Thin-Layer Immunoassay for Determination of Antibodies to Herpes Simplex Virus

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Thin-layer immunoassay (TIA) is a simple serological technique suitable for analysis of large numbers of samples. In this study, TIA was evaluated for determination of antibodies to herpes simplex virus. Herpes simplex virus antigen used in TIA was purified from material released from virus-infected cells. The results obtained by TIA were compared with those obtained by neutralization and complement fixation tests. TIA was found to be as sensitive as the neutralization test for demonstration of herpes simplex virus antibodies. No false-negative or -positive reactions were observed. In primary herpes simplex virus-1 infections, an antibody response was demonstrated by TIA, whereas antibodies could not be demonstrated in patients with primary herpes simplex virus-2 infections.

Thin-layer immunoassay (TIA) is a technically simple and accurate method for the assay of antigen-antibody reactions (1, 2). The technique is based on the principle that macromolecules may be adsorbed as a thin layer to a polystyrene surface. These macromolecules most often retain their serological reactivity and are thus capable of binding antibodies. The presence of antibodies on the antigen surface is visualized as a distinct hydrophilic condensation pattern when the surface is exposed to water vapor. TIA has been used for the determination of antibodies against purified proteins, polysaccharides, and lipopolysaccharides and has proved suitable for screening of serum samples. A quantitative TIA based on antibody diffusion in gel (DIG-TIA) has been described (1). The aim of the present study was to evaluate DIG-TIA for the serodiagnosis of herpes simplex virus (HSV) infections. A prerequisite for the application of TIA is that relatively pure antigens are available for coating of plates, and therefore a method for preparation of HSV antigen suitable for coating was developed. We have now applied TIA for determination of antibodies against HSV and have compared the results obtained with the new technique with those obtained with complement fixation (CF) (14) and neutralization (NT) (3, 11) tests as routinely performed.

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MATERIALS AND METHODS

Medium. Eagle minimum essential medium (Glasgow modification) without serum was used for maintenance of BHK-21 cell cultures.

Cells and virus. BHK-21 clone 13 was grown in 1-liter roller bottles (7, 8), for use in NT (3, 11); GMK-AH1 cells were grown in microtiter plates (7, 8). The virus strain HSV-1 F was obtained from B. Roizman, University of Chicago, Chicago, Ill. HSV-1 was inoculated onto roller bottles of BHK-21 (multiplicity of infection = 10 plaque-forming units [PFU] per cell), and the cultures were incubated at 36°C for 5 h. The medium was then discarded, and the cells were washed twice in maintenance medium. A 50-ml amount of maintenance medium was added, and the cultures were incubated until a complete cytopathic effect had developed.

Preparation of HSV antigen from culture fluid. A purification procedure described for equine infectious anemia virus antigen (4), employing treatment at low pH followed by ammonium sulfate fractionation, was adopted for preparation of HSV antigen (Fig. 1).

The BHK-21 culture fluid was centrifuged at 2,000 $\times g$ for 15 min to remove cellular debris, and the supernatant fluid was frozen at -30°C. A 2-liter amount of frozen culture fluid was thawed and then centrifuged at 10,000 $\times g$ for 1 h and at 100,000 $\times g$ for 1 h to sediment viral particles. The supernatant fluid (I) was treated by precipitation in two steps. First, the pH was adjusted to 4.2 by dropwise addition of 1.0 M acetic acid with stirring. The mixture was allowed to stand at 4°C for 15 min, then the precipitate was sedimented by centrifugation at 10,000 $\times g$ for 30 min. The pellet was suspended in a few milliliters of 0.1 M glycine-sodium hydroxide buffer (pH 8.8) and dialyzed overnight against three changes of the glycine buffer. The supernatant fluid (II) obtained after centrifugation at 10,000 $\times g$ was kept for further analysis. In a second step, ammonium sulfate (300 g/liter of supernatant fluid) was added to the acid supernatant fluid obtained after centrifugation at 10,000 $\times g$. The mixture was allowed to stand at 4°C for 1 h. The precipitate was sedimented at 10,000 $\times g$ for 30 min, and the

FIG. 1. Scheme for fractionation of HSV glycoproteins from culture fluid. NAG, *N*-acetyl-*D*-glucosamine. Roman numerals refer to the symbols used in Table 1 and in the text.

