CHAPTER IV

MATERIALS AND METHODS

1. Cell culture

Vero cell line, a continuous cell line (an aneuploid cell line), was derived from the kidney of a normal adult, African green monkey *(Cercopithecus aethiops)*. The cells were obtained from the Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand.

Vero cells were cultivated in growth medium (GM) consisting of M199 (Earle's salt, GIBCO BRL, U.S.A) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (GIBCO BRL, U.S.A.), 100 units/ml penicillin G and 100 μg/ml streptomycin (GIBCO BRL, U.S.A.), 5% L-glutamine (Sigma, U.S.A.), and 0.01 M HEPES (N_2-hydroxyethylpiperazine-N'_2-ethanesulphonic acid) (Sigma, U.S.A.). These cells were maintained in maintenance media (MM) which were prepared the same as growth media except FBS concentration was reduced to 2%.

To passage the cell into 25 cm^2 tissue culture flask (Nunclon, Denmark), the culture media was removed and the cell monolayer was washed twice with 5 ml of 0.01 M phosphate buffer saline (PBS) (see Appendix II), pH 7.5 (pre-warmed to 37^0C before use) (see Appendix II). After discarding PBS, one ml trypsin-PBS was added. The culture flask was incubated for approximately 1-3 minutes (no more than 5 minutes) at 37^0C. After that the cell sheet was observed for breaking loose from the surface. The trypsin-PBS was then discarded and tap flask sharply against palm of hand to aid in loosening the cells. The 3 ml of GM was added into the flask. The cell suspension was mixed by drawing cells and fluid up and down in a pipette. One ml of suspended cell mixture was transferred to a new culture flask. Each culture flask was added 3 ml of GM. The culture flask was incubated at 37^0C, observed daily for growth of cell (three to five days) and for changing in pH of the medium. The cell monolayers were subcultured at three or four days intervals with a splitting ratio of 1:3 as described above.

2. Viruses

(2.1) HSV standard strain

Standard HSV-1 strain KOS, and HSV-2 strain Baylor 186 were provided by Associate Professor Dr. Vimolmas Lipipun, Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

Virus stock was prepared from Vero cell monolayers infected with the virus at an approximate multiplicity of infection (MOI) of 0.01 plaque forming unit per cell (PFU/cell). After an hour of viral adsorption at 37^{0}C, the unadsorbed virus was removed; the culture was washed once with PBS and replaced with MM. The infected Vero cells were incubated further for 36 to 48 hours or until more than 75% of the cell population showed cytopathic effect (CPE). Then, they were disrupted by being repeatedly frozen (at -70^{0}C) and thawed (at 37^{0}C in water bath) for three times. The disrupted cell suspension was pelleted by centrifugation at 4^{0}C, 2,200 rpm (IEC, U.S.A.) for 15 minutes. The supernatant fluid was decanted and distributed in small aliquots into vials and kept at -70^{0}C until use. The amount of viruses was determined by plaque titration assay.

(2.2) Clinical HSV specimens

All clinical specimens which were positive for HSV isolation during January 1998 to June 2002, at Virology Laboratory Unit, King Chulalongkorn Memorial Hospital, Bangkok, Thailand were used throughout this study. They were totally 121 swabs from suspected first episode and recurrent herpetic lesions at labia, eye, skin, and genital lesion.

(2.2.1) HSV isolation

The clinical specimens were cultured in Vero cells by the method of shell vial centrifugation cell culture (SVC) method (147). The SVC method employs centrifugation of the clinical specimen in vial containing a cell monolayer. The centrifugation step enhances viral replication and shortens the time to a positive culture result. This method is used primarily for detection of HSV, CMV, and VZV (148).

The positive infected cells were determined by indirect IFA using polyclonal antibody to HSV-1 antibody. After that the positive samples were then re-cultured in Vero cells to increase the viruses enough to type by IFA using MAb HSV type specific antibodies and screen for ACV^r HSV by PRA.

