CHAPTER 1

INTRODUCTION

1. Introduction

Human immunodeficiency virus (HIV) is the etiologic agent of acquired immune deficiency syndrome (AIDS). It was discovered as the cause of AIDS in 1983. Since then, 25 years later, more than 60 million people all over the world would have been infected including more than 25 million adult and child deaths due to AIDS [183]. It is estimated that 33.2 million people were living with HIV at the end of 2007, that 2.5 million individuals became newly infected with HIV and that 2.1 million people died of AIDS in 2007 [184]. HIV continues to spread relentlessly at a rate of 6,800 new HIV infections every day, therefore a vaccine that prevent HIV infection or at least slows the progression of disease in individuals who become infected is desperately needed.

HIV is present in the blood and genital secretions of HIV-infected individuals. The transmission of HIV can occur through sexual contact by unprotected vaginal, oral or anal intercourse, through blood transfusion or exposure to HIV-contaminated needles, syringes, and other equipment, or through mother-to-child transmission (MTCT) during pregnancy, labor and delivery, or breastfeeding. HIV infects the CD4+ cells of the immune system and HIV disease is characterized by a gradual deterioration of immune function. The CD4+ T cells play a central role in the immune response, signaling other cells in the immune system to perform their functions. A healthy, uninfected person usually has 800 to 1,200 CD4+ T cells per mm^3 of blood. During HIV infection, the number of these cells in blood progressively declines, until it falls below $200/mm^3$. At this end stage of infection, the HIV-infected person becomes particularly vulnerable to the opportunistic infections and cancers that typify AIDS. However, 25 years after the discovery of the AIDS virus, both the correlates of protective immunity in HIV infection and the mechanism of HIV escape to the immune response are still poorly understood.

MTCT of HIV is the major mode of pediatric infection worldwide. The estimated number of children living with HIV was 2.1 million in 2007, with a rate of 1,200 new HIV infections a day [184]. HIV can be transmitted from mother to child at three different times: during pregnancy (*in utero*), at the time of labor and delivery (intrapartum), or postpartum through breastfeeding. In the absence of any intervention, 35% - 49% of the children born to HIV-infected mothers are infected [230; 209]. In non-breastfeeding populations it is estimated that more than 50% of

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infections occur late in pregnancy and during labour and delivery [210]. The rate of MTCT of HIV has been reduced to less than 2% in developed countries, due to highly efficient prophylaxis measures. However, MTCT of HIV remains high in resource-constrained countries, particularly Sub-Saharan African countries, where the vast majority of HIV-infected women of childbearing age reside. The reduced rates of MTCT of HIV are largely due to access to prevention interventions that include HIV voluntary counseling-testing, antiretroviral drug therapy, selective caesarean section.

Neutralizing antibodies (NAbs) have been found to be effective antiviral agents in many viral infections, as they can block viruses from infecting cells and limit viral infection *in vivo*, yet they may also bind complement or promote antibody-directed cell-mediated cytotoxicity (ADCC), thus leading to killing of infected cells. The HIV envelope (Env) glycoproteins are the target of NAbs. In natural HIV infection, primary peak viremia declines before NAbs against HIV become detectable. HIV-infected individuals may generate potent NAbs responses to their autologous isolates, but it seems that such responses only drive the evolution of the virus that escapes continuously to autologous NAbs. HIV-1 has evolved multiple mechanisms by which it generates variants in order to protect itself from antibody binding, those include nucleotide substitutions, insertions and deletions of *env* gene, modification of potential envelope glycosylation sites, and recombination. In addition, even if the NAb response matures overtime after infection, it usually lacks a broad heterologous reactivity (i.e. toward distantly related isolates). The difficulty to raise such broadly reactive antibodies is one of the major obstacles to the development of a successful vaccine. Interestingly, long-term non-progressors (LTNPs) who have remained disease-free after ≥10 years of infection have strong and frequent broadly cross-reactive NAbs responses, but their contribution to containment of the infection is still unclear [65; 67; 71; 312; 51]. Several studies in the context of the MTCT of HIV-1, a situation where babies are exposed to the virus in presence of preexisting maternal antibodies, have shown that non-transmitting mothers are more likely to have NAbs, or to have higher levels of NAbs, consistent with a role of these antibodies in reducing MTCT [32; 47; 156; 227; 357]. Passive immunization studies with human neutralizing monoclonal antibodies (HuMAbs) in the neonatal macaques model have proven that antibodies could protect against MTCT of HIV-1 infection [27; 166].

Early molecular studies of HIV in the MTCT context showed that HIV-1-infected mothers generally have a heterogeneous viral population, whereas their infected infants have a more homogeneous virus population [428; 358; 13; 301; 410]. More recent studies have clearly shown that variants from infants are more frequently resistant to neutralization by autologous maternal plasma than the overall viral population from their mothers [432; 109]. This indicates the presence of a selective pressure, probably exerted in part by the maternal NAbs, that results in the selection of a limited number of maternal viral variants for establishment of a new infection in the infants.

Most effective viral vaccines work by generating antibodies that inactivate or neutralize the invading virus, and the existing data strongly suggest that an optimally effective HIV-1 vaccine should elicit potent antiviral NAbs. Although the efficacy of antibody prophylaxis has been proven against several viruses e.g. rabies,

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poliomyelitis, measles, hepatitis A, hepatitis B, or varicella vaccine-induced NAbs responses against HIV have been disappointing in the past. Understanding the role of NAbs involved in preventing MTCT of HIV-1 and selective transmission of NAbs escape variants should provide better understanding of HIV, in term of humoral immune response and immune system evasion in HIV-1 infection. It would provide additional knowledge that might be useful in HIV vaccine development. Therefore, we focused our work on the virological and immunological characteristics of MTCT of CRF01_AE viruses, which belong to the predominant HIV-1 clade in Thai population, hoping that it will benefit to the HIV-1 vaccine development for Thai people. Our work would be also help in designing new strategies to prevent MTCT of HIV-1, for instance using passive immunization together with antiretroviral drugs to prevent the infection of infants.

2. Background

PART I. Human immunodeficiency virus (HIV)

1. History of AIDS and HIV discovery

It is over 27 years since the first recognized cases of AIDS were reported in 1981 by the U.S. Centers for Disease Control and Prevention (CDC) [1; 2], suggesting a new infectious disease. A number of young homosexual men in New York and California suddenly began to develop rare opportunistic infection of *Pneumocystis carinii* pneumonia (PCP) and Kaposi's sarcoma that seemed resistant to any treatment. At this time, the causative agent of AIDS did not yet have identified, but the first clues to the cause of AIDS derived from the immunological investigations. One consistent manifestation was a rapid decrease in level of CD4+ T cells and once those levels fell below 200 cells/mm^3, patients become vulnerable to opportunistic infections and various malignancies. The same subset of T cells is the target of the first-described human pathogenic retrovirus, human T-lymphotropic virus type 1 (HTLV-I) [317]. However, rather than inducing CD4+ T cells depletion, HTLV-I transforms these CD4+ T cells, resulting clinically in adult T-cell leukemia.

The first discovery of the cause of AIDS was in 1983 by Luc Montagnier and his colleagues at the Institut Pasteur who reported the isolation of a new human retrovirus from a lymph node biopsy from a young homosexual man with a lymphadenopathy syndrome [34]. By electron microscopy, it appeared to be different from HTLV-I and this new virus was named the lymphadenopathy-associated virus (LAV). About a year later, Robert Gallo and his colleagues at the U.S. National Institutes of Health (NIH) convincingly showed that this virus caused AIDS and they called it HTLV-III [323]. The first discovery was shared credit between two groups. Jay Levy and colleagues at the University of California, San Francisco, also independently isolated this new retrovirus, which it was called the AIDS-associated retrovirus (ARV) [231]. During the time, the virus causing AIDS was called by a different name: LAV,

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HTLV-III, and ARV. Finally, in 1986, the International Committee on the Taxonomy of Viruses recommended that this pathogen with many names should be called the human immunodeficiency virus (HIV) [87]. Soon after the identification of HIV, the first evidence of HIV-2 was described in 1985. The serum from Senegalese sex workers showed cross-reactivity preferentially to the simian immunodeficiency virus (SIV) antigens compared to HIV-1, indicating exposure to an SIV-like virus [33]. The virus was then isolated and characterized from West African AIDS patients from Guinea-Bissau and Cape Verde and referred to as LAV type 2 [83]. LAV-2 was then renamed HIV-2.

2. Current situation of HIV and AIDS epidemic

Over the past 27 years, HIV has moved from a single report [1] to a worldwide pandemic. The cumulative total of individuals infected with HIV and deaths due to AIDS since the pandemic began exceeds 60 millions and 25 millions people, respectively [183]. However, the global prevalence of HIV infection seems to have been stable since around the end of the 20th century (Figure 1). This might be due to declining of the number of new HIV infections, but also reduction in deaths due to AIDS globally over the past several years with broader access to antiretroviral therapy [184]. At the end of 2007, UNAIDS and WHO estimated that there were 33.2 million people living with HIV, within that 2.5 million individuals became newly infected with HIV, and 2.1 million people died [184]. The estimated number of adults and children to be living with HIV in 2007 is shown in Figure 2. However, the HIV epidemic has formed two broad patterns. First, the epidemics sustained in the general population of many sub-Saharan African countries. Second, the epidemic in the rest of the world is concentrated among specific groups at risk such as men who have sex with men (MSM), injecting drug users, sex workers, and their sexual partners.

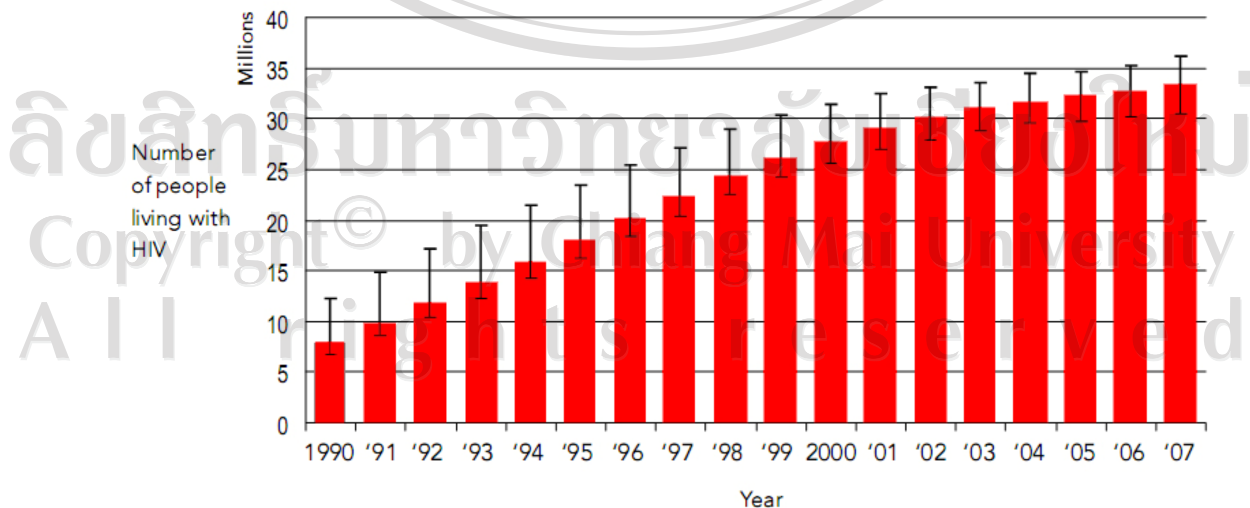

Figure 1. Estimated number of people living with HIV globally during 1990-2007. The bar indicates the range around the estimate. (Figure from UNAIDS, 2007 [184])

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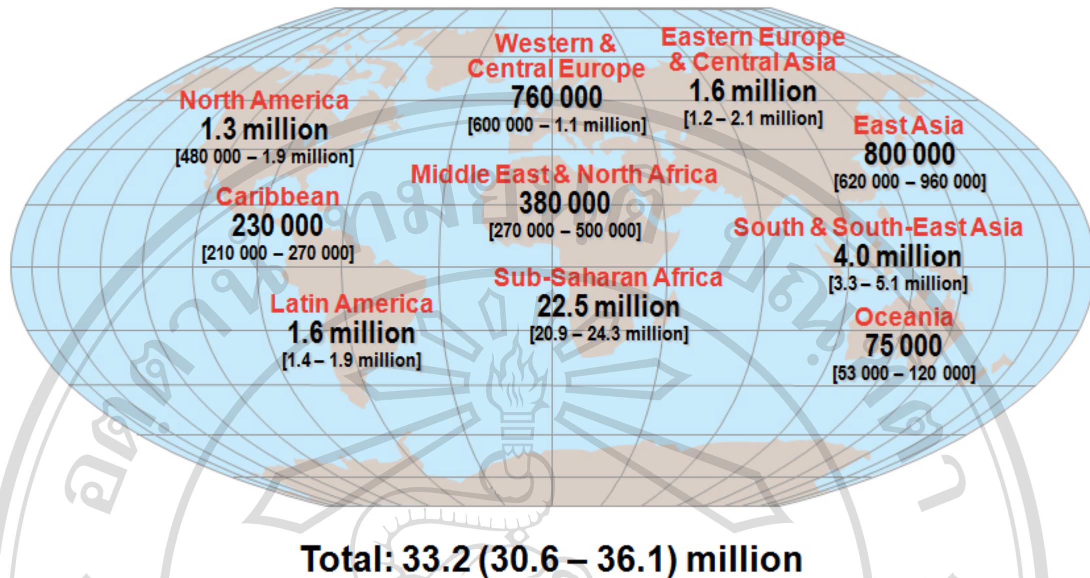

Figure 2. Estimated number of adults and children to be living with HIV in 2007. (Figure from UNAIDS, 2007 [184])

In developed countries, including North America and Western Europe, the total number of people living with HIV is increasing. This is due mainly to the life prolonging effects of antiretroviral therapy and an increase in the number of new HIV diagnoses in Western Europe since 2002, combined with a relatively stable number of new HIV infections each year in North America [184]. In the U.S., about three-quarters of newly reported cases of infection with HIV-1 are in men, most of them MSM and especially African American [8], while MTCT of HIV has been nearly eliminated in the U.S. through routine prenatal screening linked to antiretroviral therapy to those infected pregnant women [9]. In Western Europe, newly diagnosed HIV infections were mainly among MSM and injecting drug users [184]. The largest number of diagnoses were reported in France, Italy, Spain, Portugal, and the United Kingdom. Heterosexual transmission of HIV in Europe has slowly increased and mainly current infections are found among immigrants from Sub-Saharan Africa [184]. In Eastern Europe, the most-reported mode of transmission emerged among injecting drug users especially in the Russian Federation and Ukraine [184].

In low and middle-income countries, Sub-Saharan Africa is the most affected region in the global AIDS epidemic, accounts for 68% of persons living with HIV/AIDS worldwide and for 76% of all deaths due to AIDS [184]. In Southern Africa, the prevalence of HIV infection in the general population exceeds 15% and South-Africa alone has more than 10% of the population infected with HIV, with prevalence among pregnant women at 30% in 2005 and 29% in 2006 [184]. In 2007, approximately 61% of individuals infected with HIV in Africa were women and almost 90% of children who are infected with HIV live in Africa [184]. Additional most affected region outside Africa is the Caribbean, where the overall population prevalence of infection is about 1%, mainly resulting from heterosexual transmission [184].

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In Asia, national HIV prevalence is highest in South-East Asia with wide variation in epidemic trends between different countries. The HIV prevalence in Thailand, Cambodia and Myanmar all show declines, while those in Indonesia and Viet Nam are growing. The majority of HIV infection in Indonesia and Viet Nam are linked to the use of contaminated injecting equipment and unprotected paid sex [184]. In Viet Nam, injecting drug users prevalence increased from 9% in 1996 to about 34% in 2005, while more than 40% of injecting drug users in Jakarta tested HIV-positive [184]. The HIV prevalence in the countries with a large population such as India and China also concentrated in specific groups at risk, including injecting drug users, sex workers and their clients, and MSM.

In Thailand, the HIV incidence is low, about 0.03% per year [150]. The number of new HIV infections declined, however the decline in HIV prevalence has been slowing in recent years, as more people are receiving antiretroviral therapy. The new HIV infections are occurring in the most at risk population; sex workers remains an important factor in the epidemic. It is estimated that almost one in five (18%) new HIV infections in 2005 were in sex workers, their clients and those clients' other partners. The HIV prevalence among injecting drug users has remained high, over the past 15 years, ranging between 30% and 50% [184], with 3-10% being newly infected each year [197]. However, as many as one in five (21%) new HIV infections in 2005 in Thailand were in MSM [150], and HIV prevalence in this population is on the rise. The recent studies have shown increasing HIV prevalence among MSM in Bangkok from 17.3% in 2003 to 28.3% in 2005, and HIV prevalence was 15.3% in Chiang Mai and 5.5% in Puket [10]. In MTCT of HIV, there has been remarkable progress in the prevention; 98% of women who delivered their babies in public sector facilities received HIV counseling and testing, and 94% of those found to be HIV positive received antiretroviral preventive therapy [184]. The transmission risk of HIV from mothers to their children was reduced to about 10% overall and about 4% among those mothers and infants who received a complete zidovudine regimen along with other antiretroviral drugs such as nevirapine [184].

3. HIV structure, genomic organization and replication cycle

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3.1 Viral structure

HIV virion has a spherically shaped structure of approximately 100-130 nm in diameter. The structure of the mature HIV-1 virion is shown in Figure 3.

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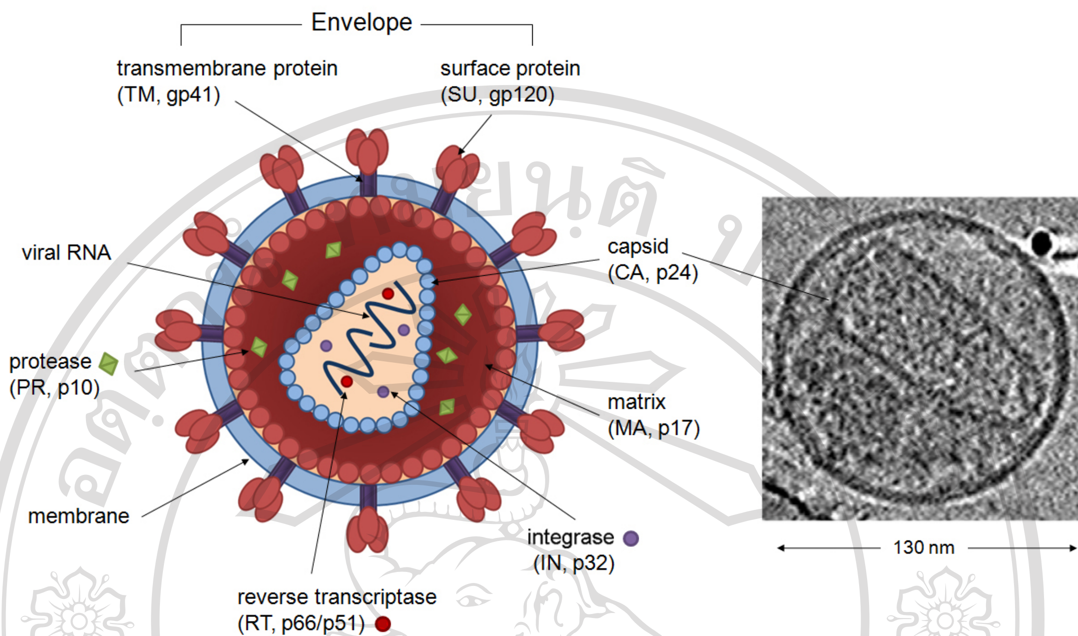

Figure 3. Schematic representation of a mature HIV-1 virion (*left*; Figure drawn for this thesis) and a mature HIV-1 particle observed by electron cryotomography (*right*; Figure from Ganser-Pornillos *et al*, 2008 [136]).

The HIV virion is enveloped by a lipid bilayer that is derived from the membranes of the host cell and harbors the viral envelope (Env) glycoproteins: the surface (SU, gp120) and the transmembrane (TM, gp41) glycoproteins. The surface glycoproteins are anchored to the virus via non-covalently links to the transmembrane (TM, gp41). The lipid bilayer also contains several cellular membrane proteins derived from the host cell, including major histocompatibility antigens, actin and ubiquitin. The viral structural proteins are referred to as Gag proteins; in mature particles, an approximately 2,000 copies of the matrix protein (MA, p17) is located under the lipid bilayer, lining the inner surface of the viral membrane, and is associated to a viral enzyme, protease (PR, p10). The conical capsid core particle comprising 2,000 copies of the protein (CA, p24) is located in the centre of the virion. The capsid particle contains two copies of the viral genomic RNA and the nucleocapsid protein (NC, p7), which is stabilized as a ribonucleoprotein complex, and also contains viral-specific enzymes: reverse transcriptase (RT, p66/p51) and integrase (IN, p32). The accessory proteins: Nef, Vif, and Vpr are also packaged within the viral particle. Three additional accessory proteins that function in the host cell, Rev, Tat, and Vpu, do not appear to be packaged in the virion.

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3.2 Genomic organization

Like other replication-competent retroviruses, HIV-1 provirus consists of three structural genes: *gag*, *pol*, and *env* with flanking long terminal repeat (LTR) sequences at each end of the genome. In addition, HIV possesses regulatory genes

(*tat*, *rev*), and accessory genes (*vif*, *vpr*, *vpu*, and *nef*). The organization and landmarks of HIV-1 genome is shown in Figure 4.

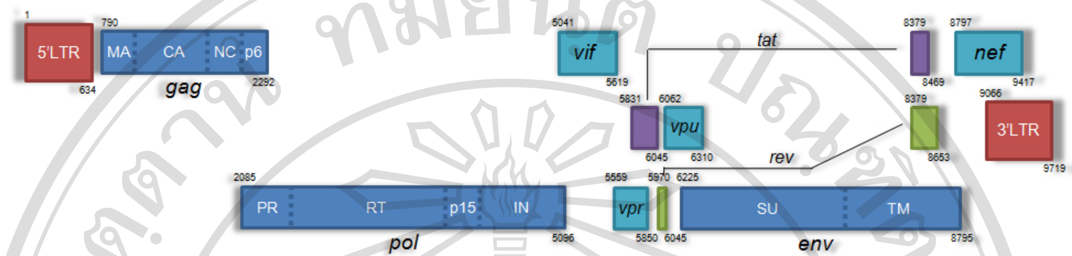

Figure 4. Organization and landmarks of the HIV-1 DNA genome. Open reading frames are shown as rectangles. The gene start is indicated by number in the upper left corner of each rectangle (ATG start codon). The number in the lower right indicates the position of the stop codon. The *tat* and *rev* spliced exons are shown in *violet* and *green*, respectively. The numbering positions in relative to HXB2 strain (GenBank accession number K03455). (Figure drawn for this thesis)

The three structural genes are *gag*, *pol* and *env*. The *gag* encodes the internal structural proteins of the virus, Gag proteins for "group-specific antigen". Gag precursor (p55) is proteolytically processed into the mature proteins: MA (matrix, p17), CA (capsid, p24), and NC (nucleocapsid, p7). *pol* encodes the viral enzymes: reverse transcriptase (RT), which contains both DNA polymerase and associated (ribonuclease H) RNase H activity, integrase (IN), and protease (PR). *env* encodes viral envelope glycoproteins as a precursor (gp160), which is then processed to a surface glycoprotein, gp120 and a transmembrane glycoprotein, gp41. The mature gp120-gp41 proteins are bound by non-covalent interactions and are associated as a trimer on the cell surface and at the virion surface.

The regulatory genes are *tat* and *rev*. They modulate transcriptional and post-transcriptional steps of virus gene expression and are essential for virus propagation. Tat acts by binding to the TAR RNA element and activates transcription initiation and elongation from the LTR promoter. Rev acts by binding to RRE and promotes the nuclear export, stabilization, and utilization of the viral mRNAs containing RRE.

The accessory genes are *vif*, *vpr*, *vpu*, and *nef*. Vif promotes the infectivity but not the production of viral particles. In the absence of Vif, the produced viral particles are defective, but the cell-to-cell transmission of virus is not affected significantly [147]. It was discovered that Vif prevents the action of the cellular APOBEC-3G protein, the potent antiretroviral cytidine deaminases. APOBEC-3G inhibits the replication of Δ*vif* HIV-1 by deaminating the minus-strand of the viral reverse transcripts, introducing numerous G→A mutations [447; 448]. Vpr is incorporated into the virion. The functions of Vpr include the targeting the nuclear import of preintegration complexes, cell growth arrest, transactivation of cellular genes, and induction of cellular differentiation. Vpu is unique to HIV-1 and some

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SIV (e.g. SIVcpz). It has two different biological functions, degradation of CD4 in the endoplasmic reticulum [423], and enhancement of virion release from the plasma membrane of HIV-1-infected cells [50]. Vpu is involved in Env maturation and is not found in the virion. It was found that Vpu antagonizes a cellular protein named tetherins that is an inhibitor of virus particle release [449]. Nef is necessary for the maintenance of high viral loads and for the development of AIDS and viruses with defective *nef* have been detected in some HIV-1 infected long term survivors [342; 352]. Vpx is found in HIV-2, but not in HIV-1. This accessory gene is a homolog of HIV-1 vpr, however the function in relation to Vpr is not fully elucidated; both are incorporated into virions at levels comparable to Gag proteins through interactions with Gag p6.

3.3 Replication cycle

The replication cycle of HIV-1 is shown in Figure 5.

Virus entry (binding and fusion)

Entry is the first step in the process of HIV infection. HIV primarily infects the CD4+ cells of the immune system. CD4 is a member of the immunoglobulin superfamily and has four extracellular immunoglobulin-like domains (D1 to membrane proximal D4). CD4 can be detected on the cell surface of about 60% of T-lymphocytes, on T-cell precursors within the bone marrow and thymus, and on monocytes/macrophages, Langerhans cells, follicular dendritic cells and microglial cells of the central nervous system. The initial step is when the viral envelope glycoprotein gp120 binds to CD4 with extremely high affinity. This results in conformational changes in gp120, which then exposes coreceptor binding sites. The coreceptors required for entry of HIV-1 are CCR5 and CXCR4, depending on the viral tropism (described below). The intracellular gp120/CD4 interaction also leads to the downmodulation of CD4 from the cell surface, rendering infected cells partially resistant to further infection [311]. Immediately following gp120 and coreceptor binding, further conformational changes take place in gp41, which allows it to expose of the fusion peptide. The fusion peptide is inserted into the target cell membrane followed by the formation of a pre-hairpin structure, exposing the N-helices. The gp41 folds to bring the N- and C-helices together, which transforms the pre-hairpin into the six-helix bundle. The formation of the six-helix bundle promotes complete fusion, in which the fusion peptide and the transmembrane segment of gp41 lie parallel on a contiguous bilayer. The steps of HIV-1 cell entry are summarized schematically in Figure 6.

