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ABILITY OF HSV REPLICATION IN HUMAN T-LYMPHOCYTES FROM HIV-INFECTED PATIENTS

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Herpes Simplex Virus (HSV) เป็นไวรัสติดเชื้อฉวยโอกาสที่สำคัญในผู้ติดเชื้อ HIV และมักจะทำให้เกิดการแพร่กระจายผ่านกระแสเลือดจนทำให้เกิดการติดเชื้อในทุกระบบ (generalized infection) ได้บ่อย ซึ่งสาเหตุน่าจะมาจากการที่ไวรัสสามารถเข้าไปเพิ่มจำนวนได้ใน T-lymphocytes ของผู้ป่วย จากการศึกษาก่อนหน้านี้พบว่า HSV สามารถเพิ่มจำนวนได้ใน T-lymphocyte ถึงแม้ว่าปริมาณไวรัสที่สร้างออกมานั้นจะต่ำกว่าใน epithelial cell แต่เมื่อทำการกระตุ้น T-lymphocyte ด้วย phytohemagglutinin (PHA) พบว่า HSV-1 สามารถเพิ่มจำนวนได้มากขึ้น ซึ่งกลไกที่ทำให้ไวรัสเพิ่มจำนวนได้มากขึ้นนั้นอาจเป็นเพราะมีการแสดงออกของ Herpes Virus Entry Mediator (HVEM) บนผิวเซลล์ของ T-lymphocyte มากขึ้นหลังจากได้รับการกระตุ้น ซึ่งการกระตุ้นด้วย PHA นั้นอาจเหมือนกับสภาพที่เกิดขึ้นในคนไข้ที่มีการติดเชื้อเรื้อรังเช่น ผู้ติดเชื้อ HIV

การวิจัยนี้จึงได้นำ T-lymphocytes ของผู้ติดเชื้อ HIV และคนปกติมาศึกษาการแสดงออกของ HVEM และยังได้นำ PBMC มาศึกษาการเจริญของ HSV-1 พร้อมทั้งจำแนกกลุ่มของ T-lymphocytes ที่ไวรัสสามารถเข้าไปเจริญเพิ่มจำนวนได้ นอกจากนั้นยังได้ทำการศึกษาผลของ HSV-1 ที่มีต่อการเพิ่มจำนวนของ HIV ด้วย

ผลการศึกษาพบว่าในผู้ติดเชื้อ HIV นั้นมีปริมาณ T-lymphocytes ที่ได้รับการกระตุ้น (activated T-lymphocytes: CD3+CD38+) มากกว่าในคนปกติอย่างมีนัยสำคัญทางสถิติ (p=0.001) ปริมาณเซลล์ที่มีการแสดงออกของ HVEM นั้นไม่มีความแตกต่างกันแต่ปริมาณการแสดงออกของ HVEM ในผู้ติดเชื้อมีน้อยกว่าในคนปกติและมีความสัมพันธ์แบบ inverse correlation กับจำนวนปริมาณของ CD4+ T-lymphocyte (p=0.03) จำนวนเซลล์ติดเชื้อ HSV-1 ใน T-lymphocytes ทั้งสองกลุ่มไม่แตกต่างกันแต่ผู้ติดเชื้อ HIV พบว่า CD4+ T-lymphocytes มีความไวในการติดเชื้อได้มากกว่า CD4+ T-lymphocytes ของคนปกติอย่างมีนัยสำคัญทางสถิติ (p< 0.001) ผลการเพิ่มจำนวนของ HSV-1 ในเซลล์ PBMC แสดงให้เห็นว่า HSV-1 สามารถเพิ่มจำนวนได้ในเซลล์ของผู้ติดเชื้อมากกว่าในคนปกติและมีการปล่อยไวรัสออกมานอกเซลล์มากกว่าที่อยู่ในเซลล์ การติดเชื้อของ HSV-1 ในเซลล์ PBMC ของผู้ติดเชื้อ HIV มีผลทำให้ปริมาณ HIV ลดลงเมื่อเทียบกับเซลล์ที่ไม่ได้รับการติดเชื้อ HSV-1

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ปีการศึกษา 2550 **ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์หลัก** .....

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HSV infection is an opportunistic infection found in HIV-infected patients. In such cases, the generalized infection is commonly occurred and may be caused by circulating HSV-infected T lymphocytes. Previous study demonstrated that HSV could replicate in T-lymphocytes but virion production has been shown to be lower than in epithelial cells. Moreover, the replication of HSV-1 increased in T-lymphocyte activated by phytohemagglutinin (PHA). One of these mechanisms may be upregulation of Herpes virus entry mediator (HVEM) receptor after PHA activation. PHA activation status may mimic *in vivo* situation such as chronic infection with other organisms for example HIV.

In this study, T-lymphocytes from HIV-infected patients and healthy individuals were determined on the expression of HVEM. Moreover, PBMC will be exposed to HSV-1. The number of HSV infected T-lymphocytes population and viral replications were investigated. In addition, HIV viral load was assayed to study effect of HSV-1 on HIV virion productions.

The results showed that the percentage of activating T-lymphocytes (CD3+CD38+) in HIV-infected patients was high significantly more than the healthy individuals (p=0.001) but not that of HVEM expressing T-lymphocytes. Although, HVEM expressing T-lymphocytes was not different but MFI of HVEM in HIV-infected patients was less than healthy donors and showed inverse correlation against CD4 counts (p=0.03). Moreover, the percentage number of HSV-1 infection in T-lymphocytes from healthy donors was barely the same as that in HIV-infected patients. CD4+ T-lymphocytes from HIV-infected patients was more statistically significant susceptible to HSV-1 infection than those from healthy donors (p<0.001). In addition, virion productions after infection with HSV-1 in PBMC of HIV-infected patients were significantly greater than those of healthy individuals and the virion production was preferred to release out from cells rather than retain in the cells. The HSV-1 infection on PBMC of HIV-infected patients resulted in decrease of the HIV production when compared to mock infected cells.

Field of study	Medical Microbiology	Student's signature .....
Academic year	2007	Principal Advisors's signature .....
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ABBREVIATIONS

ADCC	=	Antibody-Dependent Cell-mediated Cytotoxicity
APC	=	Allophycocyanin
AIDS	=	Acquired Immunodeficiency Syndrome
CD	=	Cluster of Differentiation
CPE	=	Cytopathic Effect
CTL	=	Cytotoxic T-lymphocytes
DDW	=	Double Distilled Water
DNA	=	Deoxyribonucleic Acid
E	=	Early
EDTA	=	Ethylenediaminetetraacitic
ER	=	Endoplasmic Reticulum
FITC	=	Fluorescein Isothiocyanate
gD	=	glycoprotein D
HHV	=	Human Herpesvirus
HIV	=	Human Immunodeficiency Virus
HS	=	Heparan Sulfate
HSV	=	Herpes Simplex Virus
HVEM	=	Herpes Virus Entry Mediator
ICP	=	Infected Cell Protein
IE	=	Intermediate Early
IFN	=	Interferon
Ig	=	Immunoglobulin
IL	=	Interleukin
L	=	Late
LAT	=	Latency-Associated Transcript

LIGHT	=	Lymphotoxin-like, exhibits Inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by T-lymphocytes
LTR	=	Long Terminal Repeat
MFI	=	Mean Fluorescent Intensity
MOI	=	Multiplicity of Infection
MHC	=	Major Histocompatibility Complex
NF	=	Nuclear Factor
NGF	=	Nerve Growth Factor
NLS	=	Nuclear Localization Sequences
ORF	=	Open Reading Frame
Ori	=	Origin of Replication
PBMC	=	Peripheral Blood Mononuclear Cell
PE	=	Phycoerythrin
PerCP	=	Peridinin Chlorophyll Protein
PFU	=	Plaque Forming Unit
PHA	=	Phytohemagglutinin
TAP	=	Transporter Associated with Antigen Processing
TK	=	Thymidine Kinase
TLR	=	Toll-like Receptor
TNF	=	Tumor Necrosis Factor
UV	=	Ultraviolet
VHS	=	Virion Host Shutoff Protein
VP	=	Virion Protein

CHAPTER I

INTRODUCTION

Herpes simplex virus (HSV) is a common human pathogen belonging to family *Herpesviridae,* subfamily *Alphaherpesvirinae*[1]. HSV can be divided into two serotypes, type 1 (HSV-1) and type 2 (HSV-2). HSV is a large (150-200 nm) enveloped virus with a distinct virion structure characteristic of the herpesviruses. A mature HSV virion consists of four parts; 1) an electron-opaque core containing viral DNA 2) an icosahedral capsid consisting of 162 capsomers, arrange around the viral DNA 3) an amorphous layer protein that surrounding the capsid calls tegument 4) an outer membrane envelope composes of lipids and proteins studded with viral glycoprotein spikes. The viral surface glycoproteins mediate attachment and penetration of HSV into cell and provoke host immune responses. There are at least a dozen, viral glycoprotein (g)B-N, but to date only five have been shown to have any role in viral entry. Entry of HSV into a cell proceeds in a step-wise fashion. First, virus attachment is initiated by interactions between viral gC or gB and heparan sulfate moieties on the host cell surface proteoglycans[2]. While this initial step enhances infection, it is not an absolute requirement because gC is dispensable for viral growth and replication *in vitro*. A cell that does not express heparan sulfate remains permissive at low levels. Second, virion attachment is stabilized by interactions between the gD and one of a number of recently identified cellular receptors, collectively referred to as Herpes viral entry (Hve) proteins.

The entry receptors discovered to date fall into three classes[3]. They include herpes viral entry mediator (HVEM or HveA)[4], a member of the tumor necrosis factor (TNF) receptor family ; nectin-1 (HveC) and nectin-2 (HveB), two members of the immunoglobulin superfamily and specific sites in heparan sulfate generated by certain isoforms of 3-O-sulfotransferase. Any one of these cell surface molecules can bind to gD to mediate viral entry, with each serotype having somewhat different receptor preferences. Whereas HVEM and nectin-1 are excellent entry receptors for both HSV-1 and HSV-2, nectin-2 is more active for HSV-2 than HSV-1 and 3-O-sulfotransferase is probably more active for HSV-1 than HSV-2. Finally, binding of gD to any one of these receptors triggers fusion of the viral envelope with the host cell membrane. This membrane fusion requires the action of gB and gH/gL heterodimer as well as gD and a gD receptor. Following fusion, the de-envelopment viral particle enters the cell and is subsequently targeted to the pores of the nuclear envelope[5]. The viral genome is translocated into the nucleus and a defined sequence of protein expression events commences that result in the production of viral progeny.

Productive HSV infection results in the formation of vesicular lesions in the mucosal epithelia that is the tissue tropism of its, followed by spread of the virus to the sensory neurons and establishment of a latent infection in dorsal root ganglia that may remain for the life of host. Reactivation from latency of dormant virus results in recurrent disease at or adjacent to the site of primary infection[6]. The common cold sores caused by HSV-1 and the genital herpes lesions caused by HSV-2 are not life-threatening conditions, but serious pathology can result from infections of the cornea (keratitis) or central nervous system

(encephalitis)[7]. Recurrent infection can be triggered by a multitude of different stimuli including physical trauma, fever, sunlight and stress. Many patients suffer with repeating recurrent infection which the mechanism is still unknown. HSV usually causes localized infection however the infection of newborns or immunocompromised individuals can commonly develop severe disseminated disease[8, 9].

Previous study demonstrated that both HSV-1 and HSV-2 could replicate in T-lymphocytes but virion production is lower than epithelial cells[10]. Moreover, the replication of HSV-1 increased in T-lymphocytes activated by phytohemagglutinin (PHA)[11]. One of these mechanisms may be caused by upregulation of HVEM receptor after PHA activation. PHA activation status may mimic *in vivo* situation such as chronic infection with other organisms. In case of HIV-infected patients, T-lymphocytes remain in activation state which expressed CD38 or HLA-DR more than healthy donors[12]. So, it is interesting to investigate whether the same phenomenon of HSV production will occur in T-lymphocytes of HIV-infected patients as that in a PHA activated T-lymphocytes.

CHAPTER II

OBJECTIVE

The objectives of this study are:

1. To determine HVEM and activating marker expression(CD38) in T-lymphocytes from HIV-infected patients compare with healthy individuals
2. To determine HSV replication in T-lymphocytes from HIV-infected patients compare with healthy individuals
3. To identify T-lymphocytes population to be infected with HSV

CHAPTER III

REVIEW OF LITERATURE

Herpesviruses

Herpes Simplex Virus (HSV), a member of herpesviruses belongs to *Herpesviridae* family[1]. The *Herpesviridae* is a large family, of which more than 100 herpesviruses are known to infect vertebrates. The *herpesviridae* contains several members of herpesviruses that affect humans. For the human herpesviruses, there are eight human herpesviruses have been identified to date, refered to as human herpesviruses (HHV)-1 through -8 but the original name of HHV-1 to 5 are still in common use (Table 1). All herpesviruses are capable of establishing latent infection in their natural hosts whereby they persist for a life of host.

Eight herpesviruses, differing markedly in their biologies, are known to infect humans. These include HHV-1 or herpes simplex virus types 1 (HSV-1), or HHV-2 or HSV-2, HHV-3 or varicella-zoster virus (VZV), HHV-5 or cytomegalovirus (CMV), HHV-4 or Epstein-Barr virus (EBV), HHV-6, HHV-7, and HHV-8 or Kaposi's sarcoma-associated herpesvirus. Based on genomic analysis and other biologic characteristics, the herpesviruses are classified into three subfamilies (Table 1). The *alphaherpesvirinae*, including HSV-1, HSV-2 and VZV, are neurotropic cytolytic viruses that can establish asymptomatic latent infections in neurons of the peripheral nervous system. The *betaherpesvirinae* includes CMV: so named because infected cells

become massively enlarged or “cytomegalic”. This subfamily also includes HHV-6 and HHV-7. Members of the *gammaherpesvirinae* subfamily are lymphotropic viruses which include EBV and HHV-8. Both HHV-8 and EBV are considered important cofactors in malignancies.

HSV is the first of the human herpesviruses to be discovered since ancient Greektime. The word “herpes” is derived from the Greek word “herpein” which means creep or crawl which refers to the spreading nature of visualized skin lesion[13]. HSV was isolated in 1938 by Dodd and others from the mounts of children exhibiting acute symptoms. In 1962, Schneweis KE, et al., demonstrated that there were two serotypes of HSV, designated HSV-1 and HSV-2[14].

HSV is a member of genus simplexvirus by the International Committee on the Taxonomy of Viruses [ICTV]. Alphaherpesvirus is responsible for a host of serious human ailments. Two human herpesviruses, HSV-1 and HSV-2 are the cause of cold sore and the genital herpes, respectively[7]. These two closely related viruses are most often responsible for primary and recurrent lesions in oral or genital mucosal epithelia, as well as serious lesions of the cornea that can lead to blindness, and more rare, and potential fatal, herpes simplex encephalitis. Both types exhibit tissue tropism in their *in vivo* infections. They most commonly infect epithelial cells of mucosal membranes and the sensory neurons that innervate those epithelia. The ability to infect highly differentiated cell types that are not common *in vivo* viral targets offers several advantages to the virus. First, the physical extent of the neuronal processes of the sensory neurons and their transport allows wide spread distribution of the virus throughout the host organism. Second, unlike

many viruses that kill their host cell outright, HSV has ability to become “latent” within neurons, a process by which there is down-regulation of viral progeny within the host cell, and it remains barely detectable for the life of the neuron unless reactivated. Reactivation of the latent virus by a number of factors leads to re-infection of and damage to tissue originally innervated by the sensory neurons, as well as spread of the viral infection to other area innervated by the neuron.

HSV has a broad host range and will infect a wild variety of cultured cells. They spread rapidly in cultured cells with a short reproductive cycle and efficiently destroy infected cells. HSV is endemic in virtually every human society throughout the world, from urban to remote native tribes. Human is the only natural reservoir for HSV and there are close associations recognized between HSV-1 strains and historical human populations.

Table 1: Human herpesviruses (HHV) in the *Herpesviridae* family.

Subfamily	Genus	Virus name		Biological properties		
		Official name	**Common name**	**Host range**	**Reproductive cycle**	**Latency**
Alphaherpesvirinae	Simplexvirus	HHV-1 HHV-2	Herpes Simplex Virus type 1 [HSV-1] Herpes Simplex Virus type 2 [HSV-2]	Variable, often broad	Short	Sensory ganglia
	Varicallovirus	HHV-3	Varicella-Zoster Virus [VZV]			
Betaherpesvirinae	Cytomegalovirus	HHV-5	Cytomegalovirus [CMV]	Restricted	Long	Variable in many cells include secretory glands, lymphoreticular cells, kidney and others
	Roseolovirus	HHV-6 HHV-7	HHV-6 HHV-7			
Gammaherpesvirinae	Lymphocryptovirus	HHV-4	Epstein-Bar Virus [EBV]	Limited to family of natural host	Relatively long	Specific for either B or T lymphocytes
	Rhadinovirus	HHV-8	Kaposi's sarcoma associated herpesvirus [HHV-8]			

Virion structure and genome

HSV is a large enveloped virus approximately 150-200 nm in diameter with a distinct virion structure characteristic of the herpesviruses. A mature HSV virion contains four components[1]: i) an electron-opaque core containing viral linear double-stranded (ds) DNA packaged as a tightly wrapped spool or toroidal form ii) an icosahedral nucleocapsid consisting of 162 capsomers arranged around a DNA core iii) an amorphous protein layer outside the capsid called “tegument” surrounding the capsid and iv) an outer lipid membrane envelope exhibiting viral glycoprotein spikes (Figure 1).

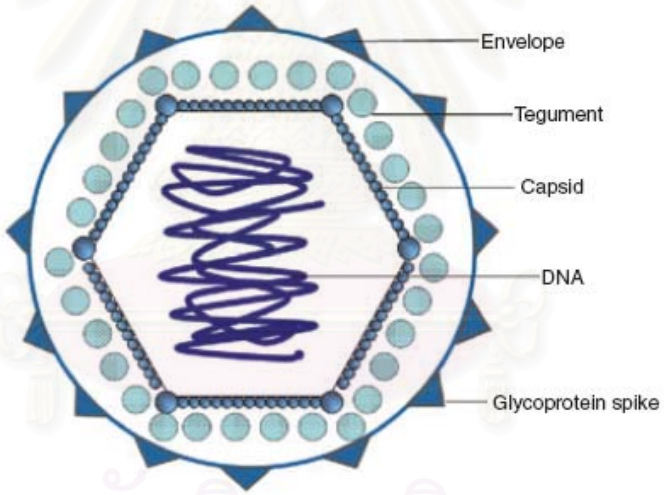


Figure 1: HSV virion structure is comprised of four components; 1) the viral DNA; 2) an icosahedral capsid shell; 3) a tegument layer; 4) lipid membrane envelope studded with viral glycoproteins.

The HSV genome is linear double-stranded DNA approximately 152 kilo-base pairs (Kbps) in length with a base composition of 67 % G+C. The genome is composed of two covalently linked segments, call

Long (L) or Short (S), base upon their relative length. These segments each consist of two segments of unique DNA, refer to as the unique long (U_L) and unique short (U_S) region. Each unique segment is flanked by inverted repeats of DNA (R_L and R_S) shows in Figure 2.

Figure 2: Schematic representation of the HSV genome. The approximately 152 kbps genome is divided into a U_L and U_S region. The unique sequences are separated by inverted repeats (shown as boxes and designated as R_L and R_S).

The HSV genome can be divided into 6 regions.

1) The ends of the linear molecules-the "a" sequences. These are important in both circularization of the viral DNA, and in packaging the DNA in the virion.
2) The 9,000 bp long repeat (R_L), encodes both an important immediate early regulatory protein (ICP0) and the promoter of and most of the "gene" for the latency-associated transcript (LAT).
3) The long unique region (U_L), which is 108,000 bp long, encodes at least 56 distinct proteins [actually more because some open reading frame (ORFs) are spliced and expressed in redundant ways]. It contains genes for the DNA replication, enzymes and the capsid proteins, as well as many other proteins.

4) The 6,600 bp short repeat (R_S) encodes the very important an immediate early protein. This is a very powerful transcriptional activator. It acts along with ICP0 and ICP27 (in the U_L) to stimulate the infected cell for all viral gene expression that leads to viral DNA replication.
5) The origin of replication of long segment (ori_L) is in the middle of the U_L region. The ori_S is in the R_S and thus, is present in two copies. All sets of ori's operate during infection to give a very complicated replication complex very similar to that seen in the replication of phage T4.
6) The 13,000 bp unique short region (U_S) encodes 12 ORFs, a number of which are glycoproteins important in viral host range and response to host defense.

These genes of HSV encode at least 84 ORFs that could potentially code for viral proteins. Of the proteins potentially encoded for, by the HSV genome, it is estimated that more than 40 accompany the viral particle itself while the remainder are thought to be involved in processes with in the host cell resulting in production of viral progeny or ensuring the survival of the progeny by interfering with the immune system. Viral genes and their products are generally named by their relative position from left to right in the U_L or U_S region (for example U_L1, U_L2 or U_S1, U_S2). However, many viral proteins are described alternated names base upon their functions, such as Infected Cell Protein (ICP) number, Virion protein (VP) number. The genes found in the unique regions are present in the genome as a single copy, but genes that are encoded in the repeat regions, such as ICP0 or ICP4, are present two copies in the genome.

Viral entry

The ability of the viruses to infect cell types *in vivo* is a function both of the specific virus-host cell interaction, as well as with the immune system effects on both infected cells and extracellular infective viruses. *In vitro* where immune properties of the animal are not playing a role, herpesviruses have a much wider, but still not ubiquitous, host cell range. Much of the work that has been done examining binding of virus to host cell surface has made use of cultured cells, such as the chinese hamster ovary-derived epithelial cell line (CHO), that are not permissive for HSV infection[15]. Introduction of plasmids coding for various candidate receptors into these cells coupled with the use of virus containing mutated or deleted viral glycoproteins has allowed considerable insight into mechanisms of viral binding.

The process of viral entry into a host cell is usually initiated by binding of the virion with various receptor molecules on the host cell plasma membrane, followed by fusion of the virion envelope with the membrane (Figure 3). Entry of HSV into cells depends upon multiple functions of viral envelope glycoproteins and multiple cell surface receptors[3]. There are a dozen of viral glycoproteins is predicted, glycoprotein (B-N), only five (gB, gC, gD, gH, gL) of which have defined roles in viral entry. First, virus attachment is initiated by interactions between virion gB and gC and heparan sulfate (HS) moieties on the host cell membrane. The interactions of HSV gB or gC with cell surface heparan sulfate are also not sufficient for viral entry. Second, virion attachment is stabilized by interactions between the gD and one of a number of cellular receptors. The HSV entry receptors divided into three classes, herpes virus entry mediator (HVEM) or HveA[4], a

member of the tumor necrosis factor (TNF) receptor family; nectin-1 (HveC)[16] and nectin-2 (HveB)[17], two members of the immunoglobulin superfamily and specific site in heparan sulfate generated by certain isoforms of 3-*O*-sulfotransferase[18]. All of them can bind to gD to mediate viral entry. Whereas HVEM and nectin-1 are excellent entry receptors for both HSV-1 and HSV-2, nectin-2 is more active for HSV-2 than for HSV-1 and 3-*O*-sulfotransferase is probably more active for HSV-1 than HSV-2[2]. Binding of gD to any one of these receptors triggers fusion of the viral envelope with a cell membrane by an undetermined mechanism that appears to require the action of gD , gB, and the gH-gL heterodimer[19]. After that, the viral tegument proteins are released into cytoplasm of the host cell.

