Detection of herpes simplex virus in cell culture stomatitis and labial lesions by the immunoperoxidase technique

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DETECTION OF HERPES SIMPLEX VIRUS IN CELL CULTURE, STOMATITIS AND LABIAL LESIONS BY THE IMMUNOPEROXIDASE TECHNIQUE

by

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B.S., Mahidol University, 1970

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Chairman, Board of examiners
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ABSTRACT

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Director: Richard N. Ushijima

The immunoperoxidase (IP) technique was compared with the virus isolation procedure for their efficiency in detection of herpes simplex virus (HSV) in tissue cultures and in clinical specimens from stomatitis and labial lesions.

Vero cell cultures infected with HSV at different multiplicity and at different culture conditions were studied. It was found that viral antigens could be detected as early as 1 hour after infection and cells infected for more than 4 hours would be suitable for the IP study. Under culture conditions which decrease the content of viable virus, its presence in cell culture was detected by the IP technique but was negative by the isolation procedure.

Of 14 clinical specimens tested for HSV, there was correlation between results by both methods, being positive or negative. Results of the two techniques were not correlated in two specimens. There was one specimen which was negative by the isolation procedure that had insufficient cells for the IP study. It was also found that the use of IP technique in detection of HSV in clinical specimens required the inclusion of good controls before the tests could be considered reliable.
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Chapter 1

INTRODUCTION

DESCRIPTION AND NATURE OF THE AGENT

The virus of herpes simplex has been known since the early days in virology. The word "herpes" has, however, been used in medicine for at least 2500 years and has referred to a variety of clinical skin conditions. Its meaning has changed considerably from that time and by the 19th century, the generally accepted use of the word was restricted to certain diseases associated with vesicular eruptions (2,65). The existence of a virus or virus like agent in the fluid from herpetic lesions was not discovered until 1912, when Gruter showed that material from human corneal herpes could produce acute keratitis when placed upon the scarified cornea of a rabbit. The causative agent came to be known as the virus of herpes simplex (28,65).

Herpes simplex virus is the prototype of the herpes virus group, in which over 50 members has been identified (44). These viruses affect mamalian, avian and amphibian species. There are at least four herpes-viruses currently known to infect man: herpes simplex virus (HSV) or herpes virus hominis (HVH), varicella-zoster (VZ) virus, cytomegalovirus (CMV), and Epstein-Barr (EB) virus. These viruses share in common many unique properties which have been summarized by Plummer (46).

The physical and biological properties of herpes simplex virus
have been studied extensively. It is characterized as a particle measuring around 100 nanometers in diameter, having an icosahedral structure with 162 subunits or capsomeres and surrounded by an envelope which is derived from host cell membranes. The enveloped particle has a total diameter between 150-200 nanometers (29,62,64). The DNA-containing core is produced in the cell nucleus and is associated with intranuclear inclusion bodies (Cowdry or Lipschutz type A) (64).

It has been estimated that one infected cell produces 1,000 new viral particles, of which 5-10% are infectious (52). Both naked and enveloped viruses are infectious, but the enveloped type appears to enter host cells more easily than does the naked type (24). Since the envelope contains essential phospholipid, the virus is inactivated by lipid solvents. The virus is very sensitive to heat, and infectivity is only maintained at 37°C for a few hours. The virus is also sensitive to acid (pH less than 6.8), organic solvents, cationic detergents, proteolytic enzymes, ultraviolet and X-radiation (61).

Two antigenic types of herpes simplex virus (HSV), type 1 and type 2, have been recognized. The two strains of HSV are closely related yet are distinguishable by their biological, serological and epidemiologic features. Type 1 HSV is associated with most non-genital infections as primary herpetic stomatitis in children and is spread primarily by the oral-respiratory route. Type 2 HSV is found predominantly to infect genital organs of males and females, can be venereally transmitted and usually causes neonatal disease. The relationship between the site of herpetic infection and HSV types is not, however, absolute (12,40,41).
The two strains of HSV share common antigens, but each type possesses one or more specific antigens (15). Cross-reacting antibodies between herpes virus types 1 and 2 have been observed (25,40). Herpes simplex virus types 1 and 2 produce different cytopathological appearance on egg chorioallantoic membrane (CAM), in tissue culture cells and differ from each other in many biological characteristics, mostly of a quantitative nature (41).

CLINICAL BACKGROUND

A report of frequent isolation of HSV in routine laboratory diagnosis of viral infections suggests that herpes simplex is probably the most common human pathogenic virus (22). Serological studies also indicate that up to 90% of the population have been infected with herpes simplex virus at some time, and there appears to be a difference in the incidence of antibodies to the virus in different age and socio-economic groups (7,8,59).

The virus causes both primary and recurrent diseases. Primary infection occurs in both adults and children having no HSV antibody. After the initial infection, another pattern commonly observed is the recurrence or chronic nature of herpetic infection. Both primary and recurrent infections can be manifested clinically or in asymptomatic form (8,26,28).

Infection with HSV may take several clinical forms but is most often inapparent. The usual clinical manifestation is the vesicular or ulcerative lesions involving the skin, oral or genital mucous membranes (26). On the mucous membranes, the virus may bring about acute
infectious gingivostomatitis, which is probably the most common primary form of herpetic infection (8,28). Two other forms, keratoconjunctivitis and vulvovaginitis are not uncommon, both of which may be either primary or recurrent infections (28,47). The virus has also been considered as a primary cause of pharyngitis and tonsilitis (35,54).

The diseases of the skin are primary herpetic dermatitis, recurrent herpes labialis, eczema herpeticum and traumatic herpes (47).

A severe generalized infection with HSV is uncommon in human beings. It can occur in newborn infants, seriously involving the eyes, the central nervous system (CNS) and the skin as well as other organs (28,47). A severity of herpetic infection may increase in certain types of hosts such as those of underlying skin disorder (atopic eczema), children with malnutrition and those with immunodeficiency (28,47).

LABORATORY DIAGNOSIS

While some infections with herpes simplex virus can be diagnosed by their clinical manifestations, laboratory tests are often required for a definite diagnosis. Laboratory techniques available for diagnosis are:

(i) Direct examination of clinical material for the presence of the virus induced intranuclear inclusion bodies (Cowdry type A), multinucleated giant cells or the ballooning degenerative type of the epithelial cells by light microscope. The pathological picture is, however, indistinguishable from that of varicella-zoster (6). Specific antigens in infected cells can be demonstrated by the immunofluorescent technique, which produces rapid (1.5-2 hours) definitive determination.
of the virus involved (14,43). In some laboratories, the electron microscope has been used for rapid differential diagnosis of herpes virus (HSV or VZ) from poxviruses infections (36,58).

