CHAPTER III

LITERATURE REVIEW

Discovery of HIV-1

AIDS (Acquired Immunodeficiency Syndrome) was the first reported in Morbidity and Mortality Weekly Report on 4th June 1981 by the Centers for Diseases Control in Atlanta. In the period October 1980 - May 1981, five healthy homosexual men were treated for biopsy-confirmed *Pneumocystis carinii* pneumonia (PCP) at three different hospitals in Los Angeles, California. However, this disease was normally occurred only in immunosuppressive person. Thus the occurrence of pneumocystosis in these healthy individuals without a clinically apparent underlying immunodeficiency is unusual. (10)

In the period June 1981 - April 1982, nineteen healthy homosexual men were treated for biopsy-confirmed Kaposi's sarcoma (KS) and/or *Pneumocystis carinii* pneumonia (PCP) in Los Angeles and Orange counties, California. A cluster of Kaposi's sarcoma (KS) and *Pneumocystis carinii* pneumonia (PCP) among homosexual male residents of Los Angeles and Orange counties, California was identified on the basis of sexual contact. One hypothesis consistent with the observation reported is that infectious agents are being sexually transmitted among homosexually active males. (11)

In July 1982, three heterosexual hemophilia A patients, who had developed *Pneumocystis carinii* pneumonia and other opportunistic infections, were reported. In the intervening 4 months, four additional heterosexual hemophilia A patients have developed one or more opportunistic infections accompanied by in-vitro evidence of cellular immune deficiency. All these patients have received Factor VII concentrates, and all but one has also received other blood components. (12) Thus these infectious agents are also being blood transmitted.

Because of this disease cause immunodeficiency syndrome but it is not a typical of any of the well-characterised congenital immunodeficiency syndromes. Thus this disease was named as AIDS or Acquired Immunodeficiency Syndrome. By the end of 1982, the distribution pattern of cases strongly suggested that AIDS was caused by an agent transmitted through sexual contact between men (11, 13) and between men and

women (14, 15) and transmitted through blood among injecting-drug users and among recipients of blood or blood products (12, 16). Cases also were identified among infants born to women with AIDS or at risk for AIDS (17).

In 1983, Robert Gallo and coworkers could isolate the novel retrovirus from patients with AIDS (18). During the same year Francois Barre-Sinoussi and coworkers could also isolate the novel retrovirus from lymph node of homosexual patient with multiple lymphadenopathies (19). Both isolates were reported in Science, 1983 and now known as Human Immunodeficiency Virus or HIV.

Origins of HIV

Many scientists now believe that AIDS appears to have started in central Africa. Because of the most subtypes and CRFs (circulating recombinant forms) are present in central Africa and HIV isolates in Africa are more genetic diverse than other continents (20, 21). Moreover, phylogenetic analysis of HIV sequences enabled the first zoonotic transmission from chimpanzee to people to be placed at around 70 years ago in central Africa (22, 23). Several monkey and chimpanzee species found in Africa are infected with retroviruses that closely related to HIV, called SIV (Simian Immunodeficiency Virus) (22, 24-26). These simian-derived strains of the retrovirus were isolated from many monkey and chimpanzee species. They are specific to monkey and chimpanzee species which is natural or indigenous host because they are apparently nonpathogenic in their natural host. (24, 26, 27) However, spontaneous infection induces a disease with clinical features that resemble human AIDS, called simian AIDS (SAIDS).

Several hypotheses have been put forward as possible mechanisms by which the simian-derived strain of the retrovirus may have been introduced into humans. Three events could explain a species jump from simians to humans. The first possibility is that the virus could have been transmitted to humans in the 1800s or early 1900s perhaps through the fact that rural Africans supplement their diet by hunting and eating small wild animals, often the simian. Those who hunt these animals occasionally injure themselves while preparing the animal carcasses, and animal blood has likely entered human tissues through accidental cuts. (28, 29)

The second possibility suggests HIV introduction by early malaria research. According to Charles Gilles, a British tropical disease expert, the early malaria research

took blood from chimpanzees, sooty mangabeys and macaques to vaccinate human volunteers against malaria; if this blood was infected with strains of SIV it might have infected the human volunteers and slowly mutated to give rise to HIV-1 or HIV-2 (28, 30).

The third possibility may have occurred from the result of contamination of early polio vaccines. Dr. Jonas Salk developed the first widely used polio vaccine in 1954. This vaccine used ultraviolet light and formaldehyde fixation to inactivate (kill) the polio virus. However formaldehyde fixation generally inactivated many contaminating viruses, one contaminant virus that was not inactivated was the retrovirus simian virus (SV40; named because it was the fortieth simian virus found contaminating the early polio vaccine) (28, 30).

In 1956, Dr. Albert Sabin developed and started human vaccination trials with a live attenuated polio vaccine which was developed in a monkey kidney cells.

During the same period an oral attenuated vaccine was also developed by Dr. Hillary Kaprowski in 1957. Kaprowski's vaccine was manufactured in a medium-primary monkey kidney epithelium. This vaccine was administered in the former Belgian Congo, Rwanda, and Burundi, a region of central equatorial Africa where seropositive to HIV is among the highest in Africa. Although, there is no good evidence that any lentivirus will infect kidney cell cultures, but there is abundant evidence that either HIV or SIV can grow in cultured lymphocytes or macrophages that may accompany industrial cultures of epithelium taken from wild caught monkeys. Thus SIV may indeed infect lymphocytes, macrophages, and other blood products, contaminants ubiquitous in primary monkey kidney culture. (28)

However, in 1996, Arifa Khan and coworkers analysed 12 monopools of oral poliovirus vaccine, which were released for use by a North American manufacturer between 1976-1986, for the presence of HIV-1 and SIV. The PCR primers used in the analyses were selected from highly conserved regions of the viral genome to detect different isolates in the HIV-1 and SIV/HIV-2 groups. The results indicate the absence of HIV/SIV in the oral polio vaccines. (31) Moreover, trypsin which is used in vaccine production was valuable for assessing non-specific binding of HIV virions to $CD4^+$ cells. Thus trypsin was helpful for eliminating input HIV under the condition used for manufacture of the vaccine. (32, 33) In addition, samples of vaccine, in storage for over 40 years, were tested at three different laboratories in the United States, Germany and

France. The tests have shown no trace of HIV or its primate antecedent HIV (34), together with the molecular epidemiological data (35).

Structure and molecular biology of HIV

As for other Retroviruses in Family Lentivirinea, HIV has unique three layer structures with roughly spherical shape, is about 110 nanometers (nm) in diameter. The outer surface of HIV particle consists of lipid envelope which is derived from host cell membrane and comprises numerous spikes. Each spike contains surface glycoprotein (SU) or gp120 and transmembrane glycoprotein (TM) or gp41. Beneath the envelope is a layer of matrix protein that surrounds the core or capsid. The capsid has a hollow truncated core shape that contains two identical strands of RNA and associated enzymes including protease (PR), reverse transcriptase (RT), RNase H (RNH), and integrase (IN). (36) (Figure 1-2)

Figure 1 Drawing of the HIV virion.
(ARIC's AIDS medical image gallery : http://www.critpath.org/aric/library/img002.htm)

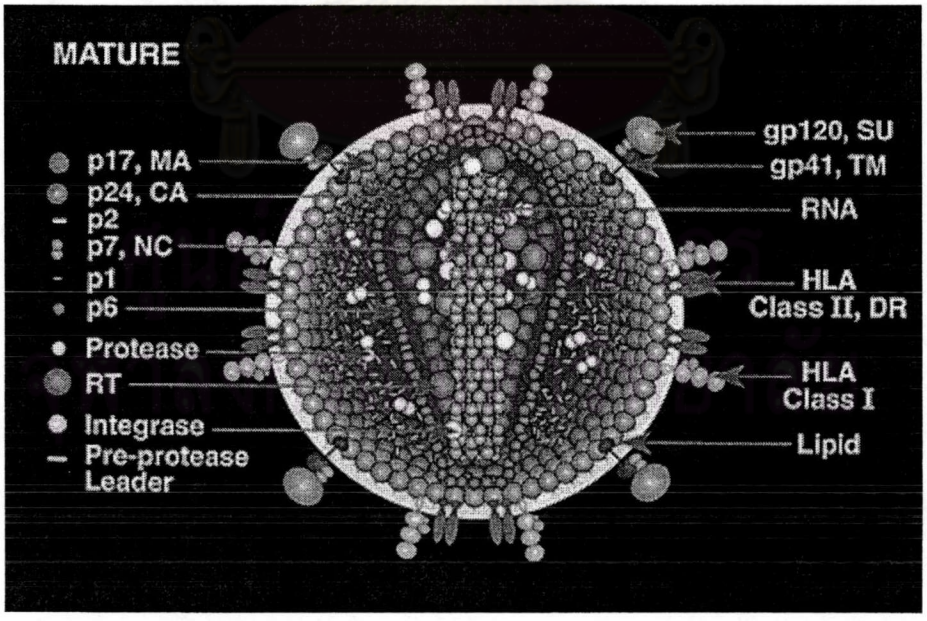
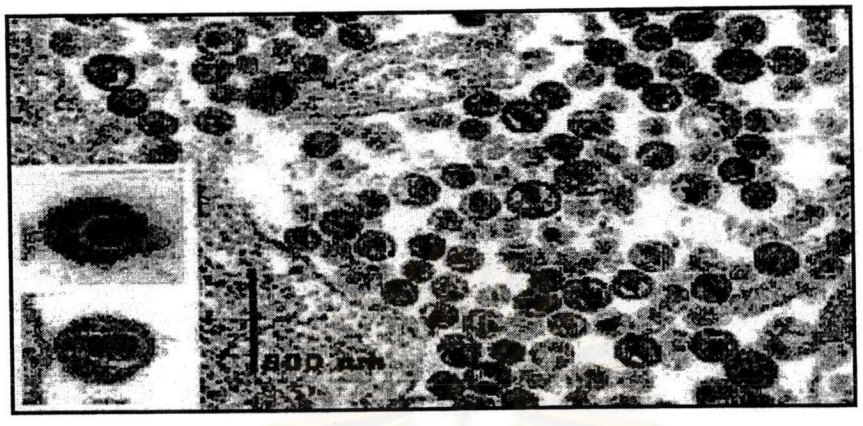