Dendritic cells (DCs) and Langerhans cells have also been implicated in transmission of HIV-1. DCs are distributed throughout the rectal, ectocervical and vaginal mucosal epithelium. It has been demonstrated that gp120 is able to attach to a C-type lectin receptor called DC-SIGN (dendritic cell specific ICAM-3 grabbing non-integrin) expressed on monocyte-derived dendritic cells, which are an *in vitro* model for DCs, but share many characteristics with primary DCs [144]. DC-SIGN is

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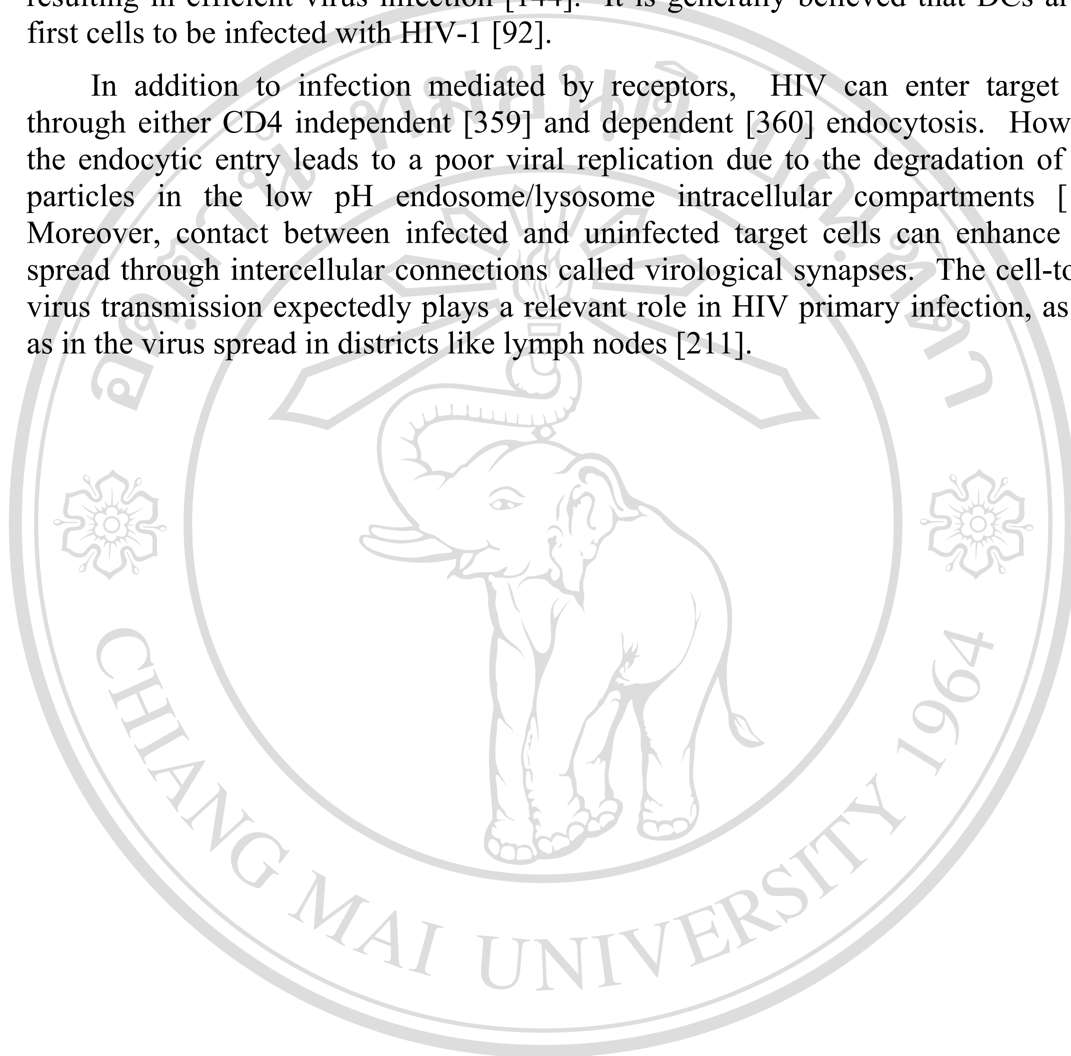
particularly efficient at retaining bound virus in an infectious state for prolonged periods of time, and in presenting bound virus to cells expressing CD4 and coreceptor resulting in efficient virus infection [144]. It is generally believed that DCs are the first cells to be infected with HIV-1 [92].

In addition to infection mediated by receptors, HIV can enter target cells through either CD4 independent [359] and dependent [360] endocytosis. However, the endocytic entry leads to a poor viral replication due to the degradation of viral particles in the low pH endosome/lysosome intracellular compartments [129]. Moreover, contact between infected and uninfected target cells can enhance viral spread through intercellular connections called virological synapses. The cell-to-cell virus transmission expectedly plays a relevant role in HIV primary infection, as well as in the virus spread in districts like lymph nodes [211].



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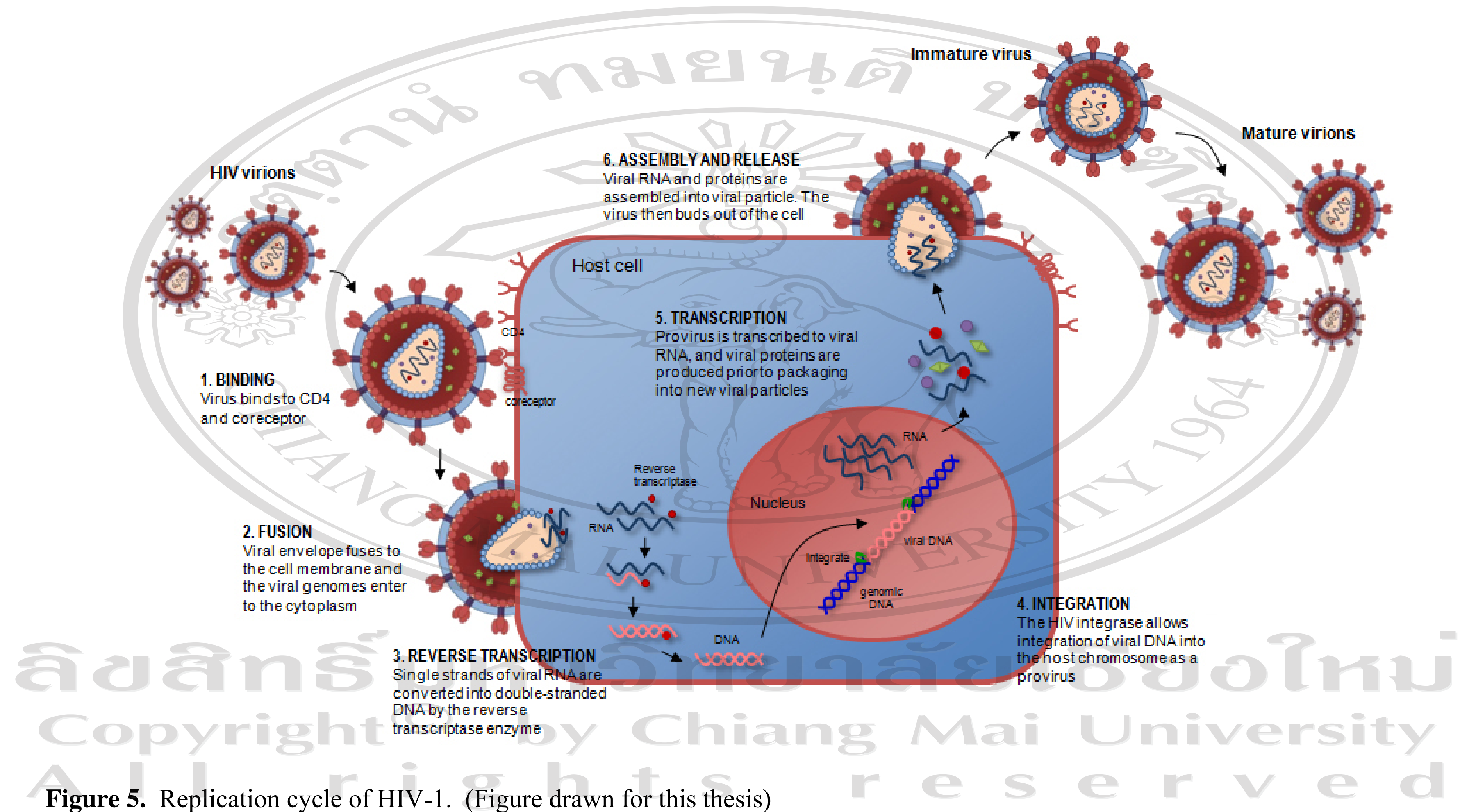

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Figure 5. Replication cycle of HIV-1. (Figure drawn for this thesis)

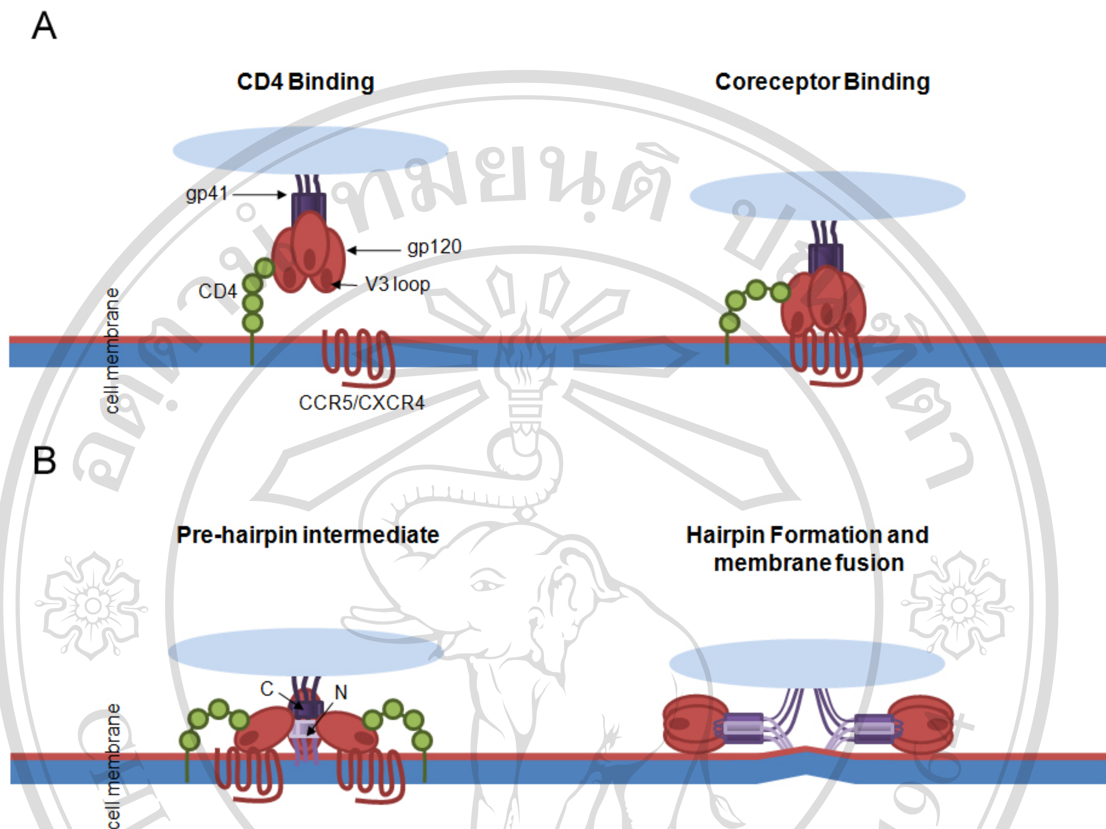

Figure 6. Schematic representation of the early steps of HIV-1 cell entry. (Figure drawn for this thesis)

A, gp120-CD4 and -coreceptor binding.

B, Steps of membrane fusion by gp41.

Reverse transcription and integration

Following membrane fusion the virus core enters and uncoats into the cytoplasm of the target cell. The virus RNA genomes are converted into double-stranded DNA. Reverse transcriptase has two distinct enzymatic activities: it is a DNA polymerase capable of copying either an RNA or a DNA template into a complementary DNA sequence; and it is an RNase H, capable of degrading the RNA strand of an RNA-DNA duplex into small pieces once it has been used as a template for the first DNA strand [450]. The reverse transcription is initiated using a molecule of the cellular transfer RNA (tRNA) that is bound to the primer binding site (PBS) located immediately downstream of the 5'R-U5 sequence on the genomic RNA, as a primer. Minus-strand DNA synthesis proceeds until the 5'end of genomic RNA, generating a DNA/RNA hybrid. The template RNA is degraded by the RNase H activity, generating a DNA fragment known as the minus-strand strong stop DNA (-sssDNA). The identical sequences known as the repeated (R) sequences mediated the -sssDNA

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jumps from 5' to the 3'end of the genome. This step is referred to as the first strand transfer. Minus-strand DNA synthesis occurs, using 3'end of the -sssDNA as a primer, accompanied by RNase H digestion of the template strand. This degradation is not complete, because the RNA genome contains a short polypurine tract (PPT) that is relatively resistant to RNase H degradation. The PPT serves as a primer for plus-strand DNA synthesis. A central PPT serves as an additional primer for plus strand DNA synthesis. The tRNA bound to the PBS is removed by RNase H, allowing a second-strand transfer of plus-strand strong stop DNA (+sssDNA) to take place between PBS sequences. Elongation continues along both DNA strands. A central termination signal (CTS) located at 3' of the central PPT allows termination of plus-strand DNA synthesis in the center, resulting in the formation of a DNA flap. The final product is a double stranded (ds) HIV-1 proviral DNA that contains the long terminal repeats (LTRs), U3-R-U5 sequences, at both end. This entire process utilizes only the virion-associated RT without host enzyme. The process of reverse transcription is shown in Figure 7.

The viral DNA is translocated from the cytoplasm to the nucleus as part of the pre-integration complex (viral DNA associated with MA, RT, and IN). The integration process begins when IN clips off several nucleotides from the 3'termini of both strands of linear viral DNA. This reaction, known as 3'-end processing, generates a molecule of DNA with 3'-recessed ends. In the nucleus, IN makes a staggered cleavage in the cellular target DNA. The 3'-recessed ends of viral DNA formed in the 3'end processing reaction are joined to the ends of the cleaved cellular DNA. This reaction is known as strand transfer. The sites for integration into cellular DNA are random. The integration process is completed when cellular repair enzymes fill in the gaps between the integrated viral DNA and the host target DNA.

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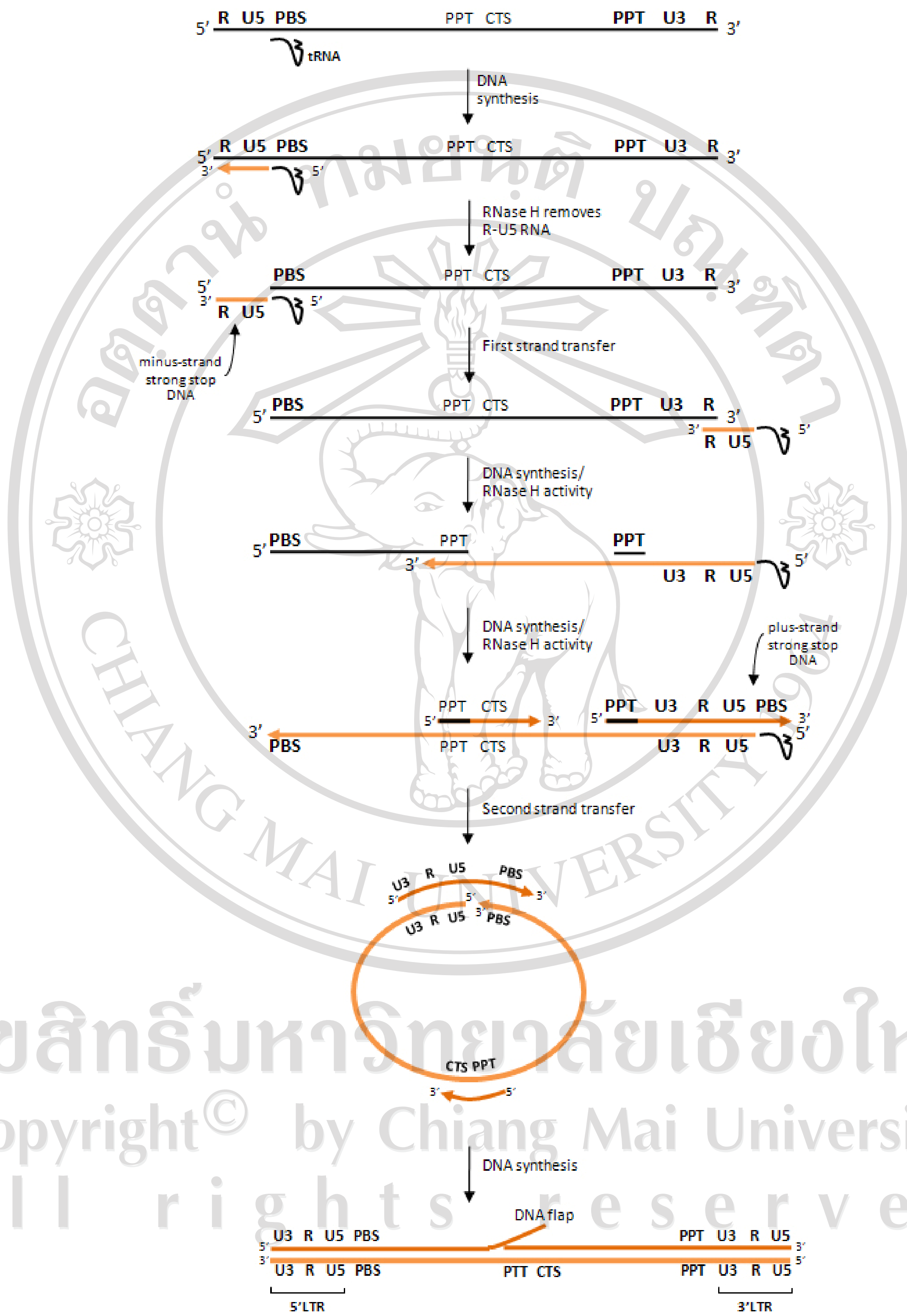

Figure 7. Process of reverse transcription of HIV genome. RNA *(Black line)*; minus-strand DNA *(light orange)*; plus-strand DNA *(dark orange)*. (Figure drawn for this thesis)

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Transcription

Transcription is mediated by the host-cell RNA polymerase II. The HIV-1 LTR serves as the site of transcriptional initiation and harbors *cis*-acting elements required for RNA synthesis. The transcription initiates at the U3/R junction that contains a consensus TATA motif (TATA is designated as the first nucleotide of the transcribed viral mRNA). Immediately upstream of TATA are three Sp-1 and two NF-κB binding sites, which are recognized by endogenous host cell transcription factors. In the R region of the LTR is a short sequence known as the transactivation response region (TAR), target for Tat. Tat plays a critical role in up-regulating transcription from the LTR by more than 100 fold. There are two possible ways that Tat can increase HIV-1 RNA synthesis, one is to augment transcription initiation and the other one is to improve the processivity of RNA polymerase. In the absence of Tat, the initiated transcripts almost terminated prematurely [96].

Viral assembly, release, and maturation

To assembly, the viral structural proteins, core, and envelope must be produced first. The HIV Env glycoprotein is synthesized in the rough endoplasmic reticulum (ER) to generate the Env precursor protein, gp160 and then transported to the Golgi complex, where it is cleaved by a host protease (furin) to give the mature envelope glycoproteins gp120 and gp41. The Env protein migrates to and anchor in the plasma membrane. The viral RNA genomes bound by p7 Gag product form a nucleoprotein complex. The newly formed nucleoprotein particle migrates to the plasma membrane at the site of Env insertion, since each particle has two viral RNA with associated Gag and Gag-Pol precursors. Then process of aligning core with Env, which is mediated by p17 Gag, and budding process occurs during which the virus core acquires a portion of the plasma membrane that contains gp41 and gp120. During or shortly after virus release from the plasma membrane, the viral PR cleaves the Gag and Gag-Pol polyprotein precursors to generate the mature Gag and Pol proteins, resulting in a cone-shaped core that contains the viral genomic RNA.

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4. HIV envelope glycoproteins

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HIV-1 envelope (Env) glycoproteins project from the viral surface and are assembled as Env spikes. They are responsible for interacting with cellular receptors and initiating the fusion of the viral and cell membranes, and therefore are a potential target for drugs aimed at blocking the first step of the viral replication cycle. Furthermore, HIV-1 Env spikes are the only viral target available for neutralizing antibodies [442]. Thus, the genetic, immunological and structural characteristics of HIV-1 envelope have been studied in order to design of new drugs as well as immunogens that will induce the broadly neutralizing antibodies, which is one of the main challenges in current HIV vaccine development.

4.1 Envelope glycoprotein synthesis and glycosylation

The Env glycoprotein of HIV-1 is synthesized as a polyprotein precursor molecule, gp160. The nascent Env protein is transported by Sec61, ER transport protein, to the endoplasmic reticulum (ER) lumen. In the ER lumen (reviewed by Scanlan *et al*, 2007 [355], the N-linked glycan precursor ($Glc_3Man_9GlcNAc_2$) is transferred from dolichol pyrophosphate (Dol-P-P) to the gp160 entering the ER. This transfer is mediated by oligo-saccharyltransferase, which recognizes N-linked glycosylation sequences (Asn-X-Ser/Thr; where X can be any amino acid) in gp160. The N-linked glycan precursor is then removed of two glucose units by glucosidases I and II into the monoglucosylated glycan ($Glc_1Man_9GlcNAc_2$). The monoglucosylated gp160 then binds to calnexin or calreticulin and promotes glycoprotein folding. The hydrolysis of the final glucose-mannose bond by glucosidase II frees gp160 from calnexin/calreticulin allows it to exit from the ER and enter the Golgi. Misfolded glycoproteins are reglucosylated by the folding sensor, UDP-glucose glucosyltransferase and can rebind to calnexin/calreticulin for further refolding cycles. Once in the Golgi, the glycoproteins undergo further processing to become complex-type glycans and gp160 is proteolytically cleaved by the cellular protease furin to gp120 and gp41. The number and position of glycosylation sites vary among HIV subtypes, as would be expected from the high degree of variation in *env* sequences. The gp120 and gp41 glycoproteins are maintained in the assembled trimer by non-covalent interactions between gp41 ectodomain and discontinuous structures composed of NH_2- and COOH-terminal gp120 sequences and transported to the cell surface, where they are incorporated into released virions and displayed on their surface as viral spikes.

4.2 Gp120 structure

Gp120 is an extensively glycosylated protein (Figure 8A) with approximately half of its mass being N-linked carbohydrates [433]. Based on comparative sequence analyses, gp120 is divided into five variable (V1-V5) and five constant (C1-C5) regions (Figure 8A and 9). The structure of HIV-1 and SIV gp120 have been determined in a series of X-ray crystallographic studies [217; 75; 216; 433; 215; 442]. The structure of CD4-bound HIV-1 gp120 in complex with a monoclonal Fab that recognizes the coreceptor binding site, has provided a framework for analyzing envelope antigenicity [217; 433]. However, the unliganded structure of HIV-1 gp120 have resisted high-resolution analysis. The structure of SIV gp120 in an unliganded conformation have been recently reported [75], and helped the understanding of the gp120 structure. The SIV gp120 core and HIV-1 HXB2 have 35% sequence identity and over 70% sequence similarity, and both have seven disulphide bonds in corresponding positions [75]. To obtain crystals (reviewed by Poignard *et al*, 2001 [318]) that diffracted with sufficient resolution, a HIV-1 gp120 core molecule was used that lacks the variable loops V1-V2, V3, and amino- (N) and carboxy- (COOH) terminal sequences (Figure 8B) and that had been enzymatically stripped of over 90% of its carbohydrates. The loops were replaced with the tripeptide linker Gly-Ala-Gly.

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The final deglycosylated ΔV3, ΔV1-V2 gp120 core retains 67% of the envelope amino acid content of the full-length molecule and has a molecular weight of 35 kDa. Despite the modifications, the core retains structural integrity as shown by its ability to bind CD4 and to interact with a number of antibodies at levels comparable to the full-length molecule. The crystal structure at 2.5 Å resolution revealed that the HIV-1 gp120 core is composed of 25 β-strands, 5 α-helices, and 10 loop segments and that it folds into an heart-shaped globular structure with dimensions of 5×5×2.5 nm [217], whereas the unliganded SIV gp120 core has the bipartite character [75] (Figure 10). These studies suggested that the conserved regions of gp120 form a central core, whereas the variable regions, with the exception of V5, are bracketed with cysteine bonds and form four loops that emerge from the surface of the protein. The gp120 core is composed of three general areas: the inner domain, the outer domain, that are linked by a four-stranded sheet termed the bridging sheet (Figure 10 and 11B). Among different clades of HIV-1, the inner domain is more conserved than the outer domain [318]. Furthermore, the gp120 core can be divided based on its binding functions (to CD4 and coreceptor binding) (Figure 11C), and based on its antigenicity and immunogenicity (non-neutralizing, neutralizing, and silent faces) (Figure 11D) [215].

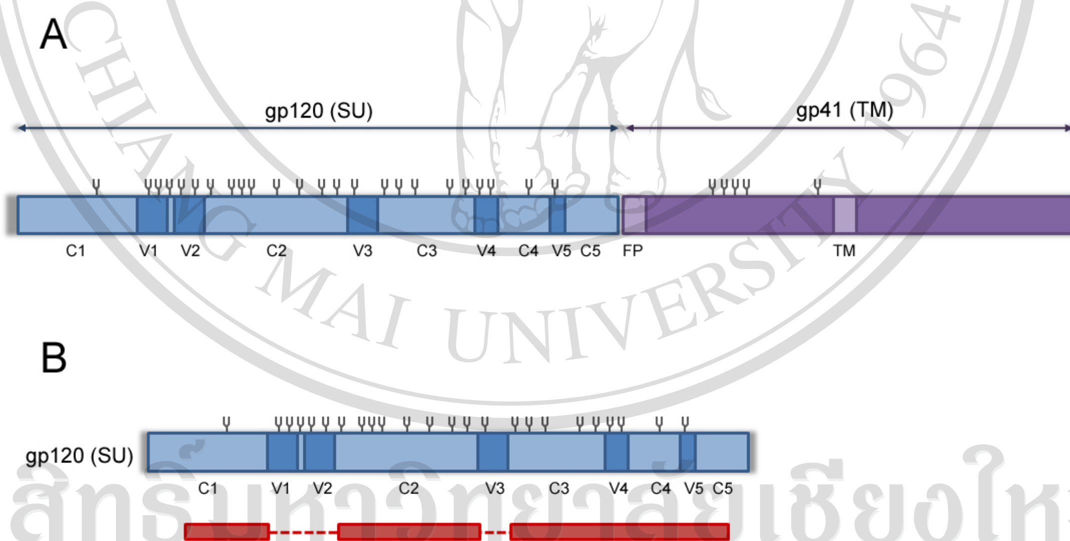

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Figure 8. Liner representation of the structure of HIV-1 envelope.

