CHAPTER IV

MATERIALS AND METHODS

Part I. Preparation of cells and stock seeds of viruses

1. Cell cultures

Vero cells, a continuous cell line, initiating from the kidney of a normal adult African green monkey (*Cercopithecus aethiops*) on March 27,1962, by Y. Yasumura and Y. Kwakita at the Chiba University in Chiba, Japan (169). These cells were obtained from the Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand. HEp-2 cells, a continuous epithelial cell line derived from human epidermoid larynx carcinoma No.2, establishing by A.E. Moore, L. Sabachewski, and H.W. Toolan in 1952 from tumors of the larynx of a 56-year-old caucasian male, were obtained from the National Cancer Institute, Bangkok, Thailand. Jurkat cells, a continuous T cell line derived from human T cell leukemia. This cell line was established from the peripheral blood of a 14-year-old boy with acute lymphoblastic leukemia (ALL) at the first relapse in 1976, were kindly provided by Dr. Pokrath Hansasuta, Oxford University, United Kingdom.

Vero cells were grown in growth medium M199 (Earle's salt) while HEp-2 cells were in MEM medium and Jurkat cells were in RPMI 1640 (all from GIBCO BRL, U.S.A.). Growth media (GM) were the medium supplemented with 10% fetal bovine serum (GIBCO BRL, U.S.A.), 100 units/ml penicillin G and 100 μg/ml streptomycin (Dumex, Bangkok, Thailand) and 0.01M HEPES (N-2-hydroxyethyl-piperaine-N′-2-ethan sulfonic acid) (Sigma, U.S.A.). Maintenance media (MM) were prepared as growth media except the concentration of fetal bovine serum was reduced to 2%.

Dispersion of cell monolayers were performed by using trypsin-PBS (see Appendix II). The culture media was removed and the cell monolayers were washed twice with 5 ml PBS

prewarmed to 37°C. After discarding PBS one ml of trypsin-PBS was added and cells were incubated for one to two minutes at 37°C, then the culture flask was gently shook until the cells were detached, GM was added. The cell monolayers were subcultured at three or four day intervals with a splitting ratio of 1:3 for Vero and 1:5 for HEp-2 cells. Jurkat cells suspensions were subcultured at three days intervals with the same ratio as in HEp-2 cells. Vero cells were grown at 37°C while HEp-2 and Jurkat cells were grown at 37°C in 5% CO_2 atmosphere.

In experiments using activated Jurkat cells, cells were stimulated with PHA at 5 μg/2x10^6 cells/ml continuously, except indicated elsewhere.

2. Viruses

Standard HSV-1 strain, KOS (isolated from a lip lesion), and HSV-2 stranin Baylor 186 were provided by Associate Professor Dr. Vimolmas Lipipun of the Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

HSV-2 strain Baylor 186, (originally isolated from a penile lesion, by Dr. Priscilla A. Schaffer of the Department of Virology and Epidemiology, Baylor College of Medicine, Texas Medical Center, Houston, Texas.)

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°C, the unadsorbed virus was removed; the culture was washed once with 0.01 M phosphate buffered saline (PBS), pH 7.5 (see Appendix II) 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°C) and thawed (at 37°C in water bath) for three times. The disrupted cell suspension was pelleted by centrifugation at 4°C, 2,000 rpm (IEC, U.S.A) for 20 minutes. The supernatant fluid was decanted and distributed in small aliquots into vials and kept at -70°C until use. 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 infected with viruses, by using a semi-solid medium, any virus particles that 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 too are killed. This process may repeat several times. The cells are then 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 of suspended Vero cell ($3x10^4$ cells) and incubated at 37°C for three hours. Then, 50 µl of overlay medium (0.8% gum tragacanth in GM) was applied. The medium was discarded after four to five days after incubating at 37°C 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.