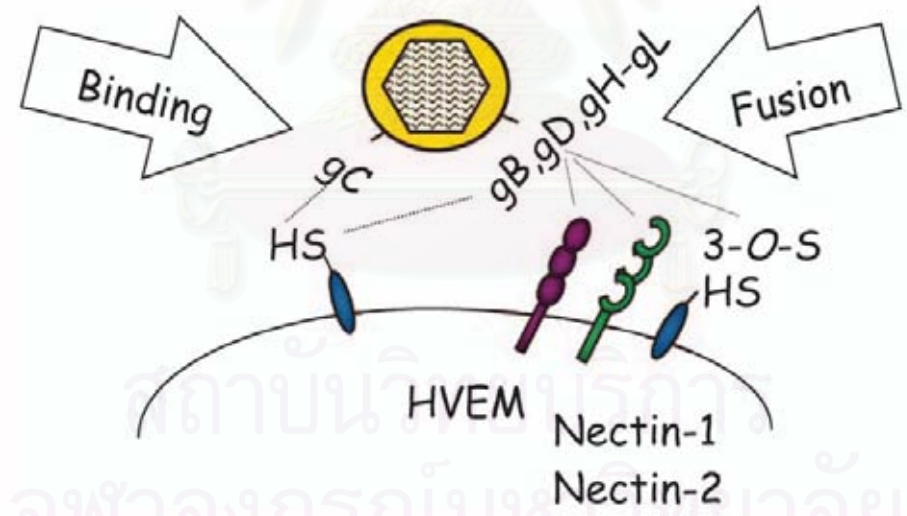


Figure 3: Cell surface receptors and viral ligands that participate in HSV entry.

Heparan sulfate binding

Heparan sulfate (HS), one several kind of glycosaminoglycan[20], is preferred and considerable to be a binding receptor. Heparan sulfate is thought to be ubiquitously expressed, at least on cells that stay put in tissues, as opposed to circulating cells of immune system. Two of the virion glycoproteins, designated gB and gC, are capable of binding to heparan sulfate. Early work clearly indicated a role for the fairly ubiquitous cell surface molecule heparan sulfate proteoglycan in viral particle binding. The binding itself seemed to be governed primarily by the actions of the gC homolog glycoproteins within the different viral envelopes. The gC glycoprotein appears to be more of a facilitator for infection rather than a strict requirement. The lack of gC on the viral envelope, or the lack of heparan sulfate on the cells does not prevent, but rather lowers the efficiency of infection[21, 22]. When gC is deleted, other envelope glycoproteins, gB or gD, are thought to be able to substitute for gC. The fact that heparan sulfate proteoglycans are found on the surface of a large number of different cell types suggests that the binding of gC to the surface is not a feature that confers tissue tropism for the herpes viruses.

Interestingly, when polarized epithelial cells Madin-Darby Canine Kidney (MDCK) cells were tested[23], it was found that gC-mutant viruses were unable to bind to or infect apical surfaces of those cells, but were quite capable of infecting the basolateral surfaces. Similarly, lack of gG prevents infection of the apical surface of MDCK cells in culture, and gC and gG both appear to be required to infect the apical surfaces of corneal epithelial cells *in vivo*. The structures in heparan sulfate to which

gB and gC bind have not been determined[24]. However, it is clear from competition studies that gB and gC bind to different structures.

gD interactions with HVEM

The viral glycoprotein, gD, appears to play a much greater role in the infective process. Both treatments of cells with soluble forms[25] of the gD protein, as well as infection in the presence of antibodies to the gD protein block viral infection[26]. At least three classes of receptors for the gD glycoprotein have been identified on mammalian cells[3]. The first receptor reported was a member of the tumor necrosis/nerve growth factor family, called HVEM (also HveA or TNFRSF14)[4]. It was originally identified by its ability to allow viral entry into infection resistant CHO cells, once the cells had been transfected with a plasmid coding for the HveA protein. HveA was later shown to directly bind to gD of HSV-1 and HSV-2[27].

HVEM is fairly abundantly expressed in lymphoid tissues including T and B lymphocytes, other leukocytes but not neurons or brain[4, 28]. HVEM is a principle receptor for HSV entry into activated human T lymphocytes, but not for a number of other human cell types. The viral ligand for HVEM is gD whereas the natural cellular ligands for HVEM are LIGHT *(*lymphotoxin-like, exhibits inducible expression, and competes with HSVglycoprotein D for HVEM, a receptor expressed by T-lymphocytes) and lymphotoxin α[29]. Glycoprotein D can interfere with the normal interaction between HVEM on LIGHT and lymphotoxin α. So, the interaction between gD and HVEM is of greater importance in regulating immune reaction to virus than viral infection of neurons. The X-ray crystal structure of the extracellular domain of HVEM bound to a

truncated soluble form of gD has been solved, and has led to some insight into the specific domain on the gD molecule that actually binds to the receptor[30]. There is some conformational flexibility in the binding domain that might account for some of the differential binding of the gD molecules from different viruses.

gD binding to nectin-1 (HveC) and nectin-2 (HveB)

A second type of receptor for the gD molecule has been found to be adhesion molecules localized to adherens junctions of epithelia as well as synaptic junctions of neurons, nectin-1 (HveC) and nectin-2 (HveB)[17, 31, 32]. Nectin, a transmembrane member of the immunoglobulin superfamily, is thought to interact with intracellular actin via a second protein afadin, and thus function as part of a cell-cell adhesion system that organizes adherens junctions or synaptic junctions[33]. Unlike HveA, nectin is abundantly expressed in a variety of tissues and cell types, including epithelial cells, fibroblasts and neurons. Nectin have four types, nectin-1 to nectin-4. Nectin-1, -2 and -3 are broadly expressed in a variety of tissues and cells. Nectin-4 appears to be localized to the placenta in humans. Only nectin-1 and netcin-2 have been shown to mediate HSV entry, through interactions with the viral ligand gD. Despite the fact that HSV, all appear to be able to utilize this receptor to effect entry, structural requirements for interaction of the gD molecule with the receptor vary with the source of the gD molecule. In addition, although there is a strict requirement for the presence of gD to effect HSV-1 entry, it does not appear to require much gD on the surface, as mutant virus expressing extremely low amounts of gD can enter cells that express high amounts of HveC[34]. Expression patterns and the

cellular localization of the nectin suggest that nectin receptors might be able to confer some degree of tissue tropism to the virus.

gD binding to modified 3-O-sulfotransferase

The third receptor for gD is produced by the modification of specific glucosaminyl 3-*O*-sulfotransferases of heparan sulfate on the surface of cells. Under activated enzyme condition, HSV-1 was able to infect CHO cells but not HSV-2[20]. This action was reversible; when the modified HS on the surface was treated with heparitinase, the cells were once again resistant to infection[18]. This binding to modified HS molecules is apparently not mediated by gC, but rather gD. Thus, even in the absence of other cellular receptors, simple modification of the HS on the outside of a resistant cell made it possible to achieve infection.

Fusion of the viral envelope to the host cell plasma membrane

After viral attachment step, the series of events that lead to cause the fusion of the viral envelope and the host cell membrane, is less well understood. Fusion of the viral envelope with the host cell plasma membrane takes place at physiological pH and temperature[35]. The ability of individual glycoproteins to effect viral entry is examined as well as the ability of those glycoproteins to allow direct cell-to-cell spread[36], exocytosis of the viral progeny or to produce syncytia in culture. These all processes require the expression of fusogenic properties, yet it appears that not all glycoproteins participate equally in each activity. For viral entry, the current evidence is that gD, gB and gH-gL complex are required for entry[19, 37], while other glycoproteins (gE, gI, gM or the U_L45 gene product) have been argued to be involved in

other membrane fusion events[38, 39]. Moreover, the binding of gD to its receptor is required as the initiating event for the fusion process between the viral envelope and the plasma membrane[40, 41]. It also appears that HVEM receptor might play a role in how gD exerts its influence in certain cell types.

In addition to gD, deletion of gB, or the gH-gL complex produces mutant viruses that can bind but not enter[42-44]. gL, which lacks a transmembrane domain, is a glycoprotein that appears to be required in the normal processing of the gH glycoprotein, which is a transmembrane protein of the viral envelope. Crosslinking studies demonstrated the adjacent localization of these four glycoproteins (along with gC) on the viral envelope. The finding that the identity of the crosslinked glycoproteins changed over the timecourse of viral entry suggested that these glycoproteins might be undergoing changes in conformation as a result of the entry process[45]. Co-expression of these four glycoproteins in COS cells increases the formation of syncytia of the cultured cells, a result that cannot be achieved through expression of any one or two proteins[37, 38]. While these four glycoproteins have thus been strongly implicated as being essential for fusion and entry, again, their specific roles and mode of interaction remain unclear.

Lytic infection

In human, lytic infection generally occurs in epithelium. Viral entry involves binding of viral glycoproteins to cell membrane receptors, penetration of the virion by fusion of the viral envelope with the cell plasma membrane, virion proteins release, and capsid transports to the nuclear pore, where viral DNA is released into the nucleus. Upon

penetration to the nucleus, a temporally program of viral gene expression mediated by RNA polymerase II of host cell begins. HSV gene expression is temporally regulated in three distinct phases, i.e., immediate early (IE) or α, early (E) or β and late (L) or γ genes.

During the early stages of the infection, at least two virion proteins induce viral control over host cell function. The first protein, virion host shutoff protein (VHS), enters the cytoplasm and remains there to cause degradation of the cellular and viral RNA. The second protein, alpha-trans-inducing factor, VP16 a component of the tegument of the virion, induces transcription of viral IE genes required for virus replication. HSV replication requires efficient and ordered expression of three temporal classes of the viral genes in a tightly regulated, coordinated cascade fashion *in vitro*. Similar transcriptional events are assumed to be regulated during latency and reactivation *in vivo*.

The first genes transcribed during viral infection are IE genes. IE genes expression does not require HSV prior protein synthesis and is stimulated by VP16-induced complex. A virion tegument protein, VP16, plays an important role in enhancing the expression of the IE proteins[46]. VP16 interacts with a number of cellular proteins. First, VP16 associates with HCF-1 in the cytoplasm and translocation of VP16 into the nucleus after that it is primed for associated with Oct-1 on "TAATGARAT" regulatory elements present in each IE-gene promoter (Figure 4)[47-49]. Five IE genes encode ICP0, ICP4, ICP22, ICP27 and ICP47. Four of the five IE proteins play critical roles in the regulation of HSV gene expression. ICP4 and ICP27 are essential regulatory proteins in all experimental systems *(in vitro* and *in vivo).* In general, ICP4 represses IE genes expression and activates E or L genes expression by

interacting with RNA polymerase II transcription factors[50, 51]. ICP27 is required for transition from IE genes expression to E and L genes expression[52, 53]. ICP0 and ICP22 are dispensable in at least some systems but the evidence indicates that each plays an important regulatory role in the viral gene regulation. ICP0 is required for IE promoter activation, but a separate domain activates E or L promoters. Thus, ICP0 can activate the expression of all classes of viral genes, in large part because it increases steady-state levels of mRNA. ICP0 also binds several cellular proteins: (i) elongation factor 1, (ii) cyclin D3, and (iii) ubiquitin-specific protease. These activities promote virus replication in differentiated cells[54]. ICP27 is also required for transition from IE genes expression to E and L genes expression[52].

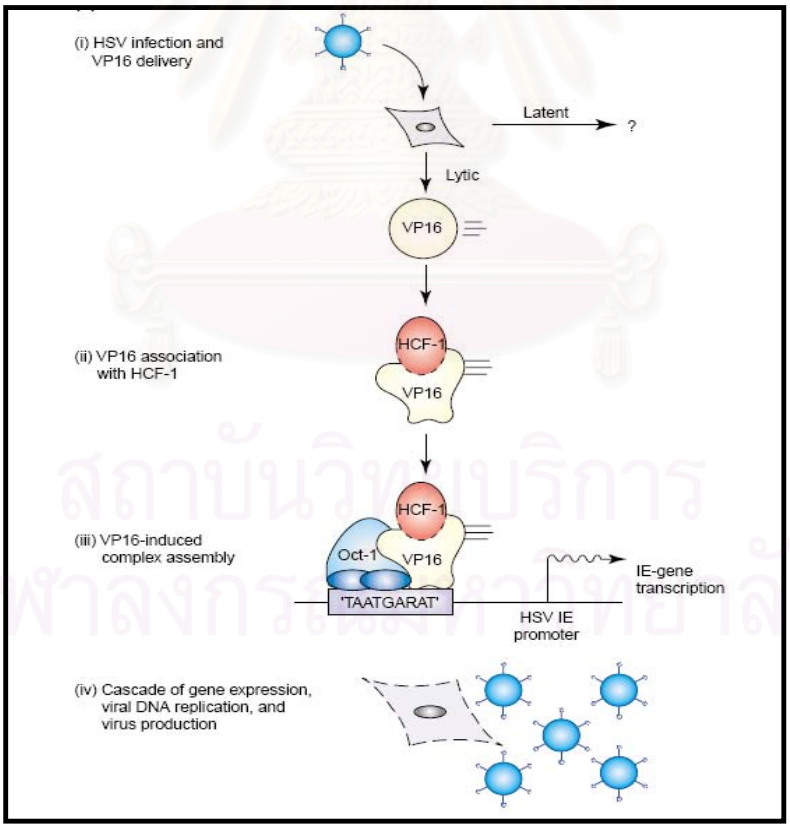


Figure 4: Role of VP16-induced complex in the HSV lytic cycle.

Activation of the host cell transcriptional machinery by the action of IE gene products, results in the expression of the E genes. The promoters for such genes (exemplified by the thymidine kinase transcript promoter) have served as models for "typical" eukaryotic promoters. IE proteins activate E genes expression, and viral DNA replication ensues. The E proteins include the enzymes that are required for replication of the viral genome: DNA polymerase; single-stranded DNA-binding protein (SSB), also known as ICP8; DNA helicase-primase; origin-binding protein (U_L9) and those enzymes involved in nucleotide metabolism.

L genes expression is also activated by ICP0, culminating in virion assembly and release. Thus, IE genes are essential for particle, ICP0 is very important for productive infection because it activates all classes of viral promoters and is expressed at high levels throughout infection. Prior to DNA replication, IE proteins initiate the transcription of not only the E genes, but also a subset of the L genes, called early/late, leaky late, or $\gamma 1$ genes. The synthesis of these proteins, while later in the infection cycle, is not strictly dependent on viral DNA replication. However, their levels are significantly enhanced upon initiation of DNA replication. A second subset of late genes, the true L or $\gamma 2$ genes, is transcribed only after the initiation of viral DNA replication.

The promoters of these genes do not provide much evidence about the control of their transcription, as there are no specific sequences recognizable by viral transcription factors. One of the important regulatory events in the cascade of gene expression is the shutdown of IE and E genes expression. There are two defined examples of this. ICP4 represses itself by binding to repressor elements in its own promoter[55]. The decrease in ICP4 level leads to a reduction of E and L genes

expression, as ICP4 is essential for those functions. ICP8 plays a role in reducing transcription from the parental genome following viral DNA replication[56, 57]. It is thought that one of the major triggers between E and L genes transcription is a movement of the transcription machinery from the parental genome to the progeny genomes. Therefore, if transcription from the parental genome is limited, IE and E gene expression is reduced.

In addition, to stimulate the expression of its own proteins, HSV shuts down host cell RNA, DNA and protein synthesis. HSV uses numerous cellular proteins during its gene expression for example: VHS[58]. Presumably, to gain access to these proteins, the virus disrupts host transcription, replication and translation.

Viral replication

HSV DNA replication occurs in specialized structures formed in the nucleus of infected cells that have been called replication compartments[59]. Viral DNA and viral DNA replication proteins are localized adjacent to cellular structures called ND10 sites, and replication compartments form at these sites as viral DNA synthesis starts[60]. The linear input viral DNA circularizes upon entry into the nucleus. Viral DNA replication initiates at one of the three origins (oriL or two of oriS) of replication within the HSV genome by U_L9, origin-binding protein, binds to specific site at an origin and starts to unwind the DNA. The single-stranded DNA binding protein, ICP8, is recruited to the unwound DNA and associated with U_L9 to recruit the five remaining replication proteins to the replication forks. DNA synthesis is believed to proceed initially via a theta replication mechanism. Once DNA synthesis begins, it

is likely that a rolling-circle replication mechanism takes over to produce the majority of the progeny genomes in an infected cell. Seven herpes genes were identified by genetic and biochemical methods as essential for viral DNA replication. These seven genes encode protein products that function as an origin binding protein (U_L9), a single-stranded DNA binding protein ICP8 (U_L29), a helicase-primase complex ($U_L5/U_L8/U_L52$) and a DNA polymerase (U_L30/U_L42). While many protein products required for viral DNA synthesis are encoded, other cellular factors, such as DNA ligases and topoisomerases, may also function in the viral DNA replication process.

In addition to seven essential viral replication proteins, HSV expresses several other early viral gene products, such as thymidine kinase (U_L23), ribonucleotide reductase (U_L39/U_L40) and uracil N-glycosylase (U_L2). These proteins are considered “nonessential” for viral DNA replication. While these gene products are dispensable for replication in some cell culture models, they are required for growth in certain tissue *in vivo* or for productive infection of non-dividing cells. Many of the “nonessential” genes found in HSV encode protein products that function in nucleotide metabolism. These activities may be critical *in vivo* because nucleotide pools, which are present in abundance in actively dividing cells, are limiting in resting cells, such as neurons. These proteins may give the virus a growth advantage either during production infection in certain tissues or during reactivation from latency in neurons.

Viral assembly and egress

Assembly of the viral capsid requires synthesis of numerous late proteins and occurs within the nucleus. Because some capsid proteins

lack nuclear localization sequences (NLS) (i.e. VP5, VP26, VP23), these proteins must form complexes with NLS-containing proteins (i.e. VP19C or pre-VP22a) in the cytoplasm for transport into the nucleus. A mature HSV capsid is comprised of an outer shell containing penton-shaped subunits of the major capsid protein, VP5, and hexons of VP5 and VP23[61, 62]. These subunits are connected by triplex structures formed by the two minor capsid proteins, VP19C (U_L38) in one copy and VP23 (U_L18) in two copies. Two non-structural genes encoding multiple proteins, U_L26 (VP21 and VP24) and $U_L26.5$ (pre-VP22a, VP22a), are also necessary for efficient capsid formation. VP21, pre-VP22a, and VP22a are scaffolding proteins. VP24 is a serine protease required for capsid maturation. Capsid assembly does not require any cellular factors and can be completed *in vitro* using purified viral protein components[63].

Empty capsid shells are loaded with viral DNA by a process that simultaneously resolves concatemers and packages genome-length monomers within the capsid to form a nucleocapsid. The mechanism of DNA cleavage and packaging is not well understood; however, it is known to require site-specific breaks to the concatemers at specific distances from the *pac1* and *pac2* sites[64, 65].

The rout of virion egress from an infected cell remains somewhat controversial. It has been observed that the nucleocapsid can bud through the inner nuclear membrane, acquiring some tegument proteins and a glycoprotein studded envelope in the process, but two pathways have been proposed from this point. A re-envelopment model[66] suggests that enveloped virions fuse with the outer nuclear membrane, thereby releasing free nucleocapsid into the cytoplasm that re-envelope by

budding into the Golgi compartment. These re-enveloped particles are secreted from the cell by a vesicular route. A lumenal pathway model proposes that enveloped virions traffic from the inner nuclear space to the Golgi in vesicles or within the lumen of the endoplasmic reticulum (ER), and are released from the cell by a normal secretory route. Recent evidence using electron microscopy and ER-retrieved glycoproteins lends considerable support to the re-envelopment model as the major route of virus egress[5, 67].

During herpesvirus infection, viral transcription, DNA replication, formation of capsids, and packaging of viral DNA occur in the nucleus. Subsequently, intranuclear capsids have to leave the nucleus to gain access to the extracellular environment. Herpesvirus maturation including primary envelopment of capsid by budding at the inner leaflet of the nuclear membrane. Two conserved herpesvirus proteins, the products of the U_L31 and U_L34 genes are involved in this process. The U_L34 gene encodes a type II C-terminally anchored membrane protein that is located in both leaflets of the nuclear membrane. The U_L31 gene codes for a nuclear phosphoprotein that is also present in the nuclear membrane of infected cells. The subsequent step in herpesvirus maturation is perinuclear primary enveloped virion in the perinuclear space and fusion of primary envelope with the outer nuclear membrane and translocation of capsids into the cytoplasm. Tegumentation occurs in the cytoplasm that follows an intricate network of protein-protein interactions with significant built-in redundancy. Tegumented capsids obtain their final envelope by budding into vesicles of the trans-Golgi network. During re-envelopment, the orientation of glycoproteins in the vesicle membrane is such that the cytoplasmic tails may make contact with tegument proteins for driving the final budding process (Figure 5).

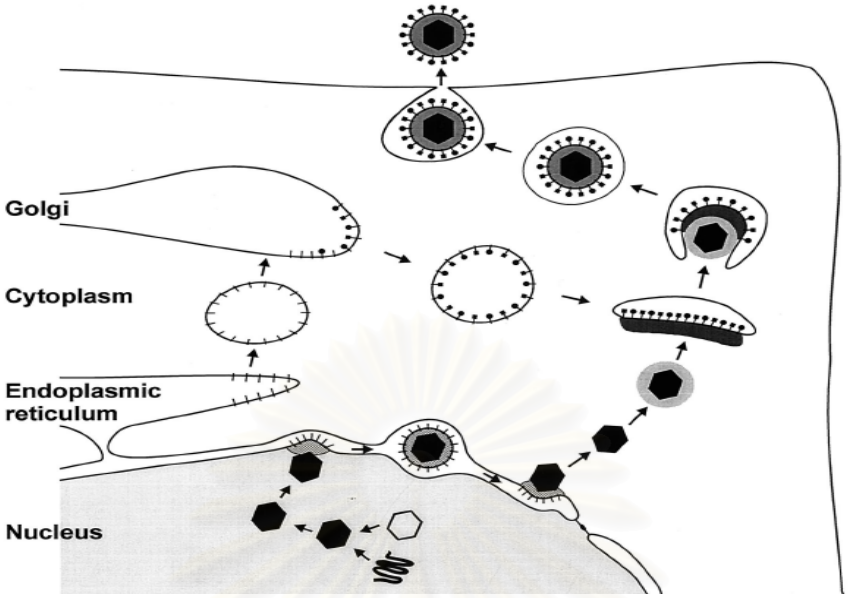


Figure 5: Summary diagram of the proposed pathway of herpesvirus egress: re-envelopment model.

