Rapid Detection of Herpes Simplex Virus in Clinical Specimens by Centrifugation and Immunoperoxidase Staining

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The effect of immunoperoxidase staining and centrifugation on the sensitivity and rapidity of herpes simplex virus detection in mink lung cell cultures was determined with 730 clinical specimens. In standard tube cultures, the use of immunoperoxidase staining resulted in detection of 31 (91%) of 34 positive cultures after overnight incubation, compared with 25 (74%) detected without the stain (P < 0.05). The effect of centrifugation of specimens onto the monolayer followed by overnight incubation and immunoperoxidase staining was studied with 431 specimens. Of 107 positive specimens, 103 (96%) were detected by this method, compared with 91 (85%) detected in standard cell cultures observed for 5 days (P < 0.02). Standard cell cultures that were examined after overnight incubation detected only 62 (58%) of the 107 positive specimens (P < 0.001).

Centrifugation of clinical specimens onto cell monolayers followed by overnight incubation and immunoperoxidase staining is more rapid and sensitive than are standard cell culture techniques for the laboratory diagnosis of herpes simplex virus infection.

The detection of herpes simplex virus (HSV) has become a major component of the workload in the diagnostic virology laboratory. Many of the specimens submitted for HSV culture are from pregnant women at high risk for genital infection who are monitored to prevent neonatal infection or from patients who might benefit from antiviral therapy. The optimal management of these patients requires rapid and accurate laboratory diagnosis of HSV infection.

Antigen detection methods are attractive for rapid HSV diagnosis since most assays require only a few hours to complete and do not require that an infectious virus be in the sample. Unfortunately, antigen detection systems with acceptable sensitivity and specificity for routine clinical use are not yet available. Alternative approaches which have been reported include the use of cell lines which allow earlier detection of viral cytopathic effect (CPE) (2, 9, 15) and the standard cell culture techniques followed by immunologic detection of viral antigens in the cell monolayer (3, 5–8, 10–13, 14). A recent report has also suggested that low-speed centrifugation might enhance the sensitivity of HSV cultures (4).

The Cellmatics HSV detection system (Difco Laboratories, Detroit, Mich.) combines mink lung (ML) cell cultures, which have been reported to allow detection of HSV CPE earlier than with other cell lines (15), with immunologic reagents for detection of HSV antigens in the cell monolayer. The purpose of this study was to compare the Cellmatics system with standard ML cell cultures. The Cellmatics reagents were then used to determine the effect of centrifugation combined with immunoperoxidase (IP) staining on the sensitivity and rapidity of HSV detection in ML cell cultures.

MATERIALS AND METHODS

Clinical material. The 730 samples used in this study were clinical specimens submitted to the Diagnostic Virology Laboratory between August 1984 and August 1985 for routine HSV isolation. Of these specimens, 672 (92%) were submitted on swabs; the remaining 58 were body fluids or biopsy materials. The swab specimens were generally received in transport media consisting of McCoy medium supplemented with 10% fetal bovine serum, gentamicin (20 μg/ml), vancomycin (2 μg/ml), and amphotericin B (2.5 μg/ml) or in viral culturette (American Scientific Products, McGaw Park, Ill.). Swab specimens were vortexed vigorously for 3 to 5 s, and all samples were diluted with transport media to a final volume of 2.0 to 2.5 ml before inoculation into cell cultures.

Cell cultures and media. ML cells (ATCC CCL 64 Mv 1 Lu; American Type Culture Collection, Rockville, Md.) were used at passages 40 through 130. The cells were grown in basal medium Eagle supplemented with 10% fetal bovine serum and maintained in Eagle minimum essential medium supplemented with 2% fetal bovine serum. These media also contained 2.8% sodium bicarbonate, penicillin (100 U/ml), streptomycin (50 μg/ml), and amphotericin B (2.5 μg/ml). ML cells prepared in the laboratory were grown in either screw-cap tubes (16 by 125 mm) or 1-dram (3.7 ml) shell vials.

