CHAPTER II

LITERATURE REVIEW

History

Acquired immune deficiency syndrome (AIDS) was first recognized in the United States in 1981, when it become apparent that an unusual number of rare skin cancers (Kaposi's sacoma) and opportunistic infections were occuring among homosexual men. These patients were found to have a markered reduction in CD4+ T lymphocytes and were subject to a wide range of opportunistic infections normally controlled by an intact immune system. In 1982, AIDS was observed in other groups such as transfusion recipients, hemophiliacs and IV drug users, thus providing further evidence of the existence of a transmissible agent. It was not until 1983, that the causative agent had been identified as a retrovirus that selectively infects certain T cell. This virus is now known as human immunodeficiency virus (HIV). [5,13,19,43]

The mode of replication of the the virus, and most of the product that it encode were characterized. It was also recognized that the extraordinary complexity and variability of the virus together with its affinity of CD4, the master cell of the immune system would make AIDS unusually difficult to control.

Etiology

Since AIDS was first recognized, the number of cases has risen swiftly worldwide. Knowledge of AIDS has also increased proportionately. Barre-Sinoussi, F Chermann JC and Montagnier L at the Pasteur Institutes in Paris and a group led by Gallo RC at the National Institute of Health, USA, independently identified the causative agent: a virus of the retrovirus family, in 1983 and 1984, respectively.[35] The French group called it LAV (Lymphadenopathy-associated virus) and the American named: HTLV-III (human T-lymphotropic virus type III) and AIDS -related retrovirus (ARV).[36,37,38] Further studies of the virus, it was found that LAV similar

to HTLV-III in infecting CD4 T-lymphocytes, but had different properties. In 1986, however, an expert committee, empowered by the International Committee on the Taxonomy of Virus, agreed on the name human immunodeficiency virus (HIV) and this name has been used ever since.[39,40] Now, the HIV can be further classified into HIV-1 (the original virus) and HIV-2 (the newly discovered variant). Both types of HIV infect patient's immune system, but the overall nucleotide sequence homology between HIV-1 and HIV-2 is only 42 percent.[41] The HIV-1 is found primarily in Central Africa, Europe and the United States whereas HIV-2 is mainly found in Western Africa .[42] HIV-2 also causes AIDS but possibly with a slower evolution than that of HIV-1.[39,43]

HIV-1 Biology

HIV-1 is classified in the Family Retroviridae, Subfamily Lentiviridae. It has a complex genomic organization which differs from that of classical retrovirus.

Like all retroviruses , HIV-1 is a single-stranded plus-sense RNA virus, icosahedral sphere with a diameter of approximately 100 nm. Electron microscopic studies have shown that they exhibit a characteristic cone-shaped core that is surrounded by a bilayered lipid envelope derived from the host cell membrane.[5,44] This envelope is studded by characteristic knobs that represent oligomeric structurs (tetramers or trimers) of the virally encoded envelope glycoprotein gp120 and gp41. The gp120 subunit comprises the extracellular portion of the viral envelope, gp41 portion spans the membrane and anchors the glycoprotein complex to the surface of virions. Below the lipid bilayer is the matrix protein p17. The inner cone-shaped core is comprised of the major capsid protein p24, which surrounds two copies of the viral RNA bound to the nucleic acid binding protein p9. The capsid structure also contains viral *pol* gene product, including the proteas, reverse transcriptase, and integrase.[44,45,46,47]

The structure of HIV-1 genome contains three known structural genes (*gag*, *pol* and *env* genes) that are necessary for replication and at least six additional genes (*tat*, *rev*, *vif*, *nef*, *vpr* and *vpu*) which serve as regulatory rather than strutural roles in

the viral replication cycle.[48] At each end of viral genome is repetitive sequences refered as long terminal repeates (LTRs).[49] In general, the retroviral LTRs do not code for proteins but contain transcription and processing signal as well as genetic regulatory elements central to the control of viral expression.[49,50]

HIV Life Cycle

Transmission

Transmission of HIV usually requires transfer of body fluids. The most important of those are blood, semen , and vaginal secretions that contain the virus, transfer of cell, especially macrophages, containing the virus.[19] In general, HIV is transmitted between humans in three ways. [13,39,51]

1. Sexual transmission : sexual contact with an infected person
   - male to male
   - male to female
   - female to male
2. Blood borne transmission : exposure to infected blood or blood component from an HIV-infected donor
   - Blood transfusion
   - sharing of needles and syringes by injecting drug user
3. Vertical Transmission
   - perínatal from an infected mother to her child
   - postpartum from nursing mother (presummably through breast feeding)

Sexual transmission accounts for the majority (75%) of case of HIV infection worldwide. The number of unprotected sexual contracts, the stage of infection (which may dictate the viral load) and the existence of genital ulceration may all increase the risk of transmission and thus play important role in the sexual spread.