A, The gp120 and gp41 domains are indicated in blue and violet boxes, respectively. N-linked glycosylation sites are indicated by U-shaped branches. (Figure drawn for this thesis)

B, The gp120 core protein that was crystallized *(red)*. It corresponds to the almost entire gp120 that was deleted of the V1-V2 and V3 regions. (Figure modified from Chen *et al*, 2005 [75])

C1 to C5, constant regions of gp120; V1 to V5, variable region; FP, fusion peptide; TM, transmembrane region.

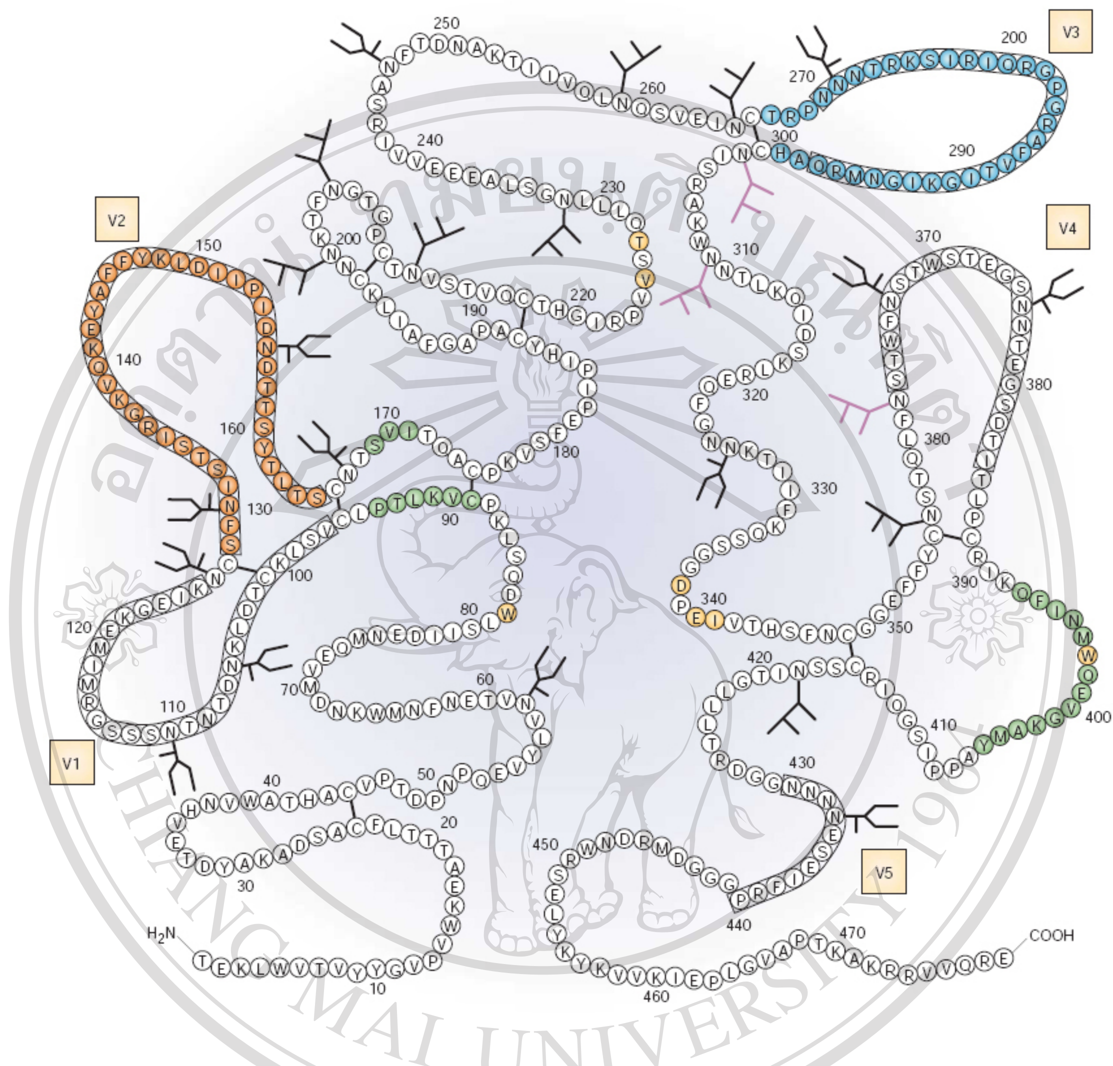

Figure 9. Diagram of the structure of HIV-1 gp120 envelope glycoprotein. The gp120 molecule with the location of the variable regions indicated in boxes (V1-V5). The glycosylation sites containing high mannose-type and/or hybrid-type oligosaccharide structures are indicated by the branched structures, and glycosylation sites containing complex-type oligosaccharide structures are indicated by the U-shaped branches. Epitopes in gp120 that induce neutralizing antibodies are highlighted in color: the highly conformational CD4-binding domain *(yellow)*, the CD4-induced epitope *(green)*, an epitope composed of α1→2 mannose residues *(purple)*, the V2 loop *(orange)* and the V3 loop *(blue)*. (Figure from Zolla-Pazner *et al*, 2004 [443])

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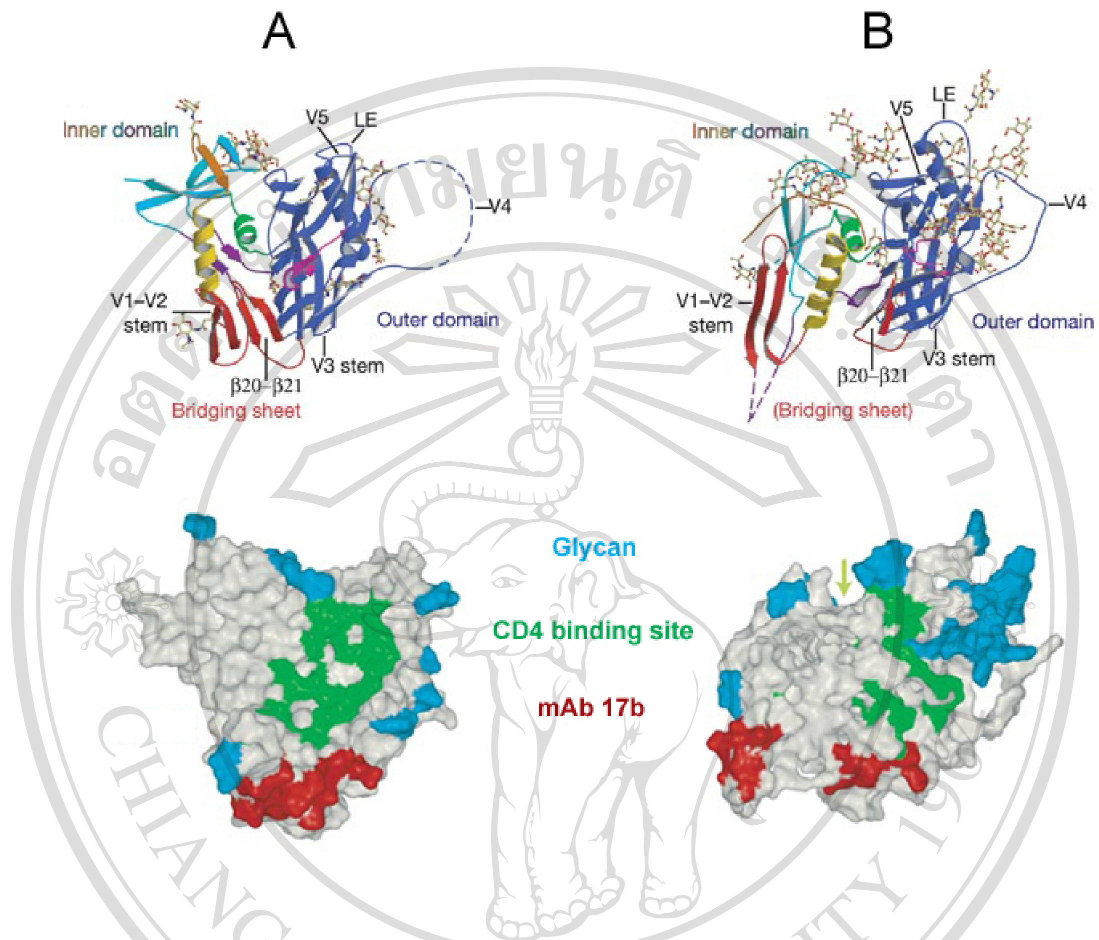

Figure 10. Comparison of the conformation and molecular surface of deglycosylated liganded HIV-1 HXB2 gp120 core with glycosylated unliganded SIV gp120 core, shows in the same view. (Figure from Chen *et al*, 2005 [75])

A, HIV-1 HXB2 gp120 core, complexed with a soluble form of CD4 and the Fab fragment of the human neutralizing monoclonal antibody 17b. CD4 and Fab have been omitted for clarity.

B, Unliganded SIV gp120 core.

Upper panels, The polypeptide chains are indicated as ribbon diagram and carbohydrates as stick models. Outer domain is in blue; inner domain, colored according to sub-structure (N terminus, *orange*; α1, *yellow*; three-strand sheet, *cyan*; outer/inner domain transition, *purple*; α5, *green*). The bridging sheet is in red and the CD4 binding loop is in pink. Lower panels, Residues in direct contact with CD4 are in green; residues contacting mAb 17b, in red; carbohydrates, in light blue. Green arrow indicates the mouth of the hydrophobic cavity.

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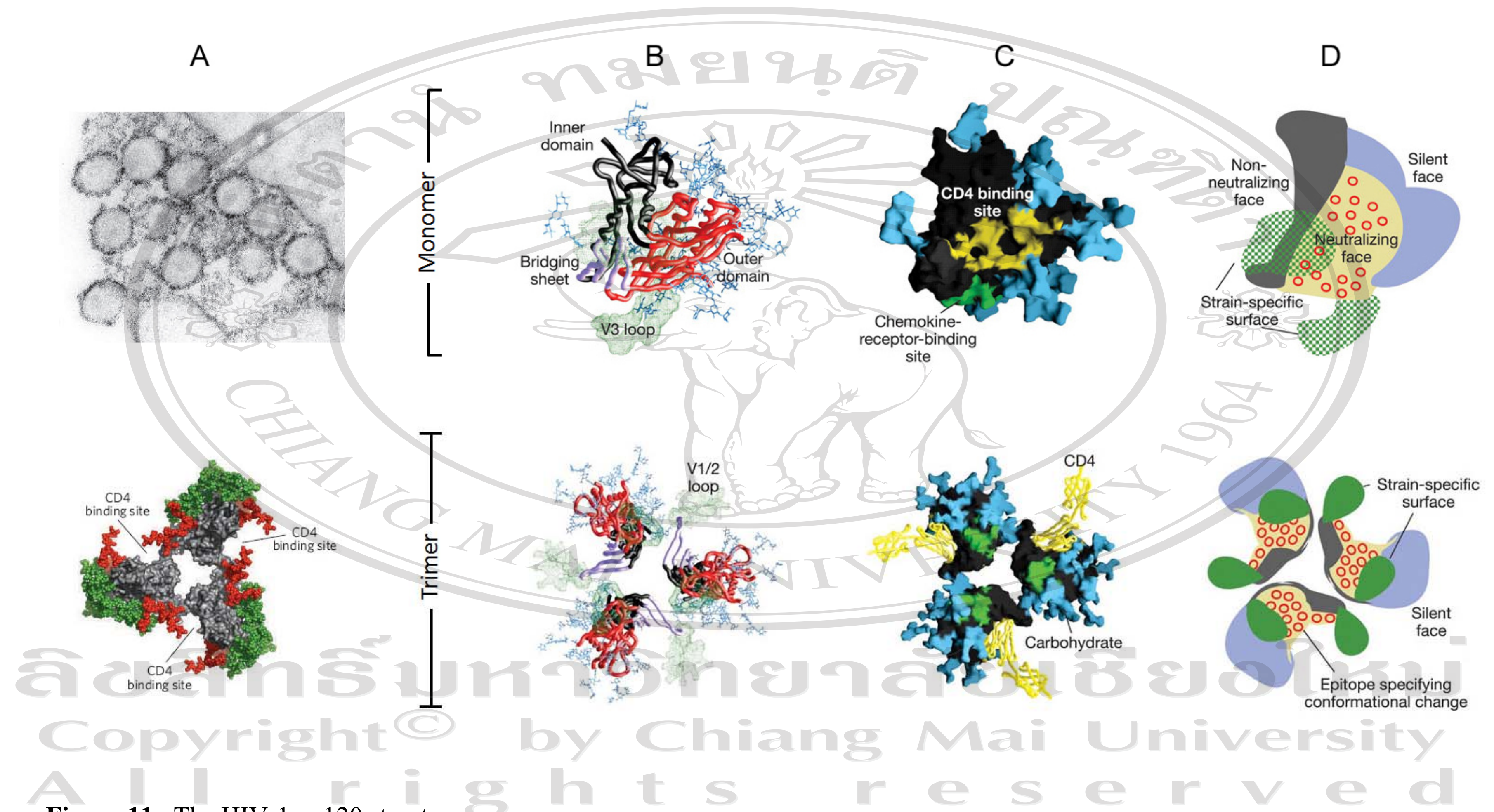

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Figure 11. The HIV-1 gp120 structure.

A, The carbohydrate shield of gp120. The upper shows an electron micrograph of virus particles. Carbohydrates are stained with ruthenium red *(dark)*. The lower shows the gp120 glycosylation surface composed of the oligomannose glycans (*green*; corresponds to the branched structures of Figure 8) which are found on the densely glycosylated outer domain, and the complex sugars (*red*; corresponds to the U-shaped branches of Figure 8) which are distributed on the more exposed receptor-binding sites and hypervariable loops. (Figure from Scanlan *et al*, 2007 [355])

B-D, The atomic structure of the HBX2 gp120 core-CD4 complex. The upper panels show monomeric gp120 and the lower panels show its trimeric form (the view corresponds to that seen from the target cell membrane). **B**, The gp120 structure. The gp120 core is displayed as Cα worm with inner domain colored in black, the outer domain in red, and the bridging sheet in violet. The approximate positions of the V1/V2 and V3 variable loops are shown in green, and the protein-proximal $(mannose)_3$ glycan cores are shown in blue. **C**, The functional surface of gp120. The HBX2 gp120 core-CD4 complex is colored by functionality: surface within 3.5 Å of CD4, in yellow; surface associated with residues identified by mutagenesis as being part of the coreceptor binding site, in green; N-linked carbohydrate, in blue; and remaining gp120 protein surface, in black. **D**, The antigenic structure of gp120: epitopes bound by non-neutralizing antibodies *(black)*, epitopes bound by neutralizing antibodies that are broadly reactive *(light green)* and neutralizing antibodies that are strain specific *(square pattern in forest green)*. The highly glycosylated gp120 surface that is rarely recognized by antibodies is colored in blue. Red circles designate those epitopes that display unusual entropy indicative of conformational change. (Figure from Kwong *et al*, 2002 [215])

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Inner domain, outer domain, and bridging sheet (Figure 10 and 11B)

The inner domain is formed mainly by the C1 and C5 regions which interact with the gp41 transmembrane unit. The inner domain surface is devoid of glycosylation [433]. In contrast, the outer domain is largely covered by glycans to shield its antigenic surface. The glycans themselves are poor target for antibodies because of their heterogeneous expression on the virus and because they are produced by the host cell, thus are self molecule. The proximal end of the outer domain includes V4 and V5 variable loops, whereas the distal end includes the base of the excised V3 loop, which interacts through hydrogen-bonds with the V1/V2 stem emanating from the inner domain. The bridging sheet stands as a peculiar minidomain in contact with, but distinct from, the inner and outer domains as well as the excised V1/V2 domain [217]. However, comparison of the liganded HIV-1 gp120 structure and the unliganded SIV gp120 shows that the respective outer domains are highly similar [75], but the conformational of the inner domain in the unliganded structure deviates significantly from those of the liganded structure (Figure 10). It also affect the formation of the bridging sheet, which links the inner and outer domains. This observation suggests that the inner domain may have significant conformational flexibility in the absence of CD4 [296]. Thus, these conformational changes from CD4 binding may be necessary to lock the coreceptor binding site and also trigger gp41 into initiating in the fusion process [296].

CD4 and coreceptor binding sites (Figure 10 and 11C)

Neither the CD4 nor the coreceptor binding sites are coherently presented on the surface of the unliganded conformation SIV gp120 core. The CD4 binding loop projects away from the centre of the outer domain and the β20-β21 segment of bridging sheet (Figure 10). The α-helices of the inner domain, the CD4 binding loop and the β20-β21 segment of bridging sheet create a long, narrow cavity, lined principally with hydrophobic side chains, in which many of the residues that are presumed to contact CD4 are located near or within this long cavity [75; 296]. The location of these conserved residues likely minimizes their immediate recognition by antibodies, while preserving the ability to contact CD4 [296]. The binding site for CD4 on the liganded HIV-1 gp120 core is located in a depression formed at the interface between the inner domain, bridging sheet, and outer domain [217]. The CD4 interacts with the face of the outer domain that is partly concealed within the hydrophobic cavity. In the conformation stabilized by CD4 binding, the bridging sheet can close up to create the coreceptor binding surface, which is flanked by the V1-V2 and V3 loops. These residues lie close together on the liganded HIV-1 gp120 structure, but the equivalent residues on the unliganded SIV gp120 structure are separated into two areas (Figure 10 and 11C). These difference are consistent with the notion that CD4 binding is required to lock these areas into a contiguous binding site, and therefore the fact that the coreceptor site is not presented until after CD4 binding suggests that the site may be susceptible to antibody recognition [296].

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Antigenicity and immunogenicity (Figure 11D)

Studies of the binding of monoclonal antibodies had led to a distinction among "neutralizing", "non-neutralizing", and "silent" faces of gp120 [433; 278] (Figure 11D). The silent face, which is composed of the outer domain, V4 and V5 variable loops, was named by Wyatt *et al*. is heavily glycosylated [433]. This heavy glycosylation may contribute to reduction of epitope exposure and to enhance viral evasion from antibodies, as they appear as self antigens to the immune system. The crystal structure of gp120 showed that most of carbohydrates are located on a single face on the outer domain of the gp120 core, and the model of gp120 trimerization suggests that this silent face is well exposed at the surface of the envelope oligomer [433; 215] (Figure 11D).

The non-neutralizing face corresponds to the inner domain of gp120 core, which is highly conserved. This face induces a strong antibody response in infected individuals, however those antibodies that bind epitopes belonging to this surface do not neutralize HIV-1 [318]. The analysis of antibodies binding to gp120 trimer suggest that this surface is buried within the gp120 trimer and is not exposed at the surface of the envelope oligomeric complex on the viral particle (Figure 11D).

The neutralizing face corresponds to the gp120 surface that interacts with receptors on the target cells [318]. However, this face is poorly accessible to antibodies (Abs). It is mainly accessible on viruses that have been adapted to immortalized T-cell line (T-cell line-adapted, or TCLA isolates). These viruses are very sensitive to neutralization, in contrast with primary isolates, which are the viruses that have been passed only a limited number of times on activated primary lymphocytes, and are much less sensitive to neutralization. This could be presumably explained by the quaternary structure of the envelope complex that differs between TCLA viruses and primary isolates. The gp120 trimer of TCLA adopts a relatively open conformation, allowing the exposure in particular of the CD4 binding site, the coreceptor binding site, and the V3 loop. In contrast, the primary isolate trimeric complex has a more closed conformation, reducing accessibility of the receptor binding sites and preventing the binding of neutralizing antibodies [278]. The primary isolates are much more representative of patient isolates than TCLA viruses.

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4.3 Gp41 structure

The HIV-1 envelope glycoprotein gp41 is a homotrimeric structure formed by three gp41 monomers, with each monomer non-covalently associated with gp120. Each gp41 molecule consists of three domains, an intracellular domain (endodomain), a transmembrane (TM) domain and an extracellular domain (ectodomain) (reviewed by Cooley *et al*, 2003 [92]).

The structure of gp41 is shown in Figures 8A and 12.

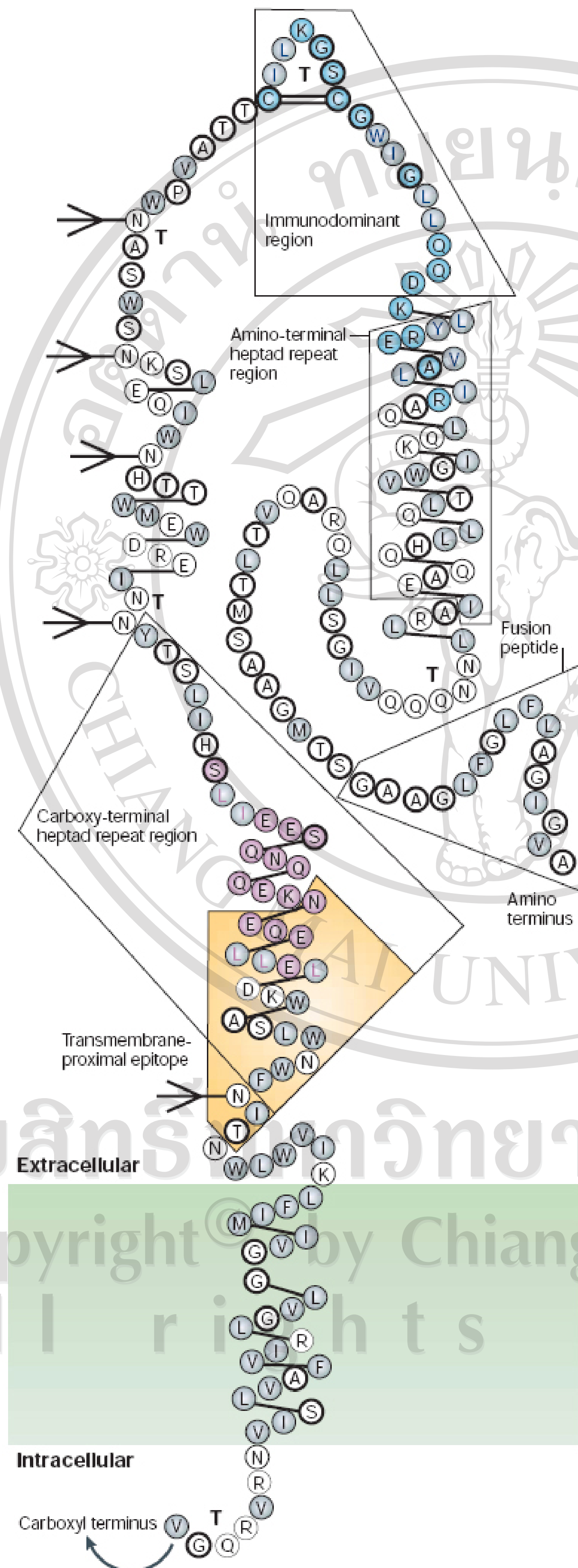

Figure 12. Diagram of the structure of HIV-1 gp41 envelope glycoprotein. The gp41 molecule with α-helices depicted as alternating three- and four-amino-acid groupings connected by single lines, hydrophobic amino acids indicated as grey circles, charged amino acids as unfilled circles and neutral amino acids as heavily outlined circles. Strong turns are indicated by 'T', and potential glycosylation sites by branched-stick figures. Key functional and structural regions are boxed, and well-characterized epitopes are shown in colour, with epitope clusters I and II shown in turquoise and pink, respectively, and the transmembrane-proximal epitope corresponding to the human broadly NAbs 2F5 and 4E10 highlighted in yellow. (Figure from Zolla-Pazner *et al*, 2004 [443])

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The ectodomain is the primary structure involved in membrane fusion. At the amino end terminus of the ectodomain is a hydrophobic fusion peptide sequence, which has been proposed to act as an insertional sequence that penetrates the cell membrane. On the carboxyl side of the fusion peptide, there are two hydrophobic heptad repeat sequences: HR1, adjacent to the amino terminus and HR2, at the carboxyl terminus and adjacent to the TM domain. These HR sequences have a leucine zipper motif suggested to form an α-helix, and are also known as N-helix (near the amino terminus) and C-helix (near the carboxy terminus). Prior to coreceptor binding the N-helices form a central three stranded coiled-coil, and are surrounded by three anti-parallel C-helices that bind to conserved grooves on the coiled-coil surface. The fusion peptide is hidden buried deep within this trimeric structure. The immunodominant region of gp41 is the region that induces high levels of antibodies that are not neutralizing but might mediate other functions such as antibody-dependent cell-mediated cytotoxicity (ADCC) and aggregation of, and complement deposition on virus particles [443].

5. HIV coreceptor usage and tropism

5.1 HIV coreceptor usage

Soon after the discovery of the CD4 molecule as the major receptor for HIV entry [100; 203], evidence started to accumulate indicating that CD4 alone was not sufficient for HIV entry into target cells. The identification of the HIV coreceptors was triggered by the studies reported in the mid-1980s and extended through the early 1990s, based on results with recombinant human CD4, indicating that the CD4 rendered cells permissive for HIV infection, but only when it was expressed on a human cell type. Maddon *et al*. showed that CD4 expressed on mouse cells allowed virus to bind but did not confer virus entry [245]. These results supported the conclusion that this restriction was due to the requirement for a cofactor of unknown identity that was specific to human cells [37]. The second phenomenon were the observations that HIV-1 isolates fell into two distinct groups depending on their phenotypes. Some HIV-1 isolates showed efficient infectivity for continuous CD4+ T cell lines, but poor infectivity for primary macrophages. This phenomenon was originally observed with isolates that had been adapted in the laboratory to replicate in T cell lines. Thus, such viruses were designated as T-cell line-tropic (TCL-tropic or T-tropic) and they were generally syncytium-inducing (SI) in assays using a highly permissive T-cell line. Other HIV-1 isolates showed the ability to infect primary macrophages much more efficiently than those T-cell lines. They were designated as macrophage-tropic (M-tropic) or non-syncytium-inducing (NSI) isolates. This characteristic was linked to the replication rate of the viruses in PBMC, where usually SI virus would replicate rapidly and highly (SI/rapid/high virus), while the replication of NSI virus would be slow and low (NSI/slow/low virus) [124].

The first HIV-1 coreceptor was identified in 1996 and was named fusin, because it mediated HIV-1 fusion [123]. The co-expression of fusin with CD4 rendered non-human cells permissive for HIV-1 Env-mediated cell fusion and infection, and the

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anti-fusin Abs potently inhibited fusion and infection of primary human CD4+ T lymphocytes. However, the Env-mediated cell fusion by fusin functioned only for T-tropic, but not M-tropic HIV-1 strains. The discovery was soon followed by the demonstration that fusin is indeed a chemokine receptor for CXC-chemokines, SDF-1α, and SDF-1β [42; 291]. Fusin was therefore renamed CXCR4 (fourth receptor for CXC-chemokines). The second HIV-1 coreceptor was identified based on the finding that the CC-chemokines (i.e. RANTES, MIP-1α, and MIP-1β) that are the natural ligands of CCR5 (fifth receptor for CC-chemokines) could block the infection of NSI HIV-1 isolate. Several groups reported CCR5 as the coreceptor for NSI viruses [20; 106; 116], while HIV-1 strains able to use both coreceptors were termed dual-tropic (D-tropic). Both CCR5 and CXCR4 belong to the superfamily of seven transmembrane (7TM) G protein-coupled receptors. More than fourteen other 7TM receptors or structural-related molecules have been identified to act as coreceptors for entry of HIV-1 *in vitro*. Currently, there is little evidence to suggest that coreceptors other than CCR5 and CXCR4 are used significantly *in vivo*.

