IN VITRO SELECTION AND CHARACTERIZATION OF DRUG-RESISTANT VARIANTS OF HIV-1

BY

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Abstract

We selected for Human Immunodeficiency Virus type 1 (HIV-1) resistance in cell culture. In order to do this, each of a wild-type laboratory strain (III_B) or a clinical isolate was passed in MT-4 cells in the presence of drug. At initial passage, the drug concentrations employed were subinhibitory for HIV-1 replication; these were gradually increased in subsequent passages. Using this system, we were able to select variants of HIV-1 that possessed up to 100-fold resistance in comparison to wild-type viruses. The drug resistance phenotype remained stable even after propagation of these variants in the absence of drug pressure for over 2 months. Genotypic analysis of drug-resistant variants revealed that HIV-1 resistance was associated with mutations occurring in the reverse transcriptase (RT)-coding region of the HIV-1 pol gene. 3'-azido-3'-deoxythymidine (AZT)-resistant variants remained sensitive to each of 2',3'-dideoxyinosine (ddI), 2',3'-dideoxycytidine (ddC), and 2',3'-dideoxy-3'-thiacytidine (BCH-189). However, cross resistance to BCH-189 in the case of ddC-resistant variants and to ddC for BCH-189-resistant variants were observed. Both wild-type and drug resistant variants behaved similarly with regard to replication rate and levels of RT activity.

We also investigated whether multiple drug resistance could be generated in cell culture. First, we demonstrated that the use of combinations of various antiviral drugs in vitro could prevent the development of HIV-1 resistance over eight weeks, even when these drugs were employed at subinhibitory concentrations. However, sequential in vitro passage of AZT-resistant HIV-1 in the presence of increasing concentrations of other drugs yielded variants that were resistant to both compounds. The multiple drug resistance phenotype also remained stable when viruses were grown in the absence of drug pressure.

Finally, we investigated whether the syncytium formation property of HIV-1 in culture could be related to the generation of multiple drug resistance through the possibility of genetic recombination. Toward this end, We used polyethylene glycol to fuse monocytic

cells that had been infected with viruses resistant to either AZT or (-) enantiomer of 2',3'-dideoxy-3'-thiacytidine (3TC). We found that viruses recovered from these hybrid cells were resistant to both AZT and 3TC. Cloning and sequencing of the RT-coding region of these variants revealed that the doubly-resistant viruses contained mutations associated with resistance to each of AZT and 3TC, suggesting that genetic recombination had occurred.

Résumé

Nous avons sélectionné pour la résistance contre le VIH-1 en culture de tissue. Pour effectuer cette expérience, nous avons développé soit des isolats cliniques du VIH ou la souche de laboratoire IIIB sur cellules MT-4 en présence de différentes drogues antivirales. Initialement, les concentrations utilisées ont été sous-inhibitrices. Ces concentrations ont été augmentées au passage subséquent. En utilisant ce systéme, nous avons sélectionné des variants du VIH-1 montrant une résistance allant jusque 1000 fois contre différents médicaments en comparaison avec les virus de type sauvage. Le phénotype de résistance est resté stable après propagation des virus en absence de drogues pendant deux mois. Les analyses génétiques de ces variants ont révélé une résistance associée à différentes mutations dans le gène encodant la transcriptase inverse du VIH. D'autres résultats ont démontré que les virus résistant à l'AZT ont été sensibles soit au ddI, ddC ou 3TC. Par contre, nous avons observé beaucoup de résistance croisée entre le 3TC et ddC. Les virus résistants ont multiplié aussi rapidement en culture de tissue en l' ansence de drogue que les virus de type sauvage, et tous deux ont produit les mêmes niveaux d'activité transcriptase inverse par mg/protéine virale.

Nous avons voulu déterminer également si la résistance contre plusieurs drogues a pu être accomplie en culture de tissue. Nous n'avons pas pu sélectionner pour les souches résistantes en utilisant des combinaisons de différentes drogues antivirales à la fois. Par contre, le sélectionnement consécutif en utilisant une seule drogue à la fois, suivi par un autre, a engendré des particules virales résistantes contre deux. Dans certains cas, nous avons même produit des variants résistant contre trois drogues différentes. Cette résistance aux différents antiviraux est restée également stable au passage en l'absence de ces médicaments.

Nous avons étudié également si la propriété de formation des syncytia en culture de tissue pouvait être reliée au développement de résistance antivirale multiple par le processus

de recombinaison génétique. Nous avons fusionné différentes cellules monocytiques infectées par différents virus résistant soit à l'AZT ou au 3TC. Les virus récupérés de ces cellules hybrides présentaient parfois une résistance aussi bien contre le AZT que contre le 3TC. Les analyses des séquences de la région transcriptase inverse de ces virus ont révélé la présence de différentes mutations associées à la résistance contre ces deux médicaments, suggérant que des échanges d'information génétique avaient lieu après ces événements de fusion.

Preface

The thesis presented is written in accordance to the particular option described in Guidelines Concerning Thesis preparation:

"Candidates have the option, subject to the approval of their Department, of including, as part of their thesis, copies of the text of a paper(s) submitted for publication, or the clearly-duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis.

