Rapid Serological Technique for Typing Herpes Simplex Viruses

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A rapid technique is described which can accurately identify a herpes simplex virus (HSV) isolate as type 1 or type 2. Filter paper disks were used to immobilize viral antigens, which were then identified by means of an 125I-labeled staphylococcal protein A immunoassay. The assay was performed in a specially designed 96-well filtration device which served as both an incubation chamber and a filter manifold. By using this system and cross-absorbed antisera to HSV types 1 and 2, 69 coded clinical isolates of HSV were correctly and unequivocally typed. HSV was also clearly distinguished from varicella-zoster virus and cytomegalovirus. This assay can be rapidly executed (<2 h) and yielded an objective endpoint; it required only minute quantities of typing sera and can be easily performed with the cells from a single infected roller tube culture. Thus, it can be used to type initial clinical isolates of HSV, yielding results within hours after the first appearance of cytopathic effects in the culture used for primary virus isolation. Moreover, it is particularly well suited to the simultaneous analysis of many specimens and is amenable to automation. These characteristics suggest that this 125I-labeled staphylococcal protein A immunofiltration technique will be applicable to the rapid identification of other herpesviruses, as well as other clinical isolates.

A number of different serological techniques have been used to distinguish between the two types of herpes simplex virus (HSV). These include microneutralization (25, 26), fluorescent-antibody staining (10, 18, 21, 22), indirect immunoperoxidase staining (2, 11), indirect hemagglutination (3), mixed agglutination (13), solid-phase radioimmunoassay (8), enzyme-linked immunosorbent assay (20), immunoelectrosmophoresis (14), counterimmunoelectrophoresis (15), and complement-dependent cytoxicity (24). Several nonserological techniques have also been developed which identify the viruses on the basis of nucleic acid hybridization (4) or restriction enzyme analysis of virus DNA (5, 17) or on the basis of various biological and biochemical differences in virus replication (16, 19, 23).

This proliferation of techniques suggests that none is completely satisfactory. Difficulties generally stem from one or more of the following problems. (i) The techniques require passage of the primary virus isolate to obtain sufficient antigen or to obtain it in the proper form for assay; (ii) the methods of processing the HSV antigen before testing are technically demanding or time-consuming; (iii) the methods of immobilizing the HSV antigen are inefficient and time-consuming; (iv) the assay procedures require large volumes of precious typing serum to achieve unequivocal results; (v) the assay procedures require long incubation periods to obtain the required sensitivity; (vi) the assays are difficult to quantitate or are subject to observer bias.

To overcome these difficulties, we have developed a sensitive 125I-labeled staphylococcal protein A (SPA) immunoassay in which viral antigens are nonspecifically immobilized on filter paper disks in an immunofiltration manifold (6). The capacity of this technique to obviate some of the above-mentioned difficulties associated with the other assays has encouraged us to adapt it to the serotyping of HSV isolates. The results are reported here.

MATERIALS AND METHODS

Tissue culture. A strain of diploid human fibroblasts (350Q), initiated from newborn foreskin at the Virus Research Unit, Children's Hospital Medical Center, Boston, Mass., was used between passages 18 and 30. These cells were grown in an atmosphere of 5% CO2 in air in Dulbecco modified Eagle medium supplemented with 100 U of penicillin and 100 μg of streptomycin per ml (DMEM) plus 10% fetal calf serum (FCS). Stationary cultures in glass roller tubes (14 by 150 mm) were seeded with 1 × 104 to 2 × 105 cells in 1.5 ml of DMEM plus 10% FCS (DMEM10).
**Virus.** Thirty-nine clinical isolates of HSV that had been previously typed by microneutralization (26) and by DNA hybridization (4) were coded for use in this study. These had previously undergone only one or two passages in human fibroblasts. In addition, we also tested 30 primary HSV isolates by using cells from the initial cultures which had been inoculated with vesicle fluid, lesion swabs, or other clinical specimens. The MacIntyre VP-3 strain (VP-539) from the American Type Culture Collection, Rockville, Md., was used as prototype HSV type 1 (HSV-1) and the MS strain (VP-540; American Type Culture Collection) was used as prototype HSV type 2 (HSV-2). The AD-169 strain of cytomegalovirus (CMV) and the CP-5262 strain of varicella-zoster virus (VZV) from the Centers for Disease Control, Atlanta, Ga., were used as prototype CMV and VZV. All of these herpesviruses were propagated in 350Q cells. Adenovirus type 4 and coxsackievirus B6 were both isolated from clinical specimens in our laboratory and characterized by neutralization tests.

