CHAPTER III

MATERIALS AND METHODS

Materials

1. Cell Culture

The HeLa cell culture, the continuous cell line established from the human cervical carcinoma, was kindly supplied by Department of Medical Sciences, Ministry of Public Health, Thailand.

2. Virus

The Herpes Simplex Virus type 2 (Strain LB), original from the University of Illinois at the Health Sciences Center, Chicago Illinois, U.S.A., was kindly supplied by Assistant Professor Pornthep Tiensiwakul, Department of Medical Technology, Faculty of Medicine, Chulalongkorn University.

3. Chemicals

Intravenous acyclovir (Zovirax, Wellcome, Lot A 3362 A) and pure chemical acyclovir (Wellcome Reference Substance, batch Q.A. 0667) were kindly provided by Professor Phairat Desudchit, Department of Preventive Medicine, Faculty of Medicine, Chulalongkorn University.

4. Culture Media

4.1 Growth Medium (GM)

The medium was used for the growth of cell cultures and was composed of ingredients which promoted rapid cellular proliferation.

4.2 Maintenance Medium (MM)

The medium was intended to keep the cell cultures in or slow steady state of metabolism during the time of viral replication, and it was less rich than GM in substances promoting rapid cellular metabolism and proliferation.

Methods

1. Preparation of Stock Virus

1.1 Preparation of Cell Culture

The procedure used for cell culture propagation were following (31, 37). The medium in cell monolayer was withdrawn and discarded. The monolayer of HeLa cells was washed with phosphate buffer saline (PBS) 2 times to remove trace amount of serum. The cells were then detached with trypsin-versine solution (TV), left at room temperature until the cells rounded up and discarded the TV-solution. The monolayer was washed 2 times with PBS. Afterwhich, the growth medium was added to the

cells, using a sucking-blowing action, and a small portion of the cell suspension was transfered to new bottle. Then incubated at 37°C until the cell sheet was confluent. At which point the cells were further passaged.

1.2 Propagation of Stock Virus

The propagation of HSV-2 from stock seed virus was carried out by the following procedure. The HeLa cells 10 mL were seeded into 8 OZ tissue culture bottles and incubated at 37°C for 24 h. Afterwhich, the monolayer of HeLa cells was washed with PBS 2 times and then infected with 2 mL of 10^{-1} dilution of HSV-2. The cultures were incubated at 37°C for 1.5 h to allow the virus to adsorb on the cells. After adsorption, the medium was discarded and 4 mL of maintenance medium was added into each culture. The cultures were again incubated at 37°C for 24-48 h, at which time the cells showed CPE 4+. The cultures were then frozen and thawed 3 times, and the cellular debris was removed by centrifugation at 3,000 rpm for 30 min. Thereafter, the supernatant fluid from the cultures were pooled, and 1 mL of the solution was dispensed into steriled vial. The stock virus was kept at -70°C until used.

1.3 Titration of Stock Virus

Quantitation of virus by plaque formation in monolayer of cell culture has been described for the determination of viral infectivity (31, 53, 54).

HeLa cells at the density of $3.5x10^5$ cells/mL were seeded onto 12 wells microtiter plate (Linbro) and incubated at 37°C in a moist chamber for 24 h. The monolayer was then infected with 0.1 mL of 10^{-3} to 10^{-6} dilutions of HSV-2, duplicated wells were inoculated for each dilution tested, and incubated at 37°C for 1.5 h with occasional tilting the plate to prevent cell drying. After this time, the infected cells were overlayered with 1.0 mL of 0.5% agar in Eagle's minimum essential medium supplement with 5% FBS, 0.15% sodium bicarbonate, 100 units of penicilllin/mL, 2 ug of amphotericin B/mL, 100 ug of streptomycin/mL and buffered with HEPES to pH 7.2. The temperature of the agar was adjusted to 40°C before being added on to the plate which was then placed at room temperature for 30 min until the agar solidified, and followed by an incubation at 37°C in moist chamber for 72 h. Thereafter, the plate was flooded with 10 % formalin for 30 min and the agar-overlays were carefully removed. The cells were then stained with dye-fixative solution for 10 min and rinsed off with tap water. Clear plaques appearing against the blue background were counted and calculated for numbers of plaque forming unit per mL (PFU) of the original virus suspension.