(2.2.2) Propagation of HSV positive samples

The virus from each clinical specimen was propagated in 12 well-plate (Nunclon, Denmark). The Vero cells were passaged into 12 well-plate ($1.5x10^5$ cells/ 2 ml/well) one day before use. Each clinical specimen was thaw rapidly. The volume of 0.2 ml of clinical specimen was mixed with the volume of 0.8 ml of MM and added to the well. The virus was allowed to adsorb for one hour at 37^0C (gently shake every 20 minutes while virus was adsorbed). After adsorption, the unadsorbed virus was removed. The culture was rinsed once with PBS and replaced with 2 ml of MM. The infected Vero cells were incubated at 37^0C and observed for five days until more than 75% of CPE were seen. The cells were scraped out from well. They were disrupted by being repeatedly frozen (at -70^0C) and thawed (at 37^0C) for three times. The disrupted cell suspension was pelleted by centrifugation (see Appendix I) at 4^0C, 2,200 rpm (ICE, U.S.A.) for 15 minutes. The supernatant fluid was decanted and distributed in small aliquots into vial and kept at -70^0C until use. If the first attempt in propagation the cells did not appear CPE more than 75% within 24 to 36 hours, the second propagation should be preformed to increase viral titer by using the virus from the first attempt. This cycle can be repeatedly until more than 75% within 24 to 36 hours of CPE was shown. Then the amount of viruses was determined by plaque titration assay.

3. Plaque titration assay

HSV is one of the viruses, which could form CPE. The CPE can be used to quantitate infectious virus particles by the plaque forming unit assay. Cells are grown as monolayer. After cells are infected with viruses, a semi-solid medium is overlayered. Therefore, any viral particles produced as the result of an infection cannot move far from the site of their production. A plaque is produced when a virus particle infects a cell, replicates, and then kills that cell. Surrounding cells are infected by the newly replicated

virus and they are killed. This process may repeat several times. The cells are stained with a dye which stains only living cells. The dead cells in the plaque appear as unstained areas on a colored background. However viruses which do not kill cells may not produce plaques. Since, these plaques originate from a single infectious virus; thus the titer of virus may be precisely estimated.

The number of viruses was titrated in 96 well-plate (Nunclon, Denmark) and the titer was expressed as PFU/ml. Briefly, the volume of 50 μl of each of the serial dilution of virus (10-fold) in MM was added in quadruplicate wells, followed by 50 μl suspended Vero cell suspension (3×10^4 cells) and incubated at 37^0C for three hours. Then, 50 μl of overlay medium (0.8% gum tragacanth in GM) (see Appendix II) was applied. The medium was discarded after four to five days after incubation at 37^0C and the infected cells were stained with 1% crystal violet in 10% formalin, for 20 minutes. The plate was washed in running water, air-dried and the number of plaques was counted. The viral titer is calculated from the data obtained in wells containing, if possible, between one-35 plaques.

$$\text{PFU/ml} = \frac{\text{Dilution} \times P_1 + P_2 + \ldots P_n \times 1}{n \qquad v}$$

where :

P = number of plaques counted in each well of one dilution

N = number of wells

V = volume inoculated in the flasks (in milliliters)

4. Typing of clinical HSV specimens by an indirect immunofluorescence assay (IFA) using monoclonal (MAb) HSV-type specific antibodies

Antigen-antibody reactions can be visualized or quantified by a variety of methods and markers. The most commonly used markers are fluorochromes, enzymes, electron-dense molecules, radiochemicals, and recently, bioluminescent and chemiluminescent markers. Viral antigens can be visualized at the cellular level using antibodies labeled with fluorochromes.

In indirect immunofluorescence, the viral-specific immunoglobulins from an animal or human are detected using conjugated antibodies prepared in another species and directed against the immunoglobulins of the first species. These antibodies can be specific for the heavy chains or the light chains, and to the Fc fragment or the Fab fragment. They are conjugated with FITC (Fluorescein isothiocyanate conjugate) and most are commercially available.

