graphically (Fig. 1). This correlation was highly significant ($P < 0.0001$).

The criteria for determining the presence of antibodies against HSV-1 were established by using a battery of 84 sera (Table 1) which, in the HSV-2 antibody test, showed absorbance indexes of less than 0.4 (HSV-2 antibody negative), yet showed A_{414} values of 0.5 or greater after absorption with uninfected-cell extracts (HSV antibody positive). When these 84 sera were tested against HSV-1 target antigens and the results were analyzed, 83 of the 84 sera (99%) were classified as HSV-1 antibody positive based on an absorbance index of

FIG. 1. Scatter diagram of results of sera analyzed for antibodies to HSV-2 by the ELISA and the MN test. The ELISA index, or absorbance index, is defined in the text. The MN index is the II/I neutralization index described by Rawls et al. (20). The broken lines at an ELISA index of 0.395 and an MN index of 0.845 represent the borders between negative and positive values for antibodies against HSV-2. The Pearson correlation coefficient (r) is 0.64; $P < 0.0001$. A number of sera could not be plotted, since specific x - y coordinates were not obtained; results of assays for these sera were recorded simply as less than or greater than. The great majority of these sera would fall in the lower left quadrant of the graph, indicating a negative diagnosis by both tests.

TABLE 3. Reproducibility of results by ELISA for determining antibodies to HSV-2

Trial no.	ELISA results ^a			
	Serum 1		Serum 2	
	<i>b</i>	<i>a/b</i>	<i>b</i>	<i>a/b</i>
1	1.581	0.32	1.759	0.83
2	1.515	0.23	1.640	0.90
3	1.300	0.19	1.389	0.94
4	1.409	0.26	1.242	0.80
5	1.332	0.31	1.415	0.89

Mean $\pm$ SD 1.43 $\pm$ 0.12 0.26 $\pm$ 0.05 1.49 $\pm$ 0.21 0.87 $\pm$ 0.06

^a Serum 1 contains antibody to HSV-1 only; serum 2 contains antibody to HSV-2 only. *a*, A_{414} generated by a serum absorbed with an HSV-1-infected-cell extract and reacted with a mixture of HSV-2 gD and gF; *b*, A_{414} generated by a serum absorbed with an uninfected-cell extract and reacted with a mixture of HSV-2 gD and gF. Coefficients of variation for *a* and *a/b* were 8 and 21% for serum 1, and 14 and 6% for serum 2.

0.5 or greater. When these same criteria were applied to the 31 sera (Table 1) which scored negative for HSV-2 antibodies and also showed A_{414} values of less than 0.5 against HSV-2 target antigens after absorption with uninfected cells (HSV antibody negative), all 31 sera scored negative for antibodies to HSV-1.

The reproducibility of the ELISA for determining antibodies against HSV-2 was confirmed by repeated testing of the same sera on different occasions (Table 3). Additional tests run on two separate occasions showed a coefficient of variation of 10% for a battery of eight HSV-1-positive, HSV-2-positive sera and 26% for a battery of nine HSV-1-positive, HSV-2-negative sera. The same degree of reproducibility was observed with the HSV-1 antibody test.

DISCUSSION

Our initial attempts to develop an ELISA were based on the assumptions that the 130,000-molecular-weight gC was antigenically specific for HSV-1 and that a test could be developed with this protein as a target antigen for detecting antibodies against HSV-1 (1, 4). These initial attempts were unsuccessful, however, since we repeatedly observed positive ELISA reactions when HSV-2-specific antisera were tested against immunoaffinity-purified gC. The reason for this discrepancy became apparent when it was shown that gC shares antigenic determinants (29, 31, 33) and maps at a position colinear (30) with the 75,000-molecular-weight glycoprotein gF (2) of HSV-2. To the best of our knowledge, a confirmed antigenically type-specific protein has not been described for either HSV-1 or HSV-2, although type-specific epitopes have been identified with monoclonal antibodies to HSV-1 (18, 24, 32) and HSV-2 (2, 13, 17, 32, 33), and type-specific antisera have been generated by absorption with heterologous viral antigens (9, 23, 25). A recent report (22) suggests that the 124,000-molecular-weight glycoprotein gG may be specific for HSV-2, since an HSV-1 counterpart has not been described.

The target antigens we employed in the ELISA are highly immunogenic glycoproteins present in viral envelopes and on the surface of infected cells (15). No attempt was made to screen all HSV-1 or HSV-2 proteins, however, and it is quite possible that other viral proteins could also serve effectively as target antigens. The protein mixtures we employed, gC and gD from HSV-1 and gD and gF from HSV-2, were selected by comparing results obtained by using individual proteins with those obtained by using protein mixtures as

target antigens. Although individual proteins could serve effectively as target antigens, the use of mixtures enhanced the sensitivity and specificity of the assay sufficiently to warrant their use.

The ELISA, as described here, required absorption of the sera for removal of cross-reactive antibodies. A relatively simple procedure with infected-cell extracts and a 30-min incubation was used for absorption. Theoretically, the absorption step could be eliminated if an antigenically type-specific viral protein was available as a target antigen or if a peptide target antigen containing one or more type-specific epitopes was available. Caution would be required with a peptide antigen, however, since any target antigen containing less than a threshold number of epitopes could reduce the sensitivity of the test below acceptable levels.

The reliability of the ELISA for detecting antibodies against HSV-2 was evidenced by the 91% index of overall agreement with the MN test. The MN test (20), in turn, was developed in conjunction with clinical isolations of HSV-2 or with clinical disease. The ELISA for HSV-1 antibodies showed a 99% index of agreement with HSV antibody-positive sera which were negative for antibodies against HSV-2. We conclude, therefore, that the ELISA described here is a reliable and sensitive test for determining the presence or absence of antibodies against HSV-1 and HSV-2 in human sera. Further, the relative simplicity of the ELISA, including the short absorption period, warrants its consideration as a routine diagnostic assay. Finally, the component parts and steps in the ELISA procedure could be readily adapted to other assay procedures.

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