5.2 Definition of HIV tropism

The earliest nomenclature of HIV tropism was designation based on the biological phenotype of the virus *in vitro*; the T-tropic/M-tropic, and SI/NSI isolates. *In vitro*, the HIV-1 infection is generally established by NSI isolates, which persist throughout infection, whereas SI isolates are found in only about 10% of new infection and over 50% of HIV-1 infected individuals that progress to AIDS [82; 176]. The presence of SI isolates is associated with accelerated CD4+ T cells decline, rapid disease progression and is predictive for development of AIDS, independent from other progression markers such as plasma viral RNA load and CD4+ T cell counts [176]. The major genotypic determinants for HIV-1 coreceptor usage lie in the V1-V2 and V3 variable loops of the gp120 Env glycoprotein [69; 172; 371], however the V3 region is the most critical determinant for the coreceptor use. Minimal changes in the V3 amino acid sequence are sufficient to switch coreceptor usage from CCR5 to CXCR4, and key mutations for CXCR4 usage have been identified, notably substitutions with basic residues at V3 positions 11 and/or 25 (positions 306 and/or 320 of *env* gene, based on HIV-1 HBXc2) [105; 104; 126; 127; 341] (Figure 13). An increased net charge of V3 is also associated with the use of CXCR4 by HIV-1 [17; 54; 127; 268]. The bioinformatic tools have been developed to predict HIV-1 coreceptor usage from the amino acid sequence of V3, taking into account the key amino acids at positions 11 and 25, plus other sites in V3 that differ between CCR5- and CXCR4-using strains [54; 175; 431]. Although the V3 amino acid sequence critically influences HIV-1 coreceptor usage, additional variations in the V1-V2 sequence could also influence HIV-1 coreceptor usage [69; 78; 154; 155], but relatively few sets of genotype-phenotype data are available for regions other than the V3 region. Furthermore, PNGS adjacent to and within V1-V2 and V3 have also been demonstrated to be important for coreceptor use [85; 293; 319; 321; 322].

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	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
HXB2 (V3)	C	T	R	P	G	N	N	T	R	K	S	I	H	M	G	W	G	R

	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
HXB2 (V3)	A	F	Y	A	T	G	Q	I	I	G	D	I	R	Q	A	H	C

Figure 13. V3 amino acid sequences and numbering according to HIV-1 HXB2 (GenBank Accession Number K03455). Residues at positions 11 and 25 are highlighted. (Figure drawn for this thesis)

After the identification of the coreceptor, CCR5 and CXCR4, it became clear that the NSI/M-tropic and SI/T-tropic phenotypes were linked to their differential use of chemokine receptors for HIV-1 entry. NSI/M-tropic viruses preferentially use CCR5, while SI/T-tropic viruses use CXCR4. Therefore, NSI/M-tropic virus has been classified as R5, SI/T-tropic virus as X4, and dual-tropic virus as R5X4. Moreover, the viral population at a given time in a given patient may contain heterogeneous mixtures composed of any combination of the three classes of virus, for example, a single HIV plasma sample may contain R5 as well as X4 or R5X4 viruses. Most population-based assays cannot readily distinguish these virus populations. For this reason it is convenient to use the term dual/mixed (D/M) to refer to these types of samples [86].

HIV tropism is now commonly defined based on the coreceptor usage which is defined as the ability of a particular HIV-1 virus to infect a target cell using a specific coreceptor, CCR5, CXCR4 or either. The HIV-1 viruses can be characterized into four classifications, inculsing R5, X4, R5X4 and mixed viruses. The HIV-1 coreceptor usage and tropism are summarized in Figure 14.

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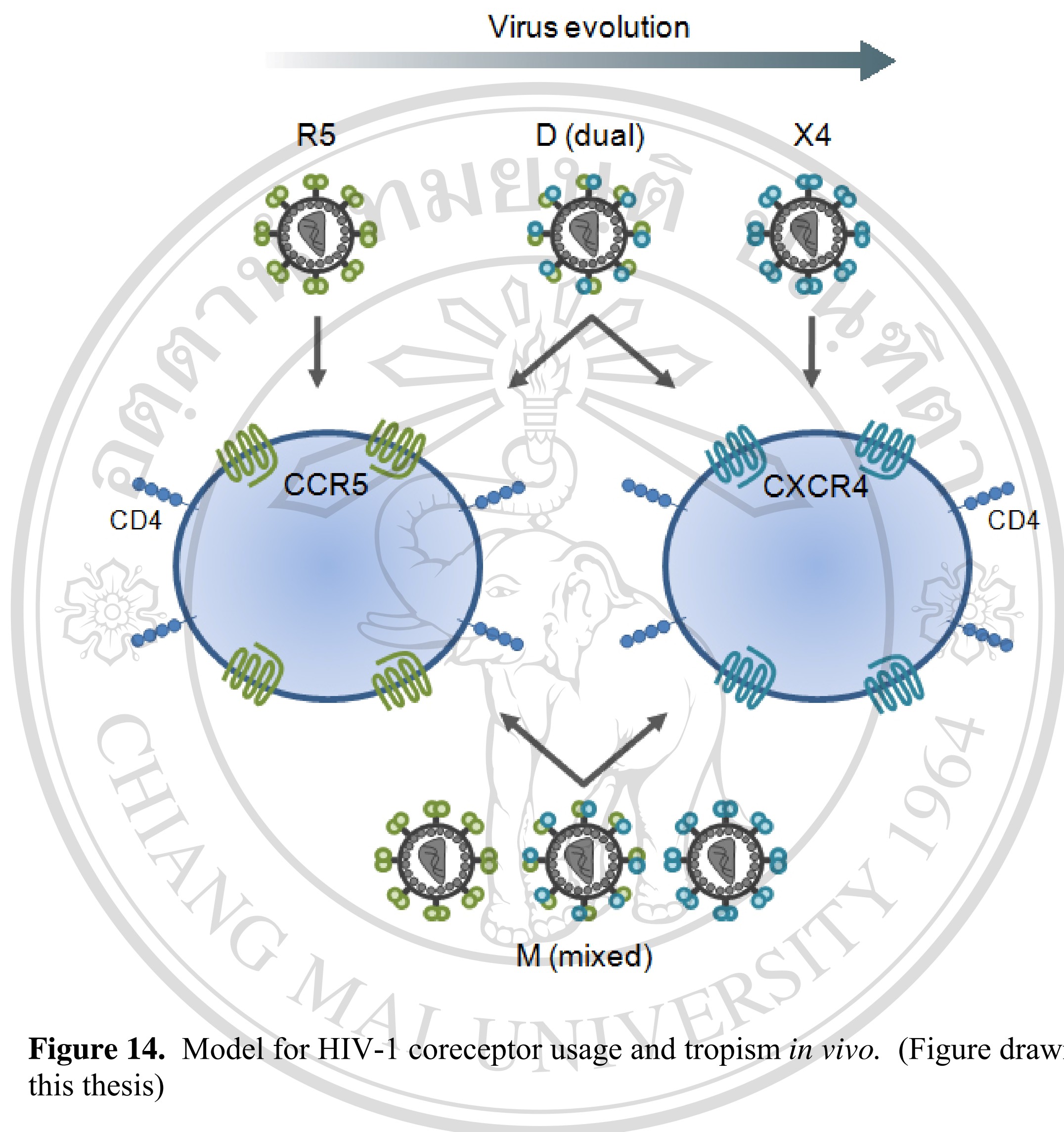

Figure 14. Model for HIV-1 coreceptor usage and tropism *in vivo*. (Figure drawn for this thesis)

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PART II. Genetic variability of HIV

1. Classification of HIV

1.1 HIV types

HIV is a member of the lentivirus genus of the *Retroviridae* family. The name lentivirus means slow virus, because these viruses take a long time before to induce the full-blown disease. Lentiviruses have been found in many African primate species, however, new world primates and Asian primates have not been found to be naturally infected with lentiviruses. Lentivirus isolates from humans are grouped into one of two types, designated HIV-1 and HIV-2, on the basis of serologic properties and sequence analysis of their viral genomes. HIV-1 and HIV-2 share 40% to 50% genetic homology, with the greatest sequence divergence in the *env* gene. HIV-1 is the result of cross-species transmission of simian immunodeficiency viruses (SIVs) isolated from chimpanzees (SIVcpz) [304; 137; 157], while HIV-2 is most closely related to a virus found in sooty mangabeys (SIVsm) [77; 76]. The worldwide epidemic of HIV and AIDS is caused by HIV-1 and generally when people refer to HIV without specifying the type of virus they will be referring to HIV-1. HIV-2 is concentrated in West Africa and is rarely found elsewhere.

1.2 HIV type 1

Groups M, O and N, and subtypes

Based on the phylogenetic analysis of numerous strains of HIV-1 isolated from diverse geographical origins, HIV-1 is subdivided into groups, subtypes, sub-subtypes, and circulating recombinant forms (CRFs) or unique recombinant forms (URFs). A phylogenetic tree representing the HIV-1 genetic diversity is shown in Figure 15. Groups refer to the very distinctive HIV-1 lineages. HIV-1 can be classified into three groups: M (major), O (outlier), and N (new, or non-M, non-O). Most HIV-1 infections globally are caused by group M viruses, whereas groups O and N are much less common. Group O has been endemic in Cameroon and neighboring countries in West Central Africa, but even there the virus represents a minority of HIV-1 strain, with a prevalence less than 10% of HIV-1 infections in Cameroon [437]. It has spread to Europe where its prevalence is still very low [74; 306; 31]. Group N has been also identified in Cameroonian patients and it is only represented by a limited number of isolates from Cameroonian patients [26; 373]. Group M has been classified into subtypes, which refer to the distinctive lineages within group M. There are 9 subtypes, denoted with letters primarily in the order of discovery, designations A to K (with no subtypes E and I). Subtypes A and F are further subdivided into sub-subtypes, denoted with numbers, including sub-subtypes A1 to A3 and the recently indentified A4 [412], and sub-subtypes F1, F2. Sub-subtype designations will be used to describe distinctive lineages that are not genetically distant enough to justify designating a new subtype. Usually the subtype should be

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assigned after analysis of the entire HIV genome; however, in most of the cases only one gene, particularly the env gene, is used for rapid identification. Within group M, the average intersubtype genetic variability is 25% for the *env* gene and 15% for the gag gene [395; 385; 58]. Subtypes within groups O and N are not yet identified. The diversity of sequences within group O viruses is nearly as great as the diversity of sequences in group M viruses, but a phylogenetic analysis of *gag* and *env* genes does not reveal clades of virus as clearly as the clades detected in the HIV-1 M group.

Intergroup recombinants

Although cases of dual infection with HIV-1 and HIV-2 have been reported, the recombination between the two types of HIV has not yet been described [98; 114]. Because of the high degree of divergence, the recombinations between group M and O viruses were not expected, but intergroup recombinants have been recently reported from Cameroon [305; 386]. Group M/O mosaic viruses can replicate well *in vivo* and *in vitro*, and can even become the predominant variant within the patient's viral population [305]. These observations illustrate how distant SIVs and HIVs can recombine, particularly in individuals who are exposed to SIV by cross-species transmission [303; 427].

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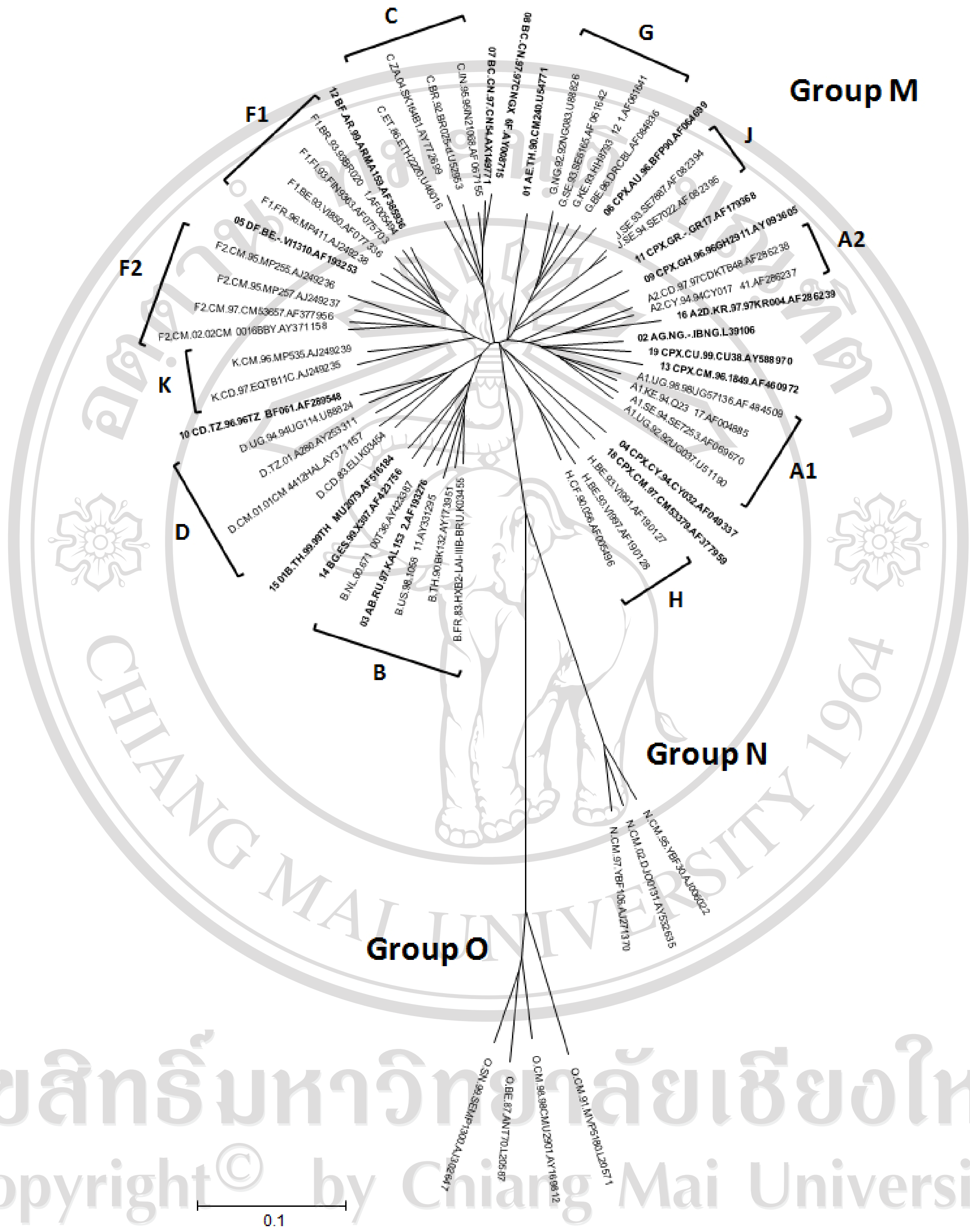

Figure 15. Phylogenetic relationship of HIV-1 group M, N, O, and CRFs. The group M subtypes A-K are indicated at the respective cluster. The tree was based on subtype and CRFs reference sequences corresponding to the *env* gene obtained from HIV database (www.hiv.lanl.gov). The groups/subtypes/CRFs, countries and years of isolate, and Genbank accession number are indicated in each reference sequences, and those from CRFs are indicated in bold characters. (Figure drawn for this thesis)

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Circulating recombinant forms (CRFs)

Sequencing full-length genomes have led to the identification of intersubtype recombinants. These are the result of recombination between subtypes, within an individual patient with HIV-1 multiple infections of two or more subtypes simultaneously. The intersubtype recombinant genomes become designated as CRFs, if; i) the identical recombinant viruses are identified in at least three epidemiologically unlinked people, ii) are characterized by full-length genome sequencing that share the same recombinant structure, and iii) form a monophyletic cluster in all regions of the genome; and as URFs, if only a single or two sequences are available [345]. The CRFs are named with a number sequential in the order in which they are reported in the literature and followed by the letters of the subtype involved, starting with CRF01_AE (Figure 16). If the recombinants consist of more than two subtypes involved, they are therefore replaced by designation "cpx", denoting complex, e.g. CRF04_cpx (A, G, H, K, U). Taxonomically, the CRFs are at the same level as the subtype. Currently, 43 CRFs (CRF01 to CRF43) have been engaged in HIV database at Los Alamos National Laboratory (www.hiv.lanl.gov), most of them having been described in Africa. Some CRFs, e.g. CRF01_AE and CRF02_AG, play important roles in regional epidemics (Figure 17A). The generation of intersubtype recombinant may occur in a setting of subtype intermixing within a population. New CRFs have often been identified in epidemics among intravenous drug users (IDU), possibly reflecting multiple intravenous exposures in the setting of relatively closed social networks, e.g. CRF03_AB, CRF07_BC, CRF08_BC, CRF14_BG, and CRF35_AD [261; 389].

Subtype "E" viruses, which are prevalent in Thailand and neighboring countries in Southeast Asia, have been first identified as a new subtype in 1992 in Thailand [262; 294]. Initially, the subtype "E" was defined based on the *env* gene sequence. Then, when the *gag* gene and the complete genome from these isolates were sequenced, it was found that regions of the genome other than the *env* gene were more similar to subtype A, so subtype E was redesignated as CRF01_AE [243; 68; 140]. Comparison of the Thai subtype E and the viruses from the Central African Republic (CAR) and Cameroon indicated that they derived from a common A/E recombinant ancestor, presumably originating in Central Africa, where both subtype A and the parental (non-recombinant) subtype E must have coexisted [283; 290; 140]. The data suggest that the CRF01_AE clade originated in Africa prior to spread in Thailand. Similarly, subtype "I" was originally described based on *env* gene fragment from a virus isolated in Cyprus and was placed in a new HIV-1 subtype I, which is now designated as CRF04_cpx. The CRF04_cpx was classified as an A/G/I recombinant [139], but it has been found recently to be an even more complex mosaic comprised of subtypes A, G, H, K and unclassified regions (U) [297].

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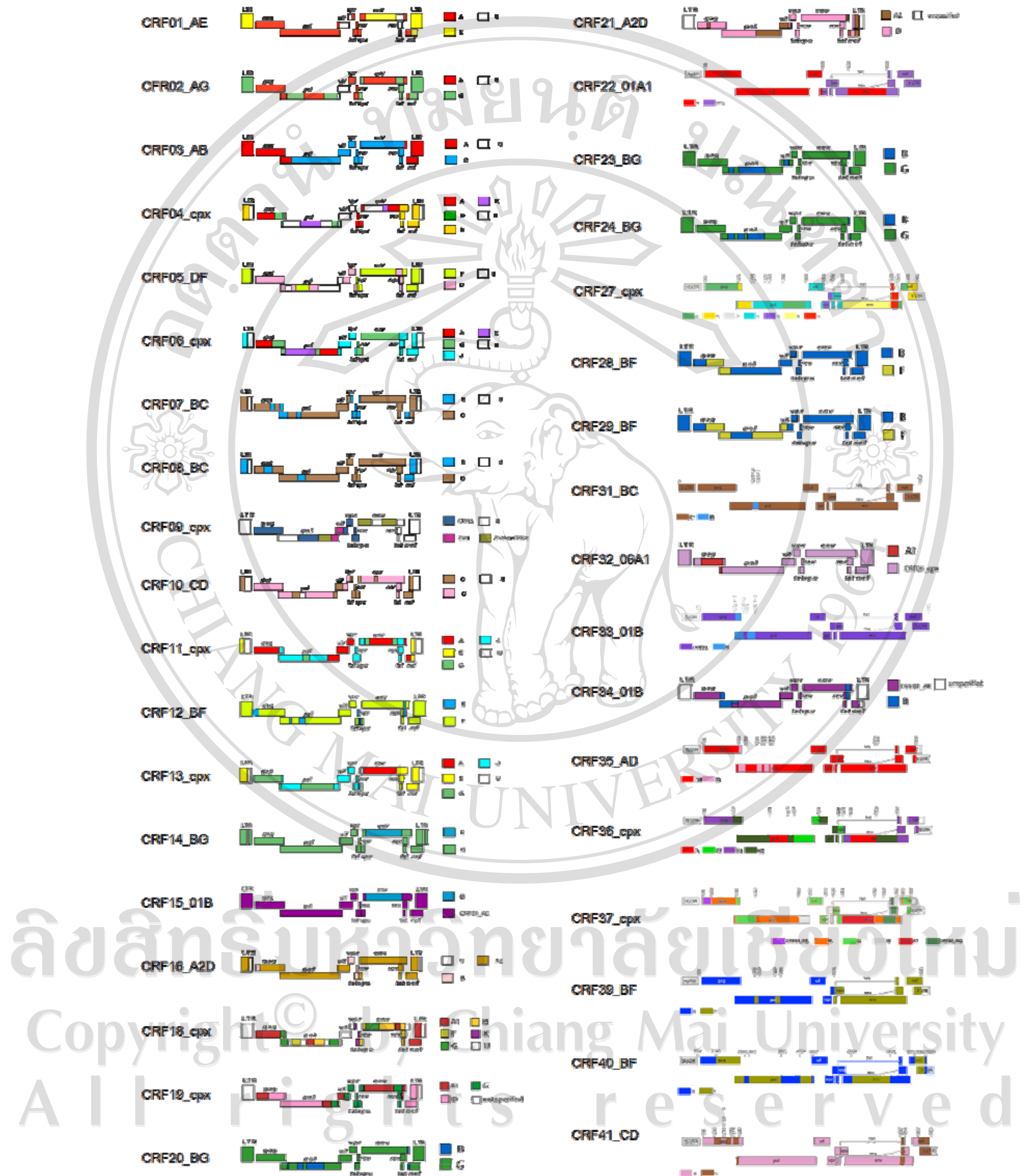

Figure 16. The mosaic pattern of the CRFs available in HIV sequence database (Figures from www.hiv.lanl.gov; 2 September 2008)

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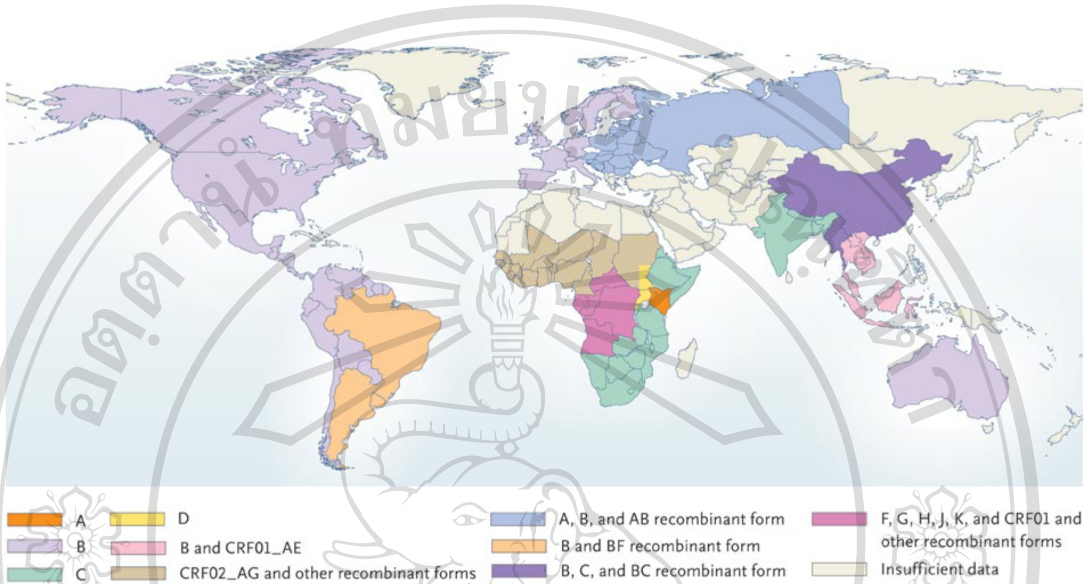
A



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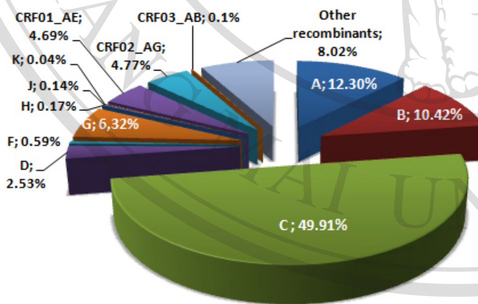

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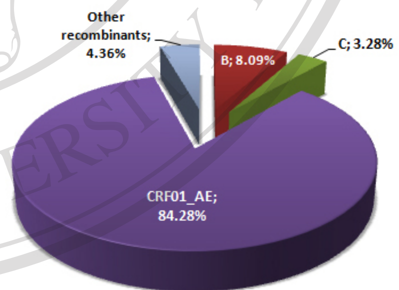

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Figure 17. Global distribution and prevalence of HIV-1 subtypes and recombinant forms.

A, Distribution within each region. (Figure modified from Taylor *et al*, 2008 [388])

B, World global prevalence.

C, Prevalence in South and South-East Asia (excluding India).

(Figures B and C from Hemelaar *et al*, 2006 [161])

1.3 HIV type 2

Like HIV-1, phylogenetic clusters have also been described for HIV-2. Currently eight groups, designated as A to H, have been reported [101]. The majority of HIV-2 sequences in the database are groups A and B, which are circulating in the human population, while groups C to H represent only few unique infections. The nucleotide and amino acid sequence diversity of HIV-2 viruses is greater than the diversity of the HIV-1 group M viruses [219]. The geographical distribution of HIV-2 is less extensive than that of HIV-1. It is restricted primarily to West Africa, although the prevalence of HIV-2 is a growing concern in certain parts of Europe and in the south-western region of India. In Guinea-Bissau, a former Portuguese colony, HIV-2 prevalence ranges 8 to 10% [326; 422] and reaches 28% among commercial sex workers [146; 226]. In addition to West Africa, countries with past socio-economical links with Portugal, including Southwest India, Angola, Mozambique and Brazil, have significant numbers of HIV-2 infections [335]. Portugal has the highest prevalence of HIV-2 infection in Europe, accounting for around 10-13% of total HIV infections and HIV-2 is responsible for 4. 5% of AIDS cases [Soriano, 2000]. In Asia, 95% of the reported HIV-2 cases are from India [194; 309].

2. Global distribution of HIV-1 strains

HIV-1 genetic subtypes are unevenly distributed in different geographical locations. According to recent studies, the most prevalent HIV-1 subtypes are subtypes A, B and C [161]. Subtype C accounts for 50% of all HIV-1 infections worldwide, while subtypes A, B, D and G account for 12%, 10%, 3%, and 6%, respectively. The subtypes F, H, J and K together account for approximately 1% of infections. The circulating recombinant forms CRF01_AE and CRF02_AG are responsible for 5% of cases, and CRF03_AB for 0.1%. Other recombinants account for the remaining 8% of infections. All recombinant forms taken together are responsible for 18% of infections worldwide. The global distribution and prevalence of HIV-1 worldwide are shown in Figure 17A and 17B. This distribution reflects the recent/present situation and might be susceptible to modifications in the next years or next decades.

