

Comparison of In Situ Hybridization and Immunologic Staining with Cytopathology for Detection and Identification of Herpes Simplex Virus Infection in Cultured Cells

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Two recently developed sensitive techniques, in situ hybridization with a biotinylated cloned DNA probe and an avidin-biotin complex immunoperoxidase assay, were compared with the appearance of cytopathic changes for the early detection of herpes simplex virus infection in cell culture. By using commercially made reagents, these detection methods were evaluated in two different cell culture systems inoculated with both high- and low-input multiplicity of virus. The results revealed that both viral antigen and viral DNA detection methods could shorten the time to diagnosis of herpes simplex virus infection in cell culture; however, these methods were most useful in specimens containing low titers of virus when a less sensitive cell system was used. In this study, the avidin-biotin immunoperoxidase method was more sensitive and much cheaper than hybridization with a biotinylated probe. Significantly, when a highly sensitive cell system was used, cytopathic changes alone were comparable in rapidity and sensitivity to viral antigen or DNA detection methods applied in a less sensitive cell system.

Rapid diagnosis of herpes simplex virus (HSV) infections has become increasingly important. In many situations, clinical criteria alone are not adequate and laboratory confirmation is essential. To date, isolation of HSV in cell culture remains the most sensitive method of diagnosis; however, the rapidity and sensitivity depend on the type of cell culture used as well as on the amount of virus present in the clinical specimen (9). To shorten the time to reporting results, sensitive viral antigen detection methods have been applied to infected cell cultures to detect the presence of virus even before viral cytopathic effects (CPE) are visible (3, 10). The recent availability of nonradioactive viral DNA probes from commercial sources has provided a potent new tool for diagnostic virologists (4, 5).

To develop and define a more sensitive method for the early detection of HSV infection, a sequential quantitative study was undertaken to detect viral antigen or viral DNA sequences in cultured cells infected with HSV type 1 (HSV-1) by using commercially made reagents. Two different cell systems, guinea pig embryo (GPE) and MRC-5 cells, were examined by using these two rapid detection methods, and the results were compared with the appearance of cytopathic changes in the infected cells.

MATERIALS AND METHODS

Virus stock. HSV-1 strain 1745 isolated in our laboratory was used in this study and has been used in various studies over the last few years (8, 11). Virus stock was prepared by infection of confluent monolayers of GPE cells at an input multiplicity of 0.001 as previously described (8).

Cell cultures. GPE cell cultures were prepared from Hartley guinea pig embryos at 30 to 40 days gestation. GPE cells at passage 1 to 3 were seeded into 24-well plastic panels (Costar, Cambridge, Mass.). MRC-5 cells were purchased from MA Bioproducts, Walkersville, Md., at passage level

23. For this study, cells at passage level 26 to 29 were seeded into 24-well panels. Cell cultures grown in panels instead of on cover slips were used in this study to avoid the tedious cover slip preparation and manipulation involved in processing large numbers of samples.

Virus infection. Confluent monolayers of GPE or MRC-5 cells were inoculated with HSV-1 at either high or low multiplicities of infection (MOI) (0.5 to 1.0 or 0.0005 to 0.0001 PFU per cell, respectively). After 2 h of adsorption at 37°C, the inocula were removed and cultures were washed three times with Hanks balanced salt solution. Maintenance media were replaced, and cultures were incubated at 37°C in a CO₂ incubator.

Virus infectivity assay. At 2- to 4-h intervals, duplicate samples of infected GPE and MRC-5 cells in culture medium were frozen at -70°C until assay. All samples were assayed at the same time in 24-well panels with GPE monolayers by the plaque formation method (6).

Fixation of monolayers for viral antigen or DNA detection. At designated time intervals after infection, monolayers in 24-well panels were examined for viral CPE, washed in phosphate-buffered saline (PBS), and then fixed in freshly prepared 4% *para*-formaldehyde for 10 min. After being fixed, cultures were rinsed in PBS and then treated with 0.25% Triton X-100 containing 0.24% saponin in PBS for 10 min at room temperature. Monolayers were again rinsed in PBS, placed in 20% glycerol-PBS, and frozen at -70°C until staining.