Target cells

HIV infects only certain types of cells. Generally, these are that possess the CD4 molecule on their external surface; most particularly, cells of the immune system called T4-lymphocytes or T-helper cells. It is these T4-lymphocytes that have been shown to be the predominant cell harbouring HIV-1 in the peripheral blood of infected individuals.[52] Several other cell types, particularly those that belong to the mononuclear phagocyte lineage and that bear the CD4 molecule. These include monocytes, macrophages, Langerhans cells of the skin, follicular dendritic cell in the lymph nodes, alveolar macrophages in the lung, retinal cells, and cell of the uterine cervix. In addition, HIV may infect microgial cells in the brain which may not bear CD4 surface protiens.[53,54,55,56]

Attachment and Entry

In order to enter a host cell, the viral particle must bind with the CD4 molecule and possible infect some co-receptor on the host cell membrane. The CD4 molecule is a surface glycoprotein whose physiological function is to bind class II histocompatibility (MHC) molecule on the surface of antigen presenting cells. It acts as a high-affinity receptor for the surface glycoprotein on the viral envelope termed gp120.[57,58] It expresses primarily on the plasma membrane of CD4+ T lymphocyte , cells of the monocyte-macrophage series , and some other cells. Once this binding is complete, the HIV-1 transmembrane gp41 is responsible for fusion of viral and cell membrane. The viral particle is thought to enter the cellular cytoplasma as a result of 'virus-to-cell-fusion', where the viral membrane joins with the host cell membrane[23,59], injecting the core of the virus into the cell. When the core of the virus is within the cellular cytoplasm, the viral RNA is converted into DNA: a process which is achieved by the action of the virus enzyme reverse transcriptare. Its called as a 'provirus' or 'proviral DNA', passes into the cell nucleus and is randomly inserted into the host cell DNA by the action of another virus enzyme called integrase.[60]

Replication

HIV starts replication by the genomic RNA is uncoated, producing a double-stranded DNA intermediate, the DNA provirus. The DNA provirus is synthesized in a complex manner by RT. The provirus is transported to the nucleus, where it is integrated into the host genome. Special sequences contained within the RNA are duplicated during the reverse transcription process so that the integrated proviral contains identical long terminal repeates (LTRs) at it ends. The LTR sequencec contains the appropriate promotor, enhancer, and other signals required for transcription of the genes by the host RNA polymerase II.[1,13,39,60,61]

Some of the RNA will be used to form the genetic material of the new particles and some will be used to encode for the structural protein for the new viral particles or the regulatory proteins, which work to control viral replication. The new virions are produced from multiple copies of the viral proteins. These proteins are formed as large precursors-long chain protein molecule which are then specifically cleaved to become the viral enzymes and structural proteins of the new virions. The assembly of the new virus particle begins with two of the precursor proteins collecting at the edge of the cell where they join together and attach themselves to the host cell membrane. They begin to form a spherical structure which bulges outwards from the cell membrane and draws two strands of viral RNA into it. An enzyme called protease, which is contained on one of the precursor protein molecules, then carries out the final steps of protein cleavage as follow: firstly, it cuts itself free from the polyprotein molecule; then, the protease works to cleave all the other viral components from the protein chain. The remaining protein segments form the protein coat that surrounds the RNA and the viral enzymes, forming the inner 'capsid', at the core of the virus particle. A third structural protein, the envelope protein, is made and transported to the cell surface independently. It contains the envelope glycoproteins, which together with elements from the host cell membrane, now totally enclose the new virus particle, which leaves the cell in a process known as budding.[60]

Latency, Activation and Trigger Factor

A unique feature of HIV-1 and other members of the Lentivirus subfamily is the ability to produce a complex array of regulatory protein that appear to be responsible for latent periods that can extend for months or years. In some cases, virus-specific DNA has even been detected in individuals who show no immunologic response, suggesting that the viral genome can exist for prolonged periods in a quiescent state without expressing viral proteins.[13]

