Detection of Herpes Simplex Virus Infection of Female Genitalia by the Peroxidase-Antiperoxidase Method Alone or in Conjunction with the Papanicolaou Stain

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The sensitive peroxidase-antiperoxidase (PXAPX) method individually and in conjunction with the Papanicolaou (PAP) stain was used to detect herpes simplex virus (HSV) in specimens from human female genitalia. Initial studies using a model system of HSV-1- or HSV-2-infected Vero cells established (i) acetone as the most effective fixative, (ii) optimal dilutions of preimmunization and anti-HSV serum for differentiation of infected from noninfected cells, (iii) optimal concentration of 3,3′-diaminobenzidine tetrahydrochloride and H₂O₂ for maximal staining of infected cells with minimal background reaction, and (iv) removal of endogenous peroxidase with absolute MeOH. These various parameters, once established, were utilized in the PXAPX or PXAPX-PAP on human specimens from the vulva or cervix. In these specimens, examined by standard light microscopy, PXAPX-positive cells were dark brown with a single nucleus or multiple nuclei. By coupling the PAP to the PXAPX, detailed nuclear observations of PXAPX-positive cells were possible and revealed nuclear changes consistent with HSV infection, including syncytium formation, chromatin condensation, and an occasional Cowdry type A inclusion. The PXAPX and PXAPX-PAP correlated (r = 0.742) over a period of 72 h with HSV isolation from these samples.

Herpes simplex virus types 1 and 2 (HSV-1, HSV-2) are ubiquitous human pathogens responsible for a variety of affections. Indeed, these viruses bring about disease states of mucous membranes, eyes, skin, nervous system, and genitalia (14). Infections by HSV-1 or HSV-2 may be primary or recurrent; that is, after the initial infection both virus types have the ability to remain latent within the host and, when conditions are favorable, to cause recurring lesions (6). This cycle may be repeated indefinitely at regular or irregular intervals and serves as an excellent source of infection for the unwitting contact.

The need for sensitive and accurate laboratory diagnostic methods for HSV detection are prompted by several factors, including: (i) frequency in the population (15, 20); (ii) association with venereal disease (11, 13, 26); (iii) association with perinatal infections (16); (iv) possible association with cervical cancer (12, 18); and (v) recent antiviral chemotherapy successes realized with adenine arabinoside treatment of HSV encephalitis (25). Accordingly, we have used the sensitive peroxidase-antiperoxidase (PXAPX) (21) method to detect HSV infections in human female genitalia. Our results demonstrate that this system is inexpensive, sensitive, and accurate, provides a permanent record, utilizes standard light microscopy, and readily interfaces with other staining techniques such as the Papanicolaou (PAP) (17), thereby increasing the parameters by which a cell may be judged infected.

MATERIALS AND METHODS

Virus. Virus stocks of HSV-1 (strain 69–85) and HSV-2 (strain 316-D) were obtained from F. Rapp, Department of Microbiology, M. S. Hershey Medical Center, Hershey, Pa. Working virus pools were prepared in primary rabbit kidney cultures or Vero cells, and virus titers were determined by the plaque assay (7).

Cells. Vero cells were obtained from the American Type Culture Collection (Rockville, Md.) and maintained in medium 199 supplemented with 5% fetal calf serum, 0.075% NaHCO₃, 10 μg of streptomycin per ml, and 100 U of penicillin per ml. Primary rabbit kidney cultures were prepared from kidneys of 4-week-old New Zealand White rabbits and maintained in the same medium as Vero cells but with 10% fetal calf serum.