2. Cell Toxicity Assay

To determine the effects of acyclovir on HeLa cell proliferation, HeLa cells at the density of $5-30x10^3$ cells in 50 uL of 2xMEM and FBS were incubated with an equal volume of acyclovir at various concentrations to obtain

final concentration of 0-4 ug/mL of acyclovir. Following an incubation at 37 °C for 24 h, the supernate was removed and the cells were trypsinized with TV-solution, and left at room temperature until the cells rounded up. Thereafter, 50 uL of PBS with 10 % FBS were added and mixed until the cells were in good suspension. The 100 uL of cell suspension was then mixed with 100 uL of 0.5 % tryphan blue. These cell suspensions were then transfered to a hemocytometer chamber. After the cells were allowed to settle down, viable cells were counted under a light microscope within 10 min after cell mixing, the viable cells were reflected the light and not being stained but the dead cells were stained blue color. Finally, the number of viable cells per well were calculated (55).

3. Antiviral Infectivity of Acyclovir by Plaque Reduction Assay

Reduction in viral plaque numbers was used to determine the antiviral activity of acyclovir on HSV-2 infected HeLa cell (9, 30).

3.1 Dose-response of Acyclovir on HSV-2 Infection

To determine the optimal concentration of acyclovir and the optimal multiplicity of infection to be used in the test, HeLa cells at the density of 2.0×10^5 cells/mL were seeded onto 96-well microtiterplate (Linbro) and incubated at 37 °C in a moist chamber for 24 h. The monolayer was then infected with 0.1 mL of HSV-2 at

different multiplicities of infection (MOI 2.5, 5, 10 and 25). After an incubation at 37 °C for 1.5 h, the inoculum was aspirated off and the infected cells were washed three times with PBS. Thereafter, acyclovir at concentrations ranging from 0-4 ug/mL were added. The plates were incubated at 37 °C for 24 h. Afterwhich, the plates were frozen and thawed three times, and were centrifuged at 3,000 rpm for 30 min. Finally, the supernate was collected for plaque assay.

3.2 Time-kinetics of Acyclovir on HSV-2 Infection

To determine the effect of acyclovir on HSV-2 at different time course of infection, HeLa cells at the density of 2.0×10^5 cells/mL were seeded onto 96-well microtiterplate (Linbro) and incubated at 37 °C in a moist chamber for 24 h. The monolayer was infected with 0.1 mL of HSV-2 at multiplicity of infection of 10. After different time intervals of infection (1.5, 6 and 12 h), the inoculum was aspirated off and the infected cells were washed three times with PBS. Thereafter, acyclovir at concentrations ranging from 0-4 ug/mL were added, the plates were then incubated at 37 °C for 24 h. Afterwhich, the plates were frozen and thawed three times and were centrifuged at 3,000 rpm for 30 min. Finally, the supernate was collected for plaque assay.

4. Assay for Effect of Acyclovir on DNA Synthesis by ^{3}[H]-Thymidine Incorporation Technique

The effect of acyclovir upon total DNA synthesis by HSV-infected cells was determined by incorporation of ^{3}H-thymidine into the cells (10).

HeLa cells at density of 2.0×10^{5} cells/mL in a volume of 0.1 mL were cultured in sterile 96-well microtiter plate (Linbro) and incubated at 37 °C for 24 h. Confluent HeLa cells were infected with 0.1 mL of HSV-2 at different multiplicities of infection (MOI of 2.5, 5, 10 and 25). At various times after infection (1.5, 6, and 12 h), the inoculum was aspirated off and acyclovir at 50 uL volume in maintenance medium was added to cell culture to give the final concentration of 0-4 ug/mL. The cell culture were then pulse with 50 uL of 20 uCi/mL of tritiated thymidine (specific activity 6.7 mCi/mMol) to give a final concentration of 0.5 uCi per well for 24 h. After pulse labeling, the medium was removed and the monolayers were trypsinized with TV-solution and precipitated with 20% trichloroacetic acid. The precipitate were collected on microglass fiber filter paper (Whatman, NJ, U.S.A.) with the automatic microcell harvester (Model CH 103, Dynatech Laboratory, U.S.A) with 10 cycles of automatic washing. The air-dried glass fiber filter paper of each well was dipped in 3 mL of scintillation fluid in the scintillation vial (Kimbel, IL, U.S.A.) for β-scintillation counting, in a β-counter (model LSC-100 C, Beckman,U.S.A). The average cpm of double-duplicate samples were calculated and the results were expressed as

a percentage of uninfected controls.

