กลไกการกระตุ้น APOPTOSIS ใน T LYMPHOCYTES โดย HERPES SIMPLEX VIRUS

นายอตินพ พงษ์พานิช

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MECHANISM OF APOPTOSIS ACTIVATION IN T LYMPHOCYTES BY HERPES SIMPLEX VIRUS

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อตินพ พงษ์พานิช : กลไกการกระตุ้น apoptosis ใน T lymphocytes โดย Herpes simplex virus (Mechanism of Apoptosis Activation in T lymphocytes by Herpes Simplex Virus) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร. จินตนา จิรถาวร อาจารย์ที่ปรึกษาร่วม : รองศาสตราจารย์ ดร. ภาวพันธ์ ภัทรโกศล ; 85 หน้า. ISBN : 974-17-5348-9

ไวรัสเฮอร์ปีส์ซิมเพล็ก (HSV) เป็นไวรัสที่มีสารพันธุกรรมดีเอ็นเอขนาดใหญ่ สามารถพบการติดเชื้อได้ในมนุษย์ทั่วไป หลังจากมีการติดเชื้อของเยื่อบุผิวหนัง ไวรัสเฮอร์ปีส์ซิมเพล็กจะทำให้เกิดการติดเชื้อแอบแฝงในเซลล์ประสาทได้ และเมื่อติดเชื้อไวรัสชนิดนี้แล้วจะทำให้เกิดการกลับมาของโรคเป็นซ้ำๆได้ ซึ่งปัจจุบันยังไม่สามารถรักษาให้หายขาด ไวรัสเฮอร์ปีส์ซิมเพล็กมีกลไกมากมายในการหลบเลี่ยงระบบภูมิคุ้มกันของมนุษย์ รวมไปถึงการชักนำให้เกิดกลไกการตาย(apoptosis) ในทีลิมโฟซัยท์ อย่างไรก็ตามกลไกการติดเชื้อและกลไกการตายของทีลิมโฟซัยท์ด้วยไวรัสเฮอร์ปีส์ซิมเพล็กยังไม่ทราบแน่ชัด ในการศึกษาครั้งนี้ ทำการตรวจสอบกลไกทางด้านโมเลกุลของการชักนำให้เกิดกลไกการตายด้วยไวรัสเฮอร์ปีส์ซิมเพล็กในเซลล์ทีลิมโฟซัยท์ โดยใช้เซลล์ Jurkat ซึ่งเป็นเซลล์มะเร็งมาเป็นตัวแทน ในการศึกษานี้ตรวจหาปริมาณเซลล์ที่ติดเชื้อด้วยวิธีอิมมูโนฟลูออเรสเซน และโฟลไซโตเมทรีของเซลล์ พบว่าวิธีโฟลไซโตเมทรีมีความไวกว่าวิธีอิมมูโนฟลูออเรสเซน คือสามารถตรวจพบเซลล์ที่ติดเชื้อได้ตั้งแต่ 2 ชั่วโมงหลังได้รับเชื้อ ในขณะที่ต้องใช้เวลาถึง 6 ชั่วโมงสำหรับวิธีอิมมูโนฟลูออเรสเซน

ผลการศึกษาการเหนี่ยวนำให้เกิด apoptosis จากเชื้อเฮอร์ปีส์ซิมเพล็กด้วยวิธีการจับด้วย Annexin V พบว่าเชื้อเฮอร์ปีส์ซิมเพล็กทั้งสองชนิดชักนำให้เกิด apoptosis ในเซลล์ Jurkat และสารยับยั้ง caspase-3, -8 และ -9 สามารถยับยั้งการเกิด apoptosis แสดงว่ากลไกที่เฮอร์ปีส์ซิมเพล็กเหนี่ยวนำให้เกิด apoptosis ในทีลิมโฟซัยท์ผ่านวิถีทาง caspase แต่อย่างไรก็ตาม อาจมีวิถีทางอื่นมาเกี่ยวข้องเนื่องจากการยับยั้ง apoptosis ด้วยสารยับยั้ง caspase ที่ใช้ไม่สามารถยับยั้งการเกิด apoptosis ได้อย่างสมบูรณ์ นอกจากนี้ยังพบว่าเชื้อเฮอร์ปีส์ซิมเพล็กชนิด II สามารถเหนี่ยวนำให้เซลล์ Jurkat เกิด apoptosis ได้มากกว่าเชื้อเฮอร์ปีส์ซิมเพล็กชนิด I ที่ 12 ชั่วโมงอย่างมีนัยสำคัญทางสถิติ (p=0.003)

การศึกษาเพิ่มเติมใน T cells จาก peripheral blood และโปรตีนของไวรัสที่เกี่ยวข้องกับการชักนำให้เกิด apoptosis น่าจะช่วยอธิบายกลไกทางโมเลกุลของการชักนำ apoptosis ด้วยไวรัสชนิดนี้ได้มากขึ้น

สาขาวิชา จุลชีววิทยาทางการแพทย์ **ลายมือชื่อนิสิต** .....

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ATINOP PONGPANICH : MECHANISM OF APOPTOSIS ACTIVATION IN T LYMPHOCYTES BY HERPES SIMPLEX VIRUS. THESIS ADVISOR : ASSISTANT PROFESSOR CHINTANA CHIRATHAWORN, Ph.D., THESIS CO-ADVISOR : ASSOCIATE PROFESSOR PARVAPAN BHATTARAKOSOL, Ph.D., 85 pp. ISBN : 974-17-5348-9

Herpes simplex virus (HSV), a large DNA containing virus, is endemic in all human population investigated. After infection of mucocutaneuos surfaces, HSV establishes a latent infection in nerve cells. Various immune evasion mechanisms have been shown to be utilized by HSV including apoptosis induction in T lymphocytes. However, the mechanisms of T cell infection and apoptosis by HSV are still unknown. This study investigated the molecular mechanisms of apoptosis induction in T cells by HSV. The Jurkat T cell line was used as a representative for T cells. The numbers of HSV-infected T cells were determined by immunofluorescent assay (IFA) and flow cytometry. The data suggested that flow cytometry was more sensitive than IFA since infected cells were demonstrated since 2 h p.i. whereas 6 h infection was required for IFA.

For apoptosis induction by HSV, Annexin V binding assay demonstrated that both HSV-1 and HSV-2 induced apoptosis in Jurkat cells and caspase-3, -8, and –9 inhibitors blocked apoptosis induced by HSV-1 suggesting that HSV-1 and HSV-2 induced apoptosis in T lymphocytes by caspase-dependent pathways. However, apoptosis might occur through other mechanism(s) since caspase inhibitors used in this study could not completely inhibit apoptosis induced by HSV infection. In addition, the data demonstrated that the numbers of apoptotic cells induced by HSV-2 was significantly higher than by HSV-1 at 12 h p.i. (p= 0.003).

Further studies in peripheral blood T cells and the proteins of viruses involved in apoptosis induction should be further performed in order to elucidate the molecular mechanism of apoptosis induced by these viruses.

Field of study Medical Microbiology Student's signature .....

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ABBREVIATIONS

°C	=	degree celsius
CPE	=	cytopathic effect
CTL	=	cytotoxic T lymphocyte
DEVD	=	Aspartyl-glutamyl-valyl-aspartic acid
DNA	=	deoxyribonucleic acid
et al.	=	et alii
FITC	=	fluorescein isothiocynate
FACS	=	Fluorescence Activated Cell Sorter
FMK	=	fluoromethyl ketone
GM	=	growth medium
gJ	=	glycoprotein J
h	=	hour
h p.i.	=	hour post-infection
ICP	=	infected cell protein
IETD	=	Isoleucyl-glutamyl-threonyl-aspartic acid
IFA	=	immunoflorescence assay
Ig	=	Immunoglobulin
IgG	=	Immunoglobulin G
kDa	=	kilodalton
LAT	=	Late-associated transcript
LEHD	=	Leucyl-glutamyl-histidyl-aspartic acid
MHC	=	Major histocompatibility complex
Ml	=	millilitre
M	=	molarity
MOI	=	multiplicity of infection
NK	=	Natural Killer
PBS	=	Phosphate Buffer Saline
PHA	=	Phytohemagglutinin
PS	=	phosphatidylserine

PFU	=	Plaque Forming Unit
PI	=	Propidium iodide
rpm	=	round per minute
TRITC	=	tetramethyl rhodamine isothiocyanate
TAP	=	Transporter associated protein
U_L	=	Unique long
U_S	=	Unique short
μg	=	microgram
μl	=	microlitre

CHAPTER I

INTRODUCTION

Herpes, from the ancient Greek meaning to creep or crawl, is the name of a family of viruses of which herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2) are human pathogens. Herpes simplex virus is a member of the herpesviridae family. The virus frequently infects human beings, causing a range of diseases from mild uncomplicated mucocutaneous infection to fatal HSV encephalitis. HSV-1 is normally associated with orofacial infections and encephalitis, whereas HSV-2 usually causes genital infections and can be transmitted from infected mothers to neonates. Both viruses establish latent infections in sensory neurons and, upon reactivation, cause lesions at or near point of entry into the body (1,2).

Host immune defenses are critical in the control of HSV infections, most of the evidence suggests that cellular immunity is important (3,4), and severe disease is seen in patients with impaired cellular immunity. Although strong humoral responses are produced in response to HSV infection, and antibodies generated during primary infection can effectively neutralize virus; however, there is little or no evidence that antisera can prevent HSV infection in human (5). In addition, there is no evidence that HSV uses antigenic variation to escape host control, it must use alternative immune escape strategies, if it is, to successfully reactivate and be transmitted. A major evasion mechanism used by HSV is the establishment of latency in dorsal root ganglion, since the nervous system is an immunopriviledged site and viral proteins are not expressed. The virus can persist and avoid the host immune system, upon reactivation, causes shedding and lesions.

However, during the reactivation process, the virus must face the host defenses. For example, HSV expresses receptors for complement and for IgG, and these may effect some degree of resistance to humoral immune response (4). When the virus reactivates and infects dermal fibroblasts and keratinocytes, it causes down-regulation

of MHC class I via inhibition of TAP by ICP47 (6,7), thus interfering with the recognition of these cells by $CD8^+$ CTL (8). Another host defense is programmed cell death or apoptosis in response to disturbance of cellular machinery by viral proteins or host immune system. Apoptosis presents a major threat to viruses, since apoptotic cells are poor hosts for viral replication (9). HSV protects infected cells from apoptosis through the action of several genes and gene products, including U_S3 (10), U_S5 (gJ) (11), ICP22 (12), ICP27 (13), and LAT (14). Together, these proteins protect infected cells from apoptosis induced directly by the virus, and also apoptosis induced by external stimuli such as CTL. In addition, there are evidences that HSV inhibits apoptosis in epithelial and fibroblast cells (15,16). However, there are reports suggested that HSV can induce apoptosis in T lymphocytes (17-19). It is well known that T lymphocytes play important roles in cell-mediated immune response. Thus, apoptosis induced in this cell may be one mechanism of HSV immune evasion.

In this study, we used Jurkat cells, a T lymphocytic cell line, as a model for investigating whether HSV-1 or HSV-2 can differently induce apoptosis in T lymphocytes. We also investigated whether apoptosis was induced via caspase-dependent pathway. In addition, indirect fluorescent assay and flow cytometry were compared for detection of HSV-infected Jurkat cells.

CHAPTER II

OBJECTIVE

The objective of this study is :

To compare molecular mechanisms of apoptosis induction by HSV-1 and HSV-2 in Jurkat cells.

CHAPTER III

REVIEW LITERATURES

History

Herpesviruses are highly disseminated in nature. Of nearly 100 herpesviruses that have been at least partially characterized, eight herpesviruses have been isolated from human, these include: Herpes simple virus type 1 (HSV-1), Herpes simplex virus type 2 (HSV-2), Vericella zoster virus (VZV), Epstein-Barr virus (EBV), Cytomegalovirus (CMV), Human herpesvirus 6 (HHV-6), Human herpesvirus 7 (HHV-7) and Human herpesvirus 8 (HHV-8) or Kaposi's sarcoma associated herpesvirus (Table1).

Human herpes simplex viruses have been documented since ancient Greek times. Greek scholars, notably Hippocrates, used the word "herpes", meaning to creep or crawl, to describe spreading cutaneous lesions. Herpes is the name of a family of viruses of which herpes simplex virus type 1 and herpes simplex virus type 2 (HSV-1 and HSV-2) are the most serious human pathogens. HSV-1 is normally associated with orofacial infections and encephalitis, where as HSV-2 usually causes genital infections and can be transmitted from infected mothers to neonates. Importantly, these viruses have a unique propensity to establish latency and recur over time. Both viruses establish latent infections in sensory neurons and, upon reactivation, cause lesions at or near point of entry into the body (1).

Herpes simplex viruses are the first of the human herpesviruses to be discovered and are among the most intensively investigated of all viruses. Their attractions are their biologic properties and their ability to cause a variety of infections, to remain latent in their host life, and to be reactivated to cause lesions at or near the site of initial infection. They serve as models and tools for the study of translocation of proteins, synaptic connections in the nervous system, membrane structure, gene

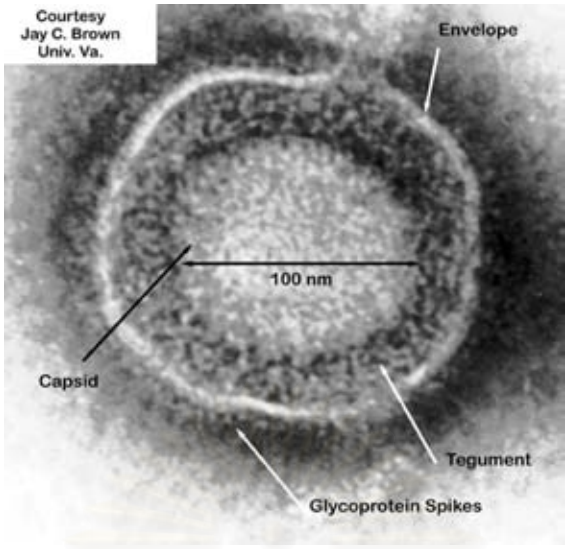
Table 1 Classification and features of herpesviridae that infect humans. (20)

Common Name	Subfamily	Typical Primary Infections	Typical Recurrent Infections	State of Latency
Herpes simplex virus type 1	α	Gingivostomatitis Keratoconjunctivitis Cutaneous herpes Genital herpes Encephalitis	Herpes labialis Keratoconjunctivitis Cutaneous herpes Encephalitis	Sensory neurons
Herpes simplex virus type 2	α	Genital herpes Cutaneous herpes Gingivostomatitis Meningoencephalitis Neonatal herpes	Genital herpes Cutaneous herpes Aseptic meningitis	Sensory neurons
Varicella-zoster virus	α	Varicella	Zoster	Sensory nerve ganglia
Epstein-Barr virus	γ	Mononucleosis Hepatitis Encephalitis	?	B lymphocytes
Cytomegalovirus	β	Mononucleosis Hepatitis Congenital cytomegalic Inclusion disease	?	Monocytes Neutrophils
Human herpesvirus 6	β	Roseola Infantum Fever and otitis media Encephalitis	?	CD4 lymphocytes
Human herpesvirus 7	β	Roseola Infantum	?	CD4 lymphocytes
Human herpesvirus 8 or Kaposi's sarcoma virus	γ	?	Kaposi's sarcoma	?

regulation, among many others, both general to viruses and specific to HSV. More than 40 years passed from the time of their isolation until Schneweiss KE *et al*.,(21) demonstrated that there were, in fact, two serotypes, HSV-1 and HSV-2, whose formal designations under International Conference for Taxonomy of Viruses (ICTV) rules are now human herpesviruses 1 and 2 (22). Not until 1961 were plaque assays published (23), and only much later were the genome sizes and the extent of homology between these two viruses reported.

Virology

Herpes simplex viruses are the members of the family Herpesviridae. They have been classified in subfamily alphaherpesvirinae, genus simplex virus. There are two antigenic types, designated HSV-1 and HSV-2, which share antigenic cross-reactivity but different neutralization patterns. As with all herpes virions, the HSV virion is 200-300 nanometer in diameter and consists of four structure elements: (i) an electron-dense core containing viral DNA, (ii) an icosapentahedral capsid surrounding the core, (iii) a tegument – an amorphous layer of proteins that surround the capsid, and (iv) an outer envelope exhibiting spikes on its surface (Figure 1). The HSV genome is a large double-stranded DNA molecule. It is approximately 150 kilobase pairs, with a G+C content of 68% (HSV-1) or 69% (HSV-2) (24-26). The DNA of HSV-1 and HSV-2 consist of two covalently linked components, designated as L (long) and S (short), with unique sequences – U_L (unique long) or U_S (unique short) flanked by large inverted repeats. Because of this arrangement, the two components can invert relative to one another, creating four different types of DNA molecule that differ only in their orientation of DNA sequences (27). The internal inverted repeat sequences are not essential for growth of the virus in cell culture; mutants from which portions of unique sequences and most of the interval inverted repeats have been deleted, have been obtained in all four arrangements of HSV DNA (28, 29). The DNAs of HSV-1 and 2 share approximately 50% homology of their base pairs, although they differ in restriction endonuclease cleavage sites and in apparent size of viral proteins (30).



www.darwin.bio.uci.edu/~faculty/wagner/hsv2f.html

Figure 1 Scanning electron micrograph of HSV

Viral Genes

HSV-1 and HSV-2 encode at least 84 different polypeptides. Each protein does many thing, hence HSV genes can encode several hundred different functions to initiate infection, HSV attaches to at least three different classes of cell-surface receptor and fuses its envelope with the plasma membrane. The capsid, minus its envelope, is transported to the nuclear pore, through which it releases viral DNA into the nucleus. HSV replicates by three rounds of transcription that yield: α (immediate early) proteins that mainly regulate viral replication; β (early) proteins that synthesis and package DNA; and γ (late) proteins, most of which are virion proteins (31).