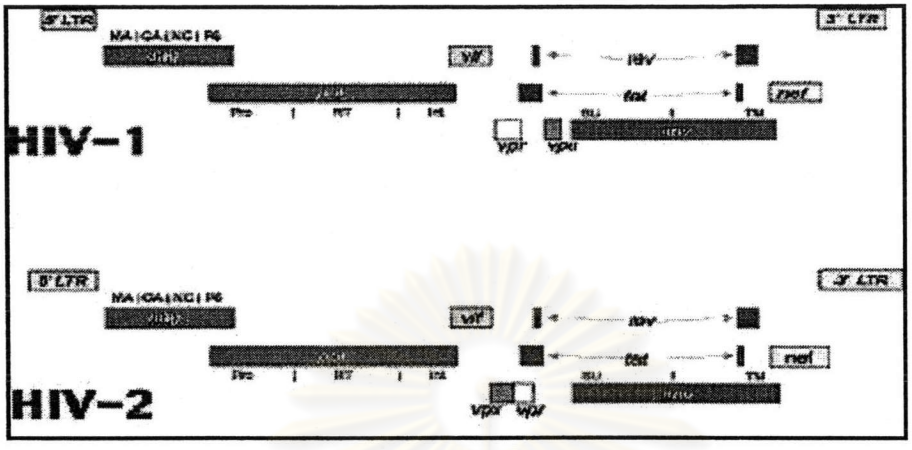
Figure 2 Electron micrograph of HIV virion.
(http://www.mbim.web.arizona.edu/academics.html)



HIV is grouped into two subtypes, designated HIV type 1 (HIV-1) and HIV type 2 (HIV-2), on the basis of serologic properties and sequence analysis of molecular cloned viral genomes. Both types has nucleotides homology about 40%. A hypervariable site is in V3 region of *env* gene so this site is used for grouped HIV-1 and HIV-2 by serology assay. The difference of nucleotides within *env* gene is used for isolate HIV-1 into 11 genetic subtypes or clades (A, B, C, D, E, F, G, H, I, J, K), and HIV-2 into 5 genetic subtypes or clades (A, B, C, D, and E). HIV-1 is the most prevalent throughout the world, and HIV-2 is the most common in Africa and less severity than HIV-1. (37)

The HIV genome is positive polarity diploid RNA which is not infectious RNA, is about 9.2 kilobases (kb) in length and has a basic arrangement of all known retroviruses. It consists of long terminal repeat (LTR) at each end of the genome; three structural gene, namely *gag*, *pol*, *env*. In addition, HIV has at least six more genes encoding viral proteins with regulatory functions (*tat* and *ref*) or accessory functions (*nef*, *vif*, *vpr*, and *vpu* or *vpx*. *vpu* is found exclusively in HIV-1, whereas *vpx* is found only in HIV-2) (Figure 3). *tat* and *ref*, are absolutely necessary for virus growyh whilst *nef*, *vif*, *vpr*, and *vpu* or *vpx* encode factors that are dispensable for virus growth in many in vitro systems (38). The LTR comprises of two short redundant (R) sequences at both termini with adjacent unique sequences, U5 and U3, found at the 5' and 3' ends respectively.

Figure 3 Genome organization of HIV-1 and HIV-2.
(http://www.aids.harvard.edu/ research/discoveries.html)



The structural proteins

1. Gag

The *gag* gene encodes a 55 kilo Dalton (kDa) precursor polyprotein, also called p55. Gag is proteolytically cleaved to four proteins, the matrix p17 (MA), the capsid p24 (CA), the nucleocapsid p7 (NC) and the low molecular mass cleavage products, p1, p2, and p6 during the maturation process (36, 39). The proteolytic processing of Gag induces a major transformation in virion structure. The MA protein remains associated with the inner face of the viral membrane whilst the CA protein condenses to form a shell around the viral RNA/NC complex.

Matrix p17 (MA)

The MA protein is about 132 residues long. The crystal structure of residues 1 through 104 shows five α-helices capped by a three-strand mixed β-sheet, with three monomers arranged like a triskylion (40). It contains N-terminal, myristoylated group basic residues within the first 50 amino acids. It is important for targeting Gag and Gag-Pol precursor polyproteins to the plasma membrane. The N-terminally myristoylated domain direct binding to the plasma membrane for the formation of viral particles. However, the N-terminal basic region is not strictly required for this process because noninfectious viral particles that lack the MA can be produced if a myristate group is placed directly upstream of CA (41). In addition, the N-terminally myristoylated MA

domain is also important in either incorporation or stable retention of Env. It interacts with the cytoplasmic tail of TM for help incorporate Env glycoproteins with long cytoplasmic tails into viral particles. (41, 42)

Capsid p24 (CA)

The CA protein is about 240 residues long. It forms a conical core of viral particle for encapsidates the viral RNA, NC protein, and key enzymes. It comprises of seven α-helices, two β-hairpins, and an exposed, partially ordered loop (36). This structure differs significantly from other RNA virus coat protein structures (43). The major homology region (MHR), a 20–amino acid sequence that is one of the most highly conserved within all retroviral Gag proteins, adopts a compact fold in which the four most conserved residues (Gln155, Gly156, Glu159, and Arg167) form a stabilizing hydrogen-bonding network. Additional, hydrophobic residues from the MHR contribute to the hydrophobic core. The MHR is essential for membrane binding and viral particle formation. The mutant was significantly impaired in its ability to self-associate on RNA or on membrane surfaces. This result suggest that Gag first binds to RNA and then assembles into a multimeric complex with a large membrane-binding face that facilitates subsequent membrane binding. (44) Furthermore, it may have a role in incorporation of Gag-Pol precursors through interactions with Gag, though not all mutants show this phenotype. (40)

Nucleocapsid p7 (NC)

The NC protein is about 55 residues long. It contains two conserved zinc finger domains "CCHC array" (Cys-X_2-Cys-X_4-His-X_4-Cys; where X = variable amino acid residue (45) as all retroviral NC protein (except for the spumaviruses). These domains are sometimes referred as "zinc knuckle" or "zinc finger like' array. (46) Both domains are separated by a short "linker sequence" (RAPRKKG) (36) and flanked by basic amino acids (40). The zinc finger domains help genome recognition and packaging through the interactions between these domains and about 120 nucleotide region of unspliced viral RNA known as the ψ-site located between the 5' long terminal repeat (LTR) and *gag* initiation codon (36). The basic residues function in vitro RNA binding (Arg7, Arg32, and Lys33) and viral replication (Lys11 and Lys14) (40).

p6

The P6 is about 51 residues long. It is a small Pro-rich protein which is located at the C terminus of Gag. It is important for incorporation of Vpr during viral assembly. Residues 32-39 and three hydrophobic residues within a highly conserved sequence motif (Leu41-X42-Ser43-Leu44-Phe45-Gly46) are important for Vpr binding. It also helps mediate efficient particle release and a region of four amino acids (Pro7-Thr8-Ala9-Pro10, PTAP) near the N terminal, has been implicated in this function. (40)

2. Pol

The *pol* gene encodes a precursor protein for several virion enzymes (PR, RT, RNase H and IN) that function at different times during the replicative cycle. The reverse transcriptase/ribonuclease H complex p51 and p66 (RT) acts in the early steps of the virus replication to form a double-stranded cDNA of the viral RNA. The integrase p32 (IN) mediates integration of the viral cDNA into the host chromosomal DNA. The protease p10 (PR) is responsible for the cleavage of the viral Gag and Gag-Pol polyproteins into function active protein during the maturation of the viral particle. (40)

Protease p10 (PR)

The PR protein is about 99 residues long (40). It is a symmetrical homodimer which is stabilized by a four-stranded antipararelle β-sheet formed by N- and C-terminal β-strands (36). The enzyme active site is formed at the dimmer interface and contains a conserved triad sequence, Asp-Thr-Gly. The dimmer contains flexible flabs that close down on the active site upon substrate binding (40). Therefore, third flexibility may be important for enzyme activity (36). The PR cleaves at several polyprotein sites to produce the final MA, CA, NC, and p6 proteins from Gag and PR, RT, and IN proteins from Pol (40) thus PR activity can have dramatic effects on virus production.

Reverse transcriptase p66 (RT)

The RT protein is a heterodimer consists of a 560-residues subunit (p66) and a 440-residues subunit (p51) (40). The p66 subunit contains both a polymerase and an RNase H domain; proteolytic cleavage of p66 removes the RNase H domain to yield the p51 subunit. Each subunit contains a polymerase domain consists of four subdomains called fingers, palm, thumb, and connection, and p66 contains an additional RNase H

domain. Even though their amino acid sequences are identical, the polymerase subdomains are arranged differently in the two subunits. The polymerase domain of p66 folds into an open, extended structure containing a large active-site cleft whilst p51 is closed and compact. Hence, it is a asymmetric homodimers. (47) The RT is both RNA-dependent DNA polymerase and DNA-dependent DNA polymerase thus it generates DNA from both RNA template and DNA template. The RNase H removes the original RNA template from the first DNA strand, allowing synthesis of the complementary strand of DNA. (40)

Integrase (p32) IN

The IN protein is about 288 residues long. It is active as an oligomer, probable a tetramer. The 288-residues monomer consists of three separate structural and functional domains. The N-terminal zinc binding domain (residues 1-55) contains a zinc-binding site. This domain facilitates oligomerization. The central catalytic core domain (residues 50-212) contains a D and E motifs which are conserved among integrases. This domain is crucial for the processing and joining reactions. The C-terminal domain (residues 220-270) has nonspecific DNA-binding activity. (40) This IN protein recognises long terminal repeats (LTR) at the 5' and 3' ends of the newly synthesized viral DNA duplex and cleaves two (sometimes three) bases from the 3' ends. The IN then ligates the 3' ends to the cellular DNA in the nucleus, with reactions appearing to occur preferentially at sites with highly bent DNA. The resulting unligated 3' ends of the cellular DNA are subsequently extend to fill gaps, and additional processing leads to the complete covalent incorporation of the proviral DNA. (36)

3. Env protein (p88) gp160

The Env (envelope) protein is about 850 to 880 residues long. It first synthesized in the endoplasmic reticulum (ER) and then migrates through the Golgi complex where it undergoes glycosylation with the addition of 25 to 30 complex N-linked carbohydrate side chains that are added at asparagines (Asp) residues. This glycosylation is required for infectivity. (48) The Env protein or gp160 undergoes cellular proteolytic cleavage into a surface (SU) subunit or gp120 and a transmembrane (TM) subunit or gp41. The gp120 is about 515 residues long. (40) It noncovalent interacts with the gp41 and exists as a trimer on the surface of infected cells and virions (49). The gp120 is five

hypervariable regions, designated V1 through V5. V1, V2, V4, and V5 is involved in CD4 binding whilst V3 is rather an important of viral tropism (50). V3 loop is also the principle target for neutralizing antibodies that block HIV-1 infectivity (51). The gp120 also interacts with DC-SIGN which is expressed on the surface of dendritic cells to increase the efficiency of infection of $CD4^+$ T cells and facilitate mucosal transmission by transporting HIV to lymphoid tissues. The gp41 is about 345 residues long (40). It forms a trimer and contains an N-terminal hydrophobic glycine-rich fusogenic domain (52) This domain mediates the fusion of the viral and cellular membranes (49, 52).