Clinical manifestations of HSV infection

HSV is a human pathogen that exhibits tissue tropism in its *in vivo* infection and most commonly infects epithelial cells of mucosal membranes. Infection with HSV can result in several diseases ranging from inapparent infections and self-limiting cutaneous lesions to fatal[7]. HSV-1 normally associates with orofacial infections and encephalitis, whereas HSV-2 usually causes genital infection and neonatal infection. Productive primary viral infection in peripheral tissue is initiated from an active infection of another individual, and limited replication occurs with ensuing tissue-associated cytopathologic changes and formation of virus-fills vesicles in the immediate vicinity of the site of original infection. The primary infection resolves within 2-3 weeks concomitant with seroconversion, and no evidence of virus infection is seen. The majority of primary oral infections is due to HSV-1 and results in mild

gingivostomatitis that often goes undiagnosed. In severe cases there can be ulceration in the mouth and pain can make swallowing difficult. HSV-2, less commonly HSV-1 can cause genital infection, leading to a sexually transmitted disease. Following primary infection and recovery, virus enters sensory nerve terminals and is transported retrogradely *via* axon to neuronal cell bodies in the trigeminal or dorsal root ganglia for HSV-1 and sacral dorsal root ganglia for HSV-2 where latency is established that may remain for a long life of host[6]. Periodic reactivation results in anterograde transport of viral particles, shedding from the neuron, and re-initiate a lytic replication cycle, which leads to asymptomatic shedding or recurrent lesions. Recurrent infection can be triggered by a multitude of different stimuli including high levels of UV light, stress, hormonal changes, physical trauma, and immunosuppressive infections with other viruses. A minority of individuals seropositive for HSV-1 suffers with recurrent infection but genital infections recur more frequently than oral infection. The genital ulcers are more painful than oral cold sores and can be a major clinical problem, although they do eventually tend to remit spontaneously.

Latency

One of the hallmarks of all herpesvirus infections is the ability of the virus to establish a latent infection that can last the lifetime of the host. Unlike a persistent infection, in which the virus is constantly replicating at some level, during HSV latency, no viral progeny is produced and only very limited gene transcription is detected. The current operational definition of latency is an infection in which the viral genome is present in a non-replicating state in an infected cell from which the virus can intermittently reactivate. The major site of HSV latent infection

is sensory neurons in ganglion tissue, either trigeminal ganglia for HSV-1 or sacral ganglia for HSV-2. After the initial primary infection, generally at an oral or genital mucosal surface, the virus travels along the innervating neuronal axon to the neuronal cell body. Once within the neuron, the virus enters a quiescent state in which the lytic gene products are not produced. During latency, the HSV genome remains in the nucleus of the neuron as circular, extra-chromosome DNA[68, 69]. In neurons latently infected with HSV, the only abundant viral RNA detected is a family of transcript referred to as the latency-associated transcripts or LATs. This transcriptional silence may allow the virus to remain hidden in the cell by avoiding immune surveillance. The virus remains in this state for the lifetime of the host, or until the proper signals reactivate the virus and new progeny is generated. Progeny virus then travels through the neuron axist to the site of the primary infection to re-initiate a lytic replication cycle. The signals and mechanisms involved in this process are poorly understood, but it appears that certain physical stresses, such as illness or exposure to ultraviolet light, increase the chance to reactivation.

The steps of the latency-reactivation cycle have been operationally divided into three major steps: establishment, maintenance, and reactivation (Figure 6). Establishment of latency includes entry of the viral genome into a sensory neuron and acute infection. Virus then enters nerve termini and moves down axon *via* retrograde transport to the sensory ganglia. Viral gene expression is then extinguished, with the exception of the LATs. Subsequently, maintenance of latency occurs and establishes for the lifetime of the host. LAT is the only known viral transcript that is abundantly expressed during this stage of latency. The HSV genome is sequestered in this nonreplicating state in the neuronal

nucleus, circular extra-chromosomal DNA or episome. This transcriptional silence may allow the virus to remain hidden in the cell by avoiding immune surveillance.

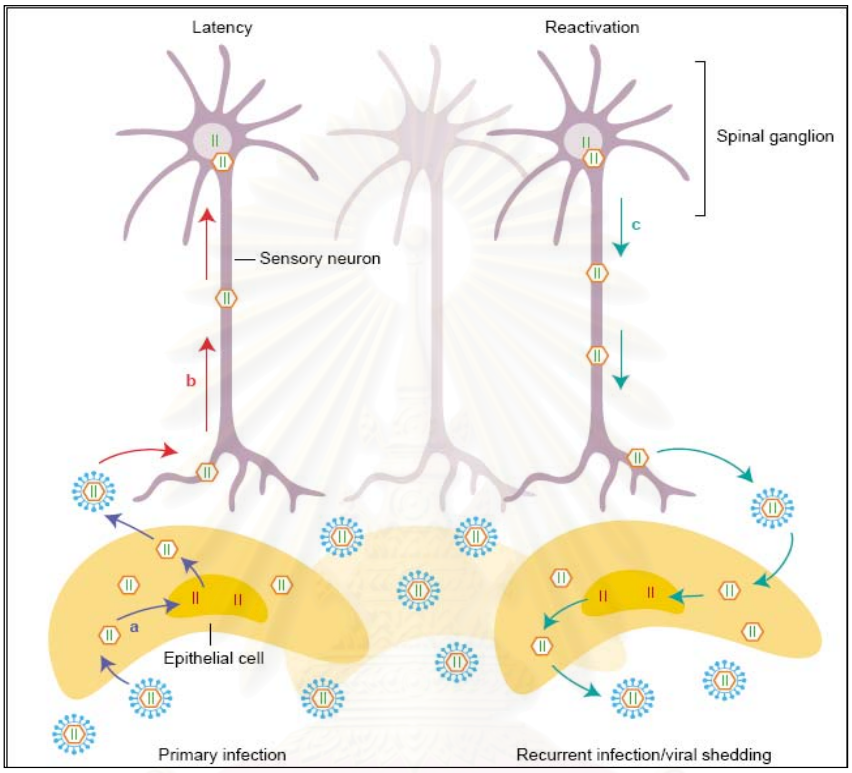


Figure 6: Stages of HSV infection. The steps of the latency-reactivation cycle have been operationally divided into three major steps: establishment, maintenance, and reactivation.

Establishment of Latency

The establishment of HSV-1 latency is preceded by virus replication at the periphery, a protective immune response mainly mediated by T-lymphocytes and macrophages, axonal transport, and entry into the cytoplasm of the neuron. The establishment of latency is regulated by viral and host (cellular and immune) factors that are neither

completely identified nor understood[70]. There is support for the idea that the establishment of latency requires viral entry into the neuron in a physical state and sufficient viral DNA copy numbers capable of replication. However, latent infection occurs when host and viral factors limit the virus's ability to replicate and the cell's ability to undergo apoptosis[71]. *In vitro* studies have shown that viral DNA replication and three functional genes (VP16, ICP4, and ICP27), essential for lytic infection *in vivo*, are not required for the establishment of latency[72, 73].

Host factors

Of the several host factors that contribute to the establishment of latency, the neuron is critical. These highly differentiated cells may contribute to the establishment of latency by restricting expression of the viral genes required for productive infection. Restriction of α genes has been suggested to arise from the absence or altered function of cellular transcription factors needed for viral gene expression[72]. Sensory neurons (i.e., adult mouse and rat) notably lack, or have in very low levels, the cellular transcription factors such as Oct-1 which are important for gene induction[74]. In contrast, neurons may express transcription factors that actively repress viral gene experssion. Two potential repressers, Oct-2[75] and N-Oct3[74], have been proposed to compete for the multicomplex binding of Oct-1 with VP16[76]. Such interaction would restrict viral immediate-early expression by inhibiting VP16 function[77].

The immune system contributes to the establishment of latency by suppressing acute HSV-1 infection and limiting the spread of virus. Immunoglobulin (Ig)G binding to antigen-expressing HSV-1-infected neurons limits virus replication, but is inefficient in cell killing because of antibody bridging with the virion Fc receptor that limits antibody function. This prolongs cell survival and allows time for HSV-1 to enter latency. Likewise, $CD8^+$ cytotoxic T-lymphocytes (CTLs) terminate viral gene expression but fail to induce cytolysis[78], because infected neurons fail to express major histocompatibility complex (MHC) class I determinants needed for effector cell functions, and the virus interferes with CTL-induced apoptosis[71]. CTLs also suppress viral infection by secreting cytokines (alpha/beta interferon) that have important antiviral properties and aid in the clearance of virus from animals also influence the establishment of latency, since they can alter the transcriptional status of the neuron[79]. Together, these findings suggest that immune function is important to animal survival and, thus, to the establishment of an effective latent phase. However, an intact immune function is not regarded as an absolute requirement for the establishment of HSV-1 latency.

Viral factors

Down–regulation of at least two viral processes (i.e., IE gene expression and DNA replication) is more than likely critical for the establishment of latency. Studies have shown that inhibition of IE gene expression reduces cellular toxicity and favors establishment of latent infection. Similarly, latent infection appears to be promoted when DNA replication is restricted by inhibiting α gene expression *in vivo*, elevating cell temperature (37^OC and 42^OC)[80].

The regulation of these processes likely involves four viral gene products: VP16, ICP4, ICP0, and LAT. The role of VP16 is suggested from studies showing that the avirulent HSV-1 that contains a mutation that reduces the viral trans-activation function of VP16 promotes the establishment of HSV-1 latency *in vitro*[80], in immunocompetent mice[81], and in mice with severe combined immunodeficiency (SCID mice)[82]. However, it is unclear how VP16 function might be altered within the neuron during latency. Investigators have postulated that VP16 levels may be reduced in the neuronal soma, because portions of the tegument are left behind when the virus migrates from terminal nerve endings to the neuron, the protein may be rendered nonfunctional by association with VHS[83], or it may be inhibited in neural cells. Certainly, the competitive inhibition of VP16 binding to α gene promoters by Oct-2[84] or N-Oct3[74] may be important. However, VP16 is not likely the sole determinant of the establishment of HSV-1 latency, since latency can be established in neurons in the presence and absence of the trans-activation function of VP16.

ICP4 is essential for the lytic growth of the virus, at least, because of its crucial function as a trans-activator of the β genes required for DNA replication. ICP0 is required for the efficient expression of ICP4 and shows the synergistic trans-activation ability of HSV β and γ genes when used in combination with ICP4[85]. Therefore, quantitative levels of ICP0 and ICP4 must decrease for acute infection to decline and latency to be established. Apparently, this can be accomplished by the absence or reduced function of VP16, the expression of LATs, and/or other cellular and viral factors not yet identified.

Maintenance of Latency

The maintenance of HSV-1 latency requires a quiescent state that is competent in providing a reservoir of infectious virus. The characteristics of this state have been partially defined from studies involving animals and certain types of cell culture, as well as clinical and autopsy studies. From these studies, it is suggested that the DNA of latent HSV-1 is maintained in a minority of infected human neurons[86]. In experimental animals the percentage of sensory ganglionic neurons that are latently infected has been reported to be higher based on detection of LAT RNA by *in situ* hybridization[87]. However, this is probably a gross underestimate of the total number of neurons that contain latent virus, since not all infected neurons express detectable levels of LAT RNA. Within the neuronal nucleus, latent HSV DNA is maintained as "circular" episomal forms folded into nucleosomes[69, 88]. The average copy number of HSV genomes *per* latently infected neuron during clinical latency is reported to range from 1 to 100[89]. However, copy numbers have been reported to exceed 1000 *per* infected neuron in experimental systems[87].

During the maintenance phase of latency, transcripts from three limited regions of the viral genome can be detected. ICP4 and thymidine kinase-specific RNAs have been detected, but in relatively low amounts of latently infected neurons compared with the LATs[90]. The abundance of LATs in latently infected neurons has resulted in their use as a marker of latency. Extensive molecular studies involving LAT have revealed that: (1) the amount of stable LAT mRNA remains constant in trigeminal ganglionic neurons for at least one year[89]; (2) there are more LAT molecules *per* latent HSV-1 genome in the trigeminal ganglion than in

the central nervous system[91]; and (3) from 10% to 30% of HSV-1-infected neurons express LAT[89, 92, 93].

Host factors

The non-dividing neuron has not been found to express any function required for the maintenance of HSV-1 latency *in vivo.* In contrast, nerve growth factor (NGF) is important for neural differentiation and the maintenance of HSV-1 latency in fetal sympathetic ganglionic neurons *in vitro*[94]. Thus, it remains unclear whether NGF is an important factor for the maintenance of HSV-1 latency, or merely reflects the growth requirements of fetal tissue. With the role of Oct-2 recently discredited, the neuronal factors that contribute to viral latency remain to be identified[76].

Viral factors

At present, no viral gene, including several essential for lytic virus replication, has been recognized to be required for the maintenance of HSV latency. These include LAT, ICP0, ICP4, ICP47, U_S9-11, and U_L24**.** The thymidine kinase (TK) gene that encodes an enzyme that phosphorylates purine pentosides and other nucleoside analogues has been postulated to play a role in the maintenance of latency because TK-HSV mutants do not express LAT efficiently in a transplantation model. However, TK is dispensable for replication in cell culture; thus, its exact role remains to be defined[95].

Reactivation from Latency

HSV-l reactivation can be viewed as the third phase of latency. Fundamentally, it involves a switch from viral quiescence to a replicative state within the neuron that may occur spontaneously or as a result of physical and in experimental animals and humans. Reactivation is associated with axonal injury traumatization of peripheral tissues, tooth extraction orofacial fracture, iontophoresis with epinephrine, timolol, and dexamethasone administration of prostaglandins, ultraviolet light radiation [UVR], cadmium, transient hyperthermia, and immunosuppression with cyclophosphamide and prednisolone. *Ex vivo*, reactivation of HSV- I occurs following explantation of latently infected human neurons and following superinfection of latently infected, explanted cultures with HSV-2 temperature-sensitive mutants. In culture, HSV-1 reactivation results when the latently infected sympathetic and sensory ganglia of mice, monkeys, and humans are subjected to NGF withdrawal, anti-NGF, 6-hydroxy-dopamine, colchicines, phorbol myristate acetate, cAMP analogs, dexamethasone, and heat stress[6, 95, 96].

Studies have shown that HSV-1 reactivation is an isolated event limited to only a few neurons within sensory ganglia[97, 98]. Whether the rate of reactivation is correlated with the number of infected neurons remains to be defined[93]. Select cells are believed to respond by inducing one or more positive factors that permit the virus to overcome repression of α genes by diluting a repressor (a copy number effect) or altering the physical state of virus[79]. Although it is unknown whether human neurons survive HSV-1 reactivation, available evidence in animals suggests that neurons die upon reactivation. If this was the case

in humans, then why is it that persons who suffer from frequent oral HSV-1 recurrences do not report dysesthesia ? This could be explained by the fact that only a limited number of neurons die after reactivation, and the plasticity of the sensory system compensates for minute peripheral sensory loss in orofacial tissues.

Host factors

Cellular stress from neuronal injury (*e.g.,* excising a trigeminal ganglion) activates a cascade of biochemical events that can lead to HSV-l reactivation. Certain cellular transcription factors appear to be key elements in these signal transduction pathways. For example, the expression of *c-fos, c-jun, c-myc,* Oct-1, and interferon regulatory factor-1 (IRF-1) is low in adult sensory neurons. However, their expression is rapidly induced as a result of reactivation events such as axotomy and axonal transport, block induction of *c-fos* by NGF in neuronal cells is blocked by 2-aminopurine (2-AP) and 2-AP inhibits HSV-l reactivation from cultured ganglionic neurons[84, 99, 100]. As stated before, Oct-1 binding promotes the trans-inducing function of VP16 on α genes. Thus, viral reactivation may occur when neurons increase expression of Oct-1 or *c-fos* following traumatic events[75]. Alternatively, axotomy and withdrawal of NGF may dilute Oct-2 activity or induce apoptotic factors conductive to HSV-1 reactivation[101]. Although several reactivation stimuli such as UVR are known to induce other transcription factors AP-1 and NF-kB, the role of these and other transcription factors in HSV-1 reactivation has not been fully explored. Consensus SP1 binding sites are located throughout the viral genome and in most viral α and β promoters. Likewise, interleukin-6, an inflammatory cytokine produced in the trigeminal ganglion, can induce transcription factors that appear to

regulate the LAT and ICP0 genes[102]. These factors activate viral α genes or multiple kinetic classes of viral genes required for reactivation.

Viral factors

α genes

There is evidence that the primary viral mediator of the switch from latency to productive infection is an α gene. ICP4 is absolutely required for the expression of HSV-1 β and γ genes that are essential for lytic infection; ICP4 transcription has been detected in latently infected trigeminal ganglia[90], and ICP4 mutants of HSV-1 are incapable of reactivation[103].

ICP0 is also a candidate gene regulator of HSV-1 reactivation. It is a positive inducer of viral replication and stimulator of expression of HSV-1 genes of all three temporal classes (α, β and γ). In some transient expression assays, ICP0 activates transcription from HSV-1 products[104]. Although ICP0 is not required for lytic infection in culture, and ICP0 is unable to activate transcription of HSV-1 β and γ genes without ICP4 during a normal viral infection, deletion of ICP0 is associated with extremely inefficient reactivation from explanted trigeminal ganglia of mice[103, 105]. ICP0 has been shown to regulate spontaneous reactivation in latently infected animals[106], and ICP0 is produced early during HSV-1 reactivation stimulated by cyclo-phosphamide and dexamethasone administration. In latency studies *in vitro*, an ICP0 mutant initiates efficient production of HSV-1 virions only

after superinfection or transfection with a vector encoding the ICP0 gene product.

β genes

HSV-1 reactivation cannot occur without β gene expression and DNA replication. However, it remains unclear whether expression of β genes follows the temporal pattern seen during lytic infection (i.e. α, β, γ) or whether DNA replication occurs at the onset of reactivation. It has been recently reported that, during reactivation, the temporal pattern of gene expression differs from that of lytic infection. Using latently infected murine trigeminal ganglia it detected β viral gene products (TK, ICP6, VP5) within the first 4 hours after explantation, whereas α gene transcripts were not detected until 8 to 24 hours after explantation[99]. This suggests that neuronal factors that cause reactivation may not act specifically on α gene, and DNA replication may be an important event that precedes α gene expression.

The importance of TK in replication and reactivation is evident from many studies. The enzyme is important for normal virus replication in experimental infection, and chemicals that block TK[107] or nucleoside transport[108] inhibit viral reactivation from HSV-1-infected and TK- HSV-1 mutant-infected trigeminal ganglion neurons, respectively. Furthermore, TK-HSV-1 mutants do not express LAT efficiently in a transplantation model[109], do not reactivate in the mouse model, and reactivate with very low efficiency in the rabbit model. Together, these studies suggest that replicative enzymes and nucleosides essential for HSV-1 DNA replication may not be in sufficient supply or

are restricted in neurons that prevent efficient virus replication until neurons are exposed to reactivation stimuli.

Latency-Associated Transcripts (LATs)

In the latency, analysis of viral gene expression revealed that all lytic genes are switched off but that one set of transcript, known as the latency-associated transcripts (LATs), accumulated to high levels. The LATs are the only major viral transcripts detected in latently infected neurons. The full-length 8.3 kilobase (kb) primary LAT transcript accumulates to low level in latently infected neurons. The major products are 2 kb and 1.5 kb RNAs, which are predominantly localized to the neuronal nucleus and are transcribed antisense to and partially complementary to the coding sequences for ICP0 (Figure 7)[86, 110]. These RNAs represent stable introns cleaved from a longer precursor that extends across the a' sequence and into the short repeat region and is complementary to all of the ICP0 coding sequences. The role of LATs in latency is not well understood, partly because the LATs are not absolutely required for the establishment and maintenance of latent infection or reactivation in most animal model systems because LAT mutants are able to establish, maintain and reactivate from latency. While no LAT-encoded protein has been conclusively demonstrated to date, the LAT transcripts may have other functions or activities. It has been shown that LAT negative viruses have increased α gene expression in neurons[111]. This function might relate to an anti-apoptotic function of the LATs that prevents neuronal death during early infection or to an ability of the LATs to downregulate lytic cycle gene expression in neurons[112], thus favoring the establishment of latency. LATs have also been ascribed a role in reactivation. Several views on the function of the LATs exist; thus,

the fundamental mechanisms by which viral gene expression is restricted in neurons remain to be elucidated.

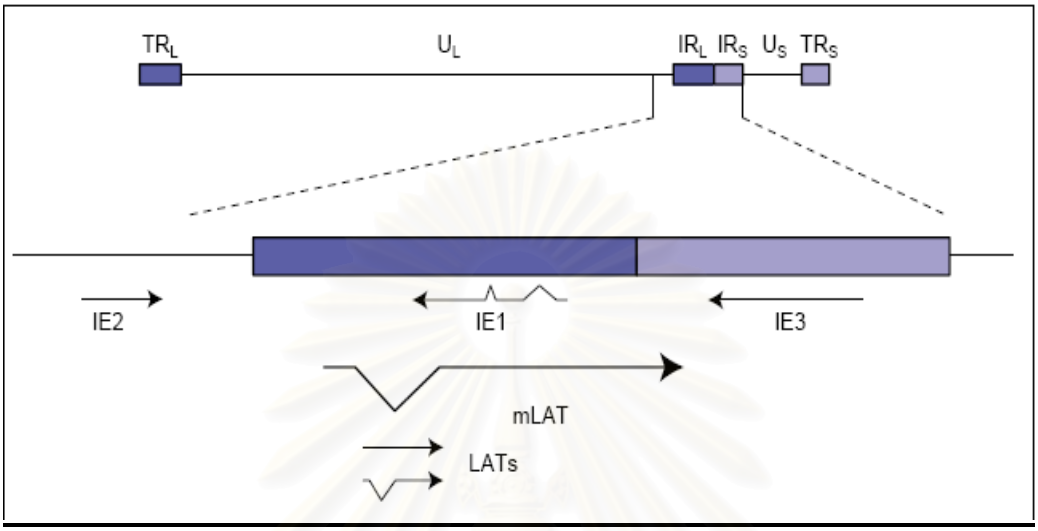


Figure 7: Organization of the HSV-1 genome, showing the origin of latency-associated transcripts. The minor (primary) latency associated transcript (mLAT; ~8.3 kb) is transcribed from the opposite strand to IE1. Major LATs are introns of ~1.5 kb and ~2 kb that are spliced from mLAT [IE1 is ICP0 and IE3 is ICP4].

