Detection of Genital Herpes Simplex Infections by a Tissue Culture–Fluorescent-Antibody Technique with Biotin-Avidin

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Several cell lines were evaluated for their suitability for rapid detection of herpes simplex virus (HSV) from clinical genital specimens. Human foreskin fibroblast (Flow 7000) cells were found to be most suitable in terms of sensitivity and adherence characteristics. HSV in clinical specimens was isolated by a standard tissue culture method by monitoring the cytopathic effect, and the titers of the HSV-positive specimens were determined. More than 65% of the HSV-positive genital specimens showed titers of \( \leq 10^4 \) 50% tissue culture infective doses per ml. The standard tissue culture-cytopathic effect method required 3 to 10 days for detection of HSV in clinical specimens of low infectivity. A more rapid technique was developed which involved a short-term tissue culture (24 h) on Lab-Tek chambers followed by staining with biotin-linked HSV antibody and avidin-fluorescein conjugate. Because of the high binding affinity of this system due to multiple binding of biotin to avidin and multiple attachment of biotin to the antibody molecule, the biotin-avidin fluorescent-antibody technique produced a quality of fluorescence far superior to that of the conventional fluorescent-antibody techniques. The tissue culture–biotin-avidin fluorescent-antibody method was as sensitive as the tissue culture-cytopathic effect test. This method provides an improved, more rapid test (26 h) for detecting HSV in clinical specimens.

Herpes simplex virus (HSV) genital infection in pregnancy may cause severe damage to the newborn (28). Most often, the baby acquires the infection from the mother at the time of delivery (4, 16, 20). If the infection can be diagnosed in the mother before delivery, cesarean section can be performed to avoid exposure of the child to the infected genital area. High-risk women are those who have had prior genital herpes or a sexual partner with genital herpes. These women should have vaginal or cervical cultures weekly for HSV beginning at week 34 of gestation (7). A child born to an infected mother should be examined for virus to determine whether the infection has been transmitted and should be treated. To diagnose a maternal or newborn infection requires the most rapid and sensitive diagnostic technique available. The objective of the present research was to develop a more rapid, sensitive technique for the detection of HSV in infected women and babies.

Tissue culture (TC) isolation of virus has been the most sensitive technique available to detect HSV (6, 8, 19, 22); however, it is relatively slow and may take up to 7 to 10 days for a final determination in specimens with low infectivity. In many instances, this is too long an interval in relation to pending delivery of a pregnancy or for early confirmation of fetal infection. Fluorescent-antibody (FA) (24), direct immunoperoxidase (8), and Papanicolaou or crystal violet (5) staining of the scrapings of HSV lesions have been tried, but these methods are less sensitive for detecting HSV than is TC virus isolation. The present report describes a combination of TC for 24 h and a highly specific and sensitive FA test with biotin-linked anti-HSV type 2 (HSV-2) antibody and avidin-fluorescein conjugate for the detection of HSV.

This work was presented in part at the 66th Annual Meeting of the Federation of American Societies for Experimental Biology, New Orleans, April 1982 [L. Nerurkar, A. Jacob, and D. L. Madden, Fed. Proc. 41:789, 1982].

MATERIALS AND METHODS

Collection of specimens. The specimens were collected by rubbing lesions in genital or vaginal areas with cotton swabs. The swabs were transported in 3 ml of Eagle minimum essential medium containing streptomycin, penicillin, and mycostatin (henceforth referred to as collection medium), refrigerated before transport, and frozen at −70°C if not studied immediately (22). Some of the cultures studied were obtained as
TABLE 1. Sensitivity of HSV-2 detection with different cell lines and the BA-FA method