The regulatory proteins

1. Tat

The Tat protein is about 72 and 101 residues long which expressed by early fully spliced mRNAs or late incompletely spliced HIV mRNA, respectively. Its name is from the function of both forms that function as transcriptional activators. It consists of four different regions that share homologies with the Tat proteins of other lentiviruses, including (from N to C terminus) a cystein-rich, a hydrophobic core, a basic, and a glutamine-rich segments. The basic segment of Tat (R49KKRRQRRR57) is essential for recognition and binding to TAR (transactivation response element) RNA, that is located at the 5' terminus of HIV RNA. This binding activates transcription from the HIV LTR at least 100-fold. (40)

2. Rev

The Rev protein is about 116 residues long. This protein contains at least three functional domains, including a basic or arginine-rich RNA binding domain (Arg35 to Arg50), a multimerization domain, and an effector or Rev activation domain. (36) The basic domain is required for mediates interactions with the RRE (Rev-response element which locates within the unspliced viral transcripts of *env* gene, the multimerization domain is required for the Rev to function, and the effector domain contains a leucine-rich specific nuclear export signal (NES) that facilitates the viral RNA from nucleus to cytoplasm through the interaction with a nuclear export receptor (namely hRIP/Rab) located at the nuclear pore. Hence, the Rev is important in the transporting of the unspliced RNA that contains the RRE to the cytoplasm where they are translated and where genomic RNA are packaged. (40)

The accessory proteins

1. Nef

The Nef (negative factor) protein is about 206 residues long (36, 40). It contains N-terminally myristoylated protein, like Vpu (40). It was first suspected to repress transcription from the LTR, hence its acronym for "negative transcription factor". It accomplishes several distinct functions. First, it is a crucial factor in HIV-1 immunopathogenesis. Nef mutant viruses exhibit decreased rates of viral DNA synthesis following infection (40). In Sydney Blood Bank Cohort, the *nef* gene deletion is the principal cause of the lack of disease progression (53). Second, it decreases the cell surface expression of CD4 receptor of infected cell via endocytotic and lysosomal degradation processes (54), and appears to involve the direct interaction of Nef with residues within the cytoplasmic tail of CD4 (36). CD4 downregulation appears to be advantageous to viral production because an excess of CD4 on the cell surface has been found to inhibit Env incorporation and virion budding (55). Third, it downregulates the cell surface expression of MHC (Major Histocompatibility Complex) class I molecules of infected cells which may help protect the infected cells from killing by cytotoxic T cell (56, 57). Finally, it alters T-cell activation pathways. The study of chimeric CD8-Nef molecule in Jurkat cells induced two opposite phenotypes which depended on the intracellular location of the fusion protein. It show a block in the early events of T cell signaling through the cell receptor (TCR) when the chimera accumulated in the cytoplasm whilst it show spontaneous activation, which led to apoptosis when the chimera high level expressed on the cell surface. (58) These results could be explained if Nef bound a cellular protein involved in activation. When kept in the cytoplasm, Nef would sequester the putative signaling molecule away from the TCR; when translocated to the cell surface, Nef and its associated protein would induce activation. (38)

2. Vif

The Vif (virion infectivity factor) protein is about 192 residues long (40). The Vif requirement is strictly cell dependent. It is essential for the replication of HIV in peripheral blood lymphocytes, macrophages, and certain cell lines. *Vif*-mutated HIV-1 is functional impaired only if produced from these cells, so called non-permissive cells. In most cell lines, the Vif is not required, designated permissive, suggesting that these cells produce a factor or factors that compensate for a lack of the Vif (40) or these cells absent

of an inhibitor of the Vif. Vif defective HIV strains can enter cells but cannot efficiently synthesize the proviral DNA (59). Compared with mature wild-type virions, Vif mutant viruses have similar protein and RNA contents but grossly altered core structures, suggesting that the Vif may play a role in viral assembly and/or maturation (40).

3. Vpr

The Vpr (viral protein R) is about 96 residues long. It is a short basic protein that is incorporated into viral particles through an interaction with the p6 and may later become associated with the nucleoprotein complexes through an interaction with the C-terminal region of the MA. It acts as a nucleocytoplasmic transport factor by directly tethering the viral genome to the nuclear pore. It contains an unusal nuclear localization signal (NLS), two putative N-terminal amphipathic α-helices instead of a canonical karyophilic NLS. (40) In addition to its nuclear uptake function, it can also induce G2 cell cycle arrest. Its C-terminal region has been shown to interact with p34cdc2/cyclinB complex, which is a regulator of the cell cycle important for entry into mitosis. This interaction leads to nuclear envelope breakdown and chromosome condensation, followed by T cell apoptosis. (40, 60, 61)

4. Vpu

The Vpu (viral protein U) is about 81 residues long. It is an oligomeric integral membrane phosphoprotein with an N-terminal 24-residue hydrophobic membrane-spanning domain and a C-terminal cytoplasmic tail (40). It is expressed by most HIV-1 isolates but not by HIV-2 or SIV, with the notable exception of SIVcpz, a close relative of HIV-1 (38). It carries a dual function, the down-modulation of CD4 and subsequent the ubiquitin-mediated degradation of CD4 molecules complexed with the Env, which interferes with virion assembly (38, 40, 62). It also promotes the release of virions from the surface of an infected cell. In the absence of the Vpu, large numbers of virions remain attached to the surface or are localized to intracellular membranes (63).

HIV life cycle (Figure 4)

Binding

To productively infect target cell, HIV must introduce its genetic material into the cytoplasm of the target cell. The process of virion entry involves the fusion of the virion envelope with the host cell membrane and requires the specific interaction of the envelope with various specific receptor molecules on the cell surface. The two viral envelope proteins, gp120 and gp41, are conformationally associated to form a trimeric functional unit consisting of three molecules of gp120 exposed on the virion surface and associated with three molecules of gp41 inserted into the viral lipid membrane. Trimeric gp120 on the surface of the virion binds CD4 on the surface of the target cell, inducing a conformational change in the envelope proteins that in turn allows binding of the virion to a specific subset of chemokine receptors on the cell surface. (64) These receptors normally play a role in chemoattraction, in which hematopoietic cells move along chemokine gradients to specific sites. Although these receptors, which contain seven membrane-spanning domains, normally transducer signals through G proteins, (65) signaling is not required for HIV infection. The binding of surface gp120, CD4 and the chemokine coreceptor (CXCR4 or CCR5, etc.) produces an additional radical conformational change in gp41. (66) Assembled as a trimer on the virion membrane, this coiled-coil protein springs open, projecting three peptide fusion domains that "harpoon" the lipid bilayer of the target cell. The fusion domains then form hairpin-like structures that draw the virion and cell membranes together to promote fusion, leading to the release of the viral core into cell interior. (66)

Entry

HIV virion can also enter cells by endocytosis. Usually, productive infection does not result, presumably reflecting inactivation of these virions within endosomes. However, a special form of endocytosis has been demonstrated in submucosal dendritic cells. These cells, which normally process and present antigens to immune cells, express a specialized attachment structure termed DC-SIGN. (67) DC-SIGN is C-type lectin which binds HIV gp120 with high affinity but does not trigger the conformational changes required for fusion. Instead, virions bound to DC-SIGN are internalized into an acidic compartment and subsequently displayed on the cell surface after the dendritic cell has matured and migrated to regional lymph nodes, where it engages T cells. (68) Thus dendritic cells expressing DC-SIGN appear to act as "Trojan horses" facilitating the spread of HIV from mucosal surfaces to T cells in lymphatic organs.

Uncoating and reverse transcription

Once the HIV virion enters the cell, the virion undergoes uncoating. Nef associates with a universal proton pump, V-ATPase, (69) which could promote uncoating by inducing local changes in pH in a manner similar to that of the M2 protein of influenza. After the virion is uncoated, the viral reverse transcription complex is released from the plasma membrane. (70) This complex includes the diploid viral RNA genome, lysine transfer RNA ($tRNA^{Lys}$) which acts as a primer for reverse transcription, Vpr which play an importance role in nuclear uptake function, viral reverse transcriptase (RT), integrase (IN), matrix (MA), nucleocapsid proteins (NC) and various host proteins. The viral RNA was generated to double-stranded DNA (dsDNA) which is compatible with the cellular DNA within the host cells's nucleus by using reverse transcriptase (RT). This process is most unusual in the nature because nearly almost living organisms transfer genetic information in only one direction, from DNA to RNA. This is why scientists have given the family name of these viruses "retrovirus", retro meaning backward.

Nuclear entry

Reverse transcription yields the HIV preintegration complex (PIC), composed of double-stranded viral cDNA, matrix, Vpr, integrase, reverse transcriptase, and the high mobility group DNA-binding cellular protein HMGI(Y). The PIC is transported to cell's nucleus where it enters by crossing the nuclear membrane. Both matrix (71) and Vpr (72), act as nuclear targeting signal.

