Comparison of Biological, Biochemical, Immunological, and Immunochemical Techniques for Typing Herpes Simplex Virus Isolates

Z. M. ZHENG, D. R. MAYO, AND G. D. HSIUNG

Yale University School of Medicine, New Haven, Connecticut 06510, and Virology Laboratory, Veterans Administration Medical Center, West Haven, Connecticut 06516

Received 23 September 1982/Accepted 21 November 1982

In this study, 102 herpes simplex virus isolates were typed by cell culture selection (chicken embryo cells and guinea pig embryo cells [CE/GPE]), (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) sensitivity, plaque reduction neutralization, and indirect immunofluorescence staining techniques. The percentages of agreement between the typing methods were as follows: BVDU sensitivity versus CE/GPE, 99% (99/102); CE/GPE and BVDU sensitivity versus neutralization, 32% (33/102); CE/GPE and BVDU sensitivity versus indirect immunofluorescence staining 17% (17/102). Results were easy to interpret when the CE/GPE and BVDU sensitivity systems were used. In contrast, when type-specific antisera prepared commercially were used, results were often obscure, even contradictory, because of antibody cross-reactions. Therefore, this study suggests that immunological and immunochromatographic methods that use presently available commercially prepared antisera cannot reliably differentiate herpes simplex virus types 1 from type 2.

A variety of serological techniques, including microneutralization (20), immunofluorescent-antibody staining (5, 14), immunoperoxidase staining (2), enzyme-linked immunosorbent assay (12), indirect hemagglutination (3), mixed agglutination (7), counterimmunoelectrophoresis (9), immunoelectrophoresis (8), radioimmunoassay (19), complement-dependent cytotoxicity (16), and monoclonal antibody analysis (17) has been used to distinguish between the two types of herpes simplex virus (HSV). Other than the monoclonal antibody analysis, all procedures require special adsorption of sera to remove cross-reacting antibodies; thus, high-titer type-specific antisera are not easily obtained. Several non-serological techniques, such as nucleic acid hybridization (4) and DNA restriction endonuclease analysis (10), have also been developed. These methods are time-consuming and expensive and cannot be applied routinely in laboratories. Because of these difficulties, typing HSV type 1 (HSV-1) and HSV type 2 (HSV-2) is not commonly done in most clinical virology laboratories.

The use of biological markers for differentiating HSV-1 from HSV-2 has been described previously (15). Since HSV-1 replicates poorly in chicken embryo (CE) cells and HSV-2 is able to form plaques in CE cells, the two virus types can be easily distinguished. More recently, it was reported that differentiation of HSV-1 from HSV-2 can be made by measuring the sensitivity of the viruses to (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) (11). To assess the applications of the biological and biochemical methods for routine use in diagnostic virology laboratories, we compared neutralization (plaque reduction neutralization) and indirect immunofluorescence staining methods for typing HSV with cell culture selection and BVDU sensitivity assay methods. The results are presented in this study.

MATERIALS AND METHODS

Virus stocks and virus assay. Most of the HSV isolates were obtained from patients in the Veterans Administration Medical Center, West Haven, Conn., from 1976 through 1982. Some isolates were received from other laboratories. Virus stocks were prepared in primary rabbit kidney (RK) cells and virus infectivity titers were determined by measurement of plaque formation in guinea pig embryo (GPE) cells grown in 24-well Costar (Belco Glass, Inc., Vineland, N.J.) plates. All virus isolates were tested during the first or second passage in cell culture.

Cell culture preparation. Primary RK cells, GPE cells, and CE cells were prepared by methods previously described (6). Briefly, RK tissues were obtained from 1- to 2-month-old rabbits, and GPE tissues were taken from guinea pigs pregnant for 30 days. For RK tissues 0.25% trypsin in phosphate-buffered saline (PBS) was used, but only 0.005% trypsin in PBS was used for GPE tissues. Cell pellets were suspended at a 1:250 dilution in Eagle minimal essential medium containing Hanks balanced salt solution and 10% heat-

396
inactivated newborn donor calf serum. Cell suspensions were seeded in 16- or 32-oz. (480- or 960-ml) bottles and incubated at 35°C for 5 to 6 days. Secondary cell cultures of RK cells were used for stock virus, and GPE cells were seeded into 24-well Costar plates. 1 ml per well for the virus assay. CE cell suspensions were prepared with 0.25% trypsin in PBS from 9- to 10-day-old CEs, and cell pellets were suspended at a 1:400 dilution in minimal essential medium containing Earle balanced salt solution and 10% newborn donor calf serum. The cell suspension was seeded into 24-well Costar plates, as for GPE cells. All plates were incubated in a 5% CO₂ incubator at 35°C for 2 to 3 days, when the cell monolayers became confluent. The cells were then used for the virus assay.