ABC-IP staining for viral antigen detection. Frozen samples of infected GPE and MRC-5 monolayers in 24-well panels of the same input multiplicity and time after infection were thawed at 37°C, rinsed three times with PBS, and assayed according to the procedure outlined in the Vectastain ABC (avidin-biotin complex immunoperoxidase [ABC-IP]) kit from Vector Laboratories, Inc., Burlingame, Calif. Polyclonal HSV-1 rabbit immune serum (Dako Corp., Santa Barbara, Calif.) at a 1:500 dilution was used. HSV-

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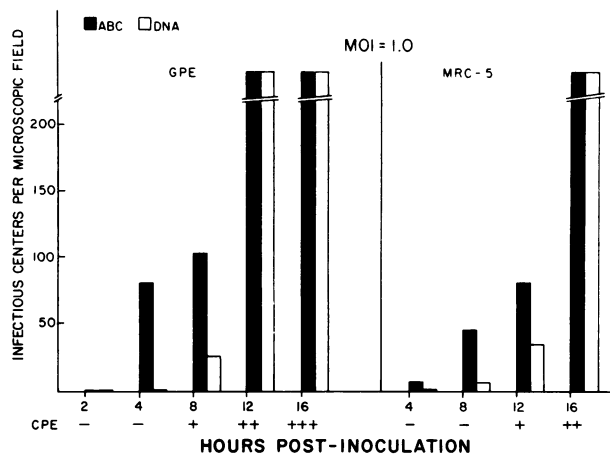


FIG. 1. Detection of infectious centers by ABC-IP (ABC) and in situ DNA-DNA hybridization (DNA) in GPE and MRC-5 cells inoculated with herpes simplex virus at a high-input MOI (MOI = 1.0). Monolayers were first examined for viral CPE at various time points p.i. Five to six monolayers were then examined by ABC and two to four were examined by DNA at each time point. Results are expressed as infectious centers detected per microscopic field (magnification, $\times 20$).

positive cells were identified by the presence of a brown precipitate. The positive cells were counted per high power field at high-input MOI or as the number of foci of positive cells per well at low-input MOI. For controls, HSV-infected wells were incubated with nonimmune rabbit serum at 1:500 dilution instead of immune serum, and the staining procedure was carried out as above. In addition, uninfected monolayers were assayed as above with HSV-1 immune serum.

In situ hybridization with biotinylated HSV DNA probe. Replicate cell monolayers on the same 24-well panels as those assayed by ABC-IP staining were also tested by in situ hybridization. Monolayers were washed three times in PBS and treated for 2 min at room temperature with 0.1 mg of autodigested pronase per ml (Calbiochem-Behring, La Jolla, Calif.) in Tris hydrochloride buffer, pH 7.5, with 5 mM EDTA (1). Immediately after this proteolytic treatment, cell monolayers were washed twice in PBS with 2 mg of glycine per ml, dehydrated through graded ethanol, and air dried. The original Enzo Bio-probe (Enzo Biochem, New York, N.Y.) protocol for in situ hybridization was followed. In each well of a 24-well panel tested, 10 μ l of biotinylated HSV probe (Enzo Biochem) in hybridization mix was added and covered with a 15-mm-diameter round siliconized glass cover slip (Bellco Glass, Inc., Vineland, N.J.). The panel was placed on a rack above a water bath for 10 min where the temperature was recorded at 85°C. After denaturation, the panel was placed in a humidified 37°C incubator overnight. After hybridization, the cover slips were removed by immersion in 2 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer; monolayers were then washed in 2 \times SSC for 5 min at room temperature, followed by 0.1 \times SSC for 10 min at 65°C and then 2 \times SSC for 5 min. Monolayers were then covered with 0.1% Triton X-100 in PBS and rinsed for 5 min. The solution was then drawn off, and the detection complex (streptavidin-biotinylated horseradish peroxidase) was added. After a 30-min incubation in a moist chamber at 37°C, panels were washed in 2 \times SSC for 5 min at room temperature, followed by 0.1% Triton X-100 in PBS for 2 min and then PBS for 3 min. The substrate (diaminobenzi-

dine hydrochloride) solution was applied, and the reaction was read as described for the ABC-IP procedure.