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Additional material (procedural and design data, as well as descriptions of equipment used) must be provided where appropriate and in sufficient detail (eg. in appendices) to allow a clear and precise judgment to be made of the importance and originality of the research reported inn the thesis.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent; supervisors must attest to the accuracy of such claims at the Ph.D. Oral Defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of the different authors of co-authored papers".

The original papers presented in chapter 2, 3, 4, and 5 are either published or submitted for publication. Chapter 1 includes literature review and a general introduction. Chapter 6 contains a general discussion.

Four original papers are as follows:

Chapter 2: Gao, Q., Z. Gu. M. A. Parniak, X. Li, and M. A. Wainberg. 1992. In vitro selection of variants of human immunodeficiency virus type 1 resistant to 3'-azido-3'-deoxythymidine and 2',3'-dideoxyinosine. J. Virol. 66:12

Chapter 3: Gao, Q., Z. Gu, J. Hiscott, G. Dionne, and M. A. Wainberg. 1993. Generation of drug-resistant variants of human immunodeficiency virus type 1 by in vitro passage in increasing concentrations of 2',3'-dideoxycytidine and 2',3'-dideoxy-3'-thiacytidine. Antimicrob. Agents Chemother. 37:130

Chapter 4: Gao, Q., Z. Gu, H. Salomon, K. Nagai, M. A. Parniak, and M. A. Wainberg. 1994. Generation of multiple drug resistance by sequential in vitro passage of the human immunodeficiency virus type 1. Arch. Virol. 136:111

Chapter 5: Gao, Q., Z. Gu, E. A. Faust, and M. A. Wainberg. 1994. Recovery of human immunodeficiency virus type 1 recombinants that possess resistance to two drugs following fusion of cells infected with distinct single drug-resistant variants. (Submitted for publication)

The candidate was responsible for all work described in this thesis, with the exception of the work of cloning, sequencing and polymerase chain reaction in chapter 2. Dr. Zhengxian Gu contributed to the cloning and sequencing in chapter 2. Ms. Alla Lvovich contributed to the polymerase chain reaction experiments in chapter 2. Dr. Hengsheng Fang helped with cloning, sequencing and polymerase chain reaction studies of chapter 5. All work was performed in the laboratory of Dr. Mark A. Wainberg.

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List of Abbreviations

AIDS: Acquired Immunodeficiency Syndrome

AZT: 3'-Azido-3'-Deoxythymidine

BCH-189: 2',3'-Dideoxy-3'-Thiacytidine

ddC: 2',3'-Dideoxycytidine

ddI: 2',3'-Dideoxyinosine

env: Envelope

HIV: Human Immunodeficiency Virus

HTLV-1: Human T-cell Leukemia Virus type 1

ID_{50}: Inhibitory $Dose_{50}$

IFA: Indirect Immunofluoresence Assay

IFN: Interferon

Kd: Kilodalton

LTR; Long Terminal Repeats

MOI: Multiplicity of Infection

NNRT Inhibitors: Non-nucleoside Analogs Reverse Transcriptase Inhibitors

PBMC: Peripheral Blood Mononuclear Cells

PCR: Polymerase Chain Reaction

PEG: Polyethylene Glycol

PFU: Plaque Forming Units

pol: Polymerase

Pro: Protease

tRNA: Transfer RNA

RT: Reverse Transcriptase

$TCID_{50}$: Tissue Culture Infection $Dose_{50}$

3TC: (-) Enantimoer of 2'.3'-Dideoxy-3'Thyiacytidine

Chapter 1

Literature Review and General Introduction

1. Overview of retroviruses

1.1. Properties of retroviruses

Retroviruses are prototypic retroelements defined by their ability to reverse the normal flow of genetic information from genomic DNA to mRNA (Weiss et al., 1985; Varmus, 1988). They can be distinguished from other retroelements which are found in many eukaryotic organisms (Temin , 1985; Weiner et al., 1986) by an extracellular phase. Retroviruses comprise a large family of viruses, which have been traditionally classified into three subfamilies, including the oncoviruses, lentiviruses, and spumaviruses, based primarily on pathogenicity rather than on genomic relationship (Teich, 1985; Coffin, 1991). Oncoviruses are usually associated with malignancies, but also can be nonpathogenic. Lentiviruses cause various slowly progressing diseases. Spumaviruses cause foamy degeneration of cells, but no apparent disease.

Retroviruses can also be divided into four different types, including type A, B, C, and D, according to their morphology and subcellular distribution (Teich, 1982; Teich, 1985). Type A oncoviruses possess only intracellular forms, while the type C produce only extracellular particles. Each of type B, C, and D viruses produces the extracellular particles with distinctive morphologies. Depending upon their type of transmission, retroviruses can be divided into two group. The exogenous group comprises retroviruses that are transmitted by horizontal infection, i.e., from one individual to another. The endogenous group includes those that can be transmitted vertically, from parent to offspring, in the form of inheritable proviruses that integrated into the chromosomes of spermacytes or oocytes (Coffin, 1982; Stoye and Coffin, 1985; Coffin, 1991). In addition, retroviruses can be classified with respect to host range. Retroviruses isolated from mice that can only replicate in cells of mice are called "ecotropic", while those that can also replicate in cells of other species are called "amphotropic" or "polytropic". Retroviruses

that can only replicate in cells of heterologous species are called "xenotropic".