Antisera. New Zealand White rabbits were prebled and sera were recovered before the initiation of immunization. Antisera to HSV-1 or HSV-2 were pre-
pared by infecting primary rabbit kidney cells with either virus type at a multiplicity of infection of 1. After 24 or 48 h, when extensive virus cytopathology had developed, the infected cells were scraped from the tissue culture bottle and sonicated to release cell-associated virus. After low-speed centrifugation (1,000 × g) to remove cellular debris, the supernatant fluid containing the virus was placed on 15 to 60% discontinuous sucrose gradients and centrifuged for 2 h at 100,000 × g (19). The partially purified, visible virus band was recovered, tested for virus infectivity on monolayers of rabbit kidney cultures, and injected subcutaneously and intramuscularly into New Zealand White rabbits. The rabbits were injected with fresh virus preparations 30 days later and exsanguinated within 10 to 21 days. The sera were tested for virus neutralizing activity by the plaque reduction test with both HSV-1 and HSV-2. Neutralizing titers were expressed as the reciprocal of the highest dilution of serum that neutralized 50% of 2,000 plaque-forming units of HSV-1 or HSV-2 in 30 min at 22°C. The anti-HSV serum selected for this study had a neutralizing titer of 1:80 for both HSV-1 and HSV-2 and did not cross-react with cells infected by Epstein-Barr virus, cytomegalovirus, varicella-zoster virus, or adenovirus type 7 or 12. In addition, this serum did not react with HEP-2 cells, human embryonic lung cells, hamster embryo cells, rat embryo cells, or Vero cells.

Other rabbit antisera used in specificity studies were as follows: (i) rabbit anti-Streptococcus bovis and rabbit anti-Streptococcus faecalis, J. Fazur, Department of Biochemistry and Biophysics, The Pennsylvania State University; University Park; (ii) rabbit anti-Vibrio cholerae (Ogawa), rabbit anti-V. cholerae (Inaba), W. Cegielski, Department of Microbiology and Cell Biology, The Pennsylvania State University; (iii) rabbit anti-bovine serum albumin and rabbit anti-human immunoglobulin G, S. Gilman, Department of Microbiology and Cell Biology, The Pennsylvania State University; (iv) rabbit anti-gypsy moth baculovirus and rabbit anti-Autographa baculovirus, W. McCarthy, Pesticides Research Laboratory, The Pennsylvania State University; (v) rabbit anti-adenovirus 12 and rabbit anti-simian adenovirus 7, F. Rapp, Department of Microbiology, M. S. Hershey Medical Center, The Pennsylvania State University, Hershey, Pa.

**Model systems.** Initial studies utilized a model system of HSV-1- or HSV-2-infected Vero cells on cover slips to assist in establishing optimum conditions for PXAPX detection of HSV antigens in cells. Vero cells (~75% confluent) on cover slips (11 by 22 mm) were infected with HSV-1 or HSV-2 at a multiplicity of infection of 0.01 so that by 24 h after infection discrete foci of infected cells surrounded by uninfected cells were evident. This procedure also resulted in asynchronously infected cells within the foci so that many stages of infection from early to late were present.

**Specimen collection and processing.** All specimens were collected from female patients ranging in age from 18 to 22 years. Specimens from an herpetiform lesion(s) of the genital area were obtained from these patients, whose chief complaints were moderate to severe pain at the site of the lesion(s) with intense itching. The herpetic lesions appeared as clusters of vesicles, tender shallow red ulcers, or dry crusted ulcers depending on the state of infection.

Samples were vigorously collected from all lesions or inflamed areas with a sterile cotton swab. The swab containing cells and virus was placed into a tube containing 5 ml of sterile transport medium (medium 199 containing 0.075% NaHCO3, 10 µg of streptomycin per ml, 100 U of penicillin per ml). The sample was processed within 60 min of collection from the patient unless otherwise specified.

**Virus isolation.** Upon receipt in the laboratory, the specimen was agitated on a Vortex mixer, and 1 ml of the sample was placed on Vero cells in 29.6-ml glass tissue culture bottles. After adsorption for 1 h at room temperature, the inoculum was removed and cell maintenance medium, further supplemented with 100 U of mycostatin per ml, was added to the cells. The cultures were incubated at 37°C and monitored daily for cytopathic effects (CPE) consistent with HSV (i.e., cell rounding, cytoplasmic stranding, syncytium formation, chromatin clumping). If no CPE developed within 4 days, the sample was considered negative for HSV. Those cultures developing HSV CPE were designated as presumptive positives, frozen (~70°C), and eventually confirmed as HSV by a neutralization test.