Cell culture selectivity. Virus assays were performed in both CE and GPE cells. Serial 10-fold dilutions of each virus stock were prepared. Samples, 0.1 ml per well, were inoculated into Costar plates containing CE or GPE cell monolayers, at two wells per dilution as previously described (15). After 1 h of adsorption at 35°C, infected cells were overlaid with 0.5% methylcellulose in minimal essential medium containing Earle balanced salt solution and 10% newborn donor calf serum. After incubation for 72 h at 35°C in a 5% CO₂ incubator, monolayers were fixed and stained. Plaques were enumerated, and virus titers were calculated.

BVDV sensitivity assay. BVDV was kindly provided by E. De Clercq (Rega Institute for Medical Research, Leuven, Belgium). The drug was suspended in sterile PBS at a concentration of 500 µg/ml, dispersed in 1-ml portions, and stored at −70°C. The sensitivity of HSV isolates to BVDV was determined by incorporating 0.7 µg of BVDV per ml into the methylcellulose overlay medium described above for GPE cell monolayers (11). In the presence of the drug a plaque reduction of more than 2 log₁₀ indicated HSV-1, whereas no effect on the plaque-forming ability of virus isolates indicated HSV-2.

Microneutralization (plaque reduction neutralization). Antisera to HSV-1 and HSV-2 were purchased commercially. Serial 2-fold dilutions, 1:40 to 1:640, of antisera were each mixed and incubated for 1 h with an equal volume of virus suspension containing 50 to 100 PFU at room temperature. Each mixture was inoculated into two wells of GPE cell monolayers. 0.2 ml per well. After 1 h of adsorption at 35°C, monolayers were overlaid with methylcellulose. After 3 days of incubation, the medium was removed, and the cells were fixed and stained. A 4-fold or greater difference in titers between the two sera determined the specific virus type.

Indirect immunofluorescence staining. Toxoplasmosis slides (clear, microscope slides with black ceramic background, eight rings, 6-mm diameter) were cleaned with 75% alcohol, washed three times with distilled water, and then autoclaved. GPE cell suspension (1 drop) was added to each circled area; the slide was placed in a petri dish and incubated at 35°C in a 5% CO₂ incubator. When the cell monolayers were confluent, they were inoculated with HSV suspensions at a multiplicity of infection of 5. After incubation for 24 h at 35°C in a 5% CO₂ incubator, scattered foci of infected cells first became apparent; then the infected cells were washed three times with PBS and fixed in acetone for 10 min at room temperature, and stored at −20°C until used. The fixed cells were overlaid with serial 2-fold dilutions of rabbit anti-HSV-1 or anti-HSV-2 serum, incubated at 37°C in a humid chamber for 1 h, and then washed three times with PBS. Slides were then overlaid with a 1:10 dilution of fluorescein isothiocyanate-conjugated swine anti-rabbit immunoglobulin, incubated at 37°C for 1 h. washed three times with PBS, covered with buffered glycerol mounting fluid (pH 7.8), mounted with a glass cover slip, and examined under a microscope with a UV light source.

RESULTS

Comparison of four methods for typing HSV isolates. A total of 52 HSV-1 and 50 HSV-2 isolates from various sources were typed by biological (cell culture selection), biochemical (BVDV sensitivity), immunological (plaque reduction neutralization), and immunochemical (indirect immunofluorescence staining) methods. The results are shown in Table 1. As the last column shows, the percentages of agreement between typing methods were as follows:

<table>
<thead>
<tr>
<th>Source of virus isolates</th>
<th>Total no. tested</th>
<th>No. of isolates typed by indicated method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HSV-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CE/GPE</td>
</tr>
<tr>
<td>Oral-facial area</td>
<td>40</td>
<td>35</td>
</tr>
<tr>
<td>Genital area</td>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>Nonfacial area (above waist)</td>
<td>18</td>
<td>9</td>
</tr>
<tr>
<td>Nongential area (below waist)</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Unknown</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>102</td>
<td>52</td>
</tr>
</tbody>
</table>

\(^a\) NT, Neutralization.
\(^b\) IF, Indirect immunofluorescence.
\(^c\) Including finger, hand, arm, eye, ear, chest, trachea, esophagus, brain, and neck.
\(^d\) Including perineum, urine, buttocks, thigh, and skin.
BVDU sensitivity versus CE/GPE, 99% (99/102); CE/GPE and BVDU sensitivity versus neutralization, 32% (33/102); and CE/GPE and BVDU sensitivity versus indirect immunofluorescence staining, 17% (17/102). Most of the isolates from facial or above-the-waist sites were HSV-1, although HSV-1 was also isolated from the genital area. HSV-2 isolates were mostly obtained from the genital area (Table 1).

The agreement between neutralization testing and indirect immunofluorescence testing with commercially prepared antisera was 24% (8/33).

**Sensitivity of HSV-1 and HSV-2 to BVDU.** The incorporation of BVDU into overlay medium to assay 52 HSV-1 isolates resulted in a plaque reduction of more than 2 log_{10} (Fig. 1). Of 52 HSV-1 isolates which replicated poorly in CE cells, 1 showed a 1.7 log_{10} plaque reduction. Under conditions identical to those used for HSV-1 isolates, HSV-2 isolates were insensitive to BVDU. A few isolates showed a plaque reduction of 0.2 to 0.4 log_{10}. Of the 50 HSV-2 isolates which could replicate in CE cells, 2 showed a plaque reduction of 0.8 log_{10} in the presence of BVDU.