supernatant fluid was discarded. The pellet was suspended and dialyzed as described above. After dialysis, any undissolved material was removed by centrifugation at $10,000 \times g$ for 30 min. The resulting supernatant fluid (III) was applied to a column (diameter, 16 mm) containing 10 ml of wheat germ lectin Sepharose 6 MB (18) (Pharmacia Fine Chemicals, Uppsala, Sweden). The gel had been equilibrated with 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride-0.15 M NaCl-0.02% (wt/vol) $\text{Na}_2\text{S}_2\text{O}_5$ (pH 8.0) and was developed at 4 ml/h with the same buffer. When the column had been washed free from nonadsorbed material 0.2 M *N*-acetyl-*D*-glucosamine (Sigma Chemical Co., St. Louis, Mo.) was applied. The eluted fractions were analyzed for HSV antigens in counter-current electrophoresis as described elsewhere (7). Antigen-containing fractions eluted with *N*-acetyl-*D*-glucosamine (IV) were pooled and concentrated on a Diaflo ultrafilter type PM 10 (Amicon Corp., Lexington, Mass.) to a volume of 4 ml and an ultraviolet absorbance value of 2.5 (280 nm – 310 nm). The concentrated glycoprotein preparation, constituting the HSV antigen, was stored at -70°C for later use in TIA.

Quantitation of HSV antigen and protein. During the preparative procedures, precipitating HSV antigens present in the different fractions were determined by testing fourfold serial dilutions of the fractions against homologous and heterologous HSV antisera in counter-current electrophoresis (7). Protein concentration was determined as the difference in ultraviolet absorbance at 280 and 310 nm. The quo-

tient between antigen titer and protein absorbance was used as an index of virus antigen specific activity.

Sera. Antisera against HSV-1 and HSV-2 were prepared in rabbits by infection with HSV-1 and 2, respectively, as previously described (9).

A panel of 100 blood donor sera tested in CF and NT for HSV antibodies was used for the evaluation of TIA. Acute- and convalescent-phase serum samples were obtained from patients with HSV-1, HSV-2, CMV, varicella-zoster and Epstein-Barr virus (5) infections. All serum samples were inactivated and stored at -20°C until tested.

Serological tests. CF tests were performed by the method of Sever (14), with minor modifications. For CF, crude heat-inactivated tissue culture-derived HSV-1 antigen was used (8). NT was performed in microtiter plates with 100 median tissue culture-infective doses of HSV-1. The technique was described by Pauls and Dowdle (11) and modified by principles described by Grillner and Blomberg (3). All samples were tested as part of the daily routine work. Sera were tested in twofold dilutions. A titer value ≥ 2 was considered positive for both techniques.

TIA. TIA was, with minor modifications, performed as previously described (1, 2). Polystyrene petri dishes (diameter, 8.5 cm; Nunc A/S; Roskilde, Denmark) were washed with 95% ethanol and dried. Antigen coating of the inner surface of the dish was performed as follows. An 18-ml amount of 0.025 M acetate buffer-0.15 M NaCl (pH 4.4) or 0.15 M NaCl was poured into the dish, followed by 100 μl of undiluted HSV antigen to which 200 μg of ovalbumin (grade V, Sigma) had been admixed. The dish was incubated for 30 min at room temperature. The antigen solution used for coating the first dish was used for coating of five further dishes in succession. Each dish received an additional exposure to ovalbumin (20 mg/liter) dissolved in saline. After the coating, the dishes were stored with saline in a humid atmosphere at 4°C until use. The dishes were rinsed with saline and 1% (wt/vol) melted agar (Noble agar, Difco Laboratories, Detroit, Mich.) dissolved in saline containing ovalbumin (1 g/liter) was poured into each dish. The agar formed a 2.5-mm-thick layer. Wells 4 mm in diameter were punched in the solidified gel and filled with 25 μl of human serum. After diffusion for 48 h at room temperature, the agar gel was rinsed off, and rabbit antiserum against human immunoglobulin G (1.8 mg of antibody per ml; Behringwerke AG, Marburg/Lahn, West Germany) diluted 1:200 in saline was poured into the dish. The antiserum was removed after 60 min at room temperature. After washing and drying, the dish was placed upside down over a container filled with water at 56°C . After 1 min of exposure to water vapor, the dish was removed and covered. Each dish was photographed, and the diameters of the zones were measured from the photographic record (Fig. 2).

RESULTS

Purification of HSV antigen. The protein content and specific HSV antigen activity after the various purification steps are shown in Table 1. The highest specific activity was found in the glycoprotein fraction obtained after wheat germ

FIG. 2. Photograph of TIA of blood donor sera, eight with and two without antibodies to herpes simplex virus. Sites of application for negative sera are indicated by white dots.