**Virus identification.** Virus isolated from human specimens on Vero cells was tested for its ability to be neutralized by HSV antiserum. Two samples of 0.2 ml each were obtained from each isolate that previously developed HSV CPE and were added to tubes containing an equal amount of a 1:4 dilution of rabbit preimmunization serum or a 1:4 dilution of rabbit anti-HSV serum. After 60 min at 22°C, the contents of each tube were placed on Vero cells, and after a 1-h adsorption period, maintenance medium was added. These cultures were monitored daily for 4 days for HSV CPE. If during this period HSV CPE developed in the preimmunization serum culture but not in the immune serum culture, the sample was designated as positive for HSV. No attempt was made to type the virus as HSV-1 or HSV-2.

**Cell preparation.** The human specimens were agitated on a Vortex mixer and passed through an 18-gauge needle 10 times to disperse cell clumps. A volume of 0.2 ml was placed in a cytospin centrifuge chamber, and the chamber was placed in a Cytospin centrifuge (Shandon Southern Instruments, Sewickley, Pa.) and spun at 600 rpm for 5 min to deposit the cells on a glass slide. The specimens were air dried for 60 s (further drying produced artifacts) and fixed in acetone (unless otherwise stated) for 5 min. The cells were stored at 4°C for no more than 21 days before the completion of all tests since it was found that storage beyond 21 days resulted in antigen decay with reduced staining intensity.

**Combined PXAPX-PAP technique.** The presence of HSV-specific antigens was detected in fixed Cytospin or cover slip cell preparations by a modification of the PXAPX (21). The preparations were covered with heat-inactivated normal goat serum (NGS) diluted 1:30 in tris(hydroxymethyl)aminomethane (Tris)-buffered saline (pH 7.4) for 5 min. After draining and blotting away excess NGS, several drops of rabbit anti-HSV serum diluted 1:10, 1:100, 1:
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500, or 1:1,000 in Tris-saline were placed on the sample for 1 h. All samples were washed twice in Tris-saline for 5 min and again overlaid with a 1:30 dilution of NGS for 5 min. Excess NGS was blotted away, and several drops of goat anti-rabbit IgG serum (heavy- and light-chain specific, Cappel Laboratories, Cochranville, Pa.) diluted 1:10 in Tris-saline were placed on the samples for 20 min. Samples were washed twice with Tris-saline for 5 min and then treated with absolute methanol for 15 min. This was followed by three 5-min washes in Tris-saline and another application of several drops of a 1:30 dilution of NGS for 5 min. With excess NGS blotted away, the samples received several drops of rabbit PXAPX serum (Cappel Laboratories) diluted 1:50 in Tris-saline with 1% NGS for 20 min. The specimens were washed twice in Tris-saline for 5 min. All samples were next treated with a freshly prepared, filtered solution of 0.0125% 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Co., St. Louis, Mo.) in Tris-saline with 0.001% hydrogen peroxide for 4 min in a light-protected Coplin jar unless otherwise stated. Samples were washed three times in Tris-saline for 5 min. If they were not to be counterstained, they were processed through graded alcohols and xylene and were mounted with Permount (Fisher) for microscopic studies. Samples to be counterstained by the PAP (17) were processed as follows: distilled water, 10 dips; distilled water, 5 min; Gill's hematoxylin (Polysciences, Inc., Warrington, Pa.) single strength (1 part Gill's no. 3 triple strength plus 2 parts 25% ethylene glycol in water), 2 min; distilled water, 10 dips; distilled water, 10 dips; 70% ethanol, 10 dips; 0.5% concentrated HCl in 70% ethanol, 1 dip to decolorize to salmon color; 70% ethanol, 10 dips; 70% ethanol, 10 dips; 3.0% concentrated ammonium hydroxide in 70% ethanol, 1 dip to a blue color; 70% ethanol, 10 dips; 70% ethanol, 10 dips; 95% ethanol, 5 dips; 95% ethanol, 2 min; Gill's modified OG-6 (Polysciences, Inc.), 2 min; 95% ethanol, 10 dips; 95% ethanol, 10 dips; Gill's modified EA (Polysciences, Inc.), 2.5 min; 95% ethanol, 10 dips; 95% ethanol, 10 dips; absolute ethanol, 10 dips; xylene, 10 dips; xylene, 5 min; Permunt and cover slip. Controls for all specimens consisted of: (i) identical treatment of Cytospin preparations as just described (PXAPX or PXAPX-PAP) with rabbit preimmunization serum at appropriate dilutions used in place of immune antiserum; (ii) Cytospin preparations which received only the PAP stain and (iii) HSV-1- or HSV-2-infected Vero cells on cover slips to serve as positive controls in the PXAPX or PXAPX-PAP procedure.