Comparison of the Cellmatics kit with ML cell culture. ML cells were provided as confluent monolayers in flat-sided tubes as part of the Cellmatics HSV detection system. Excess media in these tubes were discarded, leaving approximately 1 ml per tube before inoculation of specimens. Clinical specimens were diluted to a final volume of 2.5 ml with viral transport media, inoculated into three Difco tubes (0.6 ml per tube), and incubated at 37°C. On each of the 3 days after inoculation, one Difco tube was fixed and stained for HSV. The IP staining method was as described below except that the tubes were fixed for 35 min before staining and 0.5 ml of reagent was incubated for 20 min at room temperature for each step of the procedure.

Standard ML cell culture. The cell culture medium in the ML tubes was replaced with 1.0 ml of fresh maintenance medium. Two tubes were inoculated with 0.2 ml of sample per tube for the comparison with the Cellmatics kit, and one tube was inoculated with 0.5 ml of sample for the comparison with shell vial cultures. Inoculated tubes were incubated at 37°C and examined daily for 5 days for CPE. Cultures with apparent HSV CPE were confirmed as HSV and serotyped...
by indirect immunofluorescence with an HSV identification and typing test (Electro-Nucleonics, Inc., Columbia, Md.).

Shell vial cultures. Specimens were inoculated into a single vial by replacing the cell culture media with 0.5 ml of the specimen. The vials were then centrifuged at 3,500 × g for 15 min at 20°C in a J-6B centrifuge (Beckman Instruments, Inc., Palo Alto, Calif.) with a JS-5.2 rotor (Beckman). After centrifugation, the vials were gently agitated to suspend debris into the transport medium. The transport medium was decanted and replaced with 1.0 ml of fresh maintenance medium, and the vials were then recapped and incubated at 37°C. For the combination of centrifugation with IP staining (C-IP method), the inoculated vials were incubated overnight, fixed, and then stained with IP as described below.

IP staining of vials. Reagents for the IP stain, including the fixative, all antisera, and the chromogen, were provided as part of the Cellmatics kit and were not altered for these studies. Cell monolayers were fixed in buffered Formalin for 15 min at room temperature and then rinsed once with phosphate-buffered saline and twice with distilled water. The primary antiserum, rabbit anti-HSV, was added (0.1 ml per vial) and incubated for 15 min at 37°C. The monolayers were then washed three times with distilled water, and the secondary antiserum, peroxidase-conjugated goat anti-rabbit immunoglobulin G, was added to each vial. After a 15-min incubation at 37°C, the cultures were again washed three times with distilled water. Acetate buffer (0.1 M) containing 0.024% hydrogen peroxide was mixed 5:1 (vol/vol) with the chromogen (4 mM 3-amino-9-ethyl carbazole and 9.4 mM 4-chloro-1-naphthol in 2:1 dimethyl sulfosuccinate-ethanol). This mixture was added to the vials and incubated at 37°C for 15 min. The monolayers were then washed three times with distilled water and examined with a light microscope at magnifications of ×40 and ×100. Samples were considered positive when foci of cells with rounding or syncytia formation were present with HSV CPE and were stained with IP. The degree of positivity of the sample was ranked 1 to 4+, corresponding to 25, 50, 75, and 100% involvement of the monolayer, to provide a subjective estimate of the number of IP-stained foci seen in the monolayer. No IP staining was seen in monolayers infected with cytomegalovirus, varicella-zoster virus, or adenovirus.

Statistical methods. Proportions were compared by McNemar's test for nonindependent proportions (1). The degree of positivity of specimens was compared by the Wilcoxon signed rank test.

RESULTS

Comparison of the Cellmatics kit with ML cell culture. The sensitivity and time to positivity of standard ML cell cultures and the Cellmatics kit were compared for 145 specimens. Of the 145 specimens, 34 (23%) were positive by standard cell cultures, and 33 (23%) were positive by the Cellmatics kit method. Although the overall sensitivity of the two methods was not significantly different, the Cellmatics kit was more rapid. After overnight incubation, standard cell cultures detected 25 positive specimens (74%), and the IP method detected 31 (91%) of the 34 positive cultures (P < 0.05).