(ii) Isolation of the virus from suspected material, which can be achieved by inoculation into susceptible animals such as mice, rabbits, hamsters, guinea pigs, and into other systems as the chorio-allantoic membrane of embryonated hen's egg or susceptible tissue culture cells (61). Identification of virus isolates can be confirmed by their reaction with specific antisera. Neutralization test or fluorescent antibody (FA) technique is usually employed for the identification of HSV (18,61).

(iii) Demonstration of the presence of, or a rise in, the titer of specific antibody to HSV. There are times, especially in cases of suspected HSV-induced central nervous system diseases, when the laboratory receives only serum or cerebro-spinal fluid (CSF) specimens. In such cases, serological methods would be suitable for diagnosis. Serological tests available for diagnosis of HSV infection are: complement fixation, neutralization, fluorescent antibody, hemagglutination and hemadsorption tests (18,28,61).

RELATIONSHIP OF STOMATITIS AND LABIAL LESIONS TO HERPES SIMPLEX VIRUS

Acute herpetic gingivostomatitis is one of the several clinical patterns of disease produced by primary infection of a susceptible person with herpes simplex virus. It is estimated that about 15% of infections are manifested in this form (47). Some observers believe that this
herpetic oral lesion is by far the most common form of stomatitis encountered in children, but is frequently not recognized (32). Acute herpetic gingivostomatitis in adults has been described as rare and is often accompanied by pharyngitis (31,49,54). The etiological relationship of herpes simplex virus to acute gingivostomatitis has been substantiated by isolation of the virus in rabbit cornea (8,53), tissue culture (54), by the appearance of circulating antibodies in the convalescent sera when none exist in the acute serum (8,31,49), or by the demonstration of a characteristic histologic picture of giant cells, inclusion bodies, ballooning degeneration of cells (49).

Herpes labialis, better known as "fever blister" or "cold sore" has been considered as the recurrent form of herpes simplex infection (8,47). Human transfer studies have demonstrated the etiological relationship of recurrent herpes labialis to primary acute herpetic gingivostomatitis (4). Herpes simplex has been cultured from intact vesicles on the lips in as many as 90% of the cases (5,56). Microscopic examination of biopsy material has demonstrated characteristic inclusion bodies and the majority of patients with this condition have demonstrated antibody titers to herpes simplex virus (5,9).

Recurrent ulcerative stomatitis is often seen in adults (67), but the etiological relationship of HSV to this disease has been a subject of controversy. Since primary herpetic gingivostomatitis and recurrent herpes labialis have been shown unequivocally to be caused by herpes simplex virus (8,31,49,53) and because of the similarity of factors that can initiate recurrence (67), many attempts have been made.
to isolate the virus from recurrent stomatitis but have been unsuccessful by either inoculation into rabbit cornea (5) or infectivity in tissue culture cell (56). Serologically, only a small minority of patients with this disease manifest antibodies against the herpes virus (5,60). For this reason recurrent stomatitis of this nature are sometimes referred to as "recurrent apthous ulcers" or "canker sore" and some workers apparently consider recurrent stomatitis associated with HSV to be non-existent (6,10,56), while others have described them as being very rare (17,31,51). On occasion, however, the herpes simplex virus has been isolated from a small percentage of patients (31), and in some cases immunofluorescent technique has been used to verify the etiological relationship of HSV to recurrent ulcerative stomatitis (17).

In a number of cases, stomatitis is due to some other conditions such as trauma, food and drugs allergies, or associated with blood diseases (67). A consideration of differential diagnosis is of great importance, since some diseases including herpangina may bear a close resemblance to herpes simplex. Herpangina is a disease resembling herpetic stomatitis in some respects and has been shown to be induced by some types of Coxackie group A viruses (28).

PURPOSE OF STUDY

The detection of virus to establish its etiologic role in disease depends upon the laboratory methods used to reveal its presence. As a working hypothesis, it is assumed that the difficulties in detecting the agent by conventional virus isolation procedure may be due to mechanisms that decrease the content of viable virus. It is known that many
biological fluids contain non-specific virus inhibitors which may tend to decrease the virus population nonspecifically. The amount of inhibitors varies with the individual source and even from day to day in the same individual (19). Neutralizing activity of oral secretion to HSV has been found in some persons (11).

The method (immunoperoxidase technique) used in this study has proved useful in identifying a wide variety of viral antigens in cell cultures (1,33,38). Comparable sensitivity in detecting viral antigens has been reported between the immunoperoxidase and immunofluorescence techniques (23,63). The immunofluorescence procedure has been successfully utilized for the detection of herpes simplex virus in various types of clinical specimens (14,43); however, the use of immunoperoxidase technique in such instances has not been reported.

Therefore, it was desirable to make a preliminary evaluation of the difficulties encountered in diagnosis utilizing viral isolation by studying various parameters of the immunoperoxidase technique in cell culture systems.
Chapter 2

MATERIALS AND METHODS

VIROLOGICAL TECHNIQUES

The Virus

Herpes simplex virus (HSV) type 1, strain KOS-1 was used in this study.

A large pool of virus was prepared in Vero cells, which were grown to confluency in 32 oz glass prescription bottles. After removal of the growth medium, the cell cultures were inoculated with $1.6 \times 10^{8.0}$ TCID$_{50}$ units (50% tissue culture infective dose) of the virus in 4 ml culture fluid. Virus was allowed to adsorb for 1 hour at 37°C, then 40 ml of maintenance medium was added to each bottle. After 18 hours of incubation at 37°C, the cultures were repetitively frozen in a dry ice-acetone bath and thawed three times. After the third thawing, cellular debris was removed by centrifugation at 1,000 rpm for 10 minutes at 4°C. The clear supernatant fluid, which contained HSV virus was stored in flame-sealed glass ampules in 1 ml amounts at -70°C until used. The virus pool contained $4 \times 10^{8.5}$ TCID$_{50}$ per ml ($1 \times 10^{7.5}$ TCID$_{50}$ per 0.025 ml) when assayed as described below.

Culture Medium

Eagle's Minimal Essential Medium (MEM) was used for tissue culture. Powdered MEM purchased from Grand Island Biological Co. was

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rehydrated in triple-distilled water and NaHCO₃ was added into the medium at the concentration of 0.15%. The medium was sterilized by positive pressure filtration through a Millipore filter and stored at 4°C. To check for sterility, a medium sample was incubated at 37°C for 24 hours and then examined for possible contamination.

The culture medium was supplemented with sterile fetal calf serum (Microbiological Associates), which was inactivated at 56°C for 30 minutes to destroy heat labile, non-specific viral inhibitors. MEM contained 10% and 3% sterile fetal calf serum (FCS) and were used as growth and maintenance media respectively. Both growth and maintenance media contained penicillin (100 units/ml), streptomycin (100 mcg/ml), tetracycline (10 mcg/ml) and mycostatin (50 units/ml) in an attempt to eliminate bacterial, mycoplasmal and fungal contaminations.