RESULTS

PXAPX parameters. The Vero-infected cell system on cover slips was used to optimize three variables in the PXAPX procedure to detect HSV antigens: (i) cell fixative; (ii) dilution and specificity of anti-HSV serum; and (iii) DAB and H2O2 concentrations. Although data presented represent HSV-2-infected Vero cells on cover slips, identical results were obtained with HSV-1-infected Vero cells.

(i) Fixatives for PXAPX. The most appro-

prate fixative for detection of HSV antigens by PXAPX was determined by the degree of antigen retention after fixation. This retention was measured by the intensity of the peroxidase reaction in HSV-2-infected Vero cells, using a 1:100 dilution of anti-HSV serum as the primary antibody in the PXAPX procedure. The infected cells were fixed by a variety of methods for various periods of time as listed in Table 1. The best fixatives (i.e., 3+) included acetone for 5 min and Bouin's fix (10) for 30 min, followed by a 30-min double-distilled water rinse (Table 1). All other Bouin's fix combinations, neutral buffered Formalin, and acid alcohol fixatives resulted in reduced staining intensity (Table 1). Based on these studies, acetone for 5 min was designated as the fixative of choice due to its simplicity and speed. Although Bouin's fixed the cells in a manner that preserved HSV antigens for PXAPX, it imparted a yellow background to all cells which interfered with the reading of a positive brown HSV-infected cell.

(ii) Serum dilution and specificity. Rabbit anti-HSV serum and rabbit preimmunization serum were diluted 1:10, 1:100, 1:500, or 1:1,000 in Tris-saline and used as the primary sera in

Table 1. Effect of various fixatives on HSV antigens detected by the PXAPX method

<table>
<thead>
<tr>
<th>Fixative</th>
<th>PXAPX reaction in Vero-HSV-2*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study 1†</td>
<td></td>
</tr>
<tr>
<td>Acetone (5 min)</td>
<td>3+</td>
</tr>
<tr>
<td>Cytospr spray fix</td>
<td>1+</td>
</tr>
<tr>
<td>Neutral-buffered Formalin</td>
<td>1+</td>
</tr>
<tr>
<td>Acetone (5 min), then acid alcohol (5 min)†</td>
<td>1+</td>
</tr>
<tr>
<td>Acid alcohol (15 min)†</td>
<td>0</td>
</tr>
<tr>
<td>Study 2°</td>
<td></td>
</tr>
<tr>
<td>Acetone (5 min)</td>
<td>3+</td>
</tr>
<tr>
<td>Bouin's fix (30 min), then dd H2O2 rinse (30 min)</td>
<td>3+</td>
</tr>
<tr>
<td>Bouin's fix (24 h), then dd H2O2 rinse (30 min)</td>
<td>3+</td>
</tr>
<tr>
<td>Bouin's fix (48 h), then dd H2O2 rinse (30 min)</td>
<td>2+</td>
</tr>
<tr>
<td>Bouin's fix (72 h), then dd H2O2 rinse (30 min)</td>
<td>2+</td>
</tr>
<tr>
<td>Bouin's fix (30 min), then 50% EtOH/saturated Li2CO3 rinse (30 min)</td>
<td>2+</td>
</tr>
<tr>
<td>Bouin's fix (30 min), then 50% EtOH rinse (1 h)</td>
<td>2+</td>
</tr>
</tbody>
</table>

* 3+, Intense dark-brown reaction in infected cells (see Fig. 2C); 2+, moderate brown reaction in infected cells; 1+, weak brown-tan reaction in infected cells; 0, no difference between infected and noninfected cells.