Comparison of the C-IP method with standard ML cell culture. The C-IP method was compared with the standard ML cell cultures for 431 specimens (Table 1). Of the specimens, 107 (25%) were positive by at least one of the methods. The C-IP method, with overnight incubation, detected 103 (96%) of the 107 positive specimens, compared with 91 (85%) detected by standard cell cultures after 5 days of observation (P < 0.02). After overnight incubation, only 62 (58%) of the 107 positive specimens were detected in standard cell cultures (P < 0.001 compared with the C-IP method). The C-IP method detected HSV in 16 specimens which were negative in standard cultures, including 12 genital swabs, 3 mouth swabs, and 1 swab for which the culture site was not specified. Of these 16 specimens, 14 either came from patients with a history of genital HSV infection (eight patients) or had the virus isolate that was confirmed by cell culture passage or reisolation from the original specimen (six patients). The four specimens positive for HSV by standard cell cultures but negative by the C-IP method included a cerebrospinal fluid, a genital swab, and two swabs for which the culture site was not specified. These results indicate that the C-IP method is both more rapid and more sensitive than standard ML cell cultures.

Relative contribution of IP staining and centrifugation to the sensitivity of the C-IP method. The contribution of IP staining to the sensitivity of the C-IP method was determined for 195 specimens (Table 2). Two vials were inoculated with each specimen, one was stained after overnight incubation (C-IP method), and the other was observed daily for 5 days for the development of HSV CPE without the aid of the IP stain. HSV was detected in 54 (28%) of the 195 specimens. The C-IP method detected 52 (96%) of the 54 positive specimens, compared with 38 (70%) detected after overnight incubation (P < 0.001) and 50 (93%) detected after observation for 5 days by using centrifugation without the IP stain. Although the overall sensitivity of the shell vial cultures was not increased by the IP stain, the data on day 1 indicate that the stain contributed to more rapid detection of the CPE. This finding was examined further. All 431 cultures processed by the C-IP method were examined for HSV CPE after overnight incubation but before IP staining. Of 107 positive specimens, 103 (96%) were detected by the C-IP method, and 77 (72%) were detected by examination for CPE without IP staining (P < 0.001) (Table 3).

The contribution of centrifugation to the sensitivity of the C-IP method was examined with 149 specimens (Table 3). Each specimen was inoculated into duplicate shell vials. One

### Table 1. Detection of positive HSV cultures in 431 clinical specimens by the C-IP and ML cell culture methods

<table>
<thead>
<tr>
<th>Culture method</th>
<th>Cumulative no. of positive cultures on day postinoculation:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>C-IP cell culture</td>
<td>103&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ML culture</td>
<td>62&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total positive</td>
<td>103</td>
</tr>
</tbody>
</table>

<sup>a</sup> P < 0.001, McNemar's test.
<sup>b</sup> P < 0.02, McNemar's test.
<sup>c</sup> —, Not tested.

### Table 2. Detection of positive HSV cultures in 195 specimens by the C-IP method and centrifugation alone

<table>
<thead>
<tr>
<th>Culture method</th>
<th>Cumulative no. of positive cultures on day postinoculation:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>C-IP</td>
<td>52&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Centrifugation alone</td>
<td>38&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total positive by either</td>
<td>52</td>
</tr>
</tbody>
</table>

<sup>a</sup> P < 0.001, McNemar's test.
<sup>b</sup> —, Not tested.
TABLE 3. Detection of positive HSV cultures on day 1 postinoculation by the C-IP method, centrifugation alone, or IP staining alone in two studies

<table>
<thead>
<tr>
<th>Culture method*</th>
<th>No. positive</th>
<th>Relative sensitivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-IP</td>
<td>103*</td>
<td>96</td>
</tr>
<tr>
<td>Centrifugation alone</td>
<td>77#</td>
<td>72</td>
</tr>
<tr>
<td>C-IP</td>
<td>27</td>
<td>100</td>
</tr>
<tr>
<td>IP stain alone</td>
<td>22</td>
<td>81</td>
</tr>
</tbody>
</table>

* For C-IP and centrifugation alone, a total of 431 specimens were tested; 107 were positive by either method. For C-IP and IP stain alone, a total of 149 specimens were tested; 27 were positive by either method.