Integration and transcription

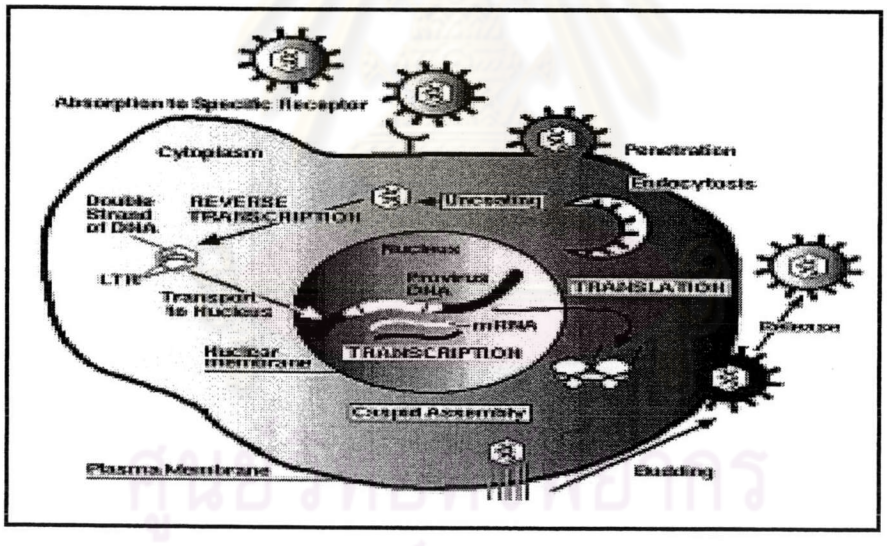
Once the PIC enters the nucleus. The viral cDNA is completely integrated into the host chromosome which is mediated by integrase. The high mobility group DNA binding cellular protein HMGI(Y) is required for efficient integration, although their precise functions remain unknown (73). Integration can lead to latent or transcriptionally active forms of infection (74). At this point, the process of viral reproduction truly begins as the viral DNA (also called "proviral DNA") within the cell's genetic material transcribes its genetic information back into viral RNA. The 5' LTR functions like other eukaryotic transcriptional units. It contains downstream and upstream promoter elements, which include the initiator (Inr), TATA-box, and three Sp1 site. These regions help

position the RNA polymerase II (RNAP II) at the site of initiation of transcription and to assemble the preinitiation complex.

Translation and assembly and budding

Within the cytoplasm, the viral proteins are processed in a process called translation. These viral proteins are then cut into smaller pieces by the viral enzyme protease. The freshly cut proteins are assembled at the plasma membrane. Each virion consist of roughly 1,500 molecules of Gag and 100 Gag-Pol polyproteins, two copies of viral RNA genome, and Vpr (75). They emerge from the host cell within a tiny section of the cell's outer lipid membrane to make its own viral envelope.

Figure 4 HIV life cycle.
(ARIC's AIDS medical image gallery : http://www.critpath.org/aric/library/img003.htm)



The genetic diversity of HIV-1

By the early 1990s, several genetic subtypes of HIV-1 had been recognised, each with a different geographic distribution. The high error rate of reverse transcriptase was identified as the main source of diversity of HIV-1, which is divided into group M (main) and two other groups named O (outlier) and N (new, non-M and non-O) (76). There is sequence variation even within patients, and this observation led to the term

"quasispecies" to reflect the presence in infected individuals of a "swarm" of viruses rather than a single isolate (77). Most HIV-1 infections in the global pandemic are caused by group M which comprises the great majority of HIV-1 isolates. It consists of nine subtypes or genotypes or clades, A, B, C, D, F, G, H. J and K, which were seperated by 25-30% amino acid distance between *env* sequence (76),(37). Whilst group O are limited to people living in or epidermiologically linked to central Africa (mainly Cameroon and some neighbouring countries), but even in this area they represent a small minority of HIV-1 infection and group N was recently identified from Cameroon (37).

By 1995, recombination had been identified as a major mechanism of HIV variation. Packaging of RNA from different subtypes into the same viral particle, coupled with the strand-switching activity of reverse transcriptase, generates recombinant HIV in co-infected individuals. This inter-subtype virus is transmitted from one patient to others and becomes one of the HIV epidemic. It identified in at least three, epidemiologically unlinked people and characterized by full-length genome sequencing, are designated as circulating recombinant forms (CRF). (37)

The most of available full-genome sequences of HIV-1 are showed in table 1. It consists of subtypes A through K, excluding E and I. Subtype E and I were reclassified as CRF when the first full-genome sequences were completed. Subtype E, which is spreading epidemically in several countries in Southeast Asia but originated from Central Africa, is a mosaic sequence consisting of A and E sequences and classified as CRF01_AE (78, 79) (Figure 5). Subtype I, which was probably circulating in Greece in the early 1980s, is a triple mosaic consisting of A, G, and I sequences (80). Besides subtype A through K, table 1 also shows nine CRF, thirty-three "unique" recombinant forms, and other five full-genome sequences which unclassified an unrelated, suggesting the existence of undiscovered subtypes (76).

The current geographic distribution of HIV subtypes and CRF is shown in Figure 6. The most prevalent strains in Africa are A, C, and CRF02_AG (IbNG), with substantial prevalence of subtypes D, F, G, H, J, and CRF01_AE. Rare strains in Africa include subtype B, a variety of other recombinants, and groups O and N. West Africa harbors mostly HIV-1 CRF02_AG (IbNG), subtype G, and HIV-2. In Asia, subtype C and CRF01_AE predominate, whilst subtype B, at least two different BC recombinants, and HIV-2 are also circulating. In Europe, subtype B predominates, but virtually all of the subtypes, prevalent CRF, group O, and HIV-2 have been found. In North America,

subtype B is the majority strain, with less than 1% of infections caused by all other subtypes and CRF combined. Subtype B dominates South America, but now up to 25% of infections may be subtype F and/or B/F recombinant. In Australia, only subtype B has been reported, and new full-genome sequences from the continent have been published. (76)

The genetic diversity of HIV-1 in Thailand

More than 80% of HIV infection in Thailand caused by subtype E or CRF01_AE , followed by 15% of subtype B' or Thai B and 1% remaining caused by other subtype (Figure 7). CRF01_AE strains from Asia and Africa are distinct (76) and Thai genotype B also differed from previously subtype B from North American and Europe with V3 motif GPGR whilst Thai genotype B possess V3 motif GPGQ, so called subtype B' or Thai B (81).

CRF01_AE and Thai B established an explosive epidemic in Thailand in the early 1990s and subsequently spread to several other countries in Southeast Asia. The genetic diversity of CRF01_AE has increased slowly in Thailand, however, the potential HIV-1 diversity in Thailand is increasing by recombination. Among Thai intravenous drug users (IVDU), the initial subtype B' epidemic has been gradually supplanted by CRF01_AE, providing for strain mixing in a highly exposed population. In 2000, the first two BE recombinant strain and the first C/CRF01_AE recombinant strain were identified in Thailand. (76)

Table 1 Full-genome sequences of HIV-1.

(McCutchan FE. Understanding the genetic diversity of HIV-1. Aids 2000;14 Suppl 3:S31-44.)

	Category*	Geographic origin
Subtypes	A	Uganda, Tanzania, Kenya, Somalia
	B	USA, Europe, China, Africa
	C	Eastern and Southern Africa, India, Brazil, USA
	D	Uganda, DRC, Kenya
	F and F2	DRC, Kenya, Congo, Cameroon
	G	Nigeria, Kenya, Congo, DRC
	H	Belgium, CAR
	J	DRC, Sweden
	K	Cameroon
CRF	CRF01_AE CM240	Thailand, China, CAR, USA
	CRF02_AG IbNG	Djibouti, Ivory Coast, Cameroon, USA
	CRF03_AB Kal153	Russia
	CRF04_cpx CY032	Cyprus, Greece
	CRF05_FD Vl1310	DRC
	CRF06_cpx BFP90	Burkina Faso, Mali
	CRF07_BC c54	China
	CRF08_BC GX-6F	China
	CRF09_cpx P2911	Senegal, USA
"Unique" recombinant forms	AC recombinant	Tanzania, Zambia, Rwanda, Ethiopia, India, Uganda
	AD recombinant	Uganda, Kenya
	BE recombinant	Thailand, USA
	Other recombinant	Various
Other	Unclassified	Cameroon, DRC, Gabon

The designations F and F2 represent sub-subtypes. CRF are numbered in the order discovered. "cpx", A complex recombination combining three or more subtypes. The structures of CRF are given by reference to a prototype strain, as indicated. The names CRF07_BC, CRF08_BC, and CRF09_cpx are proinvisional. "Unique", recombinant forms so far identified only in a single individual and without current evidence of epidermic spread. Unclassified strains fail to cluster with known subtypes in all genome regions examined.

Figure 5 CRF01_AE Reference strain : CM240 Subtypes : A, E.
(http://www.hiv.lanl.gov/content/hiv-db/CRFs/CRFs.html)

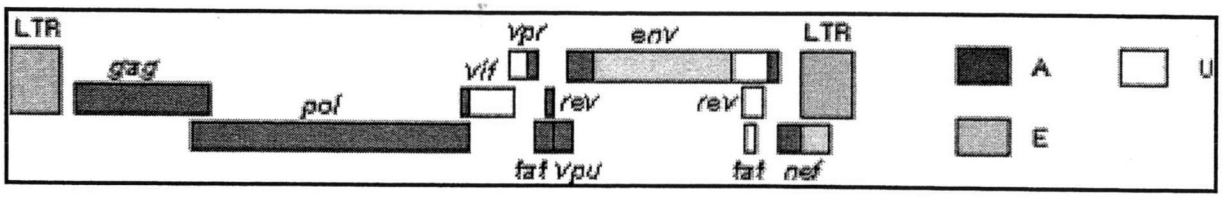


Figure 6 Geographic distribution of HIV-1 types, groups, subtypes, and CRF.
(McCutchan F, Jackson H Foundation [Rockville, Maryland]
http://www.iavireport.org/specials/specials.asp)