In primary infection with HIV, most individuals do not develop symptoms for many years. Exactly what is happening during this asymptomatic period and what causes sudden clinical deterioration is not yet clearly understood. Although small amounts of virus are present during the asymptomatic phase, the virus dose not appear to be rapidly replicating. Deterioration is , however, characterised by intensive virus replication. A variety of different factors may trigger this replication. Active replication of one or more of these possible co-factors may play a role in precipitating HIV replication and enhancing disease progression. Gene products from herpes simplex virus, cytomegalovirus, hepatitis B virus, human herpes virus type 6 and HTLV-I have been described to stimulate HIV-1 expression of viral genes in cell cultures. Additionally, any antigen that would usually elicit an immune response from the T4-lymphocytes may also lead to active viral replication following stimulation of the T4-lymphocyte. Once a T4-lymphocyte has been stimulated by the presentation of an antigen, it will usually respond by proliferation, which involves transcription of its own genetic material to form new T4-lymphocytes. This process begins when specific cellular bind with 'initiation sites' on the genome.

The HIV provirus, which is integrated into the genome of infected T4-lymphocytes, contains similar initiation sites on its long terminal repreats and the cellular proteins may mistakenly used these for their own initiation sites. One particular protein that is thought to increase transcription, appears to increase its binding to the long terminal repeats following stimulation of the infected T4-

lymphocyte. The activation of this protein may, therefore, lead to increase replication of HIV and thus trigger progression of the disease.[60]

GENETIC CONTROL OF HIV

HIV-1 contains three main genes characteristc of all retroviruses. (Table I) The order of the genes is *gag-pol-env*. The *gag* (group-specific antigen) gene encodes the strutural proteins of the virus (p24 , p1 , p9 and p7) and in some cases, the protease. The *pol* (polymerase) gene encodes the reverse transcriptase (RT), the integrase, protease, and ribonuclease. The *env* (envelope) gene encodes the two membrane glycoproteins found in the viral envelopes (gp41, gp120)

A comparision of the genetic make up of HIV-1 with that of a typical retrovirus reveals a large number of genes and a much more complex organization. HIV-1 contains, in addition to the usual ensemble of genes , an array of other genes (*tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu*). *Tat* and *rev* are regulatory genes critical for replication. Tat protein acts on the regulatory sequences of the LTRs to increase viral transcription and Rev protein binds to a region in the envelope RNA and regulates the spicing of RNA.[1,13,39] *Nef* encode for a negative regulator of transcription which the exact activity is still unknown.[62] Finally, HIV contains several other so-called accessory genes whose function are not yet completely understood. *Vif* or virion infectivity factor, may be necessary for productive infection of cell, while vpu (viral protein U) may facilitate envelope processing and viral budding. The function of viral protein R (*vpr*) remains unknown.

TABLE I. HIV-1 Genes and Gene Products

Gene	Protein	Size	Function
Structural			
gag	MA (matrix)	p16/17	Virion maturation and stability
	CA (capsid)	p24	Virion stability
	NC (nucleocapsid)	p9	Nucleic acid binding
	?	p6/7	Virion maturation and release
pol	PR (protease)	p10	Polyprotein cleavage
	RT (reverse transcriptase)	p66/51	
	IN (integrase)	p32	Provirus establishment
env	SU (external envelope)	gp120	CD4+ binding
	TM (transmembrane)	gp41	Envelop anchor , fusion with target cell
Regulatory			
	TAT	p15	Transactivator of viral genes
	REV	p19	Nuclear export , stabilization and expression of certain viral mRNAs
	TEV/NEF	p18	Unknown
	NEF	p27	Negative factor (controversy), CD4+ down-regulation
	VPR	p15	Early regulatory protein ?
Accesory			
	VIF	p23	Env polyprotein processing
	VPU	p15	Virus maturation and release

VARIATION

Genetic sequencing of portion of the HIV genome in variable regions show considerable heterogeneity. HIV-1 possesses the most error-prone reverse transcriptase (the RT enzyme lacks a proofreading capacity). Most individuals are infected with a fairly homogeneous strain of virus that with multiple cycle of replication diverges into heterogeneous quasispecies. The envelope gene is the site of the majority of these mutations, and these changes have been demostrated to confer varying cytopathic potential to different strains of HIV.[62,63]