† Study 1: DAB = 0.00625%; H2O2 = 0.00125%; Study 2: DAB = 0.0125%; H2O2 = 0.0025%; higher concentrations of DAB and H2O2 used to offset yellow background caused by picric acid in Bouin's fixative.

‡ Cytospr spray fix, Fisher Scientific Co.

§ Acid alcohol, 0.2 ml of 11.8 M HCl in 100 ml of EtOH.

‖ dd H2O2, Double-distilled water.
the PXAPX reaction on HSV-2-infected Vero cells fixed with acetone for 5 min. The DAB and H$_2$O$_2$ concentrations were 0.0125% and 0.001%, respectively. Using rabbit anti-HSV serum at a 1:10 dilution, the infected cells showed a moderate dark-brown, peroxidase-positive reaction (Fig. 1A) that was only slightly more intense than those infected cells reacted with a 1:10 dilution of preimmunization serum (Fig. 1B). However, when the anti-HSV serum was diluted to 1:100 or 1:500, HSV-infected Vero cells retained the intense dark-brown, peroxidase-positive reaction (Fig. 1C and E), whereas preimmunization serum at dilutions of 1:100 or 1:500 failed to react with the infected cells (Fig. 1D and F). At an anti-HSV serum dilution of 1:1,000, the peroxidase reaction was still positive (Fig. 1G), but slightly less intense than when using antiserum diluted at 1:100 or 1:500 (Fig. 1C and E). No positive PXAPX reaction was observed in infected cells when preimmunization serum was used at 1:1,000 (Fig. 1H). The uninfected cells did not react with either immune or preimmunization serum at any dilution.

To establish specificity of the reaction, a 1:100 dilution of rabbit antiserum produced against other viral, bacterial, or protein antigens, listed in Materials and Methods, was used as the primary antibody in the PXAPX to stain HSV-2-infected Vero cells and the cells from the cervix of one patient positive for HSV. Whereas rabbit anti-HSV sera yielded a positive PXAPX staining reaction in the infected Vero and human cells, all heterologous sera were clearly negative.

(iii) DAB and H$_2$O$_2$ concentrations. Vero cells infected with HSV-2 were fixed for 5 min with acetone and processed according to the PXAPX procedure, using a 1:100 dilution of rabbit anti-HSV serum or a 1:100 dilution of rabbit preimmunization serum as the primary antibody. However, various concentrations of DAB and H$_2$O$_2$ were tested. When DAB and H$_2$O$_2$ were utilized at concentrations of 0.05 and 0.01%, respectively, the infected cells showed a very dark brown, peroxidase-positive reaction but with a high background staining of uninfected cells (Fig. 2A). By gradually decreasing this concentration to 0.0125% DAB and 0.001% H$_2$O$_2$, the infected cells retained the dark-brown positive staining reaction; and the uninfected cells were negative (Fig. 2B). Using these concentrations of DAB and H$_2$O$_2$, the infected Vero cells were processed by PXAPX, using a 1:100 dilution of preimmunization serum as the primary antibody, and it is evident in Fig. 2C that infected cells gave a negative peroxidase reaction similar to that of uninfected cells. Therefore, all studies which followed were done using DAB at 0.0125% and H$_2$O$_2$ at 0.001%.