Controls consisted of hybridization of HSV probe with uninfected monolayers as well as hybridization of biotinylated simian virus 40 DNA probe (Enzo Biochem) to HSV-infected monolayers. Hybridizations were also performed with monolayers grown on 12-mm-diameter round glass cover slips pretreated with poly-D-lysine. Cover slips were placed in the wells of 24-well panels, seeded with cells, infected, and then fixed at various times postinfection (p.i.). Hybridizations were performed on infected monolayers on cover slips both in the panels and after transferral of cover slips to glass slides. The results were compared with those obtained from hybridizations done in panels without cover slips.

Statistical analysis. Statistical analysis was performed by using the unpaired *t* test, the Wilcoxon nonparametric test, and the chi-square test.

RESULTS

Comparison of three methods for detection of HSV infection: CPE, ABC-IP, and in situ hybridization. At a high MOI (0.5 to 1.0), viral CPE characterized by scattered rounded cells was first noted in GPE cells at 8 h p.i.; however, CPE considered reportable in a clinical laboratory was not evident until 12 h p.i. Viral antigens as detected by ABC-IP were readily apparent in infected GPE cells at 4 h p.i., and viral DNA was first detected at 8 h p.i. (Fig. 1). The majority of cells stained positively in GPE monolayers by both techniques at 12 h p.i. In MRC-5 cells, single rounded cells were first noted 12 h p.i., with reportable CPE evident 4 h later. Viral antigens were detected 4 h p.i. and viral DNA was detected 8 h p.i. in MRC-5 cells, with the majority of cells staining positively by both techniques at 16 h p.i. At the earlier time points, fewer cells stained positively by DNA hybridization than by ABC-IP. The differences between DNA and ABC-IP in both cell systems were significant when evaluated by the unpaired *t* test ($P < 0.01$ to $P < 0.05$), and showed a trend toward significance when the nonparametric test was applied ($P < 0.05$ to $P = 0.15$).

At low MOI (0.0005 to 0.0001), 23 of 24 wells (96%) of infected GPE cells examined had detectable foci of CPE at 16 h p.i., and by 20 h, all 24 wells tested were positive (Fig. 2). Positively stained cells in GPE cells were evident by ABC-IP as early as 12 h p.i. and by DNA hybridization at 16 h p.i. By 40 h, most GPE cells stained positively by both methods. In contrast, only 1 of 24 (4%) infected MRC-5 wells had detectable HSV CPE at 16 h p.i., and even at 40 h p.i., only 21 of 24 wells (88%) were positive. In MRC-5 cells, both ABC-IP and DNA hybridization first detected HSV-infected cells at 16 h p.i., but the number of positive cells detected by ABC-IP was again higher. Statistical analysis of differences between DNA and ABC-IP at the time points shown gave *P* values of < 0.01 to 0.3 by the unpaired *t* test but values of 0.15 to 0.45 by the nonparametric test due to the small numbers involved.