In this study, the detection of HSV in scraping infected cells using a specific antiserum prepared in the mouse was done. In an indirect method, the mouse antiserum is first allowed to react with the cells and then the unreacted serum is eliminated by washing. The mouse IgGs that have reacted with the viral antigens can be detected using an anti-mouse immunoglobulin conjugate prepared by injecting a goat with mouse immunoglobulin. The immunoglobulin are purified from the goat's serum and conjugated with the fluorochrome. The indirect method can also be used for the detection and guantitation of antibodies to a specific virus (149).

(4.1) Preparation of viral infected cells

The viral infected cells were prepared from Vero cell culture infected with a clinical isolate. The volume of one ml of Vero cell suspension containing 1.5×10^5 cells was plated in each well of 24 well-plate (Nunclon, Denmark). The cells were cultured one day. The adsorption of virus to the Vero cell monolayers was followed the protocol similar to propagation of HSV positive sample. After that, the infected Vero cell were

incubated until more than 75% of the cell population showed CPE. Then they were scraped from each well and cytospun by microcentrifugation at 15,000 rpm. (MicroCen 13, Germany) for one minute, the supernatant was removed. The cells were washed three times with PBS by gently resuspended the cell pellet. After discard the PBS, the infected cell suspension was smeared on 8 well of Teflon-coated slides, air-dried, fixed the cells by submerging them in acetone that has been pre-chilled to -20^{o}C for 10 minutes, removed the slide, air-dried for approximately 5 minutes and stored at -20^{o}C for at least 6 months.

(4.2) An indirect immunofluorescent staining

The acetone-fixed cells were added with 20 µl of 1:25 dilution of primary antibodies, either mouse MAb HSV-1 or HSV-2 type specific (NCL-HSV-1, HSV-2, NOVO cratra laboratory Ltd, United Kingdom) (diluted antibodies with PBS) and incubated at 37^{o}C in moist chamber for 30 minutes. Excess antibodies were removed and washed three times for five minutes with PBS and the fixed-cells were air-dried. The secondary FITC-conjugated antibodies, FITC-conjugated $(Fab')_2$ fragment of goat anti-mouse immunoglobulins (FO479, DAKO A/S, Denmark), dilution 1:20 was added to each well and incubated for 30 minutes at 37^{o}C. The fixed-cells were washed and stained with Evan's blue (1:10000) for five minutes, After that, the fixed cells were washed once with distilled water and air-dried. Then they were mounted with PBS-glycerol buffer and examined under a fluorescent microscope. The negative controls were the acetone-fixed Vero uninfected cell, applied with either MAb type specific, and the acetone-fixed HSV infected cell, applied with PBS instead of primary antibody, They were processed similar to the test system at the same time.

The observation was performed with fluorescence microscope (Olympus BX5O, Japan) with the FITC/Evan's blue (excited 495 nm/emitted 525 nm) filter and the 40X-objective.

(4.3) Interpretation of results

Positive: The apple green fluorescent staining will be observed in cytoplasm and/or nucleus of the infected cells.

To determine HSV type, the cells must be stained with one type specific MAb only. If they were reacted with both MAb HSV-1 and HSV-2 type specific, it indicated mixed infection.

5. Antiviral susceptibility testing

The basic principle of antiviral susceptibility testing is to assess the ability of an antiviral agent to inhibit viral growth. One of the most commonly used types of antiviral susceptibility assay is the plaque reduction assay (PRA).

The PRA is the first phenotypic antiviral susceptibility testing method. Unlike genotyping, this is a direct measure of viral susceptibility, reflecting the effects and interactions of all the mutations, known or unknown, on the behavior of the virus population in the presence of agents. Traditionally it has been the gold standard of resistant testing to which newer methods are compared. Then the PRA is intended to standardize susceptibility testing of HSV.

In this assay, the susceptibility of cytopathic viruses to antiviral agent is determined by the inhibition of viral plaque formation (zone of viral CPE analysis in the cell monolayer). This assay is performed in the presence of varying concentrations of antiviral agent to determine the effectiveness of the agent on the particular viral isolate, which is usually expressed as the fold-change in the IC_{50} (inhibitory concentration): the concentration of agent required to inhibit viral replication by 50 percent.