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

where:

P = number of plaques counted in all wells at this dilution

n = number of wells.

v = volume inoculated in the flasks (in milliliters)

Part II. Study of cell growth and viral growth in each cell types

1. Study of cell growth

Growth of Vero, HEp-2 and Jurkat cells were studied as followed. A total of $5x10^4$ Vero cells, $5x10^4$ HEp-2 cells or $1x10^5$ Jurkat cells was cultured in 24 well-plate (Nunclon, Denmark). Cell number was obtained by counting under the light microscope and cell viability was determined using tryphan blue staining. Cell number and viability was determined every 24 hours for six days. Each experiment was done in duplicate. Two experiments were performed.

2. Study of HSV-1 and HSV-2 growth in HEp-2 cells or Jurkat cells

Replication kinetic analysis was performed on HEp-2 cell monolayers, Jurkat cells suspension in 24 well-plate following infection with either HSV-1 or HSV-2 at three different MOIs (0.1,1 and 5 PFU/cell) and only MOI 5 in activated Jurkat cell (stimulated with PHA 5µg/ $2x10^6$cells/ml). Viral production was determined by plaque titration assay.

In brief, one ml of HEp-2 cell suspension containing $5x10^4$cells (standard plating concentration and volume throughout this study), was plated in each well of a 24 well-plate. The cells were cultured two to three days, after confluent cell monolayers approximately $2x10^5$cells were formed, they were washed twice with PBS, pH 7.5. Then 0.2 ml of each of the virus dilutions was added. After an hour of viral adsorption at 37°C, the unadsorbed virus was removed; the culture was washed once with PBS, pH 7.5 and replaced with one ml MM. HEp-2 infected cells were cultured at 37°C in 5% CO_2 atmosphere. The number of viruses was determined within the cells and supernatant (culture medium) collected at 12, 24 and 48 h.p.i. To harvest virus in the cells, after collecting the supernatant (kept at -70°C) the culture was replaced with one ml fresh MM, the cells were disrupted by being repeatedly frozen (at -70°C) and thawed (at 37°C in water bath) for three times. The disrupted cell suspension was pelleted by centrifugation (one minute at 11,000 rpm) in microcentrifuge tube (SRS, U.S.A). The supernatant fluid was then collected and kept at -70°C until assay.

In the case of Jurkat cells, cells were cultured two or three days at concentration $1x10^6$ cells/ml. Before the cells were infected with virus, Jurkat cells suspension was centrifuged at 1,500 rpm for 10 minutes at 4°C and then washed twice with PBS. The pellet was resuspended again in GM and the concentration of the cell suspension was adjusted to $2x10^6$ cells/ml. After that one ml of Jurkat cell suspension was plated in 12 well-plate, one ml of each of the virus dilutions was added then incubated at 37°C in 5% CO_2 atmosphere for one hour. The unadsorbed virus was removed by centrifugation at 1,500 rpm for five minutes at 4°C and then washed once with PBS, pH 7.5 and replaced with two ml of MM. Then 200 µl of Jurkat cell suspension containing $2x10^5$ cells was plated in each well of a 24 well-plate and adjusted volume to one ml by adding MM before incubated at 37°C in 5% CO_2 atmosphere. The number of viruses was determined with in the cells and supernatant (culture medium) collected at 12, 24 and 48 h.p.i. Viral harvesting from cells and supernatant were quantitated similar to HEp-2 cells.

Part III. Viral antigen detection.

1. Indirect-immunofluorescence detection of viral antigens

Using indirect immunofluorescence assay (IFA), it is possible to visualize or quantify the antigen-antibody reactions. The viral-specific immnoglobulins (antiviral Ig) raised from an animal are detected by using conjugated antibodies prepared in another species and directed against the immunoglobulins of the first species. These antibodies can be specific to the heavy chains or the light chains, and to the Fc fragment or the Fab fragment. They are conjugated with either fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) which are commercially available. As an example, the detection of HSV in Vero cells using a specific antiserum prepared in a rabbit. In an indirect method, the rabbit antiserum is first allowed to react with the infected cells and then the unbound serum is eliminated by washing. The rabbit IgGs that have reacted with the HSV antigens can be detected by using an anti-rabbit Ig prepared by infecting a swine with rabbit Ig. The immunoglobulins are purified from the swine's serum and conjugated with the FITC.