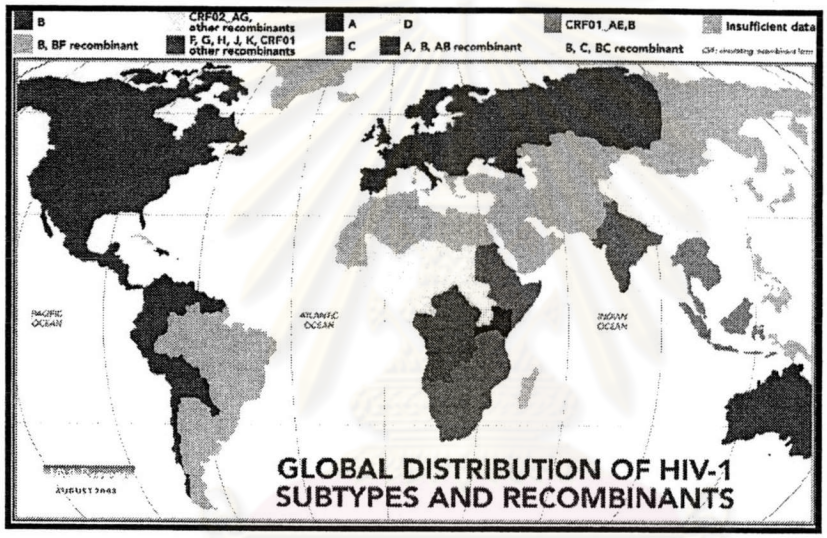
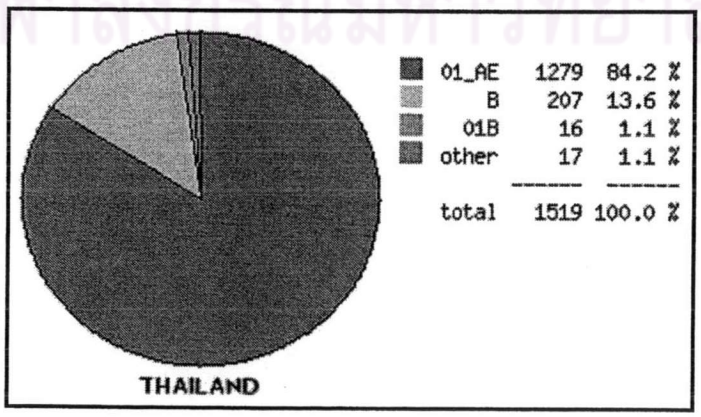


Figure 7 Subtype distributions represent the frequency in the HIV Database and not the population.
(http://www.hiv.lanl.gov/components/hiv-db/new_geography/geography.comp)



The immune responses

The mechanisms of protection against foreign substances can be divided in two main components that are complementary and modulate each other. The early reaction against foreign substances is innate immunity and the later response is adaptive immunity.

Innate immunity

Innate immunity (also called natural or native immunity) consists of mechanisms that exist before exposure to foreign substances, are capable of rapid responses to microbes, and react in essentially the same way to repeated infection. It comprises four types of defensive barriers, (1) physical and chemical barriers, such as epithelia and antimicrobial substances produced at epithelial surfaces; (2) phagocytic cells (neutrophils, macrophages), eosinophils and natural killer (NK) cells; (3) blood proteins, including members of the complement system and other mediators of inflammation; and (4) proteins called cytokines that regulate and coordinate many of the activities of the cells of innate immunity. The mechanisms of innate immunity are stimulated by structures that are common to groups of related microbes and may not distinguish fine differences between foreign substances, called pathogen associated molecular patterns (PAMPs) through the pattern recognition receptors (PRRs).

Adaptive immunity

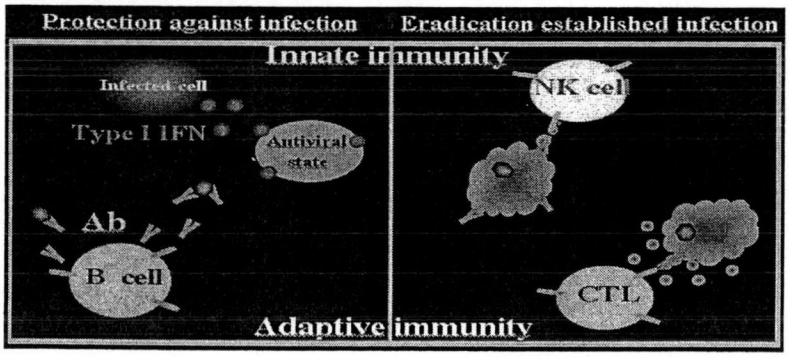
Adaptive immunity (also called acquired or specific immunity) consists of mechanisms that exist after exposure to specific foreign substances, called antigen (Ag). It is capable generating tremendous diversity in its recognition molecules, allowing it to recognise billions of uniquely different structures on antigen and is capable of remember and respond more vigorously to repeated exposures to the same antigen. It comprises two type of immune response, called humoral immunity (HMI) and cell-mediated immunity (CMI). Humoral immunity is mediated by antibodies (Ab) which are produced by B lymphocytes (B cells, the precursors of antibody-secreting cells or plasma cells) and cell-mediated immunity is mediated by T lymphocytes (T cells).

The immune responses to viral infection

Innate and adaptive immune responses to viruses are aimed at blocking the infection and eliminating the infected cells as shown in figure 8. The responses that prevent the infection are shown in the left panel of the figure 8 and consists of type I IFN (innate immunity) and antibodies (adaptive immunity). TypeI IFN comprises IFN-α or leukocyte interferon and IFN-β or fibroblast interferon. They function to inhibit viral replication in both infected and uninfected cells by inducing an "antiviral state". They also promote cell-mediated immunity against viral infection by increasing the expression of class I major histocompatability complex (MHC I) molecules, which present specific antigens to $CD8^+$ T lymphocytes, whilst antibodies are effective against viruses only during the extracellular stage of viruses. They function mainly as neutralizing antibody to prevent virus attachment and entry into host cells. In addition to neutralization, antibody may opsonize viral particles and promote their clearance by phagocytes and possibly by direct lysis of viruses with lipid envelopes.

The respond that eradicate the viral infection by killing infected cells are shown in the right panel of figure 8 and consists of NK cells (innate immunity) and $CD8^+$ T lymphocytes (adaptive immunity). NK cells are important to eradicate the viral infection before adaptive immune responses have developed. NK cells express inhibitory receptors that recognise Major Histocompatibility Complex class I (MHC I), and therefore NK cells are inhibited by MHC I-expressing cells and activated by the viral infected cells in which the virus has shut off MHC I expression. CD8+ T lymphocytes, in contrast to NK cells, recognise the viral antigens in associated with MHC T on any nucleated cells. Up to date, $CD8^+$ T lymphocytes are believed to be a critical role in control of the viral infection , including HIV-1 infection.

Figure 8 Innate and adaptive immunity against viral infection.



Mechanisms of cytotoxic T lymphocytes (CTL) mediated cytolysis

$CD8^+$ T lymphocytes are responsible for recognition and clearance of the viral infected cells through the recognition of T cell receptor (TCR) with antigenic peptide that was presented on MHC I. If the $CD8^+$ T lymphocytes happen to contact a cell expressing the MHC I/antigenic peptide complex for which it is specific, signals transduced through the TCR cause the $CD8^+$ T lymphocytes were activated and become effector cells, called cytotoxic T lymphocytes (CTL). These CTL can lyse the viral infected cells by several mechanisms, (1) perforin is exocytosed in CTL granules and polymerized in the target cell plasma membrane to form a pores that allow the entry of water and ions and result in target cell death; (2) granzymes are exocytosed in CTL granules, enter target cells through perforin pores, and induce target cell apoptosis; (3) Fas ligand (FasL) is expressed on activated CTL, engages Fas on the surface of target cells, and induces apoptosis.

The structure of MHC I

MHC I is composed of two separate, noncovalently linked polypeptide chains. The heavy chain (HC) or α-chain is an MHC-encoded, transmembrane polypeptide of 45-kDa, and the light chain or β2-microglobulin (β2-m) is a non-MHC encoded polypeptide of 12-kDa (82) (Figure 9). The α-chain is glycosylated protein of about 325 amino acid encoded on chromosome 6 in human (Figure 10). There are three class Ia loci, in humans, called HLA-A, B, and C. The α-chain consists of a cytoplasmic region (about 30 residues long), a transmembrane region, and an extracellular region (about 30 residues long), a transmembrane region, and an extracellular region composed of three domains (α1, α2, and α3) with one to three N-linked glycosylation sites. β2-m is a nonglycosylated protein of about 100 amino acid encoded on chromosome 15 in human. It has no transmembrane domain but remains associated with cells by noncovalently interacting with the extracellular region of α-chain. The membrane-proximal α3 region of the HC is an immunoglobulin-like domain that contains a binding site for the CD8 receptor on CTL. The α1 and α2 domains, which are distal to the membrane and interact with the T cell receptor on CTL, fold together to form a groove, called the cleft, that binds and displays peptides. This epitope-binding cleft, which contain a high concentration of polymorphic amino acid, consists of a β-pleated sheet floor and α-helical sides (Figure 11). The allelic

polymorphisms in HC primarily occur in those residues in and around this cleft, and in this manner, they alter the peptide-binding specificity of MHC I. (82)

The peptides that are able to successfully trigger T-cell activation should contain not only amino acid that bind MHC I by forming non-covalent bonds with both the β-pleated sheet floor and α-helical sides of the cleft but also some amino acid whose side chains point away from the cleft and are therefore available for recognition by T lymphocytes. Epitope binding clefts of MHC I have closed ends and one or more pockets in the β-pleated sheet floor to fit side chains of certain amino acid. Thus only peptides of the appropriated length can fit inside the groove. The groove is generally long enough to accommodate 8 or 9 residues in an extended conformation. Longer peptides can also fit by bulging partly out of the groove or by zigzagging within the cleft. In some cases the ends of the peptide may extend out of the groove, although this decreases the stability of interaction. Peptides contain usually two amino acid whose side chains bind noncovalently to the pockets, can bind MHC I with high affinity. One of these two rather invariant amino acid for a defined allele (called anchor residues) is usually located in the carboxyl terminal position of the nine amino acid sequence of the epitope. In the most cases it consists of a hydrophobic amino acid and binds to the F pocket of the MHC peptide-binding site. The second anchor residue is usually located in the position 2 from the N-terminus of the peptide and binds to the B pocket of the MHC peptide-binding site. The remaining amino acid of the antigenic peptides do not bind or bind to a lower extent to the cleft, and their side chains are therefore available for recognition by T cells. Moreover, amino acid located near the anchor residues, inside and outside the epitope, have also been shown to influence, either positively or negatively, the formation of stable MHC-peptide complexes. (82)

Figure 9 Drawing of HLA class I molecule.

(Klein J, Sato A. The HLA System. First of Two Parts. N Engl J Med 2000;343:702-9.)

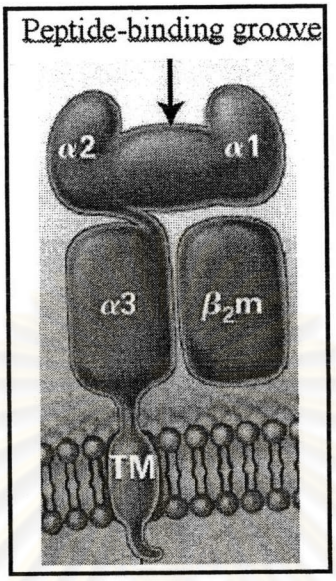


Figure 10 Location and organization of HLA complex.