**Removal of endogenous peroxidase.** The peroxidase enzyme was normally within mucus that closely adhered to cells from the female genitalia and was also contained within lymphocytes infiltrating the infected area (3, 22). Due to the nature of the enzyme system used in our studies, during initial experiments this endogenous peroxidase led to a high background stain of uninfected cells, causing false-positive reactions or difficult interpretations of PXAPX results. Obviously, the endogenous peroxidase had to be removed or destroyed while preserving the HSV antigens. Studies were first begun with the model system of HSV-2-infected Vero cells stained by PXAPX, using anti-HSV serum as the primary antibody. Various treatments to remove endogenous peroxidase, obtained from the literature (22, 23), were applied to the model system to determine which was the most feasible for application to human specimens. Preliminary studies established that the most promising treatment was absolute methanol (MeOH). Utilizing the model system, absolute MeOH was used to treat HSV-2-infected Vero cells at various steps of the PXAPX procedure to determine the effect on viral antigens. Figure 3A shows the standard PXAPX reaction of HSV-infected cells without MeOH treatment, demonstrating the intense brown peroxidase-positive reaction in infected cells. However, when these infected cells were treated for 15 min with absolute MeOH before the PXAPX procedure, the viral antigens were altered and a weak PXAPX test was observed (Fig. 3B). When treatment with absolute MeOH for 15 min was delayed until after goat anti-rabbit immunoglobulin G, but before rabbit PXAPX, the antigens were afforded a degree of protection by the primary antibody and goat antiliglobulin (Fig. 3C) without undergoing alterations themselves. This protective effect resulted in a PXAPX reaction that was indistinguishable from that of the untreated controls (Fig. 3A). When MeOH was added after the rabbit PXAPX, the reaction, as expected, was weak (Fig. 3D) due to the destruction of the peroxidase enzyme attached to the rabbit immunoglobulin.

**Application to human specimens.** Samples from the genitalia of female patients were stained by the PXAPX procedure, using rabbit anti-HSV serum or preimmunization serum as the primary antibody and absolute MeOH as described. Figure 4A shows the PXAPX stain, using anti-HSV serum, of cervical cells from one patient. Two dark-brown, HSV antigen-positive cells which appear to be fusing are quite apparent. Adjacent to these positive cells are the...
FIG. 1. Use of various dilutions of anti-HSV serum or preimmunization serum in the PXAPX procedure to detect HSV antigens in HSV-2-infected Vero cells. (A) Anti-HSV serum diluted 1:10; (B) preimmunization serum diluted 1:10; (C) anti-HSV serum diluted 1:100; (D) preimmunization serum diluted 1:100; (E) anti-HSV serum diluted 1:500; (F) preimmunization serum diluted 1:500; (G) anti-HSV serum diluted 1:1,000; (H) preimmunization serum diluted 1:1,000. ×310.
stained by PXAPX-PAP as described in Materials and Methods. When this double staining method was used, the antigen-positive cells remained dark brown (Fig. 4C), but nuclear morphology of infected cells and uninfected cells were even more apparent than when the PXAPX was used alone. The uninfected cells appeared as they would in an ordinary PAP stain and were not altered by the PXAPX procedure described here. For comparative purposes, Fig. 4D is HSV-2-infected Vero cells stained with the PXAPX-PAP, using anti-HSV serum as the primary antibody. In the center of Fig. 4D are two dark-brown, antigen-positive cells. Uninfected cells can be seen at the periphery (nuclei of uninfected cells are dark due to staining with hematoxylin).

Studies were done to select an optimal dilution of antiserum for HSV detection by PXAPX-PAP in human specimens. Using rabbit anti-HSV serum or preimmunization serum as the primary antibody at dilutions of 1:100, 1:500, and 1:1,000, dilution studies were carried out on cells from one patient. As the serum was diluted the reaction became less intense (Fig. 5A, B, C, and D). However, even at dilutions of 1:1,000, the positive cells were still evident as determined by the brown peroxidase-positive color. Indeed, at the higher dilutions nuclear morphology was even more apparent (Fig. 5C and D) than when the specimen was reacted with a lower dilution of antiserum due to a slightly less dark brown reaction. However, since a 1:100 dilution of anti-HSV serum resulted in the strongest brown positive reaction (Fig. 5A and B) and a 1:100 dilution of preimmunization serum was negative, all further studies were done with a 1:100 dilution of preimmunization or immune serum.