5. Assay for Effect of Acyclovir on Polypeptide Synthesis of HSV-2 Infected Cells.

5.1 Preparation of HSV-2 Infected Cell Extracts

HSV-2 infected cell-extracts were prepared from confluent of HeLa cells infected with HSV-2 at multiplicity of infection of 10. At different times of infection (1.5, 6.0, and 12.0 h), the supernate was removed and acyclovir at concentrations of 0.2, 0.5, 1.0, 2.0, 3.0 and 4.0 ug/mL were added. The cultures were incubated at 37 °C for 24 h. After which, the cells were trypsinized with TV-solution and wash 2 times with PBS by centrifugation at 2,500 rpm for 10 min . Cell pellets were suspended in deionized distilled water, frozen and thawed three times, sonicated for 5 min and determined for the concentration of the proteins by Lowry's method (57). Thereafter, these HSV-2 infected cell extracts were diluted to 3,000 ug/mL with PBS and stored at -70 °C until used.

Immediately before electrophoresis, HSV-2 infected cell extracts were mixed in a 1:1 ratio with sample buffer, heated at 100 °C for 3 min and centrifuged for 5 min in an Eppendrof Microfuge. Thereafter, 20 uL of each sample was applied as closely as possible to the bottom of the well using a microliter syring.

5.2 Procedure of SDS-PAGE

SDS-PAGE was used to separate the molecular weight of HVS-2 polypeptides using the system modified from the method described by Laemmli (19).

Discontinuous SDS-PAGE was performed on a 18x20x0.1 cm vertical slab gel with 3% stacking gel and 8% resolving gel.

The resolving gel was prepared by mixing the 30% stock-acrylamide with 0.375 M, Tris-HCl (pH 8.8), 0.1% SDS, and distilled water. Then, added 0.025 % of TEMED and ammonium per sulfate to polymerize the gel. Swirled gently to mix, trying not to incorporate air. Immediately, the gel was pipetted into the prepared glass plate-sandwich to a height of 14 cm, and was carefully overlayed with distilled water after pouring to give a smooth interface after polymerization. Then, let the gel stand for one hour at room temperature. After polymerization, the overlay water was aspirated off and the gel surface was washed once with distilled water.

The stacking gel was prepared by mixing the 30% stock-acrylamide with 0.125 M, Tris-HCL (pH 6.8), 0.1% SDS, and distilled water. Then, added 0.025% of TEMED and ammonium persulfate to polymerize the gel in the same way as for the separating gel. The comb was placed between the glass plate-sandwich and the gel solution was added immediately to the top of the glass plate. Let stand for one hour at room temperature. After polymerization, a

small amount of distilled water was added on the top of the gel at the corners to prevent the drying at the surface of the stacking gel. Thereafter, the gel was kept at 4 °C for overnight.

Before assembly, the glass plate-sandwich was placed in room temperature and the comb was removed by gently pulling in a vertical direction. Then, the wells were washed with distilled water and followed by an electrophoretic buffer to remove any small fragments of polyacrylamide and unpolymerized monomers. Thereafter, the glass plate-sandwich were assemblied with the upper and the lower buffer chambers. After which the electrophoretic buffer was added to the chambers. The gel was prerun with constant current of 30 mA for 30 min. After prerun, the samples were applied to the wells and the gel was electrophorosed with a constant current of 15 mA in stacking gel, followed by 30 mA in resolving gel until the marker dye merely near the end of the gel. When electrophoresis was completed, the gel was cut into two parts. One piece was stained with 0.25 % Coommassie brilliant blue and destained. The another piece, a version of the former, was soaked in Towbin buffer prior to an electrophoretic transfer.