# P < 0.001, McNemar’s test.

vial was processed by the C-IP method, and the other was handled identically except that the centrifugation step was omitted. Of the 149 specimens, 27 (18%) were positive for HSV. All of the positive specimens were detected by the C-IP method, compared with 22 (81%) detected by IP staining without centrifugation. The degree of culture positivity by each method was also compared for each specimen. The centrifuged vial was judged to have more foci of infection than the uncentrifuged vial had for 22 (81%) of the 27 positive specimens. In contrast, only one specimen had more foci in the uncentrifuged vial than in the centrifuged vial (P < 0.01). Thus, both centrifugation and IP staining appeared to contribute to the sensitivity of the C-IP method.

**DISCUSSION**

This study demonstrates that the Cellmatics HSV detection system is as sensitive as and more rapid than standard ML cell cultures for the laboratory diagnosis of HSV. Furthermore, the combination of low-speed centrifugation and IP staining with the Cellmatics kit reagents is both more sensitive and rapid than standard cell culture techniques.

The C-IP method has a number of advantages for the clinical laboratory. The method requires each specimen to be read only one time, compared with daily reading for 5 to 7 days usually required for standard cell cultures. Also, the use of the IP stain facilitates the detection of HSV CPE. Although the method of quantifying the degree of positivity in this study was not strictly controlled, IP-stained monolayers usually had more identifiable foci of HSV CPE and could be read more quickly than unstained monolayers. The use of the IP stain also resulted in a 34% increase in HSV detection. The specimens which were called positive only after staining with IP were those in which only a few small foci of infection were seen or those in which the viral CPE was not sufficiently typical of HSV to be positively identified by morphology alone. Finally, the C-IP method resulted in a 13% increase in virus isolation compared with standard ML cell cultures. All of these specimens had viral CPEs consistent with those of HSV, and 14 of the 16 specimens either were from patients with a history of HSV infection or were confirmed by reisolation of the virus. These data suggest that this is a true increase in sensitivity. A potential disadvantage of the C-IP method is that HSV typing requires a separate culture, either passage of media from the C-IP vial or reisolation from the original specimen. In practice, however, this is not a major problem, since HSV typing is rarely important for patient management.

Gleaves et al. (4) have recently reported that centrifugation of clinical specimens onto MRC-5 cell monolayers, followed 16 h later by fluorescent antibody staining, results in diagnostic accuracy for HSV equal to that of standard cell culture techniques. Although the techniques used by Gleaves et al. are similar to the C-IP method, there are some important differences. The ML cell line has been reported to produce identifiable CPE more rapidly than MRC-5 cells (15). This feature of ML cells is important to the C-IP method, since both CPE and IP staining are required for the diagnosis of HSV. In contrast, Gleaves et al. used a monoclonal antibody for HSV and do not state that viral CPE was seen in all positive cultures. Until a direct comparison is made, no conclusions can be drawn about the relative sensitivity and specificity of these two methods. A potential advantage of the C-IP method is the use of IP stain, which can be read by light microscopy and so does not require specialized equipment which may not be available in all virology laboratories.

Low-speed centrifugation of clinical specimens onto the cell monolayer has not been clearly shown to increase diagnostic sensitivity for HSV. Tenser (16), using laboratory strains of the virus, reported that centrifugation at 1,100 × g results in a 10-fold increase in HSV infectivity. No significant difference was seen in the sensitivity of HSV detection in clinical specimens in this study, although the number of positive specimens studied was relatively small. There was, however, a significant increase in the degree of positivity in the specimens which had been centrifuged. Further studies with larger numbers of specimens may more clearly define the role of centrifugation in the clinical laboratory setting.

**ACKNOWLEDGMENT**

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**LITERATURE CITED**