In the present study, HEp-2 monolayers approximately $2x10^5$ cells were grown on coverslips in 24 well-plate. The cells were infected with either HSV-1 or HSV-2 at MOI 5 and

incubated at 37 °C for two, four, six, nine and 24 h.p.i. At the end of the incubation period, the coverslips were taken out and washed twice with PBS, pH 7.5 (see Appendix II), air-dried, fixed in cold acetone (-20°C) for 10 minutes and kept at –20°C. In the cases of Jurkat and PHA-activated Jurkat cells, cells were grown in 6 well-plate at concentration of $1x10^6$ cells/ml and followed the protocol similar to HEp-2 cell. After incubating period, 200 µl of infected cells were collected in 1.5ml microcentrifuge tube. The cells were washed twice with PBS. After discard the PBS, the cell suspension was spot on 8 well slides, air-dried, fixed in cold acetone (-20°C) for 10 minutes and kept at –20°C (not more than two weeks).

For indirect immunofluorescent staining, the acetone-fixed cells were flooded with 1:80 dilution of the primary antibodies, rabbit-anti HSV-1 antibody or rabbit-anti HSV-2 antibody (polyclonal antibody, DAKO A/S, Denmark), 1:100 dilution of ICP0 antiserum, 1:250 dilution of ICP22 antiserum, 1:200 dilution of ICP47 antiserum (kindly produced by Prof. Dr. Bernard Roizman, University of Chicago, U.S.A.) and then incubated at 37°C in moist chamber for 30 minutes. Excess antibodies were removed by a 10 minutes washed with PBS, pH 7.5 and the fixed–cells were air-dried. The swine-anti rabbit antibody conjugated with FITC, dilution 1:40 was then applied to the fixed-cells and incubated for 30 minutes at 37°C. The fixed-cells were washed and stained with Evan's blue 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 (ratio 1:9) and examined under a fluorescent microscope. The negative control was acetone-fixed uninfected cell which processed similar to the test system. The infected cells were observed, scored and recorded for fluorescent pattern, localization and intensity of the fluorescent-conjugate of antiserum.

2. Flow cytometry detection of viral infected cells

Flow cytometry is a rapid, quantitative method for the multiparametric measurement of fluorescent cells. Flow cytometric analysis of HSV infected cells by immunoenzymatic system and by indirect immunofluorescence staining has been reported (170,171). By this technique, cells stained with a fluorescent dye can be detected and it is possible to correlate this parameter with other parameters of the individual cells in a population. In this study FITC-conjugated

antibodies were used to label virus infected cells so that cells expressing viral antigens could be separated from those without infection.

HEp-2 cells were infected with HSV-1 or HSV-2 at MOI 5 for 24 hours, then the culture media were removed and retained, the cells were washed with PBS and detached by two ml of trypsin about two minutes and the cells harvested in two ml trypsin. After that 0.5 ml of FBS was added and the cells were resuspended back in their culture media. The sample was centrifuged at 500 rpm for 10 minutes at 4°C, the pellet was resuspended again in cold MEM, supplemented with 1% FBS, and the concentration of the cell suspension was adjusted to $1x10^6$ cells/ml. In the cases of Jurkat and PHA-activated Jurkat cells, that infected cells suspension can be collected directly. Both HEp-2 and Jurkat infected cells were centrifuged at 1500 rpm for five minutes at 4°C, 0.02% EDTA 500 µl was added then incubated at 37 °C in 5% CO_2 for 10 minutes, washed with 3 ml cold washed buffer (see Appendix II). Then 500 µl of 4% w/v paraformaldehyde was added, vortexed and incubated at 37 °C in 5% CO_2 for 10 minutes. After these cells were washed with 3 ml cold washed buffer then 500 µl FACS permeabilizing solution was added and incubated at room temperature in the dark for 30 minutes, and washed once with three ml cold washed buffer. The infected cells being stained indirectly were then incubated with 50 µl specific antiserum (rabbit-anti HSV-1 antibody and rabbit-anti HSV-2 antibody, DAKO A/S, Denmark) at dilution 1:100 on ice for 30 minutes and washed with three ml cold washed buffer. Then infected cells were incubated with second antibody, 50 µl of diluted FITC-conjugated swine anti-rabbit antibodies (1:40) for 30 minutes on ice, washed twice as described above and added 200 µl 1% paraformaldehyde kept overnight in 4°C before analysis. For Jurkat cells, after adding second antibody and washed twice with cold washed buffer, 5 µl of periclinin chlorophyl protein-conjugated anti CD3 monoclonal antibodies (CD3/PerCP) was added and incubated at 4°C for another 30 minutes, washed once with cold washed buffer and added 200 µl 1% paraformaldehyde kept overnight in 4°C before analysis.