Cell and Care of Tissue Culture

The Vero cell (an African green monkey kidney continuous cell line) was used in both the isolation and immunoperoxidase techniques. Vero cells were maintained as a stock culture in 32 oz prescription bottles in MEM supplemented with 3% FCS. Cells were usually kept at 37°C and the medium was changed every 3-4 days or when the medium acidity increased as indicated by the yellow color of the phenol red in the medium. The cells were subcultured when a thick monolayer was formed by removing the culture medium, washing three times with Ca⁴⁺ and Mg⁴⁺-free phosphate buffered saline (Pd) and incubating the cells with 1.5 ml of versene-trypsin solution at 37°C with versene-trypsin solution covering the cell sheet. The cells usually detached from the
glass surface within 5-10 minutes and were collected by centrifugation, then mixed with growth medium and dispersed in growing flasks. Generally the cultures were split 1:3 for seeding into new containers. Fresh growth medium was added at approximately 40 ml per bottle, and the pH of the medium was adjusted with 5% CO₂ in air. For chemical composition and preparation of Ca and Mg ++ -free phosphate buffered saline and versene-trypsin solution, see appendix.

**Virus Titration and Neutralization Tests**

Virus titration and neutralizing antibody assays were conducted by microtiter methods. Tests were performed in sterile disposable microtest culture plates with 96 flat bottomed wells (Falcon Plastics, Oxnard, California; TM 3040) with sterile disposable lids (Falcon Plastics; TM 3041). Plastic pipette droppers calibrated to deliver 0.025 or 0.05 ml drops, available from Microbiological Associates, Bethesda, Maryland, were used in delivering the reagents into wells. Special wire loops (Cooke Engineering Co., Alexandria, Va.) calibrated to transfer 0.025 or 0.05 ml were used in diluting the sera.

Growth medium was used for diluting serum, virus and cells. Vero cells from confluent monolayer culture were detached from the glass by trypsinization and resuspended at 200,000 cells per ml in growth medium. This standard Vero cell suspension was used for each test and prepared just prior to use.

**Virus Titration.** HSV infectivity was performed by the method of Pauls and Dowdle (45) in the following manner:
Prior to virus isolation, 0.025 ml volume of growth medium was added to all virus dilution wells. Serial 10-fold dilutions of virus were prepared in ice-cold growth medium and 0.025 ml of each dilution was placed in each of 11 replicate wells. Immediately thereafter, 0.025 ml of cell suspension (2 x 10^5 cells/ml in growth medium) was added to each well. The last well of each dilution row, serving as the normal cell control, consisted of 0.05 ml of growth medium plus 0.025 ml of cell suspension. The plates were covered and shaken gently to insure thorough mixing, then placed in an atmosphere of 5% CO₂ in air at 37°C.

Plates were read after 48 hours incubation, using an inverted tissue microscope. The cytopathic effect (CPE) in individual wells was recorded as negative, indicating normal cells, or positive, indicating cells showing CPE.

The 50% tissue culture infective dose (TCID₅₀) was calculated according to the formula of Reed and Muench as modified and described by Lennette and Schmidt (34) and the virus titer was recorded on the basis of 0.025 ml virus dose.

Neutralization test. Test sera were inactivated at 56°C for 30 minutes and serial 2-fold dilutions (1:2 - 1:1024) were prepared in growth medium using 0.025 ml diluting loops. To each set of serum dilutions was added 0.025 ml of ice-cold virus suspension containing approximately 100 TCID₅₀ in growth medium as determined by a previous titration. The 11th well of each set served as serum control wells for cytotoxicity and 1:4 dilution of serum was prepared in 0.05 ml volume. The 12th well served as cell control and received 0.05 ml of growth medium.
medium. The virus-serum mixtures were incubated for 2 hours at room temperature, after which the 0.025 ml volumes of cells suspension (2 x 10^5 cells/ml) were added to all wells. All plates were covered and placed in a 37°C incubator with 5% CO₂ in air. Neutralizing antibody titers were recorded as the highest serum dilutions that completely inhibit CPE when the simultaneous virus titration indicated 100 TCID₅₀ virus dose in the test.

Isolation of the virus

Specimens were obtained from individuals attending the University of Montana Health Service and at dental clinics in town because of evidence of the lesions inside the mouth or of the lips.

Specimens were collected from aspirated fluid of vesicles or by swabbing the site of lesions with sterile cotton-tipped applicators and suspending the material in 1.0 ml of maintenance medium (3% inactivated fetal calf serum and antibiotics in MEM). This suspension was inoculated into Vero cells in the following manner:

The Vero cells, maintained in prescription bottles were detached from the glass by trypsinization and resuspended in growth medium (10% inactivated fetal calf serum and antibiotics in MEM) at a concentration of approximately 5 x 10^5 cells per ml. Four ml of this cell suspension was mixed with the sample suspension in a small tissue culture flask (Falcon flask 30 ml size, #3012). The culture was gasses with 5% CO₂ in air, incubated at 37°C and observed daily during a period of 5 days for the appearance of characteristic cytopathic effect (CPE) of HSV.
Preparation of Sera

Normal serum. Normal serum was derived by pooling the blood of a number of guinea pigs. Blood was drawn aseptically, permitted to clot and serum was separated. The serum showed no neutralizing activity against HSV and was stored at -20 °C until used.

Antiviral serum. Antiserum to herpes simplex virus (group-specific) was prepared in guinea pigs and was obtained from the Center of Disease Control (CDC), Atlanta, Georgia. The serum contained neutralizing antibody titer of 1:32 to HSV, strain KOS-1, when determined by the method described above. Optimal dilution for use in the immunoperoxidase study was determined by chessboard titration with peroxidase conjugate. Both normal guinea pig and antiherpes sera were adsorbed with acetone-dried, mouse liver powder for 1 hour at 37 °C and for 18 hours at 4 °C.

Antiglobulin serum. Antiserum to guinea pig gamma globulin was produced in rabbits injected intramuscularly with a mixture of guinea pig gamma globulin solution (protein concentration of 5 mg/ml) plus an equal volume of complete Freund's adjuvant at 4 weeks interval for the first two injections. Preparation of complete Freund's adjuvant is given in appendix. Subsequent injections were performed weekly with 0.2 ml of globulin solution without Freund's adjuvant until a satisfactory titer was obtained as determined by the precipitation test which was performed in the following manner:

Rabbit antiglobulin serum was drawn by capillary suction into a
non-heparinized, microhematocrit tube to rise about 1/3 of the tube. A finger was placed over the top of the tube before removing from the antiserum and the excess was wiped off. With the finger still in place, the capillary was then dipped into a tube of guinea pig serum and an equal volume was drawn up. The capillary tube was then carefully inverted several times to mix the sera. A finger was placed at the end, and the tube was placed upright in the plasticine, leaving an air space at the end between the bottom of the serum and the clay. Excess serum and finger marks were wiped off from the tube with cleansing tissue, and the tube was then placed in the incubator at 37°C for 1 hour and at 4°C overnight. The antibody content in the rabbit serum was estimated from the amount of the precipitate formed and recorded as 4+ for maximal precipitation or 0 for no precipitation. Serum with 3–4+ precipitation was chosen for use in the immunoperoxidase test.