α genes are the first to be expressed. There are five α proteins, namely, infected cell polypeptides (ICPs) 0, 4, 22, 27, and 47. α genes were initially defined as those that are expressed in the absence of viral protein synthesis. The synthesis of α polypeptides reaches peak rate at approximately 2-4 hr post-infection, but α proteins continue to accumulate until late in infection at non-uniform rate (32). To date, all α proteins, with the possible exception of α47, have been shown to have regulatory

functions. Functional proteins are required for the synthesis of subsequent polypeptide groups.

β genes are not expressed in the absence of competent α proteins. The β groups of polypeptides reach peak rates of synthesis at about 5-7 hr post-infection (32). The appearance of β gene products signal the onset of viral DNA synthesis, and most viral genes involved in viral nucleic acid metabolism appear to be in β group.

γ genes have two groups, γ_1 and γ_2, although in reality they form a continuum differing in their timing and dependence on viral DNA synthesis for expression (33-39). The prototype γ_1 gene is expressed relatively early in infection and is only minimally affected by inhibitors of DNA synthesis. In contrast, prototypic γ_2 genes are expressed late in infection and are not expressed in the presence of effective concentrations of inhibitors of viral DNA synthesis.

Assembly of the virus begins in the nucleus, with acquisition of the envelope as the capsid buds through the inner lamella of the nuclear membrane. Virus is transported through the cytoplasm to the plasma membrane, where release of progeny virions occurs. The replicative efficiency of HSV is poor, as indicated by the low ratio of infectious to incomplete virions.

Pathogenesis

The pathogenesis of HSV infections is understood through knowledge of the events of replication and establishment of latency in both animal models and humans. The pathogenesis of human disease is dependent on intimate, personal contract of a susceptible individual with someone excreting HSV. Virus must come in contact with mucosal surface or abraded skin for infection to be initiated. With viral replication at the site of infection, either an intact virion or the nucleocapsid is transported by neurons to the dorsal root ganglia, where latency is established. Although replication can sometimes lead to disease and can infrequently result in life-threatening CNS infection,

the host-virus interaction leading to latency predominates. After latency is established, a proper stimulus will cause reactivation to occur; virus becomes evident at mucocutaneous sites, appearing as skin vesicles or mucosal ulcers. Primary infection can spread beyond the dorsal root ganglia, thereby becoming systemic; however, this event is unusual (1).

Infection with HSV-1 generally is limited to the oropharynx and is transmitted by direct contact of a susceptible individual with infected secretion. Thus, initial replication of virus will occur in the oropharyngeal mucosa. The trigerminal ganglion becomes colonized and harbors latent virus. Infection of HSV-2 is usually the consequence of transmission by genital contact. Virus replicates in the genital, perigenital, or anal skin sites with seeding of the sacral ganglia (1).

Operative definitions of the nature of the infection are of pathogenesis relevance. Human definitions will be applied to both systems. For individuals susceptible to HSV infections, the first exposure to either HSV-1 or HSV-2 results in primary infection. The epidemiology and clinical characteristic of primary infection are distinctly different from those associated with recurrent infection. After the establishment of latency, a recurrence of HSV is known as reactivated infection or recurrent infection. This form of infection leads to recurrent vesicular lesions of the skin such as HSV labialis or recurrent HSV genitalis. An individual with preexisting antibodies to one type of HSV can experience a first infection with the opposite virus type at a different site. Under such circumstances the infection is known as an initial infection rather than as a primary infection. An example of an initial infection occurs in those individuals who have preexisting HSV-1 antibodies who then acquire a genital HSV-2 infection. Reinfection with different strain of HSV can occur, uncommon in the normal host and is called exogenous reinfection (1).

Epidemiology

HSV must contact mucosal surfaces or abraded skin to initiate infection. The type of HSV infection that results is a function of the host's immune status. Susceptible individuals (ie, those who are HSV seronegative) develop "primary" infection after their first exposure to HSV-1 or HSV-2. "Initial" infection occurs when an individual who has antibodies to either HSV-1 or HSV-2 is infected with the other virus type for the first time. HSV-1 and HSV-2 are usually transmitted by different routes and affect different areas of the body, but the signs and symptoms that they cause overlap (40). These viruses occur worldwide, have no seasonal variation, and naturally only infect human beings. HSV infection is rarely fatal. Most human beings have been infected and harbour latent virus that can reactivate; hence there is a vast HSV reservoir for transmission to susceptible individuals.

Many demographic factors affect acquisition of HSV-1 infection. In less developed countries seroconversion happens early in life–at 5 years in around a third of children, and in around 70–80% by adolescence. In comparison, middle-class and upper-class individuals in more developed countries become infected later on–seroconversion occurs in about 20% of children younger than 5 years; then no substantial rise in incidence happens until an increase to 40–60% at age 20–40 years. In the USA, race also affects acquisition of HSV-1. By age 5 years, more than 35% of African-American versus 18% of white children is infected with HSV-1. Incidence of infection among university students is around 5–10% annually (41).

HSV-2 infections are usually sexually transmitted. Most genital HSV infections are caused by HSV-2; however, an increasing proportion is attributable to HSV-1. Genital HSV-1 infections are usually less severe and less prone to recur than those caused by HSV-2. HSV-2 seroprevalence rises from about 20–30% at age 15–29 years to 35–60% by age 60 years. This prevalence increased dramatically from the late 1970s to late 1980s (41, 42). Factors that affect acquisition of HSV-2 infection include sex (infection is more frequent in women), race (infection is more frequent in African-Americans than whites), marital status, number of sexual partners, and place of

residence (prevalence is higher in cities than suburbs). Prevalence of antibodies to HSV-2 is highest among female prostitutes (75%) and male homosexuals (83%) (41).

As with HSV-1 infection of the mouth, HSV-2 primary, initial, or recurrent infection can be symptomless. Recurrence varies between men and women, occurring 2·7 and 1·9 times per 100 days, respectively. Women with initial genital herpes can shed the infection without symptoms; this occurs in 12%, 18%, and 23% of primary HSV-1, primary HSV-2, and non-primary-HSV-2 infections, respectively. In cells cultured from women with established genital-HSV-2 infection, symptomless shedding occurs on 3–5% of all days cultured. Analysis of swabs by PCR increases this estimation of rate to 28%, which suggests that infection is chronic rather than intermittent (43).

Cell characteristics of primary or recurrent HSV infection are those of viral-mediated cell death and associated inflammatory response. Viral infection causes cells (usually parabasal and intermediate cells of the epithelium) to swell with condensed chromatin in the nucleus, which is followed by nuclear degeneration. Plasma membranes break and infected cells form multinucleated giant cells.

Signs and Symptoms

The most common sites of HSV infection are skin and mucosal membranes, irrespective of virus type. The incubation period for HSV-1 or HSV-2 is about 4 days, and ranges from 2 to 12 days. Most people do not notice an HSV-1 or HSV-2 infection (40). Primary HSV-1 infection of the oropharynx and HSV-2 infection of the genital tract result in virus excretion for up to 23 days (average of around 7–10 days). Symptomatic oropharyngeal disease is characterised by lesions of the buccal and gingival mucosa (lasting 2–3 weeks), and by fever between 38·3 and 40°C. Intraoral ulceration lesions indicate primary infection, whereas lip lesions suggest recurrent infection.

Recurrent orolabial lesions are preceeded by pain, burning, tingling, or itching, which usually lasts for 6 hr; vesicles then appear on the vermillion border of the lip (44). Three to five vesicles normally occur, which persist for about 48 hr, then become pustules or ulcers and then form crusts within 72–96 hr; lesions are completely healed

after 8–10 days (45). Pain is most severe at the start of infection, then resolves over 96–120 hr. The frequency of recurrence varies among individuals. Other skin HSV-1 infections can occur, such as: eczema herpeticum in patients with underlying atopic dermatitis; herpes gladiatorium in wrestlers; and extensive lesions associated with Darier's and Sézary's syndromes. HSV infections of any type can trigger erythema multiforme (46).

Primary genital herpes appears as macules and papules, followed by vesicles, pustules, and ulcers (47, 48). Complications in men are rare; aseptic meningitis and urinary retention are more common in women. Other complications in men and women include paraesthesias and dysaesthesias of the legs and perineum, dysuria, localised inguinal adenopathy, and malaise. Primary perianal HSV-2 infections and proctitis are most common in male homosexuals. Non-primary initial genital infection causes milder symptoms than primary infection (fewer lesions, less pain, and less likelihood of complications), and heals in around 2 weeks. Antibodies to HSV-1 reduce disease severity of HSV-2 (49).

Recurrent male genital infection appears as three to five vesicles on the shaft of the penis; female infection causes ulcerating-vesicle genital lesions or merely vulvar irritation. The disease lasts around 8–10 days. A third of patients are estimated to have more than six recurrences per year, a third will have two per year, and the remaining third will have rare recurrences. HSV can be transmitted to sexual partners in the presence or absence of symptoms (50). Indeed, transmission usually occurs from symptomless virus shedding. Wald and colleagues (51) showed a three-fold higher frequency of HSV DNA shedding (detected by PCR) in genital secretions between, rather than during, clinical recurrences.

Genital-HSV infection is rarely transferred from mother to fetus during pregnancy; when it is transferred, it involves multiple visceral sites, and causes necrotising hepatitis with or without thrombocytopenia, disseminated intravascular coagulopathy, and encephalitis. Fetal infection occurs in about one in 300,000 live neonates in North America. It is associated with microcephaly, skin scarring, and retinitis.

Neonatal HSV has an incidence of around one in 2,000 to one in 5,000 births per year in the USA, but the incidence is substantially lower in the UK. In any country, this incidence seems to be directly related to seroprevalence of HSV-2. At least three factors affect transmission of infection from mother to fetus. Maternal primary or initial infection is transmitted to 30–50% of vaginally delivered babies—higher than the recurrent infection transmission frequency of 3% or less (52). Transplacental maternal antibodies possibly reduce severity of infection if not actually prevent transmission, and membrane rupture that lasts for more than 6 hr increases the risk of infection. Fetal scalp electrodes can also be a site for HSV transmission. Neonate infection can occur *in utero* (about 5% of infections), intrapartum (around 80%), or postnatally. Infection is nearly always symptomatic and frequently lethal. Disease can occur locally in: skin, eye, or mouth (40% of babies); cause encephalitis with or without skin infection (35%); or result in disseminated infection (25%) (40).

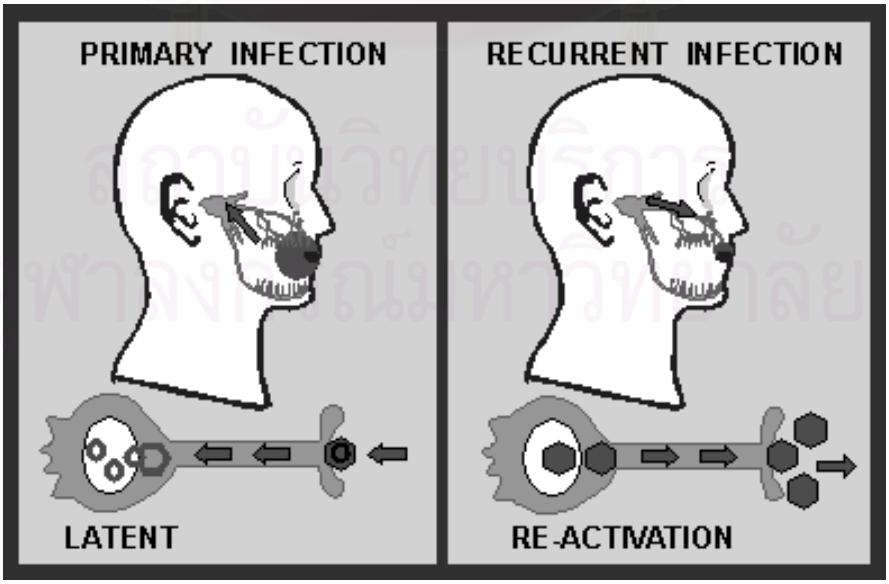
Around 300,000 cases of HSV eye infections are diagnosed yearly in the USA. Herpetic keratoconjunctivitis is associated with unilateral or bilateral conjunctivitis, which can be follicular, followed soon by preauricular adenopathy. Geographic ulcers of the cornea develop with advanced disease. Recurrences are common. Immunocompromised patients, especially organ transplant recipients, are at increased risk of severe HSV infection. These patients can develop progressive disease involving respiratory tract, esophagus, or gastro-intestinal tract (40).

HSV encephalitis is a devastating disease and is thought to be the most common cause of sporadic fatal encephalitis, which is usually focal. More than 70% of untreated patients die and only 2.5% of surviving patients return to normal neurological function (40). HSV can also affect almost all areas of the nervous system, causing meningitis, myelitis, and radiculitis, among other diseases. HSV has been isolated from the respiratory tract of adults with adult respiratory distress syndrome and acute onset bronchospasm; it increases the rates of mortality and morbidity of these problems.

Biological Properties of HSV

Herpes simple virus have two unique biological properties that influence human disease: neurovirulence or neurotoxicity and latency. The propensity of HSV to replicate in nervous system tissue can result in profound disease with severe neurological devastation, especially HSV encephalitis. Alternatively, virus can infect sensory ganglia without resulting in significant disease. When this occurs, latency is established, thus providing a reservoir for virus and its transmission to susceptible persons if reactivated (Figure 2).

HSV-1 and HSV-2 are transported by retrograde flow along axons that connect the point of entry into the body to nuclei of sensory neurons (53). Viral multiplication occurs in a small number of sensory neurons; the viral genome then remains in a latent for the life of the host. Many events cause viral reactivation, such as physical or emotional stress, fever, UV light, and tissue damage. Reactivation can occur even in hosts with cell-mediated and humoral HSV immunity. Latent virus can be reactivated from the trigerminal, sacral, and vagal ganglia of human beings either unilaterally or bilaterally in cell culture. Viral gene expression is thought to be required for reactivation but not for establishment of latency. How latency is established is not known.



www.uct.ac.za/depts/mmi/ jmoodie/hsv2.html

Figure 2 Biological property of HSV

Latency

A most property of all of the herpesviruses is their ability to become latent, persisting in an apparently inactive state for varying durations of time and then reactivating as the result of provocative stimuli (54-59). Thus, after infection, viral DNA persists in the host for the entire lifetime of the individual.

Accumulated clinical experience suggests that after primary infection, replication of virus at the portal of entry, usually oral or genital mucosal tissue, results in infection of sensory nerve endings; the virus is transported to the dorsal root ganglia (58, 60-62). No virus can be isolated from patients during interim periods (namely, those times between recurrences) at or near the usual site of recurrent lesions. Recurrences are spontaneous, but there is an association with physical or emotional stress, fever, exposure to UV light, tissue damage, and immune suppression (62-64). Latent virus was retrieved from the trigeminal, sacral, and vagal ganglia of human (54, 60, 61). In humans, the frequency of isolation of HSV from ganglia was described by Baringer (54, 60) and others (61, 65). When trigeminal and sacral ganglia were explanted and cocultivated with human embryonic lung cells, a cytopathic effect induced by HSV was detected within the newly growing cells. However, evidence of virus replication may not appear until cells have been subcultured and passed.

The state of virus during latency are currently being evaluated by many laboratories. As with other genetic mutations, the deletion of γ, 34.5 markedly impairs the ability of the virus to establish latency in mice and guinea pig models (66). Clues to the molecular pathogenesis of HSV are introduced by the demonstration of a viral transcript (LAT) in tissues of human origin. One relevant issue is the mechanism by which virus becomes latent without destroying the sensory ganglia. Factors responsible for reactivation of latent virus were studied more from a phenomenologic than from a molecular biologic standpoint. As previously noted, manipulation of the nerve root (55, 67, 68) or direct trauma to a ganglia (69) has resulted in reactivation, along with other factors (70-73). The administration of immunosuppressive agents (72, 74-76) and the

presence of documented infection causing fever also have been associated with recurrent lesions, especially pneumococcal meningitis (77). In humans, exposure to UV light was demonstrated to be associated with recurrent HSV labialis in model systems. As noted previously, this observation has led to the development of human models for studying the events of reactivation of latent virus by use of UV light exposure to select area of the skin (e.g., vermillion border of the eye) (78). The mechanisms by which events of reactivation are operative at the level of sensory ganglia remain to be determined.