Now, these are nine subtypes, designated A, B, C, D, E, F, H, I and O, have been described for HIV-1. Its based on genetic similarities and differences in the env genes, which is the most common gene region for HIV phylogenetic analysis.[64-68] Subtypes A and D have been found primarily in central and western Africa. While, subtype B is the predominant subtype in North America, Europe, Japan and Australia. Subtype C has been found mostly in southern Africa, the Central Africa Republic, and India. Subtype E can be found in Thailand and recently in the Central Africa Republic. Subtype F has been found in Romania and is a rare variant in Brazil. Isolates From Gabon and the Russian Federation were designated subtype H. An 'outlayer' subtype O contains two human and two chimpanzee isolates from Gabon and Cameroon. In Thailand, there are three distinct HIV-1 subtypes: subtype E, subtype B_{thai} , and B_{MN} were found predominantly in heterosexual (70%), IVDUs (75%), and homosexual/bisexual individuals (52%), respectively.[102,112,113]

IMMUNOPATHOGENESIS OF HIV

Although it is clear that HIV causes AIDS, the mechanisms invovled are unclear. During the early chronic asymptomatic phase, there are at least two mysteries: (1) there are fairly profound defects in laboratory measures of cell-mediated immunity, yet patients are appearently well; and (2) these laboratory defects are present even though the number of circulating CD4+ cell may be at the low normal

range and relatively few cells are infected with HIV. During acute infection, the immune system restricts the initial proliferation of HIV; subsequently, a delicate and prolonged balance of control-replication is tipped in favor of HIV. Most of the immunologic deficiency of AIDS can be explained by diminished helper lymphocyte activity but broader damage occurs than can be explained solely by the destruction of CD4+ cells. Other forms of chronic destruction of differentiated hematopoietic elements persist for decades without stem cell failure. Yet in AIDS, pancytopenia appear and there is severe disruption of all lymphoid tissue. None of this explains the unusual subset of pathogens to which HIV infection predisposes. Much of the difficulty in understanding these aspects arises from the artifacts introduced by the study of immune cells in vitro and the use of cell lines and laboratory adapted HIV. [39,71]

Immunologic abnormalities

Virtually every function of the cell-mediatedd immune system is abnormal in HIV-infected persons. In vivo, there are diminished delayed hypersensitivity response, decreased natural killer (NK) cell activity, and a progessive loss of CD4+ T cells paticularly of the memory phenotype ($CD45\text{-}RO^{+}$). In vitro, there are decreased responses to antigens and mitogens, with reduced cytokine production, proliferation, and virus-specific cytotoxic response. There are two functional subsets of CD4+ T lymphocytes: T helper type 1 (Th1) and T helper type 2 (Th 2). Th1 cells tend to secrete IL-2, interferon-gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α), and in mouse models tend to generate cytotoxic cellular secrete immune responses. Th2 lymphocytes produce different interleukins and tend to stimulate humoral immune responses (IL-4, IL-6, and IL-10). Progession of HIV disease seems to be associated with a shift from Th1 predominance to Th2 phenotype predominance.[39,73,74,75] The ability of these cell types to support HIV replication may differ, and since cytokines clearly influence viral replication, an imbalance in cytokine production may result in a relative enhancement or suppression of viral expression. [39,73,74,75] B cell function is also abnormal in infected individuals. There is decreased

ability to generate specific antibody to new antigens and a reduced ability to mount amnestic response. Paradoxically, there is a polyclonal increase in immunoglobulins. Macrophages from HIV-infected persons show altered patterns of cytokine secretion, defects in antigen presentation, and diminished chemotaxis, phagocytosis, and diminished killing of intercellular parasites. The CD4 molecule is also present on monocytes, macrophages, and other antigen-presenting cells. The role of monocytes in maintaining viral infection and transporting HIV, especially across the blood-brain barrier, is under intense investigation. Neutrophils in HIV-infected persons appear relatively normal, although they may be diminished in number. The complement system is virtually the only limb of the immune system that is completely normal in HIV-infected persons. [39]

CD4+ Cell Death

The CD4 molecule is most abundant on the T lymphocytes that serve a helper function. Its function appear to be necessary for all other lymphocyte activities, including B-cell function. CD4+ T cell numbers generally decline steadily during the clinically asymptomatic period, and symptoms usually appear when CD4+ T cell fall below a certain level at which the associated imunosuppression become severe enough to cause susceptibility to secondary infections and neoplasms. The mechanisms of the depletion in CD4+ T lymphocyte are not fully understood, the clinically observed depletion is undoubtedly multifactorial. In vivo, HIV causes CD4+ cell lysis, perphaps by depletion of membrane in the course of generation of new virions or by the accumulation of HIV proteins. CD4 molecules persent on the infected host cell may interact with the gp120 thereby the HIV replicative process, aggravating lysis. Cytopathicity has also been shown to be related to the envelope region of the virus and the amount of CD4 on the cell surface. The infected CD4+ cell may be damaged by host antibody, cell mediated immune system, or antibody-dependent cellular cytotoxicity (ADCC).[69,70] The infected CD4+ lymphocytes or uninfected lymphocytes exposed to envelope proteins undergo apoptosis under conditions that do not damage normal or unexposed cells.[71,72]

CLINICAL MANIFESTATIONS

The clinical signs and symptoms of HIV-infection are exceedingly complex. They include those of the oportunistic infections as well as those caused directly by HIV itself.