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Sensitivity of detection (TCID&lt;sub&gt;50&lt;/sub&gt; particles/ml)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>8 h</td>
</tr>
<tr>
<td>Owl monkey kidney</td>
<td>10&lt;sup&gt;4&lt;/sup&gt; - 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>SIRC</td>
<td>10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>WI-38</td>
<td>10&lt;sup&gt;4&lt;/sup&gt; - 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>HeLa</td>
<td>10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>HEP-2</td>
<td>10&lt;sup&gt;2&lt;/sup&gt; - 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Human foreskin fibroblast (Flow 7000)</td>
<td>10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mouse embryo</td>
<td>10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vero</td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rabbit kidney</td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>African green monkey kidney</td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
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</tbody>
</table>

0.1 M NaHCO<sub>3</sub> (pH 8.2 to 8.6), adjusted to a concentration of 1 mg of protein per ml, and centrifuged at 1,000 rpm at 4°C. It was then mixed with freshly prepared biotin-succinimide ester (Vector Laboratories, Burlington, Calif.; 1 mg/ml in dimethyl sulfoxide 100:12), kept at room temperature for 4 h, and extensively dialyzed to remove unconjugated biotin ester and dimethyl sulfoxide (14). The biotin-linked antibody was then titrated and frozen in small volumes at −20°C. The working dilution of this preparation ranged between 1:20 and 1:40. The biotin-anti-HSV-2 IgG preparations were found to be stable at 4°C for 3 to 4 months. Stability studies were not conducted for longer periods of time.

Fluorescein-avidin conjugate was obtained from Vector Laboratories; each batch was titrated before use.

(ii) Staining procedure. Comparison of the standard indirect FA test and BA-FA was made initially in the pilot studies. The intensity of fluorescence was always better in BA-FA, and there was no problem of background fluorescence as seen in the standard test. Therefore, the BA-FA technique was chosen for the study.

One hundred microliters of properly diluted biotin-linked antibody was added to each well and incubated for 1 to 1.5 h at 37°C in a moist chamber. At the end of incubation, the slides were washed with PBS three times, blot dried, and incubated with properly diluted fluorescein-avidin conjugate at 37°C for 0.5 h in a moist chamber in the dark. The slides were then washed in PBS three times and counterstained with Evan’s blue (0.6 mg/100 ml), followed by a PBS wash. The soft plastic bonding material was peeled off with forceps when the slides were still moist. The slides were blotted, air dried, mounted with buffered glycerol-PBS (90:10), and viewed under a Zeiss fluorescence microscope (model III RS) illuminated with an air-cooled xenon lamp (50 W) with a 40× or 100× oil lens.

Standard TC titrations of HSV. The standard TC method was also used for comparison with the TC-BA-FA method for the detection of HSV in clinical specimens and for the titration of positive specimens. Human foreskin fibroblast monolayers (Flow 7000) were infected with 0.2-ml volumes of patient specimens. After absorption of the virus overnight, fresh Eagle minimum essential medium (complete with 2% Fetal calf serum) was added. The cultures were incubated in a 5% CO<sub>2</sub> incubator for 7 to 10 days and evaluated for cytopathic effect (CPE) daily for 5 days and on alternate days thereafter. Titers of infectivity were determined by serial 10-fold dilutions, and endpoints were calculated as 50% tissue culture infective doses (TCID<sub>50</sub>) by the method of Reed and Muench (23). Virus isolation was performed in tube cultures routinely for convenience, as the tubes were easy to maintain for long periods of time without contamination.

RESULTS

The sensitivity of several different cell lines for the detection of HSV with the BA-FA system was evaluated by using serial 10-fold dilutions of laboratory-passaged HSV (MS strain of HSV-2). The results (Table 1) indicate that,
although there were some differences in sensitivity at 8 h, all the cell lines studied gave quite comparable sensitivities after 24 h of incubation (sensitivity to approximately 10 TCID₅₀ particles of virus per ml, the highest dilution evaluated). The final choice of the cell line to be used in the routine assay was made on the basis of relative adherence to the culture vessel and intactness of the monolayers with high HSV infective doses (≥10⁴ TCID₅₀ particles per ml). With the low infective doses (<10⁴ TCID₅₀ particles per ml), cell cultures generally maintained good integrity up to 24 h. Human foreskin fibroblasts (Flow 7000) or WI-38 cell lines gave the best adherence for BA-FA testing. Other cell cultures tended to round up and slough from the monolayer. The human fibroblast line (Flow 7000) was selected for all subsequent studies.