Host Immune Response

Host immune response to HSV was described initially in the early 1930s. The first studies were performed and defined the presence of neutralizing antibodies to HSV in the serum of previously infected adults (79). Host genetic background, macrophages, natural killer cells, specific T-cell subpopulations, specific antibodies, and cytokine responses have been implicated as important host defenses against HSV infection. Primary HSV infection in the mucosal epithelium is characterized by strong immune responses, including nonspecific, humoral, and cell-mediated immunity. From the limited information available, it is not absolutely clear whether humoral or cell-mediated immune responses predominate in clearance of virus, but most of the evidence suggests that cell-mediated immunity is more important (3,4). Although, strong humoral responses are produced in response to HSV infection, and antibodies generated during primary infection can effectively neutralize viruses; however, there is little or no evidence that antisera can prevent HSV infection in humans (5). Indeed, HSV can reactivate from latency and cause recurrent infections in spite of high antibody titers (80), and antibody titers do not predict the time to recrudescence or severity of secondary lesions (81).

Innate (nonspecific) immunity, macrophages and natural killer (NK) cells are the first cells to be involved in HSV clearance, and these cells limit spread while specific immune responses develop. However, macrophages and NK cells are not sufficient to

prevent infection and spread of HSV in mice (3). Nevertheless, studies of patients with defects in NK cells have suggested that NK cells are important component of the anti-HSV immune response (82, 83). Studies in mice and humans have demonstrated that T cells are important or essential to the process of containing and clearing primary and recurrent HSV infections. Patients treated with immunosuppressive agents prior to organ transplantation or with depressed T cell function frequently develop severe HSV disease (84, 85). In mice, adoptive transfer of anti-HSV T lymphocytes protected recipients and curtailed the progression of HSV infection (3). Both $CD4^+$ and $CD8^+$ T cells could protect mice from HSV challenge, and immunodepletion experiments similarly indicated important roles for both cell types *in vivo* (3).

HSV Immune Evasion

HSV has an elaborate system for blocking host responses to infection. Cells degrade some of the newly synthesized viral proteins as well as cell proteins specifically targeted for degradation. Peptides (degradation products) are transported to the endoplasmic reticulum, and those of correct size and conformation are presented by MHC-class-1 proteins. HSV blocks presentation of peptides by encoding ICP47, a small protein that binds to the transporter protein TAP1 or TAP2 and stops it from transporting peptides to the endoplasmic reticulum (86).

HSV expresses receptors for complement and for IgG, and these may effect some degree of resistance to immune responses. HSV glycoprotein C, denoted gC, binds complement factor C3 fragments (87). The HSV IgG receptors are composed of a complex of two glycoproteins, gE and gI, and bind the Fc domain of both monomeric and antigen-complexed IgG (88). There is evidence that Fc receptors on infected cells can provide resistance to IgG and complement *in vitro* (89).

Another host defense is programmed cell death in response to disturbance of cellular machinery by viral proteins or host immune system. HSV blocks programmed cell death whether caused by its own or by host proteins. At least three proteins, U_s3,

gJ, and gD, are thought to block programmed cell death caused by specific cell injury. Finally, HSV, like most viruses, makes complementary RNA that can anneal to and activate host protein kinase R. This enzyme can phosphorylate the translation initiation factor eIF-2α, which in turn blocks all protein synthesis. HSV has a counter defence; HSV protein $\gamma_1$34·5 binds protein phosphatase 1 and directs it to dephosphorylate eIF-2α with great efficiency (86). Also, cells cannot respond to infection because HSV destroys cellular mRNA, inhibits DNA transcription, and blocks splicing of mRNA. In this study, we will emphasize the most of HSV immune evasion of apoptosis.

Apoptosis or Programmed Cell Death

Apoptosis, or programmed cell death, is a normal component of the development and health of multicellular organisms. Cells die in response to a variety of stimuli and during apoptosis they do so in a controlled, regulated fashion. This makes apoptosis distinct from another form of cell death called necrosis in which uncontrolled cell death leads to lysis of cells, inflammatory responses and, potentially, to serious health problems. Apoptosis, by contrast, is a process in which cells play an active role in their own death (which is why apoptosis is often referred to as cell suicide) (90).

Upon receiving specific signals instructing the cells to undergo apoptosis, a number of distinctive biochemical and morphological changes occur in the cell. A family of proteins known as caspases are typically activated in the early stages of apoptosis. These proteins breakdown or cleave key cellular substrates that are required for normal cellular function including structural proteins in the cytoskeleton and nuclear proteins such as DNA repair enzymes. The caspases can also activate other degradative enzymes such as DNases, which begin to cleave the DNA in the nucleus. The result of these biochemical changes is appearance of morphological changes in the cell.

Apoptosis is technically defined by the morphological features of the dying cell (91), in particular, by decrease or shrinkage in cell volume, and the condensation of the

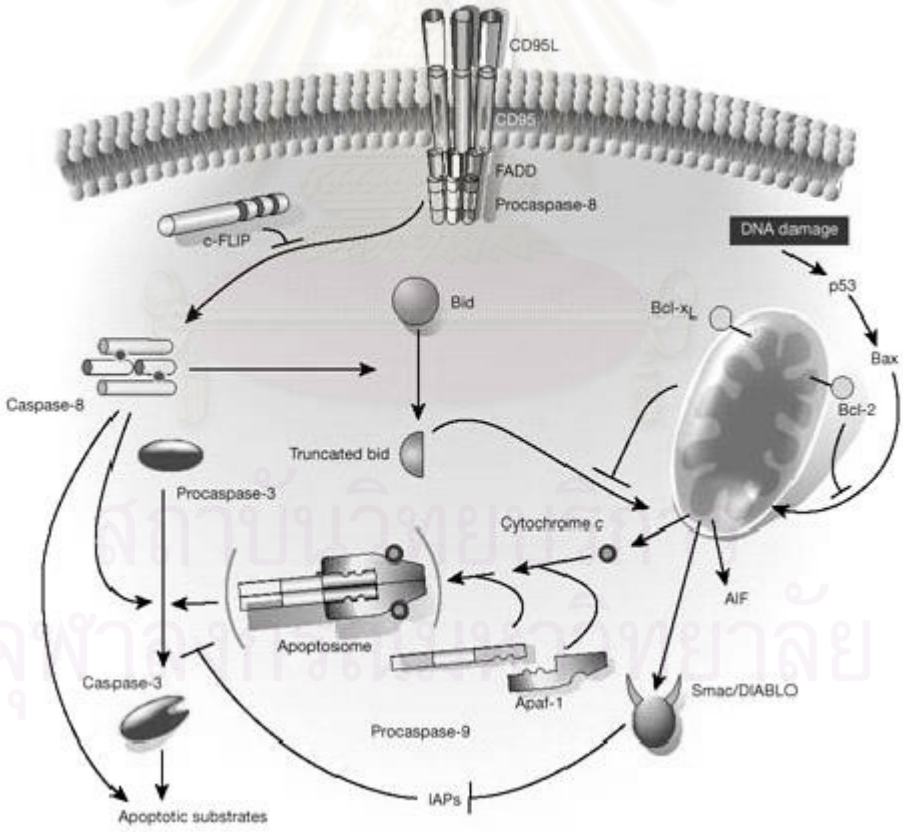
chromatin, often accompanied by fragmentation of the nucleus. The plasma membrane is frequently control into bleb, and the cell may break into discrete membrane-bound apoptotic bodies. The apoptotic cell and any apoptotic body are rapidly cleared by phagocytic cells (92), and the cell death does not trigger an inflammatory response. Obviously, apoptosis exists as a mechanism to remove cells. This removal may be because they are infected or damaged, they are in excess, they have failed a developmental test or their presence is no longer needed for a physiological process.

A variety of external or internal cell signals can initiate apoptosis. Attention has been focused recently on the group of receptors that are members of the tumor necrosis factor receptor family. They are the Fas surface protein (receptor) and the tumor necrosis factor-alpha receptor 1 (TNF-α 1) which, when stimulated by their ligands, initiate apoptosis via their intracellular “death domains” (93). Other external apoptosis signals can be delivered by protein released from cytotoxic T cells (granzyme) and by corticosteroids (94). The internal signals controlling apoptosis involve a number of influences and/or mechanisms that either induce or suppress apoptosis. Progress has been made in identifying specific genes and their products that provide the intracellular control of apoptosis. Two example of elements that appear to have a role in cancer are the p53 protein which is required for cells to initiate apoptosis following DNA damage (a genotoxic event) and the bcl2 protein which inhibits apoptosis and is a member of a family of apoptosis regulatory proteins (95, 96). Each of the signaling pathways converges with the activation of proteolytic enzyme initially known as ICE-like proteases (refers to interleukin 1 converting enzyme) now known as caspases. This family of proteases cleaves intracellular proteins both in the cytoplasm and the nucleus and thus digests the cell from within (97).

Apoptosis Pathways

Mammalian cells have two major apoptotic pathways. These are the death-receptor pathway and the mitochondrial pathway. The death-receptor pathway (left pathway in the figure 3) is triggered by members of the death-receptor superfamily

(such as CD95 and tumor necrosis factor receptor I). Binding of CD95 ligand to CD95 induces receptor clustering and formation of a death-inducing signaling complex. This complex recruits, via the adaptor molecule FADD (Fas-associated death domain protein), multiple procaspase-8 molecules, resulting in caspase-8 activation through induced proximity. Caspase-8 activation can be blocked by recruitment of degenerate caspase homologue c-FLIP (98). The mitochondrial pathway (right pathway in the figure 3) is used extensively in response to extracellular clues and internal insults such as DNA damage. These diverse response pathways converge on mitochondria, often through the activation of a pro-apoptotic member of the Bcl-2 family. Pro- and anti-apoptotic Bcl-2 family members meet at the surface of mitochondria, where they compete to regulate cytochrome *c* exit. If the pro-apoptotic camp wins, an array of molecules is released from the mitochondrial compartment. Principal among these is cytochrom *c*, which associates with Apaf-1 and then procaspase-9 to form the apoptosome (99).



Nature, 407: 2000, 770-776

Figure 3 Apoptosis pathways in mammalian cells

Apoptosis in Immune Function

Apoptosis plays a central role in immune function as the critical pathway in lymphoid development used to eliminate self-reactive pre-T cells in the thymus and self-reactive pre-B cells in the bone marrow (100, 101). In addition, it is the mechanism that controls the number of lymphocytes participating in an immune response once the foreign antigen has been successfully eliminated. Apoptosis, under these circumstances, is primarily induced via the generation of Fas protein and Fas ligand on the surface of activated T cells (102). The appearance of these surface molecules allows for the elimination of activated cells that are no longer necessary for immune protection. Thus, this pathway provides a critical means for controlling the magnitude of an immune reaction.

An additional role of apoptosis in immune function centers on the cytotoxic effector function of T cells (103, 104). Target cell destruction appears to involve the initiation of apoptosis via two different mechanisms as well as the direct induction of cell necrosis. The secretory pathway for the induction of apoptosis depends on the release of granzyme, an enzyme that induces apoptotic death. This also involves perforin, a second granular protein which upon release inserts pores into the target cell membrane. This allows granzyme intracellular entry which increases its efficiency in causing target cell apoptosis. An alternative pathway for inducing target cell apoptosis is through the expression of Fas ligand on activated cytotoxic T cells. The engagement of Fas ligand with Fas expressed on the target cell provides a death signal to the target cell.

Apoptosis and HSV

The completion of the HSV-1 replication cycle leads ultimately to the destruction of the cells in culture, and this process is generally believed to occur through a necrotic route. Consequently, productive HSV-1 replication induces major biochemical alterations in the infected cells, including the loss of matrix binding proteins on the cell surface, membrane modifications, cytoskeletal destabilization, nucleolar alterations,

chromatin margination, aggregation or damage, and a decrease in cellular macromolecular synthesis (105-110). In addition, these morphological features observed with HSV-infected cells appear to be different from those associated with cells dying from apoptosis. In this case, the cells are characterized by morphological and biochemical changes that include cell shrinkage, membrane blebbing, nuclear condensation, and fragmentation of chromosomal DNA into nucleosomal oligomers (111). Therefore, it appears that a distinction exists between cytolysis due to viral replication and the apoptosis of cells. Recent reports (10, 13, 112-117) showed that HSV-1 is also able to interfere with the process of apoptosis infected cells. HSV-1 was able to prevent apoptosis which was externally induced by various stimuli including treatment with cycloheximide (CHX) (13, 113), ceramide, tumor necrosis factor, and anti-Fas antibody (113), osmotic shock using sorbitol (118) or ethanol (115), and hypothermia and thermal shock (10, 112, 119). It was also demonstrated that HSV-1 infection itself could induce apoptosis in cells. However, there was little evidence suggested that HSV-1 was able to induce apoptosis in T lymphocytes and induced apoptosis in CD4 and CD8 lymphocytes from either cord blood or peripheral blood stimulated with PHA (17, 18). In addition, HSV-1 infection of activated CTLs were induced apoptosis (19). Numerous reports have demonstrated that T cells are necessary for clearing HSV-1 infections and that CD8 antiviral CTLs play a pivotal role in the elimination of most viruses. However, herpesviruses have evolved delicate strategies to evade the attack of T cells, enabling them to persist and reactivate in the host, usually without causing significant tissue damage. In contrast to human fibroblasts, MHC class I expression on HSV-1-infected human T lymphocytes are not disrupted. This supports the notion that the efficiency of HSV-1's interference with transport of peptides into the lumen of the endoplasmic reticulum varies not only in different species (4, 120, 121) but also in different cell types of the same species (4). As a consequence of unaltered presentation of viral antigens by MHC class I molecules, antiviral CTL populations infected with HSV-1 are rapidly eliminated.

CHAPTER IV

MATERIALS AND METHODS

Part I. Cell culture and HSV stock preparation

1. Cell culture

In this study, Vero cells were used for HSV stock preparation and Jurkat cells were a representative for T lymphocytes for the study of molecular mechanisms of apoptosis induced by HSV-1 and HSV-2.

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 was kindly provided by Associate Professor Parvapan Bhattarakosol, Ph.D , the Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. The culture medium for Vero cells was medium M199 (Earle's salt) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin G and 100 μg/ml streptomycin and 0.01M HEPES (N-2-hydroxyethyl-piperaine-N′-2-ethan sulfonic acid). Since Vero cells are adherence cells, harvesting these cells require trypsinization. The culture media was removed and the cell monolayer was washed once with 5 ml 0.01 M phosphate buffer saline (PBS), pH 7.4. After discarding PBS, two ml of trypsin (for 25 mm^3 flask) was added and cells were incubated for one to two min at 37°C, then the culture flask was gently shook until the cells were detached, and culture medium was added. Vero cells were subcultured every 3-4 days with a splitting ratio of 1:3.

Jurkat cells, a continuous T cell line derived from human T cell leukemia and established from the peripheral blood of 14-year-old boy with acute lymphoblastic leukemia (ALL) at the first relapse in 1976 was generously provided by Dr. Pokrath Hansasuta, Ph.D., the Department of Microbiology, Faculty of Medicine, Chulalongkorn

University, Bangkok, Thailand. Jurkat cells were grown in RPMI 1640 supplemented with 10% FBS, 100 units/ml penicillin G and 100 μg/ml streptomycin. Cells suspensions were subcultured at thee-day intervals with the ratio of 1:5. Both Vero and Jurkat cells were grown at 37°C in 5% CO_2 atmosphere.

2. HSV stock preparation

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

Virus stock was prepared from Vero cell monolayer infected with the virus at an approximate multiplicity of infection (MOI) of 0.01 plaque forming unit per cell (PFU/cell). After an hour of viral adsorption at 37°C, the unadsorbed viruses were removed; the culture was washed once with PBS. The maintenance media (MM, the same as culture medium except 2%FBS was used) was replaced. The infected Vero cells were incubated further for 36 to 48 h or until more than 75% of the cell population showed cytopathic effect (CPE). The cells were then disrupted by repeating freezing (at -70°C) and thawing (at 37°C in water bath) for three times. The cell suspension was collected and then pelleted by centrifugation at 4°C, 2,000 rpm (SORVALL, U.S.A.) for 20 min. The supernatant was decanted and distributed into small aliquots and kept at -70°C until use. The amount of viruses was determined by plaque titration assay.