As of January 1993, a new case definition for AIDS and a new classification system for HIV-infected persons were instituted by the CDC for use in public health surveillance. (Table II.)[5] This revised classification or staging of HIV infection incorporates the evaluation of an immunologic parameter (CD4+ T cell count) with the clinical condition of the individuals in defining stage of HIV disease.[5,76-78]

TABLE II. 1993 revised classification system for HIV infection and expanded AIDS surveillance case definition for adolescents and adults

	Clinical categories		
CD4+ T-cell categories	(A) Asymptomatic, acute (primary) HIV or PGL[a]	(B) Symptomatic, not (A) or (C) conditions	(C) AIDS-indicat conditions [b]
(1) ≥ 500/μL	A1	B1	C1
(2) 200-499/μL	A2	B2	C2
(3) < 200/μL	A3	B3	C3

[a] PGL persistent generalized lymphadenopathy. Clinical Category A inclides acute (primary) HIV infection.

[b] Kaposi' s sarcoma , opportunistic infections

The three CD4+ T-lymphocyte categories are defined as follows :

- category 1 : ≥ 500 cells/μL
- category 2 : 200-499 cells/μL
- category 3 : < 200 cells/μL

Clinical Categories

- The clinical category of HIV infection are defined as follow :

Category A

Category A consists of one or more of the conditions listed below in an adolescent or adult (≥ 13 years) with documented HIV infection. Conditions listed in categories B and C must not have occured.

- Asymptomatic HIV infection
- Persistent generalized lymphadenopathy
- Acute (primary) HIV infection with accompanying illness or history of acute HIV infection.

Category B

Category B consists of symptomatic conditions in an HIV-infected adolescent or adult that are not included among conditions listed in clinical Category C and that meet at least one of the following criteria: a) the conditions are attributed to HIV infection or are indicative of a defect in cell-mediated immunity; or b) the conditions are considered by physicians to have a clinical course or to require management that is complicated by HIV infection.

Category C

Category C includes the clinical conditions listed in the AIDS surveillence case definition (Appendix II). For classification purposes, once a Category C conddition has occured, the person will remain in Category C.

LABORATORY DIAGNOSIS

The diagnosis of HIV infection provides access to antiretroviral therapy and preventative measures, which unquestionably can be of benefit to asymptomatic HIV-1infected persons. Testing is also useful in assessing symptoms that are potentially attributable to HIV infection, management of exposures to blood and other body liquids, and many provide a public health benefit by enhancing adherence to quidelines for minimizing transmission of HIV.[39]

Detection of human Immunodeficiency virus Antibody

The most widely used is a test for HIV antibody employing the enzyme-linked immunosorbent assay (ELISA) [using whole virus lysates as the target antigens] because it is the least costly test. These have a high level of sensitivity, but because false positive can occur, all positive ELISA test must be confirmed. The confirmation test is a Western blot analysis which detects antibodies to specific viral proteins. In this procedure, viral proteins are separated by electrophoresis, transferred to nitrocellulose paper, and incubated with antisera; antibody bound to the individual proteins is detected by enzyme-labeled anti-human globulin sera. Widespread HIV testing of donor blood, for insurance and other screening purpose uncovers the 0.1% of persons who are ELISA-reactive but Western blot negative or indeterminate, these persons have had no recognized HIV risk activities, they are almost certainly HIV-uninfected. Repeated antibody testing in 3 to 6 months may be establish this with near certainty. Direct test for HIV antigen may be needed to allay to anxiety or assess persons with positive risk histories.[13,39,79]