TABLE 1. Protein content and specific activity in different fractions during preparation of HSV antigen

Antigen prepn step	Protein content by UV absorbance ^a	Specific activity (HSV antigen titer/protein concn)
Supernatant fluid after 100,000 × g (I)	680	10
Acetic acid (pH 4.2)-dialyzed precipitate (II)	250	3
Ammonium sulfate-dialyzed precipitate (III)	92	50
Glycoproteins from wheat germ lectin chromatography (IV)	10	480

^a UV, Ultraviolet.

lectin chromatography. The final yield of HSV glycoproteins was about 60% and, by means of the described procedure, a 50-fold increase in specific HSV antigen activity was achieved.

Antigenic composition of the HSV antigen (IV). The HSV antigen was tested in counter-current electrophoresis. Four precipitation lines were detected with HSV-1 antiserum, and two precipitation lines were obtained with HSV-2 antiserum. The antigen titer against HSV-1 antiserum was 1,024, and against HSV-2 antiserum it was 64. Precipitates obtained with HSV-1 antiserum were strong, whereas the precipitation lines obtained with HSV-2 antiserum were weak and could only be detected after protein staining.

Coating of TIA plates with HSV antigen. Several antigen preparations were tested for their ability to coat petri dishes. Only the purified HSV antigen preparation obtained after wheat germ lectin chromatography gave satisfactory and reproducible coating. The same antigen solution could be used to coat at least six dishes in succession. The coating property of the antigen dissolved in either acetate-buffered saline (pH 4.4) or ordinary saline was tested in preliminary experiments. Since coating at pH 4.4 gave more distinct zones than coating in saline, the former procedure was used in the following experiments.

Relation between size of TIA zone and antibody concentration. Figure 3 shows the relationship between log antibody concentration and the diameter of the TIA zone for two serum samples. Each serum dilution was tested in five replicates. A linear relationship was found between the two parameters. The regression lines and the mean diameter ± standard deviation obtained for the different dilutions are indicated in the figure. The two regression lines are parallel. In the experiment, a serial dilution factor of 3 was used, and the results indicated that a threefold difference in antibody content could be detected ($P < 0.01$).

Comparison of TIA, NT, and CF for demonstration of antibodies to HSV. A total of 100 serum samples were tested in TIA, NT, and CF for antibodies to HSV (Table 2). The area corresponding to the application well gave a hydrophilic condensation zone 4 mm in diameter

FIG. 3. Relationship between log antibody concentration and diameter of TIA zone. Regression lines for two serum samples tested in threefold dilutions. The vertical bars represent the mean ± standard deviation of five replicates. The slope values (k) of the regression lines and the correlation coefficient (r) are given in the figure.

TABLE 2. Results of TIA, NT, and CF tests for detection of antibody to herpes simplex virus in 100 blood donor sera

TIA zone diam (mm)	No. of sera with:			
	NT titer ≥ 2	NT titer < 2	CF titer ≥ 2	CF titer < 2
≥ 6	69	0	43	26
< 6	0	31	0	31

with the negative control sera. For this reason, only TIA zone diameters ≥ 6 mm were regarded as positive. In the majority of NT-positive sera, the TIA zone diameter was ≥ 10 mm. The relationship between TIA zone diameter and NT and CF titers is shown in Fig. 4a and b. There was complete agreement between TIA and NT results with regard to positive or negative results, whereas CF gave fewer positive results.

Use of TIA for diagnosis of HSV infection. Acute- and convalescent-phase sera from eight patients with HSV-1 infections were tested in TIA, NT, and CF tests. In all instances, a fourfold increase of NT as well as CF titers could be shown. When the same sera were compared in TIA, an increase of zone diameter exceeding 5 mm was noted in all instances. Paired sera from four patients with primary HSV-2 infections were also tested. Fourfold titer rises were registered when paired sera were compared in CF, whereas only low NT titers and no titer rise were noted in the same sera. No HSV antibodies were detectable by TIA in sera from patients with HSV-2 infections.

To determine whether antibodies to other viruses of the herpes group cross-react with HSV in TIA, acute- and convalescent-phase sera showing a significant titer rise in CF or immunofluorescence tests to cytomegalovirus (10 paired sera), varicella-zoster virus (5 paired sera), and to Epstein-Barr virus (3 paired sera) were tested by TIA, NT, and CF for antibodies to HSV. In none of these instances could a rise in HSV antibody titer be demonstrated.

DISCUSSION

The immunobiological importance of HSV glycoproteins has been indicated in studies in which it has been shown that antibodies against glycoprotein antigens neutralize the virus (6, 10). Antibodies to both type-common and type-specific glycoproteins are involved in neutralization of the virus (6, 12). For HSV-1, two type-specific glycoprotein antigens and for HSV-2 one type-specific antigen have been identified (15, 17). Furthermore, two type-common glycoprotein antigens have been demonstrated (15, 17).