Comparison of two cell systems, GPE and MRC-5, for rapid detection of HSV infection. When GPE and MRC-5 cells were compared (Table 1), the greatest differences in time to first detection of HSV infection were seen in detection of viral CPE. At low MOI in particular, there was a significant delay in the onset of viral CPE in MRC-5 compared with that in GPE cells (Fig. 2; $P < 0.001$, chi-square test). To examine this further, HSV-infected GPE and MRC-5 monolayer cultures were taken at successive time points p.i. and

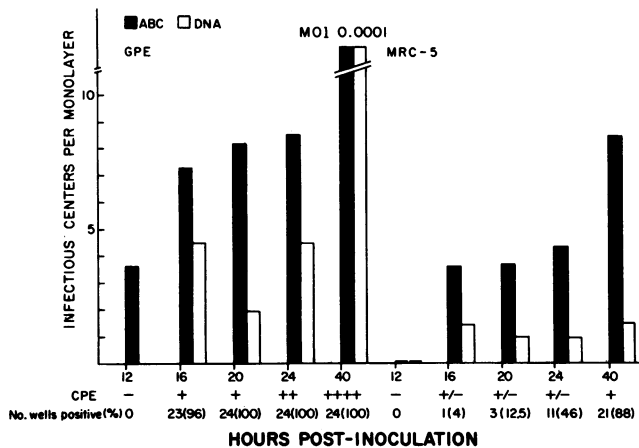


FIG. 2. Detection of infectious centers by ABC-IP (ABC) and in situ DNA-DNA hybridization (DNA) in GPE and MRC-5 cells inoculated with HSV at a low-input MOI (MOI = 0.0001). Monolayers in 24-well panels were examined for CPE at various times p.i., and the number of positive wells was determined. Five to six wells were then examined by ABC-IP and two to four wells were examined by DNA at each time point, and the number of infectious centers detected per monolayer was enumerated.

assayed for virus infectivity. Release of infectious virus occurred 8 to 12 h earlier in GPE cells than in MRC-5 cells at both high and low MOI and tended to correlate with the appearance of viral CPE (Fig. 3). Application of viral antigen and viral DNA detection methods at 4-h intervals reduced the differences between GPE and MRC-5 cells in time to first detection of viral CPE. Positive cells were first detected in both GPE and MRC-5 cells by ABC-IP (high MOI) or DNA hybridization (high and low MOI) at the same time (Table 1). However, the number of infectious centers detected per well at each time point was greater in GPE cells compared with MRC-5 cells at almost all time points tested. Of note, at low MOI, detection of CPE alone in GPE cells was equivalent to the application of viral antigen and DNA detection methods in a less sensitive cell system.

DISCUSSION

With the recognition of serious HSV infections and the availability of effective antiviral agents has come the need for development of more rapid and sensitive diagnostic tests. Conventional viral diagnosis has been based on recognition of characteristic viral CPE for different virus groups as well as selective cell susceptibility (6). Recent advances in technology have resulted in more sensitive antigen detection techniques, such as the ABC-IP method (2, 7). In addition, the development of nonradioactive cloned nucleic acid probes has allowed the tools of the molecular biologist to be applied to viral diagnosis (1, 4, 5). The application of these

TABLE 1. Comparison of three methods for detection of HSV infection in two different cell systems at high and low MOI

Cell system	First detection of HSV infection (h p.i.) with MOI of:					
	1.0			0.0001		
	CPE	ABC-IP	DNA ^a	CPE	ABC-IP	DNA
GPE	8-12	4	8	16	12	16
MRC-5	12-16	4	8	24-48	16	16

^a DNA, In situ hybridization with biotinylated DNA probe.

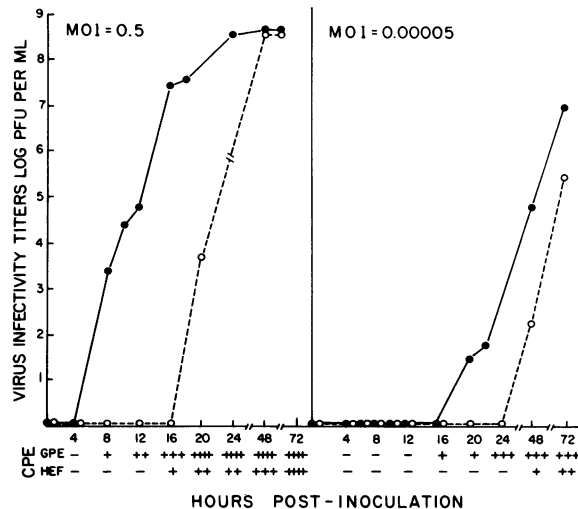


FIG. 3. Growth curves of HSV-1 at high- and low-input MOI in two different cell culture systems: GPE (●) and MRC-5, a human embryonic lung fibroblast cell strain (HEF, ○). Virus infectivity titers are expressed as log₁₀ PFU per milliliter.