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Subtype A viruses are predominant in areas of central and eastern Africa (Kenya, Rwanda, Uganda, and Tanzania) and in eastern European countries formerly constituting the Soviet Union. Subtype B, which is the most widely disseminated subtype, is predominant in North and Latin America, the Caribbean, Europe, and Australia. It is also common in several countries of Southeast Asia, North Africa, Middle East (Israel), and among South-African and Russian homosexual men. Subtype C is the overwhelming majority strain in southern Africa, Ethiopia and India. Subtype D viruses are found principally in East Africa and to a lesser extent in West Africa. CRF01_AE and subtype B co-circulate in South-East Asia, whereas CRF02_AG, along with other recombinants, dominates in West and West Central Africa. In South America, the epidemic is a mixture of subtype B and BF recombinants, with a small proportion of subtype C infections. In East Asia subtypes

B, C and BC recombinant strains dominate. Central Africa harbors a complex mixture of rare subtypes (F, G, H, J and K) and recombinants, without any predominant strain. In some regions of the world, less information is available about HIV dissemination, particularly Central Asia.

In South-East Asia, the epidemic is dominated by CRF01_AE, which is responsible for 84% of all infections (Figure 17C). Other recombinants accounted for 4%, bringing the combined proportion of CRF and other recombinants to 89%, the highest in the world. In Thailand, Cambodia and Viet Nam, CRF01_AE is responsible for more than 95% of infections, with the remaining infections caused by subtype B and other recombinants. In Myanmar, CRF01_AE accounts for 52% of infections, along with subtype B (24%), C (12%) and other recombinant (12%) [161].

3. Mechanisms of genetic diversity

The high genetic variability and rapid evolution of HIV are important factors in its worldwide spread. HIV genetic heterogeneity originates from the high mutation and recombination rates of the reverse transcriptase enzyme combined with a high turnover rate. This results in genetically diverse populations of viral species in each infected individual, term “quasispecies”. The viral quasispecies in an infected individual can differ by up to 10% [161]. Selection pressures exerted by the immune system or antiviral drugs result in further viral evolution.

3.1 High mutation rate of reverse transcriptase

Like all RNA viruses, the inability of the reverse transcriptase (RT) to correct misincorporations leads to a high error rate. The RT lacks a proofreading function. It does not contain a 3’ exonuclease activity capable of excising misincorporated nucleotides and is more error prone than cellular DNA polymerases that are capable of proofreading. The error rates of RT have been estimated in many different types of assays. A broad range of mutation rates, 10^{-3} to 10^{-4}, has been reported and it has been demonstrated that the mutation rate of HIV-1 was 3.4×10^{-5} during a single cycle of replication [251]. Recently, the mutation rate of the HIV-1 genome has also been directly measured by analyzing complete or near-full-length genomes. This high error rate was 5.4×10^{-5} mutations per base pair per replication cycle [138]. Since the HIV-1 genome is approximately 10^4 base pairs in length , this rate would translate that on average 1.1 mutations (range, 0 to 3) are generated in each viral genome during one infection cycle. In other words, most newly infected cells contain, on average, a provirus that differs from the previous cell by one mutation. Since billions of cells are newly infected every day during chronic HIV infection, the potential exists for every possible point-mutant to be generated thousands of times each day in an infected individual [89].

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3.2 High replication rate of HIV

The high replication rate of HIV increases the probability of generating viral quasispecies. It was estimated that the virus producing cells have a life-span on average of 2.2 days (half-life approximately 1.6 days) and plasma virions were estimated to have a life-span of 0.3 days (half-life approximately 0.24 days), with the production of 10.3×10^9 virions per day [307; 164]. The minimum duration of the HIV-1 life cycle *in vivo* is 1.2 days on average, and the average HIV-1 generation time, defined as the time from release of a virion until it infects another cell and causes the release of a new generation of viral particles, is 2.6 days [307]. Therefore, millions of viral variants are produced within any infected person in a single day. However, most of these generated viral mutants are probably lethal or crippled, which may explain why most viral particles in cell culture and in plasma are not infectious [310; 189].

3.3 Recombination

Requirements for the recombination process

Recombination of HIV may occur when a cell becomes infected with two or more genetically distinct HIV virions, an event also known as dual infection. These virions may be of the same subtype (producing intrasubtype recombination) or of different subtypes (producing intersubtype recombination). Although an HIV-infected cell becomes resistant to dual infection due to the down-regulation of the CD4 molecule [311], fluorescence *in situ* hybridization study of splenocytes isolated from patients showed the presence of up to eight proviruses per cell [186]. Dual infection (similar as double infection) occurs when an individual is infected with strains derived from two different individuals. The dual HIV infections can be divided into co- and super-infections. Co-infection is defined as simultaneous infection with two heterologous strains before an immune response has developed and antibodies are detectable in the blood (before seroconversion). Thus, co-infection would occur within the first month of infection. Superinfection is defined as infection with a second strain after the initial infection and the immune response to it has been established, after initial seroconversion.. A schematic representation of HIV dual infection relative to the different stages of the infection and the plasma viral load is shown in Figure 18. However, because the two parental viruses must be able to infect the same cell, the recombination presumably does not occur within all individuals living with HIV when a dual infection occurs [41]. In addition, at the level of the viral particles assembly, the different genomic RNA molecules must be able to form a functional dimer recognized by the viral packaging machinery [41].

The recombinant viruses are most easily identified and characterized if they involve viruses of different subtypes (intersubtype), but for recombinant viruses issued from the same subtype, current methods allowing reliable identification of intrasubtype recombinant viruses are still cumbersome. Unfortunately, the biological consequences of recombination, including naturally occurring recombinant viruses,

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and how it affects the viral phenotypes such as cell tropism, drug resistance, replication kinetics, disease progression, and transmission, have not been fully elucidated.

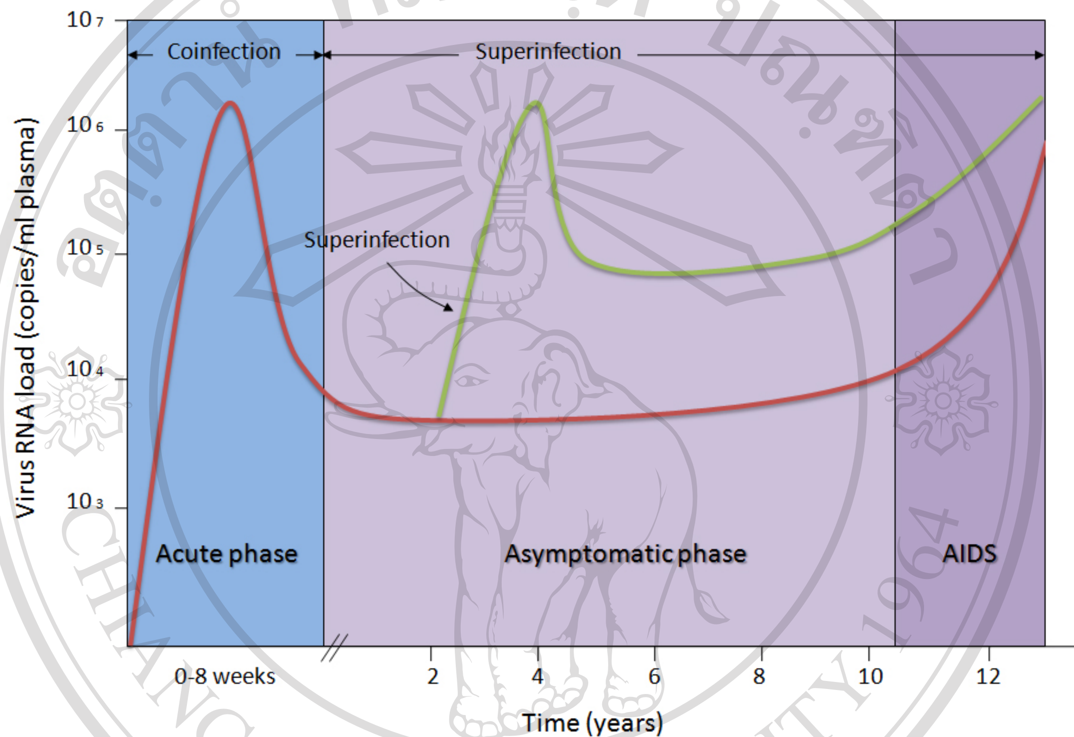

Figure 18. Type of HIV dual infection and plasma viral load at different clinical stages. The dual infection during the acute phase is called coinfection, and after seroconversion it is referred to as a superinfection. HIV superinfection often results in an increase, sometimes temporary, of the viral load *(green line)*. (Figure drawn for this thesis)

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Mechanisms of recombination

Recombination requires one round of viral replication in which one of each parental RNA genome is packaged into the same virion, producing a *heterozygous particle*. Subsequently, whenthe heterozygous particle infects a new host cell, both RNA are copied into DNA by RT and recombination occurs during this step. The generation of HIV recombinant in the context of coinfection with two HIV strains is shown in Figure 19. The mechanisms of recombinant DNA molecules have been proposed to arise during synthesis of either the first or the second DNA stand.

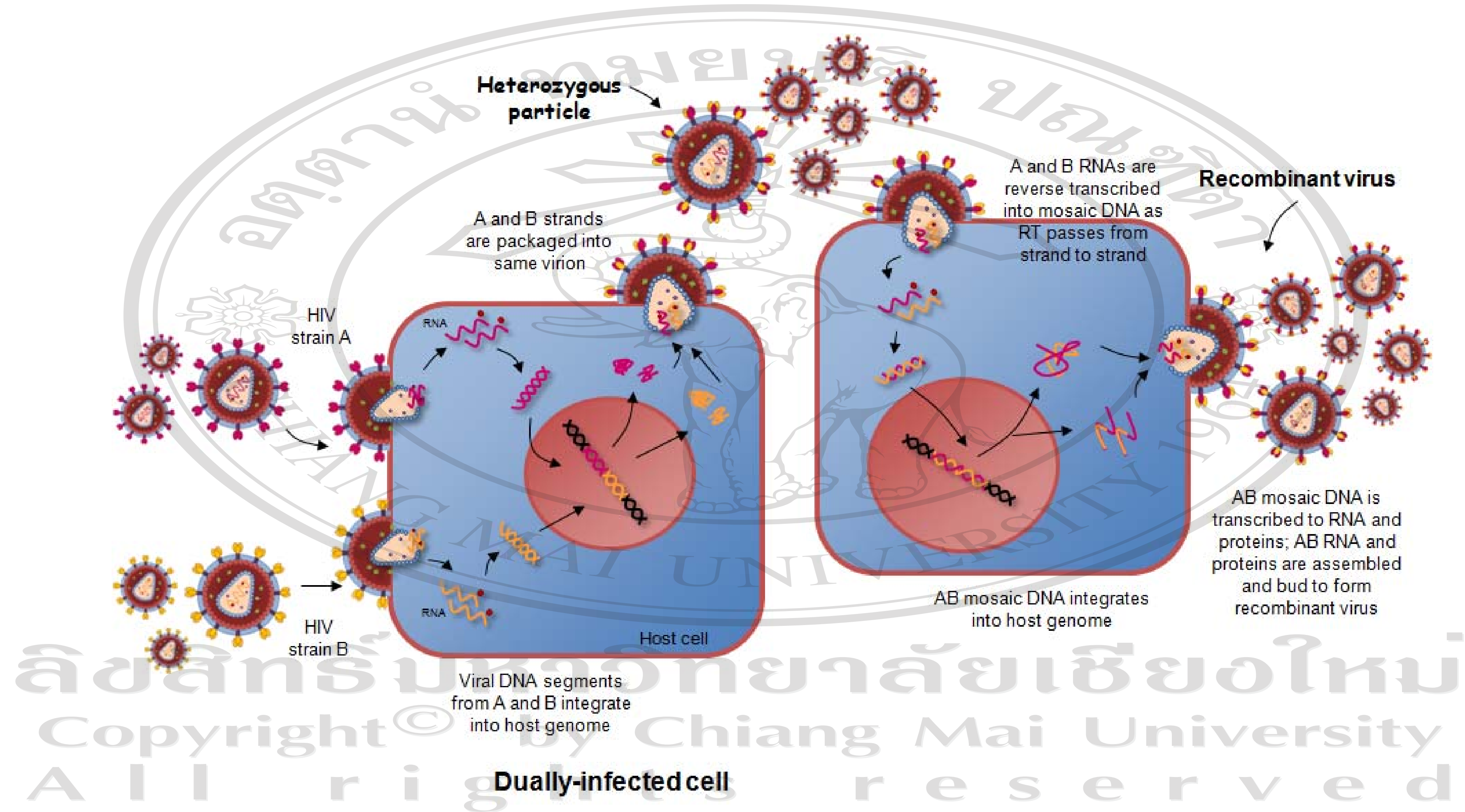

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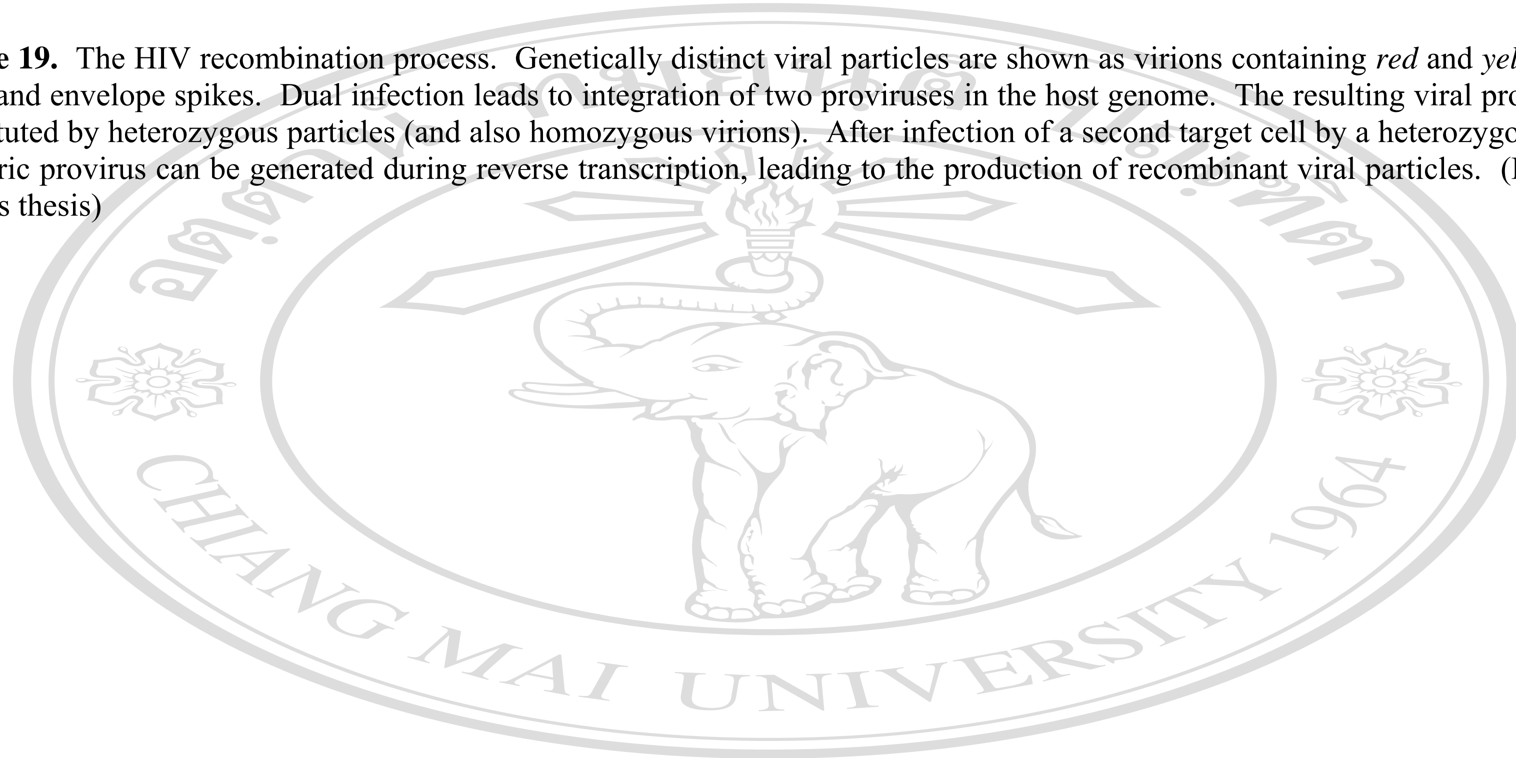
Figure 19. The HIV recombination process. Genetically distinct viral particles are shown as virions containing *red* and *yellow* genomic RNA and envelope spikes. Dual infection leads to integration of two proviruses in the host genome. The resulting viral progeny will be constituted by heterozygous particles (and also homozygous virions). After infection of a second target cell by a heterozygous particle, a chimeric provirus can be generated during reverse transcription, leading to the production of recombinant viral particles. (Figure drawn for this thesis)



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Forced copy-choice model

The forced copy-choice model, described by Coffin [88], proposes that recombinants are formed by template switching during first (minus) strand DNA synthesis from the viral RNA template. This model was formulated in part to explain how retroviruses can survive the numerous breaks typically observed in virion RNA preparations. Since the breaks are random, the reverse transcriptase can switch template from one to the other copy of genomic RNA. If the two molecules are not identical, this leads to genetic recombination. This model can also explain why one virus particle may give rise to only one DNA provirus [169; 295]. The forced copy-choice model is shown in Figure 20A. The RNA template that is copied before the switch is defined as the donor RNA, while the one onto which synthesis is transferred is called the acceptor. During reverse transcription the annealing of the nascent DNA to the acceptor RNA, a process often referred as docking, the RNase H activity of the RT degrades the genomic RNA once copied, generating an extensive region of single-stranded DNA complementary to the second copy of the acceptor RNA. For the transfer to occur, the acceptor RNA must not have been reverse transcribed in the region of transfer. This would ultimately lead to the transfer of DNA synthesis on the acceptor RNA (strand transfer), resulting in template switching. Several mechanisms have been proposed as triggers for template switching. First, the presence of breaks on the genomic RNA within the viral particle would force DNA synthesis to be transferred onto the other copy of genomic RNA (Figure 20B) [196; 451; 452]. The break would allow extensive degradation of the RNA template by the RT-encoded RNaseH activity, leading to shortening of the heteroduplex and, eventually, to the melting of the nascent DNA from the donor RNA. The nascent DNA would then anneal onto the acceptor RNA, and DNA synthesis would be restored. Second, the presence of a strong pause site or stop signal (pause-driven) triggers for template switching (Figure 20C) [451; 452]. Third, the presence of structured regions on the RNA template in which the template switching occurred within the hairpin region (Figure 20D) [451; 452].

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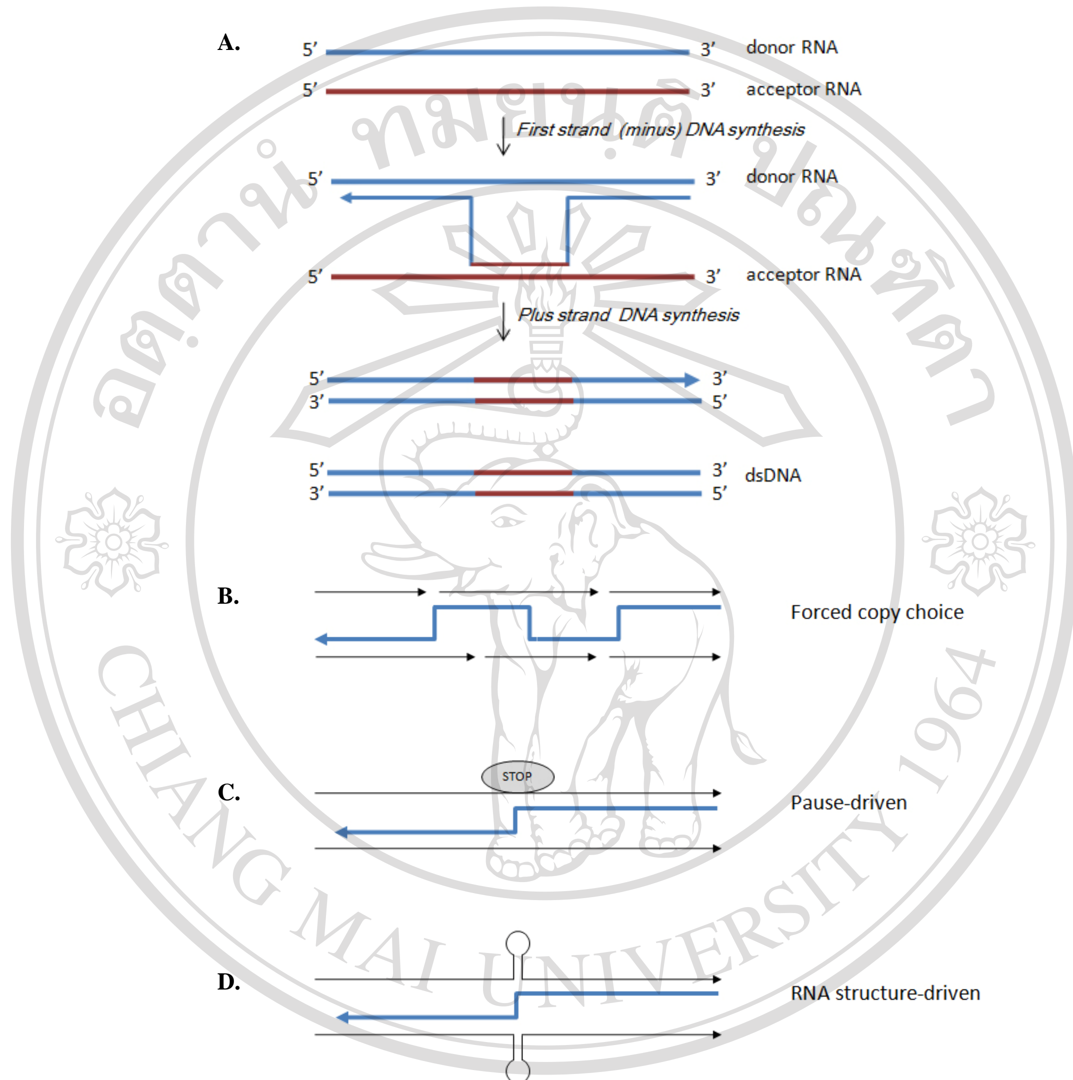

Figure 20. Forced copy-choice model. (Figures modified from Galetto *et al*, 2005 [452])

A, Schematic representation of copy-choice.

B, Forced copy-choice: the break on the genomic RNA are shown as discontinuity in the lines.

C, Pause-driven: the stop signal is indicated only on one RNA, although it might be present on both templates.

D, RNA structure-driven: a structured region of the genomic RNA is schematically indicted as hairpin, and is drawn in both templates.

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Strand displacement-assimilation model

This model proposes that recombination occurs by plus strand viral DNA exchange. It is based on the observation that plus DNA synthesis is discontinuous and "H-like" structures were observed by electron microscope after disruption of virions that had been permeabilized to allow reverse transcription [187]. The strand displacement-assimilation model is shown in Figure 21. This model is consistent with several biochemical features of retrovirus replication. The DNA minus strand copied from the RNA genome starts at a unique primer (tRNA) and is continuous. However, internal plus strand DNA synthesis starts at many sites on the minus strand DNA template from RNA primers that are produced by RNaseH digestion of the genomic RNA. The newly synthesized DNA plus strands are at first short and incomplete, and single-strand gaps are present [187; [188]. As plus strand DNA synthesis continues these gaps are filled. However, DNA synthesis does not stop when the 5' end of an adjacent plus strand is encountered. Instead, the 5' end strand is displaced by continued synthesis, forming a single-stranded tail. Thus, the "H-like" structure is formed when these free tails invade the DNA being synthesized on the second genome.

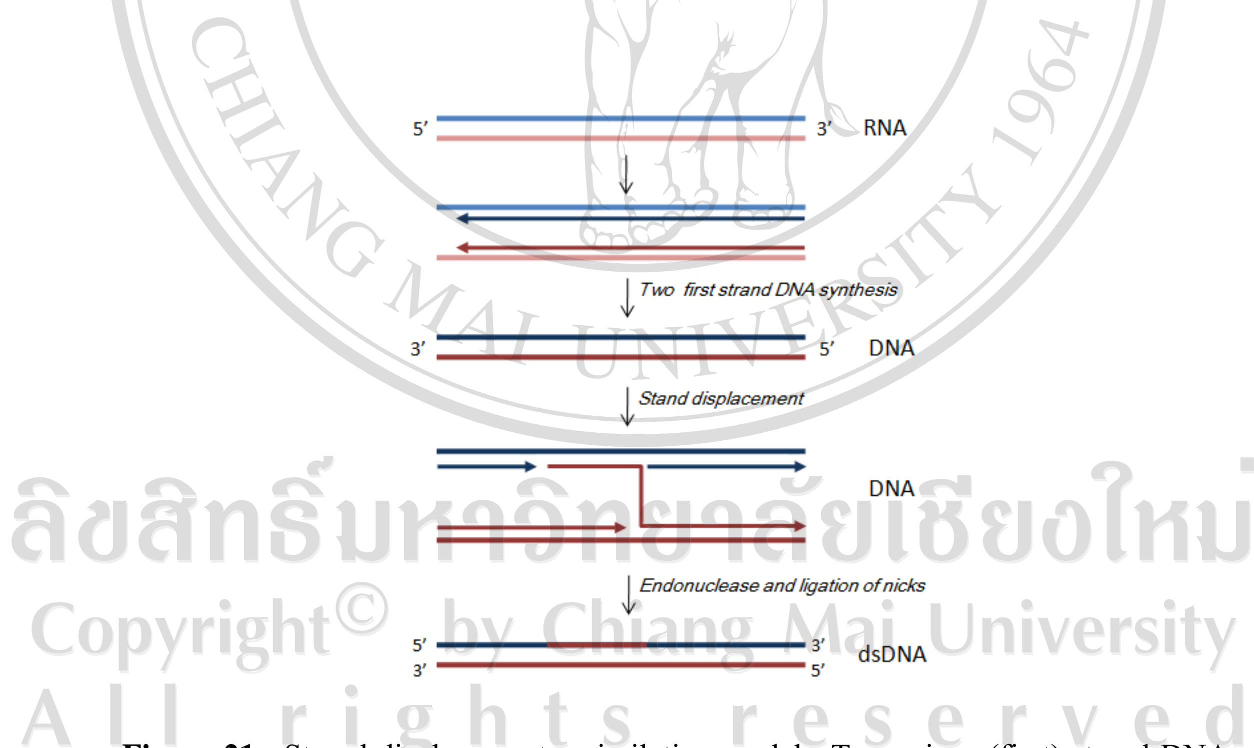

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Figure 21. Strand displacement-assimilation model. Two minus (first) strand DNA (*dark blue and red arrows*) are synthesized utilizing two different RNA templates (*blue* and *pink*, respectively). Plus (second) strand DNA synthesis accompanied by strand displacement and assimilation of single strand DNA tail onto another DNA. After endonucleolytic cut and ligation of the nicks, the resulting product will be a DNA heteroduplex. (Figure modified from Galetto *et al*, 2005 [452])

Distribution of recombination breakpoints

The HIV-1 recombination rate was approximately two to three recombination events per genome per replication cycle, and crossovers or breakpoints were identified throughout the viral genome [177]. A recent study found that recombination breakpoints are non-randomly distributed across the genomes of HIV-1 intersubtype recombinants by analyzing the position of the breakpoints found in sequences of the Los Alamos Database (http://www.hiv.lanl.gov/) [453]. Two recombination prone regions or “hot spots” were identified. They are located at the borders of the *env* gene around the first exon of *tat*, *vpu* and the beginning of *env*, and the second exon of *tat*, *rev*, and the 3’ end of *env* (Figure 22). However, comparing the recombination breakpoints across gp120 indicated that that the C2 region also is a hotspot for recombination [29; 135].