As demonstrated by Kaplan et al. (10), culture

fluid from HSV-infected cells contains soluble, mostly type-specific HSV glycoproteins. The stability of HSV glycoproteins at low pH provided a convenient means for precipitating irrelevant proteins from the culture fluid. The concentrated and partially purified material obtained after ammonium sulfate precipitation was subjected to further purification by affinity chromatography on wheat germ lectin Sepharose (18). A tenfold increase in specific HSV antigen activity was achieved by this procedure. The antigen concentration of the solution used for

FIG. 4. Relationship between results obtained from analysis of blood donor sera by means of NT (a), CF (b), and TIA tests. The time of antibody diffusion in the TIA test was 48 h for all sera. Only sera positive in TIA and NT have been included.

the coating was found to be of importance for the appearance of positive reactions, with higher concentrations giving more distinct reactions. In preliminary tests to demonstrate HSV antibodies by TIA, the contrast between the positive reaction zones and the surrounding background areas was low due to the high wettability of the latter. The wettability of the background could be decreased by coating the plates with HSV antigen to which a small amount of ovalbumin had been added. By this procedure, an enhanced contrast was obtained.

A clearly demarcated hydrophilic condensation zone exactly corresponding to the application well was seen with negative control sera. The cause for this serologically nonspecific phenomena is presently unknown. Sera which were positive in NT and/or CF gave a zone diameter of at least 6 mm, thus well exceeding the size corresponding to the application well.

In TIA as developed for the determination of HSV antibodies, a linear relationship was found between log antibody concentration and the diameter of the hydrophilic zone. This seems to be in contrast with results obtained with the bovine serum albumin-anti-bovine serum albumin system, in which a linear relationship between log antibody concentration and area of demonstrable antigen-antibody reaction was noted except with low antibody concentrations (1). This difference is probably due to different antibody levels in the two systems, the anti-bovine serum albumin antibodies being present at higher concentration. Preliminary studies performed with a human serum with a very high content of HSV-1 antibodies indicate that the same type of relationship exists between the two parameters in both systems.

In paired acute- and convalescent-phase sera from patients with HSV-1 infection showing a titer rise against HSV in NT and CF, a significant increase in antibody concentration could also be revealed by TIA. In primary HSV-2 infections, titer rises against HSV were demonstrable by CF, but no antibody activity could be demonstrated by TIA. Negative reactions obtained with serum from HSV-2-infected patients may be explained by the finding that humans with no evidence of prior infection with HSV-1 develop mainly type-specific antibody when infected with HSV-2 (13). This might imply that the HSV-1 antigen used in TIA reveals only antibodies against type 1-specific glycoprotein antigens. However, since the antigen also contains type-common determinants, a specific reaction cannot be guaranteed until type-common determinants have been removed from the antigen preparation. Results obtained by TIA and

NT were in complete agreement with regard to the presence or absence of HSV antibodies, whereas CF performed with crude heat-inactivated HSV antigen was less sensitive for demonstration of antibodies. Zone diameters obtained in TIA showed no clear relation to NT or CF titer values except in the high range. One explanation for the discrepancy between the results obtained with TIA and those obtained by NT or CF might be that the purified antigen used in TIA represents only a fraction of all the different HSV antigens reactive in CF and NT. It should also be remembered that TIA reflects the primary antigen antibody interaction whereas, in NT and CF, secondary manifestations of the antigen antibody reactions are registered.

The relationship between HSV and other herpesviruses (an antigenic relationship has been proposed between varicella-zoster and HSV [16]) could possibly cause cross-reactions in TIA. However, with the limited number of sera tested, no such cross-reactions could be detected.

TIA has several advantages over conventional serological techniques such as NT and CF. It is technically easier to perform. It is also less time consuming than both CF and, particularly, NT. Antigen-coated plates may be stored at 4°C in a humid atmosphere for several months without notable effects on the antibody-binding properties. In conventional serological techniques, e.g., NT and CF, the quantitation of antibodies is based on testing twofold serial serum dilutions, and only fourfold changes in titer are considered to be significant. In DIG-TIA, serial dilution of the samples is not performed. Due to the higher accuracy of quantitative diffusion techniques as compared to that of serological techniques employing end point titrations, the former technique probably detects relatively small changes in antibody content. The clinical significance of registering such small changes, however, has to be established. In this study, the reactions occurring on the plate were reinforced by supplementary treatment of the plates with rabbit anti-human immunoglobulin G. By using antisera which are specific for immunoglobulin A or M antibodies, TIA might be adopted for determination of antibody activity in different classes of immunoglobulins.

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