The clinical genital HSV specimens were then tested with the standard TC-CPE method, and the sensitivity was compared with that of the TC-BA-FA method. It was possible to test only 41 of 381 negative specimens (Table 2). The titers of specimens which were found to contain HSV by the TC-CPE method were then determined by the same method. The CPE included the enlargement and rounding up of cells, the development of inclusions, and cell fusion. The TCID₅₀ titers were calculated as described previously (23) (Table 2). Of 416 specimens tested, 35 were found to be culture positive, and more than 65% of the culture-positive specimens had viral titers of ≤10⁴/ml, with a mean titer of 10⁴.8 TCID₅₀/ml. Only five specimens were from patients with primary infections, and their viral titers were relatively high compared with those obtained from patients with recurrent infections.

The comparison of the TC-CPE method with the TC-BA-FA technique with TC-grown reference HSV showed that low-titer virus cultures (<10⁴ TCID₅₀/ml) required 3 to 7 days and high-titer virus cultures (≥10⁴ TCID₅₀/ml) required 1 to 3 days to show CPE evidence of infection with the TC-CPE method. The genital cultures with titers of <10 required up to 10 days to show CPE. With the TC-BA-FA method, HSV was detected in low-titer virus cultures in 26 h and in high-titer virus cultures in 10 h. Care was taken to examine the microscope fields which had monolayers and not multilayered areas, because the latter gave false high intensity of fluorescence. At least 15 to 20 high-power fields were screened before a specimen was considered negative. The results of 24 h of incubation were considered more reliable than 8 h, as this allowed sufficient viral replication to detect small detect small amounts of virus.

The TC-BA-FA method completely correlated with the TC-CPE method for the detection of HSV in clinical specimens. With both methods, 24 positive and 37 negative specimens were detected in swab cultures taken from patients with suspected genital herpes.

The biotin linking of anti-HSV IgG or the Fab’2 fragment retained the affinity for HSV binding, unlike other procedures in which an enzyme or marker protein is conjugated with immunoglobulin (1). The biotin linking was stable for at least 3 to 5 months at 4°C, and no dissociation was noticed, as indicated by the checkerboard titration analysis performed at different time intervals. The quality of fluorescence on positive specimens was far superior to the conventional indirect FA technique (IgG-fluoresceinated anti-IgG), which was initially used for comparison. The intensity of fluorescence was distinctly bright in positive specimens, and the negative specimens were devoid of any fluorescence (Fig. 1). The clear-cut demarkation of positive and negative results is an important criterion of a good staining technique. Figure 1 includes a positive and a negative specimen studied by the TC-BA-FA method with the human fibroblast cell line. The nonfluorescing negative results indicate that avidin reagent does not bind to the fixed preparations directly. However, its usage on unfixed preparations is not yet advocated, as preliminary results have indicated that certain cell lines show some affinity for nuclear binding.

**DISCUSSION**

We observed that titers of most of the clinical genital herpes specimens ranged from <10 to 10⁷.2 TCID₅₀, with a mean of 10².8 TCID₅₀/ml. Guinan et al. (13) reported comparable mean HSV titers (10².2) in specimens collected from women with genital herpes. The wide range of HSV titers in genital specimens may have resulted from the differences in severity of infection and the manner and time of specimen collection (5, 8). The high titers of HSV in primary infections were of particular interest. A rapid and sensitive test is necessary for detection of HSV.