5.3 Procedure for Staining of the Gel

After electrophoresis or electrophoretic transfer, the gels were stained overnight in 0.25 %

Coommassie brilliant blue, and then destaind with several changes of destaining solution until background was cleared. The stained gel was fix in fixative solution for 2 h before drying at 80 °C under vacuum (LKB 2003 Slab Gel Dryer).

5.4 Western Blotting Technique

Electrophoretic transfer of proteins from the gel to nitrocellulose membrane was carried out using a modification of the method described by Towbin, et al. and Bernette (20, 60).

5.4.1 Preparation for Blotting

Following electrophoresis, the gel was equilibrated in the transfer buffer by changing the buffer several times during a relatively short pre-equilibration period. The nitrocellulose was wetted by slowly sliding it at a 45 °C angle into transfer buffer and allowed it to soak for 30 min. This was important to insure proper binding of the protein to the membrane. Two pieces of filter paper and two pieces of fiber pads were simultaneously soaked in transfer buffer.

5.4.2 Procedure of Western Blotting

After pre-equilibration, a pre-wetted fiber pad was placed on the gray panel of the holder and a piece of saturated filter paper was placed on top of the pad. The pre-equilibrated gel was then place on top of the

filter paper and a pre-wetted nitrocellulose was placed on top of the gel by holding the nitrocellulose at opposite ends so that the center portion was sagging and allowing the center portion to contact the gel first. Gradually lowered the ends of the nitrocellulose. This process was to expel any air bubbles. Carefully rubbed the nitrocellulose from side to side to push out all air bubbles. Thereafter, completed the sandwich by placing a piece of saturated filter paper on top of the nitrocellulose and a saturated fiber pad on top of the filter paper. The gel holder was closed and placed in the Trans-Blot tank so that the gray panel of the holder was on the cathode side of the tank. The tranfer buffer was added to fill the chamber. Electrophoretic transfer was performed at 250 mA for 3 h and followed by 50 mA for 18 h. After transfer, the nitrocellulose was removed, then rinsed with 0.1% Tween 20 in phosphate buffer saline and air dried. The nitrocellulose was kept at 4 °C in a plastic bag until used.

5.4.3 Immunostaining on Nitrocellulose Membrane

Prior to use, the nitrocellulose was cut into strips and blocked in 5% non-fat dry milk at 37 °C for 3 h. The nitrocellulose was then rinsed in 0.1% Tween 20 in phosphate buffer saline (PBS/T) 2-3 times and was incubated with rabbit anti-HSV-2 diluted 1:20 at 37 °C for 3 h and followed by an overnight incubation at 4 °C. Thereafter, the nitrocellulose was washed, five time with

10 min soaking time in PBS/T with constant shaking to remove the excess unbound antiserum and incubated with 1:50 peroxidase conjugated swine anti-rabbit globulin at 37 °C for 30 min. The excess unbound antiserum conjugated was washed with PBS/T three times (10 min soaking time) with shaking. After washing, the nitrocellullose was soaked in a mixture of 4-chloro-1-napthol substrate and H_2O_2 and further incubated at room temperature (in the dark) for 30 min. The reaction was stopped by rinse with deionized distilled water. The nitrocellulose was dried and stored in dark to prevent fading of the pattern prior to photograph.

5.5 Procedure for India ink Staining

After transfer, the nitrocellullose paper with the blotted proteins was washed 4 times for 10 min each with PBS containing 0.3% Tween 20 and then stained with a solution of Pelikan fount india drawing ink, 1 uL india ink/mL PBS-0.3% Tween 20, for 2-18 h (59). Agitation was required during the staining period. Afterwhich, the blotted nitrocellulose sheet was destained by rinsing with deionized distilled water. Record the migration distance of each protein band and determined the relative mobility (Rf) of the proteins by dividing distance of protein migration by distance of tracking dye migration. The Rf values were plotted against the known molecular weights on semilogarithmic paper and then estimated the molecular weight of unknown protein from calibration curve.