Immune response to HSV

When HSV infects host, the host's immune system employs a variety of strategies to eliminate the virus. Innate immunity, the first line of defense during both primary and recurrent infection, is essential during this period of acute infection to limit initial viral replication and to facilitate an appropriate adaptive immune response. Innate responses to virus infection consist of three phases: ever-present secreted proteins such as complement and natural antibody, early-induced responses such as interferons produced by infected epithelia and resident dendritic cells (DC), and recruited cellular effectors including neutrophils, macrophages, and natural killer (NK) cells, secreted substances[113]. HSV virions can

activate complement, likely by way of the lectin activation pathway and natural IgM secreted into the vaginal lumen limit HSV entry into the epithelia by neutralizing virions. Other, HSV infection can stimulate interferon (IFN) α/β production by epithelial cells to prime uninfected cells for an antiviral state. DC also produce large amounts of IFN α/β and take up HSV particles. IFNα/β and other cytokines activate macrophages to produce cytokines and chemokines such as interleukine (IL)-1, -8, -12, -15, -18, and RANTES (regulated upon activation, normal T cell expressed and secreted). Subsequently, proinflammatory cytokines and others recruit and activate neutrophils, monocytes/macrophages, NK cells phagocytose virions and secrete antiviral products including TNFα and defensins. Macrophages also phagocytose virions and secrete antiviral products including tumor necrosis factor (TNF)α, IFNα/β, and nitric oxide. NK cells lyse infected cells and secrete cytokines including IFNγ. These proinflammatory cytokines and other products develop of type-1 and type-2 immunity with DC. The alternate way, HSV can stimulate type 1 IFN response, tissue-specific chemokines that recruit and activate inflammatory cells, and cytokines that drive lymphocyte activation and Th1 immunity, all of which are induced by Toll-like receptor (TLR) signaling through TLR2 and TLR9[114].

In adaptive immunity, both the cellular and humoral arms of the immune response play a key role in limiting the severity of the HSV acute infection. The peripheral CD4+ T-cells seem to be particularly important in recruiting and activating macrophages, and in bulk cultures of human lymphocytes, CD4+ are predominant CTLs that respond upon stimulation with HSV antigen. In the murine nervous system, in contrast, CD8+ T-cells response and IgG seem to play a key role in mediated viral

clearance and in blocking the spread of the virus at synaptic junctions within the peripheral nervous system. While both CD4+ and CD8+ cells are present within HSV-infected nervous tissues, there is no CTL response directed toward infected neurons and it is likely that the targets of these cells are non-neuronal cells. Perhaps an even more important role of CTLs is the secretion of chemokines. IFNα/β is detectable within the first few days of HSV infections and acts as an inhibitor of viral replication as well as an activator of macrophages. In addition, activated CD4+ cells produce IFNγ, which also is a strong inducer of macrophages. In turn, activated macrophages (as well as cells of the nervous system such as microglia and oligodendrocytes) produce cytokines such as TNFα and IL-1, which have global effects on both other immune cells as well as on the transcriptional status of neurons. IFNγ has been shown to specifically facilitate the clearance of viral infection from HSV-infected mice. In addition to their role in limiting acute viral infections, it is intriguing to consider possible influences on the permissively of neurons for establishment of latency[115, 116].

Immune evasion by HSV

Coexistence of the viruses and their hosts imposes an evolutionary pressure on both the virus and the host immune response. Viruses have evolved strategies to evade immunity. The mechanisms for HSV immune evasion can broadly categories.

Interfere with humoral immune response

HSV is capable of evading immune attack by interfering with MHC class I antigen presentation, inhibiting activities mediated by complement (C) components C3, C5, and properdin (P) and interfering with activities mediated by the Fc domain of IgG antibodies[117]. HSV-1 glycoprotein gE binds the IgG Fc domain and interferes with C1q binding and antibody-dependent cell-mediated cytotoxicity (ADCC)[77]. HSV-1 gC binds C component C3 and its activation products, C3b, iC3b, and C3c, and accelerates the decay of the alternative C pathway C3 convertase. HSV-1 gC also interferes with C5 and P binding to gC. HSV-1 gC and gE interfere with different aspects of host immunity, including blocking C activation at different stages of the cascade; therefore, these glycoproteins may be additive or synergistic in mediating immune evasion[118].

Interfere with cellular immune response

The HSV ICP47 gene product is a small cytosolic protein of 88 amino acid of which only residues 3-34 are necessary to inhibit Transporter Associated with Antigen Processing (TAP) function[119]. ICP47 inhibits peptide binding to TAP, but does not affect ATP binding. ICP47 acts as a competitive inhibitor of peptide binding to TAP and is thought to bind directly to the peptide-binding site[120]. ICP47 does not behave like a normal peptide as it is not translocated across the membrane and it remains associated with TAP. Futhermore, whereas peptide binding by TAP stimulates ATP hydrolysis and causes a conformational rearrangement of TAP, these events are inhibited by ICP47 binding.

The alternative mechanism, HSV-1 infection targets the molecular coplayers of MHC class II processing, HLA-DR (DR), HLA-DM (DM), and invariant chain (Ii)[121]. HSV-1 infection strongly reduces expression of Ii, which impairs formation of SDS-resistant DR-peptide complexes. Residual activity of the MHC class II processing pathway is diminished by viral envelope gB. Binding of gB to DR competes with binding to Ii. Both, gB-associated DR and DM heterodimers are exported from the endoplasmic reticulum, as indicated by carbohydrate maturation[122].

A novel immune evasion mechanism is utilized by HSV inhibition of T-cell proliferation. By recombinant soluble gD protein or gD-expressing mouse fibroblasts inhibited T-cell proliferation that was induced by OKT3 [anti-CD3 monoclonal antibody (mAb)][123]. The co-expression of gD and HVEM resulted in the inhibition of the nuclear factor (NF)-κB activation that was induced by the HVEM overexpression. Taken together, the results suggest that the inhibitory effect of gD may be due to its ability to actively inhibit the signaling pathway that is mediated by HVEM on the cell surface level.

Modulation of apoptosis

The prevention of apoptosis during HSV-1 infection is dependent upon the accumulation of β and γ gene products. ICP27 stimulates the expression of these factors. It is conceivable that because ICP27 orchestrates the synthesis of multiple viral factors which block apoptosis in HSV-1-infected cells, it is a central regulatory player in the prevention process. Comparison, viruses producing nonfunctional forms of ICP4

accumulate large amounts of IE proteins including ICP27, but are strictly blocked from synthesizing gene products of the later kinetic classes[124].

Interfere with interferon

The tegument also contains VHS, the virion host shutoff protein encoded by HSV gene U_L41. VHS is an mRNA-specific RNase that triggers rapid shutoff of host cell protein synthesis, disruption of preexisting polyribosomes, and degradation of host mRNAs in the absence of de novo viral gene expression[58]. This global shutoff stem comes from at least two distinct inhibitory pathways. First, the levels of most host mRNAs undergo a precipitous decline, curtailing synthesis of the corresponding proteins[125]. VHS contributes to this decline by globally increasing the rate of mRNA degradation in the cytoplasm. The effect of VHS is magnified by virus-induced suppression of host mRNA synthesis, mediated through repression of primary transcription and pre-mRNA splicing. As described below, the multifunctional IE protein ICP27 plays a particularly prominent role in the inhibition of host mRNA biogenesis at both of these levels. ICP27 thus collaborates with VHS to reduce the abundance of host mRNA during infection. Second, HSV alters the function of the host translational apparatus, such that translation of the residual portion of many of the down-regulated cellular mRNAs is strongly impaired. This effect is probably due to impaired initiation, as a significant fraction of the residual mRNAs is found in 48S translational preinitiation complexes.

The ICP34.5 protein of HSV-1 functions to block the shutoff of protein synthesis involving double-stranded RNA-dependent protein kinase (PKR)[126]. In this process, the ICP-34.5 protein recruits cellular

protein phosphatase 1 (PP1) to form a high-molecular-weight complex that dephosphorylates eIF-2α without any other viral proteins[127-129]. Secreted IFN binds IFN receptor to activate the signaling pathway. In the case of IFN-γ, the receptor-associated kinases Jak1 and Jak2 are activated and phosphorylate Stat1[130]. These and associated proteins are translocated into the nucleus, where they induce antiviral proteins. The activation of antiviral response is mediated by promyelocytic leukemia (PML). In wild-type-virus-infected cells, Jak1 disappears, PML is degraded, and the constituents of ND10 are dispersed. As a result, the infected cell does not respond to exogenous IFN to curtail viral replication.

Replication of HSV in T-lymphocyte

The interaction of HSV with human lymphocytes is complex. Cellular immune mechanisms are important in controlling herpetic infection, and there is evidence that HSV in turn can functionally affect lymphocytes. In addition, these cells may be sites of growth and carriage of the virus *in vivo*. Previous studies have demonstrated that HSV replicates preferentially in stimulated T cells. Although freshly isolated T cells are nonpermissive for viral replication, mitogen-stimulated T cells[131] and IL-2-stimulated T cells replicate and produce infectious HSV[132]. Activated CD4 and CD8 positive T-cell subsets were equally capable of replicating HSV[133]. In addition, MHC class II-expressing T cells actively replicate virus without prior mitogen stimulation. Kirchner and Schrod have reported the replication of HSV in EBV-stimulated human B cells[134], and Daniels *et al.* have shown that macrophages cultured *in vitro* for several days are capable of replicating HSV[135]. Additionally, Naraqi ***et al.*** have reported the successfully isolation of

HSV from the leukocytes of acutely infected patients[136], suggesting the possibility of a spread of viral infection mediated by infected lymphocytes.

Previous study demonstrated that both HSV-1 and HSV-2 could replicate in lymphocytes but virion production is lower than epithelial cell[10]. Moreover, the replication of HSV-1 increased in T-lymphocyte activated by phytohemagglutinin (PHA)[11]. One of these mechanisms may be the upregulation of HVEM receptor after PHA activation.

Interaction between HSV and HIV-1 in T cells

Several mechanisms by which HSV could affect the life cycle of HIV-1 have been described. The first is a direct effect of herpesvirus gene products on the transcriptional and post-transcriptional regulation of the HIV-1 provirus. The second is an indirect stimulation of HIV-1 expression, mediated by chemokines released from HSV-infected cells or by viral antigen stimulated T cells. Thirdly, HSV could cause an alteration of the HIV-1 cell tropism as the result of phenotypic mixing between HIV-1 and HSV. This will allow infection of cells that are not normally susceptible to HIV-1 infection, such as CD4 negative cell.

Transcriptional and post-transcriptional transactivator

HIV-1 proviral gene expression is driven by its 5'-LTR and depends on two virus-encoded, non-structural RNA-binding proteins[137], Tat and Rev. The main effect of Tat is at the level of transcriptional initiation and processing. In contrast, the Rev protein acts post-transcriptional to increase the transport of un-spliced and single-

spliced viral transcripts from the nucleus to the cytoplasm. It has also been suggested that Rev augments translation of the gag-pol and vpu/env mRNAs[138].

The transcriptional activity of the HIV-1 LTR can also be enhanced by a Tat-independent mechanism. A large number of cellular stimuli such as mitogens, stress cytokines and viruses, were shown to transactivate the HIV-1 LTR and stimulate expression of the HIV-1 provirus[139, 140]. The binding of NF-kB and Sp1, which are transcription factors of cellular origin, to the LTR seems to be important in this process[141]. The HSV-1 α genes, ICP0, ICP4 and ICP27 have been demonstrated to play a role in transactivation, although the occurrence of this phenomenon depends on the experimental model and cell line used[142-145]. In particular, the HSV-1 transactivator ICP0 has been shown to increase expression form many viral and non-viral promoters *via* a mechanism that is independent to its binding to specific DNA sequences. Weber *et al*.[146] described a dominant-negative mutant of the ICP0 protein, which suppressed the expression of both an HIV-1 protein and a chloramphenicol acetyltransferase gene. Chapman *et al*.[147] showed that transfected ICP0 activated HIV-1 LTR expression from an integrated chimeric HIV-1 provirus. In addition, ICP0 has been demonstrated to co-operate effectively with Tat in the stimulation of the LTR-driven transcription. The co-operation between ICP0 and Tat is specific for the HIV-1 LTR[148].

The role of HSV IE proteins was also examined in HIV-infected cells, using HSV-1 mutants carrying deletions in ICP0, ICP4 and ICP27 genes, respectively. ICP0 and ICP27 deletion mutants were found to stimulate HIV-1 replication to the same extent as wild-type HSV-1, whereas infection with the ICP4 mutant failed to enhance HIV-1

replication[144]. In contrast, when ACH-2 cells that harboured a latent HIV-1 provirus were used as target cells, the infection with ICP4 and ICP27 mutants stimulated the HIV-1 provirus. These data suggest that α gene products may differentially affect HIV-1 replication and that the difference in the effect could be related to the HSV-1 status (i.e. productive or latent infection)[149].

In addition, the nuclear level of the cellular protein leader binding protein-2, that can bind DNA near the transcriptional initiation site of the HIV-1 promoter, are enhanced markedly in T cells following HSV-1 infection[150]. Leader binding protein-2 was associated with HSV-1 induced transcriptional activation of HIV-1, independently of NF-kB and Sp1 activity. Contrasting with the role of the IE proteins, the HSV strong transactivator VP16 which is present in the viral particles, does not contribute to HSV-mediated transactivation of the HIV-1 LTR.

With regard to post-transcriptional regulation, the product of the HSV-1 IE gene ICP27 has been demonstrated to influence HIV-1 mRNA levels, by affecting the stability and splicing of certain mRNAs[151]. Recent experiments have shown that the U_S11 gene product of HSV-1, a true late gene product packaged within the virion, was able to bind the Rev-responsive elements on HIV-1 mRNAs[152]. U_S11 exhibited a nucleocytoplasmic localization shortly after infection and subsequently accumulated in the nucleoli. In HIV-1 infected cells, U_S11 can act as a chaperone and escort unspliced retroviral mRNAs form the nucleus to the cytoplasm, where they associate with polyribosomes for translation. U_S11 thus resembles the HIV-1 Rev protein in terms of its method of transactivating HIV-1 expression. In particular, U_S11 has been shown to

act at the post-transcriptional level to up-regulate the retroviral envelope glycoprotein expression.

Cytokine release and antigen recognition

Macrophages and CD4 positive cells are among the first cells to infiltrate herpetic lesions. The presence of activated CD4 positive cells in herpetic lesions creates an ideal micro-environment for HIV replication. Additionally, indirect stimulation of HIV-1 expression could take place through cytokine release from either HSV-infected cells or viral antigen-stimulated cells.

Several pro-inflammatory and immunoregulatory cytokines profoundly affect HIV replication in T lymphocytes and mononuclear phagocytes[153]. HSV-infected cells release cytokines that, *via* paracrine effects, could activate HIV-1 proviral DNA through different signal transduction pathways, as proviral gene expression can be activated by immune stimulation in HIV-1-infected T cells. Since some of these HIV-1-infected T cells may be committed to the recognition to HSV cognate antigens, antigen presentation form any HSV-infected cell could provide the right stimulus to activate HIV replication[154]. Tremblay et al. showed that peripheral blood mononuclear cell exposure to HSV-1 or HSV-2 stimulated the cells to become active producers of HIV-1[155]. This increased production of HIV-1 particles appeared to be a consequence of both HSV-encoded transactivating factors and mitogenic proliferation.

HIV tropism enhancement

HIV-1 infection is mediated by CD4, which is the major viral receptor. The presence of this molecule is essential but not sufficient of HIV-1 entry. Since CD4 is the major component of the cellular HIV receptor, viruses could affect HIV-1 replication by modulating CD4 expression. Such an effect has been demonstrated for HHV-6[156]. In contrast, no modulating of CD4 expression was seen in human CD4 positive lymphocytes, which had been chronically infected by HSV-1, indicating that HSV-1 is not able to influence the HIV-1 replication cycle at this level[157]. Moreover, since some cellular chemokine receptors act as the second signal required for HIV-1 internalization after CD4 binding, the same function could be served by homologue encoded by herpesviruses. Indeed, the U_S28 gene product of human CMV has been proven to mediate HIV-1 infection in otherwise resistant CD4 positive cells[158]. In addition, some herpesviruses have been demonstrated to modulate chemokine receptor expression on the surface of infected cells, thus influencing HIV infection. As far as HSV-1 infection of T-lymphocytes is concerned, the expression of CXCR4, which is the major HIV-1 co-receptor for this cell type, is not altered[157].

It is also possible that herpesviruses could either encode proteins of their own or activate cellular proteins that are capable of acting as alternative receptors for HIV-1. HIV-antibody complexes can infect monocytes and macrophages by means of complement receptors or receptors for the Fc portion of IgG. The expression of immunoglobulin Fc receptors can be induced in cells infected with HSV and human CMV[159]. The Fc receptors induced by human CMV allows immune

complexes of HIV-1 to infect otherwise resistant fibroblasts. The same phenomenon could also be possible for HSV-encoded Fc receptors.

Finally, a double infection of the target cells by HIV-1 and a second virus could result in the production of pseudotypes, i.e. of viral particles containing the HIV-1 genome enveloped with the surface glycoproteins derived from the second virus. These pseudotypes would be able to infect HIV-1 resistant cells by exploiting non-HIV specific cellular receptors. This could result in an expansion in HIV-1 tropism because once the retroviral genome had gained access to the cell, it would then be able to replicate. Indeed, HIV-1 pseudotyping by the HSV-1 envelope has been described[157, 160]. These pseudotypes are able to infect a significant enlarged spectrum of cells, including many CD4 negative cells. This phenomenon could be extremely relevant for the pathogenesis of HIV-1 infection *in vivo*, because it could help to explain the high HIV-1 load during the terminal phases of AIDS, when the CD4 positive cells number is low. Large amounts of wild-type HIV-1 particles could in fact be released from CD4 negative cells that are infected by pseudotypes.

CHAPTER IV

MATERIALS AND METHODS

Part I. Cell sample preparation

1. Study population:

15 healthy volunteer blood donors were enrolled into this study as the control group. 25 HIV- infected patients with known history of HIV infection for at least one year who attended The Anonymous Clinic, Thai Red Cross were recruited into this study as the subject. All of them are antiretroviral drug naïve with no sign of opportunistic infection.

2. Specimen collection:

5 ml of EDTA blood and 7 ml of heparinised blood were obtained from each volunteer. After collecting, the blood sample was immediately processed.

3. Preparation of peripheral blood mononuclear cell (PBMC):

PBMC were obtained from heparinised blood by density-gradient centrifugation. Brieftly, the blood (7 ml) was resuspended with equal volume of RPMI 1640 (Gibco, BRL, USA) and overlayered on ficoll-hypaque (density gradient 1.077 g/l), followed by centrifugation at 1,500 rpm for 30 minutes at room temperature. After that, PBMC were harvested and washed twice with RPMI 1640 and then resuspended in

RPMI 1640 supplemented with 10% fetal bovine serum (R10) and processed further for analysis.

Part II. Preparation of cells and stock seed of viruses

1. Cell culture

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. These cells were obtained from the Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand. Vero cells were grown in growth medium (GM) M199 (Earle's salt) supplemented with 10% fetal bovine serum (Hyclone, UK), 100 units/ml penicillin G and 100 µg/ml streptomycin (Dumex, Bangkok, Thailand), 2mM L-glutamine and 0.01M HEPES (N-2-hydroxyethyl-piperaine-N′-2-ethan sulfonic acid) (Sigma, USA). Maintenance media (MM) were prepared as growth media except the concentration of fetal bovine serum was reduced to 2%.

Dispersion of cell monolayer was performed by using trypsin-versene (see Appendix II). The culture media was removed and the cell monolayer was washed twice with 5 ml 0.01M phosphate buffered saline (PBS), pH 7.5 prewarmed to 37ºC. After discarding PBS, one ml of trypsin-versene was added and cells were incubated for one to two minutes at 37ºC, then the culture flask was gently shook until the cells were detached, GM was added. Vero cells were subcultured at three or

four-day intervals with a splitting ratio of 1:3. Vero cells were grown at 37ºC.

2. Virus

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

Virus stock was prepared from Vero cell monolayer infected with HSV-1 at the approximate multiplicity of infection (MOI) of 0.01 plaque forming unit per cell (PFU/cell). After an hour of viral adsorption at 37ºC, the unadsorbed virus was removed; the culture was washed once with 0.01M 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 -70ºC) and thawed (at 37ºC in water bath) for three times. The disrupted cell suspension was centrifuged at 4ºC, 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 -70ºC until use. The amount of viruses was determined by plaque titration assay.

Part III. Cell surface receptor detection

1. Antibodies

Several anti-human monoclonal antibodies (mAbs) and their conjugated fluorochromes such as anti-human CD3 conjugated with peridinin chlorophyll pretein (PerCP), anti-human CD38 and anti-human CD4 conjugated with allophycocyanin (APC) and anti-human CD8 conjugated with phycoerythrin (PE) were purchased from Becton Dickinson (BD, USA). Monoclonal antibody against HVEM (anti-HVEM) was obtained from Abcam (Abcam, UK). The secondary antibody for detecting anti-HVEM was goat polyclonal to mouse immunoglobulin conjugated with PE was obtained from Dako, Denmark. Moreover, anti-HSV-1, polyclonal antibody raised from rabbit was used for detection of HSV-1 infecting cells followed by goat anti-rabbit immunoglobulin conjugated with fluorescein isothiocyanate (FITC) as secondary antibody purchased from Dako (Dako, Denmark).

2. Detection of cell surface molecules (HVEM, CD3 and CD38) by Flow cytometry

Flow cytometry is a rapid, quantitative method for multiparametric measurement of fluorescent cells. Flow cytometric analysis of HSV infected cells by immunoenzymatic system and by indirect immunofluorescence staining. 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 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.

PBMC from either healthy blood donor or HIV patient will be determined for the presence of CD38 and HVEM expression on their T-lymphocytes. Briefly, $5x10^5$ cells of PBMC were first stained with anti-HVEM. After the addition of the mAbs, samples were gently mixed by vortex, and incubated in the dark at 4ºC for 30 minutes. The stained samples were washed with 2 ml of washing buffer (0.5%BSA in PBS) and centrifuged at 1,500 rpm for 5 minutes at 4ºC. The supernatant was discarded and then cell pellet was stained again with goat anti-mouse-PE and washed with 2 ml of washing buffer. After HVEM staining, cell pellet was then incubated with a mixture of anti-CD3-PerCP and anti-CD38-APC (5 µl each) and washed twice. Finally, the cell pellet was resuspened in 500 µl of 1% paraformaldehyde in PBS and subjected to flow analysis. The number of cells in each sample was analyzed by FACScan (BD, USA) and calculated with the Cell-Quest software . The frequency (percentage) was determined by two-color dot plot analysis and density (mean fluorescent intensity; MFI) was determined utilizing a histogram plot. The non-staining PBMC was run in parallel as negative population.