**Fractionation and estimation of antiglobulin**

**Fractionation.** The globulin portion of rabbit anti-guinea pig gamma globulin serum was fractionated by precipitation with ammonium sulfate at 35% saturation (20). To precipitate the globulin, freshly prepared solution of 70% (v/v) of saturated ammonium sulfate solution (SAS) was slowly added to an equal volume of rabbit antiglobulin serum while stirring gently. The reaction mixture was mixed well and allowed to stand at room temperature for a minimum of 4 hours and then centrifuged to pack the precipitated globulin at 2,500 rpm for 30 minutes at 4°C. The supernatant fluid was removed and the precipitate was resuspended and dissolved in distilled water to a final volume of the
original volume of the serum. For the second and third precipitations, an equal volume of 70% SAS was added to the protein solution in the same manner but the reaction mixture was centrifuged immediately. After the third precipitation, the precipitated globulin was dissolved in distilled water to a final volume of about \( \frac{1}{2} \) of the original volume of the serum and was dialyzed against frequent changes of 0.85% NaCl solution (pH 8.0) at \( 4^\circ C \). Dialyzation was terminated when sulfate was no longer detected by adding a small amount of saturated BaCl\(_2\) solution to an equal volume of the saline solution. The globulin was considered free from sulfate when no cloudiness was observed. Merthiolate, as a preservative, at a final concentration of 1:10,000 was added to the globulin solution which was then stored at \(-20^\circ C\) in several small portions.

**Protein estimation.** Prior to the peroxidase conjugate, the protein content of fractionated globulin solution was estimated by Folin-Ciocalteu reagent as modified and described by Lowry et al (37), using bovine serum albumin as a standard.

The globulin solution was diluted to give a concentration of approximately 40-100 mcg of protein in 1.0 ml volumes with distilled water. To the diluted globulin, 5 ml of alkaline copper tartrate solution was added, mixed and allowed to stand at room temperature for 10 minutes. To this mixture, 0.5 ml of diluted Folin-Ciocalteu reagent was then added and stirred immediately on the Vortex. The reaction mixture was held at room temperature for 30 minutes before reading in a Beckman DU Spectrophotometer at a wave length of 540 um. The globulin
content was estimated from the reading of light absorption when checked against a standard curve of bovine serum albumin which was run simultaneously. Chemical compositions and preparation of the reagents are given in appendix.

**Labeling of antiglobulin with peroxidase enzyme**

The antiglobulin fraction recovered from rabbit anti-guinea pig globulin serum with ammonium sulfate precipitation was conjugated to the peroxidase enzyme by the method described by Miller et al (38).

Five mg of horseradish peroxidase (Type VI, Sigma Chemical Co., St. Louise, Mo.) was dissolved in 1.0 ml of 0.3 M sodium bicarbonate, and 0.1 ml of 1% fluorodinitrobenzene (v/v in absolute ethanol) was added. The mixture was stirred gently for 1 hour at room temperature, 1.0 ml of 0.08 M sodium periodate was then added and the incubation was continued as before for 30 minutes. One ml of 0.16 M ethylene glycol was added into this mixture and stirred gently for 1 hour at room temperature and it was then dialyzed against 3 changes of 0.01 M carbonate buffer, pH 9.5 at 4°C. The dialyzed peroxidase aldehyde could be kept at 4°C for up to 1 month.

To 8 mg of the rabbit antiglobulin, 3.3 ml of peroxidase aldehyde was added and stirred gently for 3 hours at room temperature. The peroxidase-antiglobulin conjugate was dialyzed against phosphate buffered saline (PBS), pH 7.1 at 4°C overnight and then against PBS containing 4 mM sodium borohydride for 8 hours at 4°C. Finally it was dialyzed against four changes of PBS, pH 7.1 at 4°C to remove unbound peroxidase.

The dialyzed material contained peroxidase-labeled antiglobulin,
was stored at -20°C in small portions and was used without further modification. Chemical composition of PBS and carbonate buffer are given in appendix.

**Preparation of slides**

Slides were prepared by placing drops of glycerin in three rows of six on standard, clean microscope slides, which were then sprayed with a Teflon-like compound (Fluoro-Glide, film bonding grade, Chemplast Inc.) and allowed to dry. The glycerin drops were rinsed off by running hot tap water on the slides for 3-5 minutes. The slides were then dipped briefly in distilled water and air dried. By this means the slides gave a ground glass-like appearance except for the clear areas originally protected by the glycerin. The result was 18 wells per slide, each 2-3 mm in diameter.

For the preparation of tissue cells, infected and uninfected monolayers were scraped off the glass with Pasteur pipettes and centrifuged at 1,000 rpm for 5 minutes at 4°C. The culture medium was removed and the cells were washed twice with PBS, pH 7.1. After being washed, the cells and cell debris were suspended in several drops of PBS, and one drop of this suspension was placed into each of three wells on a slide. Each slide consisted of six different samples of tissue cultures. The slides were air dried, immediately fixed in acetone for 10 minutes at 4°C, dried and stored at -70°C for later staining.

Preparation of slides of clinical specimens were handled as described for tissue culture.
**Immunoperoxidase staining**

The reaction for localizing HSV antigens in cell cultures and in clinical specimens was performed by the indirect method (16). One drop of antiherpes serum at its optimal dilution was added to cover completely each well in the first horizontal row (6 different samples in each well per row) on a slide. Similarly one drop of normal serum was placed over each well in the second row to serve as negative serum control. Cells in each well of the last row received 1 drop of PBS for the negative saline control. The preparations were left to react at 37°C for 1 hour in Petri dishes with lids lined with moistened paper and then given four 10-minutes washes with PBS at room temperature. After washing the slides were gently blotted dry, and one drop of peroxidase conjugate at its optimal dilution was added to each well. The preparations were reincubated for 60 minutes at 37°C in humidified Petri dishes and the slides were washed as before and air dried.

Negative antigen control consisting of slide preparation of uninfected cells were similarly treated, in parallel with infected cells.

The detection of the immuno-complex was achieved by the reaction of the peroxidase enzyme with its specific substrate in Kaplow stain (30). One drop of Kaplow stain was placed in every well for 1 minute and the slides were then rinsed with water, air dried and mounted in permanent mounting medium (Harelco synthetic resin). For chemical composition and preparation of Kaplow stain, see appendix.