(Klein J, Sato A. The HLA System. First of Two Parts. N Engl J Med 2000; 343:702-9.)

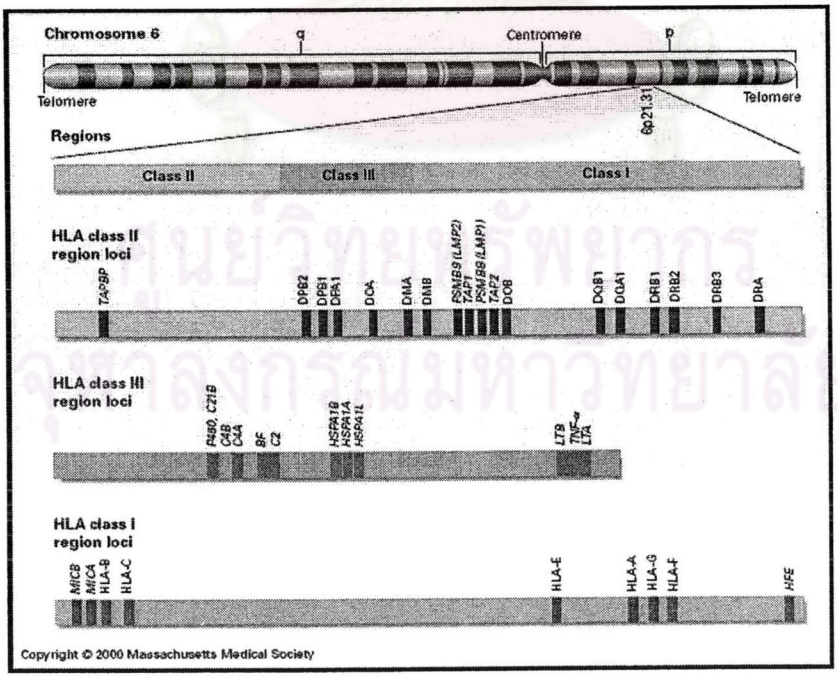
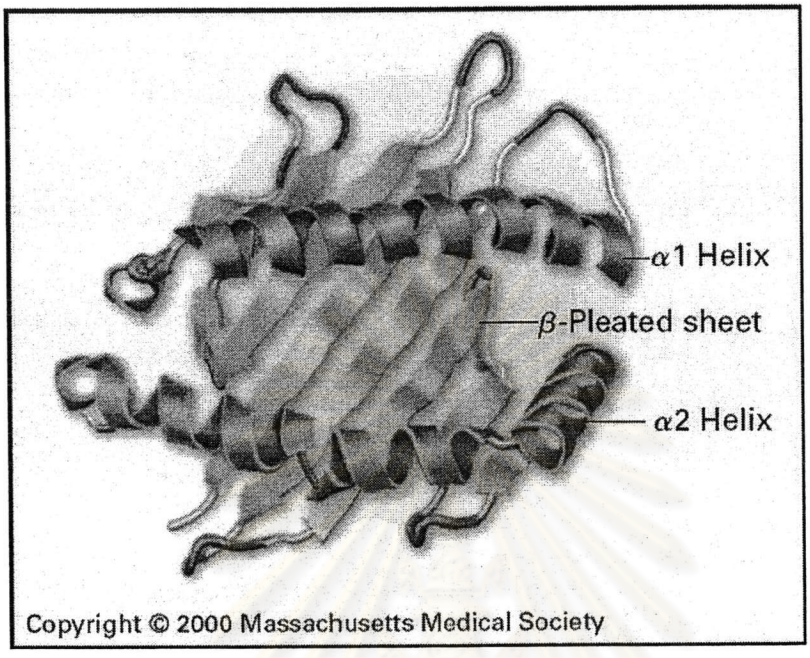


Figure 11 Ribbon model of the tertiary structure of HLA Class I peptide-binding module. (Klein J, Sato A. The HLA System. First of Two Parts. N Engl J Med 2000; 343:702-9.)



Cytolytic pathway for antigen processing and presentation to MHC I

Proteolysis is a basic function of the cell. It contributes to the acquisition of the final, mature form of a protein, or it is responsible for its degradation when it is defective or no longer needed (83). A large proportion of polypeptides might never attain their native structure because of the errors known as defective ribosomal products (DRiPs), were found to rapidly ubiquitylated and degraded. This mechanism mediated by 26S proteasome which comprises two components, a 20S catalytic core and two 19S regulator (Figure 12). (82, 84)

The 20S catalytic core consists of 28 (14 different) subunits, 21-31 kDa in molecular mass, which are arranged as four heptameric staggered rings. The two outer rings contain the α subunits (α1- α7) which form the gate through which substrates enter and products are released. The two inner rings contain two copies of the β-subunits (β1- β7), three of which (β1, βb2, and β5) harbour the six active sites, function in catalytic activities. The catalytically active residue is a single threonine located at the amino acid termini of three β-subunits. Each of the three β-subunits has a different preference of cleaving after acidic, basic or hydrophobic residues, respectively.

The 19S regulator consists of two multisubunit components, a base and a lid. The base, which attaches to the two α-rings, is composed of six ATPase and two non-ATPase subunits. The ATPase has a chaperone-like activity. They are believed to help unfold and de-ubiquitinating ubiquitinnated protein substrates and channel them into the 20S core, therby controlling access of the protein substrates to the protease within. The lid, which contains up to ten non-ATPase subunits, is responsible for protein substrate binding and has poorly understood exactly functions. (84)

Viral proteins synthesized intracellularly are also degraded to antigenic peptides via proteolysis but they are generated in the cytosol by immunoproteasome which was conformational induced by IFN-γ (Figure 13). Three β-subunits (β1i or LMP2, β5i or LMP7, and β2i or MECL-1) of the 20S proteasome, which are IFN-γ inducible, are replaced by the constitutive proteases, β1 (δ), β2 (ζ), and β5 (MB1). IFN-γ not only induces the formation of immunoproteasomes, but also induces the proteasome activator (PA28) which can influence proteasome behavior by substituting for one of the two 19S regulator complexes. (84) The PA28 contains three subunits, α, β, and γ. PA28α and PA28β subunits form a heteropolymer, whilst PA28γ subunits form a homopolymer. The notion that the PA28αβ complex is involved in proteasome-dependent MHC I antigen processing is supported by the main localization of PA28α and PA28β on the endoplasmic reticulum (ER) with immunoproteasomes and by the upregulated expression of PA28α and β subunits, but not PA28γ, by IFN-γ. By the binding directly to the end of the 20S proteasome, the PA28 induces conformational changes on the catalytic sites of the 20S proteasome and activate selectively the peptidase activities of the 20S proteasome. (85)

The α-subunit tails of proteasome prevent the exit or entry of substrates, however, binding of the PA28 or the 11S regulator forces proteasomes to selectively shift the positions of their α-subunit tails, enhancing the uptake of substrates or the release of peptide products. The 11S regulator recruits and unfolds ubiquitinated protein substrates whilst the PA28αβ complexes opens the exit gate at the end of the immunoproteasome. (85)

Small peptide fragments are then transferred across the membrane of the ER by an ATP-dependent heterodimeric complex of two proteins, TAP1 (Transporter associated with antigen 1) and TAP2 (Transporter associated with antigen 2), which are noncovalently associated. For several individual epitopes, it is well documented that TAP

cannot transport the optimal final size epitopes. Instead, only peptide fragments longer than seven residues are transported (82, 83) and the efficiency of TAP drop off dramatically with peptide fragments longer than about twelve residues but there is no clear upper limit. (82) These longer peptide fragments need further trimming at the ER (83). Inside the lumen of the ER, the assembly of MHC I can process through two different pathways, HC and β2-m associate and then peptide fragments are added, or HC and peptide fragments associate, followed by β2-m. (82) These peptide/MHC I trimeric complexes are then transported to the Golgi apparatus and reach the cell surface to become available for recognition by T cell receptor (TCR) via exocytosis pathway (figure 14).

Figure 12 Proteasome composition.
(Kloetzel PM. Antigen processing by the proteasome. Nat Rev Mol Cell Biol 2001;2(3):179-87.)

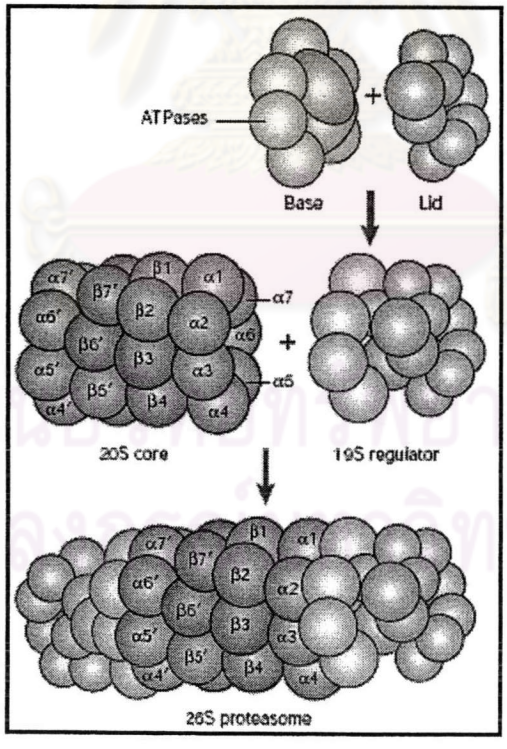


Figure 13 Formation of the immunoproteasome.

(Kloetzel PM. Antigen processing by the proteasome. Nat Rev Mol Cell Biol 2001;2(3):179-87.)