**Preparation of viral antigen.** Roller tube cultures containing approximately 4 x 10^5 350Q cells and 1.5 ml of DMEM were inoculated with 0.1 ml of untitrated virus (in the form of the clarified supernatants from primary virus isolation cultures which had been frozen at -70°C when more than half the cells exhibited cytopathic effects) or with 0.1 ml of virus transport medium containing vesicle fluid or a clinical swab. When approximately one-half of the cells displayed cytopathic effects (usually 24 to 48 h), the cells were scraped into the medium with a rubber policeman and sonicated for 2 min in the cuphorn of a Heat Systems sonicator, as previously described (6). In later experiments the tube cultures were processed by freezing them at a slant and then mixing the contents with a Vortex apparatus as they were thawing. This method produced larger fragments of cellular debris, and more antigen was trapped on the filters.

In addition, large pools of monodisperse HSV-1, HSV-2, VZV-, and CMV-infected 350Q cells, as well as uninfected 350Q cells, were prepared as previously described (6, 28) and frozen in samples at -70°C. More than 90% of the HSV-1 and HSV-2-infected cells contained viral antigens on their surface, as determined by both direct and indirect immunofluorescence (6). Each experiment included, as control antigens, aliquots from single pools of prototype HSV-1-infected, HSV-2-infected, and uninfected 350Q cells.

**Antisera.** The HSV antisera used were reference rabbit antisera to HSV-1 (MacIntyre VP-3, lot 12) and HSV-2 (MS, lot 2), which were the generous gift of K. Herrmann of the Centers for Disease Control. These sera had been prepared by inoculating rabbits with the VR-3 or MS strain of HSV, which had been grown in primary rabbit kidney cultures as described by Pauls and Dowdle (25). These sera were diluted 1/100 and then cross-absorbed with packed monodisperse 350Q cells which had been inoculated 24 h earlier with the heterologous HSV type at a multiplicity of infection of 3 PFU per cell. The absorption was performed by incubating equal volumes of diluted serum and packed cells at 37°C for 1 h and then overnight at 4°C. The supernatant fluid was clarified by centrifugation at 1,400 x g for 15 min and then at 46,900 x g for 2 h to remove virus and virus-antibody complexes.

Typing sera for CMV and VZV were human convalescent sera selected for their high titers of antibody to the homologous virus and for the relative absence of antibody to the other human herpesviruses. At the dilutions used (anti-VZV, 1/5,000; anti-CMV, 1/2,000), these sera were specific for their respective virus antigens.

**Preparation of [125I]SPA.** Purified freeze-dried SPA was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, and radioiodinated by the chloramine-T method of Hunter and Greenwood (12) as modified by Dorval et al. (7). The [125I]SPA had a specific activity (assuming 100% recovery) of 525 Ci/mmol.

**[125I]SPA immunofiltration assay.** The [125I]SPA immunofiltration assay has previously been described in detail (6, 25a). Briefly, it is performed in a specially designed 96-well microfiltration device which serves as both an incubation chamber and a filtration manifold (V&P Enterprises, San Diego, Calif.). The bottom of each of the wells contains an 0.2-ml-diameter, 0.2-mmd-thick filter paper disk (S89 Blue Ribbon, Schleicher & Schuell, Inc., Keene, N.H.) glued to a rubber policeman. Because the hole is small, surface tension prevents liquid within the well from draining through. When vacuum is applied, the liquid is quickly drawn through the filter disks and out of the wells. This efficiently removes soluble material and leaves particulate matter trapped on the filters. All 96 filters can then be rapidly washed by adding 0.3 ml of buffer to each well, using a Cornwall repeating syringe with an eight-prong dispensing manifold. After assay, the filters are conditioned for 10 min with 0.1 ml of FCS buffer (0.01 M phosphate-buffered saline, pH 7.4, containing 10% FCS, 1% bovine serum albumin, 0.3% gelatin, and 0.01% sodium azide). The vacuum is applied, removing the fluid, and 175 µl of a sonicated cell suspension from a roller tube culture infected with a clinical specimen or from an uninfected cell culture is added to each of eight wells. This corresponds to cellular debris from 20,000 to 40,000 cells per well. Cellular debris is trapped on the filters, which are washed twice with 0.3 ml of gelatin buffer (phosphate-buffered saline containing 0.3% gelatin and 0.01% sodium azide). A 50-µl portion of each cross-absorbed HSV typing serum (diluted in FCS buffer) is added to triplicate wells, and the remaining two wells receive 50 µl of control serum. After incubation at 37°C for 30 min, the unbound serum is removed by filtration, and the filters are washed three times with gelatin buffer. Next, 10^3 cpm of [125I]SPA in 50 µl of bovine serum albumin buffer (phosphate-buffered saline with 1% bovine serum albumin, 0.3% gelatin, and 0.01% sodium azide) is added to each well, and incubation is continued at 37°C for 60 min. The unbound [125I]SPA is removed by filtration, and the filters are washed three times with gelatin buffer. The filters are dried by allowing the vacuum to draw air through them for 3 min and placed in tubes, and the bound [125I]SPA is determined with a Searle model 1195 automatic gamma counter.