The presence of peroxidase marker in infected cells was seen as bright blue granules, whereas the cellular cytoplasm stained pink.
and nuclei stained red when viewed under light microscope. The preparations could be kept for several days for subsequent examinations.
Non-specific staining of the peroxidase conjugate due to binding of immunoglobulins nonspecifically to the tissue may be reduced or eliminated by dilution of the antiserum and/or conjugate. Therefore, optimal dilution of the antiherpes serum and of the enzyme-antiglobulin conjugate used in this study were determined by the so-called chessboard titration (3) in which a series of dilutions of guinea pig sera and peroxidase conjugate were tested against each other (see Table 1).

Dilutions of negative control serum (normal guinea pig serum), positive antiherpes serum and peroxidase conjugate were made in phosphate buffered saline (PBS), pH 7.1, which was also included in the titration. The HSV-infected Vero cells at 6 hours after infection were used as the antigen.

As demonstrated in Table 1, the plateau-titer (PT) or the highest dilution of antiherpes serum which gave a definite positive reaction with the highest dilution of the peroxidase conjugate was 1:640. The plateau end point (PEP) or the highest dilution of conjugate giving this plateau titer appeared at the conjugate dilution of 1:40. This dilution of conjugate yielded the highest titer of antiherpes serum without concomitant non-specific staining and was, therefore, chosen for future
## Table 1

**Titration of immunoglobulin reagents for the immunoperoxidase test**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Reciprocal of Sera Dilution</th>
<th>Reciprocal of Peroxidase-conjugate dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>PBS</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NGPS 20</td>
<td>1+</td>
<td>±</td>
</tr>
<tr>
<td>60</td>
<td>1+</td>
<td>-</td>
</tr>
<tr>
<td>HSV-infected cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPAS 20</td>
<td>4+</td>
<td>4+</td>
</tr>
<tr>
<td>40</td>
<td>4+</td>
<td>4+</td>
</tr>
<tr>
<td>80</td>
<td>4+</td>
<td>4+</td>
</tr>
<tr>
<td>160</td>
<td>3+</td>
<td>3+</td>
</tr>
<tr>
<td>320</td>
<td>2+</td>
<td>2+</td>
</tr>
<tr>
<td>640</td>
<td>1+</td>
<td>1+</td>
</tr>
<tr>
<td>1280</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NGPS 20</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>60</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>Uninfected cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPAS 20</td>
<td>1+</td>
<td>±</td>
</tr>
<tr>
<td>40</td>
<td>1+</td>
<td>-</td>
</tr>
<tr>
<td>80</td>
<td>±</td>
<td>-</td>
</tr>
</tbody>
</table>

**PBS** = Phosphate Buffered Saline, pH 7.1  
**NGPS** = Normal guinea pig serum  
**GPAS** = Guinea pig antiherpes serum

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The antiherpes serum at the dilution of 1:40 was also chosen on the basis that the antiherpes serum gave a maximum staining with the selected conjugate at this dilution. Negative control serum at the dilution corresponding to the optimal dilution of positive antiherpes serum was also selected for the test.

**SPECIFICITY OF ANTIHERPES SERUM**

To insure that the antiherpes serum used in the immunoperoxidase test was specific to HSV antigens, both immunoreagents were tested at their working dilutions with:

a) Preparations of uninfected tissue cultures of rabbit corneal cell line (SIRC) and of cell lines from human origin (Hep-2 and Fl cells)

b) Preparations of Vero cell cultures infected with Respiratory syncytial virus (RSV) and Adeno 1, 12, 18 viruses.

No cross-reactivity was observed in these preparations as shown in Table 2. Therefore, it seemed reasonable to conclude that the antiherpes serum was specific to HSV antigens.

**DETECTION OF HSV IN TISSUE CULTURES**

To determine the relative efficiency of the immunoperoxidase (IP) and virus isolation procedures in detection of HSV, tissue cultures infected with HSV and incubated with or without antibody containing medium were used as a model. At varying times after infection, the cell cultures and supernate fluids were examined for HSV. The experiments were performed as follows:

Two groups of Vero cell cultures (28 cultures/group) which had
Table 2

Controls for establishing specificity of IP staining

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Reagent step 1</th>
<th>Reagent step 2</th>
<th>Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV</td>
<td>PBS</td>
<td>conjugate</td>
<td>negative</td>
</tr>
<tr>
<td></td>
<td>NGPS</td>
<td>conjugate</td>
<td>negative</td>
</tr>
<tr>
<td></td>
<td>GPAS</td>
<td>conjugate</td>
<td>positive</td>
</tr>
<tr>
<td>Heterologous</td>
<td>GPAS</td>
<td>conjugate</td>
<td>negative</td>
</tr>
<tr>
<td>Uninfected</td>
<td>GPAS</td>
<td>conjugate</td>
<td>negative</td>
</tr>
</tbody>
</table>

PBS = Phosphate Buffered Saline, pH 7.1
NGPS = Normal guinea pig serum
GPAS = Guinea pig antiherpes serum
formed monolayer sheets of approximately $1.2 \times 10^5$ cells in 16 x 125 mm screw cap culture tubes were overlaid with 0.5 ml of HSV. To one group 150 TCID$_{50}$/cell of HSV was added and 30 TCID$_{50}$/cell was added to the other. The virus was allowed to adsorb at room temperature for 60 minutes and at 37°C for an additional 60 minutes, after which the unadsorbed virus was removed by washing the cell cultures 4 times with Hanks' balanced salt solution. One-half of the cultures in each group was incubated at 37°C with 1 ml of MEM containing 10% FCS and the other half was incubated with 1 ml of MEM containing 40% HuAS (Human antiherpes serum, neutralizing titer of 1:16).

At 1 hour intervals thereafter, for a period of 6 hours, 2 cultures from each group (4 groups at this step) and one uninfected control culture tube were studied. The culture medium of each tube was removed and centrifuged at 1,000 rpm for 5 minutes, and 0.5 ml of the supernate was used for virus isolation by the method described. The monolayer cells of each tube were examined for viral antigens by the immunoperoxidase technique. Two cultures of each group were further incubated at 37°C. Characteristic CPE of HSV was observed in all cultures 24 hours after infection.

Comparison of virus detection by IP and isolation procedures in tissue cultures grown in the presence and absence of antibody is given in Table 3.

Viral antigens could be detected as early as 1 or 2 hours after infection, depending on the multiplicity of the virus, by the IP method. This finding was comparable in time to that observed by Ross et al (50),
using the immunofluorescent technique. At this time, the viral antigens appeared as fine discrete blue granules at the cell membrane and in the cytoplasm. As time elapsed, the intensity of the staining and number of positive cells increased as shown in Table 3 and Table 4. The percentage of the positive cells indicate the average number of infected cells showing blue granules, of a total of 300 cells counted. The degrees of intensity shown in Table 3 indicates the intensity of the stain observed in the majority of the positive cells. The infection was not synchronous, even with high multiplicity of the virus. The peroxidase marker ranged from fine blue granules to irregular masses depending on the intensity of the antigen. The maximal intensity of the stain was observed at 4-5 hours after infection.