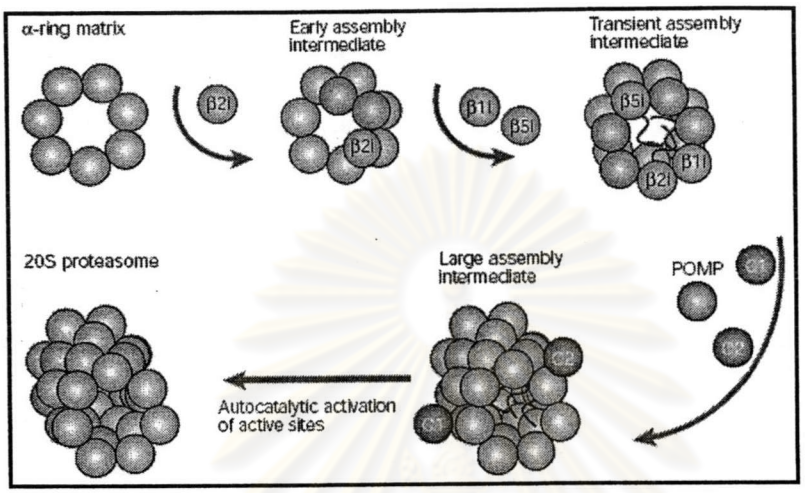
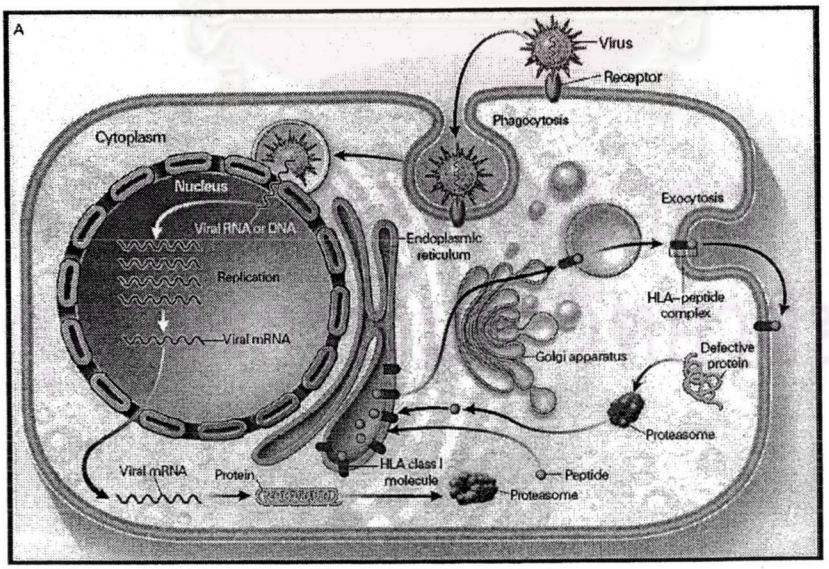


Figure 14 Processing and presentation of viral antigen to $CD8^+$ T lymphocyte through MHC Class I Molecules.

(Klein J, Sato A. The HLA System. First of Two Parts.N Engl J Med 2000; 343:702-9.)



HIV-1 specific $CD8^+$ T lymphocyte responses

Individuals infected with HIV show both cellular and humoral (antibody) immune responses to the virus. Neutralizing antibody responses against HIV are not strong, and are followed in rapid sequence by the emergence of viruses resistant to the neutralizing activity of these antibodies (86) whilst $CD8^+$ T lymphocyte are believed to have an ability in limiting HIV replication. $CD8^+$ T lymphocyte inhibits HIV replication both indirectly, by producing soluble chemokine antiviral factors. These include IFN-γ, which can render nearby cells relatively resistant to productive viral infection via a complex cascade of receptor-mediated binding and activation. It also produces beta chemokines MIP-1α (macrophage inflammatory protein-1 alpha), MIP-1β (macrophage inflammatory protein-1 beta), and RANTES (regulated on activation, normal T expressed and secreted), which inhibit HIV from infecting new cells by blocking HIV coreceptors CCR5 (87). $CD8^+$ T lymphocyte also inhibits HIV replication directly, by recognising and killing infected cells through cytolytic pathway.

Studies in the human peripheral blood lymphocyte (PBL)-reconstituted Severe Combined Immunodeficiency (SCID)/beige mouse model which the mice lacks mature T, B, and natural killer cells showed HIV resistance in mice which have been reconstituted with PBL from Highly Exposed Persistently Seronegative (HEPS) individuals (88). Studies in Simian Immunodeficiency Virus (SIV)-macaque model show that $CD8^+$ depleted SIV-infected monkeys were more progressive to AIDS than control monkeys which their $CD8^+$ T lymphocytes were not depleted and the reappearance of $CD8^+$ T lymphocytes coincided with a decrease in plasma viral load to levels seen before $CD8^+$ T lymphocytes depletion (1). In humans, evidence supporting a protective role of $CD8^+$ T lymphocytes in HIV-1-infection is less direct. In clinical study, the appearance of HIV-1-specific $CD8^+$ T lymphocytes was closely associated with the drop in plasma viral load during acute infection (2). In vivo, Gag was most predominantly recognised by CTL during asymptomatic HIV-1 infection and Gag-specific $CD8^+$ T lymphocyte responses gradually decreased during progression to AIDS (3) and a significant inverse correlation was observed between Gag-specific $CD8^+$ T lymphocytes frequency and plasma viral load (4). The study of HIV-1-specific cytolytic responses against all expressed HIV-1 proteins also showed negatively correlation between breadth of responses and viral load and disease progression (89).

In addition, weak HIV-1-specific $CD8^+$ T lymphocyte responses were demonstrated in some sex workers who remain HIV-seronegative despite definite exposure to HIV (5), called highly exposed persistently seronegative (HEPS) individuals and vigorouse HIV-1-specific $CD8^+$ T lymphocyte response were also detected in five HBV-infected patients who accidental exposured to HIV and HBV infected blood derived from an HIV- and HBV-co-infected patient with high HIV and HBV viremia (90). In Thailand, the weak HIV-1-specific cytolytic responses were also detected in HEPS with Nef responses dominating over all structural proteins (91). However, antigenic specificity of $CD8^+$ T lymphocytes might differ in control of HIV-1 infection. HIV-specific responses in Highly Exposed Persistently Seronegative (HEPS) African sex workers focused strongly on epitopes rarely or never recognised in HIV-1-infected individuals. Seroconverted HEPS individuals switched to recognise epitope which were preferentially recognised by HIV-1-infected individuals. (6, 7)

Cytotoxic T-Lymphocyte (CTL) Assays

1. Limiting dilution assay (LDA)

This assay detects and quantifies antigen specific cytotoxic T cell number in given cell population. These assay provides an estimate of the 'precursor frequency' of a given cell type. Positive results in this assay (proliferation or cytotoxicity, see below), indicate the presence of antigen-specific precursor cells in the PBMC population at the start, which have become activated and have subsequently divided during the period of cell culture. The function of these cells is then measured in the assay by either proliferation, cytokine production or cytotoxicity. In LDA, many micro-lymphoproliferation assays are prepared in vitro, using a range of dilutions of the cell population under investigation, with at least 24 replicate cultures at each dilution. Other factors (such as growth factors, antigen and APC) need to be added to the microtitre wells in excess, so that the only parameter that is limiting is the number of responding antigen-specific cells that are present in the cell population at the start. Under these conditions, and assuming that 'single-hit' kinetics apply, the number of non-responding cultures follows the Poisson distribution. A semi-log plot of the percentage of non-responding cultures plotted against the number of input cells per culture should produce a straight line, and the input number of cells at the start that contained on average one specific

precursor cell can be calculated from the zero term of the Poisson distribution. Several different statistical approaches for calculating the precursor-cell frequency have been described, including minimum χ^2 and maximum likelihood analysis. A variation on the lymphoproliferation LDA, which can be used to assay helper T-cell frequencies, is one that measures cytokine production from each micro-culture. Supernatant is harvested from the stimulated cultures, and assayed for the presence of cytokine, either by enzyme-linked immunosorbent assay (ELISA) or by bioassay. This assay has the advantage that it can be faster than assessing cell division by the incorporation of ^{3}H-Thy. (92)

2. Interferon gamma (IFN-γ) ELISpot Assay

This assay measure the ability of T cells to secrete IFN-γ in response to a specific antigen. They are based on ELISA methodology. PBMCs from the individual being tested are added along with specific HIV peptides to a 96-well nitrocellulose plate that has been coated with antibodies to IFN-γ. Control wells containing PBMCs and medium alone, without the HIV peptides, are assayed in parallel. During an overnight incubation at 37°C HIV-specific T cells present among the PBMCs will be stimulated by their cognate HIV peptide to secrete IFN-γ. The plate-bound anti-IFN-γ antibody captures this IFN-γ. The following day, the PBMCs are removed and the wells washed before addition of a second anti-IFN-γ antibody, which is labeled with biotin. This second antibody recognizes a different epitope on IFN-γ than the first coating antibody. A "sandwich" of IFN-γ between two anti-IFN-γ antibodies is thus produced. Excess unbound antibody is removed by washing before addition of a streptavidin-enzyme conjugate. Streptavidin binds with extraordinarily high avidity to the biotin linked to the second anti-IFN-γ antibody, creating a very stable biotin/streptavidin complex bound to the enzyme (typically horseradish peroxidase, HRP). The enzyme substrate is added, which results in the formation of a colored product, or spot, where the streptavidin/biotin complex resides. Each spot corresponds to an antigen-specific cell that has secreted IFN-γ in response to its cognate HIV peptide. The number of spots corresponds to the number of antigen-specific T cells present among the PBMCs. Because antigen-specific effector T cells secrete a number of cytokines and chemokines in addition to IFN-γ that may play an antiviral role, measurement of IFN-γ alone is not ideal, since it is likely that discrete populations of cells secreting other cytokines and chemokines will be missed. ELISpot

assays have been developed to enumerate cells secreting a number of other cytokines, such as IL-2, tumor necrosis factor alpha (TNF-α) or granzyme. (93)

3. Intracellular Cytokine Staining (ICS)

Intracellular cytokine staining (ICS) is a flow cytometry-based method for enumeration of antigen-specific, cytokine-secreting T cells. PBMC from the subject being tested are stimulated with HIV peptides in the presence of costimulatory antibodies against CD28 and CD49d. Negative control stimulations with PBMC and costimulatory antibodies but no HIV peptide, and positive control stimulations with superantigens such as staphylococcal enterotoxin B (SEB) are set up in parallel. Stimulations are allowed to proceed for 6 hours at 37°C in the presence of brefeldin A or monesin, which inhibit protein transport through the golgi. Thus any cytokines induced by the HIV peptides in HIV-specific T cells will be prevented from being secreted and will accumulate within the cells. Responding cells can be visualized using fluorescently labeled antibodies that are specific for the cytokines of interest. Responding cells can be further characterized by additional, lineage-specific antibody markers (eg, CD3 and CD8 antibodies for $CD8^+$ T cells). Once fluorescently labeled, the cells are analyzed using a flow cytometer, which possesses lasers and filters to excite specific fluorescent antibodies and measure the released light, permitting enumeration of the responding T cells. (93)