Part IV. The adsorption ability of virus

Adsorption of virus

HEp-2, Jurkat and one day PHA-activated Jurkat cells were grown in 24 well-plate. The number of cells was $2x10^5$ cells per well. The HEp-2 cells were inoculated with 0.2 ml of either HSV-1 or HSV-2 at MOI 5 while Jurkat and PHA-activated Jurkat cells were 0.5 ml inoculum (MOI 5). Adsorption process was allowed to occur at 37°C for one hour. After that, the inoculum was collected and kept at -70°C. The number of viruses was assayed by plaque titration.

Part V. Study of Hve A gene expression by RT-PCR

1. Samples

Jurkat cells were grown in RPMI 1640 GM (GIBCO BRL, U.S.A.) at 37°C in 5% CO_2 atmosphere for three days. The PHA activated Jurkat cells were cultured in the presence of 5 µg per $2x10^6$ cells/ml of PHA at 37°C in 5% CO_2 for 24 hours. Jurkat and PHA-activated Jurkat cells were collected into the sterile centrifuge tubes. The number of viable cells was determined by tryphan blue exclusion and hemocytemeter, at approximately $1x10^6$ to $5x10^6$ cells/ml. After centrifugation for one minute at 11,000 rpm and discarding supernatant, the cell samples were washed once with PBS before RNA isolation and RT-PCR analysis were performed.

2. Total RNA isolation

RNA isolation was done as previously described by Warner MS., *et al* 1998 (18). Total RNA was isolated using the RNeasy Kit (Qiagen, U.S.A.) according to the manufacturer's instructure. Briefly, each cells pellet approximately $1x10^6$ to $5x10^6$ cells, in a 1.5 ml eppendorf tube was added by 350 µl of lysis GITC buffer (buffer RLT) then homogenized the lysis mixture by a vortex mixer for two minutes. The 350 µl of 70% ethanol was added in the homogenized lysate and mixed it well by pipetting and vortexing. A precipitate may form after the addition of ethanol, but this will not effect the RNeasy procedure. Total of 700 µl of sample, including any

precipitate which may form, was applied to a RNeasy mini spin column sitting in a 2-ml collection tube, and the tube was centrifuged for 15 seconds at 11,000 rpm. After centrifugation and the supernatant was discarded, 700 µl washed buffer (buffer RW1) were pipetted on the RNeasy column, followed with centrifugation for 15 seconds at 11,000 rpm. Then, RNeasy column was transferred into a new 2-ml collection tube and washed once with 500 µl washed buffer (buffer RPE) for 15 seconds at 11,000 rpm. After that 500 µl washed buffer (buffer RPE) was added to RNeasy column again and centrifuged for two minutes at 11,000 rpm to dry the RNeasy membrane. Finally, RNeasy column was transferred into a new 1.5 ml collection tube, and 50 µl of RNase free water was added directly onto the RNeasy membrane. To elute the extracted RNA, the column was centrifuged for one minute at 11,000 rpm. The extracted RNA was aliquoted and stored at -20°C. The purified RNA concentration was determined by measurement of optical density (OD) at 260 nm and the purity of prepared RNA determined by ratio of OD at 260:OD 280, should have the ratio equal to or higher than 1.8.