Human immunodificiency virus antigen

Circulation HIV antigen, consisting mostly of the major core p24, in this assay monoclonal anti-p24 antibody is bound to a solid phase and serum is allowed to react, bound antigen is detected by use of an enzyme-tagged anti-HIV Ig that is visualized in a colorimetric reaction. The disadvantage of this test is that although p24 antigenemia may be present prior to the development of antibodies, the

antigen may not be detectable in substantial proportion of healthy HIV-infected antibody-positive patients. Finally, the direct culture of HIV from plasma or peripheral blood of infected individuals may be useful in monitoring the effects of therapy. The most efficient method for isolation is the use of target cells, either T cell lines or mitogen stimulated PBMCs from normal donors, cocultivated with PBMCs or plasma from the HIV-infected individual. Cultures are kept for up to a month, and the virus is detected in the supernatant either by p24 antigen detection. [80,81,82]

PCR method in the diagnosis of HIV-1 infection

In most cases the diagnosis of human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) infection can be based upon serological test[83,84], however in certain cases such tests are not appropriate :

1. The immediate post-exposure/pre-seroconversion period before the development of the antibodies to HIV antigen that are the basis of most all commercial test.
2. Infection of infant from vertical transmission, where materal antibodies may be co-circulating.[85,86,87]
3. Screening of possibly contaminated blood products.[88]
4. Confirmatory screening of sera giving ambiguous western blot results.

In these particular cases diagnostic PCR methods can be useful for demonstrating the present of HIV genome. The PCR is usually performed on a DNA sample prepared from PBMCs, detecting the presence of proviral DNA, integrated into the host genome , which is a feature of the retrovirus life cycle. Detection of free virus is also feasible of the technically more demanding since it requires the preparation of viral RNA, followed by the synthesis of a cDNA copy. Thus both serological and PCR-based methods can be used for diagnosis, but PCR will probably be used only in the special cases outline above. PCR is a repetitive process consisting of three distinct steps: (I) denaturation of dsDNA, (ii) annealing of specific primers, and (iii) extension of annealed primers. [84]

Nested PCR methods using nested sets of primers can successfully detect single molecules of DNA, and are therefore the method of choice for a single diagnostic work. In common with any PCR technique the nested PCR with be fundamentally affected by the quality of the sample avilable, the degree of match of the primers used to the target sequence, and the length of the PCR product being generated. The degree of primer match to the target is a fundamental limitation of any PCR technique. In practice, the degree of mismatch is minimized by targeting the least variable of the genome under study, which are the reverse transcriptase (RT) region of *pol* and *gag* gene. For maximal sensitivity of detection it should be targeted to these regions. The using of several primers pairs simultaneously in the first round of the nested PCR ("multiplexing") dose not lead to any loss in sensitivity, and hence,several such sets of primers can be used. [89,90,91]

RT-PCR methods are identical to DNA methods once the initial template has been copied by reverse transcriptase, but the lower stability of the RNA template, together with the requirement for a cDNA synthesis, make the use of RNA as a routine source of PCR template in a diagnostic setting. However, a monitoring viral levels in HIV-infected individuals during anti-retroviral therapy depends upon an accurate measurement of the amount of circulating virion, which may be possible only by RT-PCR.[92,93] In the traditional RNA extraction method, plasma or serum is ultracentrifuged and the pelleted virion lysated, treated with a RNase-free DNase, and finally, after heat inactivation of DNase, cDNA is synthesized from the HIV-1 genomic RNA. There are several disadvantages of this method, including the expense and inconvenience of ultracentrifugation, the time consuming of the procedure, and the development for large volumes of starting material. This had led to the development of alternative method. Briefly, the RNA was extracted by treating plasma with a lysis solution containing the poly (rA) which both facilitates the precipitation of viral RNA and reduces intersample variability. Then, the RNA was precipitated with isopropanol and centrifugation, washed with 70% ethanol, and resuspended in DEPC-treated water.[114]

HIV isolated from individuals during long-term treatment with AZT frequently shows reduced susceptibility, as determined by cell culture assay of virus isolate.[94,95] The phenotypic changes in AZT-resistant viruses are associated with a set of mutation at five codons in the RT of HIV at the following residues: Met ^{41}-->Leu, Asp ^{67}-->Asn, Lys ^{70}--> Arg, Thr ^{215} --> Phe or Tyr, and Lys ^{219} --> Gln. To investigate the clinical significance of resistance, rapid, large-scale susceptibility assessment of all individuals is required ,preferably without the need to isolate HIV by coculture of peripheral blood mononuclear cells (PBMCs). The proven association between the degree of AZT resistance and the number of specific mutation in RT has provided a rational basis for using genetic assays to AZT sensitivity. The develpoment to detect these point mutations is a modification of a "selective" PCR procedure. In this approach, oligonucleotide primers are used to enable differential priming of DNA synthesis from wild-type and mutant virus. These primers are used separately with appropriate paired, common primers, and a specific PCR product matches the target sequence. [26,94]