Part IV. Efficiency of HSV-1 replication in PBMC

1. HSV-1 replication in PBMC

The 1 x 10^6 cells of PBMC from either normal healthy donor or HIV patient were cultured in 24-well plate and infected with HSV-1 at

MOI of 5 for 24 hours. Then the infected cells and culture supernatant were separately collected by centrifugation at 2,000 rpm for 5 minutes at 4ºC. The cell pellets were then added with 1 ml of MM and further lysed by freezing and thawing for three times. The cell lysate was centrifuged at 2,000 rpm for 20 minutes at 4 °C and the lysate supernatant was collected. Both culture and lysate supernatant were kept -70 °C until determining the amount of viruses by plaque titration assay.

2. Flow cytometry detection of HSV-1 infected cells

The 1 x 10^6 PBMC infected cells were prepared similarly to HSV-1 replication except that cell pellets spin at 1,500 rpm for 5 minutes at 4ºC, 0.02% EDTA 500 µl was added and incubated at 37ºC in 5% CO_2 for 10 minutes. After that these cells were washed with 3 ml of cold washing buffer (Appendix II). Then 500 µl of 4% w/v paraformaldehyde was added, mixed and incubated at 37ºC in 5% CO_2 for 10 minutes. After washing step, 500 µl of FACS permeabilizing solution (BD, USA) was added and incubated at room temperature in the dark for 30 minutes, and the cells were washed once. The infected cells being stained indirectly were then incubated with 50 µl rabbit-anti HSV-1 antibody at dilution 1:100 on ice for 30 minutes, and washed with 3 ml of cold washing buffer. Then the 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 with cold washing buffer and 5 µl of each anti-CD3-PerCP, anti-CD4-APC and anti-CD8-PE was added and incubated at 4ºC for another 30 minutes, washed once and added 500 µl 1% paraformaldehyde kept overnight in 4ºC before analysis. The number of cells in each sample was analyzed by FACScan (BD, USA) and calculated with the Cell-Quest software. The frequency of stained cells

was determined by two-color dot plot analysis and density was determined utilizing a histogram plot. The non-staining PBMC were run in parallel as negative population.

3. Detection of HSV virions by 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 virus 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 37ºC 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 37ºC 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 V. Quantitative HIV viral load

HIV RNA in plasma can be quantitated by nucleic acid amplification technology, such as the Polymerase Chain Reaction (PCR). The AMPLICOR HIV-1 MONITOR Test, v1.5 (Roche Diagnostic) is an *in vitro* nucleic acid amplification test for the quantification of HIV-1 RNA in human plasma on the COBAS AMPLICOR Analyzer (Roche Diagnostic). This test uses PCR technology to achieve maximum sensitivity and dynamic range (50-750,000 copies/ml) for the quantitative detection of HIV-1 RNA by using a combination of two specimen preparation procedures, the Standard and Ultrasensitive procedures, in EDTA or ACD anti-coagulated plasma. The quantitation of HIV-1 viral RNA is performed using the HIV-1 quantitation standard (non-infectious RNA transcript). The AMPLICOR HIV-1 MONITOR Test, v1.5 is based on the four major processes:

1) Specimen preparation

The AMPLICOR HIV-1 MONITOR Test, v1.5 can be used with either of two specimen preparation procedures, the Standard procedure or the Ultrasensitive procedures. In the Standard specimen preparation procedure, HIV-RNA is isolated directly from plasma by lysis of virus particles with chaotropic agent followed by precipitation with alcohol. With the Ultrasensitive specimen preparation, HIV-1 viral particles in plasma are first concentrated by high speed centrifugation (23,600 x g for an hour at 2-8ºC), followed by the Standard procedure. A known number of quantitation standard RNA is incorporated into each individual specimen at a known copy number and is carried through all steps along with the HIV-1 target and is amplified together.

2) Reverse transcription and amplification

Processed specimens are added to the amplification mixture in the amplification tubes (A-ring) in which both reverse transcription and amplification occur. The reverse transcription are performed with the thermostable recombinant enzyme *Thermus thermophilus* DNA polymerase in the presence of manganese (Mn) and under the appropriate buffer conditions to forming a DNA strand complementary (cDNA) to the viral target. Following reverse transcription, the reaction mixture is heated to denature the RNA and PCR process is started. The COBAS AMPLICOR Analyzer automatically repeats this process for a designated number of cycles, each cycle effective doubling the amount of amplicon DNA. Amplification occurs only in the region of the HIV-1 genome between the primers.

3) Hybridization

Following PCR amplification, the COBAS AMPLICOR Analyzer automatically adds denaturation solution to the A-tubes to form single-stranded DNA. A suspension of magnetic particles coated with an oligonucleotide probe specific for either HIV-1 amplicon or HIV-1 quantitation standard amplicon is added to each of four HIV-1 amplicon dilutions in D-cups. The biotin-labeled amplicon are hybridized to the target–specific oligonucleotide probes bound to the magnetic particles.

4) Detection

Following the hybridization reaction, the COBAS AMPLICOR Analyzer washes the magnetic particles in the D-cups to remove unbound material, and then adds avidin-horseradish peroxidase conjugate. The avidin-horseradish peroxide conjugate binds to the biotin-labeled amplicon hybridized to the target-specific oligonucleotide probes bound to the magnetic particles. The COBAS AMPLICOR Analyzer washes unbound conjugate and then a substrate solution containing hydrogen peroxidase and 3,3',5,5' tetramethybenzidine (TMB) to each D-cups. In the presence of hydrogen peroxide, the bounded-particles catalyses the oxidation of TMB to form a colored complex, the absorbance of which is measured by the COBAS AMPLICOR Analyzer at the wavelength 660 nm.

The amount of HIV-RNA in each specimen is then calculated from the ratio of the total HIV-1 absorbance to the total HIV quantitation standard absorbance and the input number of HIV-1 quantitation standard RNA molecules.

PART VI. Data analysis

Data for each assay are presented as mean±SD of sample. The Mann-Whitney U test and paired-samples T-test were used to determine the statistical significance of the different observed between groups. The Pearson correlation coefficient test was used to analyze the association observed between different parameters. *P*-value ≤ 0.05 was considered statistic significant.

CHAPTER V

RESULTS

Characteristics of study population

Total of 25 HIV antiretroviral drug naïve patients was recruited. All of them have no sign of opportunistic infections and at least one year of HIV infection. There are 16 male (64%) and 9 female (36%). The range of age is between 22 to 62 years old (mean±SD = 33.92±8.98). The number of CD4+ cells count was varied between 59-878 cells/mm^3 (mean±SD=358.96 ± 185.21). The amount of viral RNA was determined on the day of collecting sample. The results showed that the HIV viral load ranged from less than 50 to 194,618 copies/ml as shown in Table 2a (mean±SD=38,969.96 ± 54,103.52).

Blood was also obtained from 15 healthy donors. There are 7 male (46.67%) and 8 female (53.33%). The range of age is between 21 to 32 years old (24.40±2.82) as shown in Table 2b.

Table 2a. Characteristics of HIV infected patients

Patient	Sex	Age (years old)	CD4 count (cell/mm^3)	Viral Load (copies/ml)	Years after known seropositive
P01	M	38	59	181,819	>1
P02	M	34	69	56,166	>1
P03	M	24	176	48,929	1
P04	M	23	497	<50*	1
P05	M	30	214	9,981	>1
P06	M	35	202	12,550	10
P07	F	35	314	9,543	>1
P08	F	32	201	89,357	4
P09	F	39	397	1,574	2
P10	M	39	403	50,936	9
P11	F	30	214	9,981	1
P12	F	36	261	59,154	7
P13	F	27	292	83,737	2
P14	M	22	296	4,919	1
P15	F	27	307	10,831	5
P16	M	32	313	194,618	2
P17	M	32	408	17,119	2
P18	M	32	449	12,482	4
P19	F	31	331	96,825	5
P20	M	42	417	3,944	4
P21	M	30	511	13,421	2
P22	M	28	506	5,854	1
P23	M	55	878	<50*	12
P24	M	33	601	<50*	1
P25	F	62	658	359	10
Mean±SD		33.92±8.98	358.96±185.21	38,969.96±54,103.52	

M : male F : female

* To evaluate mean±SD, this number used as equal to 50 copies/ml

Table 2b. Characteristics of Donors

Donor	Sex	Age (years old)	CD4 count (cell/mm^3)
D01	F	23	788
D02	M	29	395
D03	F	23	378
D04	M	23	629
D05	F	24	464
D06	M	21	ND*
D07	F	21	438
D08	F	23	772
D09	M	25	ND*
D10	M	32	856
D11	F	23	ND*
D12	F	26	624
D13	M	32	375
D14	M	23	257
D15	F	29	ND*
Mean±SD		24.4±2.82	543.27±200.20

M : male F : female

*ND = Not Done

Detection of CD38 and HVEM expression on T-lymphocytes from healthy individuals and HIV-infected patients

Previous study of *Chimma* and co-worker reported upregulation of HVEM mRNA was observed when Jurkat, T-lymphocyte cell lines, was activated with PHA and suggested that this phenomenon might mimic the situation occurs in chronic infection such as HIV patients[11]. Therefore, an attempt to evaluate the percentage of the number of CD38+ and HVEM+ expressing T-lymphocytes *ex vivo* comparing between HIV-infected patients and healthy individuals was done. PBMC from 25 HIV-infected patients and 15 healthy individuals were stained with anti-human CD3 conjugated with PerCP, anti-human CD38 conjugated with APC, and anti-HVEM conjugated with PE and the percentage of cells and density of HVEM expression was determined. Table 3 and 4 showed the results of the percentage of CD3+CD38+, CD3+HVEM+ and CD38+HVEM+ cells from HIV-infected patients and healthy donors. The representative flow cytometry two-color dot plots were shown in Figure 8 and 9.

The results showed that the percentage of CD38+ T-cells from HIV-infected patients (46.51±17.54 %) is higher than the healthy donors (27.54±14.12 %) with statistic significant (*p*-value = 0.001). However, the percentage of HVEM+ T-cells was very low and not different (*p*-value = 0.282) between two groups (0.50±0.53 % and 0.57±0.34%) as shown in Figure 8. Moreover, the percentage of cells that co-expression of CD38+HVEM+ was very low in both groups and not different similar to that of HVEM expressing T-lymphocytes (CD3+HVEM+) even though the CD38+ cells increased.

As the number of HVEM expressing cells were the same, the intensity of HVEM molecule on each cell may be different. Thus, MFI of HVEM was determined. MFI of HVEM expression on lymphocytes in HIV-infected patients (3.75±1.57) was less than the healthy individuals (4.92±2.11). However, the different was not statistically significant (*p*-value = 0.142) as shown in Figure 11 and 12.

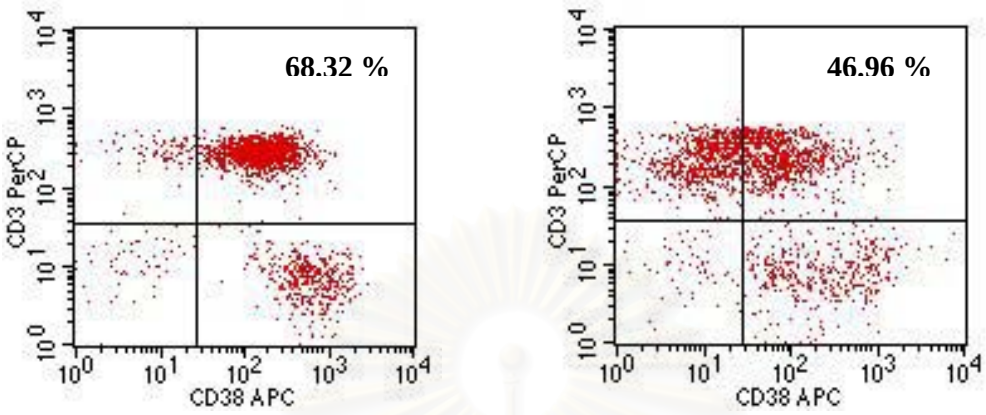
Table 3. Percentage number of activated T-lymphocytes, HVEM expression and MFI of HVEM in T-lymphocytes of HIV-infected patients

Patients	Number Staining cells (%)			MFI of HVEM
	CD3+CD38+	CD3+HVEM+	CD38+HVEM+	
P01	68.32	0.31	0.46	5.18
P02	36.51	0.46	0.70	5.13
P03	61.29	1.39	2.54	4.31
P04	48.45	0.17	0.30	3.63
P05	38.42	0.73	1.32	6.19
P06	56.82	0.41	1.13	5.83
P07	18.39	0.01	0.01	2.36
P08	53.62	0.73	0.59	3.86
P09	47.93	0.16	0.08	2.57
P10	96.67	0.03	0.01	2.96
P11	31.32	0.03	0.01	2.52
P12	32.51	0.02	0.02	2.10
P13	57.90	1.25	2.58	4.69
P14	62.52	1.54	2.97	4.89
P15	55.80	0.45	0.47	2.98
P16	49.63	0.15	0.23	4.51
P17	35.61	0.58	1.09	3.28
P18	21.78	0.04	0.03	1.75
P19	53.03	0.27	0.97	4.6
P20	15.38	0.00	0.00	1.58
P21	54.89	1.88	2.42	6.94
P22	46.96	0.58	0.09	4.72
P23	50.94	0.95	1.02	1.01
P24	32.82	0.39	0.58	4.44
P25	35.22	0.02	0.01	1.75
Mean±SD	46.51±17.54	0.50±0.53	0.79±0.92	3.75±1.57

Table 4. Percentage number of activated T-lymphocytes, HVEM expression and MFI of HVEM in T-lymphocytes of healthy donors

No.	Number Staining cells (%)			MFI of HVEM
	CD3+CD38+	CD3+HVEM+	CD38+HVEM+	
D01	12.35	0.52	2.28	7.03
D02	8.15	0.98	3.09	8.53
D03	8.00	0.64	1.77	6.76
D04	34.53	0.18	1.22	7.16
D05	48.49	0.15	1.75	7.99
D06	31.99	0.56	0.23	2.97
D07	31.03	0.27	0.08	2.49
D08	28.46	1.05	0.46	3.49
D09	18.97	0.59	0.08	4.18
D10	17.32	0.40	0.03	3.40
D11	35.89	0.30	0.02	3.62
D12	29.54	0.88	0.11	3.46
D13	29.45	1.01	0.09	3.92
D14	20.21	0.02	0.01	2.55
D15	58.76	0.93	0.78	6.24
Mean±SD	27.54±14.12	0.57±0.34	0.80±0.99	4.92±2.11

HIV-infected patients



Healthy Donors

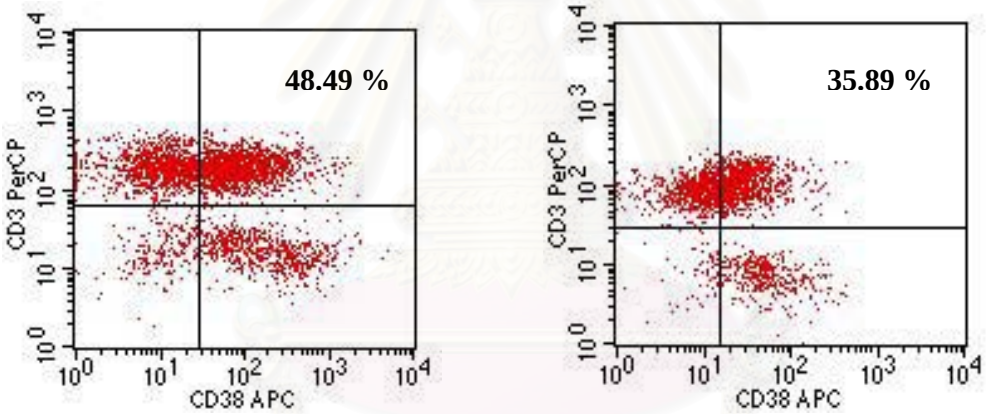
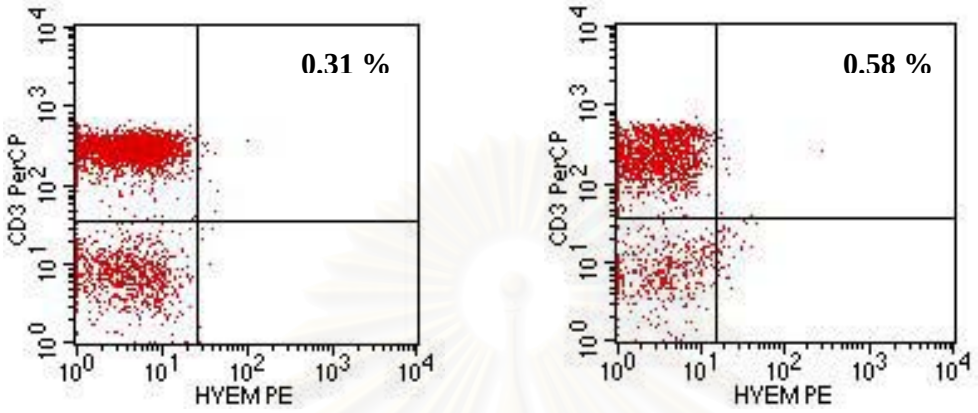


Figure 8. Representative profile comparing CD3+CD38+ cells from 2 HIV-infected patients (P01 and P22) and 2 healthy donors (D05 and D11) from flow cytometry two-color dot plot.

HIV-infected patients



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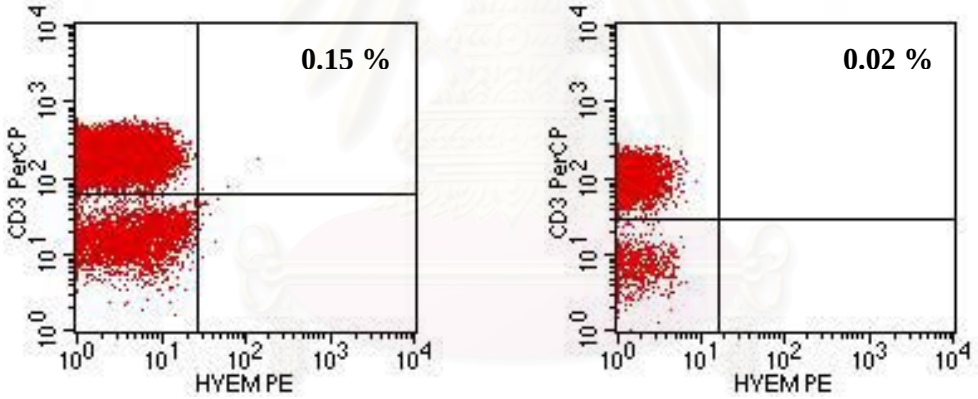


Figure 9. Representative profile comparing CD3+HVEM+ cells from 2 HIV-infected patients (P01 and P22) and 2 healthy donors (D05 and D14) from flow cytometry two-color dot plot.

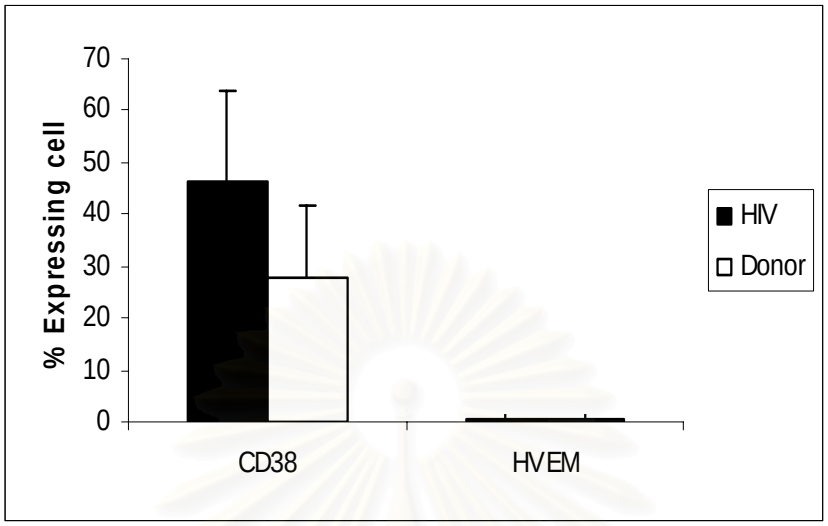


Figure 10. Comparison the mean percentage of CD38+ and HVEM+ T lymphocytes of HIV-infected patients (black column) and healthy donors (white column).

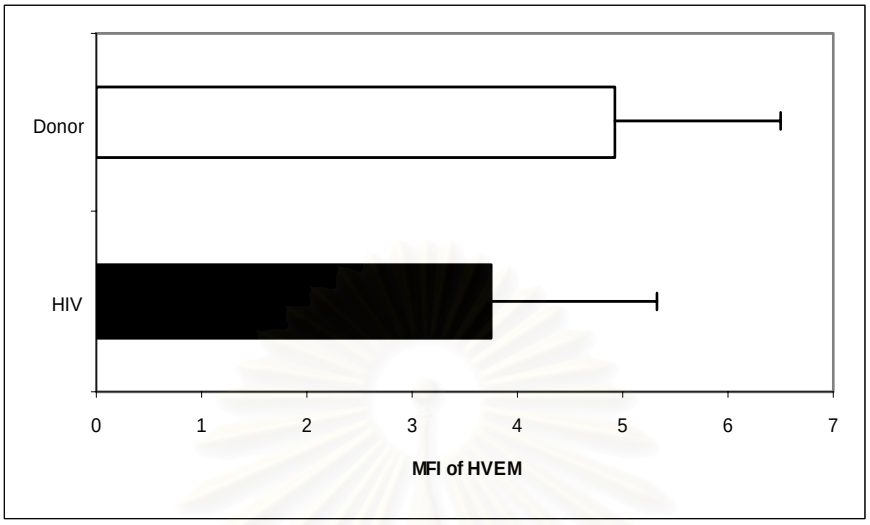
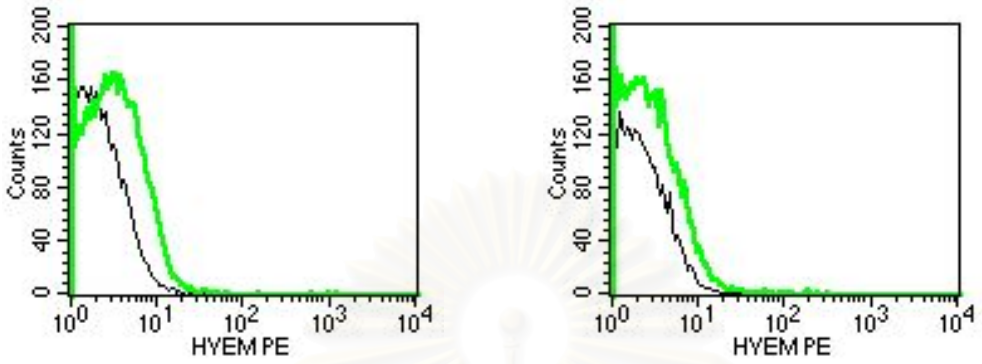


Figure 11. Comparison the mean fluorescence intensity (MFI) of HVEM expression in lymphocytes of HIV-infected patients (black column) and healthy donors (white column).