4. Cytotoxic T-Lymphocyte (CTL) Assay

Chromium release assay determines the ability of $CD8^+$ T cells to lyse target cells expressing specific HIV antigens. Briefly, freshly isolated PBMC from an infected or vaccinated individual are stimulated in vitro with HIV antigens for 2 weeks in order to expand HIV-specific T cells. Such expansion is required because HIV-specific $CD8^+$ T cells are normally present at frequencies that are too low to detect by this method if cells are used directly ex vivo. These expanded T-cell lines are then incubated with autologous target cells (normally B-lymphoblastoid cell lines (BLCL) derived by transformation of autologous B cells with Epstein-Barr virus) that have been labeled with the radioisotope ^{51}Cr and infected with vaccinia expressing the HIV antigens of interest. HIV-specific T cells will recognize and lyse the target cells, resulting in liberation of the radioactive label into the culture supernatant. Spontaneous lysis is measured in replicate wells containing radiolabeled targets in the absence of effector cells, and maximum lysis is measured in

replicate wells containing detergent. Radioactivity of the supernatants is measured using scintillation counting. Specimens with HIV-1-specific lysis exceeding 15% [calculated as: (lysis minus spontaneous lysis) / (maximum lysis minus spontaneous lysis) x 100] after correcting for lysis against mock-infected target cells are considered positive. (93)

5. MHC Tetramer Binding Assay

Whilst the cellular assays mentioned thus far measure effector function, tetramers measure the absolute number of cells that recognize a particular epitope, without providing any information regarding the functionality of these cells. HIV-infected cells digest HIV proteins into short peptidic fragments (epitopes), which are bound to MHC class I molecules and displayed at the cell surface. T cells recognize their targets through the interaction of their T-cell receptors (TCR) with these MHC/HIV epitope complexes. MHC tetramers are reagents consisting of four MHC class I molecules bound to the HIV peptide of interest, linked together by a fluorescently labeled streptavidin molecule. These reagents will bind specifically to the TCR of all T cells that recognize that particular MHC/peptide complex. This is a very powerful tool for precise, easy, and rapid enumeration of HIV-specific T cells. Tetramer technology is limited by several factors. The exact HIV peptide and its restricting MHC class I molecule must be known in order to make the tetramers. Since there are hundreds of different HIV epitopes whose restricting MHC molecules are not defined, this constitutes a major limitation. In addition, tetramer staining gives no information regarding the functionality of the stained T cells. (93)

HIV Viral Load Assays (94)

1. The HIV-1 Quantiplex (bDNA) Assay

The Quantiplex HIV-1 RNA assay (Chiron Diagnostics, Norwood, MA) is a branched DNA (bDNA) sandwich method which quantifies plasma HIV-1 RNA by amplifying the signal rather than the target nucleic acid. The bDNA assay does not require viral RNA purification or PCR amplification steps. Instead, virions are concentrated by centrifugation and disrupted by detergent and Proteinase K, releasing viral RNA. This lysate is incubated with two sets of oligonucleotides. The first set captures viral RNA, hybridizing to both conserved regions of the HIV-1 *pol* gene and to

oligonucleotides bound to the microwell. The second set of oligonucleotides provides signal amplification. This set consists of four components: oligonucleotides with homology to both the target RNA and to preamplifier oligonucleotides; preamplifier oligonucleotides, amplifier oligonucleotides, and oligonucleotide probes bound to alkaline phosphatase (AP). Each of these components binds by hybridization to the next at multiple sites. In this way, the signal is amplified without copying the target RNA. Detection is by chemiluminescence using an AP-specific substrate. The amount of light detected is directly proportional to the amount of bound nucleic acid. The absolute quantity of HIV-1 RNA is determined from an external standard curve run on the same plate.

An important advantage to this strategy of signal amplification is the avoidance of quantification errors inherent in extracting and amplifying the target nucleic acid sequence. In addition, plasma substances and anticoagulants such as heparin that may impair RT-PCR (see below) do not adversely affect the bDNA assay. The bDNA assay oligonucleotide probes bind equally to HIV-1 Group M subtypes A-F allowing viral load measurements of diverse HIV-1 strains. Disadvantages of the bDNA assay include the absence of an internal quantitation standard for each sample and the requirement of a relatively large sample volume (at least 1 ml of plasma), which may pose a problem when sample volume is limited, such as in pediatric specimens.

There have been three generations of the HIV-1 Quantiplex assay. Version 3.0 is currently in use. In general, the Quantiplex HIV-1 assay is highly reproducible and can discern 2-3 fold changes in HIV-1 RNA. Version 3.0 allows accurate quantification to levels as low as 50 copies/mL, with a 4 log dynamic range of up to 500,000 RNA copies/mL.

2. The AMPLICOR HIV-1 MONITOR Assay

The AMPLICOR HIV-1 MONITOR assay (RT-PCR, Roche Diagnostics Corporation, Branchburg, NJ) is the only FDA-approved HIV-1 RNA quantification assay. It is based on reverse transcription (RT) of the target HIV-1 RNA, and polymerase chain reaction (PCR) amplification of the resulting cDNA. Viral RNA is extracted from either acid citrate dextrose (ACD) or EDTA anticoagulated plasma with guanidine isothiocyanate, and nucleic acid from the relatively impure lysate is precipitated with isopropanol. Reverse transcription and PCR amplification occur in a single step using the

thermostable recombinant enzyme *Thermus thermophilus* DNA polymerase (*rTth* pol) which has both RT and DNA polymerase activities. Segments of the viral cDNA are amplified exponentially to a high copy number (amplicons) with repeated cycles of heating and cooling in the presence of biotinylated oligonucleotide primers specific to a conserved region of HIV-1 *gag*, *rTth pol*, deoxynucleotide triphosphates, and the appropriate buffer components. Amplicons are denatured and single-stranded DNA is bound to microwells coated with HIV-specific oligonucleotide probes. An avidin-horseradish peroxidase (HRP) conjugate is added, binding to the biotin-labeled amplicon. The amount of bound amplicon is determined after the addition of an HRP-specific colorimetric substrate.

The AMPLICOR HIV-1 MONITOR assay uses an internal quantitation standard (qs) at a known concentration that is added to each sample before extraction, both to quantify the sample HIV-1 RNA and to compensate for plasma inhibitory factors affecting extraction and amplification. The qs consists of RNA transcribed in vitro that is identical in size to the target amplicon and uses the same HIV-1 gag primer binding sites, generating an amplicon that is also captured on the microtiter well. A colorimetric readout occurs through an enzyme-linked detection system, differentiating the internal standard and target amplicons.

The Standard AMPLICOR HIV-1 MONITOR assay version 1.0 can quantify HIV-1 over the range of 400-750,000 RNA copies /mL and requires 0.2 ml of plasma. An FDA-approved modification known as the "UltraSensitive" assay allows detection of lower RNA levels. The modification consists of an increased initial input volume (0.5 mL of plasma), an ultracentrifugation step to concentrate virions, and a decreased resuspension volume. This procedural modification decreases the lower limit of quantitation to 50 RNA copies/mL, but the upper limit also decreases to 75,000 RNA copies/mL. Thus, with the two different sample preparation procedures, the combined linear range for the AMPLICOR HIV-1 MONITOR assay is 50-750,000 RNA copies/mL. There is good agreement in calculated RNA concentrations between the Standard and UltraSensitive procedures within the shared dynamic range. The two methods generally differ by less than 2-fold, with the UltraSensitive method slightly under-representing the HIV-1 concentration (median decrease, 22% compared with Standard procedure.) The next generation of the AMPLICOR HIV-1 MONITOR assay is designated version 1.5 but is currently available for research use only in the United States.

3. The NucliSens HIV-1 Assay

The NucliSens HIV-1 QT assay is based on target amplification using NASBA (nucleic acid sequence based amplification) technology (Organon Teknika, Duram, NC). The NASBA assay selectively and directly amplifies HIV-1 RNA without PCR in a one-step sandwich hybridization procedure using two oligonucleotide primers, three enzymes, nucleoside triphosphates and the appropriate buffers. First, highly purified RNA is extracted using guanidine thiocyanate and silicon dioxide particles. The RNA is amplified by repeated cycles of synthesis and transcription off a double-stranded DNA intermediate. An oligonucleotide primer (P1) specific to a region in HIV-1 *gag* is used to synthesize cDNA from the specimen RNA template using Avian Myeoblastosis Virus (AMV) reverse transcriptase. The RNA strand is degraded by RNAseH, allowing the oligonucleotide primer P2 to bind and initiate second-strand DNA synthesis. Anti-sense RNA is then transcribed off the double-stranded DNA via a T7 polymerase promoter (originally incorporated by P1). This cycle is repeated resulting in exponential amplification (1 million to 1 billion-fold) under isothermal conditions. The amount of nucleic acid is determined directly by chemiluminescence, which is characterized by very high sensitivity and a broad dynamic range. Quantitation of HIV-1 viral load is accomplished by co-amplification of three internal RNA quantitation standards specific for HIV-1 *gag* and part of *pol*.

The advantages of HIV-1 quantification by the NucliSense HIV-1 QT assay are that a relatively small plasma sample volume is used (0.1 ml is standard, although the assay can accommodate a wide range of sample volumes (0.01-2.0 ml), and there is a large dynamic range (80-1,000,000 RNA copies/mL). The reaction is rapid (90 minutes), takes place in a single tube, and does not require temperature cycles. In addition, the extracted RNA product is relatively pure and free of interfering substances that inhibit PCR amplification, such as those in heparin-treated blood plasma or seminal plasma. For this reason, the NucliSens HIV-1 QT assay is commonly used for determining viral load in many types of tissues in which interfering substances may be present.

The second generation NucliSens HIV-1 QT assay is currently in use. Version 2.0 allows increased sensitivity over the first generation assay (80 RNA copies/mL for version 2.0 versus 400 RNA copies/mL for version 1.0). Both versions demonstrate equal specificity, reproducibility, accuracy and precision in a comparative study using plasma samples containing known concentrations of Group M, subgroup B HIV-1. Results from

studies of Group M non-B subgroups show efficient quantification of A, C, and D, but poor or no quantification of E, F, and G subgroups.