ANTIRETROVIRAL THERAPY

Since the first cases of AIDS were reported in the early 1980s, several drugs have been developed against the human immunodeficiency virus (HIV). Each time HIV infects a new cell, it needs to make a double-stranded (DNA) version of its single-stranded genetic code (RNA) in order to take over the production machinery of the infected cell. The first antiretroviral drugs developed were designed to stop this process, and are called reverse transcriptase inhibitors (RTIs).[96]

Nucleoside analog Reverse Transcriptase Inhibitors

These drugs inhibit the action of HIV's reverse transcriptase enzyme by blocking the assembly of the nucleotide chain. After entry into target cells, these drugs are converted into the active triphosphorylated form by cellular enzyme. Viral reverse transcriptase may be inhibited by these compounds or may take up the compounds and insert them in the growing DNA proviral chain. Chain termination results because subsequent nucleoside do not have the deoxyribosylhydroxyl group for attachment of the next link. Though 1993, three antiretroviral drugs had been licensed in the United States : azidothymidine [Retrovir (AZT)], didanosine [Videx (ddI)], and zalcitabine [HIVID(ddC)]. Two addition numbers of this class are available under expanded access investigational new drug (IND) regulations: Stavudine [ZERIT(d4T)] and lamivudine [Epivir(3TC)]. Experience with these drugs in treatment of HIV infection has been only partially successful, productively modest temporary increases in T cells and modest temporary reductions in the viral load. [39,96]

ZIDOVUDINE (Azidothymidine : AZT)

The dideoxynucleoside; 3'-azido-2'-3'-dideoxythymidine (Zidovudine) was first found to have activity against HIV in 1985. [97,98]

Zidovudine, an analogue of thymidine with the substitution of an azido (-N3) group at the 3' position. It inhibits proviral DNA synthesis in two ways: competitive inhibition and chain termination. It was the first nucleoside analoge registered for use in patients with HIV infection. Registration for this indication was based on trials which demonstrated that AZT had antiretroviral activity and that it prolonged survival and delayed disease progression in patients with AIDS and symptomatic HIV infection. [97-101]

DIDANOSINE (2',3'-dideoxyinosine:ddI)

Didanosine was licensed in 1992. It became the second antiretroviral agent to receive regulatory approved in the U.S. and Canada. On the strength of trial ACTG 116B/117, which studied moderate to severe HIV illness in persons who received AZT for 16 or more weeks. This study demonstrated that in asymptomatic patients and those with AIDS-related complex, didanosine significantly delayed the time to the first AIDS-defining event or death, compared to AZT. [102,103]

ZALCITABINE (2',3'-dideoxycytidine:ddC)

Zalcitabine was licensed in 1992 for use in with zidovudine on the strength of a comparison AZT trial with patients in a separate trial zidovudine and zalcitabine a small randomized indicated that the addition of zalcitabine to zidovudine improved the CD4+ cell counts. However, with zalcitabine , peripheral neuropathy is the side effect which most often limits dosage in zalcitabine monotherapy.[14]

STAVUDINE (2', 3-Didehydro-2', 3'-dideoxythymidine : d4T)

Stavudine, another nucleosine analogue in clinical trial , also has potent anti HIV activity in vitro. In phase I trials, it increases in CD4+ cell counts and suppression of p24 antigenemia. Toxicities include neuropathy, elevations of liver transaminases, and anemia. [104]

AZT RESISTANCE

Within several months of begining therapy with nucleoside analogue reverse trascriptase inhibitors, isolated patients began to require increasing concentrations of drugs to inhibit the replication of HIV in vitro. The clinical significance of resistance to individual antiretroviral agents has not been early determined. Resistance often coexists with severe immunosuppression, high viral burdens, other phenotypic characteristics and poor clinical status; all of these factors may be predictors of disease outcome and may confound analyses.[105-108]

It is now generally understood that the short-lived nature of the antiretroviral response is due to the virus' ability to change its molecular structure (mutate) in order to avoid the effects of a given drug.[96] For example, in AZT-monotherapy: within 6 months, the T cell counts and viral load begin to return toward pretreatment levels (baseline).[96,107,108] Specific mutation that leads to a change of the amino acid sequence of the reverse transcriptase enzyme can produce a new version of HIV. Because the role of reverse transcriptase is to convert the HIV RNA genome into ssDNA and to convert ssDNA to dsDNA. Unlike human DNA polymerase, HIV-RT dose not correct transcription error by exonucleolytic proofreading. The average error rate has been estimated as being one per 1700 incorporated nucleotide. In addition, some template positions may be more prone to error; with rate as high as one per 70 polymerised nucleotide.[22,104,109]