Utilizing the PXAPX-PAP stain described above and virus isolation and neutralization, 35 specimens from 21 patients were studied. Of the 35 specimens, 26 gave positive PXAPX-PAP reactions and 26 gave positive HSV isolation and neutralization tests (Table 2). In 25 instances, the specimen was positive for HSV by both PXAPX-PAP and virus isolation. The exceptions were one patient who was positive for HSV by PXAPX-PAP and negative by HSV isolation and another patient who was negative by PXAPX-PAP and positive by HSV isolation.

Time study. Studies were designed to mimic various delays specimens may undergo before processing to determine if the PXAPX-PAP procedure would continue to detect HSV antigens in human specimens. Accordingly, specimens were collected from patients and processed immediately and at 24-h intervals for 72 h. During the experimental period, the samples were kept at 4°C. These results are presented in Table 3.
FIG. 3. PXAPX detection of HSV antigens in HSV-2-infected Vero cells after treatment with MeOH. Anti-HSV serum was used as the primary antibody at a 1:100 dilution. (A) No MeOH treatment; (B) MeOH treatment for 15 min before PXAPX procedure; (C) MeOH treatment after goat anti-rabbit immunoglobulin G but before rabbit PXAPX; (D) MeOH treatment after the rabbit PXAPX. ×310.

FIG. 4. PXAPX or PXAPX-PAP method of detection of HSV infection of the female genitalia. Anti-HSV serum was diluted 1:100 and used as the primary antibody in the PXAPX procedure. (A, B) PXAPX stain of human cervical cells using anti-HSV serum; (C) PXAPX-PAP stain of human cervical cells using anti-HSV serum; (D) PXAPX-PAP stain of Vero-HSV-2 cells using anti-HSV serum. ×310.
and demonstrate that six specimens from four patients yielded a positive PXAPX-PAP and positive virus isolation at all time periods for all specimens but one. The one exception was the lack of a positive PXAPX-PAP at 48 h in patient 3461C. Interestingly, infectious virus was also recovered at each time period and correlated with the PXAPX-PAP (r = 0.742). It was observed that the more rapidly infectious virus was recovered, the more PXAPX-PAP-positive cells were found in the specimen (Table 3). However, even though a positive correlation coefficient (r = 0.742) was calculated between infectious virus recovery and a positive PXAPX-PAP, it should be noted that the intensity of the peroxidase reaction observed in infected cells and the rapidity of virus recovery decreased as the age of the specimen increased. Consequently, samples processed when received produced superior PXAPX-PAP results than those processed at 24, 48, and 72 h.

**DISCUSSION**

The results of this study demonstrate that the PXAPX procedure is a reliable and sensitive method of detecting HSV infection of cells from the female genitalia. The coupling of the PAP stain to PXAPX, although not a requirement, provides an additional parameter by which cells are judged as HSV infected. The PXAPX (or PXAPX-PAP) procedure correlated (r = 0.742) over a period of 72 h with virus isolation and was highly specific when tested against several unrelated rabbit antisera.

The most common rapid method of examining cells for HSV infection is either direct or indirect immunofluorescence. However, the immunofluorescent system may be plagued by problems of low sensitivity and non-specificity (5, 8). This may, in part, reflect the nonspecific binding of immunoglobulin G molecules to Fc receptors on HSV-infected cells (24). As a consequence, preimmune and immune sera can bind to HSV-infected cells in a similar manner, making the establishment of specificity difficult. As an alternative to immunofluorescence, Benjamin and Ray (2), as well as Benjamin (1), have successfully utilized the indirect immunoperoxidase procedure to detect HSV antigens in brain cell suspension and mucocutaneous lesions. In our study we have used the PXAPX procedure, which is reportedly 20 times more sensitive than indirect immunoperoxidase (4) and up to 1,000 times more sensitive than indirect immunofluo-
Table 2. Comparative results of PXAPX-PAP and virus characterization studies of specimens from human patients