**Cross-reactions between anti-HSV-1 and anti-HSV-2 sera for typing HSV.** When commercially prepared type-specific antisera were used for neutralization and indirect immunofluorescence tests, HSV-1 isolates and most HSV-2 isolates could not be differentiated because of cross-reactions between the two sera. The anti-HSV-2 sera contained high titers of HSV-1 antibody; therefore, none of the 52 HSV-1 isolates could be typed when the results of the neutralization or indirect immunofluorescence test of isolates with the two antisera were compared (Table 1). Of the 50 HSV-2 isolates, 33 were typeable by the neutralization test (66%), and 17 were typeable by the indirect immunofluorescence test (34%). In addition, only 8 of 102 HSV isolates could be typed by both neutralization and indirect immunofluorescence methods (25 isolates were typeable only by the neutralization test, 9 isolates were typeable only by the indirect immunofluorescence test, and 60 isolates were nontypeable by either test).

**DISCUSSION**

How to type HSV has been a difficult problem to resolve. Although many investigators have tried to develop more specific serological methods, it has been questioned whether serological techniques can be used for typing HSV-1 and HSV-2 because of the antigenic homogeneity of the two types. Finding differences between the biological markers and BVDU sensitivity of HSV-1 and HSV-2 appears to resolve this problem.

In this study, 102 HSV isolates were typed by cell culture selection and BVDU sensitivity assay. One of the HSV-1 strains was not quite as sensitive to BVDU as the other HSV-1 tested, and two of the HSV-2 strains were slightly more sensitive to BVDU than the other HSV-2 strains tested. These three isolates were obtained in 1976 and 1977 and were passaged at least five times. All new isolates, passaged only one or two times, agreed completely when tested with the CE/GPE and BVDU sensitivity systems (11). An additional 100 new isolates of HSV have recently been typed by the CE/GPE and BVDU sensitivity systems, and results are in 100% agreement as to HSV types (data not shown). In addition, 100% agreement was obtained when BVDU sensitivity and nucleic acid analysis methods were compared (E. Kern, personal communication). Thus, cell culture selection or BVDU sensitivity assay can be conveniently applied for distinguishing HSV-1 from HSV-2 in case typing difficulties arise when one of the above-mentioned immunological methods is used. These non-immunological methods are simple, reliable, and economical and can be routinely used in most diagnostic virology laboratories.

Because of the close immunological relationship between antigens specified by HSV-1 and HSV-2, type-specific animal sera which are available commercially exhibit considerable cross-reactivity and may vary from lot to lot. Consequently, when quantitative differences between HSV-1 and HSV-2 microneutralization titers are compared, the serological typing of virus isolates is equivocal since these differences in titers are relatively small, often less than fourfold. Mosely et al. (13) examined material from herpetic lesions by direct immunofluorescence with commercially available antisera to HSV-1 and HSV-2. They observed that 37 of 54 (69%) specimens reacted with HSV-1 conjugate and

![FIG. 1. Plaque reduction of HSV-1 and HSV-2 in the presence of BVDU (0.7 μg/ml) in methylcellulose overlay medium. Numbers in parentheses indicate the number of strains tested.](image-url)
that 46 of 54 (85%) specimens reacted with HSV-2 conjugate, suggesting that the conjugates were not strictly type specific. In the present study, because anti-HSV-2 serum cross-reacted with HSV-1 isolates, none of the HSV-1 isolates could be typed by neutralization and indirect immunofluorescence staining methods. Anti-HSV-1 serum also cross-reacted with most HSV-2 isolates. Therefore, we consider that neutralization and indirect immunofluorescence staining methods cannot be reliably applied for differentiating HSV-1 from HSV-2 when type-specific antisera are used without specific adsorption treatment. Such specific adsorption can be expected to improve results but not as much as the described non-immunological methods.

Monoclonal antibody techniques have been recently developed for typing HSV-1 and HSV-2 (1, 17, 18). Because of the specificity of monoclonal antibodies and the antigenic complexity of the two HSV types, some monoclonal antibodies may only react with certain HSV isolates when a particular method is used, and other monoclonal antibodies may always cross-react with HSV-1 and HSV-2. Furthermore, some monoclonal antibodies may fail to react with some strains which lack the specific antigenic determinant, resulting in some typeable strains and some nontypeable strains. Therefore, pooled monoclonal antibodies for typing HSV-1 and HSV-2 in clinical laboratories may be necessary, as suggested by Pereira et al. (17).

ACKNOWLEDGMENTS

This study was partially supported by research grant HD10609 from the National Institute of Child Health and Human Development and by the Veterans Administration Research Fund.

Z.M.Z. is a Yale-China visiting fellow from the Virus Research Institute, Hubei Medical College, Wuhan, Hubei, China.

LITERATURE CITED