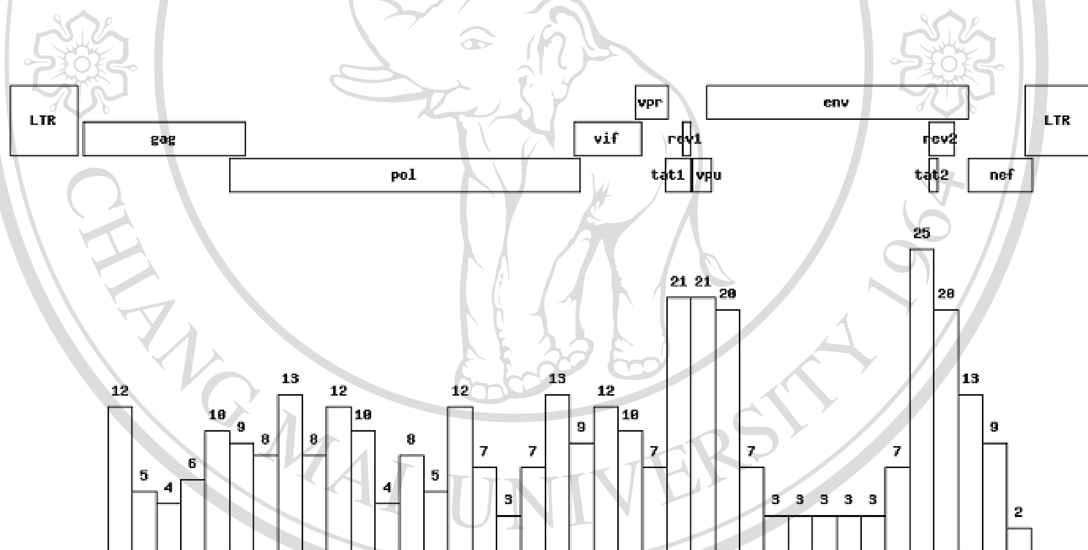

Figure 22. The distribution of breakpoints across HIV-1 genome. The diagrammatic representation of HIV-1 genome is shown in upper panel. The bars and the number of breakpoints detected in sequences of the Los Alamos Database (http://www.hiv.lanl.gov/) is shown in lower panel. (Figure from Jun *et al*. 2007 [453])

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4. Implications of HIV variability

4.1 Transmission and disease progression

The association between HIV-1 subtypes and risk of transmission or disease progression has been difficult to define. This was due to the fact that most studies concerned small numbers of patients or short follow-up. However, the HIV-1

epidemic is sustained by different subtypes and within each region, segregation of subtypes to different risk groups has been reported. For example, the co-circulation of subtype B among IDUs and CRF01_AE among heterosexuals was originally described in Thailand [140]. The segregation of subtype B to homosexuals and subtype C to heterosexuals was also described in South Africa [408], and more recently in Argentina among MSM, sustained by subtype B, and heterosexuals and IDUs, sustained by BF recombinant [25]. Several studies have reported that HIV-1 subtypes may differ with respect to viral load [168; 151], coreceptor usage [402], and transcriptional activation levels [174]. The HIV strains capable of using CCR5 are more frequently transmitted than strains that use CXCR4, however X4 viruses emerge later in infected patients and are associated with more rapid disease progression [36]. It was found that HIV-1 subtype D used CXCR4 more frequently in early infection [170], whereas subtype A mostly used CCR5 even in late infection [389]. This may explain why HIV-1 subtype D-infected patients had more rapid progression that those infected with subtype A in Uganda, Kenya, and Tanzania [190; 201]. The percentage of X4 virus appears lower in subtype C than in subtype B, even when the viruses are obtained from patients with advanced AIDS [80]. A previous study in Tanzania suggested that subtypes A, C and recombinants are more likely to be perinatally transmitted than subtype D [339], and that pregnant women infected with subtype C were more frequently susceptible to transmit HIV to their children than those infected with subtype B [338]. The study of chimeric SIVmac239 containing clade-specific consensus promoters from HIV-1 group M, showed that the chimeras containing a subtype C LTR region predominated when used for simultaneous co-infection of rhesus macaques with clade B and the putative clade E LTR [72]. This observation provides a potential explanation for the epidemiological predominance of subtype C worldwide. Moreover, the preferential transmission by breastfeeding of inter-subtype recombinant forms in the LTR has been recently reported [208].

HIV-1 subtype differences in disease progression have been studied in several cohorts. For instance, a study in Senegal reported that women infected with non-A subtypes were 8 times more likely to develop AIDS than those infected with subtype A [193]. A recent study of a Kenyan cohort showed that patients infected with subtype D had a higher mortality rate and a faster decline in CD4+ count than those infected with subtype A or C [28]. The propensity of subtype D to exhibit a greater degree of using dual coreceptor (CCR5/CXCR4) than other subtypes [170] may help to explain the observation that subtype D appears to be associated with a more rapid rate of disease progression than other subtypes [389]. Conversely, no difference in disease progression was found between patients infected with subtypes B and C in Israel [421], among patients infected with subtypes A, B, C and D in Sweden [15], subtypes B and CRF01_AE in Thailand [21] or subtypes CRF02_AG or other subtypes in Cameroon [228].

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4.2 Diagnostics

Detection of HIV infections can be accomplished by several techniques, including i) serologic detection of HIV antibodies, ii) serologic detection of HIV

antigens (p24), iii) viral isolation, or iiii) molecular detection of viral DNA or RNA. HIV variability has enormous impact on these diagnostics since commercial assays must detect all known variants of HIV-1 and HIV-2. Serologic assays are the currently preferred initial screening techniques, based on detection of antibodies to structural proteins p24 *gag* and mainly gp41 *env*, utilizing inactivated virus (1st generation tests), or a mixture of recombinant virus proteins or synthetic peptides (2nd and further generation tests) as antigens. Early diagnostic tests were not able to reliably detect HIV-2 infection, as well as the HIV-1 group O that failed to be diagnosed accurately by some serologic tests due to gp41 variation [333; 300; 242]. Thus, current commercial HIV ELISA assays most commonly include both HIV-2 antigen, and either broadly cross-reactive HIV-1 group M antigens or group O specific antigens. In order to detect antigens prior to the formation of antibodies or so-called reduce seroconversion window, the 4th generation immunoassays are designed to detect both the p24 antigen and antibody in a single test [418]. Recent comparative studies have shown that most of these immunoassays, and especially rapid tests, are sensitive and specific for diagnosing persons with chronic infections with HIV-1 group M subtypes [300; 206; 347], but they display significantly lower sensitivity for detection of non-B subtypes infections, mainly due to the fact that these assays were developed based on HIV-1 subtype B strains [204].

4.3 Antiretroviral therapy

HIV genetic variability may influence susceptibility to antiretroviral (ARV) drugs by involving genes for viral proteins targeted by ARV drugs. These are *pol* gene that encodes the enzymes (protease and reverse transcriptase, and more recently integrase) as well as *env* gene coding for the transmembrane envelope glycoprotein gp41. Those HIV-1 proteins are targets for protease inhibitors (PI), RT inhibitors (nucleoside and non-nucleoside reverse transcriptase inhibitors; NRTIs and NNRTIs) and fusion inhibitors, respectively. These ARV molecules are usually developed based on the HIV-1 subtype B. Until the past few years, antiretroviral treatment had been not widely used in many developing countries, where non-B subtypes of HIV-1 predominate. The polymorphisms, if conferring drug resistance, can be selected by drug-selective pressure and dramatically influence the therapeutic outcome. Alternatively, the polymorphisms do not confer drug resistance but may change the "genetic barrier" which is defined as the number of viral mutations required to develop escape mutations able to overcome the drug-selective pressure. In general, several mutations are generally required for the virus to become resistant to protease inhibitors (high genetic barrier), whereas a single amino acid substitution can induce resistance to NNRTIs (low genetic barrier) [35]. For instance, one of the most relevant findings is the reduced genetic barrier for the V106M substitution in subtype C, which has been observed in different studies and which confers high-level resistance to all NNRTIs [53; 403; 406]. In particular, the V106M mutation in RT is facilitated in subtype C by a single transition (GTG to ATG), compared to two transitions in viruses of other subtypes (GTA to ATG). Moreover, natural resistance to NNRTIs is a characteristics of both HIV-1 group O and HIV-2, [108; 330].

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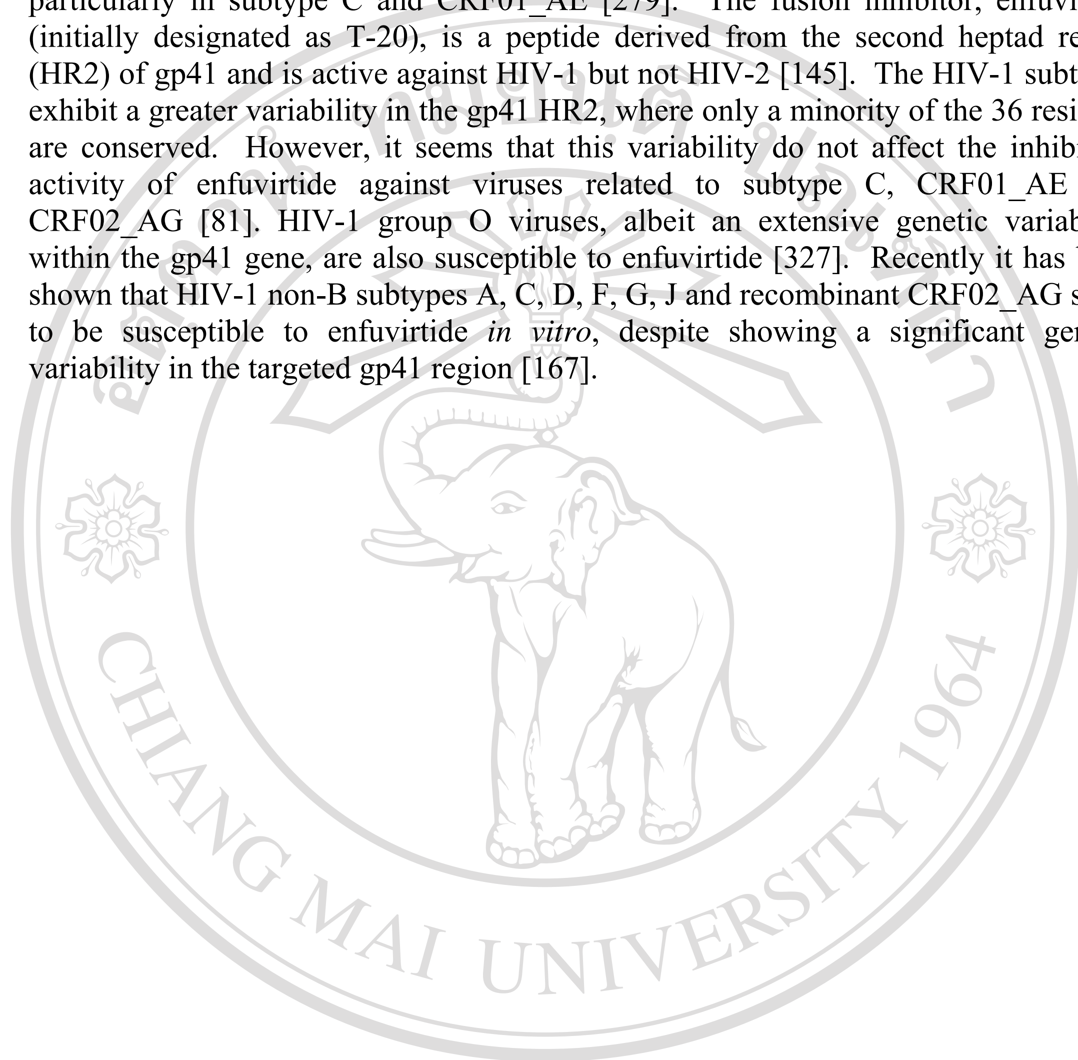
The *env* variability may modulate susceptibility to entry inhibitors. The natural resistance can occur with BMS-378806, the gp120–CD4 interaction inhibitor, particularly in subtype C and CRF01_AE [279]. The fusion inhibitor, enfuvirtide (initially designated as T-20), is a peptide derived from the second heptad repeat (HR2) of gp41 and is active against HIV-1 but not HIV-2 [145]. The HIV-1 subtypes exhibit a greater variability in the gp41 HR2, where only a minority of the 36 residues are conserved. However, it seems that this variability do not affect the inhibitory activity of enfuvirtide against viruses related to subtype C, CRF01_AE and CRF02_AG [81]. HIV-1 group O viruses, albeit an extensive genetic variability within the gp41 gene, are also susceptible to enfuvirtide [327]. Recently it has been shown that HIV-1 non-B subtypes A, C, D, F, G, J and recombinant CRF02_AG seem to be susceptible to enfuvirtide *in vitro*, despite showing a significant genetic variability in the targeted gp41 region [167].



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PART III. Neutralizing antibody against HIV infection

1. Antibody responses to HIV infection

Following acute HIV infection, a massive viremia triggers an immunologic responses. The time cause of HIV infection is represented schematically in Figure 23. The median viral load is generally high, approximately 5 million RNA copies/ml of plasma. After first few months of infection, the viremia declines to set point, and by one year the average HIV viral load is around 30,000 copies/ml [244]. The viral load set point is a strong predictor of how rapidly an individual will develop AIDS [264; 265], and thus this evidence indicates that differences in immune responses account for the differences in viral load set point and disease progression in an infected individual. The fall in the viremia coincides with the onset of the cytotoxic T lymphocyte (CTL) response. Antibody present at this time is generally vigorous, but predominantly directed to the structural proteins, such as p24 and p17 in Gag, and may not have a strong antiviral effect. Neutralizing antibodies (NAbs) are defined mechanistically for their ability to block viral entry into cells [202], since they may also bind complement or promote Ab-directed cell-mediated cytotoxicity (ADCC), thus leading to killing of infected cells [12; 14]. Most NAbs are directed against the gp120 of HIV envelope as well as against a neutralization face on gp120 (as reviewed in part I). The primary peak viremia in an infected individual usually declines prior to the detection of NAbs (Figure 23). Thus, it was deduced that NAbs do not play a major protective role once the infection has occurred. Rather, cellular immune responses, especially a CTL response, which arise early in infection and target infected cells, are thought to play a critical role in controlling an established infection by decline of primary viremia, but are ineffective in preventing initial infection [43; 171]. However, a few HIV-1-infected individuals, described as long-term nonprogressors (LTNPs), remain asymptomatic, stable CD4+ T-cell counts and low level of HIV viremia for more than 10 years without ARV treatment [57; 65]; some of those persons had strong NAb responses against multiple primary HIV-1 isolates *in vitro* [65; 274; 312]. NAbs can prevent cell-free virus spread, and most individuals rapidly develop NAbs to their autologous HIV strain, typically within two months of infection [132; 152; 343] , and usually lacking of broad reactivity, can be overcome by the emergence of neutralization-escape mutants.

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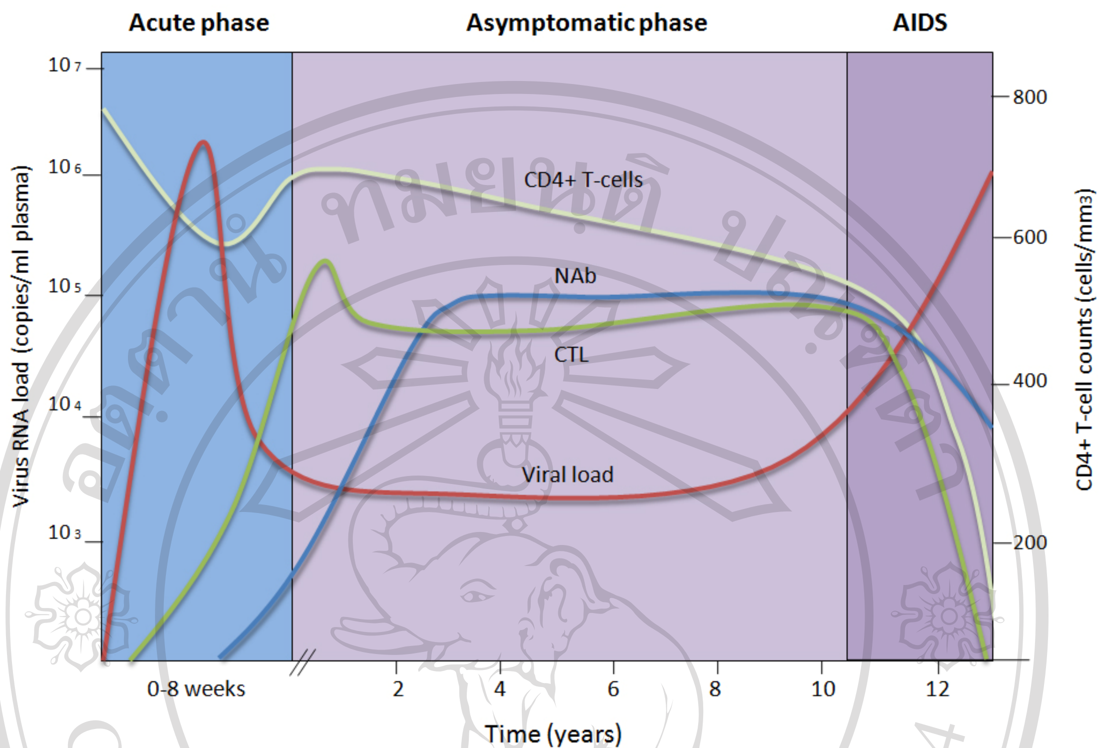

Figure 23. Time cause of HIV infection. The rate decline in CD4+ T-cells (*light green*), rate of plasma RNA load (*red*), CLT response (*green*), and NAb response (*blue*) during HIV infection and disease progression. CTL, Cytotoxic T lymphocyte; NAb, Neutralizing antibody. (Figure drawn for this thesis)

2. Mechanisms of antibody-mediated protection

Antibodies (Abs) can protect against various viral infections by several mechanisms. The main mechanism that have been found to be effective antiviral seem to be mediated by NAbs. Neutralization is the ability of Abs to reduce infectivity of a virus particle by interfering at one of several steps in the virus life cycle [443]. Abs can target both cell-free virions and virus-producing cells by binding to molecules on the surface of virions or infected cells and prevent their interaction or control viral replication. The viral entry is an essential step in HIV replication and mediated by the HIV Env glycoproteins, therefore the primary targets for NAbs. The NAbs bind specific for various region of gp120 and gp41 include the CD4 binding site (CD4bs), the CD4-induced (CD4i) epitope, and the V2, V3 loops of gp120; and the fusion domain in gp41 that are exposed during membrane fusion (as review in part I; Figure 11D). The NAbs may also bind to cellular receptor sites implicated in HIV entry such as the chemokine receptor CCR5. The Abs can also exert also exert their virus effect by simply cross-linking virus particles, leading to high molecular aggregates that can be eliminated by phagocytosis [113], and lysis of virus particles by activating complement cascade [379]. In addition, in many viruses, Abs can also mediate post-fusion neutralization such as preventing the primary

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uncoating of the virus in the cytoplasm, transcription steps in the nucleus, and virus assembly and budding at the cell membrane [443].

Abs might also target HIV-infected cells mediated by ADCC as well as by activating complement cascade leading to killing of infected cells *in vivo* [12; 11; 275]. Abs of IgA or IgM isotypes have been reported to transverse the mucosal epithelium *via* polymeric immunoglobulin (Ig) receptor binding [382], suggesting a mechanism for polymeric anti-HIV Abs to block HIV transcytosis [425]. On the other hand, Abs may indeed have infection enhancing effects, especially after immunization with whole virus particles [411]. The protective mechanisms of Abs to HIV infection are summarized in Table 1 and Figure 24.

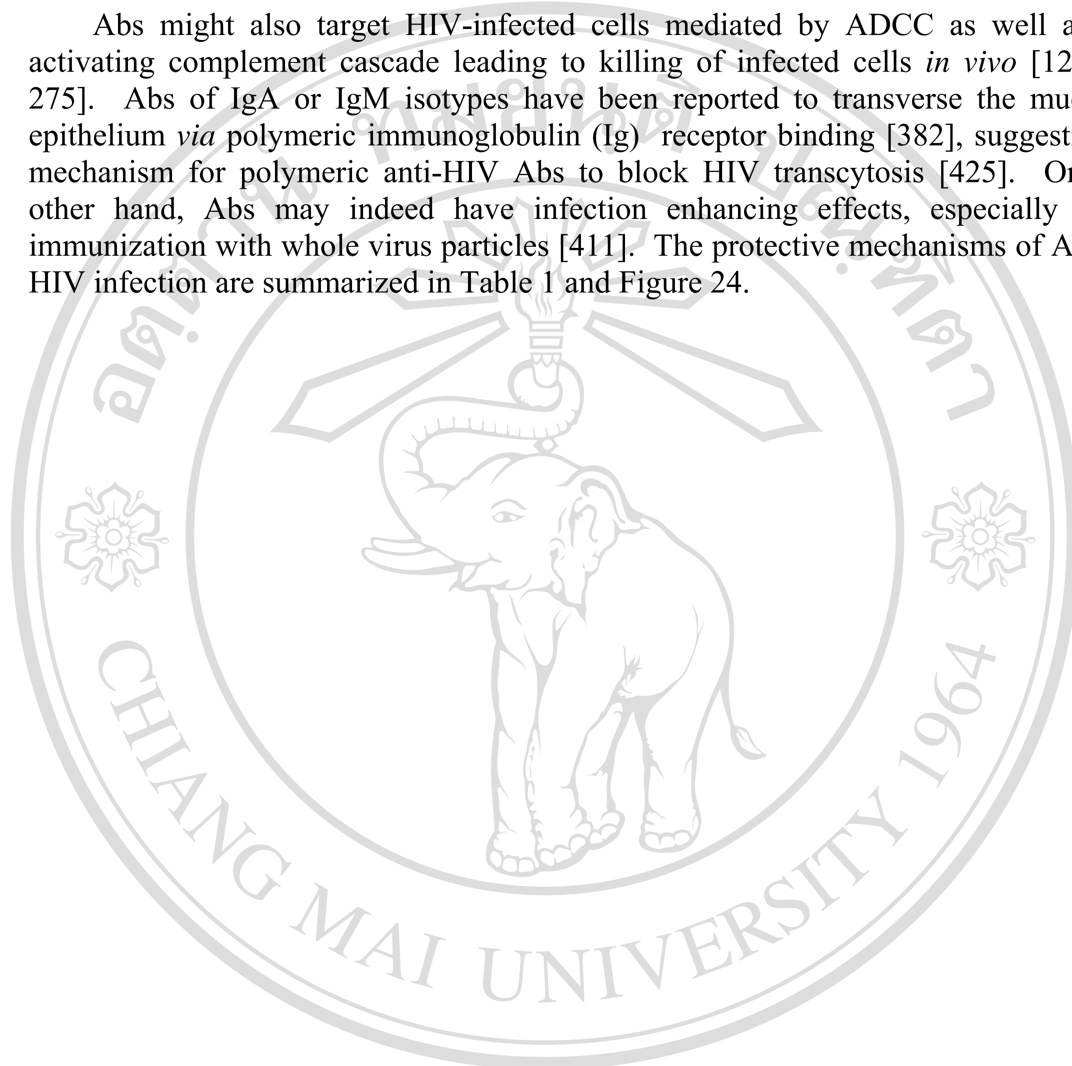

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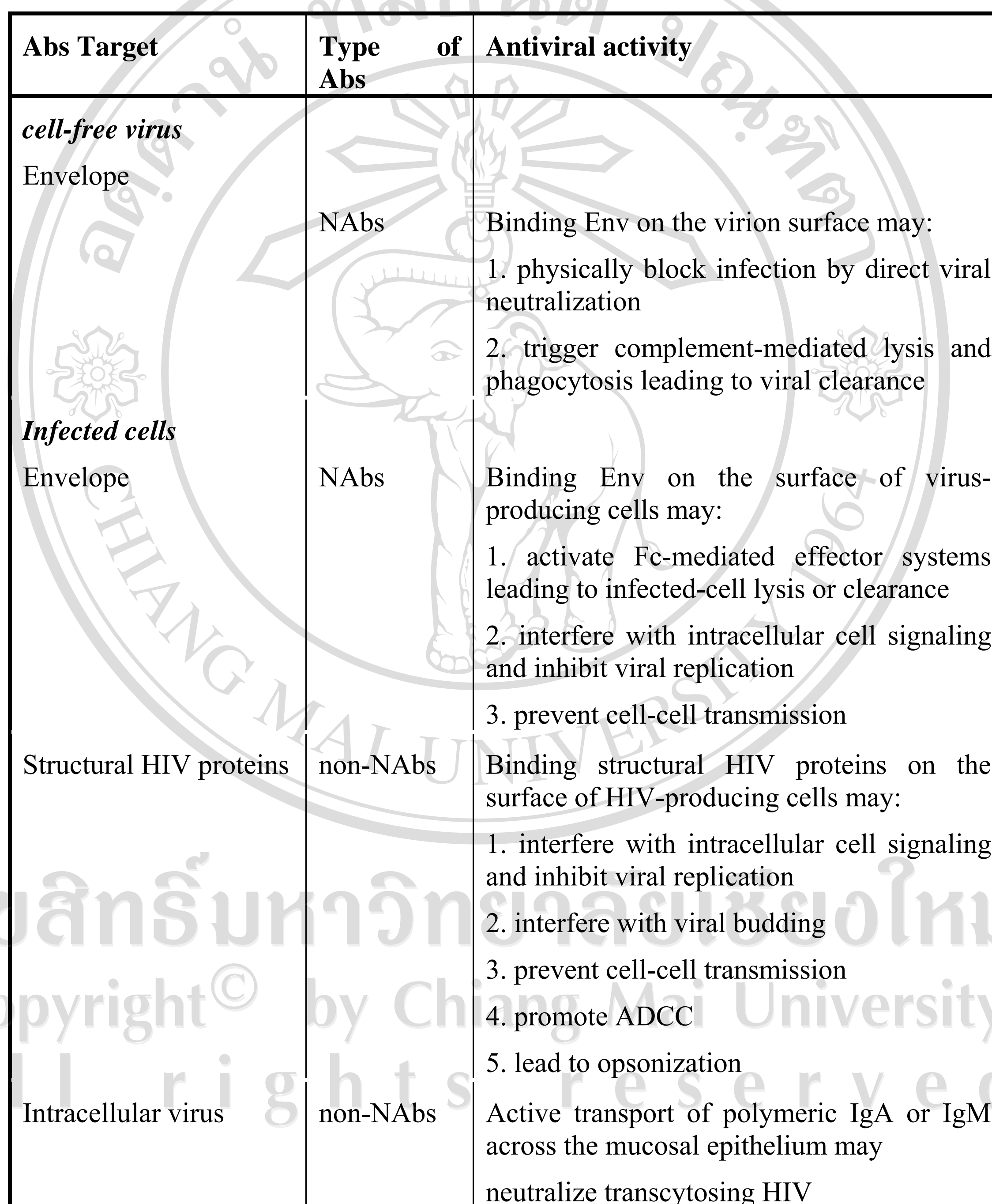
Table 1. Potential mechanisms by which neutralizing and non-neutralizing Abs modulate the HIV life cycle (modified from McCann, *et al*. 2005 [259]).