**Expression of results and statistical analysis.** The results were expressed as mean counts per minute of [125I]SPA bound ± standard deviation. The significance of the differences in [125I]SPA binding between control and immune sera was determined by using Student’s t test. Each value was corrected for nonspecific binding (i.e., background corrected) by subtracting the mean counts per minute of [125I]SPA bound to
TABLE 1. Effect of absorption with heterotypic HSV antigens on the type specificity of antisera to HSV-1 and HSV-2

<table>
<thead>
<tr>
<th>Virus antigen</th>
<th>Total cpm* of [125I]SPA bound with antiserum to:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HSV-1*</td>
</tr>
<tr>
<td>Before absorption</td>
<td>HSV-2*</td>
</tr>
<tr>
<td>HSV-1</td>
<td>13,171 ± 1,049</td>
</tr>
<tr>
<td>HSV-2</td>
<td>4,120 ± 629</td>
</tr>
<tr>
<td>Control</td>
<td>518 ± 12</td>
</tr>
<tr>
<td>After absorption*</td>
<td>HSV-2*</td>
</tr>
<tr>
<td>HSV-1</td>
<td>3,192 ± 107</td>
</tr>
<tr>
<td>HSV-2</td>
<td>513 ± 31</td>
</tr>
<tr>
<td>Control</td>
<td>386 ± 4</td>
</tr>
</tbody>
</table>

* Mean ± standard deviation of three replicates.

HSV-1 Control = 386 ± 4 cpm; HSV-2 Control = 3,043 ± 242 cpm; Control = 452 ± 98 cpm.

HSV-1: 1/100; HSV-2: 1/10,000; control: 1/4,000.

After absorption:

- HSV-1: 1,068 ± 16 cpm
- HSV-2: 3,043 ± 242 cpm
- Control: 452 ± 98 cpm

* Dilution at which each antiserum was used: anti-HSV-1, 1/4,000; anti-HSV-2, 1/8,000; control, 1/4,000.

** Each antiserum was first diluted 1/100 and then absorbed with an equal volume of packed cells infected with the heterologous HSV type. It was then further diluted to achieve the final dilution noted above.

RESULTS

Preparation of type-specific antisera. Preliminary experiments demonstrated that at low dilution the cross-absorbed anti-HSV-1 and anti-HSV-2 sera still possessed detectable antibodies to common HSV antigens. However, at higher dilutions these cross-reacting antibodies were diluted out, and the cross-absorbed sera became relatively type specific (Table 1). Similar results were also obtained with the anti-VZV and anti-CMV sera, which were essentially monospecific when used at high dilution (anti-VZV, 1/5,000; anti-CMV, 1/2,000). Nonspecific binding of serum and [125I]SPA to the infected and uninfected cells was low (Table 1). We have previously shown that this nonspecific binding is primarily to the filters and not to the cells (6).

** Herpesvirus identification by [125I]SPA immunoassay.** The specificity of the typing sera at higher dilutions was determined by checkerboard assays, using target cells infected with each of four human herpesviruses (HSV-1, HSV-2, CMV, and VZV) as well as with uninfected target cells. The results clearly demonstrated the specificity of each typing serum at the dilution used (Table 2).