Between 1-3 hours after infection, the number and intensity of positive cells, in cultures incubated with medium containing FCS differ from those in medium containing HuAS, as shown in Table 4. This difference was clearly seen in the cultures with lower virus multiplicity. This decreased ability to detect virus could be due to coating of some of the antigenic sites of HSV, by antiherpes immunoglobulin resulting in fewer positive cells and lesser degree of intensity when the cells were stained with the peroxidase labeled anti-guinea pig globulin. If peroxidase labeled anti-human globulin were applied to these preparations, it might have provided an answer to this hypothesis. None of these preparations showed cross-reactivity with the peroxidase labeled anti-guinea pig globulin. There appeared to be no difference between the two groups at 4, 5 and 6 hours after infection, with respect to the percentage of
Table 3

Detection of HSV by IP and isolation techniques in tissue cultures incubated with MEM + FCS and MEM + HuAS media at various times after infection

<table>
<thead>
<tr>
<th>Multiplicity Medium</th>
<th>Method</th>
<th>Detection (time after infection-hr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>FCS IP</td>
<td></td>
<td>1+</td>
</tr>
<tr>
<td>Isolation</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td><strong>150 TCID50</strong></td>
<td></td>
<td>1+</td>
</tr>
<tr>
<td>HuAS IP</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Isolation</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>FCS IP</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Isolation</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td><strong>30 TCID 50</strong></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>HuAS IP</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Isolation</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Uninfected FCS IP</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

* = degrees of intensity of the stain observed in majority of positive cells
FCS = fetal calf serum
HuAS = human antiherpes serum

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Table 4

Percentage of peroxidase positive cells recorded from infected tissue cultures with different multiplicity and culture conditions at various times after infection

<table>
<thead>
<tr>
<th>Multiplicity</th>
<th>Medium</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>150 TCID₅₀</td>
<td>FCS</td>
<td>12</td>
<td>67</td>
<td>81</td>
<td>79</td>
<td>88</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>HuAS</td>
<td>9</td>
<td>49</td>
<td>59</td>
<td>75</td>
<td>86</td>
<td>93</td>
</tr>
<tr>
<td>30 TCID₅₀</td>
<td>FCS</td>
<td>0</td>
<td>28</td>
<td>56</td>
<td>71</td>
<td>69</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>HuAS</td>
<td>0</td>
<td>8</td>
<td>33</td>
<td>72</td>
<td>68</td>
<td>85</td>
</tr>
<tr>
<td>Uninfected</td>
<td>FCS</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

FCS = fetal calf serum
HuAS = human antiherpes serum
positive cells and intensity of the stain.

At 6 hours after infection as shown in Table 3, the virus was detected by both methods in tissue cultures incubated with FCS containing medium, but in tissue cultures incubated with HuAS, virus could not be isolated although its presence was detected by the IP technique. This observation lends support to the hypothesis that the isolation technique may be less efficiency under some circumstances which decrease the content of viable virus.

The uninfected, negative control preparations did not show non-specific staining when tested simultaneously with the infected preparations.

CLINICAL SPECIMENS STUDY

Table 5 summarizes results from cultures of 51 clinical specimens from individuals who had stomatitis and labial lesions suspected of being caused by HSV infection. Of 31 labial lesions cultured, 20 yielded herpes simplex virus, and 11 gave no evidence of HSV activity. Of a total of 20 specimens cultured, HSV was isolated from 5 cases of stomatitis, in which one of these had labialis lesions at the same time. The other 15 specimens of stomatitis were negative for HSV.

To determine the relative efficiency of IP and isolation techniques for detection of HSV in clinical specimens, 0.5 ml of suspension from clinical materials was inoculated into tissue culture and the remainder of the suspension was used in the IP technique.
Table 5

HSV isolations from stomatitis and labial lesions

<table>
<thead>
<tr>
<th>Culture</th>
<th>Labialis</th>
<th>Stomatitis</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>20</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>Negative</td>
<td>11</td>
<td>15</td>
<td>26</td>
</tr>
<tr>
<td>Total</td>
<td>31</td>
<td>20</td>
<td>51</td>
</tr>
</tbody>
</table>

Table 6 and Table 7 represent the correlation between the IP and isolation methods for detection of HSV in 14 patients tested. Specimens from eye brow, back and finger lesions suspected of HSV infections were included. As indicated, of 10 cases positive by isolation, 9 were positive by IP and of 3 cases negative by isolation, 1 was positive by IP. One specimen from a finger lesion which was negative by isolation had insufficient cells for IP method and was not included in Table 7.

In the staining procedure, negative control wells receiving normal guinea pig serum or PBS and subsequently treated with peroxidase conjugate were included for each specimen. None showed any evidence of non-specific staining. HSV infected cells and uninfected Vero cells preparations were included as positive and negative controls respectively.
Table 6

Examinations of Clinical Specimens by IP and Isolation Procedures

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Sources</th>
<th>Isolation</th>
<th>IP</th>
</tr>
</thead>
<tbody>
<tr>
<td># 46</td>
<td>labialis</td>
<td>positive</td>
<td>negative</td>
</tr>
<tr>
<td># 47</td>
<td>labialis</td>
<td>positive</td>
<td>positive</td>
</tr>
<tr>
<td># 48</td>
<td>labialis</td>
<td>positive</td>
<td>positive</td>
</tr>
<tr>
<td># 51</td>
<td>labialis</td>
<td>positive</td>
<td>positive</td>
</tr>
<tr>
<td># 52</td>
<td>labialis</td>
<td>positive</td>
<td>positive</td>
</tr>
<tr>
<td># 53</td>
<td>labialis</td>
<td>positive</td>
<td>positive</td>
</tr>
<tr>
<td># 56</td>
<td>labialis</td>
<td>positive</td>
<td>positive</td>
</tr>
<tr>
<td># 57</td>
<td>labialis</td>
<td>positive</td>
<td>positive</td>
</tr>
<tr>
<td># 55</td>
<td>stomatitis</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td># 58</td>
<td>stomatitis</td>
<td>positive</td>
<td>positive</td>
</tr>
<tr>
<td># 59</td>
<td>stomatitis</td>
<td>positive</td>
<td>positive</td>
</tr>
<tr>
<td># 43</td>
<td>eye brow</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td># 44</td>
<td>back</td>
<td>negative</td>
<td>positive</td>
</tr>
<tr>
<td># 54</td>
<td>finger</td>
<td>negative</td>
<td>QNS</td>
</tr>
</tbody>
</table>

QNS = insufficient cells for IP study
Table 7

Correlation of IP with virus isolation in 13 patients tested

<table>
<thead>
<tr>
<th>IP</th>
<th>Virus isolation</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>3</td>
</tr>
</tbody>
</table>
Chapter 4

DISCUSSION

The principle problem associated with the immunoperoxidase technique experienced in this study was to obtain the maximal specificity as well as sensitivity of the immunoreagents used in the system.