HIV-infected patients



Healthy Donors

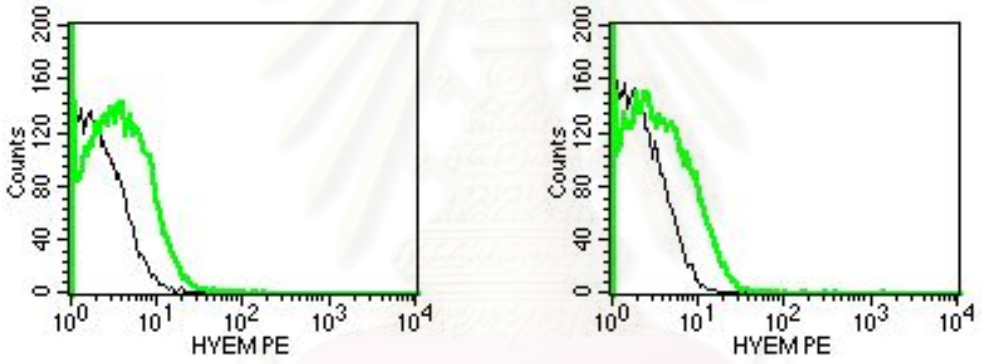


Figure 12. Representative profile comparing HVEM expression in T-lymphocytes from 2 HIV-infected patients (P01 and P22) and 2 healthy donors (D11 and D14) from flow cytometry histogram.

Correlation between the percentage of activated T-lymphocytes, HVEM expressing T-lymphocytes and MFI of HVEM against CD4 count

To analyze whether there was any correlation between the number of CD3+CD38+ cells and CD4+ cell count in HIV-infected patients, scattered plot with linear correlation was done. In addition, a correlation between HVEM expressing T-lymphocytes and MFI of HVEM against CD4+ cell count was also tried. The results showed an inverse correlation of percentage of CD38 expressing T cells but no statistical significant correlation (R= -0.138, *p*-value=0.511) as shown in Figure 13. No correlation between the percentage of HVEM expressing T-lymphocytes and CD4 count (R= 0.015, *p*-value=0.942) was found (Figure 14). But the intensity of HVEM (MFI of HVEM) and CD4 count showed statistic significant correlation (R= -0.435, *p*-value=0.030) (Figure 15).

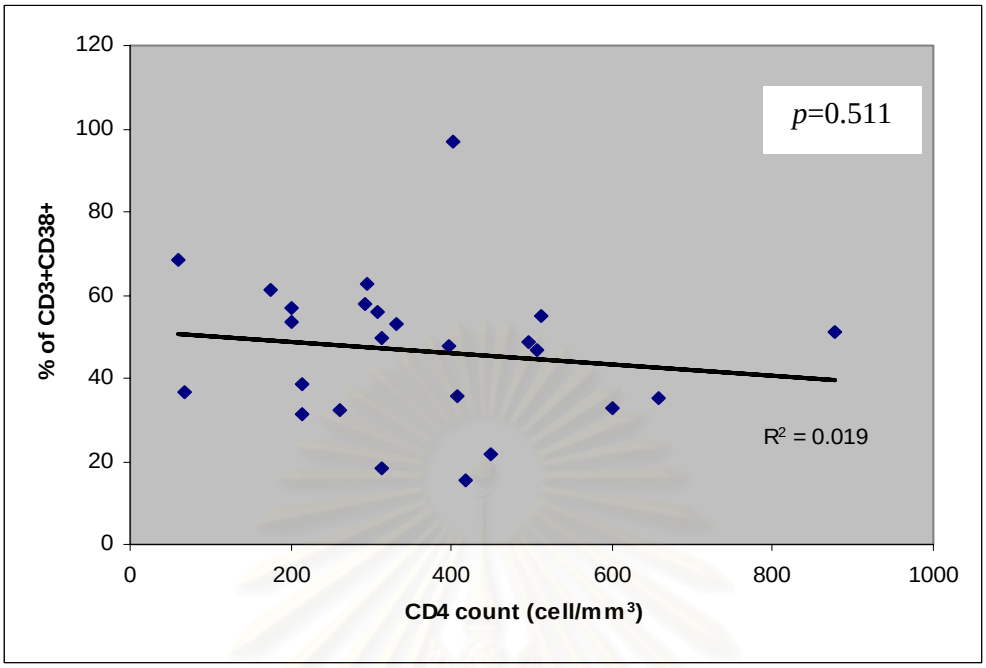


Figure 13. Correlation between the percentage of CD3+CD38+ cells and CD4 count in HIV- infected patients

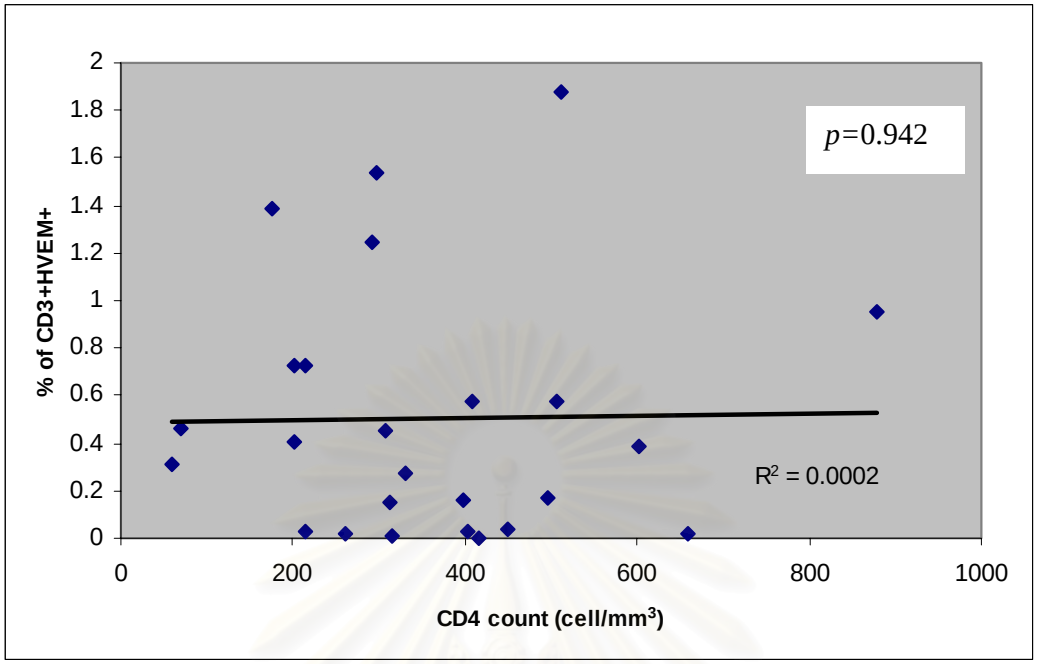


Figure 14. Correlation between the percentage of CD3+HVEM+ cells and CD4 count in HIV- infected patients

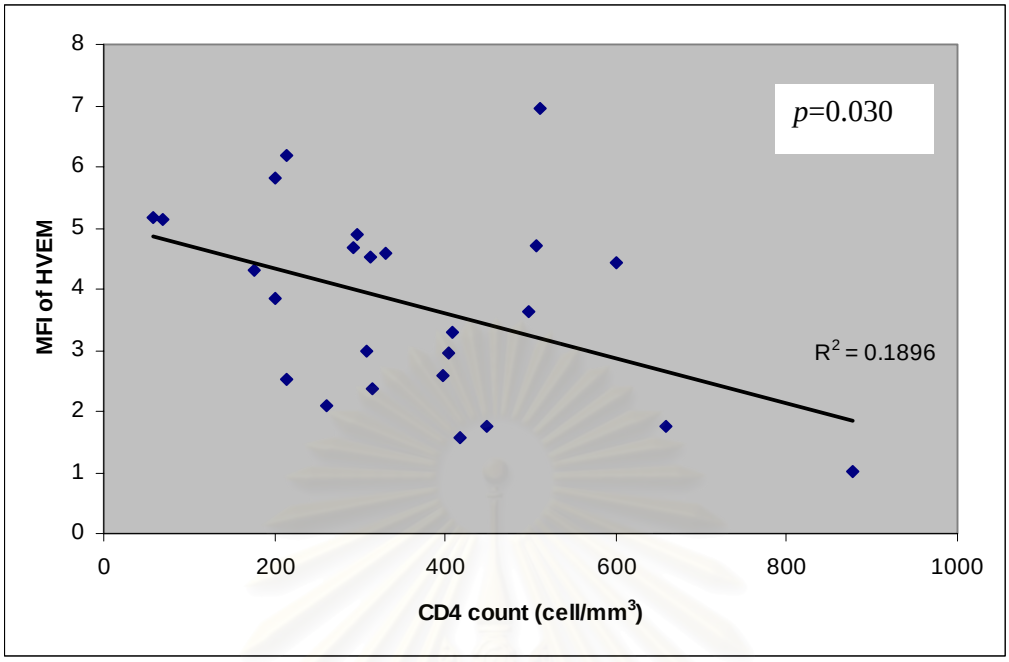


Figure 15. Correlation between the percentage of MFI of HVEM and CD4 count in HIV- infected patients

Determination of HSV-1 infected T-lymphocyte subpopulation by Flow Cytometry

To investigate which subpopulation of T-lymphocytes was susceptible to HSV-1 infection, PBMC from those volunteers were infected with HSV-1 at MOI of 5 for 24 hours and infected cells were stained with anti-CD3, anti-CD4, anti-CD8 and anti-HSV-1. The number of infected T-lymphocytes in each sample was analyzed and divided to the percentage of each infected CD4+ and infected CD8+ as shown in Table 5 and 6. The representative of flow cytometry two-color dot plots were demonstrated in Figure 16-18.

The results showed that HSV-1 could infect both CD4+ and CD8+ T-lymphocytes of HIV-infected patients and healthy donors. Moreover, the percentage number of HSV-1 infected T-lymphocytes from healthy donor (80.76±7.13) was barely the same as that from HIV-infected patients (79.25±14.63) as shown in Table 5 and 6. However, the percentage of infected CD4+ T-lymphocytes from HIV-infected patients (97.60±2.20) was higher than those from healthy donors (92.93±3.90) (p-value < 0.001) which was similarly to those observed in the CD8+ T-lymphocytes (97.7±3.62 vs 91.89±9.33, p-value = 0.001) as shown in Table 7 and 8 suggesting that either CD4+ T-cells or CD8+ T-cells of HIV-infected patients was more statistically significant susceptible to HSV-1 infection than those of healthy donors.

Table 5. Percentage number of HSV-1 infection in T-lymphocytes subpopulation of HIV-infected patients

Patient	HSV-1 infected cells (%)		
	CD3+	CD3+CD4+	CD3+CD8+
P01	81.61	4.67	94.76
P02	43.09	6.37	93.00
P03	92.03	24.90	64.99
P04	85.81	10.09	77.55
P05	91.77	32.97	57.59
P06	90.64	32.15	59.04
P07	83.48	11.01	85.59
P08	94.90	43.26	45.53
P09	96.80	26.88	60.89
P10	72.32	43.95	17.14
P11	92.85	31.73	48.56
P12	69.70	27.84	64.95
P13	83.25	32.65	61.98
P14	86.60	33.79	58.89
P15	39.85	21.51	57.52
P16	87.30	11.73	85.19
P17	75.68	24.01	39.61
P18	86.78	32.38	53.99
P19	85.32	30.24	61.14
P20	81.90	14.03	69.78
P21	60.29	30.20	68.84
P22	66.54	44.75	6.63
P23	84.56	59.13	33.67
P24	69.88	20.45	63.12
P25	78.24	21.29	61.66
Mean±SD	79.25±14.63	26.88±13.07	59.66±20.73

Table 6. Percentage number of HSV-1 infection in T-lymphocytes subpopulation of healthy donors

Donor	HSV-1 infected cells (%)		
	CD3+	CD3+CD4+	CD3+CD8+
D01	93.61	37.97	52.18
D02	79.56	37.88	57.41
D03	74.58	32.62	58.03
D04	81.80	60.95	35.34
D05	82.04	55.44	24.72
D06	76.08	47.51	19.09
D07	76.11	48.52	25.98
D08	72.18	52.16	12.68
D09	73.01	49.58	28.89
D10	81.09	46.43	18.84
D11	75.03	43.44	33.75
D12	92.12	46.81	44.65
D13	76.00	45.02	41.04
D14	88.77	55.56	29.82
D15	89.55	39.83	34.50
Mean±SD	80.76±7.13	46.65±7.62	34.46±13.94

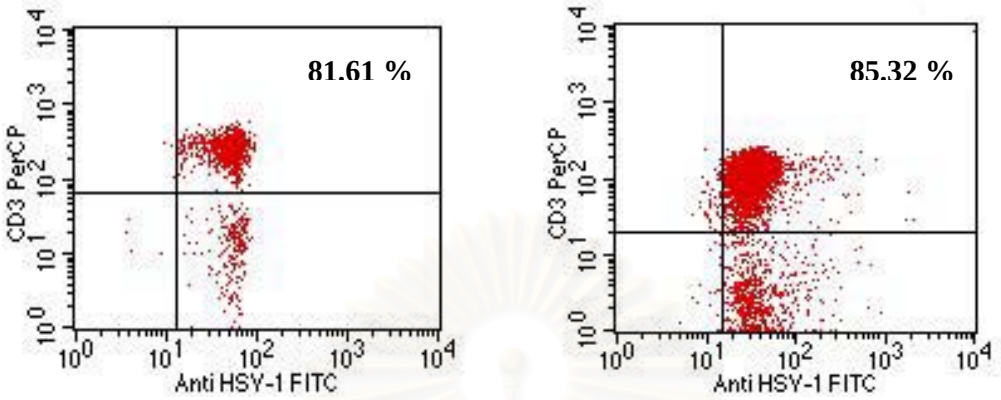
Table 7. Percentage of HSV-1 infection in CD4+ and CD8+ T-lymphocytes of HIV-infected patients

Patient	% / Total CD4		% / Total CD8	
	CD4+HSV+	**CD4+HSV-**	**CD8+HSV+**	**CD4+HSV-**
P01	96.29	3.71	99.29	0.71
P02	98.76	1.24	99.83	0.17
P03	98.65	1.35	98.37	1.63
P04	99.70	0.30	99.60	0.40
P05	99.70	0.30	99.86	0.14
P06	99.84	0.16	100.00	0.00
P07	94.83	5.17	100.00	0.00
P08	97.00	3.00	97.39	2.61
P09	98.97	1.03	98.64	1.36
P10	93.63	6.37	87.63	12.37
P11	100.00	0.00	99.77	0.23
P12	97.72	2.28	99.92	0.08
P13	99.00	1.00	98.95	1.05
P14	98.69	1.31	99.63	0.37
P15	99.91	0.09	99.88	0.12
P16	97.67	2.33	99.67	0.33
P17	98.69	1.31	97.83	2.17
P18	96.8	3.20	98.34	1.66
P19	95.06	4.94	98.41	1.59
P20	98.04	1.96	97.11	2.89
P21	100.00	0.00	99.87	0.13
P22	92.63	7.38	86.33	13.67
P23	98.88	1.12	99.67	0.33
P24	93.89	6.11	93.89	6.11
P25	95.64	4.36	94.83	5.17
Mean±SD	97.60±2.20	2.40±2.20	97.79±3.62	2.21±3.62

Table 8. Percentage of HSV-1 infection in CD4+ and CD8+ T-lymphocytes of healthy donors

Donor	% / Total CD4		% / Total CD8	
	CD4+HSV+	**CD4+HSV-**	**CD8+HSV+**	**CD8+HSV-**
D01	98.60	1.40	99.05	0.95
D02	99.34	0.66	99.46	0.54
D03	98.40	1.60	98.59	1.41
D04	94.51	5.49	98.17	1.83
D05	89.71	10.29	96.71	3.27
D06	89.12	10.88	74.08	25.92
D07	93.70	6.30	85.24	14.76
D08	87.36	12.64	67.55	32.45
D09	92.10	7.90	92.60	7.40
D10	92.32	7.68	94.77	5.23
D11	91.47	8.53	92.14	7.86
D12	94.41	5.59	96.17	3.83
D13	92.98	7.02	93.91	6.09
D14	93.63	6.37	94.61	5.39
D15	86.21	13.79	95.30	4.70
Mean±SD	92.93±3.90	7.07±3.90	91.89±9.33	8.11±9.33

HIV-infected patients



Healthy donors

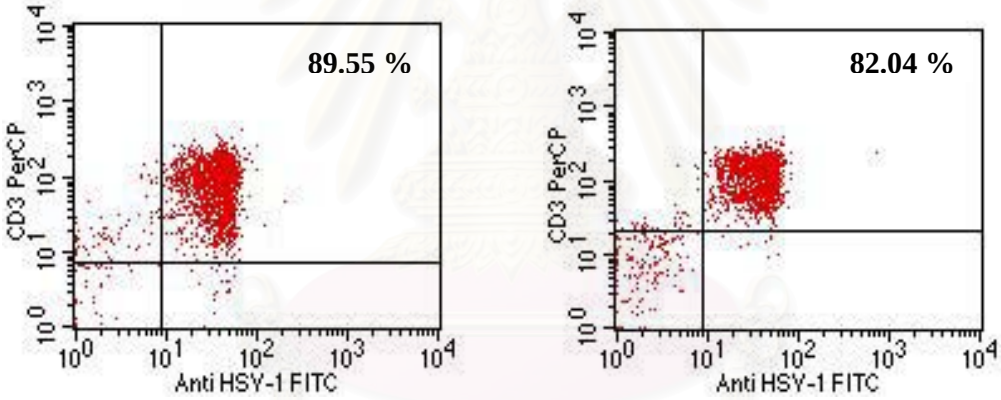
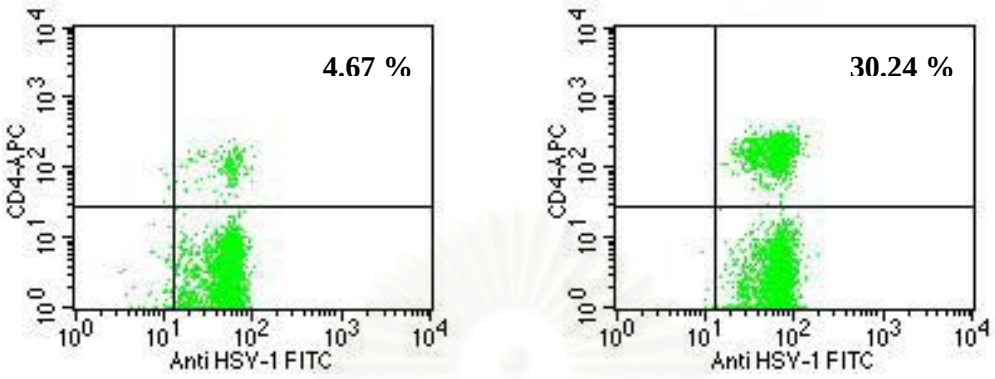


Figure 16. Representative profile comparing HSV-1 infection in T-lymphocytes from 2 HIV-infected patients (P01 and P19) and 2 healthy donors (D02 and D 07) from flow two-color dot plot.

HIV-infected patients



Healthy donors

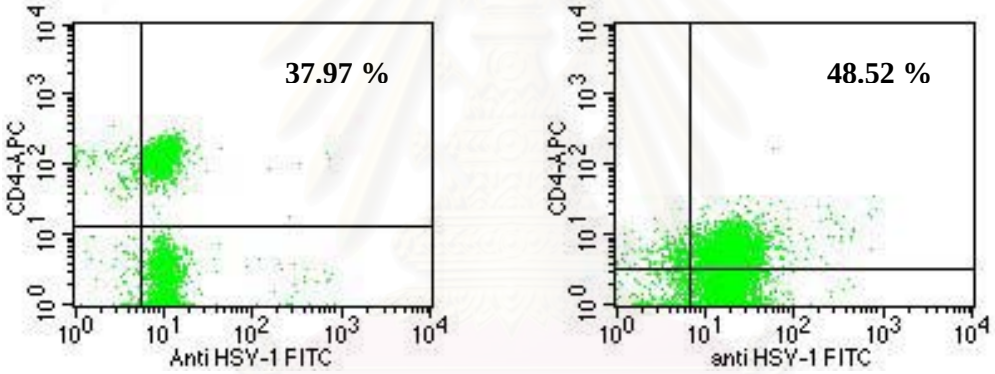
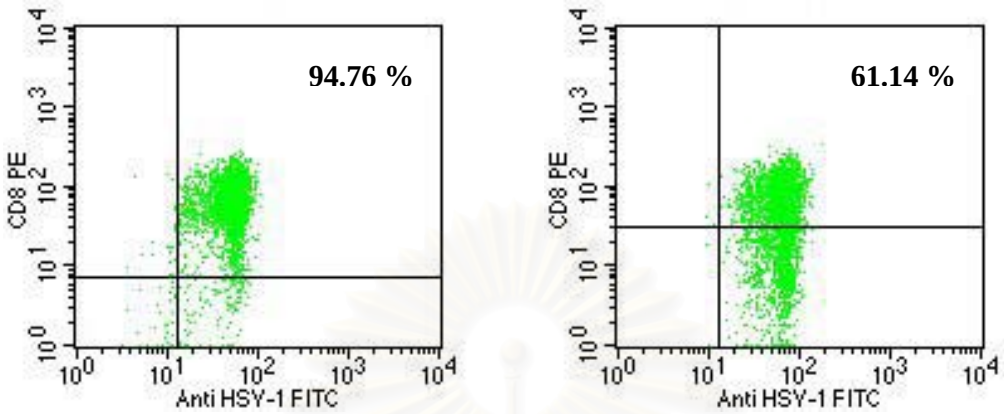


Figure 17. Representative profile comparing HSV-1 infection in CD4+ T-lymphocytes from 2 HIV-infected patients (P01 and P19) and 2 healthy donors (D01 and D 07) from flow two-color dot plot.