3. Plaque titration assay

HSV can form CPE so infectious virus particles in the plaque unit forming assay can be estimated by observing CPE. Vero cells grown as a monolayer were used in plaque titration assay. Cells are infected with viruses and plaque formation is observed. By using a semi-solid medium, any virus particles in the cells that were 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 be repeated several times. The cells are then stained with a dye which stains only living cells. The dead cells in the plaque appear as unstained area on 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 can be precisely estimated.

Plaque titration assay was performed in 96 well-plates (Nunclon, Denmark) and the titer was expressed as PFU/ml. Briefly, 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 (3 x 10^4 cells) and incubated at 37°C for 3 h. Then, 50 μl of overlay medium (0.8% gum tragacanth in GM) was applied. The medium was discarded after four to five days after incubating at 37°C and the infected cells were stained with 1% crystal violet in 10% formalin, for 20 min. The plate was washed in running water, air-dried and the number of plaque was counted. The viral titer is calculated from the data obtained in wells containing, if possible, between 1-35 plaques, using the formula as followed.

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

Where :

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

n = number of wells

v = volume of inoculum (in milliliters)

Part II. Detection of infected cells

1. Indirect-immunnofluorescence assay (IFA)

IFA was performed to demonstrate infected cells as previously described (122). Jurkat cells were grown in a 24 well-plate at a concentration of $1x10^6$ cells/ml. The cells were then infected with either HSV-1 or HSV-2 at MOI 0.5, 1, 5, or 10 and incubated at 37°C for 2, 4, 6, 12 and 24 h. After indicated incubation time, cells were collected and transferred to 1.5 ml microcentrifuge tubes and centrifuged at 13,400 rpm (EPPENDROF, Germany) for 30 seconds. The cells were washed twice with PBS by centrifuging at 13,400 rpm for 30 seconds. After the PBS was discarded, the suspension was spot on 8-well slides, air-dried, fixed in cold acetone (-20°C) for 10 min and kept at -20°C (not more than two weeks).

For indirect immunofluorescent staining, the acetone-fixed cells were flooded with 1:100 dilution of primary antibodies, rabbit-anti HSV-1 antibody or rabbit-anti HSV-2 antibody and then incubated at 37°C in moist chamber for 30 min. Excess antibodies were removed by 5-minute wash for 3 times with PBS, pH 7.5 and the fixed-cells were air-dried. The swine anti rabbit antibody conjugated with FITC, dilution 1:80 was then applied to the fixed-cells and incubated for 30 min at 37°C. The fixed-cells were washed and stained with Evan's blue for five min, after that, the fixed-cells were washed once with distilled water, air-dried and mounted with PBS-glycerol buffer (ratio 1:9). Cells were examined under a fluorescent microscope. Numbers of infected cells were scored and recorded. Localization and intensity of the fluorescence were also observed.

2. Flow cytometry

Flow cytometry is a rapid, quantitative method for the multiparametric measurement of fluorescent cells. By this technique, cells stained with a fluorescent dye can be detected and numbers of stained cells can be obtained. In this study, rabbit

anti-HSV-1 or HSV-2 was used to stain cells, then followed by FITC-conjugated antibodies This way, cells expressing viral antigens could be separated from those without viral antigens.

Jurkat cells were grown and infected with HSV as mentioned above in IFA. The cells were collected and transferred into microcentrifuge tubes and centrifuged at 13,400 rpm for 30 seconds. Cell were then stained for Flow cytometry detection as described by Steele-Mortimer, et al (123). Briefly, 500 μl of 4% w/v paraformaldehyde was added and cell suspension was vortexed and incubated at 37°C in 5% CO_2 for 10 min. Then, cells were washed with cold PBS and 500 μl FACS permeabilizing solution was added and incubated at room temperature in the dark for 30 min. After washing with cold PBS, cells were then incubated with 50 μl rabbit-anti HSV-1 antibody or rabbit-anti HSV-2 antibody at the dilution 1:100 on ice for 30 min followed by washing with cold PBS. Then, cells were incubated with second antibody, 50 μl of FITC-conjugated swine anti-rabbit antibodies (1:40) for 30 min on ice. After washing with cold PBS, cells were resuspended in 500 μl 1% paraformaldehyde and kept overnight at 4°C in the dark before analysis. Cells were then analyzed using Flow cytometry (Beckman Coulter, U.S.A.). For each sample, 10,000 cells were measured and data analysis was performed with SYSTEM II of EpicsXL.

Part III. Apoptosis detection

1. Annexin V staining assay

Apoptotic cells undergo rapid morphological alterations that indicate the progression of cell death. These include changes in the cytoskeleton and plasma membrane, condensation of the cytoplasm and nucleus and internucleosomal cleavage of DNA. An early indicator of apoptosis is the rapid translocation and accumulation of the membrane phospholipid phosphatidylserine (PS) from the cytoplasmic interface to the extracellular surface (124, 125). This loss of membrane asymmetry can be detected by utilizing the binding properties of Annexin V. Annexin V is a 35 kDa calcium

dependent phospholipid binding protein that preferentially binds to negatively charged phospholipids including PS. According to this property, Annexin V labeled with FITC was widely used for apoptotic cell detection. Cells progressing though apoptosis are monitored according to their Annexin V-FITC and propidium iodide staining pattern using fluorescent microscopy or flow cytometry. Viable cells will be negative for Annexin V FITC and PI. Staining cells that are induced to undergo apoptosis will be positive for Annexin V FITC but negative for PI. Both cells in later stages of apoptosis and necrosis will be stained positive for both Annexin V FITC and PI.

Annexin V binding assay was performed according to the manufacturer's instruction. Jurkat cells (200 μl of $5x10^5$ cells/ml) were plated in a 24 well-plate. The cells were then either mock infected or infected with either HSV-1 or HSV-2 at MOI 0.5, 1, 5 or 10 and incubated at 37°C for 2, 4, 6, 12 and 24 h. As a positive control, cells were incubated with camptothecin, an alkaloid isolated from the stem wood of the Chinese tree, *Camptotheca acuminate* (126), this compound selectively inhibits the nuclear enzyme DNA topoisomerase, it ultimately causes cell death, at the concentration 4 μg/ml at 37°C for 4 h. At the end of the incubation period, cells were collected in 5-ml plastic tubes and washed twice with cold PBS at 1,500 rpm for 5 min. After PBS was discarded, the cell pellet was resuspended in 100 μl of 1x Assay buffer, and 1 μl of Annexin V FITC (0.2 μg/μl) and 2 μl of PI (0.05 μg/μl) was added. Cells were incubated for 15 min at room temperature in the dark. After that, 400 μl of 1x Assay buffer was added and cells were analyzed immediately by Flow cytometry. For each sample, 20,000 cells were measured.

2. Inhibition of apoptosis by caspase inhibitors

Caspases exhibit catalytic and substrate-recognition motifs that have been highly conserved (127). These characteristic amino acid sequences allow caspases to interact with both positive and negative regulators of their activity (127). The substrate preferences or specificities of individual caspases have been exploited for the development of peptides that successfully compete for caspase binding (127). In

addition to their distinctive aspartate cleavage sites, the catalytic domains of the caspases require at least four amino acids to the left of cleavage site as the prominent specificity-determining residue (127-129).

DEVD, IETD and LEHD (each letter represents an amino acid residue) are peptides that preferentially bind caspase-3, caspase-8 and caspase-9, respectively. It is possible to generate reversible or irreversible inhibitors of caspase activation by coupling caspase-specific peptides to certain aldehyde, nitrite or ketone compounds. Fluoromethyl ketone (FMK)-derivatized peptides act as effective irreversible inhibitors with no added cytotoxic effects. Inhibitors synthesized with a benzyloxycarbonyl group (also known as Z) at the N-terminus and O-methyl side chains exhibit enhanced cellular permeability.

For experiments performed to indicate the involvement of caspases in apoptosis induction, caspase-3, -8 and –9 inhibitors were used. DEVD-FMK (Aspartyl-glutamyl-valyl-aspartic acid-Fluoromethyl ketone), IETD-FMK (Isoleucyl-glutamyl-threonyl-aspartic acid-Fluoromethyl ketone) and LEHD-FMK (Leucyl-glutamyl-histidyl-aspartic acid-Fluoromethyl ketone) are used as caspase-3, caspase-8 and caspase-9 inhibitors, respectively. The binding by these peptides inhibits caspase activity resulting in inhibiting apoptosis involving caspases targeted of those peptides.

Jurkat cells were either mock infected or infected with MOI 5 of HSV-1 or HSV-2 in the presence or absence of various concentrations of caspase 3, 8 and 9 inhibitors (10 μM, 50 μM and 100 μM were used) as indicated. After 18 h of infection, the cells were collected and apoptosis detection was performed using Annexin V assay as described above.

Part IV. Statistical analysis

Data were analyzed with the paired sample *t* test, using the statistic package in SPSS for windows version 11.5. A *p* value less than 0.05 was considered significant.

CHAPTER V

RESULTS

1. Detection of HSV infected cells by IFA

IFA was used in order to estimate the numbers of infected cells. In addition, the localization of viral protein expression at various time points can be observed using this approach. Jurkat cells were infected with HSV-1 or HSV-2, the amounts of viruses and times indicated in Figure 4 and 5. Polyclonal antibodies to HSV-1 and HSV-2 were used to indicate infected cells.

The numbers of infected cells were estimated and shown in Tables 2 and 3. For both HSV-1 and HSV-2, the number of infected cells detected was increased when the inoculum size and incubation time were increased and viral antigen was detected since 2 h when 5 and 10 MOI of viruses were used. For 0.5 and 1.0 MOI of viruses used, 6 h of infection time was required so 5% or more of infected cells could be demonstrated. In addition, according to numbers of infected cells obtained by IFA, the numbers of Jurkat cells with HSV-1 antigens detected were higher than those with HSV-2.

Localization of stained HSV antigens was also observed. There was no difference in localization of antigen at 2, 4 and 6 h p.i. However, for HSV-1, HSV antigens were mostly detected within the cytoplasm at 12 h p.i. whereas, for HSV-2, HSV antigens were demonstrated at the periphery of the cells at 12 and 24 h p.i.

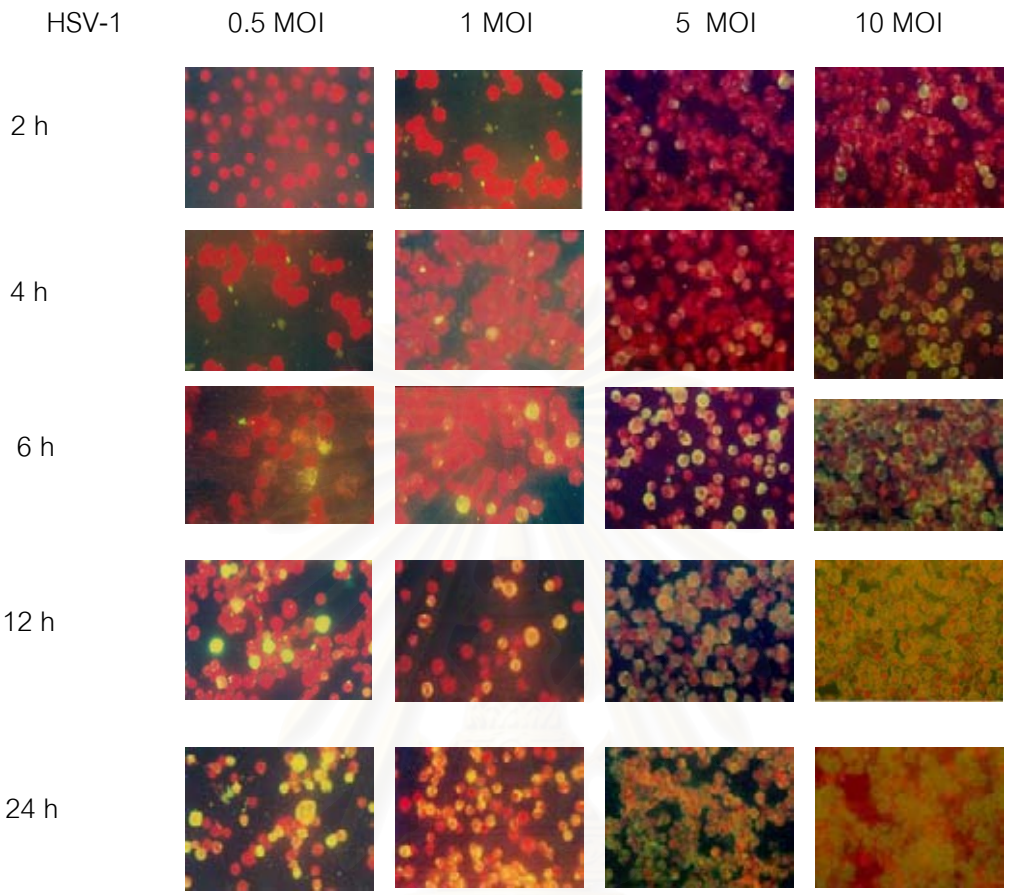


Figure 4 Fluorescent staining of HSV-1 infected Jurakat cells. Jurkat cells were infected with HSV-1 at 0.5, 1, 5 or 10 MOI for 2, 4, 6, 12 and 24 h. Cells were then stained with rabbit anti-HSV-1 antibodies as mentioned in Materials and Methods. (magnification 400X).

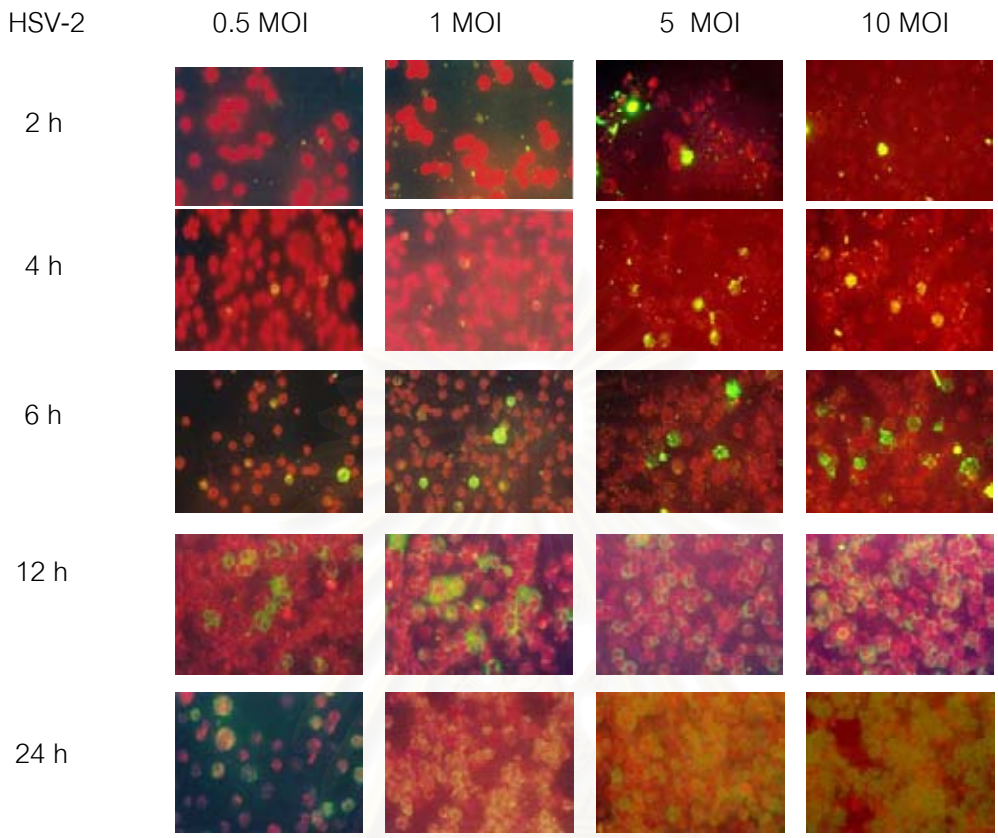


Figure 5 Fluorescent staining of HSV-2 infected Jurakat cells. Jurkats cells were infected with HSV-2 at 0.5, 1, 5 or 10 MOI for 2, 4, 6, 12 and 24 h. Cells were then stained with rabbit anti-HSV-2 antibodies as mentioned in Materials and Methods. (magnification 400X).

Table 2 Estimated numbers of HSV-1 infected Jurkat cells obtained by IFA.

HSV-1	MOI			
h p.i	0.5	1.0	5.0	10
2	0 %	0 %	1 %	5 %
4	0 %	1 %	5 %	30 %
6	5 %	10 %	30 %	60 %
12	10 %	40 %	70 %	80 %
24	40 %	50 %	80-90 %	80-90 %

Table 3 Estimated numbers of HSV-2 infected Jurkat cells obtained by IFA

HSV-2	MOI			
h p.i	0.5	1.0	5.0	10
2	0 %	0 %	1 %	1 %
4	1 %	1 %	2 %	2 %
6	2-5 %	5 %	5 %	5 %
12	5-10 %	10-15 %	20 %	50 %
24	30-50 %	70 %	70-80 %	80-90 %

2. Detection of HSV infected cells by flow cytometry

In addition to IFA, flow cytometry was performed in order to quantitate the numbers of HSV-infected Jurkat cells. Cells were infected with HSV-1 or HSV-2 at the amounts of viruses and times as shown in Figure 6 and then stained for flow cytometry detection as described in Materials and Methods. Uninfected control cells were included in each experiment to allow determination of the specificity of staining reaction. The number of cells in each group was calculated with the System II software (coulter Epics XL, BECMAN COULTER) and expressed as the percentage of total population. Results shown were the mean of thee independent experiments.

The results in Figure 6, Tables 4, 5, and 6 demonstrated that the percentage of Jurkat cells positive for HSV-1 and HSV-2 antigens were increased when the amounts of viruses and infected time were increased. Viral antigens could be detected since 2 h p.i. for both HSV-1 and HSV-2 even only 0.5 MOI of viruses was used.

Although, it seems that the numbers of Jurkat cells infected with HSV-1 were higher than those with HSV-2 corresponding to data obtained by IFA, there were some experiments with certain amounts of viruses and incubation times used did not support the conclusion (such as at 12 h p.i. with 1.0 MOI).

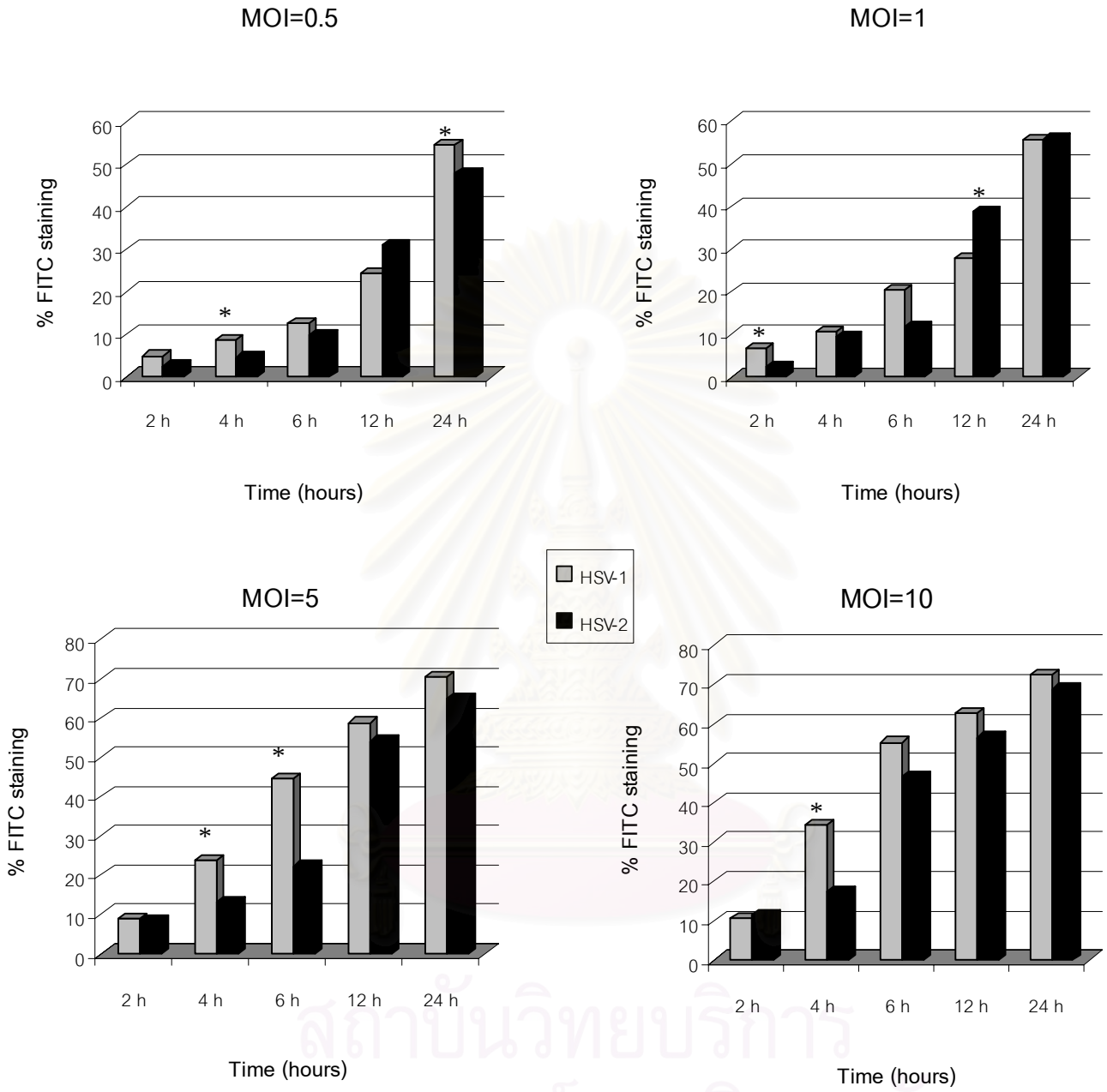


Figure 6 HSV infected cells detected by flow cytometry. Jurkat cells were infected with indicated amounts of HSV-1 or HSV-2 for 2,4,6,12 and 24 hours and stained for flow cytometry detection as described in Materials and Methods. The data are mean ± SE from three independent experiments. *$p<0.05$

Table 4 Results of flow cytometry for detection of HSV-1 infected cells. Various amount of viruses and infected times were used. The data demonstrated the percentage of HSV-1 infected cells from three independent experiments.

HSV-1		h.p.i.				
MOI		2	4	6	12	24
1	exp#	5.69	8.9	13.3	26.3	54.6
		4.2	5.7	12.2	22.5	53.6
0.5	exp#2					
	exp#					
3		4.69	11.1	12.3	24.3	55.6
1	exp#1	7.55	12.63	22.8	31	57.8
	exp#2	6.1	9.1	16.6	23.2	54.4
	exp#3	6.5	10.48	21.8	29.6	54.3
5	exp#1	8.53	20.36	45.78	57.78	69.78
	exp#2	9.43	26.63	46.73	59.03	71.23
	exp#3	8.5	23.8	40.5	59.1	70.2
10	exp#1	9.16	30.36	56.53	64.36	72.96
	exp#2	11.33	36.93	55.42	60.73	72.43
	exp#3	10.8	35.2	53.78	62.9	72.14

exp# = experiment number

Table 5 Results of flow cytometry for detection of HSV-2 infected cells. Various amount of viruses and infected times were used. The data demonstrated the percentage of HSV-2 infected cells from three independent experiments.

HSV-2		h.p.i.				
MOI		2	4	6	12	24
1	exp#	2.1	5.6	10.3	26.5	47
		3	4.8	9.5	30.5	46.86
0.5	exp#2					
	exp#					
3		2.5	3	9.8	35.7	49.7
1	exp#1	2.4	7	10	40.5	50
	exp#2	2.5	11.6	12.1	36.4	61.6
	exp#3	2.5	10.3	13.7	39.21	55.9
5	exp#1	6.67	11.74	21.34	58.04	62.64
	exp#2	11.1	13.8	21.1	49.4	68.3
	exp#3	7.8	13.66	24.1	54.4	63.77
10	exp#1	11	14.2	41.34	53.6	67.6
	exp#2	12.34	19.24	48.74	57.54	66.34
	exp#3	10.37	18.6	49.2	58.32	72.6

exp# = experiment number

Table 6 Percentage of Jurkat cells positive for HSV-1 and HSV-2 antigens detected by flow cytometry. Data are mean ± SE of three independent different experiments (shown in Tables 4 and 5).

HSV-1	MOI			
h p.i	0.5	1	5	10
2	4.86 ± 0.44	6.72 ± 0.43	8.82 ± 0.31	10.43 ± 0.65
4	8.57 ± 1.57	10.74 ± 1.03	23.59 ± 1.81	34.16 ± 1.97
6	12.60 ± 0.35	20.40 ± 1.92	44.34 ± 1.94	55.24 ± 0.80
12	24.37 ± 1.10	27.93 ± 2.40	58.64 ± 0.43	62.66 ± 1.05
24	54.60 ± 0.58	55.50 ± 1.15	70.40 ± 0.43	72.51 ± 0.24

HSV-2	MOI			
h p.i	0.5	1.0	5.0	10
2	2.53 ± 0.26	2.47 ± 0.03	8.52 ± 1.33	11.24 ± 0.58
4	4.47 ± 0.77	9.63 ± 1.37	13.07 ± 0.66	17.35 ± 1.58
6	9.87 ± 0.23	11.93 ± 1.07	22.18 ± 0.96	46.43 ± 2.55
12	30.90 ± 2.66	38.70 ± 1.21	53.95 ± 2.50	56.49 ± 1.46
24	47.85 ± 0.92	55.83 ± 3.35	44.90 ± 1.73	68.85 ± 1.91

3. Apoptosis of Jurkat cells induced by HSV

Jurkat T cells were infected with various amounts of HSV-1 or HSV-2 for indicated times. Apoptotic cells were then detected using Annexin V binding assay as mentioned in Materials and Methods.

The data shown that both HSV-1 and HSV-2 induced apoptosis in Jurkat cells and the induction was dose dependent. As shown in Table 7, 8 and Figure 7, for HSV-1, the apoptosis induction can be observed significantly (compared with non-infected cells) at 24 h p.i. since 0.5 MOI of viruses was used (p = 0.005). For HSV-2, significant number of apoptotic cells was detected since 12 h p.i. at 1 MOI (p = 0.038). When the numbers of apoptotic cells were compared between Jurkat cells infected with HSV-1 and HSV-2, at 12 h p.i., the number of apoptotic cells from HSV-2 infection was higher than that from HSV-1 (p = 0.003). There was no significant difference when results from 24 h p.i. were compared (p = 0.286).

The additional experiments with 18 h p.i. included were done to investigate apoptosis induction. As shown in Table 9,10 and Figure 8A, for HSV-1, corresponding to previous experiment, there was no significant number of apoptotic cells detected at 12 h p.i. (p = 0.068), but the significant numbers were obtained at 18 and 24 h p.i. (p = 0.007 and 0.005, respectively). For HSV-2, significant numbers of apoptotic cells compared with non-infected cells, were detected when results from 12, 18 and 24 h p.i. were analyzed (p = 0.015, 0.029 and 0.013, respectively). At 12 h p.i., numbers of apoptotic cells were higher when HSV-2 was used than HSV-1 (p = 0.043). For 18 h p.i. at 10 MOI, there was significant difference in apoptotic cell numbers from HSV-1 and HSV-2 infection when the data at 18 h p.i. was investigated (p = 0.021). However, there was no significant difference when the data at 24 h p.i. was investigated (p = 0.18).

Examples of dot plots from annexin V-FITC binding assay were shown in Figure 8B

Table 7 Data from Annexin V-FITC staining of Jurkat cells infected with various amounts of either HSV-1 and incubation times as indicated . Data shown are from three independent experiments.

HSV-1/0.5 MOI	exp#1	exp#2	exp#3	Mean ± SE
2 h p.i.	0.5	0.42	1.8	0.91 ± 0.45
4 h p.i.	1.5	2.73	2.9	2.38 ± 0.44
6 h p.i.	2	3.74	2.1	2.61 ± 0.56
12 h p.i.	1.5	3.81	3.5	2.94 ± 0.72
24 h p.i.	7	5.38	6.2	6.19 ± 0.47

HSV-1/1 MOI	exp#1	exp#2	exp#3	Mean ± SE
2 h p.i.	1.4	1.52	0.94	2.29 ± 0.18
4 h p.i.	1.75	1.85	1.27	2.71 ± 0.18
6 h p.i.	1.8	1.05	1.19	2.01 ± 0.23
12 h p.i.	1.9	1.68	1.82	2.47± 0.06
24 h p.i.	10	8.86	6.57	8.47 ± 1.01

HSV-1/5 MOI	exp#1	exp#2	exp#3	Mean ± SE
2 h p.i.	2.1	3.4	1.24	2.25 ± 0.63
4 h p.i.	2.8	1.1	2.31	2.07 ± 0.51
6 h p.i.	2.2	2.47	1.45	2.04 ± 0.31
12 h p.i.	2.4	2.24	2.87	2.5 ± 0.19
24 h p.i.	18	12.65	14.08	14.91 ± 1.60

HSV-1/10 MOI	exp#1	exp#2	exp#3	Mean ± SE
2 h p.i.	2.3	2.42	1.31	2.01 ± 0.35
4 h p.i.	3.5	3.51	1.65	2.89 ± 0.62
6 h p.i.	3.1	2.49	1.39	2.33 ± 0.50
12 h p.i.	3.8	3.33	3.32	3.48 ± 0.16
24 h p.i.	24	17.2	19.16	20.12 ± 2.02

exp#=experiment number

Table 8 Data from Annexin V-FITC staining of Jurkat cells infected with HSV-1 or HSV-2. Jurkat cells were infected with various amounts of viruses and incubation times as indicated. Data shown are three independent experiments.

HSV-2/0.5 MOI	exp#1	exp#2	exp#3	Mean ± SE
2 h p.i.	2.15	1.07	1.23	1.48 ± 0.34
4 h p.i.	1.27	2.13	1.65	1.68 ± 0.25
6 h p.i.	1.55	2.36	1.38	1.76 ± 0.30
12 h p.i.	2.37	2.35	1.72	2.15 ± 0.21
24 h p.i.	7.35	8.3	6.6	7.41 ± 0.49

HSV-2/1.0 MOI	exp#1	exp#2	exp#3	Mean ± SE
2 h p.i.	1.45	2.06	1.35	1.62 ± 0.22
4 h p.i.	1.21	2.97	1.16	1.78 ± 0.60
6 h p.i.	1.64	2.23	1.31	1.73 ± 0.27
12 h p.i.	4.11	4.39	4.36	4.29 ± 0.09
24 h p.i.	8.98	10.19	8.18	9.12 ± 0.58

HSV-2/5 MOI	exp#1	exp#2	exp#3	Mean ± SE
2 h p.i.	1.52	2.56	1.23	1.77 ± 0.40
4 h p.i.	2.08	2.95	1.35	2.13 ± 0.46
6 h p.i.	2.29	2.39	1.35	2.01 ± 0.33
12 h p.i.	7.15	4.73	5.11	5.66 ± 0.75
24 h p.i.	23.22	18.63	15.12	18.99 ± 2.34

HSV-2/10 MOI	exp#1	exp#2	exp#3	Mean ± SE
2 h p.i.	2.42	3.02	1.39	2.28 ± 0.48
4 h p.i.	2.59	2.98	1.42	2.33 ± 0.47
6 h p.i.	2.56	2.21	1.42	2.06 ± 0.34
12 h p.i.	8.84	5.3	8.33	7.49 ± 1.10
24 h p.i.	27.6	17.76	20.78	22.05 ± 2.91

exp#=experiment number

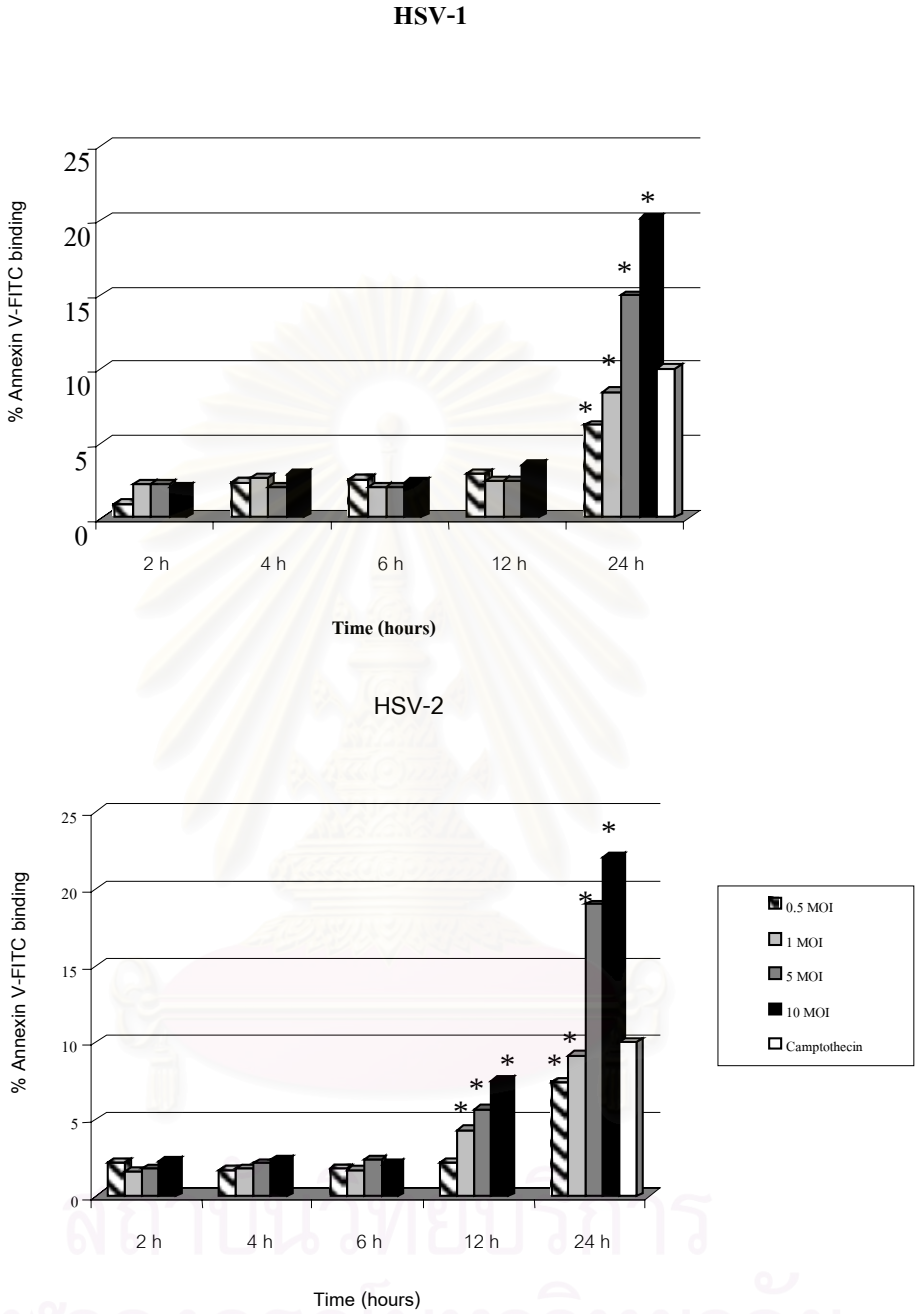


Figure 7 Apoptosis of Jurkat cells induced by HSV. Jurkat cells were infected with HSV-1 or HSV-2 for 2, 4, 6, 12 and 24 h at 0.5, 1, 5 and 10 MOI as indicated. Cells then were subjected to Annexin V staining assay as described in Materials and Methods. The data shown are mean of numbers of cells positive for Annexin V obtained from three independent experiments. * $P<0.05$

Table 9 Data from Annexin V-FITC staining of Jurkat cells infected with HSV-1 (include 18 h). Jurkat cells were infected with various amounts of viruses and incubation times as indicated . Data shown are from three independent experiments.

HSV-1/0.5 MOI	exp#1	exp#2	exp#3	Mean ± SE
2 h p.i.	0	1	0	0.33 ± 0.33
4 h p.i.	0	0.5	0	0.17 ± 0.17
6 h p.i.	0	1	0	0.33 ± 0.33
12 h p.i.	0	1.7	0	0.57± 0.57
18 h p.i.	1.6	1.8	1.7	1.7 ± 0.43
24 h p.i.	5	3.81	5	4.6 ± 0.40

HSV-1/1 MOI	exp#1	exp#2	exp#3	Mean ± SE
2 h p.i.	1.5	0.5	0	0.67 ± 0.44
4 h p.i.	0.2	0.3	0.16	0.22 ± 0.04
6 h p.i.	0	1	0	0.33 ± 0.33
12 h p.i.	0.9	0.89	1	0.93 ± 0.04
18 h p.i.	2.67	2.86	3.01	2.85 ± 0.10
24 h p.i.	11	8	5.16	8.05 ± 1.69

HSV-1/5 MOI	exp#1	exp#2	exp#3	Mean ± SE
2 h p.i.	0.2	0.57	0	0.26 ± 0.17
4 h p.i.	0	0.37	1.2	0.52 ± 0.35
6 h p.i.	0.2	0.43	0.18	0.27± 0.08
12 h p.i.	2	0.45	2.05	1.5 ± 0.53
18 h p.i.	16.8	15.2	14.66	15.55 ± 0.64
24 h p.i.	20.4	21.6	20.6	20.86 ± 0.37

exp# = experiment number

Table 9 (continued)

HSV-1/10 MOI	exp#1	exp#2	exp#3	Mean ± SE
2 h p.i.	0.2	0.2	0.3	0.23 ± 0.03
4 h p.i.	0.9	0.68	0.54	0.71 ± 0.11
6 h p.i.	1.1	0.45	0.12	0.55 ± 0.28
12 h p.i.	3.8	1.54	2.5	2.61 ± 0.65
18 h p.i.	19.88	21.5	20.73	20.7 ± 0.47
24 h p.i.	22	25.63	28.75	25.46 ± 1.95

exp# = experiment number

Table 10 Data from Annexin V-FITC staining of Jurkat cells infected with HSV-2 (include 18 h). Jurkat cells were infected with various amounts of viruses and incubation times as indicated . Data shown are from three independent experiments.

HSV-2/0.5 MOI	exp#1	exp#2	exp#3	Mean ± SE
2 h p.i.	0.53	0.52	0	0.35 ± 0.18
4 h p.i.	0.11	0.4	0.54	0.35 ± 0.13
6 h p.i.	0.26	0.32	0.11	0.23 ± 0.06
12 h p.i.	1.37	2.35	0.9	1.54 ± 0.43
18 h p.i.	2.57	4.01	3.29	3.29 ± 0.42
24 h p.i.	6	8.3	6.19	6.83 ± 0.73

HSV-2/1.0 MOI	exp#1	exp#2	exp#3	Mean ± SE
2 h p.i.	0	0.51	0.5	0.34 ± 0.17
4 h p.i.	0.05	0.24	0	0.1 ± 0.07
6 h p.i.	0.35	0.2	0.24	0.26 ± 0.04
12 h p.i.	2.06	2.39	1.54	2 ± 0.25
18 h p.i.	3.75	4.25	5	4.3 ± 0.36
24 h p.i.	7.6	10.19	7.77	8.52 ± 0.84

HSV-2/5 MOI	exp#1	exp#2	exp#3	Mean ± SE
2 h p.i.	0.5	0.35	0.25	0.37± 0.07
4 h p.i.	1.02	0	0.24	0.42 ± 0.31
6 h p.i.	2	0.35	0.8	1.05 ± 0.49
12 h p.i.	6.11	4.73	3.29	4.71 ± 0.81
18 h p.i.	12.8	14.2	13.5	13.5 ± 0.40
24 h p.i.	21.84	18.63	14.71	18.4 ± 2.06

exp# = experiment number

Table 10 (continued)

HSV-2/10 MOI	exp#1	exp#2	exp#3	Mean ± SE
2 h p.i.	0.8	0.47	0	0.42 ± 0.23
4 h p.i.	0.53	0.3	0.31	0.38 ± 0.08
6 h p.i.	1.27	0.2	0.15	0.54 ± 0.37
12 h p.i.	7.8	5.3	3.51	5.54 ± 1.24
18 h p.i.	14.8	16.9	17.7	16.5 ± 0.86
24 h p.i.	24.3	17.76	20.37	20.81 ± 1.90

exp# = experiment number

A.

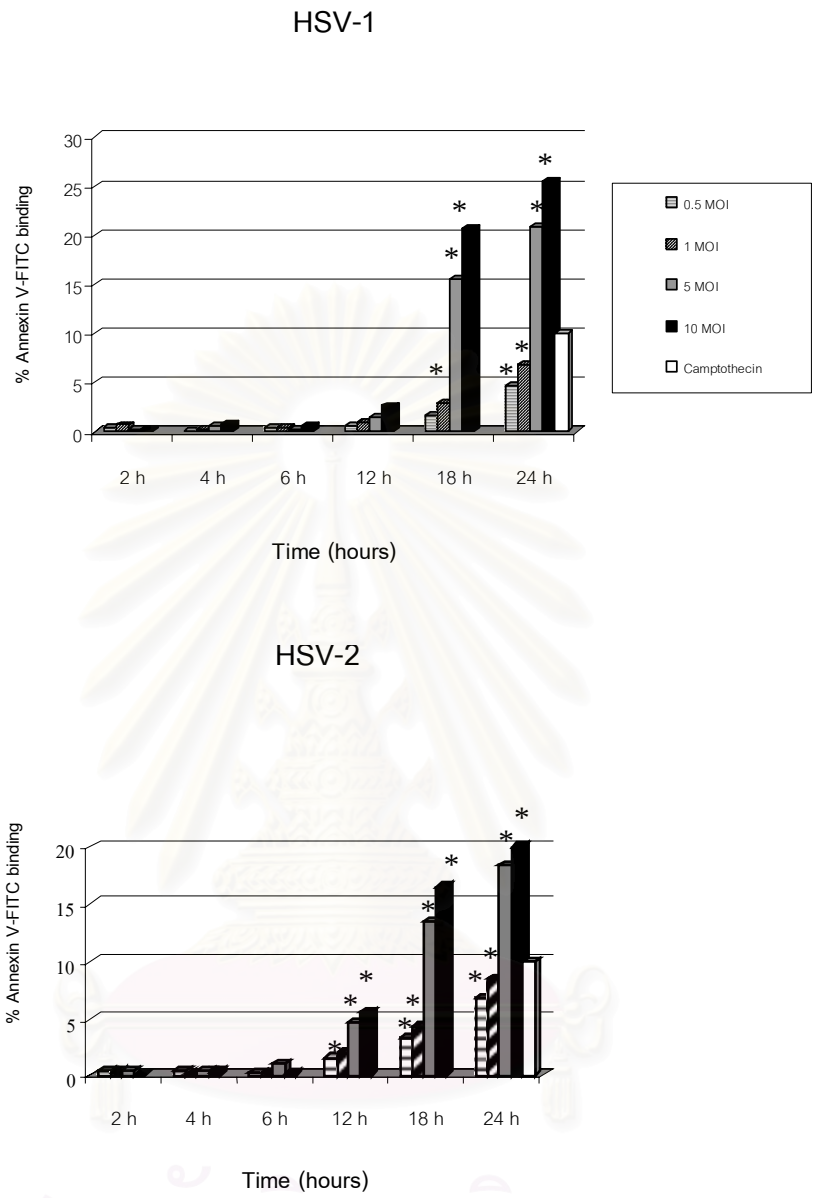
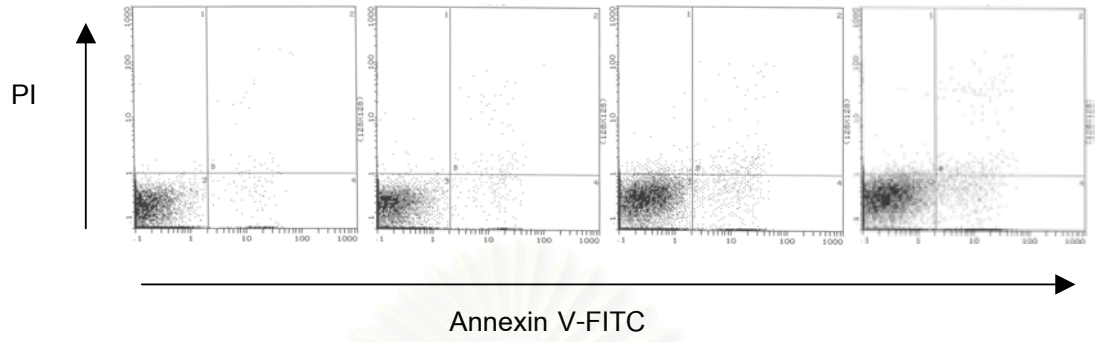


Figure 8A Apoptosis of Jurkat cells induced by HSV. Jurkat cells were infected with HSV-1 or HSV-2 for 2, 4, 6, 12, 18 and 24 h at 0.5, 1, 5 and 10 MOI as indicated. Cells then were subjected to Annexin V staining assay as described in Materials and Methods. The data shown are mean of numbers of cells positive for Annexin V obtained from three independent experiments. * $P<0.05$

B. HSV-1 0.5 1 5 10 MOI



HSV-2 0.5 1 5 10 MOI

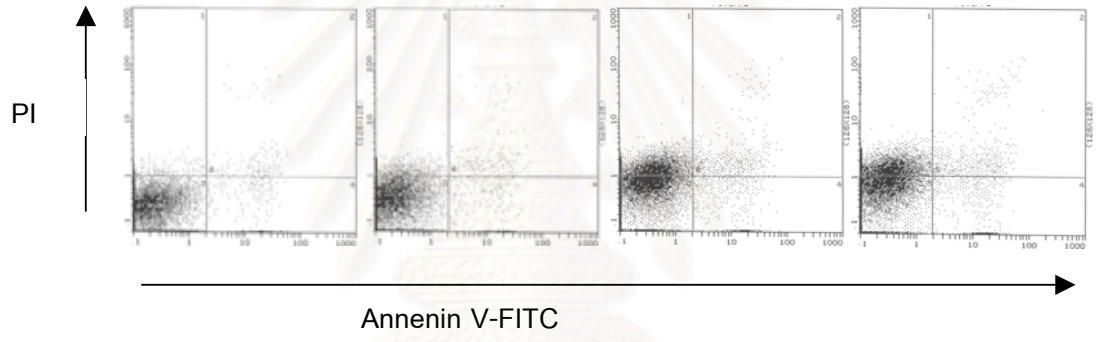


Figure 8B Dot plots of annexin V-FITC binding assay from Jurkat cells infected with the various amount of HSV-1 or HSV-2 for 18 h p.i.

4. Apoptosis of Jurkat cells induced by HSV is caspase-dependent

To determine whether caspases are involved in HSV-induced apoptosis in Jurkat cells, the effects of the cell permeable caspase-3 inhibitor Z-DEVD-FMK, caspase-8 inhibitor Z-IETD-FMK, and caspase-9 inhibitor Z-LEHD-FMK on apoptosis induction were examined. Each inhibitor was used at the concentrations of 10, 50 and 100 μM. Jurkat cells were infected with HSV-1 or HSV-2 for 18 h in the presence or absence of caspase inhibitors prior to Annexin V-FITC staining. Figures 9, 11 and 13 were from experiments using caspase-3, -8 and -9 inhibitors, respectively and results shown in these figures are mean of numbers of Annexin V positive cells obtained from three independent experiments (data shown in Tables 11, 12 and 13) and the percentage of inhibition shown in Table 11, 12 and 13 and Figure 10, 12 and 14. Apoptosis inhibition by all caspase inhibitors was does dependent. All concentrations of all inhibitors reduced the numbers of apoptotic cells. However, only at concentrations 50 and 100 μM of all inhibitors significantly reduced numbers of apoptotic cells induced by HSV-1 or HSV-2 ($p < 0.05$).

Table 11 Data of the effect of caspase-3 inhibitor (Z-DEVD-FMK) on apoptosis of Jurkat cells induced by HSV. Jurkat cells were infected with HSV-1 or HSV-2 in the presence or absence of caspase-3 inhibitor for 18 h, then Annexin V-FITC staining was performed. Data demonstrated are from three independent experiments.

	exp#1	exp#2	exp#3	Mean ± SE	% Inhibition
Mock	1.5	2	1.9	1.80 ± 0.15	-
HSV-1	18	25	25	22.67 ± 2.33	-
10 uM Z-DEVD-FMK	15.5	25	23.5	21.33 ± 2.95	7
50 uM Z-DEVD-FMK	12.8	18.9	18.8	16.83 ± 2.02	26
100 uM Z-DEVD-FMK	12.6	16.2	17	15.27 ± 1.35	32
Camptothecin	13	11.5	11.5	12.0 ± 0.50	-

	exp#1	exp#2	exp#3	Mean ± SE	% Inhibition
Mock	1.5	2	1.9	1.80 ± 0.15	-
HSV-2	12.3	13.4	13.5	13.03 ± 0.37	-
10 uM Z-DEVD-FMK	10.7	11.2	12.8	11.57 ± 0.63	11
50 uM Z-DEVD-FMK	6.6	9.1	9	8.23 ± 0.82	37
100 uM Z-DEVD-FMK	6.2	9.1	8.4	7.90 ± 0.87	38
Camptothecin	13	11.5	11.5	12.0 ± 0.50	-

exp# = experiment number

Table 12 Data of the effect of caspase-8 inhibitor (Z-IETD-FMK)on apoptosis of Jurkat cells induced by HSV. Jurkat cells were infected with HSV-1 or HSV-2 in the presence or absence of caspase-8 inhibitor for 18 h, then Annexin V-FITC staining was performed. Data demonstrated are from three independent experiments.

	exp#1	exp#2	exp#3	Mean ± SE	% Inhibition
Mock	0.8	1	1.1	0.97 ± 0.09	-
HSV-1	30	26.6	26.4	27.73 ± 1.13	-
10 uM Z-IETD-FMK	25.5	28	27.2	26.90 ± 0.74	5
50 uM Z-IETD-FMK	20	20.6	19.1	19.90 ± 0.44	28
100 uM Z-IETD-FMK	20	18.9	19.7	19.53 ± 0.33	29
Camptothecin	15	13	13	13.67 ± 0.67	-

	exp#1	exp#2	exp#3	Mean ± SE	% Inhibition
Mock	0.8	1	1.1	0.97 ± 0.09	-
HSV-2	18	15.4	15.5	16.27 ± 0.87	-
10 uM Z-IETD-FMK	16	13.3	13	14.10 ± 0.95	17
50 uM Z-IETD-FMK	11.5	10.7	12	10.73 ± 0.43	30
100 uM Z-IETD-FMK	11	9.5	9.2	9.9 ± 0.56	39
Camptothecin	15	13	13	13.67 ± 0.67	-

exp# = experiment number

Table 13 Data of the effect of caspase-9 inhibitor (Z-LEHD-FMK) on apoptosis of Jurkat cells induced by HSV. Jurkat cells were infected with HSV-1 or HSV-2 in the presence or absence of caspase-9 inhibitor for 18 h, then Annexin V-FITC staining was performed. Data demonstrated are from three independent experiments.

	exp#1	exp#2	exp#3	Mean ± SE	% Inhibition
Mock	0.8	1	1.1	0.97 ± 0.09	-
HSV-1	24.8	26.6	26.3	26.0 ± 0.60	-
10 uM Z-LEHD-FMK	21.2	24.7	24.5	23.4 ± 1.10	11
50 uM Z-LEHD-FMK	14.5	18	15.7	16.07 ± 1.03	38
100 uM Z-LEHD-FMK	16	17.8	16.7	16.83 ± 0.52	35
Camptothecin	13.4	13	13	13.13 ± 0.13	-

	exp#1	exp#2	exp#3	Mean ± SE	% Inhibition
Mock	0.8	1	1.1	0.97 ± 0.09	-
HSV-2	13.5	15.4	15.2	14.77 ± 0.63	-
10 uM Z-LEHD-FMK	12.7	12.8	13.3	12.97 ± 0.17	11
50 uM Z-LEHD-FMK	7.9	10.6	10.6	9.70 ± 0.90	38
100 uM Z-LEHD-FMK	7.1	9.4	9.5	8.67 ± 0.78	41
Camptothecin	13.4	13	13	13.13 ± 0.13	-

exp# = experiment number

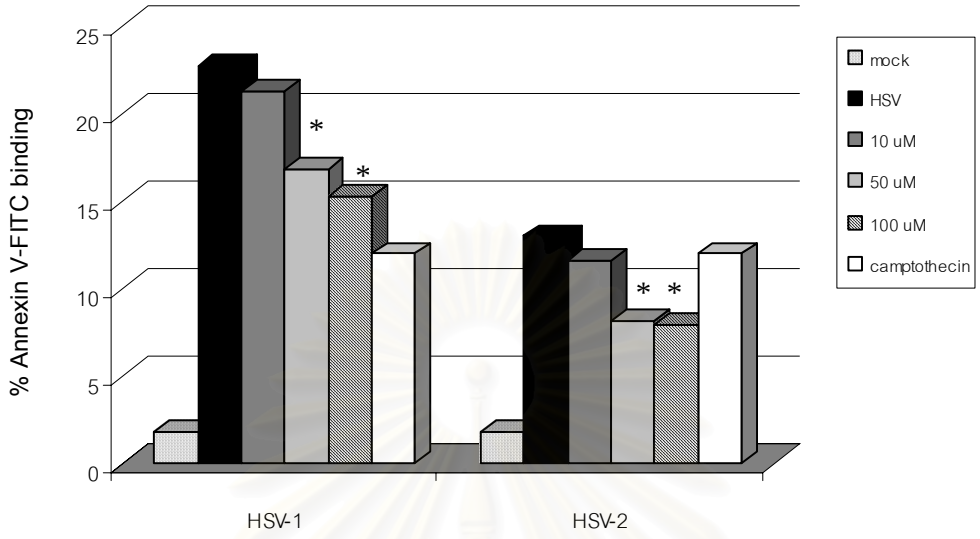


Figure 9 The effect of caspase 3 inhibitor on HSV-induced apoptosis of Jurkat cells. Jurkat cells were infected with HSV-1 or HSV-2 in the absence or presence of caspase inhibitors for 18 h. Apoptosis was then determined using Annexin V assay as mentioned in Materials and Methods. Data are mean of three independent experiments. * $p<0.05$

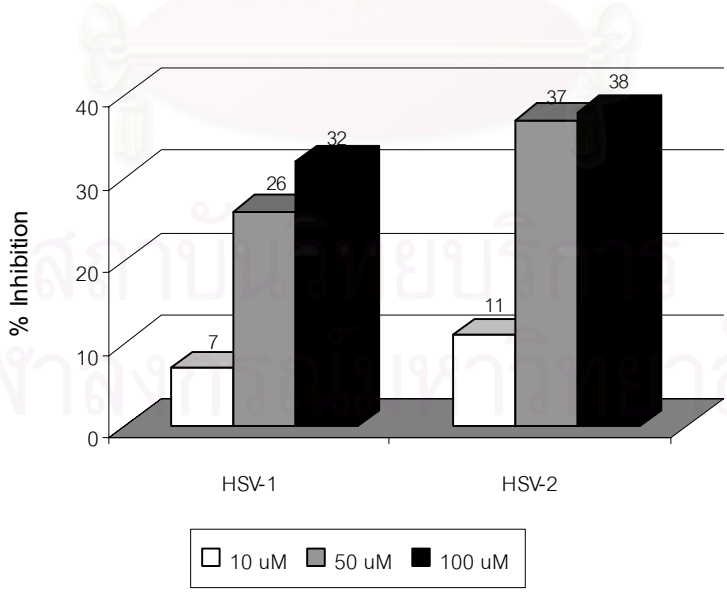


Figure 10 The percentage of apoptosis inhibition by caspase-3 inhibitors. Data are mean of three independent experiments.

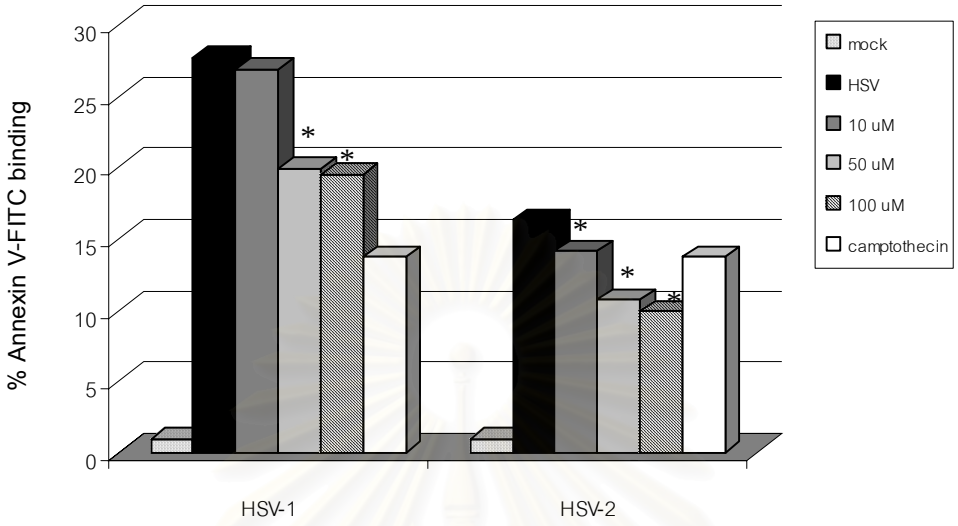


Figure 11 The effect of caspase 8 inhibitor on HSV-induced apoptosis of Jurkat cells. Jurkat cells were infected with HSV-1 or HSV-2 in the absence or presence of caspase inhibitors for 18 h. Apoptosis was then determined using Annexin V assay as mentioned in Materials and Methods. Data are mean of three independent experiments. * $p<0.05$

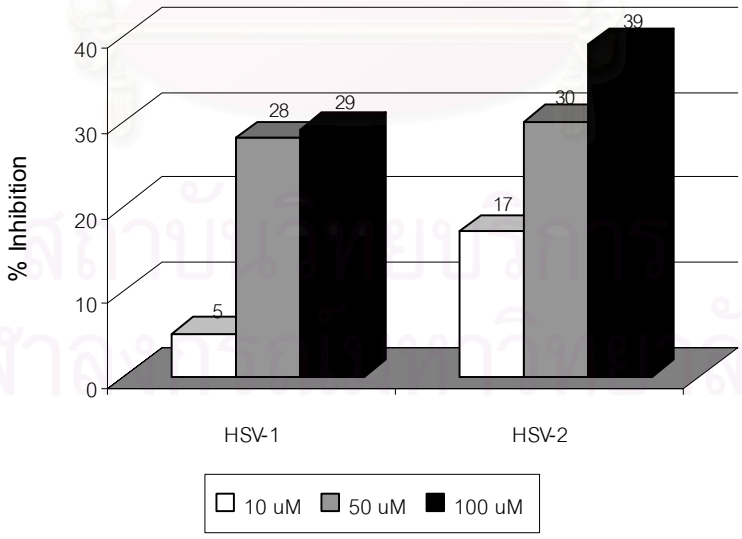


Figure 12 The percentage of apoptosis inhibition by caspase-8 inhibitors. Data are mean of three independent experiments.

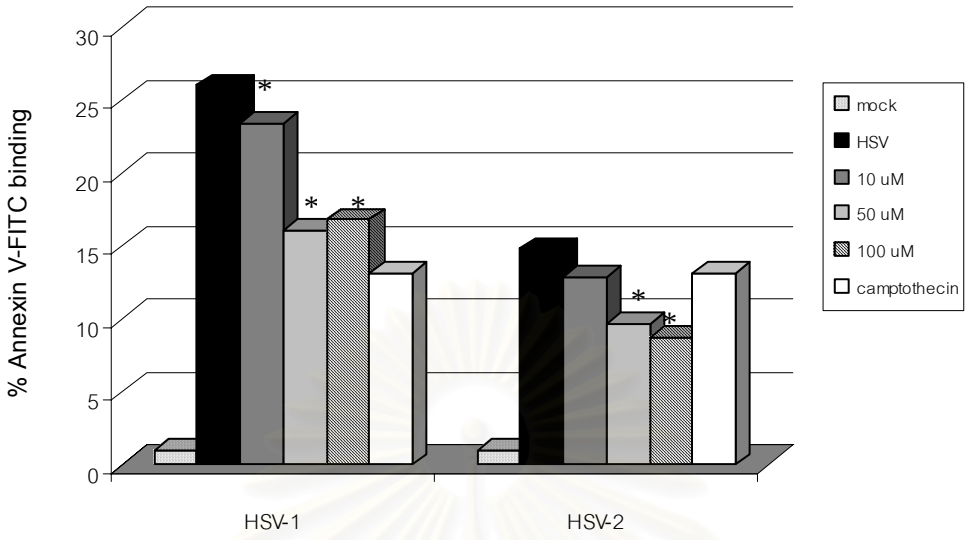


Figure 13 The effect of caspase 9 inhibitor on HSV-induced apoptosis of Jurkat cells. Jurkat cells were infected with 5 MOI of HSV-1 or HSV-2 in the absence or presence of caspase inhibitors for 18 h. Apoptosis was then determined using Annexin V assay as mentioned in Materials and Methods. Data are mean of three independent experiments. * $p<0.05$

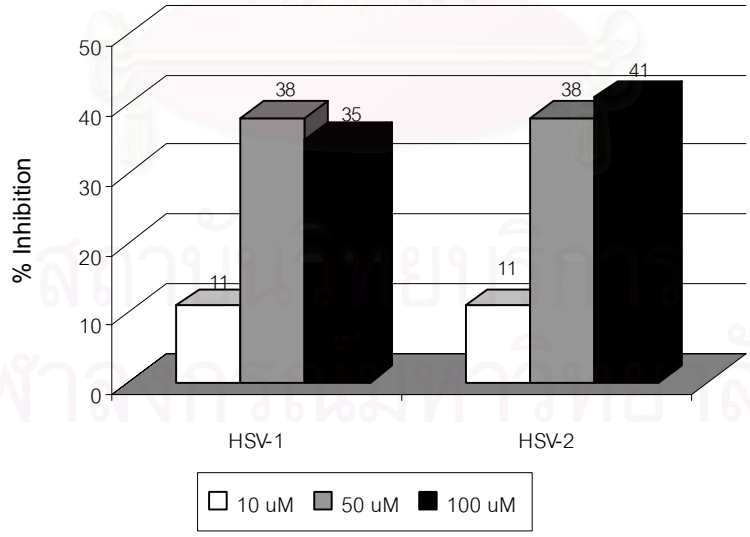


Figure 14 The percentage of apoptosis inhibition by caspase-9 inhibitors. Data are mean of three independent experiments.

According to the data obtained above, apoptosis of Jurkat cells induced by HSV is caspase-dependent. We further investigated whether the combination of caspase inhibitors provide any additional information. Jurkat cells were infected with HSV-1 or HSV-2 in the presence or absence of combinations of caspase inhibitors for 18 h before AnnexinV-FITC staining was performed. For HSV-1, as shown in Table 14 and Figure 15, there was no significant difference when the numbers of apoptotic cells using each inhibitor was compared. However, the combination of three inhibitors reduced significant numbers of apoptotic cells when compared with results with either caspase-3 or -8 inhibitor was used alone or with caspase-8 and -9 inhibitors were used together (p=0.003, 0.036, and 0.034, respectively). For HSV-2, only combination of caspase-8 and -9 inhibitors reduced the significant number of apoptotic cells (p=0.014) when compared with caspase-8 inhibitor alone. The percentage of apoptosis inhibition shown in Figure 16.

Table 14 Data demonstrating the effect of combinations of caspase inhibitors on apoptosis of Jurkat cells induced by HSV. Jurkat cells were infected with 5 MOI of HSV-1 or HSV-2 for 18 h in the presence or absence of combination of caspase inhibitors. Apoptosis was demonstrated by Annexin V-FITC staining. Data are from three independent experiments.

	exp#1	exp#2	exp#3	Mean ± SE
Mock	2	2.3	2.5	2.27± 0.15
HSV-1	23	29.8	29.1	27.30± 2.16
Caspase 3 inhibitor	19.2	22.9	21.9	21.33 ± 1.11
Caspase 8 inhibitor	17.6	25.1	21.6	21.43 ± 2.17
Caspase 9 inhibitor	13.6	16	18.7	16.10 ± 1.47
Caspase 8+9 inhibitor	13.6	16.6	17.7	15.97 ± 1.23
Caspase 3+8+9 inhibitor	11.5	13.2	13.5	12.73 ± 0.62
Camptothecin	12.9	13.5	13	13.13 ± 0.13

	exp#1	exp#2	exp#3	Mean ± SE
Mock	1.1	1.17	1.5	1.26 ± 0.12
HSV-2	14	13.3	16.9	14.73 ± 1.10
Caspase 3 inhibitor	6.2	9.1	8.4	7.90 ± 0.87
Caspase 8 inhibitor	8.9	9.5	9.2	9.20 ± 0.17
Caspase 9 inhibitor	7.1	9.4	9.5	8.67 ± 0.78
Caspase 8+9 inhibitor	8.3	8.6	8.5	8.47 ± 0.09
Caspase 3+8+9 inhibitor	8.7	7.7	7.5	7.97 ± 0.37
Camptothecin	12.9	13.5	13	13.13 ± 0.13

exp# = experiment number

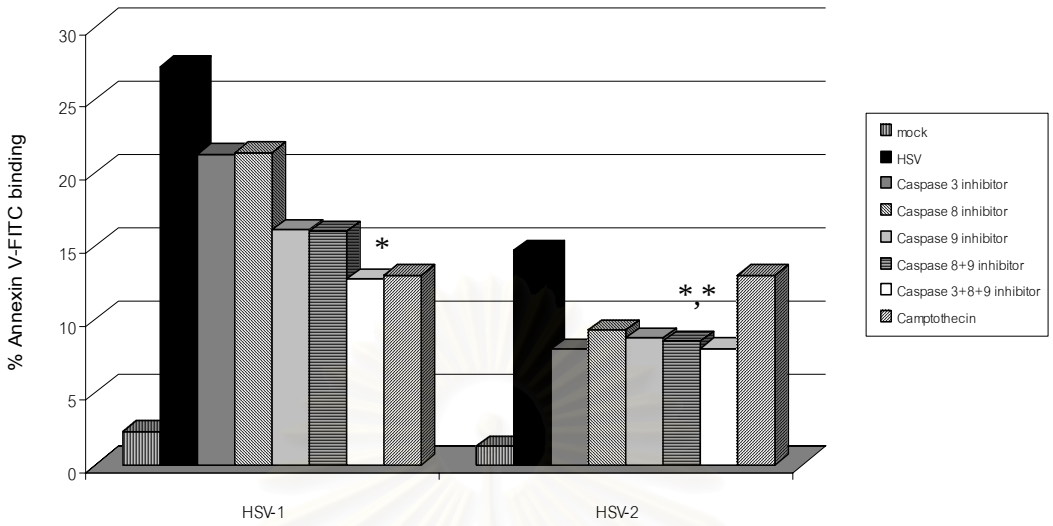


Figure 15 Effects of combinations of caspase inhibitors on apoptosis induction by HSV. Jurkat cells were infected with 5 MOI of HSV-1 or HSV-2 for 18 h in the presence or absence of various combinations of caspase inhibitors. Apoptosis detection was then performed by Annexin V staining. Data are mean of three independent experiments. *p = 0.003, 0.036, and 0.034 when compared with caspase 3, caspase 8 alone and caspase 8+9, respectively. *'*p = 0.014 when compared with caspase 8 alone.

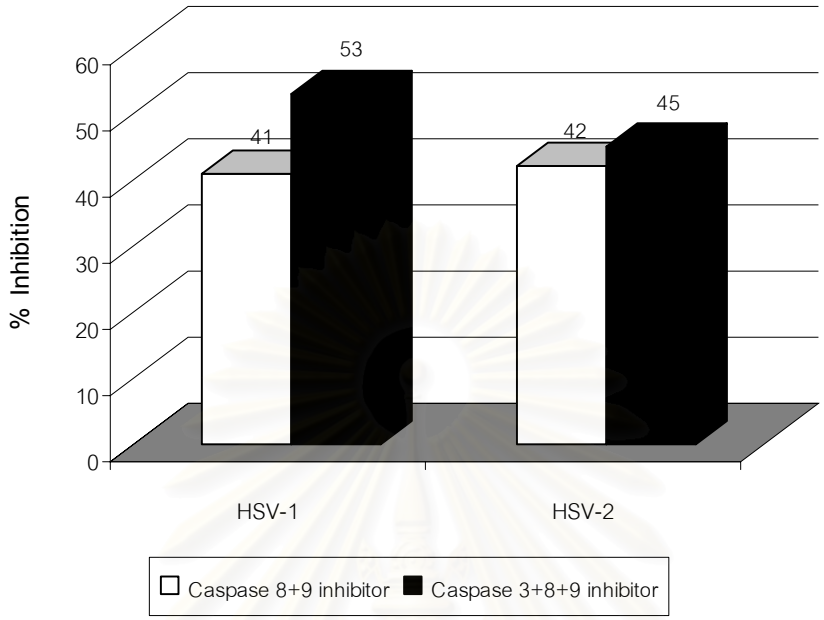


Figure 16 The percentage of apoptosis inhibition by combination of caspase inhibitors. Data are mean of three independent experiments.

CHAPTER VI

DISCUSSION

Although HSV infection and its ability to establish latent infection have been recognized for a period of time, the molecular mechanisms involved in pathogenesis of this organism is still not clearly understood. The major areas of interest in the study of HSV infection involved mechanisms of reactivation and immune evasion. As mentioned earlier that HSV utilizes various approaches for evasion of immune response and apoptosis induction of T lymphocytes was suggested to be one of those . Apoptosis of T lymphocytes may result in immnosuppression and lymphocytopenia seen in response to HSV infection.

Various evidences on mechanisms of apoptosis inhibition in various cell types were demonstrated, however, there have been a few reports on apoptosis induction and its molecular mechanisms in T lymphocytes. In 1988, Heyward *et al*. demonstrated that HSV-1 could replicate in HLA-DR-positive T lymphocytes and this infection induced apoptosis in those cells activated with phytohemagglutinin (PHA) (130). In 1997, Ito *et al*. demonstrated that HSV-1-induced apoptosis in both PHA-activated CD4 and CD8 lymphocytes from cord blood (17). In 1997, Ito *et al*. investigated that HSV-1 induced apoptosis in PHA-activated peripheral blood T lymphocytes and apoptosis induction occurred only in CD4 but not in CD8 lymphocytes (18).

Since most studies in T lymphocytes used HSV-1, we were interested in comparing apoptosis induction induced by HSV-1 and HSV-2. The significant findings of our study can be summarized as followed. We showed that HSV-1 and HSV-2-infected Jurkat cells underwent apoptosis monitoring by Annexin V-FITC staining, and these processing events were detected in the infected cells for 18 h at 1 MOI for HSV-1 and 12 h at 1 MOI for HSV-2. The data also suggest that HSV-2 induced apoptosis better than HSV-1.

In 1999, Galvan *et al*. demonstrated that HSV-1 blocked apoptosis via both caspase-independent and caspase-dependent pathways (131). We demonstrated that HSV-1 and HSV-2 infection induced apoptosis via a pathway which activates and process caspase-dependent. Even though caspase-3 is the downstream effector of caspase-8 and –9, its inhibitor could not inhibit apoptosis more than the inhibitor of caspase-8 or –9. It is probably because there are additonal downstream effectors such as caspase-6 and –7. In this study, HSV could not induce apoptosis in early time of infection which is probably became infected cell proteins between 3 and 6 h p.i. In 2003, *Goodkin* et al., demonstrated that the nuclear translocation of transcription factor NK-kappaB between 3 and 6 h p.i. was necessary to prevent apoptosis in wild-type HSV-1-infected human HEp-2 cells (132).

Since each inhibitor used in our study could not completely inhibit apoptosis induced by HSV-1, we were interested in whether the increase in apoptosis inhibition could be observed when combinations of caspase inhibitors were used. The data suggest that other mechanism (s) such as the caspase-independent pathway could be involved since the inhibitors of caspase-8 and –9 and their downstream effector, caspase-3 all together were not able to completely inhibit apoptosis. Caspase-idependent pathway have been shown to execute apoptosis in many types of neuronal injury. In addition, apoptosis-inducing factor (AIF) is an important factor involved in the regulation of this caspase-independent neuronal cell death (133).

Even Jurkat cells have been widely used as a model for T lymphocytes, further study using peripheral blood T lymphocyted should be done to confirm our observation. In addition, viral and host proteins involved in apoptosis induction will further elucidate the molecular mechanisms of apoptosis induction. Most genes and gene products of HSV reported are involved in inhibition of apoptosis. For examples, Leopardi *et al*. in 1996; demonstrated that a viral protein known as ICP4 blocked apoptosis induced by HSV-1 in Vero cells (119) and in 1997, they demonstrated that the U_S3 gene of HSV-1 was necessary

to prevent apoptosis induced by the virus (10). The previous study suggested that U_S3 gene of HSV-1 correlated with phosphorylation of BAD, a BH3-only proapoptotic Bcl-2 family member. Phosphorylation of BAD has been demonstrated to abrogate its proapoptotic activity (134). The HSV latency-associated transcript (LAT) is a complex transcription unit expressed primarily in neurons containing latent genomes and has been shown to inhibit cell death by blocking caspase-8 and caspase-9 pathways (135). Moreover, Perkin *et al*., 2003 reported that ribonucleotide reductase (R1) protein (ICP10 protein kinase) of HSV-2 blocked apoptosis in cultured hippocampal neurons by activating the extracellular signal-regulated kinase (ERK) survival pathway and involving a c-Raf-1-dependent mechanism and induction of antiapoptotic protein Bag-1. In contrast, HSV-1 activated c-Jun N-terminal kinase (JNK), c-Jun,and ATF-2, induces the proapoptotic protein BAD, and trigger apoptosis in hippocampal neurons consistent with disease pathogenesis supporting the evidence that HSV-1 causes encephalitis more than HSV-2 (136). Other viruses, such as human papillomaviruses, its proteins (E2) are pro-apoptotic. E2 induces apoptosis through the extrinsic pathway, involving the initiator caspase-8. In addition, E2 is cleaved by caspases during apoptosis, providing an example of an apoptotic inducer, which is itself a target for caspase cleavage. The cleaved E2 protein exhibits an enhanced apoptotic activity, suggesting that it may participate in an amplification loop (137).

Other target site for HSV is at the level of the antigen-presenting cell (APC). The most potent form of APC known is the dendritic cell (DC). DC plays a key role in the induction of the primary cellular immune response to intracellular pathogens, as they are the main cell type that stimulates naive T cells in the draining lymph nodes (138). In 2003, Jones CA *et al*., found that HSV induces rapid cell death in murine bone marrow-derived DC, with HSV-2 being more potent than HSV-1 (139). This study demonstrated that HSV induces immature DC to undergo apoptosis by a mechanism involving caspase-8. In general, cellular FLICE-inhibitory protein (c-FLIP) inhibits death receptor signal by blocking caspase-8 (98). It has been demonstrated that HSV-infected immature DC down-regulated

long c-FLIP and up-regulate p53 where as other apoptosis-regulation proteins (e.g. Bcl-2) were not affected (140).

Bhattarakosol et al, 2002, previously demonstrated that HSV-1 and -2 can replicate in either PHA- activated or non-activated Jurkat cells. However, in their study, only 1 MOI of viruses was used. In our study, IFA and flow cytometry for detection of HSV-infected Jurkat cells using various amounts of HSV and infection times were compared.

Indirect fluorescence assay is a technique commonly used for demonstration of viral infected cells. Numbers of infected cells can be estimated under a fluorescent microscopy. In addition, localization of stained viral antigens in cells can be demonstrated using IFA. However, the numbers of infected cells obtained by this method can be subjective and varied between one observer to another. Flow cytometry has been widely used for detection and enumeration of fluorescently labeled cells or particles. The data can be analysed and numbers of cells or particles of interest can be obtained by using the computer program. Several samples can be performed within a shorter time than IFA.

Steele-Mortimer et al, 1990 (123), has demonstrated the use of flow cytometry for analysis of HSV-1 and influenza C virus infected cells. Both viruses were detected in cells using flow cytometry since 12 h p.i. and the percentage of fluorescent labeled cells increased with both time p.i. and amounts of viruses. This study indicated that flow cytometry can provide additional information on activity of antivirals as compared to conventional methods such as the plague reduction assay and other techniques. Flow cytometry allows the simultaneous assessment of such parameters as DNA content, RNA content, cell volume, both general and specific viral antigen expression, and viability of large numbers of individual cells. Our results provided additional information that flow cytometry was more sensitive than IFA since the infection time required for detection of viral antigens using flow cytometry was shorter than using IFA. However, results obtained by

flow cytometry lack the information on localization of antigens detected. IFA will certainly be able to fulfill that purpose.

Since Jurkat cells used were composed of both $CD4^+$ and $CD8^+$ cells (data not shown), we performed additional experiments to investigate which population of Jurkat cells were infected with HSV-1 or HSV-2. We found that there was no significant difference in the percentage of $CD4^+$ Jurkat cells infected with HSV-1. However, the percentage of $CD8^+$ Jurkat cells infected with HSV-2 was higher than with HSV-1 ($p = 0.001$) (data not shown). This is quite an interesting observation since a populaition of $CD8^+$ T cells is cytotoxic T cells responsible for killing viral infected cells.

The informations on how HSV induces apoptosis in T lymphocytes, which population of T lymphocytes are preferentially infected, where viral antigens are located during a course of infection and which viral and host proteins are involved in those processes are inevitable required for understanding of pathogenic mechanisms of HSV infection.

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APPENDICES

Appendix I

Chemical Agents and Instruments

A. Chemical substrances

Annexin V FITC (Santa Cruz Biotecnology, USA)

Assay buffer (Santa Cruz Biotecnology, USA)

Caspase-3 Inhibitor (R&D systems, USA)

Caspase-8 Inhibitor (R&D systems, USA)

Caspase-9 Inhibitor (R&D systems, USA)

Disodium hydrogen phosphate (Merck, Germany)

Fetal bovine serum (Gibco BRL, USA)

HEPES (Amresco, USA)

Hydrochloric acid (Merck, Germany)

L-Glutamine (Gibco BRL, USA)

Paraformaldehyde (Sigma, USA)

Penicillin G (Gibco BRL, USA)

Potassium chloride (Merck, Germany)

Potassium dihydrogen phosphate (Merck, Germany)

Propidium iodide (Santa Cruz Biotecnology, USA)

M199 (Earle's salt) (Gibco BRL, USA)

Rabbit-anti HSV-1 and HSV-2 antibody (DAKO A/S, Denmark)

RPMI 1640 (Gibco BRL, USA)

Sodium chloride (Merck, Germany)

Sodium hydroxide (Merck, Germany)

Streptomycin (Gibco BRL, USA)

Swine anti-rabbit antibody conjugated with FITC (DAKO A/S, Denmark)

B. Glasswares

Beaker (Pyrex, USA)

Cylinder (Witeg, Germany)

Serological pipette (Pyrex, USA)

12 well-glass slide (Diasolin, USA)

24 well-plate (Nunclon, Denmark)

96 well-plate (Nunclon, Denmark)

25 mm^3 Culture flask (Nunclon, Denmark)

C. Instruments

CO_2 Incubator (Jencons, UK)

Eppendorf minispin (Eppendorf, Germany)

Flow cytometer Epic XL (Beckman coulter, USA)

Mixer Vortex-Genie (Scientific industries, USA)

PHM 83 Auto cal pH meter (Radiometer, Denmark)

Refrigerated centrifuge model RT7 (Sorvall, USA)

Water bath (Precision, USA)

Appendix II

MEDIA, REAGENTS AND PREPARATIONS

A. MEDIA

1. 2X M199 medium

Medium 199 powder	9.5	g
Deionized distilled water	500	ml

Shake until medium has dissolved. This media was sterilized by passing it through a 0.22 μm filter. Store the medium at 2-8 °C.

2. RPMI 1640 medium

Per Liter:

To 900 ml of deionized distilled water, add:

RPMI medium powder	10.4	g
$NaHCO_3$	2.0	g

Shake until the solutes have dissolved. Adjust the pH to 7.2-7.4 with 1N HCL (~1 ml). Adjust the volume of the solution to 1 liter with deionized distilled water. Sterilize by passing it through a 0.22 μm filter. Store the medium at 2-8 °C.

3. Plaque overlay medium

3.1 Solution A

10X M199 with Earle's salt with L-glutamine with $NaHCO_3$	20 ml
Heat inactivated fetal bovine serum	20 ml
Penicillin G	0.2 ml
Streptomycin	0.2 ml
1M HEPES	2 ml
1M $NaHCO_3$	1.5 ml
Add deionized distilled water to	100 ml

3.2 Solution B

Gum tragacanth	2 g
Add deionized distilled water	100 ml

Boil until the solute has dissolved. This solution was sterilized by autoclaving. Store at 2-8 °C. Plaque overlay medium was prepared by solution A : solution B in ratio 1:1.

4. 2% Fetal bovine serum + M199 medium

2X M199 medium	50 ml
Heat inactivated fetal bovine serum	2 ml
Penicillin G	1 ml
Streptomycin	1 ml
1M HEPES	1 ml
1M $NaHCO_3$	0.5 ml
Add deionized distilled water to	100 ml

5. **10% Fetal bovine serum + M199 medium**

2X M199 medium	50	ml
Heat inactivated fetal bovine serum	10	ml
Penicillin G	1	ml
Streptomycin	1	ml
1M HEPES	1	ml
1M $NaHCO_3$	0.5	ml
Add deionized distilled water to	100	ml

B. REAGENTS

1. **HSV stock preparation**

1.1 1X Trypsin

10X Trypsin	90	ml
Add deionized distilled water	10	ml

2. **Reagents for indirect immunofluorescence and flow cytometer**

2.1 0.15 M PBS pH 7.4 (Washing buffer)

NaCl	8.0	g
KCL	0.2	g
Na_2HPO_4	1.15	g
KH_2PO_4	0.2	g
Add deionized distilled water to	1,000	ml

2.2 Evan blue (100X) stock

Evan blue	0.5	g
Add deionized distilled water	133.5	ml

2.3 Evan blue (1X) 100 ml

100X Evan blue stock	1	ml
Add deionized distilled water	99	ml

2.4 1% Crystal violet in 10% Formalin

Crystal violet	1	g
10% Formalin	100	ml

2.5 FACS permeabilizing (1X) 10 ml

FASC perm (10X)	1	ml
Add deionized distilled water	9	ml

3. Reagent for annexin V staining and caspase inhibitor assay

3.1 Assay buffer (10X) 10 ml

1M HEPES pH 7.4	1	ml
5M NaCl	2.8	ml
0.1M $CaCl_2$	2.5	ml
Add deionized distilled water to	10	ml

BIOGRAPHY

Mr. Atinop Pongpanich was born on March 23, 1977 in Saraburi, Thailand. He graduated with the Bachelor degree of Science in Medical Technology from the Faculty of Allied Health Science, Chulalongkorn University in 1997. He is currently a member of Immunology Unit, Department of Microbiology, King Chulalongkorn Memorial Hosipital and was given the opportunity to pursue his MS degree in the Inter-Department of Medical Microbiology, Faculty of Graduate School, Chulalongkorn University since 2001.