The indirect immunoperoxidase (IP) test, like the immunofluorescent technique (FA), requires the participation of two antibodies (16). The first, guinea pig antiherpes serum, binds specifically with HSV antigens and then acts in turn as an antigen combining with the second antibody, an anti-guinea pig globulin. The test is positive when HSV antigens react with specific guinea pig globulin which in turn reacts with peroxidase-labeled antiglobulin. Sufficient peroxidase molecules are attached to HSV antigens to make them visible under appropriate staining. Because of this nature of the reaction, its individual characteristics play a key role in the test results, and this implies that the immunoglobulins used in the system should be strong and specific.

An attempt was made to prepare antiserum against HSV in guinea pig, but the low titer of specific antibody and the presence of non-specific reactivity which could not be removed by dilution or adsorption with tissue powder rendered them unsatisfactory for the immunoperoxidase study. Adsorption of the antiserum with tissue powder was helpful for reducing non-specific staining, but it involved loss of
the reagent and reagent activity. The problems of non-specificity and cross-reactivity encountered in the IP have been reported to be analogous to that found in FA technique, being dependent on the qualities of the two reagents (38,63), but the peroxidase conjugate was found to give fewer non-specific reactions (23).

The chessboard titration as recommended by Beutner et al (3) for standardization of immunofluorescent conjugate was found useful in this study as a means to predict the relationship of specific and non-specific staining properties of the reagents. The titer so obtained provided a convenient and meaningful indication of the potency of the reactants. The use of the proper dilutions of the reagents not only affords the advantage of economy use but also abolishes non-specific staining.

The guinea pig antiherpes serum prepared by CDC contained a high level of specific antibody as determined by chessboard titration. Non-specific staining was observed in this antiserum, but it was easily removed by dilution. As shown in Table 2, the antiherpes serum at its working dilution had no cross-reactivity with heterologous antigens. Therefore, it can be assumed that the material in both infected tissue cultures and in clinical specimens which combined with antiherpes serum and peroxidase conjugate consisted of antigens associated with HSV infections.

The disease (herpetic stomatitis) considered in this study presents clinical similarity to those caused by allergies and traumatic, metabolic or psychogenic disturbances (67). It is variously
estimated that between 20-50% of the population are affected with recurrent herpes labialis, recurrent stomatitis and related conditions (13,55,57), the frequency of recurrences varying widely from one lesion every few years to weekly episodes. For many of the so-called uncomplicated conditions, it is merely distressing and annoying. In the other categories, the symptoms can be alarming and required medical attention, particularly in the recurrent stomatitis group as, in some cases, the condition is so severe that as many as 100 lesions occur at the same time (13). The combined problems of various etiologies and lack of specific therapy in most cases have made this disease a difficult challenge. Numerous agents, such as topical antibiotics, antihistamines, smallpox vaccine, gamma globulin, corticosteroids and iododeoxyuridine have been used for therapy of these diseases with varying degrees of success (28,66,67). These agents are helpful in reducing the associated pain and severity of the lesions but do not prevent the recurrence.

Differentiation of the etiologic factors of these diseases by laboratory means is important, especially in view of the current trend toward the use of topical steroids and also in order to gain guidelines by which these diseases can be managed. In cases in which herpes simplex virus proves to be the etiologic agent, treatment with iododeoxyuridine is beneficial to the patients but the steroids seem to facilitate the spread of the virus. Treatment with steroid in conjunction with iododeoxyuridine was found useful in some cases (28).

A variety of laboratory procedures are available for detection
or diagnosis of HSV infections, but there are some technical or inherent difficulties that give a degree of inaccuracy to any one method or under some circumstances make it inapplicable.

In attempting to isolate HSV from clinical specimens by the culture method, the manner of obtaining specimens and the stage of illness are important in obtaining sufficient virus for detection. It is generally agreed that the specimen is richest in virus when taken as close as possible to the onset of the illness, but a more important and generally significant problem may be the loss of viability of the virus due to unsuitable transport media or effect of temperature (42) during its trip to the laboratory. The result of this study in tissue culture system (Table 3) suggests that the immunoperoxidase technique may serves as a better means to detect the virus in such instances.

Different types of tissue culture cells are used in diagnostic laboratories and these cells may vary significantly in their susceptibility to a small amount of HSV. It has been reported that rabbit kidney cell (RK) is superior to human embryonic lung fibroblast cell (WI-38), which in turn is superior to primary human embryonic kidney cell (HEK) for isolation of HSV (21,35). Moreover, physical and chemical factors also influence virus detection by culture method, as it was found that glutamine is required for RSV induction of classical syncytial cells (39) whereas glutamine inhibits giant cell formation by measles virus (48). The inability to isolate HSV from 11 of 31 specimens of labialis lesions (Table 5) could be due to these factors.

Speed and accuracy have always been of great importance in the
clinical laboratory. However, evidence of viral infections is most frequently obtained by serological or isolation procedures, which are expensive, complex and time consuming. It would be reasonable to assume that the IP technique could provide a possible answer to this need. As shown in this study, evidence of HSV infection could be detected as early as 4 hours (maximal staining), at which time neither the release virus nor CPE of HSV was observed. The time required for the IP technique to identify the virus was about 4-5 hours. This suggests that the IP could hasten the identification and/or typing in tissue culture of viral isolates that produce CPE slowly or not at all such as is the case with varicella-zoster. The use of IP for rapid identification and typing of human myxoviruses and paramyxoviruses in tissue culture has been reported (1).

There is an urgency to effect such early, correct etiologic diagnosis as in cases of encephalitis suspected of HSV infection, which is often a fatal disease. Treatment with iododeoxyuridine may be curative, and FA technique has proved useful to provide rapid diagnosis from brain biopsy specimens (27). In cases of pregnant women who may deliver in about 24 hours and have HSV infection in the birth canal, the availability of a rapid and accurate diagnostic test would be of great value, since it would allow the physician an opportunity to decide upon performing a cesarean section for prevention of disseminated herpes infection of the newborn.