Analysis of the HIV-RT gene from clinical isolates revealed that resistance was due to multiple nucleotide changes confering specific amino acid substitutions in RT at the following residues : Met ^{41} --> Leu, Asp ^{67} --> Asn, Lys ^{70} --> Arg , Thr ^{215} --> Phe or Tyr and Lys ^{219} --> Glu [22]

A substitution at codon 70 appears transiently before the emergence of mutation at codon 215. Mutation at Codon 215 have been noted as being the most stable variant , though both Thr ^{215} --> Tyr and Thr ^{215} --> Phe substitutions have been described and may coexist. The mutation at codon 70 may then reappear or a substitution at codon 41 may appear subsequently, being joined by Larder and colleugues assessed the interaction between different mutation sites: a mutation at codon 215 alone in parts a 16-fold increase in the 50% inhibitory concentration (IC_{50}) of virus isolates whereas a mutation at codon 41 alone results in only a fourfold rise in IC_{50}. However, viruses with substitutions at both codon have a 60-fold increase in IC_{50}. The addition of a mutation at codon 41 in virus with mutations at codon 67 and 70 as well as the 215 codon reduces sensitivity 31- to 179 fold. Viruses with mutations at codon 67, 70, 215, and 219 were also noted to have IC_{50} values > 100-fold higher than wild-type virus.[110] Although acting as genotypic markers of

resistance, the presence of codon changes may not accurately predict the degree of susceptibility. [104] (Table III.)

Resistance depends on the genetic code of the virus (its genotype) and on how the virus' physical and chemical make up responds to its environment or the drug (its phenotype). The genotype can change through mutation, and the phenotype can change in its drug sensitivity. Mutations occurring on specific gene locations can affect resistance, and the phenotypic effects the degree of sensitivity.

Viral strains resistant to AZT exhibit cross-resistance to other nucleoside analogue containing the 3'-azido group , but generally do not show cross-resistance to other nucleoside analoges or non-nucleoside reverse transcriptase inhibitors (NNRTIs). [104]

TABLE III : Effect of Mutation on Drug Susceptibility to AZT-monotherapy

Amino acid change	Phenotypic effect fold increase in IC_{50}
41	4
215	16
41,215	60-70
67,70,215,219	120
41,67,215	180

HIV Protease Inhibitors

The HIV-1 protease enzyme is an important target for the design of anti-HIV drug. Because mutation on the active site of the protease molecule have been found to prevent complete processing of the virus. From this and other studies, viral load

reduction in response to treatment is now regarded as an important tool in monitoring antiretroviral therapy.[111]

Several of the protease inhibitors have been shown in studies to reduce HIV load by up to 99% and increase CD4+ T cell counts by over 100 cells/mm.^{3} of blood. It improves clinical well being, decay disease progression, and extened survival. The three protease inhibitors currently licensed by the FDA for use in the United States are saquinavir, ritronavir, and indinavir. Many other protease inhibitor, including Agouron's nelfinavir and Glaxo Wellcome's 141 W94 , are in various stages of human study and not yet approved in the United States. [96]

COMBINATION THERAPY

The HIV infection may be attacked at different levels: antiviral and immunological, and it may be advantageous to combine drugs that interact at different levels or at different target. The combination chemotherapy may result in higher overall efficacy, lower toxicity and possibly diminish the risk of drug resistance. Several drug combinations have been shown to act synergistically against HIV in vitro. Not all drug combinations will necessarily lead to synergism, however , and some may simply have an additive effect whilst other may have an antagonistic effect. There is also a rational basis for combining antiviral drugs that act at different stages of the HIV replication cycle. When combined, such drugs may provide a double barrier; prevent shedding of the virus from its reservoirs and prevent spread of the virus and recruit of new cells, so keeping the HIV infection under complete control.

There may also be good reasons for combining two nucleoside analogues ,both of which work by inhibiting reverse transcriptase. In recent study has shown that combining zidovudine with ddI in either simultaneous or alternating fashion may be an appropriate strategy to prevent resistance to either drug.

For maximal suppression of HIV replication provides the best chance for prolonged benefit from anti-HIV drug therapy by delaying resistance, slowing disease progression , and extending survival.[111]