It has been found that many disorders in either animal species or in human are associated with retroviruses, including rapid and long-latency malignancies, wasting diseases, neurological disorders, and immunodeficiencies (Teich, 185; Goffin, 1991). Retrovirus-related proviruses (endogenous retroviruses) have been detected in all mammalian genomes that have been investigated. It has been estimated that in some species of mice as many as 1000 to 2000 copies of endogenous proviruses or provirus-like elements per haploid genome exist (Varmus and Brown, 1989; Coffin, 1991). Despite the variety of interactions with host, all retrovirus isolates are quite similar in virion structure, genome organization, and mode of replication.

Retroviruses are particles with a relatively simple structure measuring about 100 nm in diameter. They consist of an inner electron-dense core (nucleoid) and an outer envelope. The core contains ribonucleoprotein assembly surrounded by an outer protein shell which is, in turn, surrounded by an envelope. The envelope is derived from host membranes enriched with viral envelope glycoproteins, surface glycoprotein and transmembrane protein.

The retroviral genome comprises two copies of single-stranded RNA molecules that are capped and polyadenylated in ways similar to those of cell mRNAs. The genome may thus be considered diploid (Varmus, 1988; Coffin, 1991). This unique situation may facilitate formation of heterozygotes and genetic recombination that is associated with observed retroviral diversity. Retroviruses share features of genetic organization and genetic content. They all contain three essential replicative genes: the first, gag, encodes the structural proteins of the virus capsid and core ; the second, pol, encodes the reverse transcriptase (RT) and integration protein (IN); and the third, env, encodes the virus surface glycoproteins required for binding to specific receptor molecules on the surface of cells during subsequent infections. The order of the genes is invariably gag-pol-env (Coffin, 1991). These replicative genes are flanked at both ends by long terminal repeats

(LTR). The features that distinguish retroviruses from all other viruses involve conversion of genomic RNA into a double-stranded DNA intermediate by the virion-associated reverse transcriptase and mandatory integration of viral linear DNA into host chromosomal DNA, resulting in the formation of proviruses, by the viral integrase (Varmus, 1988; Coffin, 1991).

1.2. Retroviral reverse transcriptase

Before recognition of the existence of reverse transcription, it was generally believed that genetic information only flowed from DNA to RNA. Although the provirus hypothesis, described by Howard Temin in early 1960s (Temin, 1964), proposed that RNA tumor viruses could have a DNA genome when present in cells (provirus) and an RNA genome when present in virions, and that the RNA-containing viruses could be integrated into host genomes by a form of provirus, few scientists were convinced by this hypothesis.

The earliest evidence for existence of an RNA-dependent DNA polymerase in RNA tumor virions were by Temin and Baltimore in 1970. They found that virions of several RNA tumor viruses contain an enzyme with RNA-dependent DNA polymerase, reverse transcriptase, which can catalyze the conversion of viral genome from a single-stranded RNA to double-stranded DNA (Temin and Mizutaui, 1970; Baltimore, 1970). Thus, the discovery of RT enzyme aroused much attention, particularly because it constituted a molecular proof that genetic information can sometimes flow "backward" i. e., from RNA to DNA. It also provided a mechanism for the incorporation of cancer gene, carried in the form of RNA by RNA tumor viruses, into the host-cell genome . These viruses with RT were therefore termed retroviruses.

Following the discovery of reverse transcriptase, extensive studies on retroviral RTs have been done. These studies revealed that the reverse transcriptase of all retroviruses is encoded by the pol gene, which is located downstream of the gag gene. They are

synthesized as part of gag-pol polyprotein precursor translated from a full-length viral mRNA. After incorporation into the nascent virion particle, the gag-pol polyprotein is cleaved posttranslationally by protease to yield several viral proteins (Yoshinaka & Luftig, 1977; Eisenman & Vogt, 1978).

All retroviral RTs possess three distinct enzymatic activities: 1) an RNA-dependent DNA polymerase activity involved in the synthesis of the minus strand of the proviral DNA; 2) a DNA-dependent DNA polymerase activity that catalyzes the synthesis of the plus strand DNA strand; and 3) an RNase H activity that degrades the RNA primer used for the synthesis of the plus DNA strand and removes the tRNA primer used to initiate first strand synthesis (Varmus, 1988; Coffin, 1991). Although reverse transcriptase was an unusual DNA polymerase, many of its intrinsic properties were found to be similar to those of bacterial DNA polymerase. These properties included the absolute requirement for a primer to initiate DNA synthesis as well as the requirements for template, triphosphates, divalent cation, and physiological salt, pH, and temperature (Hurwitz and Leis, 1972; Leis and Hurwitz, 1972; