CHAPTER II

LITERATURE REVIEW

1. Herpes simplex virus

Herpes simplex viruses (HSV) are the first of the human herpesviruses to be discovered and are among the most intensively investigated of all viruses (Roizman and Knipe, 2001). The investigations into the HSV first began in ancient times, 25-plus centuries ago (Nahmias and Dowdie, 1968). Hippocrates used the term "herpes" to describe the lesions that appeared to creep or crawl along the skin (Beswick, 1962; Wildy, 1973). Herodotus was the first to draw an association between these cutaneous eruptions and fever, the association that has survived to this day. Galen recognized that HSV recurrences develop at the same anatomical site (Roizman and Whitley, 2001). Several observations made in the late 19^{th} and early 20^{th} centuries brought and end to the imprecise descriptive era of HSV infections. Numerous points substantiate this conclusion. First by, histopathologic studies described multinucleated giant cells associated with herpesvirus infections (Unna, 1886). Second by, the unequivocally nature of HSV infections was determined (Lowenstein, 1919).

The advances in major laboratory in the past 25 years have provided a foundation for the recent application of molecular biology technologies of the study of human disease such as restriction enzymes, DNA cloning, and DNA sequencing. One significant advance was the detection of antigenic differences between HSV types. Although suggested by Lipschitz (1921) on clinical grounds more than 60 years ago and by others from laboratory observations (Plummer, 1964), in 1968 Nahmias and Dowdle and in 1971 Schnewels and Nahmias demonstrated biologic and antigenic differences between two types of HSV. These investigators proposed that human herpes simplex virus type 1 (HSV-1) was more frequently associated with oral and nongenital infections; whereas human herpes simplex virus type 2 (HSV-2) was associated with genital tract disease. This observation was pivotal for many of the clinical, serological, immunological, and epidemiological studies. Obviously, other critical advances made over the past decade have contributed to our understanding of the natural history of HSV infections. These include the

following, among others. First came the descriptions of herpes genitalis (De Morbis Veneresis, 1736) and herpes encephalitis (Smith *et al.*, 1941), then successful antiviral therapy was established unequivocally for HSV encephalitis by vidarabine therapy (Whitley *et al.*, 1977) and, subsequently, for genital HSV infections (Bryson *et al.*, 1983) and HSV infections in the immunocompromised host (Meyers *et al.*, 1980). In fact, the real therapeutic advance for mucocutaneous and visceral HSV infections was the discovery of acyclovir and the demonstration of its mechanism of action by Elion in 1982 (Whitley and Gnann, 1992). The differences between strains of HSV were demonstrated by restriction endonucluease technology, which has become an important molecular epidemiologic tool (Buchman *et al.*, 1978). Type-specific antigens were used for seroepidemiologic studies of HSV infections (Roizman *et al.*, 1984). Many studies have focused on the replication of HSV and the resultant gene products. A principal goal of these investigations is to define the biological properties of these gene products that affected the nature and pathology of HSV infection. The engineering of HSV and the expression of specific gene have been expected to provide technology for new vaccines (Roizman *et al.*, 1985; Stevens *et al.*, 1987), as well as for the use of HSV for gene therapy in cancer (Andreansky *et al.*, 1998; Markert *et al.*, 2000) and CNS diseases (Roizman and Whitley, 2001). Finally, extensive effort has been spent on the study of HSV latency with incremental advances (Stevens, 1989; Roizman and Sears, 1993).

2. Characteristics of herpes simplex virus

Herpes simplex viruses (HSV) are classified in the genus *Simplexvirus* (White and Fenner, 1994; Taylor *et al.*, 2002), in the subfamily *Alphaherpesvirinae*, and in the family *Herpesviridae* by the International Committee on the Taxonomy of Viruses (ICTV) (Roizman *et al.*, 1967, 1981, 1992; Van Regenmortel *et al.*, 1991). The members of this subfamily are classified on the basis of a variable host range, relatively short reproductive cycle, rapid spread in culture, efficient destruction of infected cells, and capacity to establish latent infections primarily but not exclusively in sensory ganglia (Roizman and Pellet, 2001).

HSV is a large enveloped virus; 150-200 nm in diameter; with a distinct virion structure characteristic of the viruses (Figure 1). A mature HSV particle contains four structural features (Wildy *et al.*, 1960): (i) an electron-opaque core containing viral DNA, (ii) an icosahedral capsid surrounding the core, (iii) an amorphous\tegument surrounding the capsid, and (iv) an outer membrane envelope studded with viral glycoprotein spikes on its surface. The core contains the linear double strand DNA (dsDNA) genome wrapped as a toroid or spool structure (Furlong *et al.*, 1972; Zou *et al.*, 1999). The ends of the genome are probably held together or are in close proximity in as much as a small fraction of the packaged DNA appears to be circular and the bulk of the linear DNA circularizes rapidly in the absence of protein synthesis after it enters the nuclei of infected cells. DNA extracted from HSV virions contains ribonucleotides, nicks, and gaps. The tegument, a term introduced by Roizman an Furlong to describe the structures between the capsid and the envelope (Roizman and Furlong, 1974), has no distinctive features in thin sections, but it may appear to be fibrous on negative staining. The tegument is frequently distributed asymmetrically, and its thickness may vary depending on the location of the virion within the infected cell; when the amount is variable, there is more of it in virions accumulating in cytoplasmic vacuoles than in those accumulating in the perinuclear space. The available evidence suggests the amount of tegument is more likely to be determined by the virus than by the host. The variability in the thickness of tegument results in the variation in size of virions. The capsid is approximately 100 nm in diameter and composed of 162 capsomers arranged in icosahedral symmetry. The hexameric capsomers are 9.5 x 12.5 nm in longitudinal section; a channel 4 nm in diameter runs from the surface along their long axis. Finally, the envelope consists of a lipid bilayer with about 12 different viral glycoproteins called spikes embedded in it. The envelope

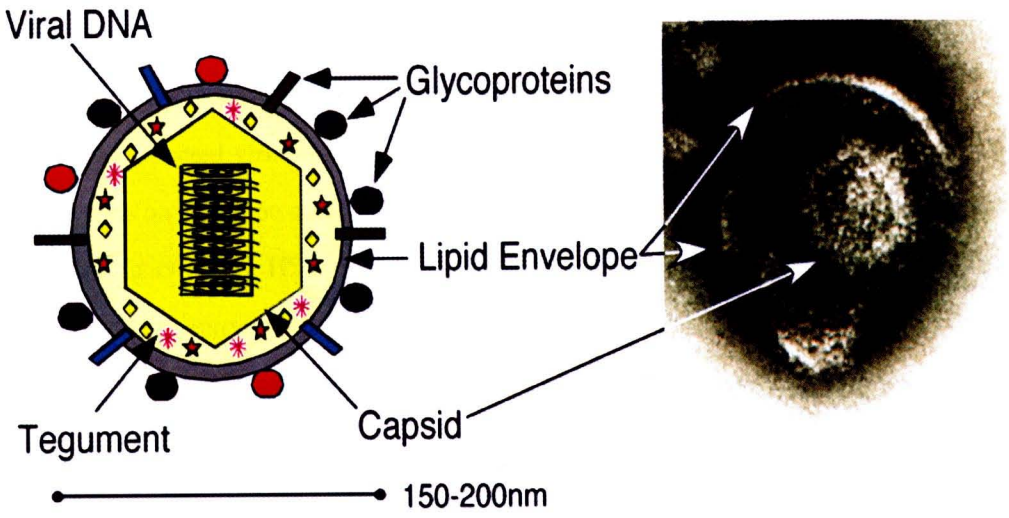


Figure 1. Structure of herpes simplex virus virion (Taylor *et al.*, 2002).

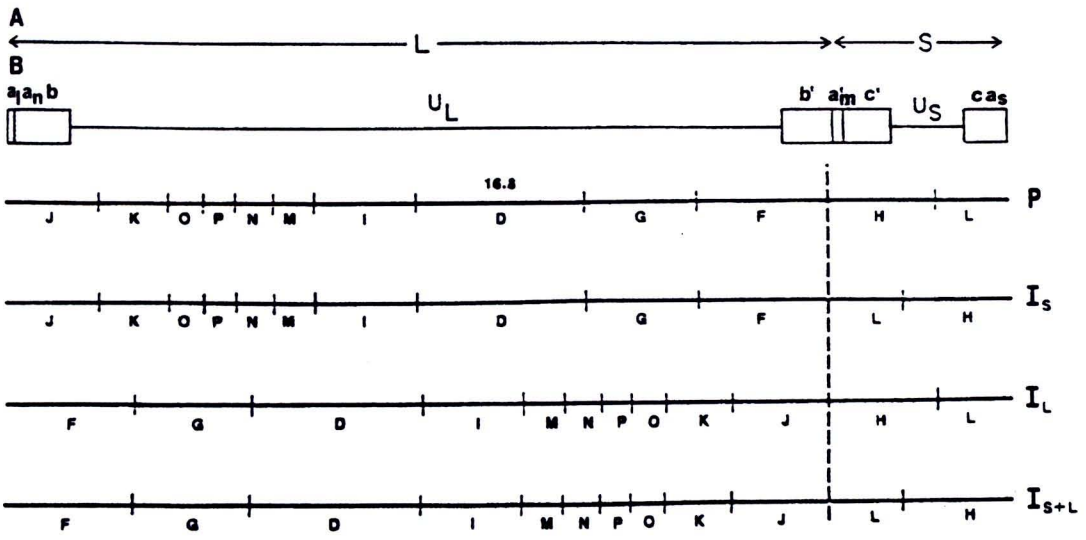


Figure 2. The arrangement of DNA sequences in HSV genome (Riozman and Philip, 2001).

appears to be derived from patches of altered cellular membranes. The presence of lipid was demonstrated by analyzing of virions and by the sensitivity of the virions to lipid solvents and detergents. Early studies on purified HSV virions suggested that they contain more than 30 distinct proteins, which were designated as virion polypeptides (VP). Of the about 30 known and another 10 suspected virion proteins, at least 12 are on the surface of the HSV virion. The viral glycoproteins on envelope surface are gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM, and gN which have many functions in HSV infection (Roizman, 1996; Roizman and Philip, 2001). The gB is required for viral entry and induces neutralizing antibody of host immune response. Glycoprotein C (gC) is involved in cell attachment and plays a role in blocking host immune response to infection. Since it has a C3b complement component receptor, gC can protect infected cells from antibody-dependent cellular cytotoxicity (Friedman *et al.*, 2000; Lubinski *et al.*, 2002). The gD is required for postattachment entry of virus into cells. The gE has an immune escape function by binding with IgG via Fc receptor in the form of gE-gI complex. This Fc receptor can protect both host cell and viruse against immunologic attack by steric hindrance resulting from the binding of normal IgG or from bipolar bridging of HSV antibody which can attach to gE/gI by its Fc end and simultaneously to another HSV glycoprotein by one Fab arm. The gG plays a role in egress and cell-to-cell spread, whereas gH forms complex with gL. This complex is required for fusion of host membranes, and cell-to-cell spread. The gJ is reported to block apoptosis, and gK is important for efficient viral exocytosis. However, the function of gN is still unknown (Roizman and Knipe, 2001).

The HSV genome consists of 152,000 base pairs, with a G+C content of 68.3% for HSV-1 and 69% for HSV-2 (Roizman, 1992). The homology between the two types of HSV genome is about 50%. The genome is composed of two covalently linked segments, designated as Long (L) and Short (S), based upon their relative length (Figure 2). Each segment consists of unique sequences regions (U_L or U_S) flanked by inverted repeat sequence (Wadsworth *et al.*, 1975; Whitley and Roizman, 2001). This large genome allows the virus to encode at least 80 gene products (Taylor *et al.*, 2002). Most of the polypeptides specified by one virus type are antigenically related to the polypeptides of the other type (Corey and Spear, 1986). They are designated as either infected cell specific polypeptide (ICSPs) or infected cell polypeptides (ICPs). The three groups of HSV proteins, α, β, and γ, are synthesized in a sequential order. There are six α-proteins namely ICP0, ICP4, ICP22, ICP27, ICP47, and $U_S1.5$. The synthesis of

α-polypeptides occurs very soon after infection, about 2 to 4 hours postinfection, but some α-proteins continue to be produced throughout the period of infection (Honess and Roizman, 1974; Roizman and Knipe, 2001). The β-polypeptides reach peak rate of synthesis about 5 to 7 hours after infection (Honess and Roizman, 1975), and are generally involved in viral DNA replication. These proteins are divided into two groups: β_1 and β_2. The β_1 proteins occur very early after infection, exemplified by polypeptides ICP6, the large component of the viral ribonucleotide reductase (Huszar and Bacchetti, 1981), and ICP8, the major DNA binding protein (Conley *et al.*, 1981). They are differentiated from α-proteins by their requirement for functional ICP4 protein for their synthesis (Honess and Roizman, 1975). The β_2 proteins are synthesized later; they include the viral thymidine kinase (TK) and DNA polymerase. Most of the β proteins are responsible for viral nucleic acid metabolism and are the main target of antiviral chemotherapy (Whitley, 2001). The appearance of β gene products signals the onset of viral DNA synthesis. The γ-proteins are primarily structural polypeptides, including viral glycoproteins, capsid, and some tegument components. They are divided into two classes: γ_1 and γ_2. The γ_1 polypeptides are synthesized early in the absence of viral DNA replication, while the γ_2 polypeptides occur after the viral DNA replication has initiated. These γ-proteins also act as a major target for host immune response (Silver and Roizman, 1985; Roizman and Knipe, 2001; Taylor *et al.*, 2002). Virulence of HSV that control the course of human HSV infection is composed of invasiveness and neurogrowth. Invasiveness is the capacity of virus to reach a target organ, including nervous system tissues, from the portal of entry. In order to disseminate to the target organ, it is necessary for the virus to multiply at peripheral sites. Neurogrowth is the viral ability to grow in nervous system tissue. Thus, HSV virulence requires at least two distinct sets of viral function. The first comprises viral genes responsible for access and injury of host cells whose destruction is responsible for the disease. The second are viral genes and gene functions that turn off host responses to infection (Whitley and Roizman, 2001).

3. Multiplication of herpes simplex virus

The events of HSV replication (Figure 3) involve several major steps, including entry, viral gene expression, viral DNA synthesis, virion assembly, and egress of progeny virions. The entry of HSV into cell is affected in three stages (Figure 4). The first involves the attachment of the virion to the cell surface. The second step involves the interaction of gD with one of the several cellular receptors. In the last stage, the viral envelope and the plasma membrane fuse to release the capsid-tegument structure into cytoplasm.

The initial virus attachment depends on the interaction of viral envelope gC, and, to a lesser extent gB, with the glycosaminoglycan moieties of cell surface heparin sulfate (WuDunn and Spear, 1989; Herold *et al.*, 1991; Shieh *et al.*, 1992). In addition, gB also binds to cell surface independently of heparin sulfate and allows viral entry into cell (Bender *et al.*, 2005). While this initial step enhances infection (Banfield *et al.*, 1995), it is not an absolute requirement for either entry or viral replication, and cells that do not express heparin sulfate remain permissive of HSV entry at low levels. After the initial step, virion attachment is stabilized by binding to a coreceptor. This sequential step involves the interaction of gD with one of the several cellular molecules that belong to three structurally unrelated molecular families (Spear *et al.*, 2000, 2003, 2006). The first of these coreceptors is a member of the tumor necrosis factor (TNF) receptor family originally called herpesvirus entry mediator (HVEM) but renamed HveA (Montgomery *et al.*, 1996; Whitbeck *et al.*, 1997). HveA is present primarily in lymphoid cells, but also found in other cell types such as lung, liver, and kidney (Kwon *et al.*, 1997). It serves as a receptor for entry of HSV-1 and HSV-2, but not for the related alphaherpes virus (Spear *et al.*, 2006). The second family of HSV coreceptors belongs to the immunoglobulin (Ig) superfamily, including nectin-1 or HveC (Geraghty *et al.*, 1998) and nectin-2 or HveB (Warner *et al.*, 1998). These cellular proteins were shown to act as intercellular adhesion molecules and to be localized at adhesion junctions (Aoki *et al.*, 1997; Lopez *et al.*, 1998; Takahashi *et al.*, 1999; Satoh-Horikawa *et al.*, 2000). Nectin-1 is broadly expressed in cells of epithelial, fibroblast, neural, and hematopoietic cells. It mediates entry of all HSV-1 strains, HSV-2 and also mediates cell-to-cell spread of HSV (Cocchi *et al.*, 2000). Nectin-2 is expressed in gall bladder, kidney, and testis. It acts as entry receptor for HSV-2 selectively, but not for wild-type HSV-1 (Lopez *et al.*, 2000). The third coreceptor is specific sites in heparin sulfate generated by certain isoforms of 3-*O*-sulfotransferases (3-OSTs)

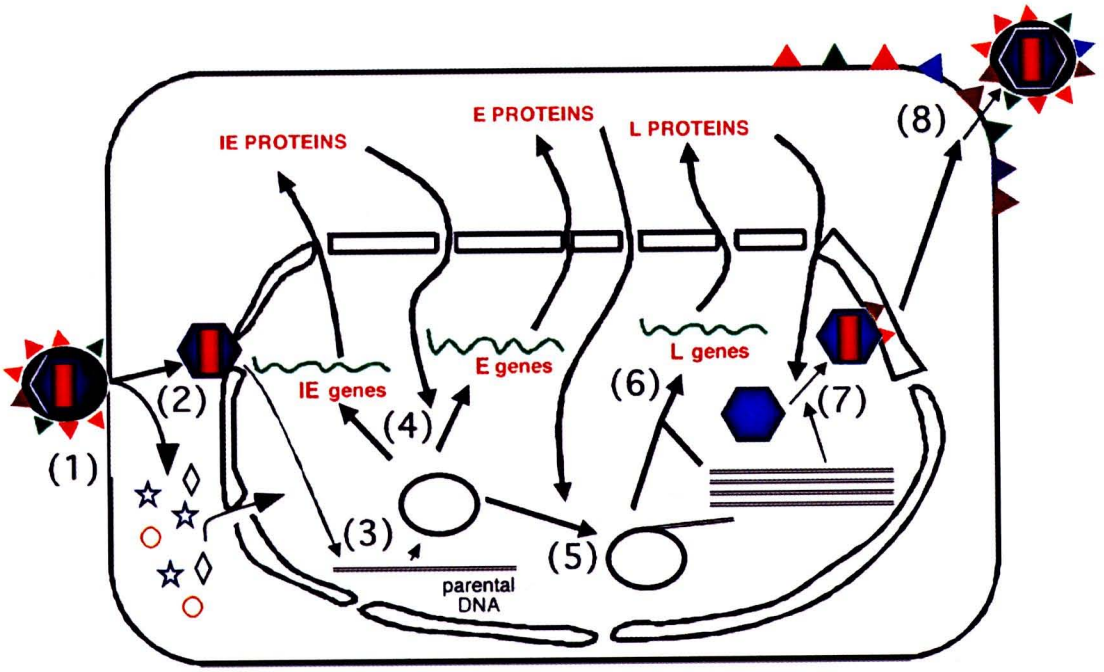


Figure 3. The cycle of productive HSV replication in a cell (Taylor *et al.*, 2002).

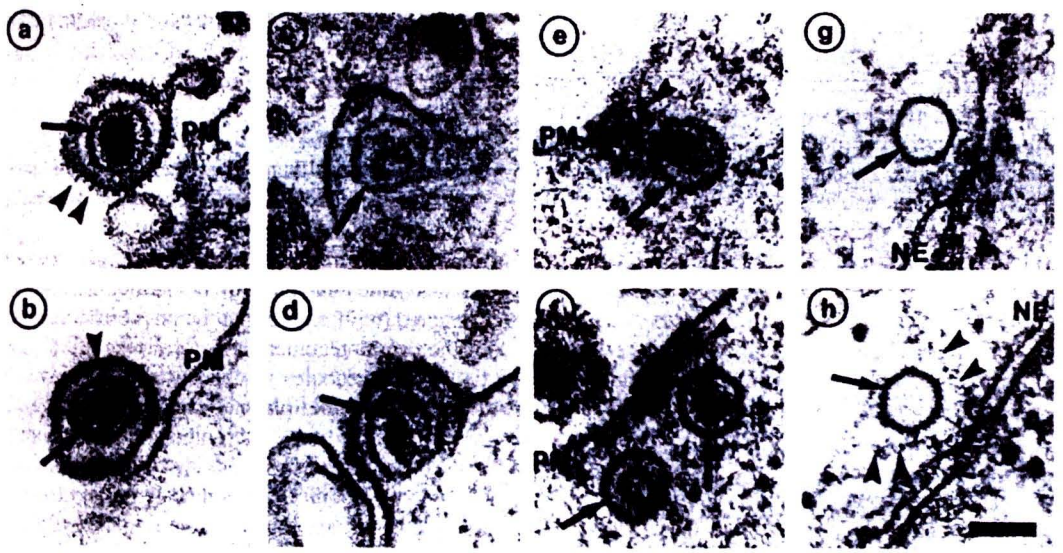


Figure 4. The entry and uncoating of HSV in infected cell. These figure show the stages of viral binding (a and b), fusion (c and d), release of the capsid into the cytosol (e and f), and binding of capsid to the nuclear pore (g and h) (Roizman and Philip, 2001).

called 3-*O*-sulfated heparin sulfates. The 3-*O*-sulfated heparin sulfates generated by 3-OST isoforms 2, 3A, 3B, 4, 5, and 6 can efficiently mediate HSV-1 entry but not HSV-2 entry. These coreceptors are broadly distributed on human tissue, primarily in heart, brain, lung, kidney, liver, skeletal muscle, and placenta (Shukla *et al.*, 1998, 1999; Liu *et al.*, 1999; Shworak *et al.*, 1999; Xia *et al.*, 2002; Tiwari *et al.*, 2004, 2005; Xu *et al.*, 2005; O'donnell, 2006). Moreover, recent work has identified a novel type II cell surface membrane protein, designated B5, which can serve as a receptor for HSV entry into the cells. However, it has not yet been shown that B5 interacts directly with HSV virions or with gD (Perez *et al.*, 2005). The last step in viral entry is the fusion of the HSV envelope with the plasma membrane of the host cell by an undetermined mechanism. Current evidence indicates that virus-cell fusion requires gD (Ligas and Johnson, 1988), gB (Sarmiento, 1979), and gH-gL heterodimer (Forrester *et al.*, 1992; Perez-Romero *et al.*, 2005). Nevertheless, intact virions may also enter via endocytic pathway into some cell types in certain conditions (Nicola and Straus, 2004; Milne *et al.*, 2005; Nicola *et al.*, 2005).

Following the fusion, viral nucleocapsid and tegument proteins are released into cytoplasm of the host cell. The nucleocapsid and some tegument proteins, such as VP16 and VP1-2, are transported through the microtubules network to the nuclear pore (Sodeik *et al.*, 1997) while other tegument proteins remain in the cytoplasm. At the pore, the nucleocapsid releases its DNA into the nucleus, leaving an empty capsid at the cytoplasmic side of the complex.

The bulk of incoming viral DNA circularizes rapidly after infection and in the absence of viral protein synthesis (Garber *et al.*, 1993), and then the transcription of HSV gene begins. Host RNA polymerase II is responsible for synthesis of all viral mRNAs (Alwine *et al.*, 1974; Costanzo *et al.*, 1977). While cellular proteins are sufficient for the synthesis of viral transcripts, viral proteins are sufficient for the synthesis of viral transcripts, viral proteins are necessary for the initiation and enhancement of transcription of certain genes. These proteins act in concert with an abundance of cellular proteins to produce the full range of viral gene products needed for productive viral infection and replication. The first gene transcribed during viral infection are the immediate early (IE) or α genes. Initiation of transcription of these genes proceeds by recruitment of cellular transcriptional machinery to IE gene promoters that contain numerous host regulatory sequences. IE gene expression does not require prior HSV protein synthesis; however, an HSV protein brought in with the virion tegument, VP16, plays an important role in enhancing the expression of the α-proteins (Batterson and Roizman, 1983). The α proteins include several multifunctional proteins that play essential roles in the regulation of later viral gene expression as well as in the control of host cell. Expression of the next set of HSV genes, the early (E) or β genes, requires at least 3 IE proteins, ICP0, ICP4, and ICP27, but this expression is not associated with the onset of viral DNA synthesis (Watson and Clements, 1980; Stow and Stow, 1986; Uprichard and Knipe, 1996). The β-proteins are generally involved in viral DNA replication. These proteins block further synthesis of α proteins and lead to transcription of the third set of viral RNAs (γ genes). Prior to DNA replication, α proteins initiate the transcription of not only the E genes, but also a subset of the late (L) or γ genes, called early-late, leaky-late, or γ_1 genes. The synthesis of these proteins later in the infection cycle is not strictly dependent on viral DNA replication. However, their levels are significantly enhanced upon initiation the initiation of DNA replication. A second subset of late genes, the true late or γ_2 genes, is transcribed only after the initiation of viral DNA replication.

Viral DNA replication occurs in nucleus initiated at the origins of replication within the HSV genome and is believed to proceed initially via theta replication mechanism (Figure 5). Once DNA synthesis begins, it is likely that a rolling-circle replication mechanism takes over to produce concatemeric molecules. Thus, most of the viral progeny DNA molecules that accumulated in the infected cell nucleus are head-to-tail concatemers (Jacob *et al.*, 1979). Seven herpes genes were identified as essential for viral DNA replication (Challberg, 1986).

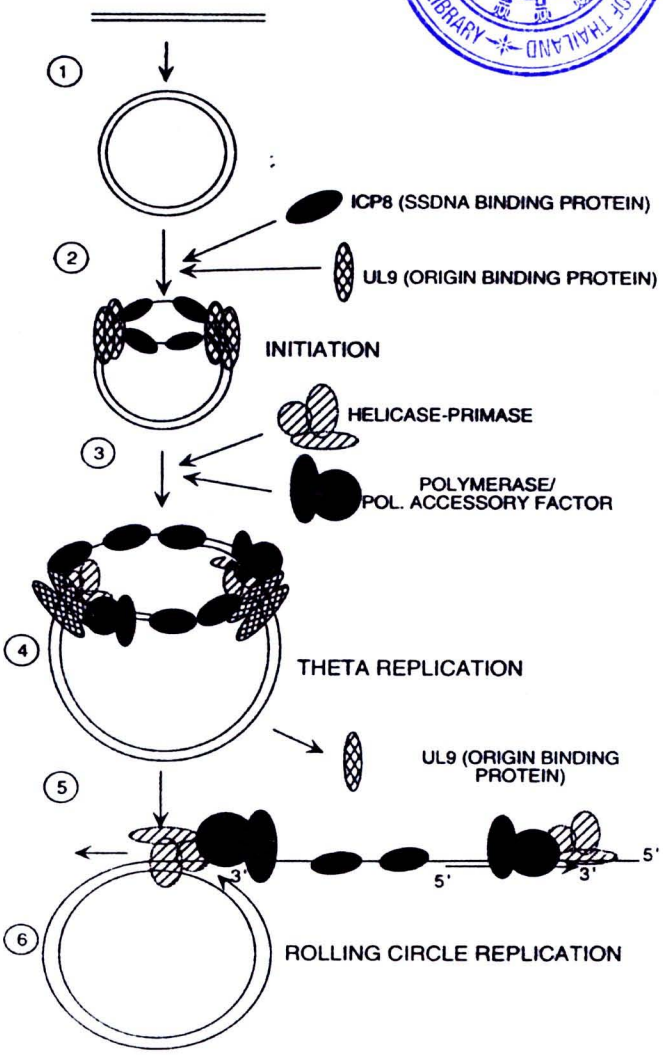


Figure 5. A model of HSV DNA replication (Riozman and Philip, 2001).

These seven genes encode protein products that function as an origin binding protein (U_L9) (Elias *et al.*, 1986), a DNA binding protein ICP8 (U_L29) (Conley *et al.*, 1981), a helicase-primase complex (U_L5, U_L8, U_L52), and a DNA polymerase (U_L30, U_L42) (Purifoy *et al.*, 1977). In addition, HSV expresses several other early viral gene products, such as thymidine kinase (Kit and Dubbs, 1965), ribonucleotide reductase (Bacchetti *et al.*, 1986), and uracil N-glycosylase (Caradonna *et al.*, 1987). These proteins are considered nonessential for viral replication, but are required for nucleotide metabolism and viral DNA synthesis and repair in resting cells, such as neurons.

Assembly of viral capsid requires synthesis of numerous late proteins, and occurs within the nucleus. These capsid proteins, such as VP5, VP19c, VP21, VP22a, VP24, and VP26, are synthesized in cytoplasm and transported into the nucleus. Empty capsid shells are loaded with viral DNA by a process that simultaneously cleaves of HSV progeny DNA concatemers and packs genome-length monomers within the capsid (Ladin *et al.*, 1980, 1982). The mechanism of DNA cleavage and packaging is not well understood; however, it is known to require site-specific breaks to the concatemers at specific distances from the packaging signal (Varmuza and Smiley, 1985; Deiss *et al.*, 1986; Smiley *et al.*, 1990).

After encapsidation of full-length viral genomic DNA molecules, the nucleocapsids are capable of budding through the inner nuclear membrane (Vlazny *et al.*, 1982). Interactions between capsid and tegument proteins and between tegument proteins and viral glycoproteins promote this budding process. It is clear that nucleocapsids acquire some tegument proteins and a glycoprotein studded envelope upon budding through the inner nuclear membrane. From this point, the route for egress of the virion particle from the space between the inner and outer nuclear membranes to the exterior of the infected cell remains controversial. Two general pathways have been hypothesized for virion egress. A re-envelopment pathway suggested that enveloped particles fuse with the outer nuclear membrane; thereby de-envelop the nucleocapsids and release free nucleocapsids into the cytoplasm. These nucleocapsids are re-enveloped by budding into the golgi compartment and the re-enveloped particles are secreted from the cell through secretory vesicles (Siminoff and Menefee, 1966; Stackpole, 1969). A luminal pathway has been proposed in which the enveloped particles traffic from the inner nuclear space through the cytoplasm in the lumen of the endoplasmic reticulum or in vesicles to the golgi where final maturation of the virion glycoprotein occurs. These mature virions are released from the cell by a

normal secretory route (Enquist *et al.*, 1999). However, recent evidences lend considerable support to the re-envelopment model as the major route of virion egress (Granzow *et al.*, 2001; Skepper *et al.*, 2001).

The eclipse period is 5 to 6 hours in monolayer cell cultures, and virus increases exponentially until approximately 17 hours after infection. Each cell has then made 10^4 to 10^5 physical particles, of which about 100 are infectious (Roizman and Knipe, 2001; Taylor *et al.*, 2002).

4. The fate of the infected cells

HSVs require living cell for their replication. The longer the infected cells remain alive, the more progeny viruses are made, and ultimately the more the virus spreads. Cells productively infected with herpesviruses do not survive. Cell death is the result of not only irreparable injury caused by viral replication but also cellular responses to infection. Almost from the beginning of the reproductive cycle, the infected cells undergo major structural and biochemical alterations that ultimately result in their destruction.

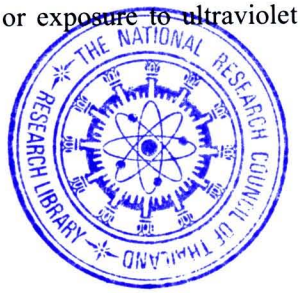
The structural alterations in infected cells occur in many ways. First, the nucleolus becomes enlarged and finally disaggregates. Concurrently, host chromosomes become marginated, and later in infection, the nucleus becomes distorted and multilobed (Roizman and Furlong, 1974). The viruses then induce changes in appearance of cellular membranes. The duplication and folding of intracellular membranes are characteristic of cells in late HSV infection (Morgan *et al.*, 1959; Epstein, 1962; Leestma *et al.*, 1969). HSV also causes a cell-type-dependent fragmentation and dispersal of golgi vesicles throughout the cytoplasm (Campadelli-Fiume *et al.*, 1993). In addition, viral proteins especially glycoproteins are inserted into cellular membranes (Spear and Kellejmroian, 1970; Roizman and Spear, 1971) resulting in alterations of cellular membrane structure and antigenicity (Roizman and Roane, 1961; Roane and Roizman, 1964; Roizman and Spring, 1967). Moreover, polykaryocytosis is another character of HSV infected cells. Both HSV-1 and HSV-2 cause cells to round up and adhere to each other or fuse into polykaryocytes (Hoggan and Roizman, 1959; Ejereito *et al.*, 1968; Ruyechan *et al.*, 1979). Rearrangements of the microtubular network are apparent very early in infection. Thus, the microtubules at the junction of the network with the plasma membrane appear to be disrupted in infected cells (Ward *et al.*, 1998). Finally, the accumulation of viral replication proteins, progeny viral DNA, and nucleocapsid components within nucleus (De Bruyn Kops and Knipe, 1988) may cause the formation of intranuclear inclusion bodies in herpesvirus-infected cells (Schwartz and Roizman, 1969; Smith and Sutherland, 1986). These large eosinophilic intranuclear inclusion bodies are one of the characters of HSV infections that can usually be found both in herpesvirus-infected tissues and in appropriately stained cell cultures (White and Fenner, 1994).

It has been known for many years that HSV shuts down host cell RNA, DNA, and protein synthesis (Roizman and Tognon, 1983) because the virus uses numerous cellular proteins for its own protein synthesis (Taylor *et al.*, 2002). Thus, host DNA synthesis is shut off (Roizman and Roane, 1964), host protein synthesis declines rapidly (Roizman and Borman, 1965; Sydiskis and Roizman, 1966), and glycosylation of host proteins ceases (Smiley *et al.*, 1992). Furthermore, host macromolecular metabolism is altered in HSV infected cells in at least three ways. First, the virion virion-host shut-off (vhs) protein causes the degradation of mRNA present in infected cells early in infection (Kwong *et al.*, 1988; Zelus *et al.*, 1996; Karr and Read, 1999). Next, multiple viral genes are involved to impede cellular transcription (Preston and Newton, 1976) and translation (Wagner and Roizman, 1969) to facilitate the cellular to viral gene transcription and translation. In one example, ICP27 inhibits RNA maturation by redistributing host splicing factors (Sandri-Glodin *et al.*, 1995; Sandri-Goldin and Hibbard, 1996). Thus, HSV regulatory protein ICP24 inhibits host RNA splicing and contributes to the decrease in cellular mRNA levels during infection (Hardwice and Sandri-Goldin, 1994). This effect has little or no effect on viral RNA synthesis because very few viral transcripts are spliced. Another viral protein, ICP22, has been shown to be required for modification of host RNA polymerase II following infection (Spencer *et al.*, 1997), perhaps altering the ability of this complex to transcribe from the genome. Finally, the virus selectively destabilizes (Advani *et al.*, 2000) and degrades (Everett *et al.*, 1994) a variety of cellular proteins, especially those involved in regulation of host cell cycle (Advani *et al.*, 2000; Roizman and Knipe, 2001).

5. Latent infection

One of the hallmarks of all herpesvirus infections is the ability of the virus to establish a latent infection that can last the lifetime of the host. The major site of HSV latent infection is sensory neurons in ganglion tissue, either trigeminal ganglia for HSV-1 or sacral ganglia for HSV-2. After the initial primary infection, generally at an oral or genital mucosal surface, the virus enters nerve ending and is transported retrograde along axon to the neuronal cell body (Cook and Stevens, 1973; Hill, 1981, 1985). During latency, the HSV genome remains in the nucleus of the sensory neuron as circular, extra-chromosomal DNA (Rock and Fraser, 1985;

Mellerick and Fraser, 1987). In neurons latently infected with HSV, no viral progeny is produced and only very limited gene transcription is detected. This viral mRNA, called LATs or Latency Associated Transcripts, may limit viral gene expression (Garber *et al.*, 1997) or protect neurons from apoptosis (Perng *et al.*, 2000). Clearly, HSV in latent infections is not cause lytic infection, as occurs in mucous membranes and still promote neuronal survival during its latency. The virus remains in this state for the lifetime of host, or until the proper signal periodically reactivate the virus and new progeny viruses are generated. Infection virus travels anterogradely to peripheral tissues by axonal transport (Colberg-Poley *et al.*, 1981), usually to cells at or near the site of initial infection (Carton and Kilbourne, 1952; Roizman, 1966). The signal and mechanisms involved in this process are poorly understood, but it appears that host immune status (Roizman and Knipe, 2001) and certain physical stresses, such as illness or exposure to ultraviolet light, increase the chance of reactivation (Taylor *et al.*, 2002).



6. Pathology of herpes simplex virus infection

The pathologic changes induced by the replication of HSV are similar for both primary and recurrent infection but vary in the quantitative extent of cytopathology. These changes represent a combination of virally mediated cellular death and associated inflammatory response because HSV causes cytolytic infections. Lesion induced in the skin and mucous membranes by HSV-1 and HSV-2 are the same and resemble those of varicella zoster virus.

HSV productive infections are lytic, as a result of virus-induced shutdown of host protein and nucleic acid synthesis, and obvious when microscopically detected. The histopathologic changes induced by viral infection include ballooning of infected cells and the appearance of condensed chromatin within the nuclei of cells, followed by subsequent degeneration of the cellular nuclei of cells, followed by subsequent degeneration of the cellular nuclei, generally within parabasal and intermediate cells of the epithelium. Cell fusion which appears as multinucleated giant cells provides an efficient method for cell-to-cell spread for HSV, even in the presence of neutralizing antibody (Brooks *et al.*, 2001).

With cell lyses, clear fluid called vesicular fluid containing large quantities of virus appears between the epidermis and dermal layer. The vesicular fluid contains cell debris, inflammatory cells, and multinucleated giant cells. In dermal substructures, there is an intense inflammatory response, usually in corium of the skin, more so with primary infection than with recurrent infection. With healing, the vesicular fluid becomes pustule with the recruitment of inflammatory cells, and then it scabs. Scarring is uncommon but has been found in some patients with frequently recurrent lesions. Vascular changes in the area of infection include perivascular cuffing and areas of hemorrhagic necrosis. These histopathologic findings become particularly prominent when the infection occurs in organs of the body other than skin, for example HSV encephalitis or disseminated neonatal HSV infection.

HSV is transmitted by contact of a susceptible person with an individual excreting virus. The virus must come in contact with mucosal surfaces or abraded skin for infection to be initiated. The site of primary infection depends on the way in which the patient acquires the virus. It is often noted that HSV-1 usually causes infection at oropharyngeal tract, and is spread by respiratory droplets or by direct contact with infected saliva. HSV-2 is usually sexually transmitted and replicates in the genital, perigenital, or anal skin sites. This reflects the mode of transmission rather than any intrinsic property of virus (Sack *et al.*, 2004).

Once epithelial cells are infected, there is replication of the virus around the lesion and entry into the innervating neuron. Because of infection with HSV-1 generally to oropharynx, initial replication of virus occurs in the oropharyngeal mucosa. The trigeminal ganglion becomes colonized and harbors latent virus. Acquisition of HSV-2 infection is usually the consequence of transmission by genital contact. Virus replicates in genital mucosa with seeding of the sacral ganglion (Bastein *et al.*, 1972; Stevens and Cook, 1974; Stevens, 1975). After the establishment of latency as described above a recurrence of HSV infection is known as reactivated infection or recurrent infection. This form of infection leads to recurrent vesicular lesions of the skin such as herpes labialis or recurrent genital herpes (Baringer and Swoveland, 1973). Reinfection with a different strain of HSV can occur but extremely uncommon in the normal host. It is called exogenous reinfection (Brook *et al.*, 2001).

Primary HSV infections are usually mild. In fact, most are asymptomatic. Only rarely virus can spread beyond the dorsal root ganglia, thereby becoming systemic. Such circumstances include multiorgan disease of pregnancy, disseminated neonatal HSV infection, and disseminated HSV infection in immunocompromised host. It is likely that the widespread organ involvement is a result of viremia in the host incapable of limiting viral replication to mucosal surfaces.

The natural history of HSV infections is influenced by both nonspecific and specific host defense mechanisms (Lopez *et al.*, 1993). It has been reported that host genetic background (Lopez, 1975; Lopez *et al.*, 1981), macropharges (Starr *et al.*, 1976; Schneweis *et al.*, 1982), natural killer cells, specific T-lymphocyte subsets (Kohl *et al.*, 1989), specific antibodies (Eberle and Courtney, 1981; Bernstein *et al.*, 1985; Kahlon *et al.*, 1987), and lymphokine responses (Sheridan *et al.*, 1982; Cunningham and Merigan, 1983) are the important host defenses against HSV infection.

During primary infection, IgM antibodies directed against envelope glycoprotein gB and gD appear transiently and are followed by IgG (Kurtz, 1974) and IgA (Friedman and Kimmel, 1982) that persist over time. The more severe the primary infection or the more frequent the recurrences, the greater the level of antibody response. However, the pattern of antibody response does not correlate with the frequency of recurrent disease. Cell-mediated immunity and nonspecific host defense mechanisms are also important in controlling both primary and recurrent infections of HSV. All these responses may well influence on the acquisition of disease, the severity of infection, and the host resistance to subsequent HSV reactivation. However, spontaneous reactivations can occur in spite of the presence of both host's HSV-specific humoral and cellular immunity. This immunity helps in limiting local viral replication, so that recurrent infections are less extensive and less severe. Accordingly, many recurrences are asymptomatic and detected only by viral shedding in secretions. When symptomatic recurrent infections occur, episodes of recurrent HSV-1 infection are usually manifested as cold sores or fever blisters near the lip. More that 80% of the human population harbors HSV-1 in a latent form, but only a small portion experience recurrences.

Passively transferred maternal antibodies are acquired in many newborns. These antibodies are lost during the first 6 months of life, and the period of greatest susceptibility to primary herpes infection occurs between 6 months and 2 years. Clearly, humoral immunity does

not prevent either recurrences or exogenous reinfection. Thus, transplacentally acquired antibodies from the mother are not totally protective against infection of newborns (Sullender *et al.*, 1987, 1988; Kahlon and Whitley, 1988). HSV-1 antibodies begin to appear in early childhood and are present in most persons by adolescence. Antibodies to HSV-2 rise during the age of sexual activity and adolescence.

7. Epidemiology of herpes simplex virus infection

HSV-1 and HSV-2 occur worldwide, have no seasonal variation, and naturally only infect human beings. HSV spreads principally by close person-to-person contact with lesions or mucosal secretions. Most human beings have been infected and harbor latent virus that can be reactivated; therefore there is a vast HSV reservoir for transmission to susceptible individuals. Although HSV-1 and HSV-2 are usually transmitted by different routes and involve different areas of the body, there is a great deal of overlap between the epidemiology and clinical manifestations of infection. HSV-1 is probably constantly present in humans than any other viruses. Primary HSV-1 infections usually occur in the young child, less than 5 years of age, and most often asymptomatic. With clinical illness, the mouth and lips are the most common sites of this virus infection. Gingivostomatitis usually is the clinical manifestation in young children while pharyngitis is associated with HSV-1 primary infection in young adults.

Many demographic factors influence the acquisition of HSV-1 infection including geographic location, socioeconomic status, and age. In developing countries, seroconversion happens early in life (Bader *et al.*, 1978). By 5 years of age, approximately one third of children seroconverted and this frequency increased to 70% to 80% by early adolescence (Nahmias *et al.*, 1970). In comparison, middle-class and upper-class individuals in more developed countries become infected later. Seroconversion occurs in about 20% of children younger than 5 years; then no substantial rise in incidence happens until an increase to 40% to 60% at age 20-40 years (Wentworth and Alexander 1971). In the United States, race also affects acquisition of HSV-1. By 5 years of age, over 35% of African-American versus 18% of white children is infected with HSV-1. Incidence of infection among university students is about 5% to 10% annually (Nahmias *et al.*, 1990). These studies suggested that the frequency of direct person-to-person contact,

indicative of crowding encountered with lower socioeconomic status, appears to be the major mediator of HSV-1 infection. The largest reservoir of HSV-1 infections in the community is recurrent herpes labialis. The frequency of recurrent HSV-1 infection is approximately 33% in several studies (Friedman *et al.*, 1977; Ship *et al.*, 1977). Recurrent infections may occur in the absence of clinical symptoms, but still have viral shedding. At any given time approximately 1% to 5% of normal adults will be excreting HSV (Sheridan and Hermann, 1971; Hatherley *et al.*, 1980).

Usually, HSV-2 is transmitted by sexual intercourse and results in genital HSV infection, so antibodies to this virus are seldom found before puberty. Although most genital HSV infections are caused by HSV-2, an increasing proportion is attributable to HSV-1 (Wolontis and Jeansson, 1977; Corey *et al.*, 1983). Genital HSV-1 infections are usually less severe and less prone to recur than those caused by HSV-2 (Reeves *et al.*, 1981). HSV-2 seroprevalence rises from about 20% to 30% at age 15-29 years to 35% to 60% at age 60 years (Fleming *et al.*, 1997). Factors that affect the acquisition of HSV-2 infection include sex, race, marital status, number of sexual partners, and place of residence. HSV-2 infection is more frequent in women than men (Rawls *et al.*, 1971; Poste and Howkin, 1972) and in African-Americans than whites. This infection prevalence is higher in cities than in suburbs. Importantly, the number of sexual partners greatly influences the acquisition of HSV infection (Rawls and Gardner, 1972; Rawls *et al.*, 1976) according to the highest prevalence of antibodies against HSV-2 among female prostitutes (75%). As with HSV-1 infection of the mouth, primary and recurrent HSV-2 infection may be symptomatic or asymptomatic. Either situation also provides a reservoir of virus for transmission to susceptible persons. HSV-2 infection tends to recur more often than HSV-1 infection (Whitley and Roizman, 2001).

Maternal infections of genital HSV pose risks to both mother and fetus. Rarely, pregnant women may develop widely disseminate maternal disease after HSV infection (Anderson and Nicholls, 1972; Peacock and Sarubbi, 1983) that led to life-threatening disease, such as hepatitis, thrombocytopenia, leucopenia, and encephalitis. The mortality rate among these pregnant women is greater that 50%. The major risk to the fetus is maternal primary or initial genital HSV infection (Kulhanjian *et al.*, 1992). Primary infection before 20 weeks of gestation has been associated with spontaneous abortion; however, recurrent infection is the most common form of HSV infection during gestation. Neonate infection can occur in uterus (about 5% of infections),

intrapartum (around 80%), or postnatally infection (Whitley, 2001). Transmission of infection to the fetus is most frequently related to the actual shedding of virus from infected maternal secretions in the mother's birth canal at the time of delivery. Estimates of the frequency of cervical shedding of virus among pregnant women vary widely. Nevertheless, the majority of infants (70%) who develop neonatal disease are born to women who do not have a history of genital herpes and are asymptomatic at the time of delivery (Brooks *et al.*, 2001). The rate of occurrences of neonatal HSV infections is approximately 1 in 3,000 to 1 in 5,000 deliveries per year (Nahmias *et al.*, 1989). Neonatal HSV infection is almost symptomatic and frequently lethal from disseminated infection especially CNS infection. Mortality in the absence of therapy exceeds 80% and all of few survivors have neurologic impairment (Whitley *et al.*, 1988).

The clinical manifestations of primary HSV-1 infection range from totally asymptomatic to gingivostomatitis in young child and pharyngitis or tonsillitis in adult (Glezen *et al.*, 1975). Following recovery from primary oropharyngeal infection, the individual retains a chance of suffering from recurrent attacks of herpes labialis, otherwise known as fever blisters or cold sores. Recurrent oralabial lesions are preceded by a prodome of pain, burning, tingling, or itching which generally lasts for 6 hours. Vesicles, normally three to five, appear most commonly on the vermilion border of the lip. These lesions are completely healed after 8 to 10 days. The frequency of recurrence varies among individuals. Other skin HSV-1 infections can occur, such as eczema herpeticum in atopic dermatitis patients, herpes gladiatorium in wrestlers, and herpes whitlow in dentists and nurses.

Primary genital herpes appears as macules and papules followed by vesicles, pustules, and ulcers (Corey *et al.*, 1983) on vulva in female, penis in male, and perianal region in male homosexuals. Systemic complications in men are rare; however, aseptic meningitis and urinary retention are common in women. Recurrent genital herpes appears as three to five vesicles on genital and is characterized by a prodrome and localized irritation. Genital HSV infection is rarely transferred from mother to fetus during pregnancy and causes neonatal herpes. This disease can result in, skin, eye, or mouth infections, encephalitis with or without skin infection, or disseminated disease involving multiple organs (Whitley *et al.*, 1981).

Herpetic keratoconjunctivitis is HSV-1 infection of eye beyond the newborn age (Binder, 1977). Primary infection is associated with either unilateral or bilateral conjunctivitis, while

recurrent infection is usually unilateral. The clinical symptom includes photophobia, tearing, and eyelid edema accompanied by dendritic lesions. Geographic ulcers of the cornea develop with advanced disease and lead to blindness from repeat HSV infection.

Herpes encephalitis is a rare manifestation of HSV, usually type 1, infection; nevertheless, the virus is thought to be the most common cause of sporadic fatal encephalitis. The temporal lobes are principally involved and mortality rate in untreated cases is over 70%. HSV can also affect almost all areas of the nervous system, causing significantly meningitis, myelitis, and radiculitis (Whitley and Roizman, 2001). Moreover, patients compromised by immunotherapy, underlying disease, or malnutrition are at increased risk for potentially lethal disseminated HSV infection. These patients can develop progressive disease involving respiratory tract, commonly esophagus, or gastrointestinal tract.

8. *In vitro* methods for antiviral test

The methodology used in the determination of the antiviral activity as well as the interpretation of the results have been virtually specific to each laboratory and are consequently not comparable to one another, so simple procedures and guidelines for evaluation of antiviral or virucidal activity of compounds are urgently needed. This is a series of experimental assays through which the anti-HSV activity of candidate antiviral compound may be evaluated *in vitro*, the potency of the compound can be assessed, and the antiviral mechanism of the candidate substance may be investigated.

HSV grows well in a wide variety of cell types to yield high titers of virus stocks. Cell lines routinely used to grow HSV and test for the anti-HSV activity of compounds include BHK (baby hamster kidney cell line), RK13 (rabbit kidney cell line), Vero (monkey kidney cell line), and CV1 (monkey kidney cell line) (Harland and Brown, 1997). Antiviral compounds may block HSV replication cycle at different stages (Figure 6), thus virus replication in cell cultures may also be monitored by the detection of viral products, for example viral DNA, RNA, or glycoproteins.

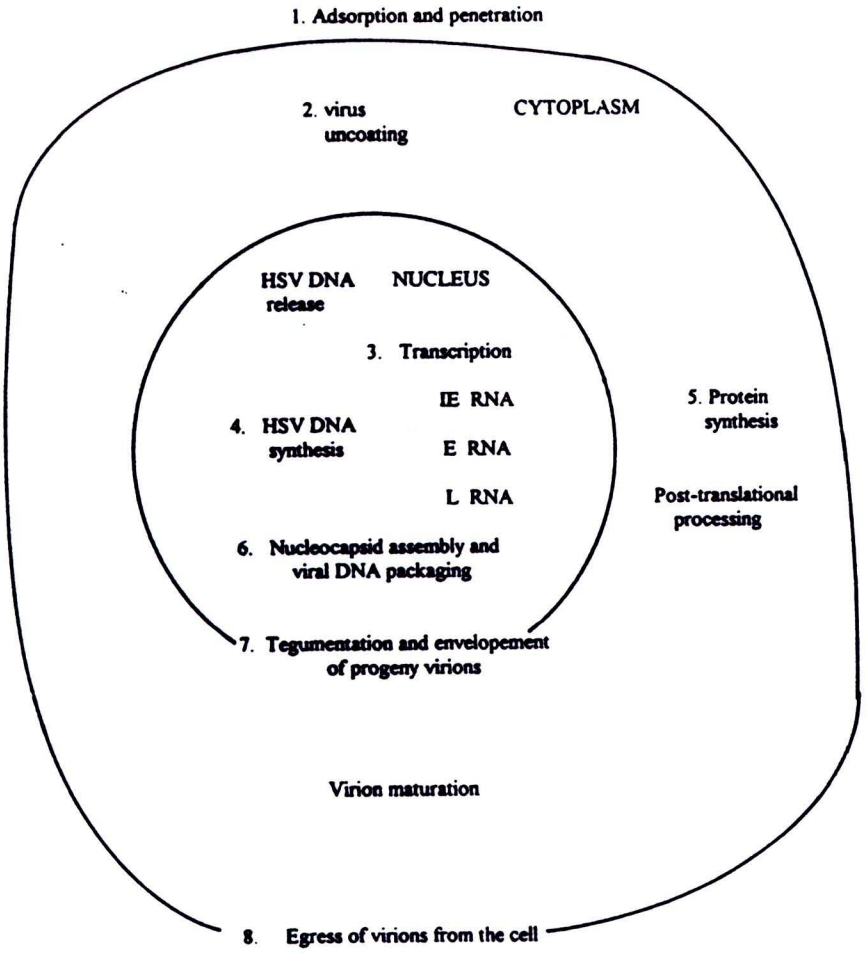


Figure 6. Stages in the HSV replication cycle that might be blocked by antiviral agents (Dargan, 1998).

In order to study the antiviral activity of a new drug, it is important to determine whether antiviral activity can be uncoupled from the effect of cellular toxicity. Cytotoxicity tests define the upper limit concentration of candidate compounds which can be used in subsequent antiviral tests. The simplest cytotoxicity tests *in vitro* are the vital-staining techniques. These methods are easy to perform, need little equipment, and give reliable results (Galt *et al.*, 1990; Sydiskis *et al.*, 1991). In these tests, cells are treated with trypan blue or neutral red dyes. Trypan blue is excluded by live cells, but stains dead cells blue (Anderson *et al.*, 2003; Washman *et al.*, 2003). In contrast, neutral red is taken up by live cells, staining them a brownish-red color, whereas dead cells remain colorless. In addition to vital-staining test, cell viability can be confirmed by additional experiment, such as measuring incorporation of ^{3}H thymidine into cellular DNA, and measuring cell ability to cleave tetrazolium salt by mitochondrial enzymes to give a color product (Konsula and Bariel, 2005). Absence of cytotoxicity in *in vitro* test does not necessarily mean that test compound does not have toxicity *in vivo*. Similarly, a moderate level of cytotoxicity in *in vitro* test may not necessarily exclude *in vivo* use of the compound.

The method most commonly used for evaluation of *in vitro* antiviral activities is based on the different abilities of viruses to replicate in culture cells in which test substances appeared and determine ED_{50}. The ED_{50} is the effective concentration or dose of test compounds that eliminates 50% of the viral infectivity. As previously mentioned, HSV can cause cytopathic effects (CPE) or form plaques in cell cultures, thus ED_{50} is the dose or concentration of the test compound which inhibited CPE, reduced or inhibited plaque formation, and reduced virus yield or other viral functions by 50% (Vlietinck and Berghe, 1991).

Generally, plaque reduction or inhibition assay is considered as a reference or standard for antiviral assay (Ellis *et al.*, 1987). This assay uses a constant number of viral particles and varying the non-toxic concentrations of test substance (Abou-Karam and Shier, 1990; Liu *et al.*, 2004). Typically, a monolayer of cultured cells is allowed to bind virus and then overlaid with a layer of semisolid medium which prevents spreading of virus from the area of originally infected cells. The test substance can be added into cell monolayer before or after virus adsorption is accomplished. The infected cultures are incubated further for an appropriate period of time, then they are fixed, stained with dye, and plaques (the areas of infected cells) are counted. By

reference to the number of plaques observed in virus control or untreated culture, ED_{50} is calculated and expressed.

Histochemical staining for plaque assay using a cell line, Vero ICP6LacZ#7, that expresses β-galactosidase activity was established (Tebas *et al.*, 1995). Antiviral compound was added into HSV-infected cell monolayers, followed by pooled human immunoglobulin which limited the spread of virus to the surrounding cells. Two days later, the cell monolayers were stained for β-galactosidase activity. The plaques appear blue against a clear background of unstained, uninfected cells. The procedure of the CPE inhibition assay is similar to the plaque inhibition assay except the semi-solid substance is not included in the culture medium. The infectivity of virus could be examined by microscopic observation of characteristics of viral CPE (Yip *et al.*, 1991) or by the dye uptake assay (Marchetti *et al.*, 1996). The therapeutic index (TI) can be computed by the ratio of the maximum drug concentration at which 50% of the growth of normal cells is inhibited to the minimum drug concentration at which 50% of virus is inhibited.

Another method in measuring antiviral activity is virus yield inhibition assay (Yoosook *et al.*, 1999; Tenser *et al.*, 2001; Washman *et al.*, 2003). In this method cell monolayer is infected with virus, and increasing concentrations of test compound are added after virus adsorption. Following a cycle of viral replication, the harvested cell cultures are disrupted by three cycles of freeze-thaw, supernatants are kept, and virus yields are determined by plaque assay. Plotting the diminishing infectious virus yields provides drug dose-response curves that yield more information than can be obtained from EC_{50} value alone.

The major goal in the investigation of the antiviral mechanism is to identify the virus gene product that is the target of test compound. According to the concept of this assay, it its first necessary to identify and isolate drug resistant virus mutants from treated cultures by various methods, including single round selection, mapping the drug resistance gene, or DNA sequencing. However, if no drug-resistant variant can be isolated, it will be important to investigate the point in the virus replication at which drug-sensitive function operates.

Virucidal assay is the method to examine the elimination of infectivity when virus particles are mixed with increasing concentration of test compound in solution. Viral titers are obtained from the titration of this mixture solution in cell monolayer. Virucidal activity may be caused by disintegration of the entire virus particle, solubilization of the herpersvirus envelope, or

the chemical modification, degradation, or masking of some essential envelope proteins. HSV particles treated with a virucidal agent are blocked at adsorption and/or penetration, the earliest stage of the virus replication cycle.

To investigate the stage of HSV adsorption to target cells, two techniques have been used. The first technique indirectly measures virus adsorption by quantifying the rate of decrease in infectivity of the inoculum applied to cell monolayers (Hayashi *et al.*, 1992). The second technique quantified virus particles bound to cell surfaces by measuring accumulation rate of the radioactivity associated with ^{35}S-methionine labeled virus particles. Both techniques should be performed at 4^{o}C, since at this temperature HSV particles can bind to the receptors at cell surfaces but cannot penetrate into target cells.

Penetration assay is the test to investigate the effect of test compound on the rate of HSV entry into target cells (Rosenthal *et al.*, 1984). This assay depends on the observation that HSV binds to cells at 4^{o}C but does not penetrate until the temperature is raised. After virus attachment at 4^{o}C, the medium containing test compound is added to cell monolayer and then temperature is shift to 37^{o}C to allow virus penetration. Virus particles that have not yet penetrated the cells are inactivated, at various times after temperature upshift, by treating the infected cell cultures with low pH solution. The infectivity that becomes resistant to low pH inactivation with time is the rate of virus penetration.

The assays to examine the stage of HSV replication cycle inhibited by test compound at a point subsequent to virion entry include electron microscope studies, which show morphological changes during viral replication, or single step HSV growth experiments in which a single antiviral concentration of test substance is added to infected cell cultures at progressively later times throughout the virus replication cycle. If the target protein of antiviral agents has function in early stage of infection, late addition of test compound will have no inhibitory effect on infectious virus yield. On the other hand, if test compound inhibits protein function that is required in late infection, or throughout the replication cycle, the viral infectivity will decrease whenever the drug is present.

Viral DNA synthesis detection is the assay that determines the effect of test compound on HSV-DNA synthesis. In the past, HSV-DNA synthesis has been studied by separation of ^{3}H-thymidine labeled infected-cell DNA into viral and cellular fractions by cesium chloride gradient

centrifugation (Dargen and Subak-Sharpe, 1986). More recently, this technique has been substituted by a dot-blot, Southern blotting method (Kuo *et al.*, 2001; Evers *et al.*, 2004). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is another technique that can be used to investigate the effect of antiviral compound on viral protein synthesis and post-translational processing. The inhibition in HSV replication is usually reflected in changes in the quantity or the apparent molecular weights of HSV specific polypeptide bands on SDS-PAGE gels (Wachsman *et al.*, 2003). These reductions may resulte from test compounds that inhibit viral gene transcription, viral mRNA translation, or reduce viral mRNA or protein products stability (Dargan, 1997). In addition, other antiviral assays based on measurement of specialized function and viral products have been studied using recently developed methods such as flow cytometric analysis (Pavic *et al.*, 1997), nucleic acid hybridization (Lin *et al.*, 2000), and enzyme-linked immunosorbent assay (ELISA) (Anderson *et al.*, 2003).

9. Antiherpes virus agents

There are three categories of anti-herpesvirus drugs in current clinical use (Andrei *et al.*, 1995). The first category is pyrophosphate analogues such as phosphoacetic acid (PAA) and phosphonoformic acid (PFA), known as foscarnet or trisodium phosphonoformate. These drugs act as a direct inhibitor of viral DNA polymerase by reversibly blocking the pyrophosphate binding site of viral polymerases (Oberg, 1989; Chrisp and Clissold, 1991). This binding site involves in releasing the pyrophosphate product of DNA synthesis during DNA polymerization process. PFA inhibits this cleavage of pyrophosphate groups from the deoxynucleoside triphosphates (dNTPs), a crucial step in DNA chain elongation, thus PFA inactivates virus by interfering with the elongation of the viral DNA chain. According to very poor absorption after oral administration, foscarnet used in the form of cream has been reported to accelerate healing of recurrent facial and genital herpes lesion, and given systematically it can halt the progression of HSV, varicella zoster virus (VZV) and cytomegalovirus (CMV) infection. However, the most common side effect of foscarnet is renal toxicity, associated with tubular intestinal lesions (Snoeck, 2000).

The second category is the nucleoside analogues that can be divided into two groups. One is a variety of purine nucleoside analogues including acyclovir (ACV), valacyclovir (VACV), penciclovir (PCV), and famcyclovir (FCV). Antiviral activity and selectivity of drugs in this group are based on their specific activation by herpesvirus-encoded thymidine kinase, which converts these drugs intracellularly to their monophosphate metabolites. Viral thymidine kinase has broader specificity than cellular thymidine kinase which unable to phosphorylate these compounds, therefore these drugs are non-toxic to uninfected cells. After initial conversion of the purine nucleoside analogues to their monophosphate, then further activation to diphosphate and triphosphate metabolites are catalysed by cellular enzymes, such as kinases, involved in nucleotide metabolism. These drugs in triphosphate forms inhibit the DNA polymerase reaction (Naesens and Clercp, 2000). Valacyclovir is the L-valyl ester of acyclovir that was developed as an oral prodrug of ACV to increase oral bioavailability and provide higher plasma ACV levels (Beutner *et al.*, 1995). VACV is rapidly and extensively metabolized to ACV and L-valine after oral administration. Penciclovir is also a guanine analogue that is used topically as a cream to treat cold sores. Famciclovir is the diacetyl ester of 6-deoxy-penciclovir, an oral prodrug of PCV.

FCV was synthesized to improve oral absorption of PCV because FCV is rapidly and extensively adsorbed in the upper intestine. After adsorption, FCV undergoes substantial first pass metabolism via deacetylation and oxidation in liver to yield PCV (Vere Hodge, 1993; Perry and Wagstaff, 1995). Another group comprises pyrimidine nucleoside analogues, for instances idoxuridine (IDU), trifluridine (TFT), and brivudin (BVDU). Similar to purine nucleoside analogues, drugs in this group require specific phosphorylation to their monophosphte forms by viral enzyme; however, the monophosphate is converted to the diphosphate form by HSV or VZV encoded thymidine kinase (Clercq, 2004). Upon further phosphorylation by cellular kinases, nucleoside triphosphate can then interact with viral DNA polymerase, either as competitive inhibitor with natural substrate or as an alternative substrate incorporated into growing viral DNA chain and affect both the stability and functioning of the DNA. Idoxuridine and trifluridine in the form of eye drops or ophthalmic cream have been used to treat HSV keratitis (Clercq, 2005), while both oral and topical administration of brivudin can be used as a therapeutic agent in herpes labialis, herpes keratitis, and herpes zoster (Clercq, 2004).

The third class consists of those drugs that are independent from viral thymidine kinase for their activation. These drugs include the acyclic nucleoside phophonates, such as cidoforvir (HPMPC; 3-hydroxy-3-phosphonylmethoxypropyl-cytosine). Cidoforvir (CDV) has a potent and broad spectrum anti-DNA virus activity that includes all human herpesviruses (Naesens *et al.*, 1997). CDV targets the viral DNA polymerase. After intracellular phosphorylation by cellular kinase to their monophosphate and diphosphate derivatives, acyclic nucleoside analogues incorporate at the 3'-end of the viral DNA chain and act as chain terminators or competitive inhibitors of further DNA chain elongation (Neyts *et al.*, 1994).

Acyclovir (ACV), 9-(2-hydroxyethoxymethyl) guanine or acycloguanosine, has been a drug of choice for the prophylaxis and treatment of HSV infections for many years. This acyclic guanosine analogue is a selective inhibitor of HSV-1, HSV-2, and VZV DNA replication with low host cell toxicity. Following uptake of ACV by virus-infected cells, the first phosphorylation of the drug to ACV-monophosphate occurs via HSV-encoded thymidine kinase. Subsequent conversion to ACV-diphosphate and ACV-triphosphate is catalysed by host cell enzymes. ACV-triphosphate is a potent inhibitor of HSV DNA polymerase and is a competitive inhibitor of cellular deoxyguanosine triphosphate resulting in viral enzyme inactivation. The inactivation of viral DNA polymerase occurs due to lack of the 3'-hydroxy group required for subsequent 5' to

3'phosphodiester linkages to elongate viral DNA chain; thereby, DNA chain termination appears once the drug enters the viral DNA (Figure 7).

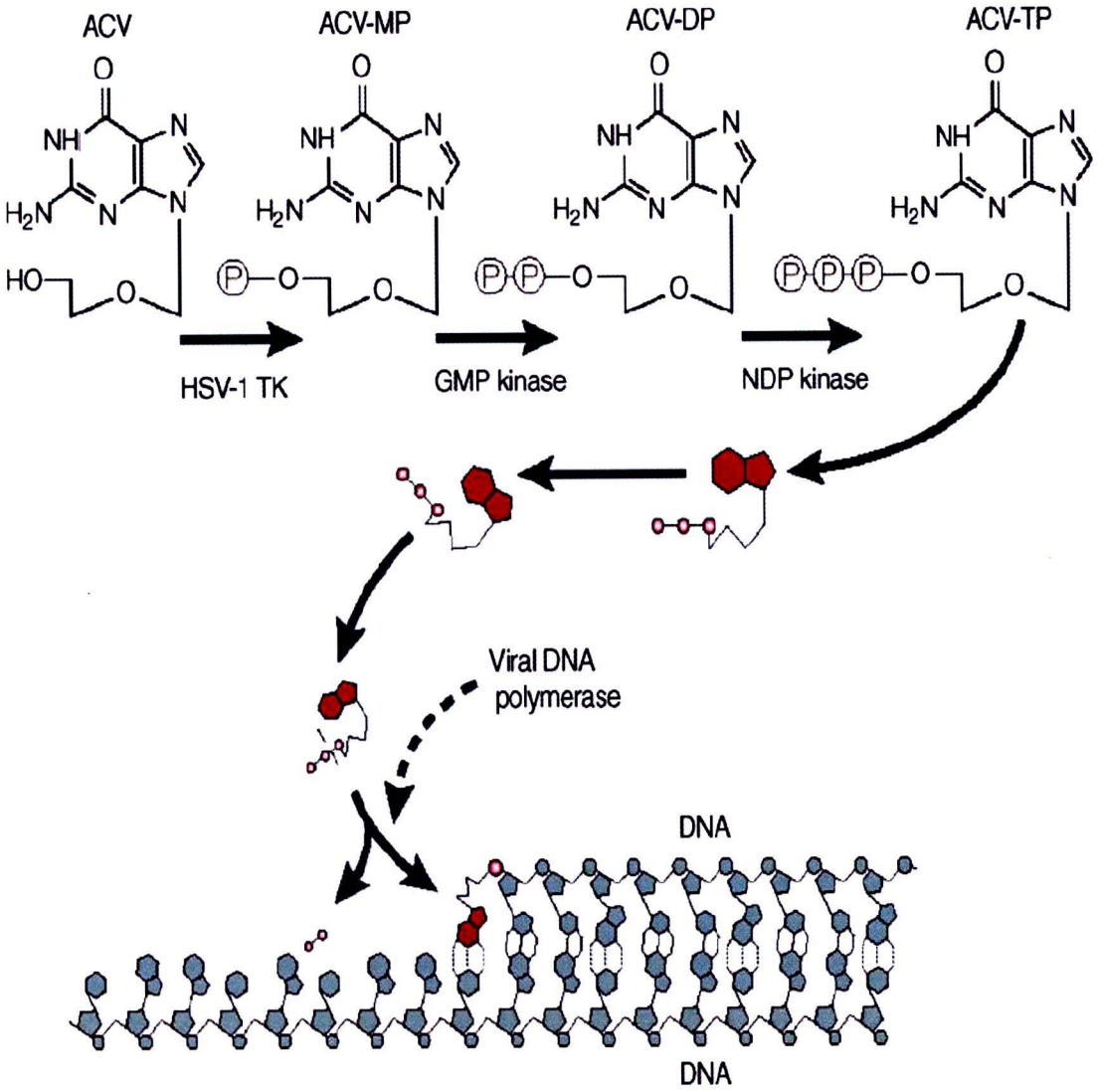


Figure 7. Mchanism of antitviral action of acyclovir (ACV) (Clercq, 2004).

ACV is clinically used in the treatment of mucosal, cutaneous, and systemic HSV-1 and HSV-2 infection and VZV infection, and may be administered orally, topically as an aqueous cream, or by slow intravenous infusion. Nevertheless, this widespread use of ACV has led to the emergence of HSV strains resistant to ACV. ACV resistant mutants of HSV can be recovered from both *in vivo* and in cell cultures. The first case of clinical viral resistant strains was published in 1982 (Crumpacker *et al.*, 1982; Silbrack *et al.*, 1982) and the incidence had continued increasing mainly among immunocompromised patients and particularly allogenic bone marrow transplant patients of which the prevalence reaches 5% and 30%, respectively (Morfin and Thouvenot, 2003). The mutation is most frequently located in the viral gene encoding thymidine kinase, but more rarely occurs in viral DNA polymerase gene. There are three kinds of ACV-resistant mutants. Firstly, thymidine kinase deficient virus is the mutants that lose thymidine kinase activity or fail to produce this enzyme, and 95% of ACV-resistant isolates from patients represent a thymidine kinase deficient phenotype (Hill *et al.*, 1991). Secondly, the group of mutant that can produce the enzyme but has altered thymidine kinase substrate specificity by point mutation is called thymidine kinase altered virus. Thirdly, some viruses mutate by alteration of DNA polymerase activity (Larder *et al.*, 1983). These mutations leading to resistance occur spontaneously during viral replication and resistant viruses are then selected by antiviral treatment. Management of drug-resistant HSV infections depends on the mechanism of resistance of mutant strains. Foscarnet and cidoforvir that directly act on viral DNA polymerase without requiring activation by viral thymidine kinase are still active against viruses resistant to ACV because of a mutation in their thymidine kinase gene; however, they may be associated with a significant level of toxicity in clinical practice (Morfin and Thouvenot, 2003).

Vaccination remains the ideal method for prevention of HSV infection; nevertheless, prevention of HSV infections has unique problems because of its recurrences even in the presence of humoral immunity. However, protection from life-threatening infection can be achieved in animal models with avirulent, inactivated, or subunit glycoprotein vaccines. The first vaccine developed was killed whole-virus HSV-1 and HSV-2 vaccines which were licensed in Germany (Allen and Rapp, 1982). Nevertheless, the results from many clinical studies in the efficacy of these vaccines differ widely. Inactivated virus vaccines may benefit some patients with recurrent infection, but long-term benefit could not be established, since there are potential risks of inactivated vaccine especially as a cofactor in the development of cervical carcinoma. These were

followed by a partially purified HSV-1 vaccine, glycoprotein HSV-1 and HSV-2 subunit vaccines, and recombinant glycoprotein vaccines. Some recombinant glycoprotein vaccines appear highly immunogenic and now a few of them are being tested in clinical trails, particularly recombinant HSV-2 vaccine. One of these is a recombinant HSV-2 glycoprotein D vaccine (Straus *et al.*, 1993) and another is glycoprotein B and glycoprotein D recombinant vaccine (Stanberry *et al.*, 1987). However, to demonstrate high antibody titer and protective effects against HSV-2 recurrent infection, these two vaccines require efficient adjuvant, such as complete Freund's adjuvant, lipophilic muramyl tripeptide (Bernstein *et al.*, 2005), or immune enhancer (Quenelle *et al.*, 2006). In addition, these vaccines did not provide 100% protection in all patient groups (Corey *et al.*, 1999), especially in men (Spruance, 2000), even in the presence of adjuvant. Other two promising HSV vaccines are genetically engineered, live-attenuated HSV-deletion mutant vaccine and glycoprotein H deletion mutant vaccine. The former was made by carefully selected gene deletions to remove putative neurovirulence sequences in HSV gene. The latter, known as the HSV-DISC vaccine, is a vaccine deleted of an essential glycoprotein H, thus allowing only a single cycle of infectious HSV replication, and has been studied in phase I clinical trail in the United Kingdom (Markert *et al.*, 2000).

10. Antiherpes virus activity of natural substances

There are a number of natural options available for the prevention and treatment of herpes simplex virus infection. Natural remedies that show promises either for prophylaxis or treatment include lysine, vitamin C, vitamin E, zinc, adenosine monophosphate, glutathione, copper, and medicinal plants. These substances have different antiviral mechanisms, and some of them also have biological functions in human such as amino acid, vitamin, trace element, and antioxidant.

Various kinds of essential oils from medicinal plants have a long history of use. They have been used widely in both developing and developed countries for the treatment of various human diseases such as respiratory infection, asthma, atopic dermatitis, allergic rhinitis, and gastrointestinal disease (Buckle, 1997). These uses of medicinal plants are gaining popularity because of several advantages, including often fewer side effects, better patient tolerance, local

availability, relative acceptance due to long-time use, and less prone to the emergence of drug resistance strains. It has been reported that essential oils show not only anti-bacterial and anti-fungal activities but also anti-viral activity. Sandalwood oil, the essential oil of *Santalum album*, has antiviral activity against HSV-1 and HSV-2 virions, thus preventing adsorption of virions to host cells and inhibition of cell-to-cell virus spread *in vitro* (De Logu *et al.*, 2000). Other essential oils from medicinal herbs that completely inhibited HSV replication include *Cupressus sempervirens* (cypress), *Juniper communis* (juniper), *Melaleuca alternifolia* (tea tree), *Eucalyptus globulus* (eucalyptus) (Schnitzler *et al.*, 2001), *Ocimum basilicum album* (tropical basil), *Mentha piperita* (peppermint), *Origanum majorana* (majoram), *Ravensara aromatica* (ravensara), *Lavandula latifolia* (Lavender), *Citrus limon* (lemon), *Rosmarinus officinalis* (rosemary), and *Cymbopogon citratus* (lemongrass). These essential oils, especially lemongrass oil, possessed strong activity according to its complete inhibition of HSV-1 growth at a concentration of 0.1% *in vitro*, inactivation against viral particles by interaction with the virions, and binding to viral envelopes or glycoproteins (Minami *et al.*, 2003). In clinical use, there are two medicinal plant extract preparations used as topical treatment for HSV infection. First, the extract of the leaves of *Melissa officinalis* or lemon balm is used as a lemon balm cream containing 1% extract of the leaves. Lemon balm cream can reduce the symptoms (Koytchev *et al.*, 1999) and promote rapid lesion healing (Wolbling and Leonhardt, 1994). Second, leaf extract of *Clinacantus nutans* Lindau displayed anti-HSV-1 anti-HSV-2, and anti-inflammatory activity (Lipipun *et al.*, 2003). The cream containing 40 mg of the extract of *Clinacantus nutans* Lindau commercially produced by Government Pharmaceutical Organization (GPO) could effectively treat HSV lesions in herpes labialis patients (เกศริน บุษรานนท์, 2550).

Lysine, an essential amino acid, has been shown to inhibit normal replication of HSV (Betsy *et al.*, 2005), block the binding of HSV-1 by interfering with cellular receptor function (Langeland *et al.*, 1988), and antagonize the growth-promoting action of arginine on HSV replication in tissue cultures (Griffith *et al.*, 1981). The proteins synthesized by HSV normally contain more arginine than those synthesized by host cells (Kagan, 1974), and arginine is required for HSV replication. Lysine appears to antagonize arginine by several mechanisms. For examples, lysine acts as an antimetabolite of arginine, increases arginine excretion by competing with arginine reabsorption at renal tubule, competes with arginine absorption in the intestine, induces arginine degradation via enzyme arginase acrivation, and competes with arginine transportation

into cells (Miller and Foulke, 1984). Treatment with oral and topical lysine preparations in patients with orofacial or genital herpes could shorten the course and duration of the disease, reduce the severity of lesions, and reduce the frequency of recurrences.

Zinc ions at concentration of 0.1 mM have been reported to almost completely inhibit the replication of HSV-1 and HSV-2 *in vitro* (Gupta and Rapp, 1976). The inhibition appeared to result from the selective inhibition of viral DNA polymerase (Gordon *et al.*, 1975; Fridlender *et al.*, 1978). Topical application of various zinc preparations has been shown to be effective in the treatment of cutaneous human HSV infections. Most of the studies using zinc sulfate (Brody, 1981; Finnerty, 1986) or zinc monoglycerolate (Apisariyakulm *et al.*, 1990) indicated that topically applied zinc could shorten the duration of HSV skin infection and possibly prevent both spontaneous and sunlight-induced recurrent infections with a few adverse effects such as irritation, unpleasant dryness, or nausea (Godfrey *et al.*, 2001). Besides topical zinc treatment, oral zinc supplement also could reduce the duration and severity of HSV infection (Jones, 1979) and might prophylaxis recurrences induced by sun exposure (Fitzherbert, 1979); however, long-term zinc supplementation should be accompanied by a copper supplement in order to prevent zinc-induced copper deficiency (Fosmire, 1990).

Copper or cupric ions have been shown to inactivate several types of viruses, including members of *Herpesvirus* (Sagripanti, 1992) and completely inhibit HSV plaque formation when combined with reducing agents such as ascorbic acid (Sagripanti *et al.*, 1997). The killing of HSV by copper followed a pattern in which Cu (II) ions bound with high affinity to viral DNA favoring guanosine residues (Sagripanti and Kraemer, 1989; Sagripanti, 1991). This binding produced oxidative base damage and gave the products including single and double DNA strand breakages as well as base modifications, mainly 8-OH-deoxyguanosine, and free radicals (Toyokuni and Sagripanti, 1996).

Vitamin C or ascorbic acid is an important antioxidant and has been shown to inactivate a wide range of viruses both *in vitro* and *in vivo* including HSV (White *et al.*, 1986; Betanzos-Cabrera *et al.*, 2004). There are numerous studies that reported the effect of oral or topical ascorbic acid treatment on HSV infection in both healthy and immunocompromised patients. These results suggested that vitamin C accelerated the healing of HSV lesions (Klenner, 1940), reduced the mean time until remission of symptoms (Terezhalmy *et al*, 1978) and severity of

symptom, and significally reduced HSV yield after the first day of treatment (Hovi *et al.*, 1995). The vitamin C treatment was most effective when initiated drug during the prodromal period and the antiviral effect of ascorbic acid was more pronounced at higher dose especially in treatment of an acute infection (Betsy, 2006).

Vitamin E or α-tocopherol, a lipid soluble antioxidant, protects cell membranes from oxygen free radical causing damage. Like other antioxidants, vitamin E has been reported to enhance the healing of wounds (Slater and Block, 1991; Martin, 1996). In various *in vivo* (Martin, 1995) and clinical studies, topical application of vitamin E oil (Starasoler and Haber *et al.*, 1978) or capsule (Fink and Fink., 1980) relieved pain, aided in the rapidly healing of oral herpetic lesions, reduced lesion development and severity of symptoms when compared to control (Sheridan *et al*, 1997)

Resveratrol (3, 5, 4'-trihydroxystilbene) is a non-flavonoid phenol compound produced naturally by some spermatophytes, such as grapes, in response to injury or fungal attack. This compound has antioxidant (Stivala *et al.*, 2001), anti-cancer (Jang *et al.*, 1997), antimicrobial (Jeandet *et al.*, 1995), and anti-HSV activities. Resveratrol effectively inhibits HSV *in vitro* by targeting immediate early events in HSV replication (Docherty *et al.*, 1999). The precise mechanism of action of resveratrol is unknown, but it has been reported that resveratrol disrupted the cell cycle by inhibition cell cycle factors (Schang *et al.*, 1998) or phosphorylation (Stewart *et al.*, 1999). Because HSV requires cellular function associated with cell cycle progression in order to replicate (Hossain *et al.*, 1997), this affect might contribute to the inhibitory effects of this compound on HSV replication. Topical application of resveratrol cream effectively suppressed the development of HSV wide type and ACV resistant strain induced cutaneous lesion without dermal toxicity *in vivo* (Docherty *et al.*, 2004).

Glutathione (γ-glutamyl-cysteinyl-glycine or GSH) an SH group containing tripeptide, is the most prominent intracellular low-molecular weight thiol found in eukaryotic cells. GSH serves as an important intracellular water-soluble antioxidant and detoxifying agent (Droge *et al.*, 1994). Many viral infections, including hepatitis C virus (Boya *et al.*, 1999), human immunodeficiency virus (HIV) (Kalebic *et al.*, 1991), parainfluenza-1, sendai virus, and HSV (Vogel *et al.*, 2005) have been shown to associate with marked depletion of extra-cellular and intracellular GSH levels. Furthermore, it has been reported that exogenous GSH was able to

induce a strong concentration-dependent inhibition of HIV, sendai virus, and HSV replication *in vitro*. In clinical use, GSH has been suggested for use as a dietary supplement in HIV patients (Lyn, 2000). Although the mechanism of antiviral activity of GSH needs to be fully elucidated, all data indicated that GSH inhibit HSV replication by interfering with very late stages of HSV life cycle (Palamara *et al.*, 1995).

11. α-Lipoic acid and derivatives

α-lipoic acid (LA) or thioctic acid (chemical name: 1,2-dithiolane-3-pentanoic acid) (Figure 8) is present in all plant and animal species primarily in mitochondria. In human being, this compound acts as coenzyme in the α-keto acid dehydrogenase complex that takes part in energy formation (Lyn, 2000). As a cofactor, LA linkes to lysine residues of the 2-oxo acid dehydrogenase multienzyme complexes by binding with acyl groups and transfering them from one part of the enzyme complex to another (Morris *et al.*, 1995; Fujiwara *et al.*, 1996). In this process LA is reduced to dihydrolipoic acid (DHLA). LA contains a five-membered ring which contains two sulfur atoms and a carboxylic acid group. It is insoluble in water, but soluble in organic solvents such as methanol and ethyl ether. LA is not only found in vegetable such as potato, spinach, and tomato, but also synthesized in mammalian cells (Glantzounis *et al.*, 2006). Food derived from tissue with a high metabolic activity has a high LA content (Herbert and Guest, 1975), thus this indicated that most LA in diet originates from the multienzyme complex. Therefore, it has been suggested that LA from mammalian diet is absorbed in the form of lipoyllysine because proteolytic enzymes do not effectively cleave the peptide bond between LA and lysine. In addition LA can be obtained by *de novo* biosynthesis in mitochondria from fatty acids and cysteine which required enzyme lipoic acid synthase (Cakatay, 2006). Because it is a low molecular weight substance (molecular weight: 206), LA is readily absorbed from gut and passes through the blood-brain barrier (Packer *et al.*, 1995). Exogenous LA supplement enters the cells and converts easily to its reduced from (DHLA) by cytosolic enzymes including GSH reductase, thioredoxin reductase, and also mitochondrial enzyme E3.

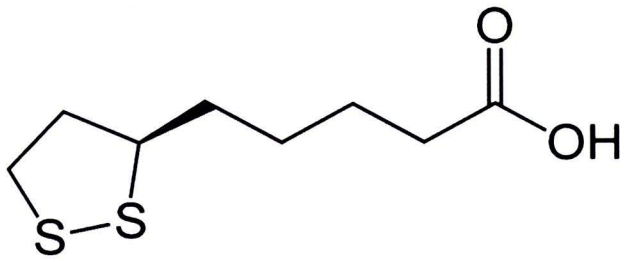


Figure 8. Structure of α-lipoic acid.

LA is a potent antioxidant in both fat-soluble and water-soluble environments. Furthermore, its antioxidant activity extends to both its oxidized and reduced forms. Both LA and DHLA can act as antioxidants against reactive oxygen species, including superoxide radicals, hydroxyl radicals, hypochlorus acid, peroxyl radicals, and singlet oxygen. However, the mechanism of the free radical scavenging process performed by these compounds still remains controversial, and there is also evidence indicating that LA and DHLA may exert prooxidant activity (the activity of antioxidant in a situation which it produces more oxidative stress) both *in vitro* (Maini *et al.*, 2002) and *in vivo* (Cakatay *et al.*, 2005). For example, DHLA can easily reduce Fe^{3+} to Fe^{2+} which promotes lipid peroxidation (Bast and Haenen, 1988). Another important property of LA is its ability to regenerate other antioxidants, such as vitamin C, glutathione, and vitamin E. LA, after reduction to DHLA, is able to contribute to the nonenzymatic regeneration of GSH and vitamin C (Rose and Bode, 1995) and regenerate vitamin E in a cascade of regenerating reactions (Bast and Haenen, 1988). These interactions of LA with other antioxidants result in membrane protection from lipid peroxidation at lipid bilayer. Many researches have also reported that LA can increase coenzyme Q10 levels (Kagan *et al.*,

1990). LA appears capable of chelating certain metals. It binds and forms stable complexes with copper, manganese, and zinc (Sigel *et al.*, 1978). In animal models, it has been found to protect against arsenic poisoning (Grunert, 1960), and reduce cadmium-induced hepatotoxicity in both animal and *in vitro* studies (Muller and Menzel, 1990). In addition, LA chelated mercury from renal tissue *in vitro* (Keith *et al.*, 1997). LA and DHLA can reduce the pathology of several diseases of which it is assumed that reactive oxygen species (ROS) or oxidative stress accompanies their etiology in animal models. For example, LA administration minimizes oxidant generation and macromolecular damage in skeletal muscle of aged rats. It also dose-dependently prevented the development of clinical signs in a rat model for multiple sclerosis and acute allergic encephalomyelitis resulted from ROS (Schreibelt *et al.*, 2006). Both LA and DHLA could provide protection from the harmful effects of free radicals including ischemia and reperfusion injury in heart (Serbinova *et al.*, 1992) and brain tissues (Prehn *et al.*, 1992). DHLA was able to inhibit the peroxidation of linoleic acid and of the non-HDL fraction catalyzed by rabbit reticulocyte 15-lipoxygenase (Lapenna *et al.*, 2003). LA enhanced glucose utilization in isolated rat diaphragm (Haugaard and Haugaard, 1970), heart (Singh and Bowman, 1970), and myotubes (Bashan *et al.*, 1993); furthermore, it protected rat pancreatic islet cells from destruction by ROS (Heller *et al.*, 1997).

LA administration has been used as therapeutic agent for numerous clinical indications. In diabetes, LA has potential applications for many aspects of diabetic pathology. These include the improvement of insulin sensitivity in type 2 diabetes (Jacob *et al.*, 1999), slowing the development of cataractogenesis (Maitra *et al.*, 1995; Ou *et al.*, 1996), treatment of diabetic neuropathy (Reljanovic *et al.*, 1999; Ametov *et al.*, 2003), and prevention diabetic nephropathy (Melham *et al.*, 2001). Exogenous LA administration has been reported to improve the biochemical parameters of glaucoma and visual function in open-angle glaucoma, and be beneficial in the treatment of mushroom poisoning (Packer *et al.*, 1995). Moreover, LA may have a positive effect on patients with Alzheimer's disease and other types of memory dysfunction by decreasing and protecting oxidative damage in the central nervous system. LA administration, alone or together with vitamin E, is an effective treatment for radiation injury by lessening indices of oxidative damage and normalizing organ function (Ramakrishnan *et al.*, 1992; Korkina *et al.*, 1993).

Another therapeutic use of LA and DHLA is protection of HIV viral activation in AIDS patient. Oxidative stress has been reported to occur in several different viral infections, including parainfluenza, HSV, and HIV (Ciriolo *et al.*, 1997). In HIV infection, oxidative stress induces both HIV replication and DNA damage, leading to immunosuppression (Marmos *et al.*, 1997), and the role of antioxidants as antiviral in the treatment of HIV has been supported in multiple studies (Kalebic *et al.*, 1991; Israel *et al.*, 1992). Oral LA interrupted HIV replication by completely blocking the activation of nuclear factor kappa-B (Susuki *et al.*, 1992; Packer and Susuki, 1993), a protein that functions as a nuclear factor and appears to play a role in inflammation, and inhibition of reverse transcriptase (Lyn, 2000). LA treatment resulted in increases in CD_4 T-helper cells and CD_4 T-helper cell/CD_8 T-suppressor cell ratio (Packer *et al.*, 1995).

LA appears to be safe in dosages generally prescribed clinically. It was no acute toxicity and did not show any adverse effects in rat. LD_{50} was 400 to 500 mg/kg after an oral dosage in dogs (Parker *et al.*, 1995). There was no evidence of mutagenic activity and genotoxic activity, but there have not been sufficient studies to guarantee the safety of LA supplement in pregnant women (Cremer *et al.*, 2006). Although few adverse effects were noted, LA at 20 mg/kg given intraperitoneally to severely thiamine-deficient rats proved fatal. This effect was prevented by administration of thiamine prior to LA administration (Gal, 1965). In human, allergic skin reactions are among the few reported side effects of LA supplementation (Parker *et al.*, 1995). It is estimated that humans can tolerate several grams of LA administered orally (Biewenga *et al.*, 1997); however, therapeutic dosages of LA range from 600 to 1800 mg daily. In addition, it is critical to select the appropriate pharmacological doses of LA for use in oxygen-related disease according to balance between its prooxidant and antioxidant activity of thiol compound (Cakatay, 2006).

Lipoamide (6, 8-dithiooctanoic amide or thioctic acid amide) (Figure 9) is a functional form of lipoic acid which linked to lysine side chain of multienzyme complexes that catalyzed oxidative decarboxylation of keto acid. Similar to LA, lipoamide is a small substance with molecular weight of 205, is found in the inner membrane of mitochondria, and is a powerful antioxidant. It has been reported that lipoamide, which is the analogue that more closely resemble the cellular protein-bound lipoyllysine than free LA, could effectively prevent oxidant-mediated apoptosis of lysosomes (Persson *et al.*, 2001), and inhibit nitric oxide production in macrophages better than free LA (Guo *et al.*, 2001).

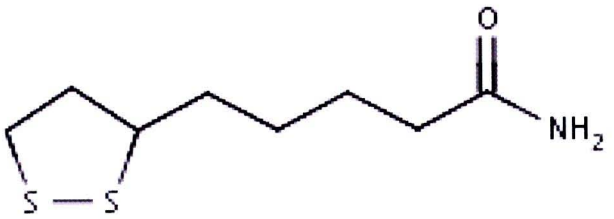


Figure 9. Structure of lipoamide.