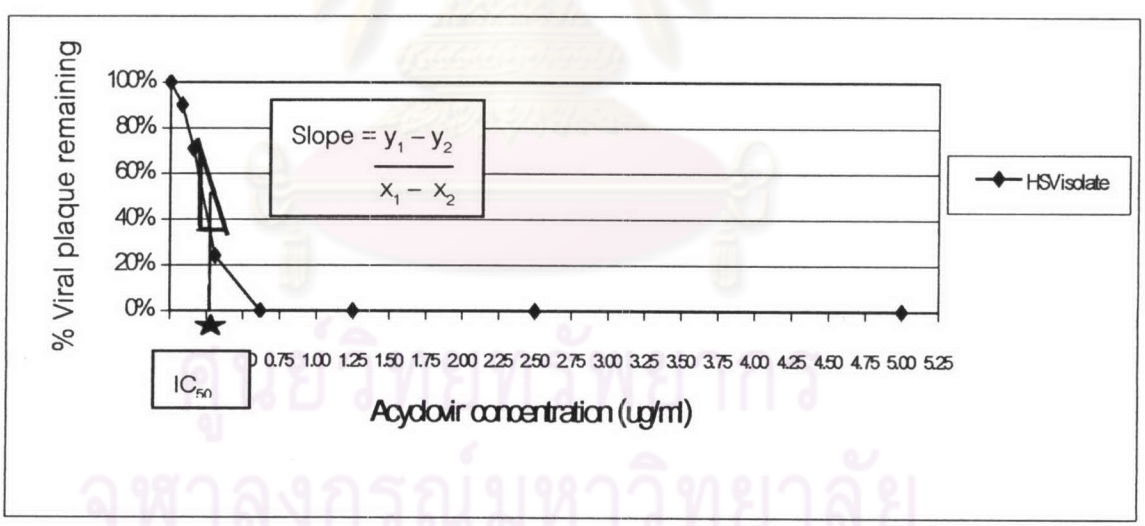
Antiviral susceptibility testing: In brief, HSV isolates were tested for their sensitivity to ACV by PRA in Vero cell to determine IC_{50} of ACV in 96 well-plate (Nunclon, Denmark). Cell monolayers (6×10^5 cells/ml) were infected with approximately 30 PFU of HSV isolate. After adsorption for three hours at 37^0C, the wells were overlaid with overlay medium (0.8% gum tragacanth in growth medium), which were already mixed with diluted ACV (Acycloguanosine, Sigma, U.S.A.), two-fold dilution starting from

concentration of 5 to 0.08 μg. Each dilution was done in quadruplicate. The plate was incubated for three to four days at 37^0C. Then, the overlay was removed, fixed and stained with a mixture of 1% crystal violet and 10% formalin for 20 minutes, washed and air-dried. Plaques were counted and IC_{50} was calculated from linear equation.

Interpretation of results:

The number of plaque was counted. The mean number of plaque in each dilution was converted to percentage of remaining plaque (mean number of plaque at various ACV concentration×100/ mean number of plaque at ACV=0). This number (%) was plotted against ACV dilution (Figure 5).

Figure 5: Determination of the IC_{50} value of ACV by plotting of relation between the percent of viral plaque remaining after incubation and each concentration of ACV.



★ = IC_{50} value

The IC_{50} value was calculated by linear equation

$$y = ax + C$$

y = Vertical; showed the percent viral plaque remaining

x = Horizontal; showed ACV concentration

C = constant value

a = slope; calculated from $\frac{y_1 - y_2}{x_1 - x_2}$

HSV isolates were considered susceptible to ACV when the IC_{50} was < 3 μg/ml and resistant when the IC_{50} was ≥ 3 μg/ml (127). The standard HSV-1 strain KOS and HSV-2 strain Baylor 186 were tested in each assay.