It is unlikely, however, that the IP could be economically used for diagnosis of most ill-defined human diseases which are caused by a large number of virus serotypes.
Although the immunoperoxidase has proved useful in detection of viral antigens in cell culture system, there may be limitations of the method that could prevent detection of HSV infection in clinical specimens. As shown in Table 6, specimen #46 might have been interpreted as negative for HSV infection had the IP technique only been performed. Negative immunoperoxidase in this specimen could be due to not having enough virus-infected cells, even though a sufficient number of cells were on the smears. A specimen was considered suitable for IP if it contained at least 8 cells per well. The results shown in Table 3 and Table 4 also suggest that cells of more than 4 hours infection period would be suitable for the IP study. One of 14 specimens tested had insufficient cells for the study. The problems encountered in cytological diagnosis as to the quality of specimens have been discussed by Nahmias et al (143), who found that when all specimens were considered regardless of their cells population, FA technique was inferior to isolation in diagnosis of HSV infection.

In addition to the problem of obtaining suitable specimens, there is the problem of interpretation of IP preparation. This requires the inclusion of good controls before the tests can be considered reliable. Due to the problem of cross-reactivity encountered in the early part of this study, the IP technique was not applied to those 11 specimens (Table 5) of labialis lesions which were negative by the isolation technique. It would be interesting to know the relative efficiency of the two techniques in diagnosis of labialis lesions which are commonly accepted as recurrent forms of herpes infection.
The IP technique was able to detect HSV in one specimen from lesions on the back which were negative for HSV by isolation (Table 6). It is likely that this is not an instance of a false positive reaction but of other factors which may have resulted in negative virus isolation. Although comparison between the two techniques was performed on a small number of clinical specimens, it could be expected that the use of IP in conjunction with isolation technique in diagnosis of HSV infections would provide an increase in number of positive diagnosis.

It would appear that ulcerative stomatitis associated with HSV infection in adults is not as rare as it has been thought. The manner of obtaining specimens, mode of preservation, stage of illness and methods used in detection of the virus play a significant role in diagnosis of this disease.
Chapter 5

SUMMARY

The etiologic role of HSV in ulcerative stomatitis has been a subject of controversy whereas labial lesions are commonly accepted as recurrent forms of herpes simplex infection. Because of the lack of success in isolating the virus in most cases of stomatitis some observers believe that recurrent ulcerative stomatitis associated with HSV is uncommon.

The immunoperoxidase (IP) technique and virus isolation procedure were compared for their efficiency in detection of HSV infection in tissue culture and in clinical specimens. The comparison of the two techniques resulted from a hypothesis that the difficulties in finding the virus isolation procedure could be due to loss of viability or concentration of the virus in clinical specimens.

Vero cell cultures infected with HSV at different multiplicity and at different culture conditions were used as a model. The results of this study lend some support to the hypothesis and also suggest that cells infected for more than 4 hours would be suitable for IP study in clinical specimens.

Of 13 clinical specimens tested for HSV, there was correlation between results by both methods in 11 specimens, being positive or negative. One specimen from labial lesions which was positive by isolation was negative by the IP method. One specimen from back lesions
was positive by IP, whereas it was negative by isolation for HSV.

It is suggested that the use of IP in conjunction with standard isolation procedure could provide an accurate diagnosis in most cases. It is likely that ulcerative stomatitis associated with HSV is not as rare as it has been thought.
LITERATURE CITED


APPENDIX

1. **Ca\(^{++}\) and Mg\(^{++}\)-free Phosphate Buffered Saline (Pd)**

   Concentrated (10x) stock solution
   - NaCl 80.0 g
   - KCl 2.0 g
   - Na\(_2\)HPO\(_4\)·7H\(_2\)O 21.5 g
   - KH\(_2\)PO\(_4\) 2.0 g
   - Triple-distilled water 1,000.0 ml

   Working solution, pH 7.4
   - Conc. stock solution 100.0 ml
   - Triple-distilled water 900.0 ml

   This solution was sterilized by autoclaving at 18 lbs pressure, for 15 minutes and stored at room temperature.

2. **Chemical Composition of Versene-Trypsin Solution**

   In 1 liter of triple-distilled water
   - NaCl 8.0 g
   - KCl 0.4 g
   - NaHCO\(_3\) 0.58 g
   - Glucose 1.0 g
   - Trypsin 1.5 g
   - Versene 0.2 g

   To prepare versene-trypsin solution, all ingredients except versene and NaHCO\(_3\) were mixed with triple-distilled water, stirred for
1 hour and then the other two ingredients were added. This solution was sterilized by filtration through a Seitz-filter with a porosity of 20 μm, and stored in small quantities at -70°C.

3. Phosphate Buffered Saline (PBS), pH 7.1, 0.01 M

\[
\begin{align*}
\text{Na}_2\text{HPO}_4 \text{ (anhydrous)} & \quad 1.07 \text{ g} \\
\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O} & \quad 0.35 \text{ g} \\
\text{NaCl} & \quad 8.5 \text{ g} \\
distilled \text{ water to} & \quad 1,000.0 \text{ ml}
\end{align*}
\]

4. Carbonate Buffer, pH 9.5, 0.01 M.

Thirty-five ml of a solution of 0.1 M \text{Na}_2\text{CO}_3 was mixed with 65 ml of 0.1 M \text{NaHCO}_3 and 900 ml of distilled water was added to the mixture.

5. Reagents for protein estimation with Folin-Ciocalteu reagent

Solution 1: 1% \text{CuSO}_4 \cdot 5\text{H}_2\text{O} in distilled water
Solution 2: 2% Sodium tartrate in distilled water
Solution 3: 2% \text{Na}_2\text{CO}_3 in 0.1 N \text{NaOH}
Reagent A: Alkaline copper tartrate solution

100 parts of solution 3 plus 1 part of solution 1 and 1 part of solution 2. Freshly prepared solution was used for the test.

Reagent B: Folin-Ciocalteu phenol reagent obtained from Fisher Scientific Co., New Jersey, was diluted with distilled water to make it 1 N in acid.
6. Preparation of Freund's Adjuvant
   a) 8 parts light mineral oil
   b) 2 parts emulsifier (Arlacel A)
   c) 1 mg/ml of dried tubercle bacilli (TBC)

Two parts of Arlacel A was ground together with dried tubercle bacilli (1 mg/ml of Freund's Adjuvant) in a mortar, and 8 parts of light mineral oil was added and mixed well. An equal volume of guinea pig gamma globulin was then added drop by drop while continue grinding until a smooth water in oil emulsion was formed.

7. Kaplow stain

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% ethanol</td>
<td>100.0 ml</td>
</tr>
<tr>
<td>benzidine dihydrochloride</td>
<td>0.3 g</td>
</tr>
<tr>
<td>0.132 M (3.8% w/v) ZnSO₄·7H₂O</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>sodium acetate (NaC₂H₃O₂·3H₂O)</td>
<td>1.0 g</td>
</tr>
<tr>
<td>3% H₂O₂</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>1 N NaOH</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>Safranin</td>
<td>0.2 g</td>
</tr>
</tbody>
</table>

The ingredients were added in order listed, the pH was adjusted to 6.0 ± 0.05, filtered and stored at room temperature.