TABLE 2. Human herpesvirus identification by [125I]SPA immunoassay: specificity of antisera to HSV-1, HSV-2, VZV, and CMV

<table>
<thead>
<tr>
<th>Virus antigen</th>
<th>Background-corrected cpm* of [125I]SPA bound with antiserum to:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HSV-1*</td>
</tr>
<tr>
<td></td>
<td>HSV-2*</td>
</tr>
<tr>
<td></td>
<td>VZV*</td>
</tr>
<tr>
<td></td>
<td>CMV*</td>
</tr>
<tr>
<td>HSV-1</td>
<td>990 ± 15</td>
</tr>
<tr>
<td>HSV-2</td>
<td>23 ± 10</td>
</tr>
<tr>
<td>VZV</td>
<td>-51 ± 37</td>
</tr>
<tr>
<td>CMV</td>
<td>21 ± 78</td>
</tr>
<tr>
<td>Control*</td>
<td>0 ± 28</td>
</tr>
</tbody>
</table>

* 350Q cells infected with HSV-1, HSV-2, VZV, or CMV or uninfected 350Q cells (control): sonicated suspension of 25,000 cells per well.

** Mean ± standard deviation of three background-corrected replicates.

* Dilution at which each antiserum was used: anti-HSV-1, 1/5,000; anti-HSV-2, 1/10,000; anti-VZV, 1/5,000; anti-CMV, 1/2,000.

* The control serum was a normal rabbit or human serum used at the same dilution as the corresponding antiserum.

** Nonspecific background binding to control (uninfected) 350Q cells with the various antisera was as follows:

- anti-HSV-1, 332 cpm
- anti-HSV-2, 378 cpm
- anti-VZV, 458 cpm
- anti-CMV, 1,308 cpm
TABLE 3. Effect of target cell number on HSV typing with the [\(^{125}\)I]SPA immunoassay

<table>
<thead>
<tr>
<th>HSV type</th>
<th>No. of infected cells(^a)</th>
<th>Background-corrected cpm(^{d}) of [(^{125})I]SPA bound with antiserum to:</th>
<th>HSV-2/HSV-1 binding ratio(^e)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HSV-1(^c)</td>
<td>HSV-2(^d)</td>
</tr>
<tr>
<td>1</td>
<td>25,000</td>
<td>1,423 ± 45</td>
<td>333 ± 58</td>
</tr>
<tr>
<td></td>
<td>12,500</td>
<td>973 ± 92</td>
<td>198 ± 28</td>
</tr>
<tr>
<td></td>
<td>6,250</td>
<td>730 ± 78</td>
<td>195 ± 20</td>
</tr>
<tr>
<td></td>
<td>3,125</td>
<td>501 ± 95</td>
<td>119 ± 22</td>
</tr>
<tr>
<td>2</td>
<td>25,000</td>
<td>102 ± 18</td>
<td>1,780 ± 363</td>
</tr>
<tr>
<td></td>
<td>12,500</td>
<td>29 ± 48</td>
<td>1,343 ± 180</td>
</tr>
<tr>
<td></td>
<td>6,250</td>
<td>23 ± 17</td>
<td>775 ± 47</td>
</tr>
<tr>
<td></td>
<td>3,125</td>
<td>16 ± 34</td>
<td>583 ± 130</td>
</tr>
</tbody>
</table>

\(^a\) The HSV antigen preparation in each well consisted of a sonically disrupted suspension of the stated number of infected cells.

\(^b\) Mean ± standard deviation of three replicates.

\(^c\) Anti-HSV-1, diluted 1/5,000.

\(^d\) Anti-HSV-2, diluted 1/10,000.

\(^e\) Background-corrected cpm of [\(^{125}\)I]SPA bound with antiserum to HSV-2/background-corrected cpm of [\(^{125}\)I]SPA bound with antiserum to HSV-1.

**Effect of target cell number on serotyping results.** To determine the effect of the amount of cell debris present on this assay, we varied the number of target cells used to create the cell debris from 3,125 to 25,000 per well. Variation in target cell number in the range tested had no effect on the capacity of the assay to distinguish between HSV-1 and HSV-2 (Table 3).