| TABLE 2. Quantitation of infectivity of HSV in genital herpes specimens (n = 416) |  |
|---|---|---|---|
| TCID₅₀ | No. of specimens | Primary | Recurrent |
| particles/ml | | | |
| ≥10⁴⁻¹ | 3 | 1 | 2 |
| 10⁻².1-10⁻⁴.0 | 2 | 2 | 0 |
| 10⁻⁴.1-10⁻⁶.0 | 6 | 2 | 4 |
| 10⁻⁶.1-10⁻⁸.0 | 5 | 0 | 5 |
| 10⁻⁸.1-10⁻¹⁰.0 | 2 | 0 | 2 |
| 10⁻¹⁰⁻¹-10⁻¹².0 | 4 | 0 | 4 |
| ≤10 | 13 | 0 | 13 |

*Mean titer of HSV-positive specimens = 10².8 TCID₅₀/ml.*
in specimens in maternal and newborn infections in the perinatal period, in which immediate clinical decisions must be made.

We have developed and evaluated a TC-BA-FA method for the detection of HSV infections. The method required only 26 h for completion and had sensitivity comparable to that achieved with the long-term TC-CPE method. We select-

FIG. 1. Monolayers of human foreskin fibroblasts, HSV-2 infected (A) and uninfected (B), stained with BA-FA.
ed a human fibroblast line for the test to provide good initial growth of the virus and adherence of the cells to the culture surface. This was followed by the highly sensitive and specific FA test with the BA system. This test reduced the time required for diagnosis of HSV to 24 h of growth in TC and 2 h of staining.

The choice of tissue culture cell lines for standard TC-CPE detection of HSV varies in different laboratories. Human embryonic lung fibroblasts (WI-38), primary rabbit kidney cells, and Vero cells are the more commonly used cell lines (22). Recently, guinea pig embryonic cell cultures have been shown to be equally sensitive for HSV isolation (15). With all of these cell lines, cultures of HSV must be observed for 7 to 10 days before they can be reported as negative.

Direct staining of scrapings from genital lesions is a rapid and easy method. The outcome, however, depends on the number of intact cells showing morphological changes or the presence of viral antigens or both. Hence, the proper timing and manner of specimen collection becomes very important. In most studies, these tests detect only 40 to 80% of the virus-positive specimens (5, 8, 24).

Short-term TC followed by the detection of viral antigens (TC-BA-FA) is a reliable, relatively rapid method. It permits detection of both extracellular and cell-associated infectious virus present in specimens. The method amplifies small amounts of virus present in some specimens, thus improving the sensitivity of the test. TC in combination with either immunological staining or electron microscopy has been used in the past for detection of HSV (10, 17, 18) or other viruses (9, 25, 26). These studies have used conventional methods, employing direct or indirect FA techniques with fluorescent or peroxidase labels.

We believe this is the first report of the use of the BA-FA method for the detection of viral antigens (L. Nerurkar, A. Jacob, and D. L. Madden, Fed. Proc. 41:789, 1982). This system should be able to improve the detection of other viral antigens and antibodies if the proper choice of reagents is made (12). There has been a preliminary report describing the use of this reagent in the detection of antibodies to hepatitis B antigen (C. Kendall, I. Ionescu-Matiu, and G. R. Dreesman, Fed. Proc. 41:789, 1982). The high affinity of binding of biotin to avidin, multiple binding of biotin to antibody, multiple binding of avidin to individual biotin molecules, which is almost irreversible (3, 11), and ease of preparation of reagents may eventually cause this system to replace the use of antoglobulin conjugates in the conventional indirect staining procedures. The combination of TC followed by staining with the BA system seems to reduce both false-positive and false-negative results, which makes the detection more reliable as compared with the direct tissue staining procedures previously described (8, 19). Furthermore, it appears to minimize or eliminate interference by Fc receptors binding to the Fc portion of the antibody (2, 21, 27), which may give some false-positive results.

Emphasis in the future will have to be on further shortening the time required to accurately diagnose HSV infections. In the meantime, this method appears to be particularly valuable for clinical situations which require the rapid detection of HSV.

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