HIV-infected patients



Healthy donors

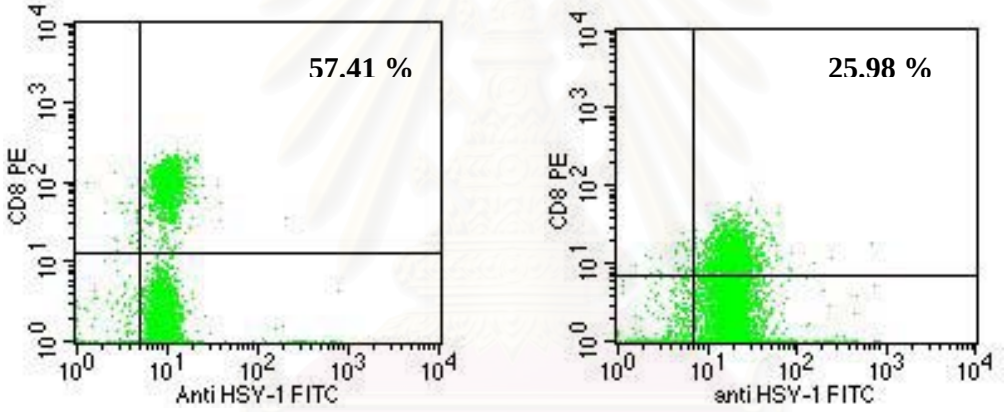


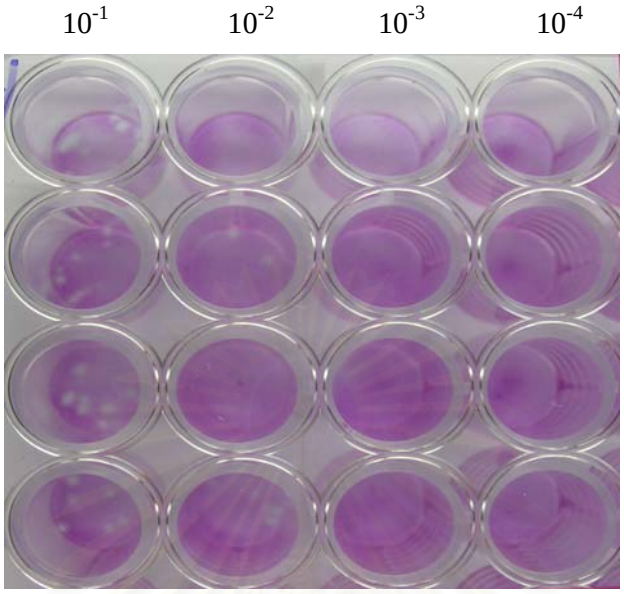
Figure 18. Representative profile comparing HSV-1 infection in CD8+ T-lymphocytes from 2 HIV-infected patients (P01 and P19) and 2 healthy donors (D02 and D 07) from flow two-color dot plot.

Replication efficiency of HSV-1 in PBMC

The PMBC from those volunteers (both HIV-infected patients and healthy donors) were infected with HSV-1 at MOI of 5 for 24 hours after that cell lysate and culture supernatant were collected. The efficiency of HSV-1 production in PBMC was quantitated by plaque titration assay (Figure 19, 20). The results were demonstrated in Table 9 and Table 10.

The amount of HSV production in cell lysate from healthy individuals (2,170±900 PFU/ml) was significant lower than that from HIV-infected patients (3,540±1,210 PFU/ml) (*p*-value = 0.001) similar to culture supernatant (32,000±8,190 vs 43,800±11,300) (*p*-value = 0.002). It is clearly shown that the virion production in PBMC preferred to release out rather than retained in cell (Figure 21).

a. cell lysate



b. culture supernatant

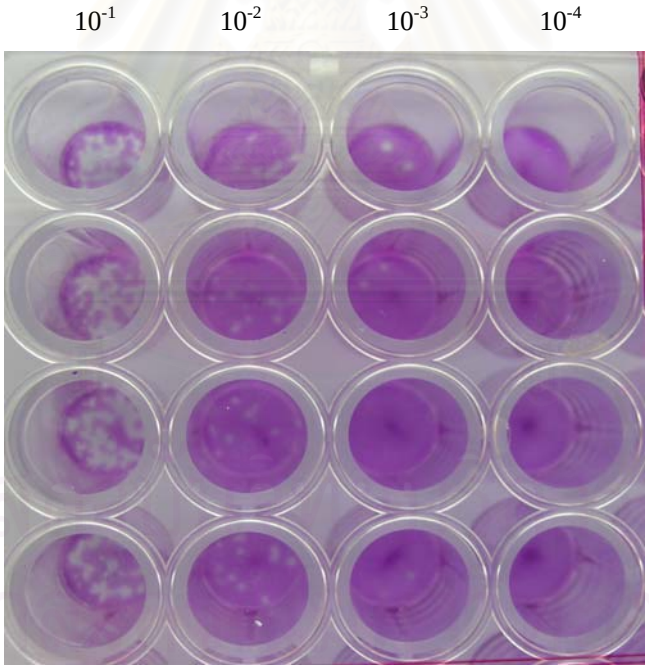
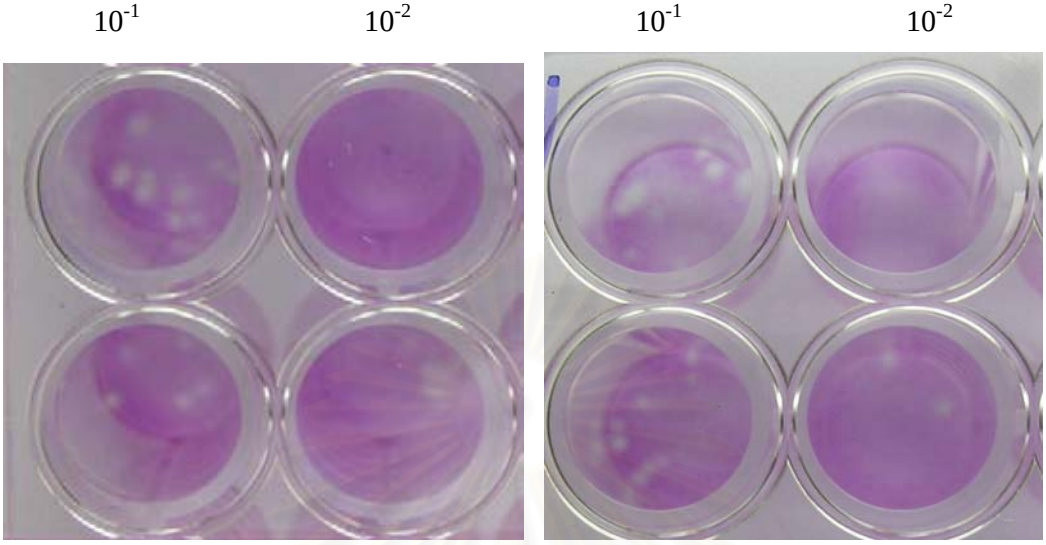
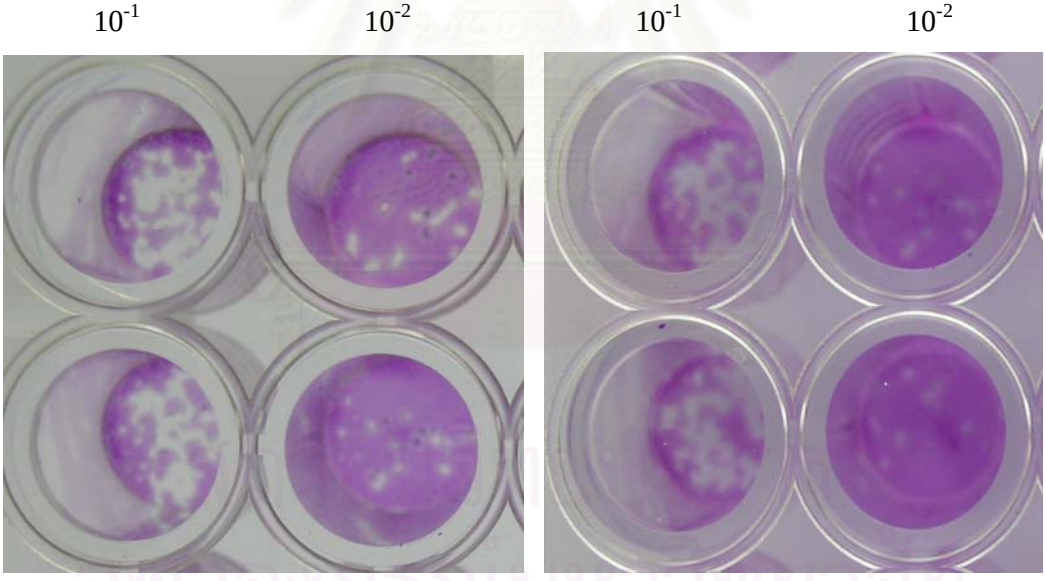


Figure 19. Plaque titration assay from cell lysate (a) and culture supernatant (b) in 4 dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4})

a. cell lysate



b. culture supernatant



HIV-infected patients **Healthy donors**

Figure 20. Plaque titration assay from cell lysate (a) and culture supernatant (b) from HIV-infected patients and healthy donors at dilution 10^{-1} and 10^{-2}

Table 9. The amount of HSV-1 virions produced from PBMC in HIV-infected patients

Patient	Amount of viruses (x 10^3 PFU/ml)		
	Inside cells	**Supernatant**	**Total**
P01	3.5	55	58.5
P02	4.0	35	39.0
P03	1.5	40	41.5
P04	4.0	55	59.0
P05	2.5	25	27.5
P06	2.0	50	52.0
P07	4.5	65	67.5
P08	3.5	40	43.5
P09	5.5	35	40.5
P10	3.0	60	63.0
P11	4.5	45	49.5
P12	4.0	40	44.0
P13	6.5	25	31.5
P14	3.0	35	38.0
P15	2.5	60	62.5
P16	3.0	55	58.0
P17	4.5	45	49.5
P18	4.0	55	59.0
P19	2.0	50	52.0
P20	2.5	45	47.5
P21	4.0	45	49.0
P22	5.5	35	40.5
P23	3.0	40	43.0
P24	2.5	35	37.5
P25	3.0	25	28.0
Mean±SD	3.54±1.21	43.80±11.30	47.34±11.14

Table 10. The amount of HSV-1 virions produced from PBMC in healthy donors

Donor	Amount of viruses (x 10^3 PFU/ml)		
	Inside cells	**Supernatant**	**Total**
D01	1.5	30	31.5
D02	1.0	25	26.0
D03	2.0	20	22.0
D04	1.5	30	31.5
D05	1.0	20	21.0
D06	3.0	35	38.0
D07	2.5	45	47.5
D08	3.0	25	28.0
D09	1.5	40	41.5
D10	2.0	30	32.0
D11	4.0	35	39.0
D12	2.5	25	27.5
D13	1.5	45	46.5
D14	2.0	35	37.0
D15	3.5	40	43.5
Mean±SD	2.17±0.90	32±8.19	34.17±8.48

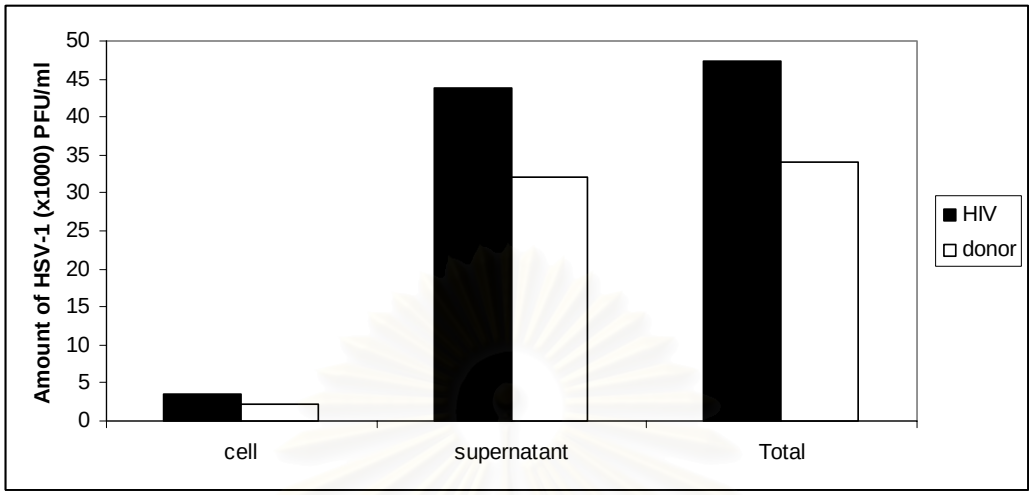


Figure 21. Comparison of the amount of virus in PFU/ml by plaque titration assay in cell lysate and culture supernatant after infecting with HSV-1 at MOI of 5 in healthy individuals (white column) and HIV-infected patients (black column)

Effect of HSV-1 on HIV virions production from HIV-infected PBMC

The effect of HSV-1 infection on PBMC obtained from HIV-infected patients was observed comparing to PBMC without HSV infection. The PMBC from each HIV-infected patient was infected with HSV-1 at MOI of 5 for 24 hours after that culture supernatant was collected. The amount of HIV production (HIV load) in PBMC was quantitated by The AMPLICOR HIV-1 MONITOR Test, v1.5. The results were demonstrated in Table 11 and Figure 22.

Mean $\log_{10}$ amount of HIV production from the non-infected HSV-1 PBMC (3.85±1.09) is greater statistical significant than that from the HSV-1-infected PBMC (3.54±1.05) (*p*-value < 0.001).

Table 11. The amount of HIV virions produced from PBMC infected and non-infected (mock) HSV-1 in HIV infected patients

No.	HIV viral load (copies/ml)		Log_{10} HIV viral load	
	Mock	Infected HSV	Mock	Infected HSV
P01	32,000	12,400	4.51	4.09
P02	38,200	2,960	4.58	3.47
P03	23,500	9,580	4.37	3.98
P04	50	84	1.70	1.92
P05	3,720	2,550	3.57	3.41
P06	33,100	11,100	4.52	4.05
P07	43,900	8,860	4.64	3.95
P08	128,000	90,600	5.11	4.96
P09	7,960	14,000	3.90	4.15
P10	60,200	39,300	4.78	4.59
P11	2,540	1,860	3.40	3.27
P12	13,900	7,420	4.14	3.87
P13	51,300	19,600	4.71	4.29
P14	2,190	376	3.34	2.58
P15	8,420	5,740	3.93	3.76
P16	112,800	34,900	5.05	4.54
P17	5,650	2,130	3.75	3.33
P18	8,910	5,690	3.95	3.76
P19	78,200	96,400	4.89	4.98
P20	603	59	2.78	1.77
P21	63,700	18,100	4.80	4.26
P22	29,300	23,700	4.47	4.37
P23	50	50	1.70	1.70
P24	95	50	1.98	1.70
P25	50	50	1.70	1.70
Mean±SD	29,933.52±35,658.77	16,302.36±25,560.51	3.85±1.09	3.54±1.05

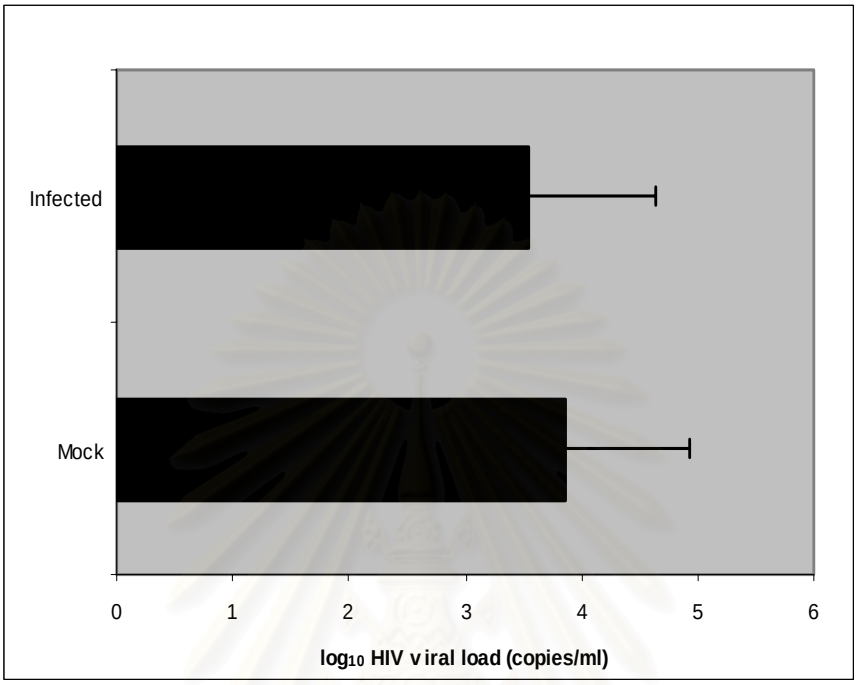


Figure 22. Comparison the mean $\log_{10}$ HIV load between mock and HSV-1 infected PBMC of HIV-infected patients.

CHAPTER VI

DISCUSSION

HIV infection is an important public health problem in many countries around the world. The progression of HIV infection depends on the immune status in each individual. When the immune status declines, a number of pathogens can cause opportunistic infections such as *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Cryptococcus neoformans*, *Pneumocystis carinii*, *Candida albicans*, cytomegalovirus, and HSV[161]. In HIV patients, HSV reactivation can often occur and HSV infection is able to cause not only localized infection but also disseminated or systemic infection. This phenomenon is easily found especially in immunocompromised host including HIV-infected patients[8, 9].

Beside epithelial cells, HSV can grow in macrophage and lymphocytes[10, 162]. HSV can replicate in T-lymphocytes with very low efficiency of virion production comparing to efficiency of virion production in epithelial cells[10]. However, the yield viral production can be increased equal to that of epithelial cells after activating T-lymphocytes with mitogen, PHA[11]. In order to achieve entry to host cells, HSV requires many host cell surface receptors and HVEM is one of these receptors that restrict expression on immune cell especially lymphocytes. Upregulation of HVEM mRNA has been demonstrated in PHA activating T-lymphocyte (Jurkat) cell lines[11]. Therefore, HSV viremia may come from HSV infection in activated lymphocytes. Chronic infection such as HIV infection may mimic this viremia phenomenon

since under chronic infection, the lymphocytes especially T cells have already been activated by several cytokines. Several studies reported the high percentage of activated T-lymphocytes (CD3+CD38+) present in HIV-infected patients[12]. And recent data confirmed that the percentage of activated T-lymphocytes (CD3+CD38+) was significantly higher in HIV-infected patients (46.51±17.54%) than that in healthy individuals (27.54±14.12%, Table 3 and 4). According to *in vitro* study of *Chimma P et al*. [11] suggested that the more activated T cells the more HVEM expressing cells should be detected. But the results of *ex vivo* study showed no difference in the percentage of HVEM expressing T-lymphocytes (CD3+HVEM+) as well as HVEM expressing in activated cells (CD38+HVEM+) between two study groups (Table 3 and 4). This suggested that although the number of activated T lymphocytes increased, the number of HVEM expressing T cells did not change. In this study, the number of positive HVEM staining cells was quite low, although optimization of flow cytometry staining conditions using multiple antibodies including anti-HVEM antibody were done (see Appendix III). This was not expected since HVEM is mainly expressed on immune cells including T-lymphocytes. Reduction in HVEM+ cells detection was possibly due to the interference of other antibodies staining on cell surface of the same cell. Even though, the results could be analyzed.

Interestingly, the percentage of CD38+HVEM+ expressing cells in both groups was higher than those of CD3+HVEM+ suggesting other cell types beside CD3+ cells were activated or present CD38+ and HVEM+ on their surface. These cells were B-lymphocytes and NK cells[12]. Moreover, the number of HVEM molecule on the cell surface determining by measuring MFI in HIV-infected patients was lower than in healthy donors but not statistically significant (Table 3, 4 and Figure

11). One possible explanation of this phenomenon is that the activated T-lymphocytes obtained from those HIV-infected patients might already have lost HVEM on their surface. HVEM is a member of tumor necrosis factor receptor superfamily that binds to LIGHT (natural ligand of HVEM). LIGHT is a tumor necrosis factor (TNF) superfamily ligand that regulates T cell immune responses by signaling through the HVEM and the lymphotoxin β receptor (LβTR). The interaction of LIGHT-HVEM is co-stimulatory T-cells. HVEM are constitutively expressed in freshly isolated peripheral blood T and B lymphocytes, monocytes, and cultures of immature dendritic cells and cells that express LIGHT is activated lymphocytes, natural killer (NK) cells and immature dendritic cells. LIGHT and HVEM are expressed at different times on the surface of T lymphocytes. LIGHT transcripts are measurable by RT-PCR shortly (1–2 hours) after T cell activation. Cell surface levels of LIGHT are detectable by 4 hours following T cell activation, peak at 24–48 hours and gradually decline by day 5. In contrast, HVEM levels are high on resting T cells and decrease rapidly over time following T cell activation. Thus, as LIGHT levels increase, HVEM levels coordinately decrease at roughly the same rate. At day 5 post-activation, as LIGHT levels decline to baseline, HVEM begins to appear on the surface again, gradually reaching resting levels by day 6–7 post-activation[163]. So, HVEM expression from HIV-infected patients was less than healthy donors possibly due to T-lymphocytes from HIV-infected patients were activated whereas T-lymphocytes from healthy donors were less or non-activated.

There are many reasons to explain why the results did not correlate to *in vitro* study of HVEM expression in Jurkat T-lymphoblastoid cell lines. First, T-lymphocytes were used in these 2 studies were different. Jurkat cells, used in *in vitro* study, are transformed T-lymphoblastoid cell

lines whereas normal T-lymphocytes were used *ex vivo*. Second, activation status between *in vitro* and *in vivo* may not be the same. T-lymphocytes *in vitro* were activated with PHA as mitogen whereas T-lymphocytes *in vivo* were activated with many stimuli such as cytokine and receptor and ligand interaction. Thus, different activation may effects the response of the cells. Third, T-lymphocytes from HIV-infected patients were repeatedly activated for long time unlike mitogen activated T-lymphocyte just one time. So, many surface receptors on T-lymphocyte from both groups might be different including HVEM. Beside those reasons, group of studied patients may play some role. Most of HIV-infected patients have CD4 counts more than 200 cells/ml and no sign of opportunistic infection implying that their immune status was also good and probably similar to healthy persons, HVEM expression from healthy donors and HIV-infected patients was not significant different. But some report they found that when T-lymphocytes were activated, the expression of HVEM was decrease and LIGHT (the ligand of HVEM) was increase instead of the HVEM [163].

The inverse correlation between the number of CD38+ T-lymphocytes and CD4+ T cells has already been demonstrated in HIV patients as well as the number of CD38+ T cells was used as a marker to predict the severity of the HIV infection in patients [12]. Inverse correlation between these two parameters was illustrated similar to those previously reports. An attempt to explore the correlation between other parameters such as CD3+HVEM+ cells and MFI of HVEM against CD4+ T cell count has been done. Interestingly, the significant correlation was observed between MFI of HVEM and CD4+ T cell count (R= -0.44, p-value=0.03; Figure 15). The more CD4+ T cells, the less molecule of

HVEM present on the surface of T-lymphocytes. After cells activation for awhile, HVEM will disappear. Under low CD4+ cells, HIV patients have chance to get more opportunistic infections. In such situation, T-lymphocytes might be repeatedly activated causing lost of HVEM on cell surface.