**Typing HSV isolates with cross-absorbed antisera.** Thirty-nine previously typed clinical isolates of HSV, as well as single isolates of VZV, CMV, adenovirus type 4, and coxsackievirus B6, were inoculated into duplicate tube cultures of 350Q cells. One tube culture of each isolate was harvested, and all 43 were tested under code in a single experiment, as described in Materials and Methods. The duplicate cultures were frozen at -70°C, and the typing assay was repeated again under code 2 weeks later. Twenty of the isolates were reinoculated into tissue culture and retested under code 4 weeks later. The typing results of all three experiments were identical. The isolates fell into two distinct groups consisting of 25 HSV-1 and 14 HSV-2. The HSV-1 isolates bound an average of 884 cpm of [\(^{125}\)I]SPA (background corrected) after incubation with HSV-1 antiserum and 201 cpm after incubation with HSV-2 antiserum. The mean HSV-2/HSV-1 binding ratio of the HSV-1 isolates was 0.228 (range, 0.145 to 0.400). The HSV-2 isolates bound an average of 118 cpm of [\(^{125}\)I]SPA (background corrected) after incubation with HSV-1 antiserum and 1,140 cpm after incubation with HSV-2 antiserum. The mean HSV-2/HSV-1 binding ratio was 9.7 (range, 4.8 to 15.0) (Fig. 1). These typing results agreed completely with those previously obtained by microneutralization (26) and by DNA-DNA hybridization (4) for these same isolates. The CMV, VZV, adenovirus, and coxsackievirus cultures, as well as 40 uninfected cultures, each bound less than 400 total (i.e., not background corrected) cpm of [\(^{125}\)I]SPA with either HSV antiserum.

The 30 primary HSV isolates also fell into two distinct groups consisting of 10 HSV-1 and 20...
HSV-2 (Fig. 1). As in the case of the 39 previously typed isolates, none had intermediate HSV-2/HSV-1 binding ratios.

**DISCUSSION**

This [\(^{125}\)I]SPA immunofiltration assay was previously shown to be more sensitive for the detection of antibody to HSV than were complement fixation, microneutralization, or direct or indirect fluorescent-antibody assays (6). The studies presented here indicate that the [\(^{125}\)I]SPA immunofiltration assay is rapid, sensitive, and accurate in differentiating HSV-1 from HSV-2 and show that it can also readily distinguish HSV from CMV and VZV. Each of the 39 coded HSV clinical isolates that had been previously typed by microneutralization (26) and DNA-DNA hybridization (4) was unequivocally identified and correctly typed with this [\(^{125}\)I]SPA immunofiltration assay. In addition, the 30 primary HSV isolates were clearly separated into two distinct groups with comparable HSV-2/HSV-1 binding ratios. This ability to discriminate clearly between HSV-1 and HSV-2 was made possible by the sensitivity of the [\(^{125}\)I]SPA immunofiltration assay, which permitted us to use the cross-absorbed typing sera at high dilutions at which they were essentially type specific. Furthermore, the sensitivity of this technique permits the assay to be carried out with a small number of infected cells, and this allows it to be performed directly with the primary isolate.

This assay has a number of additional advantages over other techniques for HSV typing. (i) It is rapid (<2 h); thus an isolate can be typed on the same day that cytopathic effect is recognized in the primary culture. (ii) The steps required to prepare and immobilize the viral antigens are technically simple and rapid. (iii) The assay is economical of valuable typing serum. (iv) Because it is performed in modified microtiter plates, the assay is amenable to the screening of large numbers of isolates. (v) The assay is quantitative and is not subject to observer bias. (vi) Because SPA binds to the immunoglobulins of many mammalian species, it is not necessary to prepare all typing sera in the same species. (In this study the antisera to HSV-1 and HSV-2 were from rabbits, whereas the antisera to VZV and CMV were from humans; we have also shown that this assay can be used with HSV type-specific mouse monoclonal antibodies to serotype HSV isolates [unpublished data].) (vii) The use of SPA rather than antoglobulin has a theoretical advantage in the immunodiagnosis of viruses of the herpesvirus group because these viruses induce immunoglobulin Fc receptors on the membranes of infected cells (1, 9, 27). These receptors pose a problem in standard immuno-

fluorescence assays because labeled antibody is nonspecifically bound to the virus-induced Fc receptors (9). SPA, in contrast to antoglobulins, binds to the Fc portion of certain immunoglobulins without binding to Fc receptors. Furthermore, an immunoglobulin bound by its Fc region to an Fc receptor on a herpesvirus-infected cell may not bind SPA, as it does antoglobulin, because the Fc region to which the SPA attaches may not be available. We have previously shown (6) that nonspecific binding of immunoglobulin G to HSV-1-infected cells is not a problem in this [\(^{125}\)I]SPA immunassay.

The many features enumerated above suggest that this new immunofiltration assay may be useful in the clinical laboratory for the rapid detection and differentiation of HSV-1, HSV-2, VZV, and CMV antigens. Other studies concerning the detection and identification of HSV antigens directly from vesicle fluid and other clinical specimens are presently under investigation.

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**LITERATURE CITED**