Abs Target	**Type of Abs**	**Antiviral activity**
cell-free virus		
Envelope		
	NAbs	Binding Env on the virion surface may:
		1. physically block infection by direct viral neutralization
		2. trigger complement-mediated lysis and phagocytosis leading to viral clearance
Infected cells		
Envelope	NAbs	Binding Env on the surface of virus-producing cells may:
		1. activate Fc-mediated effector systems leading to infected-cell lysis or clearance
		2. interfere with intracellular cell signaling and inhibit viral replication
		3. prevent cell-cell transmission
Structural HIV proteins	non-NAbs	Binding structural HIV proteins on the surface of HIV-producing cells may:
		1. interfere with intracellular cell signaling and inhibit viral replication
		2. interfere with viral budding
		3. prevent cell-cell transmission
		4. promote ADCC
		5. lead to opsonization
Intracellular virus	non-NAbs	Active transport of polymeric IgA or IgM across the mucosal epithelium may
		neutralize transcytosing HIV

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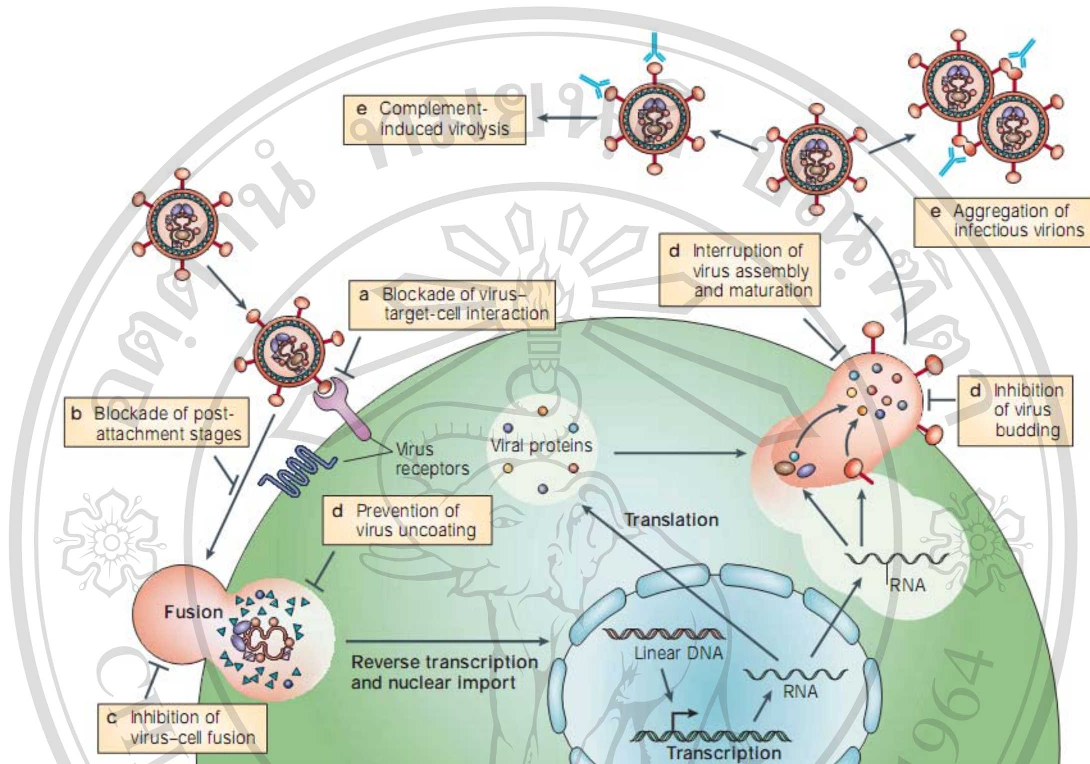

Figure 24. Steps at which antibodies can potentially interfere with HIV-1 replication. (Figure from Zolla-Pazner, *et al*. 2004 [443])

A, Antibodies can block the virus-cell interaction by inhibiting the binding of virion to CD4 and coreceptors on the cell surface; and preventing conformational changes of the virus envelope that are required for subsequent steps in the virus life cycle.

B, After attachment of the virus to target cells, antibodies can inhibit further conformational changes in the virus envelope glycoproteins that create or expose domains involved in virus-cell fusion.

C, Antibodies can also block the protein domains that are involved in virus-cell fusion.

D, Antibodies might be involved in: preventing virus uncoating after entry; interrupting virus assembly; preventing maturation of the virus particle; and inhibiting virus budding.

E, Additional mechanisms of antibody-mediated neutralization include complement-induced virolysis and aggregation of infectious virions.

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3. HIV-1 neutralizing monoclonal antibodies

Since the HIV-1 envelope glycoproteins are present on the exterior of the virus, expose location at the surface and due to their critical role for viral entry, they are the major target for NAbs [433]. Several human monoclonal antibodies (mAbs) with broad neutralizing activities both against laboratory-adapted HIV-1 strains and various primary isolates *in vitro* have been isolated. These mAbs originally characterized from HIV-1 clade B infected individuals and have targeted several epitopes on HIV-1 envelope glycoproteins, gp120 and gp41 (Figure 25). On the gp120, the mAb IgGb12 (b12) and the weakly neutralizing mAb F105 bind to the CD4-binding site (CD4BS) and competes with the HIV-1 primary receptor, CD4 [62; 325]. The other gp120-directed broadly neutralizing mAb, 2G12, binds to a cluster of glycans on the gp120 outer domain [401]. The mAb 2G12 is the only mAb that bind to the silent face, which is heavily glycosylated (Figure 11 and 25). Other gp120 regions that have been suggested as potential sites for NAbs include the V3 region that is implicated in binding to the coreceptor CCR5. The V3 loop elicits Abs that are often strain specific, however, one exception is the V3-directed mAb 447-52D, which exhibits greater breadth of neutralization [149; 40]. Another MAbs include 17b, X5, and 412D recognize a region close to the coreceptor binding site, which is better exposed after gp120 binding to CD4 (CD4i epitope) [217; 383; 331; 282; 392].

The gp41-directed broadly neutralizing mAbs 2F5 and 4E10 bind to a region close to the viral membrane called the membrane proximal external region (MPER), which is hydrophobic and highly conserved across clade [285; 381; 446] (Figure 12, highlighted in yellow, and 25). Of these, the mAb 2F5 recognizes the ELDKWA epitope [285; 298], while mAbs 4E10 and Z13 map to an epitope containing the NWF(D/N)IT sequences [446] (Figure 12).

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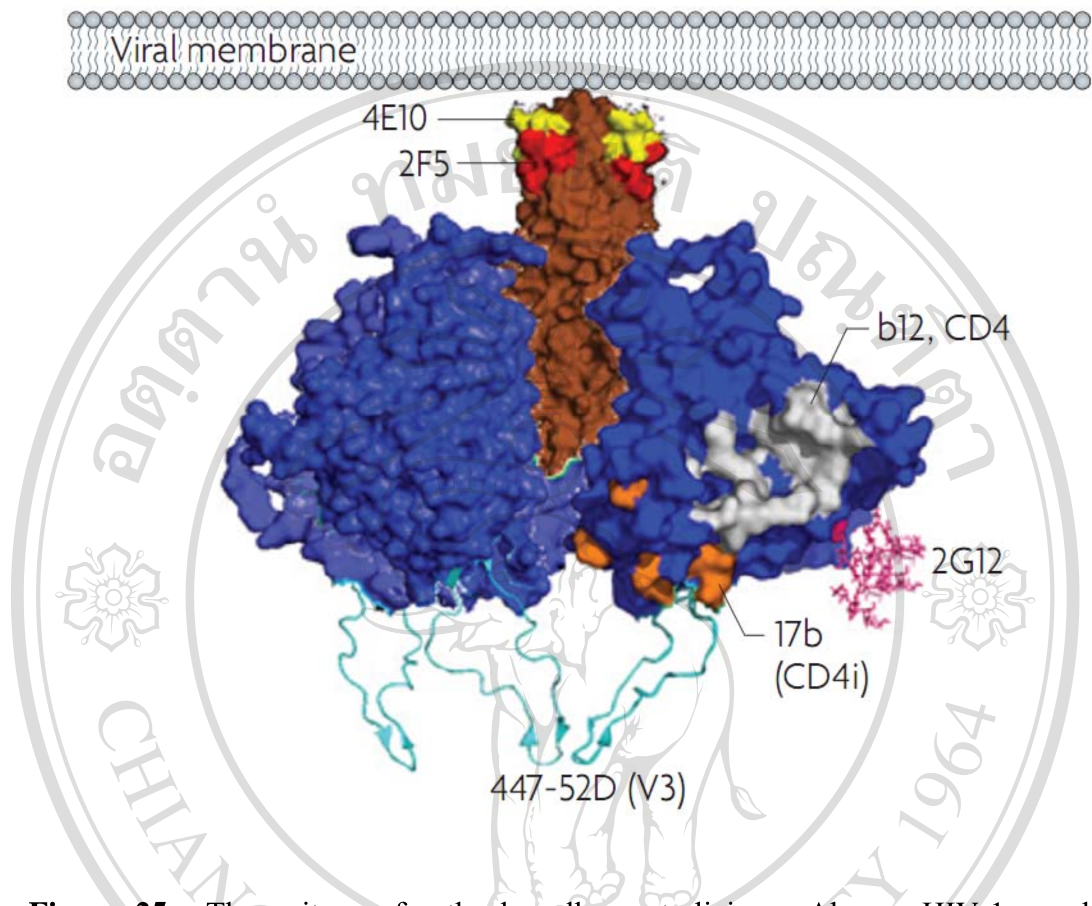

Figure 25. The epitopes for the broadly neutralizing mAbs on HIV-1 envelope glycoprotein. The epitopes for the mAbs 4E10 (*yellow*) and 2F5 (*red*) that comprise the membrane proximal external region; the epitopes for two mAbs 2G12, which recognizes a glycans cluster (*pink*), and b12, which recognizes a surface that overlaps with the CD4BS (*gray*); and the epitopes that are recognized by the CD4i-directed mAb 17b (*orange*) and the V3 loop-directed mAb 447-52D (*blue*), are shown. (Figure from Karlesson Hedestam *et al*., 2008 [195])

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4. Primate challenge models and passive immunization

The efficacy of human neutralizing mAbs was evaluated in passive immunoprophylaxis studies in primates challenged with chimeric simian-human immunodeficiency virus (SHIV) strains, which encode HIV *env* gene in simian immunodeficiency virus (SIV) backbones. The administration of mAbs either pre- [27; 254; 257; 166; 299] or post-exposure [125; 165] to the virus provided effective protection to macaques against SHIV infection or disease. The passive immunization with combinations of mAbs F105, 2G12 and 2F5 completely protected neonatal macaques against oral challenge with SHIV-*vpu*+ when given first to the pregnant dams by intravenous infusion five days prior to scheduled cesarean section, then to newborns prior to virus exposure (day 0), and 8 days later [27], or when mAbs infusion to the pregnant dams was omitted [165], or when two doses of the triple mAb

combination were given as post-exposure prophylaxis 1 hour after oral inoculation with the virus [165]. The pharmacokinetic analysis revealed that cord blood contained neutralizing levels of all three mAbs, which were IgG1 subtype, indicating effective transport of these mAbs across the placenta [27]. The post-exposure prophylaxis studies in neonatal macaques orally challenge with pathogenic SHIV89.6P, a chimera that encodes the *env* gene of the primary dual-tropic HIV89.6 and that became acutely pathogenic after passage through several monkeys [337], were also successful [125; 165]. The quadruple combination of mAbs b12, 2G12, 2F5 and 4E10 completely protected two of four neonatal macaques and contained acute viremia and/or prevented acute disease in the other two [125]. In addition, this quadruple mAbs combination effectively neutralized primary HIV-1 clade A, B, C, and D isolates *in vitro* [200].

Phase I clinical trials were conducted in HIV-infected individuals to evaluate safety, immunogenicity and pharmacokinetics of human mAbs [426; 70; 24; 23]. Single mAb F105 [427; 70], 4E10 [23], and combinations of 4E10/2F5/2G12 [23] and 2F5/2G12 [24] were safe and well tolerated in humans when administered at concentrations necessary to achieve >99% neutralization *in vitro*. Furthermore, 2F5/2G12 combinations showed a transient decrease in viral burden in some treated HIV-infected adults [24]. Recently, the protective efficacy of passively administered mAb combinations of 2G12, 2F5 and 4E10 was evaluated in chronically HIV-infected individuals undergoing interruption ARV therapy. Preliminary results from this study demonstrated a delay or a decrease in rebound viremia in some individuals and support a role for neutralizing mAbs in controlling HIV infection in vivo [399].

5. Viral strategies to avoid neutralizing antibodies

During the course of infection, the virus survives by mutating to escape antibody recognition. The viral escape variants elicit subsequent NAb responses. Interestingly, Richman *et al*. have shown that potent NAb responses are mounted against the autologous virus during the acute phase of HIV infection and also to subsequent variants that emerge over the cause of disease [343]. These findings suggest that NAbs exert a selective pressure on the virus, continually driving it to evolve in order to escape neutralization.

There are multiple mechanisms by which HIV-1 generates variants in order to evade host immune responses. Such mechanisms include nucleotide substitutions, insertions and deletions, modification of Env potential N-linked glycosylation sites (PNGS), and recombination [259] (Figure 26). HIV Env is heavily glycosylated, with almost 50% of the total mass consisting of poorly or non-immunogenic carbohydrate moieties ([229]; reviewed in part I). Wei *et al*. suggested that mutations in the HIV-1 envelope glycoproteins contribute to the acquisition and rearrangement of sugar moieties, an "evolving glycans shield" that potentially masks underlying peptide motifs and physically hinders Ab binding without reducing viral fitness [240]. The removal of specific glycans sites on the HIV-1 glycoprotein can dramatically increase viral sensitivity to NAbs [207; 260; 329].

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The mutations in the variable loops of gp120 alter amino acid sequences, the pattern of glycosylation and the length of the loops are thought to be involved in neutralization escape. The virologic determinants that influence HIV transmission were evaluated in heterosexual transmission pairs, and results demonstrated that newly transmitted viruses isolated from recipients had a modified HIV Env that contained shorter V1-V4 regions with fewer PNGS when compared with viral quasispecies present in the linked donor [107]. Furthermore, newly transmitted viruses were uniquely sensitive to neutralization by antibody from the transmitting partner. One may hypothesize that a modified Env containing shorter variable loops with reduced glycosylation may represent a phenotype that may be required to overcome the bottlenecks of mucosal transmission. These findings may suggest a maintaining between a neutralization-resistant phenotype (late/end stage virus) and a phenotype that is more readily transmitted but sensitive to neutralization (early virus).

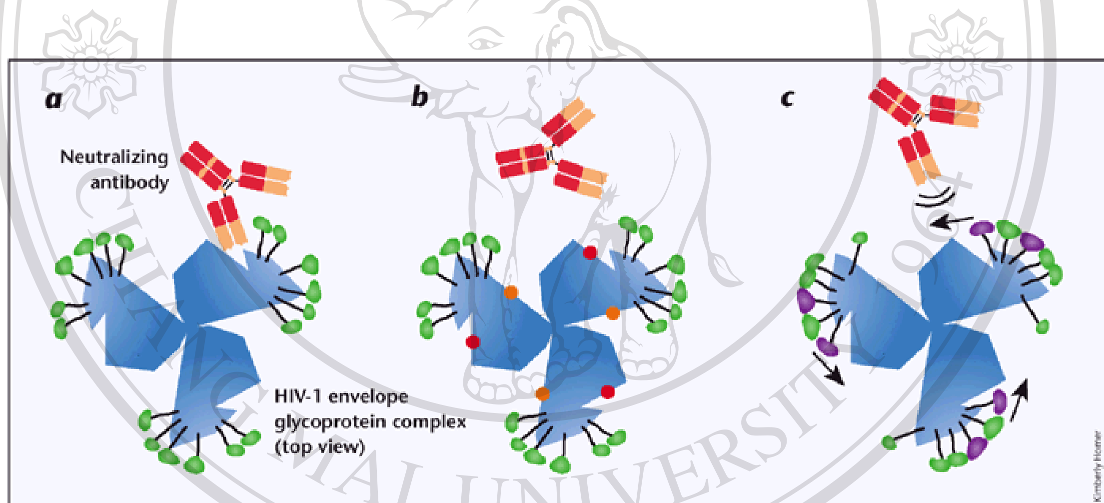

Figure 26. Comparison of the direct epitope variation and the glycan shield models of HIV-1 immune escape. (Figure from Mascola *et al*., 2003 [256])

A, Neutralizing antibodies bind to the HIV-1 envelope glycoprotein complex, which consists of 3 identical gp120-gp41 heterodimers.

B, Traditional viral escape involves random mutations (resulting envelope changes depicted with *red* and *orange* dots) that alter the neutralizing epitope (*red* dots) and diminish antibody binding.

C, The glycan shield model proposed by Wei *et al*. [420] suggests that steric hindrance rather than epitope variability prevents neutralizing antibodies from binding their cognate epitopes. Added or repositioned glycans are shown in *purple*.

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PART IV. Mother-to-child transmission of HIV

1. Current situation

Mother-to-child transmission (MTCT) is the principal cause of HIV infection in children. According to the UNAIDS and WHO, the estimated number of children living with HIV and the estimated new infections among children were 2.5 million and 420,000 in 2007, respectively. The deaths due to AIDS among children were 330,000 in 2007. Nearly 90% of infected children live in Sub-Saharan Africa. The HIV epidemic among children mirrors that among women, as the vast majority of children acquire infection vertically from their mothers. Despite the estimated numbers of women living with HIV worldwide declined from 17.7 million in 2006 to 15.4 million in 2007, the proportion of women versus men has been increasing in each region (Figure 27), especially in Sub-Saharan Africa where almost 61% of adults living with HIV in 2007 were women. Most HIV-infected women worldwide are of childbearing age. They become infected mainly as a result of heterosexual transmission and injecting drug use [183]. The decrease in rate of MTCT of HIV that has been observed during the last decade can be due to different factors: more HIV-infected pregnant women received HIV counselling and testing, use of ARV treatment during pregnancy and delivery, associated to ARB treatments to their infants after birth, and use of other efficacious interventions, including cesarean section before labour and before rupture of membranes, and complete avoidance of breastfeeding. In the United States, the MTCT of HIV has been nearly eliminated, with the rate less than 2% [93], through routine prenatal screening linked to antiretroviral therapy to those infected pregnant women [9]. In Thailand, the program for prevention of mother-child HIV transmission (PMCHT) was implemented nationally since 2000. There has been remarkable progress with 98% of women who delivered in public sector facilities receiving HIV counselling and testing, and 94% of those found to be HIV positive received antiretroviral preventive therapy [183]. The transmission risk of HIV from mothers to their children was reduced to about 10% overall and about 4% among those mothers and infants who received a complete zidovudine regimen along with other antiretroviral drugs such as nevirapine [184].

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Percent of adults (15+) living with HIV who are female 1990–2007

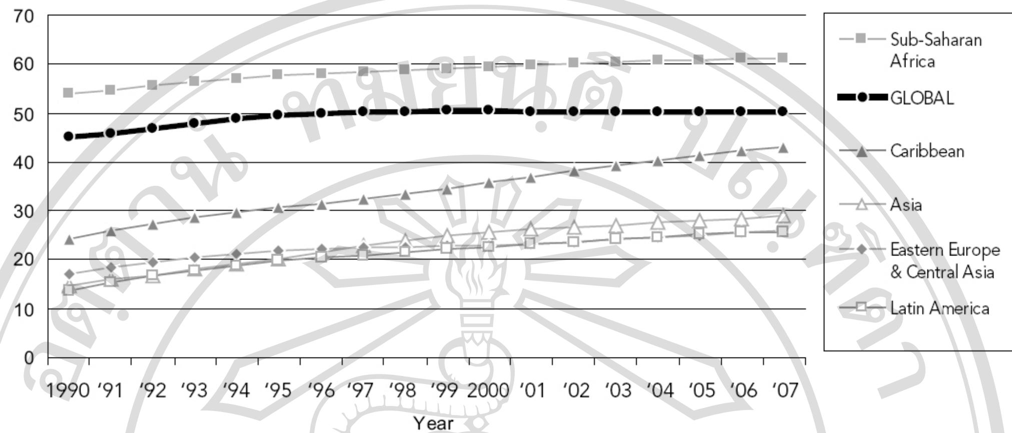

Figure 27. Percentage of women living with HIV from 1990 to 2007. (Figure from UNAIDS, 2007[183])

2. Timing and mechanisms

HIV-1 can be transmitted from mother to child at three different times: during pregnancy (*in utero*), at the time of labour and delivery (intrapartum), or postpartum through breastfeeding. In the absence of any intervention, 35% - 49% of the children born to HIV-infected mothers are infected with approximately 8% becoming infected during pregnancy, 15% during labour and delivery, and 12% - 26% during breastfeeding [230; 209].

2.1 *In utero* transmission

2.1.1 Morphology of the human placenta

The human placenta is a temporary organ that consists of a fetal part, the chorion (chorionic plate and chorionic villi) and a maternal part, the decidua basalis. The placenta is the site of exchange between mother and fetus during fetal development. It provides oxygen and nutrients to the fetus, disposes fetal waste products, synthesizes and secretes hormones, growth factors, cytokines and other bioactive molecules, protects the fetus against pathogens and forms an immunologic barrier between mother and fetus [133]. At four weeks of development, the basic structure of the placenta has been formed with maternal blood begin delivered to the forming placenta via spiral arteries while being drained away via uterine veins. The maternal arteries form spiral to make contact with the placenta in which they end in open channels, bathing maternal blood into the intervillous space. The fetal circulation enters the placenta via the umbilical arteries embedded within the umbilical cord. The

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fetal circulation branches into units liked a tree. The terminal branches are made up of capillary loops within the chorionic villi, the functional units of the placenta responsible for maternal-fetal exchange processes. Once nutrients have been absorbed and waste products released, the fetal blood ultimately collects into the umbilical vein, where it returns to the fetus via the umbilical cord. The basic structure of the human placenta at four week of development is shown in Figure 28A.

Early in fetal development the trophoblast differentiates into double layers: the inner layer of mononucleated cytotrophoblasts and the outer layer of syncytiotrophoblasts (syncytial layer), separating the fetal circulation from the maternal circulation. The stroma contains mesenchymal tissue (connective tissue), macrophages (Hofbauer cells) and fetal capillaries. During the first trimester these capillaries are initially small and embedded in the center of the stroma. As pregnancy progresses the chronionic villous exhibits increased numbers of fetal capillaries, the cytotrophoblasts layer becomes discontinuous and only few cytotrophoblasts are found underneath the syncytiotrophoblasts at term. The surface area of the syncytiotrophoblasts expands faster than its volume and therefore it is locally thinned and aggregated nucleus into clumps or syncytial knots. Thus the placenta barrier consists of three cell layers in the early placenta (the syncytiotrophoblasts, the cytotrophoblasts, the fetal endothelial cells), but of only two layers (the syncytiotrophoblasts and the fetal endothelial cells) in the at-term placenta [133]. The terminal chorionic villi in first and third trimester of pregnancy are shown in Figure 28B, C and D.

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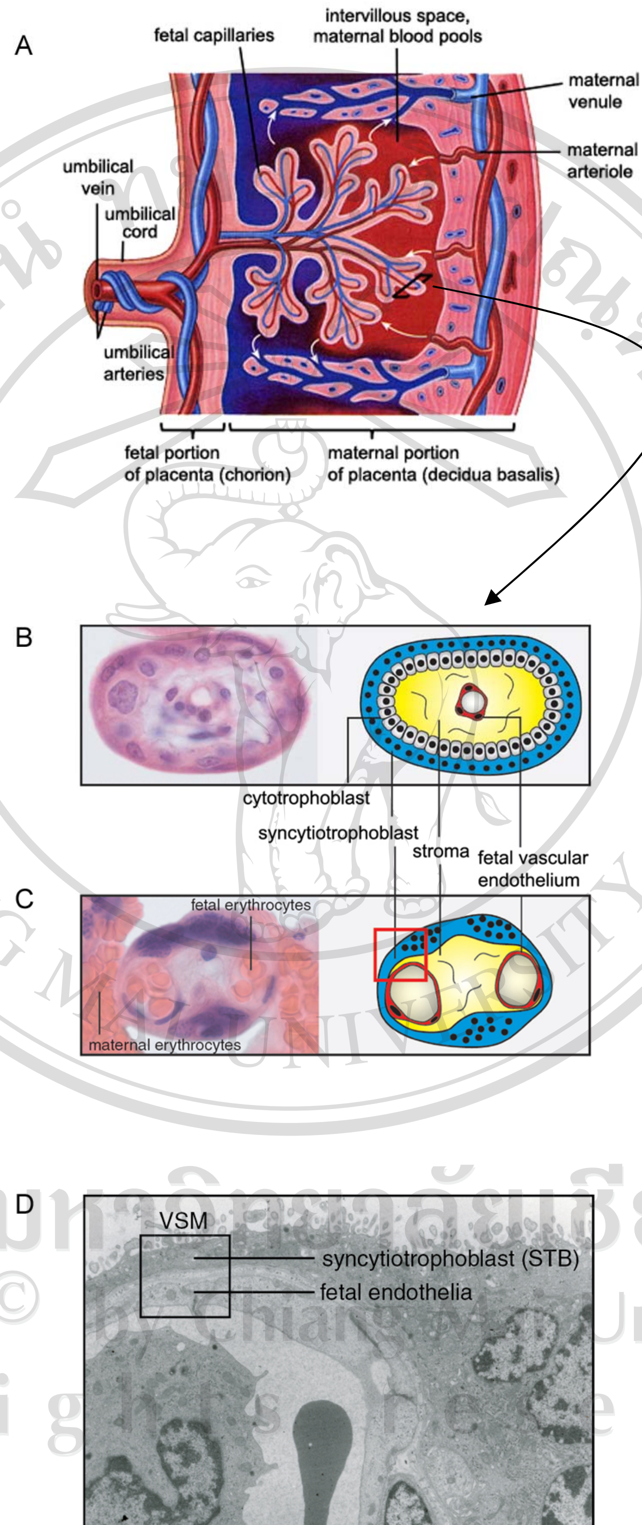

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Figure 28. Schematic representation of placenta structure. (Figure A from http://instruct1.cit.cornell.edu/; March 2008; figures 1B-D from Fuchs and Ellinger, 2004 [133])

A, The basic structure of the human placenta consists of the fetal portion and the maternal portion,

B-C, Cross-sections of a chorionic villi in first (B), and third trimester (C): hematoxylin/eosin stained paraffin section (left) and corresponding schematic drawing (right) of the villi demonstrating the syncytiotrophoblasts (blue), the cytotrophoblasts (gray), and the mesenchymal tissue (yellow) that contains small fetal vessels lined by endothelial cells (red).