new viral antigen or DNA detection methods to infected cell cultures should provide more rapid and specific results without sacrificing sensitivity.

In the present study, we compared two recently developed sensitive techniques for the early detection of HSV in cell cultures and, in addition, attempted to correlate the results with two important variables, the cell culture system used and the input multiplicity of virus. In this study, both ABC-IP and in situ hybridization provided an earlier, more specific diagnosis of HSV infection in cell culture. At both high and low MOI and in both cell culture systems examined, however, positively stained cells were noted later or were less numerous (or both) when detected by DNA hybridization than when detected by ABC-IP (Table 1). In additional experiments in our laboratory with GPE cells alone and a broader range of input MOI (data not shown), the greater sensitivity of ABC-IP was repeatedly demonstrated. In addition to being less sensitive, in situ hybridization also produced less consistent results. For example, the number of infectious centers detected by DNA hybridization unexpectedly decreased at 20 h (Fig. 2). This variability was noted repeatedly at other time points in different experiments and was not seen with ABC-IP. To ensure that the decreased sensitivity and sometimes variable results seen with hybridization were not caused by the use of 24-well panels, hybridization with infected monolayers on cover slips was compared with hybridization with monolayers in panels, and no difference was found (data not shown). Hybridization was routinely carried out overnight to allow maximal time for reannealing to occur but was still not as sensitive as ABC-IP. In addition, Carnoy fixative was compared with *para*-formaldehyde, but hybridization was not increased. It is not known whether denaturation of DNA in sample and probe was complete under the conditions recommended or whether other potential changes in the hybridization protocol could result in increased sensitivity.

In a previous report, we found that GPE cells were equal to primary rabbit kidney cells in sensitivity to HSV and that both were superior to the three different human fibroblast cell strains tested, including MRC-5 cells (9). Recently, we have pursued this work further and found that the differ-

ences in sensitivity were most apparent when cell cultures were inoculated with clinical specimens containing low-infectivity titers of HSV (L. S. Zhao, E. S. Balkovic, M. L. Landry, and G. D. Hsiung, *Abstr. Annu. Meet. Am. Soc. Microbiol.* 1986, C-52, p. 336). The present study also found a significant delay in onset of CPE in less sensitive cell cultures at low MOI; however, the application of viral antigen or DNA detection methods lessened the differences between cell systems.

From the results of the present study, it is apparent that viral antigen and DNA detection methods can shorten the time to diagnosis of HSV infection in cell culture. In our hands, the ABC-IP method was technically simpler, more sensitive, more consistent, and much cheaper than in situ hybridization with a biotinylated probe (reagent cost per test: \$0.30 for ABC-IP versus \$3.38 for Enzo Bio-probe). Commercial kits with cell culture inoculation followed by IP staining with HSV antibody are available. However, a variety of cell culture systems are available in kits from different suppliers. The comparative sensitivity of the cell cultures used and the optimum time for staining after inoculation of clinical specimens is the subject of current studies in our laboratory. From data presented in the present report, viral antigen and DNA detection methods appear to be most useful in specimens containing low titers of virus, especially when less sensitive cell systems, such as MRC-5, are used. When highly sensitive cell systems are used, such as GPE or rabbit kidney cells, CPE alone may be equivalent in rapidity and sensitivity to viral antigen or DNA detection methods applied in less sensitive cell systems.

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