3. cDNA synthesis

A reverse transcription (RT) reaction was performed on each mRNA sample, using up to an estimated 250 ng of total mRNA per tube and was reverse transcribed using oligo-dT $_{12\text{-}18}$ (Invitrogen, U.S.A.) as a primer for reverse transcription. The reaction took place in a total volume of 20 µl containing the following components: 5 mM $MgCl_2$, 1xRT buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl], 0.5 mM each of dNTPs, 0.3 µM Oligo $(dT)_{12\text{-}18}$, 0.01M DTT, 2.5 U/µl SUPERSCRIPT II RT, 2 U/µl RNase inhibitor, 0.1 U/µl *E.coli* RNase H and adjusted to the final volume by using DEPC-H_2O (all reagents were purchased from Invitrogen, U.S.A.). First, RNA sample, Oligo $(dT)_{12\text{-}18}$ primers and dNTPs were mixed and followed by denaturing at 65°C for five minutes and then annealing at 42°C for two minutes. After that, one µl of SUPERSCRIPT II RT was added and incubated at 42°C for 50 minutes to allow cDNA synthesis and terminated the reaction at 70°C for 15 minutes and removed RNA by adding one µl (two Units) RNase, incubated at 37°C for 50 minutes. The cDNA was then used for PCR or stored at -20°C for further use.

4. PCR amplification

The PCR amplification of HveA region using consensus primers, HVENT03 (5′) and HVEM228 (3′), nucleotide sequences of these specific primers were 5′ CAG GTT ATC GTG TGA AGG AG 3′ and 5′ TCT TCT TTT CAC ACA TAT GAT 3′, respectively (18). The human ß-actin primers used as an internal control primers, their nucleotide sequences were as followed: the sense primer (5′) was 5′ CCT TCC TGG GCA TGG TGT CCT3′ and the antisense primer (3′) was 5′ GGA GCA ATG ATC TTG ATC TTC 3′. The amplified HveA product was approximately 509 bp whereas, human ß-actin gene product was 202 bp. Since ß-actin is an ubiquitous housekeeping gene, it was used as a control in each of the samples to insure that the extracted samples have the total RNA messages in the similar amounts and no inhibitors were present in the PCR reaction. The ß-actin PCR carried out with a total volume of 50 µl containing 1X PCR buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl], 1.5mM $MgCl_2$, 0.2 mM each dNTPs, 0.2 µM 5′ß-actin primer, 0.2 µM 3′ß-actin primer, 2 units of Taq polymerase (BRL, U.S.A.). Before running PCR, 2 µl of cDNA was added. The DNA amplification was done using DNA thermocycler (Hybaid, U.S.A.). Each PCR cycle included 95°C for three minutes for one cycle, 95°C for two minutes and then at 60°C for one minute for 25 cycles. The PCR products were stored at 4°C until analysis.

Each cDNA sample obtained from 10,25,50,100 or 200 µg of total RNA was subjected for PCR analysis of HveA primers. For PCR reaction: two µl of RT product was used in a total volume of 50 µl containing the following components (Promega Corp.) in final concentrations: 1X PCR buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl] , 1.5 mM $MgCl_2$, 0.2 mM each dNTPs, 0.2 µM 5′HveA (HVENT03) primer, 0.2 µM 3′HveA (HVEM228) primer and two units of Taq polymerase (BRL, U.S.A.). The PCR was started at 94°C for two minutes, one cycle and followed by denaturing at 94°C for one minute, annealing at 58°C for another one minute and followed by extension at 72°C for one minute. The step was repeated for 35 cycles. In the last cycle, the extension period was allowed for five minutes.

5. Detection of amplification products

The amplified products were analyzed by gel electrophoresis (GE). This is a standard method used for separation, identification and purification of DNA fragments based on the principle of different DNA fragment mobility. Ten microliters of amplified product were performed in horizontal gel electrophoresis by using 1% agarose gel, consisting of 0.5 μg per ml ethidium bromide in 0.5X Tris borate EDTA buffer (TBE) (see Appendix II). The electrophoresis was carried out at 100 volts for 40 minutes and PCR products were visualized under UV light and a photograph was taken by a polaroid camera.