D, Transmission electron micrograph of a terminal chorionic villous at term demonstrating a vasculo-syncytial membrane (VSM), an area of maternal-fetal transport processes and numerous microvilli at the apical surface of the syncytiotrophoblasts that contact maternal blood.

2.1.2 Mechanism of HIV *in utero* transmission

The mechanism of HIV transmission from mother to child *in utero* is poorly understood, but it seems that the transmission occurs primarily when HIV crosses the placenta, and it can infect the placenta at all stages of pregnancy. This is supported by the evidence that HIV-1 antigens and nucleic acids have been detected in trophoblastic and villous Hofbauer cells of the placenta by immunocytochemistry and in-situ hybridization, as early as 8 weeks gestation [233]. HIV-1 DNA has been detected in chorionic villi of first and second trimester placentas and their corresponding cord blood by PCR [103]. Although the presence of the virus has been detected in thymus, spleen, and peripheral blood mononuclear cells (PBMCs) from second trimester aborted fetuses [250; 95; 55], the majority of *in utero* transmission occurs during the third trimester.

HIV-1 has been shown to productively infect trophoblasts both *in vitro* [218; 415; 413] and *in vivo* [267; 112; 440]. However, they exhibit a much lower susceptibility to productive HIV-1 infection than CD4+ T cells [415; 439; 102]. During pregnancy, the apical side of the syncytiotrophoblasts is in contact with infected maternal blood which contains both cell-free HIV-1 and HIV-1 infected cells. The syncytiotrophoblasts form a polarized epithelium-like monolayer presenting of polarized endocytic pathways (early, late, and recycling endosomes). On this contact, cell-free HIV-1 virions are rapidly and massively internalized by the trophoblasts through endocytic pathway independently of gp120/CD4 and coreceptor interactions [413; 18]. CD4 is weakly expressed or absent on the trophoblastic cells whereas the expression of CCR5 and CXCR4 may vary during the course of pregnancy [439; 214; 273]. Once HIV-1 is endocytosed, some internalized virions are degraded by the lysosomes, some are transcytosed to the basal pole and recycled back to the apical pole, while others escape the endosomes to reach the cytoplasm and establishe replication in the trophoblasts [413; 414; 46]. In contrast to cell-free virions, the contact between HIV-1 infected cells and trophoblastic cells induced a massive and rapid budding of HIV-1 virions into the apical pole of the trophoblastic cells followed by virions internalization and then transcytosis into the basal pole [46; 218]. This

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results in fusion between infected cells and trophoblastic cells leading to virus replication inside the cells [218]. The mechanisms of *in utero* transmission of HIV-1 is summarized in Figure 29.

An alternative mechanism of *in utero* transmission would be that the HIV-1 infected cells or cell-free viral particles from the mother circulation can directly pass through breaches in the trophoblast layer to reach the fetal blood circulation. These might occur spontaneously or as a result of chorioamnionitis, smoking and the abuse of illicit drugs [456-458]. However, it seems that the presence of minor injuries in the trophoblast layer is not associated with the transmission of HIV [64]. The HIV binding lectins DC-SIGN and DC-SIGNR are expressed in the human placenta and might play a role in *in utero* HIV transmission [376]. DC-SIGN expression has been found on maternal deciduas macrophages and fetal Hofbauer cells [142; 377], and DC-SIGNR is expressed in the liver, lymph nodes and placenta [316]. Whatever the mechanism of infection through the placenta, the risk of *in utero* transmission of HIV-1 is directly related to viremia. Therefore, reducing the presence of HIV-1 within the placenta cells would be associated with reducing the risk of vertical transmission of HIV.

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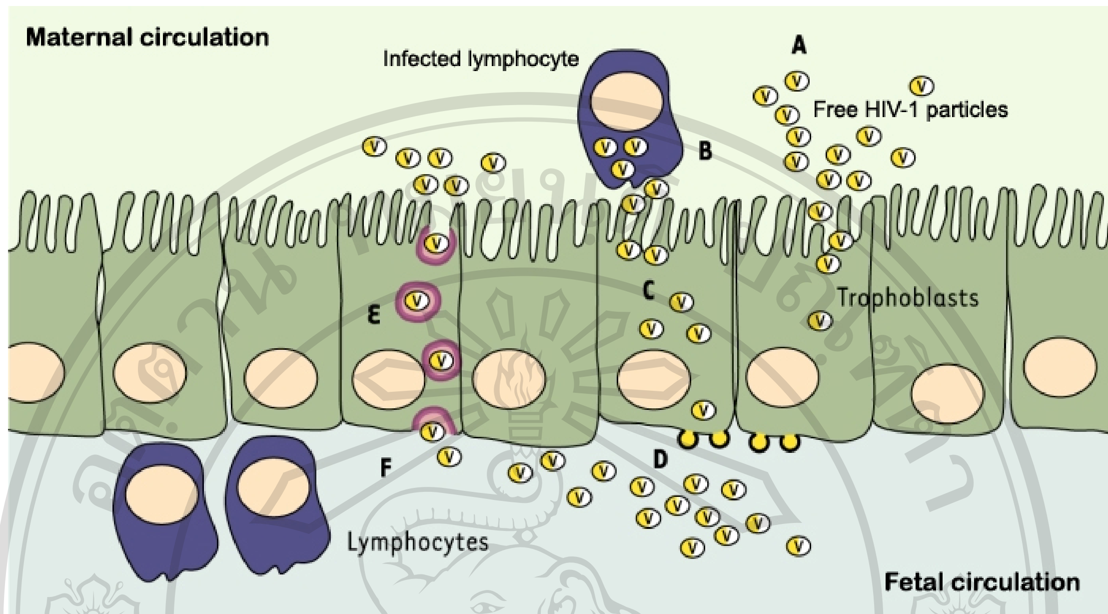

Figure 29. Mechanisms of *in utero* transmission. (Figure modified from Vidricaire *et al*., 2004 [454])

A-B, Cell-free virion or infected cells (or macrophages) in the maternal circulation come in contact with trophoblastic cells.

C, HIV enters trophoblastic cells through mechanisms still hypothetical: fusion of virions with the membrane or internalization by endocytosis.

D, Viral replication in the trophoblastic cells and buds of new virions into the fetal circulation.

E, HIV could also enter into the trophoblast by endocytosis, and be transported directly from apical pole to basolateral pole without infection of trophoblastic cells (transcytosis).

F, In both cases (infection of trophoblastic cells or transcytosis), HIV reached cells underlying such as lymphocytes and macrophages fetal for infection.

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2.2 Intrapartum transmission

Transmission during labour and delivery can occur via direct contact of the fetus/neonate with free HIV particles or infected cells from maternal blood and genital secretions during passage through the birth canal. It has been shown for instance that first-born twins are at increased risk of infection compared with the second twin [117], through ascending infection from the vagina or cervix to the fetal membranes, and through the amniotic fluid, and absorption in the fetal/neonatal digestive tract.

The aminotic fluid has three main functions: to protect the fetus physically, to provide chamber for fetal movements, and to regulate fetal body temperature. The aminotic fluid is derived from dialysis of maternal and fetal blood through blood vessels in the placenta, from the fetal urinary tract and respiratory tract. The fetus swallows about 400 ml of amniotic fluid per day in the final stages of pregnancy. Alternatively, the transmission can also occur during uterine contractions in labour via a breakdown in the maternal-fetal barrier followed by placental maternal-fetal microtransfusions. Placental microtransfusions have previously been suggested as a route of HIV-1 transmission [361; 192; 39; 238], and they are considered a plausible route of hepatitis B, C, and G vertical transmission [239; 238; 237]. Long labour and prolonged membrane rupture may increase the risk of transmission [247; 270; 225], although this has not been found in all studies. In the French perinatal cohort, haemorrhage during labour and the presence of bloody amniotic fluid substantially increased the risk of vertical transmission [247], and in one small study, bloody neonatal gastric aspirate was associated with infection in the infant [289]. Prepartum and intrapartum bleeding may expose the fetus to maternal blood, but could also be associated with placental disruption and transplacental transmission. Therefore both birth cannal disinfection and elective caesarean section delivery would be appropriate to avoid exposure in the birth canal, although prophylaxis of the fetus/infant with antiretrovirals would be the intervention of choice to prevent infection through microtransfusions.

HIV-1 has been cultured from cell-free and cellular fractions of vaginal and endocervical secretions of HIV-1 infected women [416], and HIV-1 infected cells have been found in female genital tract secretions by polymerase chain reaction amplification [179; 146; 281]. The excretion of HIV has been found significantly higher in pregnant than in non-pregnant women [163]. The presence of HIV in cervicovaginal secretions may be a risk factor for perinatal transmission. It has been found that chlorhexidine gluconate inactivates HIV *in vitro* at a concentration of 0.2% [159; 276]. The vaginal lavages with chlorhexidine during labour have been suggested as a method to reduce MTCT, either by inactivating the virus or by decreasing the viral concentration in the birth canal. However, trials in Malawi [38] on the use of vaginal cleansing with 0.25% chlorhexidine solution every 4 h during labour, and washing of the baby with the same solution at birth, and in Kenya [134] with 120 ml of 0.2% chlorhexidine on alternating weeks of enrolment, later increased to 0.4% every 3 h from admission to delivery, demonstrated that cleansing had no significant impact on HIV transmission rates, except when membranes were ruptured for more than 4 h before delivery.

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2.3 Postpartum transmission

The mechanisms of HIV-1 transmission through breastfeeding, or of protection from infection, are not well understood. During breastfeeding, the infants are daily exposed to high amounts of cell-associated and cell-free HIV-1via their oral and gastrointestinal mucosa. This exposure is estimated to be more than 700,000 viral particles per day [232]. HIV could theoretically be introduced into the

gastrointestinal tract submucosa of the infant by a breach in the integrity of the epithelial cell layer, or by concomitant infectious agents. Since gastric acid production in the neonate is reduced, more virus, rather cell-free or cell-associated, may reach the monostratified intestinal mucosa [266]. Although HIV has been detected in both the liquid phase of breast milk and breast milk cells [232; 286; 363; 424], the origin of virus in breast milk is still unclear. Free virus can be derived, at least in part, from blood (either from plasma or from infected lymphocytes/monocytes) and then released into breast milk. Alternatively, it can be produced by local replication in macrophages and in ductal and alveolar mammary epithelial cells [397]. The origin of HIV in breast milk is summarized in Figure 30.

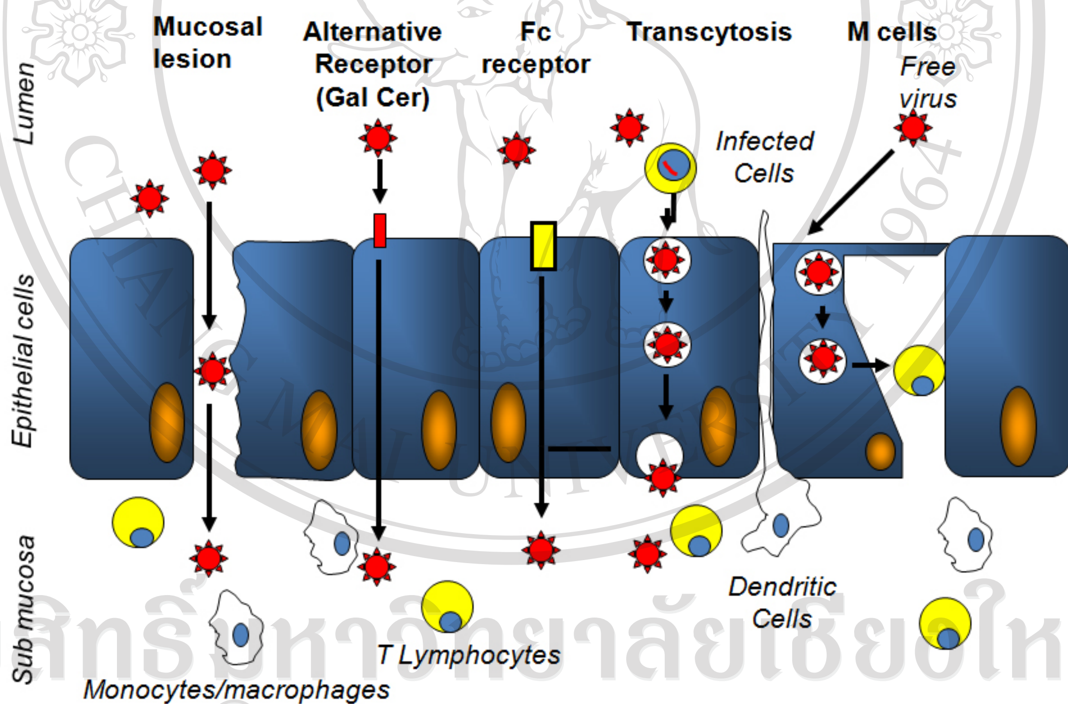

Figure 30. Origin of HIV in breast milk. (Figure from Van de Perre *et al.*, 2007 [455])

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3. Risk factors

There are numerous factors that increase the risk for MTCT of HIV infection, including maternal, obstetrical, and neonatal factors.

3.1 Maternal factors

Maternal viral load

Maternal plasma viral load is the strongest independent predictor of MTCT of HIV [49; 122; 380; 110; 185]. In recent studies, maternal HIV-1 RNA level at delivery remains the most consistent predictor of MTCT [61; 156]. Increasing geometric mean level of plasma HIV-1 RNA is generally associated with increasing rates of perinatal transmission. The highest rate of transmission is found among women whose HIV plasma level is more than 100,000 copies/ml, and conversely, the transmission is a rare event when viral load is undetectable. A study in New York showed a mean viral load of 16,000 copies/ml in transmitters and 6,600 RNA copies/ml in non-transmitters [393]. Women in this study with measurable viral loads were almost six times more likely to transmit HIV than those in whom the virus was undetectable, after controlling for the CD4+ count. In a French study, transmission rates increased with increasing viral load, 12% in those with less than 1,000 copies/ml compared with 29% in those with more than 10,000 copies/ml [258]. Moreover, the most recent study of 1,473 Thai pregnant women and their non-breastfed infants enrolled in the clinical trial of the efficacy of various durations of zidovudine (ZDV) prophylaxis (PHPT-1) indicated that *in utero* transmission was independently associated with HIV-1 load >35,000 copies/ml and delayed initiation of maternal ZDV prophylaxis until >31.4 weeks gestation, whereas variable associated with intrapartum transmission were HIV-1 load >10,000 copies/ml, induction of labour and premature labour with tocolysis [185].

The local viral load in cervicovaginal secretions and in breast milk may also be an important determinant of intrapartum transmission and transmission through breastfeeding [178; 84, 241]. HIV-1 levels in these fluids have been shown in most studies to be correlated with CD4+ cells count and plasma viral load [178; 286; 405]. The presence of sexually transmitted infections or other causes of inflammation, vitamin A deficiency and local immune response may affect viral shedding [224]. In Rwanda, postnatal transmission was associated with the presence of HIV-1 infected cells in breast milk [405].

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Maternal immunological status

Transmission from mother to child is more likely with decreased maternal immune status, reflected by low CD4+ cells counts, low CD4+ cells percentages or high CD4+/CD8+ ratios [270; 128]. These in turn may be markers for higher viral loads, as opposed to risk factors in themselves, although an interaction between viral

load and immune response may be present. In the European Collaborative Study (ECS), there was an increased risk of MTCT when maternal CD4+ cells counts were below 700 cells/mm^3 [3]. Transmission increased almost linearly in this study with decreasing CD4+ cells counts [287]. Several other studies have also noted similar associations [287; 314; 220; 430]. In the WITS study, the association between low CD4+ cells percentages and transmission was only seen in women without persistently positive viral cultures. Where there were at least one negative culture and high CD4+ cells percentages, transmission rates were in the range of 1-4% [314].

There have been conflicting results about the role of neutralizing antibodies in preventing transmission. Some studies have shown that high levels of maternal neutralizing antibody are associated with lower rates of transmission, while in others no association was observed [357; 162; 227; 48; 32]. Little is known about the role of mucosal HIV-1 antibodies and viral shedding in the genital tract which may affect intrapartum transmission rates. Infection through breastfeeding has been associated with a lack of IgM and IgA anti-HIV-1 in breast milk [405; 286].

Maternal nutritional factors

Vitamin A levels in plasma of HIV-1 positive mothers have been correlated with the risk of transmission in a Malawi study [364]. The mean vitamin A level in those mothers who transmitted the virus to their children was significantly lower than in those who did not transmit. The women with vitamin A levels below 1.4 μmol/l had a 4.4 fold increased risk of transmission, which dropped with increasing vitamin A levels. One US study showed no relationship between low vitamin A levels and transmission [60], while another cohort study did show a correlation [153]. The mechanism of vitamin A effect is uncertain, but the influence of vitamin A on the integrity of the vaginal mucosa or placenta and the immune stimulatory properties of the vitamin have been suggested [224; 364]. Alternatively, low vitamin A levels may be a marker for other deficiencies or behavioural factors, which influence transmission. Other micronutrients, including zinc and selenium, have been suggested as having a possible role .

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Placental factors

Placental factors have been implicated in transmission of the virus from mother to child [271; 390; 368; 22]. An association between increased transmission and the presence of chorioamnionitis was described early in the epidemic. Chorioamnionitis, an infection of the chorionamnion space, increases the risk of MTCT [407; 320]. Breaks in the placental surface can occur at any stage of pregnancy and may be related to transmission, although the significance of these may, in turn, depend upon the maternal viral load [64]. Smoking and drug use, both associated with increased transmission, may exert this effect through placental disruption [271]. Moreover in areas of high malaria prevalence, infection of the placenta is common in pregnancy. Placental *P. falciparum* infestation has been associated with poorer survival in infants born to HIV-1 positive mothers in Malawi, which may represent increased

transmission rates [45], and with higher rates of transmission from mother to child in Kenya [97].

3.2 Obstetrical factors

Prolonged rupture of membranes has been shown to be a risk factor for HIV transmission among women treated and untreated with antiretroviral therapy during pregnancy [141; 246]. The risk of infection is related to the duration of membrane rupture and is particularly increased when this duration is more than 4 hours [225]. Prolonged rupture of membranes appears to be a particular risk among women with low CD4+ cell counts [270; 6] and women at preterm gestation [247]. Preterm delivery is an additional risk factor for intrapartum HIV transmission [212]. Some studies that do not adjust for plasma HIV-1 RNA level have demonstrated increased transmission with intrapartum maternal hemorrhage [247], maternal sexually transmitted infections [247], and amniocentesis [247; 391].

Delivery be ceasarean section decreases the risk of transmission by about 25%. In twins, the firstborn has a higher likelihood of being infected that the second, presumably because of more prolonged neonatal exposure to maternal mucocutaneous vaginal secretions [117].

3.3 Neonatal factors

Neonatal variables such as premature birth at less than 35 weeks' gestation [287] and birth weight less than 2500 g [141; 225] have been found associated with neonatal HIV acquisition. Breastfeeding is responsible for a high proportion of MTCT in developing countries, where 30% or more of HIV infections will occur through breast milk. This is less common in the developed world, where most HIV-positive women will not breastfeed. There is decreased acidity, decreased mucus, lower IgA activity and thinned mucosa in the newborn gastrointestinal tract, which may facilitate transmission [288; 272].

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4. Prevention of mother-to-child transmission of HIV

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4.1 Antiretroviral therapy

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Antiretroviral therapy (ART) recommendations for HIV infected pregnant women are based on the principle that therapies of known benefit to women should not be withheld during pregnancy unless the risk of adverse effects to the mother, foetus or infant outweighs the expected benefit to the woman concerned [388]. The first drug that was used to prevent MTCT of HIV was ZDV in 1994. According to the WHO guidelines, the women who need ART for their own health should receive ZDV, following revised ART guidelines recently posted by WHO [459] (reviewed in Appendix B). The use of ART when indicated during pregnancy will improve the

health of the mother and substantially decrease the risk of transmission of the HIV to the infant. In case of women who do not need treatment, or do not have access to treatment, should be offered antiretroviral prophylaxis to prevent MTCT using one of the drug regimens known to be safe and effective. The most efficacious regimen among those recommended for prevention of MTCT for women with HIV who do not need ART is ZDV from 28 weeks with single dose nevirapine (NVP) at onset of labour for the mother and single dose NVP plus one week ZDV for the infant. Alternative but less efficacious regimens include one based on ZDV alone (from 28 wk of pregnancy and through labour for the mother and for one week for the infant), one using the combination of ZDV plus lamivudine (3TC) (from 36 wk of pregnancy, through labour and one week postpartum for the mother, and for one week for the infant), and a regimen comprising a single dose of NVP to the mother and to the infant (which does not need to be initiated until labour). The PACTG 076 study in a non-breastfeeding population which HIV-infected pregnant women were given ZDV intravenously starting between 14 to 34 weeks of gestation and continuing through to delivery, and then the newborn infant was given ZDV for 6 weeks, indicated that this regimen decreased transmission by about 67%, from 25.5% to 8.3% [90]. However, it has also been shown that the transmission rate can be reduced to as low as 1-2% with the PACTG 076 regimen plus elective caesarean delivery before onset of labour [248; 5]. In Thailand, the PHPT-1 study examined the efficacy of different lengths of ZDV prophylaxis during pregnancy and to the neonate, it showed an *in utero* transmission rate of 5.1% when ZDV was initiated at 35 weeks versus 1.6% when initiated at 28 weeks [221].

4.2 Mode of delivery

Labour and delivery management of HIV infected pregnant women should focus on minimizing the risk for both perinatal transmission and the potential for maternal and neonatal complications. Caesarean delivery performed before onset of labour and rupture of membranes has been found to be associated with a significant decrease in perinatal HIV-1 transmission in which it protects the baby from direct contact with the mother's blood and genital tract secretions. This procedure is called *elective cesarean delivery*. The European Mode of Delivery Collaboration was a randomized controlled trial of elective cesarean delivery *vs* labour among 436 HIV-infected women [4]. The cesarean delivery would normally be scheduled at 38 weeks of pregnancy, rather than 39 weeks, to avoid the initiation of labour or rupture of membranes. Transmission occurred in 10.5% of women without caesarean delivery compared with 1.8% in the elective cesarean delivery, which represents 80% efficacy, independent of the use of prophylactic antiretroviral therapy with ZDV. The International Perinatal HIV Group performed a meta-analysis of 15 prospective cohort studies, including 8533 mother-child pairs [5]. Those also found a decreased risk of transmission in women undergoing cesarean delivery. However, there was no clear information on HIV-1 RNA levels. More recently, among women with predelivery HIV-1 RNA levels less than 1,000 copies/ml, no difference was found in transmission prevalence among women with vaginal deliveries (0.8%), elective cesarean deliveries (0.8%), or nonelective cesarean deliveries (1.1%) [367]. Other studies have found a

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potential protective role of elective cesarean delivery among women with HIV-1 RNA levels greater than 1,000 copies/ml [375; 120]. The American College of Obstetricians & Gynecologists (ACOG) has chosen 1,000 copies/ml as the threshold above which to recommend elective caesarean delivery as an adjunct for prevention of transmission [7]. Many study results point to significant morbidity associated with cesarean delivery among HIV-infected women [365; 417; 252; 334], and some physicians will not recommend this approach because of the risks to the mother's health.

4.3 Breastfeeding interventions

Breastfeeding is normally the best way to feed infants with its benefits going far beyond sound nutrition, however, HIV is found in breast milk as both cell-associated and cell-free virus, as described above. Therefore HIV-infected women are advised not to breastfeed when they have access to safe milk substitutes. Without any other interventions, the overall rate of MTCT of HIV is about 15% to 25% among HIV-positive women who do not breastfeed and 25% to 45% among women who breastfeed [308]. The risk of transmission is associated with the duration of breastfeeding and possibly the mode of breastfeeding and breast health [118; 180]. Current WHO/UNAIDS/UNICEF guidelines recommend that women with HIV infection should be fully informed of both the risks and benefits of breastfeeding and be supported in their decision about feeding practices [183]. If safe alternatives to breastfeeding are not available in resource-limited settings, exclusive breastfeeding for the first several months of life is recommended. If children born to women infected with HIV can be ensured uninterrupted access to nutritionally adequate breast milk substitutes that are safely prepared, they are at less risk of illness and death if they are not breastfed. However, when these conditions are not fulfilled, in particular in an environment where infectious diseases and malnutrition are the primary causes of death during infancy, artificial feeding substantially increases children's risk of illness and death [183]. The new approaches involving prophylaxis for HIV-exposed breastfeeding infants are being investigated. In the HIVNET 023 study, a phase 1/2 open-label randomized trial with three different 6-month regimens of nevirapine prophylaxis (once weekly, twice weekly and once daily) for breastfeeding, HIV-exposed infants were investigated and the regimens found to be safe and well tolerated [369].

Further, ZDV, 3TC and NVP, have all been detected in the breast milk of HIV-infected women on treatment [277; 284]. This could probably lower the viral load in breast milk and be associated with a reduced risk of HIV transmission. However, there is a possibility of only some drugs penetrating the breast milk, some only in sub-optimal concentrations that may not be sufficient to decrease viral replication, probably promoting the development of drug resistant virus in the milk, which could be transmitted to the infant. Moreover, the toxicity of chronic ARV exposure of infants via breast milk is unknown.

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4.4 Others interventions

Vitamin A prophylaxis

Vitamin A is an essential micronutrient for normal immune function. Vitamin A deficiency is found to be common among HIV-infected pregnant women and was associated with higher MTCT of HIV-1 and increased infant mortality [362]. The biological mechanisms by which vitamin A deficiency could influence MTCT may include impairment of immune responses in both mother and infant, abnormal placental and vaginal pathology and increased HIV viral burden in breast milk and blood [364]. However, there is no conclusive evidence to say that vitamin A supplementation can reduce MTCT.

Vaginal disinfection

Most HIV infections in children occur during delivery. Both free and cell bound viruses have been found in cervical and vaginal secretions. Theoretically, cleansing the vagina with an antiseptic or virucidal agent such as chlorhexidine could reduce this mode of transmission. Again, there is scarcity of evidence to conclusively say that inexpensive modalities like cleaning/disinfection of the birth canal can reduce MTCT. However, the incidence of neonatal sepsis is definitely cut down by these strategies.

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