Among T-lymphocytes, both CD4+ and CD8+ cells were able to be infected with HSV-1 (Table 5 and 6). More than 90% of either CD4+ and CD8+ T cells of both groups were infected. Significant difference of HSV-1 infection in CD4+ and CD8+ T cells between HIV-infected patients and healthy individuals was demonstrated (Table 7 and 8) implying that the susceptibility of CD4+ and CD8+ T cells of HIV-infected patients were higher than those of healthy donors. In addition, the ratio of HSV-1 infected CD4+ to CD8+ cells in HIV-infected patients was only 0.46 whereas that in healthy donors was 1.37. This difference was due to the different in ratio of CD4+ to CD8+ cells between two study groups. In normal adult population, the ratio of CD4+ to CD8+ cells should be greater than 1 (approximately 2:1) but the invert ratio of CD4+ to CD8+ (less than 1) was commonly found in HIV-infected patients especially in the progress stage[164]. The ratio of CD4+ to CD8+ in Asian population was mostly below 2:1 (communication to Dr. Pokrath).

After evaluation of the yield HSV virions production in equal number of PBMC, it was found that HSV production in HIV-infected patients was significant higher than that in healthy individuals (47,340$\pm$11,140 PFU/ml vs 34,170$\pm$8,480 PFU/ml, Table 9 and 10). This observation might cause by two reasons, one was the cells obtained from

HIV-infected patients might be in the activated state supporting HSV growth and/or another reason was the number of HSV-1 infected cells in HIV group was higher than those in normal group. The results from this study support possibility of first reason. First, the activated (CD38+) cells in HIV-infected patients were higher than those from healthy donors. Therefore, the efficiency of HSV replication increased. La S. and his colleague showed that resting T cells had HVEM receptor and allowed HSV to enter but no replication was observed. Only activated T cells supported growth of the virus[123]. Second, although the number of HSV-1 infected T cells in HIV-infected patients was lower than in normal group (79.25$\pm$14.63 vs 80.76$\pm$7.13, Table 5 and 6). However the second reason could not be excluded since the other cells type (non-CD3+ cells) in PMBC such as B-lymphocyte, monocytes and NK cells could be hosts for HSV. Unfortunately the total number of infected cells could not be able to calculate due to lack of white blood count.

Different characteristic in HSV release from cells was observed in this study. The yield virion production in PBMC either from cell lysate or supernatant of HIV-infected patients was greater significantly from those in healthy individuals. Moreover, virion productions in PBMC were detected in supernatant greater than inside the cells. This phenomenon probably caused by type of cells. Virion releasing from epithelial cells is by cell-to-cell spread because cell membrane is very close contact[36]. In case of PBMC, cells float individually so virions were released out from cell to supernatant. The ability of releasing HSV-1 virions from circulating white blood cells into blood stream might be one mechanism to enhance disseminated infection in immunocompromised host.

A number of studies revealed synergistic co-pathogens between HIV-1 and HSV-2. Nagot *et al* [165]underlines the association of HSV-2 with significantly higher amounts of HIV-1 in plasma and in genital secretions in women with sexually acquired HIV-1. Multiple studies have shown that a persistent of HSV-2 infection increase of 0.5 $\log_{10}$ copy/ml in plasma HIV-1 level. Recently, treatment of HSV-2 infection reduced HIV replication[166]. Similar events were happened to HSV-1, HSV-1 infection was capable to activate HIV-1 proviral DNA to replication[147]. Double infection between HSV-1 and HIV-1 could result in the production of psuedovirions[157]. From those accumulated data, HIV virion production from PBMC of HIV-infected patients should increase after HSV-1 inoculation. In contrast, the result showed HIV-1 production from PBMC infected with HSV-1 was significant lower than that from mock infection (Table 11 and Figure 22) which was unexpected results. However, Calistri *et al*[167] showed that positive or negative influence on HIV production depended on the multiplicity of HSV-1 superinfection. MOI of 10 PFU/ml suppressed HIV-1 production while MOI of 1 PFU/ml enhanced HIV-1 replication. In this study, MOI of 5 PFU/ml was applied suggesting that the results should be similar to MOI of 10 PFU/ml. In addition, the environment *ex vivo* might differ from *in vivo*, at least absence of cytokines and chemokines. Those factors affect viral production both HSV and HIV virions[153].

In conclusion, HSV-1 can infect both CD4+ and CD8+ T lymphocytes. The efficiency of HSV-1 replication in these T-lymphocytes was not depend solely on the presence of HVEM receptor. HSV-1 can grow much better in PBMC of HIV-infected patients and its growth affects the HIV virion production. Enhancing of HSV release out from infected PBMC cells might play an important role for HSV viremia

(Table 12). For further study, the number of CD4+ HVEM+ cells and CD8+HVEM+ cells should be determined to support HVEM entry receptor. To see the effect of HSV infection to HIV production, vary of MOI is necessary. Detection of cytokines release in the culture system may help to understand the role in HSV or HIV replications.

Table 12. Summary data

	Mean±SD		
	HIV-infected patients	**Healthy donors**	***P*-value**
HVEM in activated cells			
% CD3+CD38+	46.51±17.54	27.54±14.12	0.001
%CD3+HVEM+	0.50±0.53	0.57±0.34	0.282
%CD38+HVEM+	0.79±0.92	0.80±0.99	0.890
MFI of HVEM	3.75±1.57	4.92±2.11	0.142
Correlation against CD4+ cells			
CD3+CD38+	*R= - 0.138		0.511
CD3+HVEM	R= 0.015		0.942
MFI	R= - 0.435		0.030
Susceptibility to HSV-1 infection			
%CD3+HSV-1+	79.25±14.63	80.76±7.13	0.922
%CD4+HSV-1+/ Total CD3+	26.88±13.07	46.65±7.62	<0.001
%CD8+HSV-1+/ Total CD3+	59.66±20.73	34.46±13.94	<0.001
%CD4+HSV-1+/ Total CD4+	97.60±2.20	92.93±3.90	<0.001
%CD8+HSV-1+/ Total CD8+	97.79±3.62	91.89±9.33	0.001
Yield HSV-1 production ($x10^3$)			
Inside cell	3.54±1.21	2.17±0.90	0.001
Supernatant	43.80±11.30	32.00±8.19	0.002
Total	47.34±11.14	34.17±8.48	0.001
Yield HIV-1 production (log_{10})			
Mock	3.85±1.09		
HSV-1 infection	3.54±1.05		<0.001

*R = Correlation

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APPENDICES

APPENDIX I

REAGENTS, METERIALS AND INSTRUMENTS

A. MEDIA AND REAGENTS

Absolute ethanol	(Merck, Germany)
Allophycocyanin-conjugated anti-human CD4 (CD4/APC)	(BD, U.S.A.)
Allophycocyanin-conjugated anti-human CD38 (CD38/APC)	(BD, U.S.A.)
Bovine serum albumin	(Sigma, U.S.A.)
Ethylenediamine tetraacetic (EDTA)	(Amresco, U.S.A)
Fetal bovine serum (FBS)	(Hyclon, UK.)
FACS Permeabilizing solution with saponin	(BD, U.S.A.)
Fluorescein isothiocyanate-conjugated goat polyclonal to mouse antibody	(Abcam, UK)
Gum tragacanth	(Sigma, U.S.A.)
L-glutamine	(Sigma, U.S.A.)
Medium 199 (M199)	(GIBCO, U.S.A.)
Mouse monoclonal anti-HVEM	(Abcam, UK)
Paraformaldehyde	(Sigma, U.S.A.)
Penicillin	(Dumex, Thailand)
Periclinin chlorophyl protein-conjugated anti-human CD3 (CD3/PerCP)	(BD, U.S.A.)
Potassium chloride (KCl)	(Merk, Germany)
Phycoerythrin-conjugated anti-human CD8 (CD8/PE)	(BD, U.S.A.)
Rabbit-anti HSV-1 antibody	(DAKO,Denmark)

RPMI 1640 (with L-glutamine)	(GIBCO BRL, U.S.A.)
Sodium Azide (NaN_3)	(Sigma, U.S.A.)
Sodium chloride (NaCl)	(Merk, Germany)
Sodium hydrogencarbonate ($NaHCO_3$)	(Merk, Germany)
Swine-anti rabbit antibody conjugated with FITC	(DAKO, Denmark)
Streptomycin	(Dumex, Thailand)
Trypsin	(Sigma, U.S.A.)

B. MATERIALS

Microcentrifuge tube	(SRS, U.S.A.)
Tissue culture flask	(Nunclon, Denmark)
Tissue culture plate	(Nunclon, Denmark)
12x75 mm polystylene tubes with caps	(BD, U.S.A.)

C. INSTRUMENTS

Autoclave (model S-90N)	(Tomy seiko, Japan)
FACSort Flow cytometry with Cell Quest software	(BD, U.S.A.)
Incubator type 80	(Memmert, Germany)
Microcentrifuge	(Fotodyne, U.S.A.)
Mixer-Vertex-Genic	(Scientific industries, U.S.A.)
Refrigerator	(Toshiba, Japan)

APPENDIX II

REAGENTS PREPARATION

REAGENTS AND MEDIA FOR CELL CULTURE

1. 10% M199 medium

2X M199 with Earle's salts, with L-glutamine, without $NaHCO_3$	50	ml
1 M HEPES	1	ml
Pen/Strep. Antibiotic (10^5 unit/ml)	0.1	ml
10% $NaHCO_3$	1	ml
5% L-glutamine	1	ml
Fetal bovine serum	10	ml
DDW	46	ml

Sterilized by filtration and stored at 4ºC

2. 1X RPMI 1640

RPMI 1640 with L-glutamine	10.4	g
$NaHCO_3$	2	g
HEPES	2.383	g
DDW	1,000	ml

Sterilized by filtration and stored at 4ºC

3. 10% RPMI 1640 (R-10)

1X RPMI 1640 with L-glutamine	90	ml
Fetal bovine serum	10	ml
Pen/Strep. Antibiotic (10^5 unit/ml)	0.1	ml

Stored at 4ºC

4. 10X PBS (phosphate-buffer saline)

NaCl	40	g
KCL	1	g
$NaHPO_4$	5.75	g
KH_2PO_4	1	g
DDW	300	ml

Adjusted to pH 7.4 and adjusted volume to 500 ml with DDW and sterilized by autoclaving

5. 1X PBS

10X stock PBS	100	ml
DDW	900	ml

6. 10X Trypsin

Trypsin	0.5	g
EDTA	0.2	g
NaCl	9.0	g
DDW	100	ml

Sterilized by filtration and stored at -20ºC

7. 1X Trypsin

10X stock trypsin	10	ml
DDW	90	ml

Stored at 4ºC

8. Plaque overlay medium

Solution A

10X M199 with Earle's salts, with L-glutamine, without $NaHCO_3$	20	ml
FBS	20	ml
1 M HEPES	2	ml
Pen/Strep. Antibiotic (10^5 unit/ml)	0.2	ml
10% $NaHCO_3$	3	ml
5% L-glutamine	2	ml
DDW	42	ml

Solution B

Gum tragacanth	1.6	g
DDW	100	ml

Solution A and B were mixed at the ratio 1:1 before use.

REAGENTS FOR FLOW CYTOMETRY

1. Washing buffer

Bovine serum albumin	5	g
NaN_3	1	g
1X PBS	1,000	ml

2. FACS Permeabilizing solution (1X)

10X stock FACS Permeabilizing solution

Dilute 1:10 with DDW only

Stored at Room temperature for one month and at 4ºC for one year

APPENDIX III

OPTIMIZATION OF FLOW CYTOMETRY

The Jurkat cells were preliminary used for setting the staining condition for HVEM expression by flow cytometry. The cells which previously be activated by PHA 5 µg per 1×10^6 cell for 1 day and 2 days were stained with anti-HVEM and rabbit anti-mouse IgG conjugated with PE. The number and intensity of the stained cells were determined compared to normal non-activated Jurkat cells (Figure 1). The intensity of activated Jurkat cells was greater than that of non-activated Jurkat cells (Figure 1). The condition was applied for further staining in PBMC.

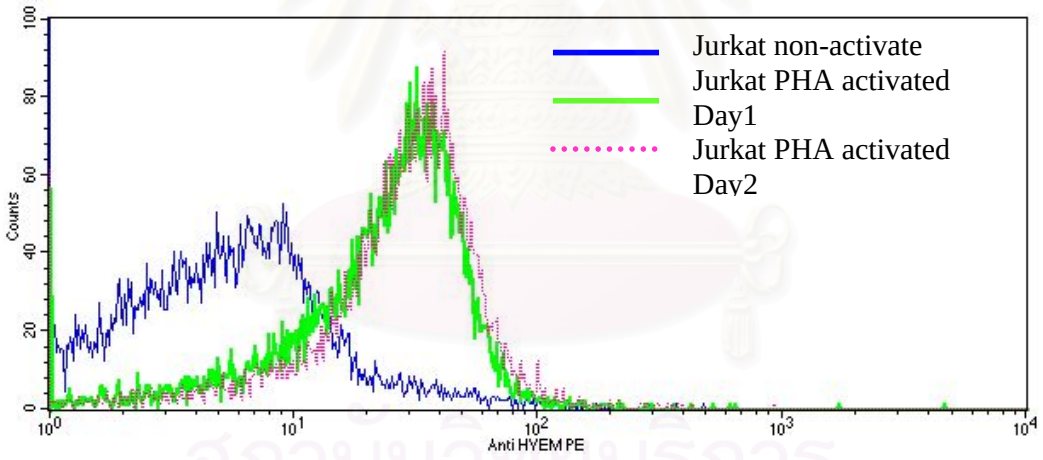
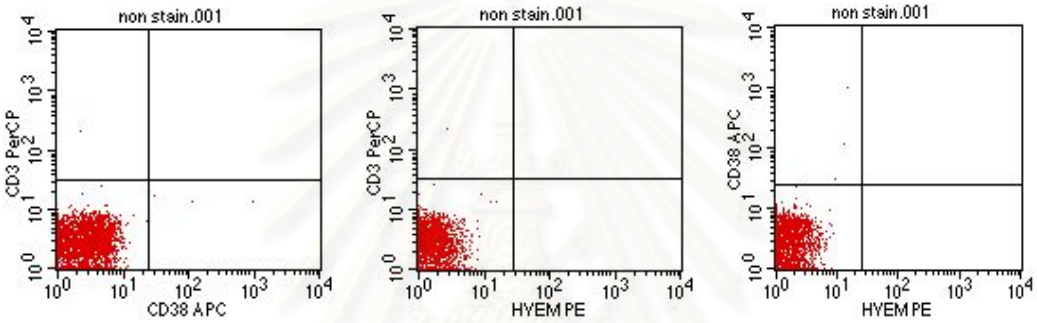


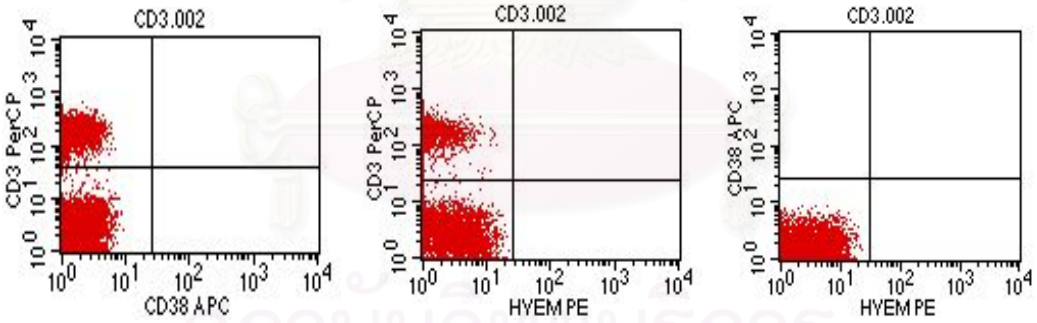
Figure 1. Jurkat cells were activated with PHA for 1 day and determined the presence of HveA on the surface membrane by flow cytometry

Before the experiments of patients were done, the setting flow cytometry were used in PBMC of patients and stained with one, two and three antibodies to analyze the pattern expression. PHA activated PBMC were used as positive control. The following picture represents optimization conditions in analyzing CD3, CD38 and HVEM expression on PBMC from flow cytometry two-color dot plot (Figure 2).

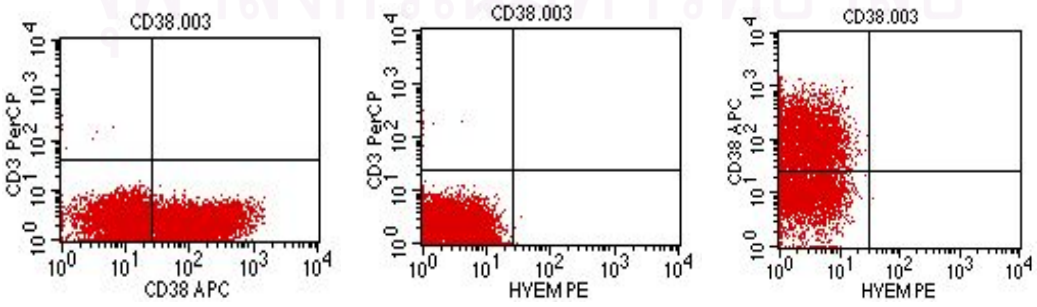
a. isotype control



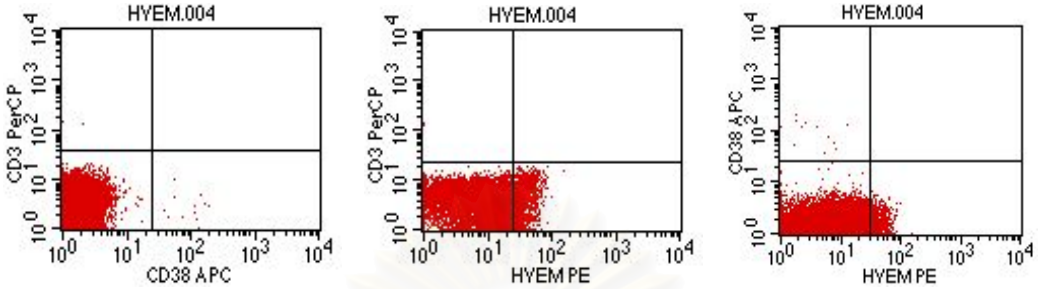
b. CD3 staining



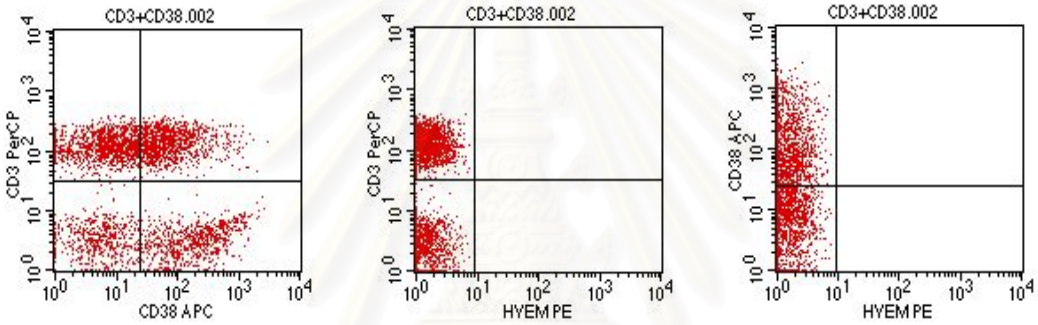
c. CD38 staining



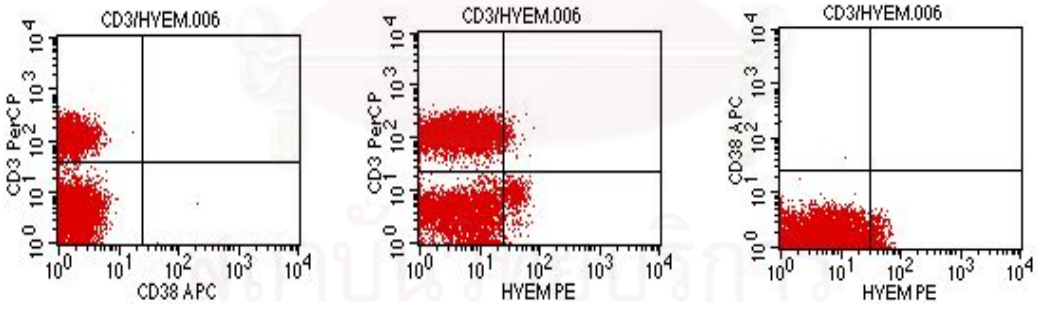
d. HVEM staining



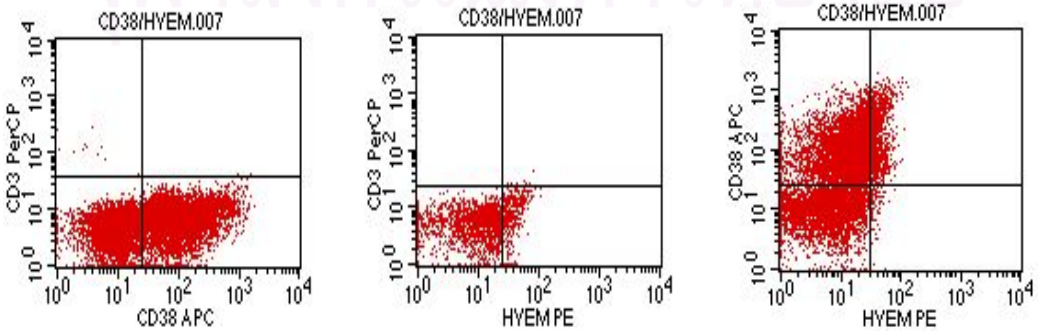
e. CD3 and CD38 staining



f. CD3 and HVEM staining



g. CD38 and HVEM staining



h. CD3, CD38 and HVEM staining

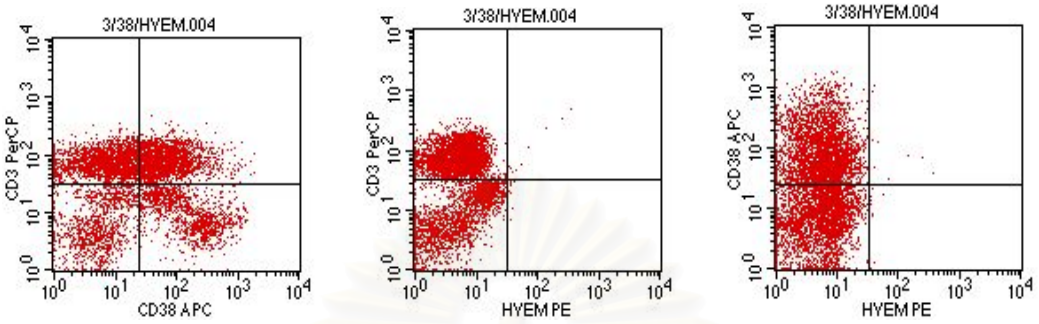


Figure 2. Representative two color dot plots CD3-CD38, CD3-HVEM and CD38-HVEM in PBMC that was stained with a.) normal human Ab b.) Anti-CD3 PerCP c.) Anti-CD38 APC d.) Anti-HVEM followed by anti-mouse IgG-PE e.) mixture of CD3 and CD38 f.) HVEM staining followed by anti-CD3 g.) HVEM staining followed by anti-CD38 h.) HVEM staining followed by mixture of CD3 and CD38

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