<table>
<thead>
<tr>
<th>Total no. of samples examined (site of isolation)*</th>
<th>No. of samples confirmed as HSV^</th>
<th>No. of isolates positive by PXAPX-PAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>19 (cervix)</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>16 (vulva)</td>
<td>15</td>
<td>15</td>
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</table>

* Samples were from patients whose clinical description was variable and may or may not have included the following: (i) no herpetic lesions; (ii) beefy red cervix, no isolated lesions; (iii) multiple herpetic vesicles, early shallow ulcers, or late-stage healing ulcer.

^ Specimens were designated as HSV positive if characteristic HSV CPE (i.e., cell rounding, cytoplasmic stranding, chromatin condensation) occurred in the primary isolates and if the virus from the primary culture was neutralized by HSV antiserum.

rescence (21). In a direct comparison between indirect immunofluorescence and PXAPX to detect HSV antigens in tissue culture cells, it has been shown that PXAPX was 400 times more sensitive than indirect immunofluorescence (9). Additionally, in our current study the use of higher antiserum dilutions assisted in the resolution of specificity problems due to nonspecific binding of immunoglobulin G to HSV-infected cells. When our preimmunization or immune serum was used at a dilution of 1:10, each gave comparable results, negating the obvious difference between the two. However, due to the greater sensitivity of the PXAPX procedure, we were able to dilute our serum beyond the point at which nonspecific binding occurred with preimmunization serum. At serum dilutions of 1:100, 1:500, or 1:1,000, the anti-HSV serum was strongly positive, whereas preimmune serum failed to react.

The addition of the standard PAP to the PXAPX provides a second parameter to assist in identifying an HSV-infected cell. By coupling the PAP to PXAPX, an immunologically positive cell can be studied for nuclear pathological changes consistent with HSV infection. Using this procedure, we observed nuclear changes in PXAPX-positive cells, including pyknotic nuclei, chromatin condensation, syncytium formation, and Cowdry type A inclusion in some, but not all, PXAPX-positive cells. The PAP was selected since it is common to most laboratories and is not altered by the PXAPX. It is possible that other counterstains may be utilized to satisfy a particular need.

We found that the coupling of the PXAPX to the PAP called attention to HSV-infected cells that may be overlooked in the routine screening when cells are only PAP stained. The most apparent cell in a specimen from an HSV lesion is the syncytium, which is readily seen when stained by PAP. However, our virus-positive specimens most frequently contained cells with a single nucleus that were PXAPX positive. When replicate samples that were only PAP stained were studied, these cells were easily overlooked; however, when stained by PXAPX-PAP the immunological reaction dramatically called attention to the HSV-infected cell with the single nucleus. Upon further microscopic study it was observed that the single nucleus of these PXAPX-positive cells had alterations consistent with HSV infection.

Whereas our results demonstrate that the PXAPX-PAP procedure is an asset in detecting HSV infection of the female genitalia, it is of value to reiterate several factors which we consider important to the successful completion of this test: (i) MeOH treatment to remove endog-
enous peroxidase in mucus and lymphocytes; (ii) a dilution of serum sufficient to eliminate nonspecific binding of preimmunization serum so that specificity may be established; (iii) titration of each new lot of DAB and H2O2 to give a maximum peroxidase reaction with minimal background; and (iv) titration of enzyme reaction time since the amount of time permitted for the enzyme to react dictates the intensity of the final product.

By careful consideration of the above factors, it is our experience that the PXAPX-PAP system is sensitive, specific, permanent, and inexpensive. Owing to the greater recognition of HSV in a variety of human abnormalities and the damping of antiviral chemotherapy, a system with such attributes will be a valuable adjunct to successful diagnosis and treatment.

LITERATURE CITED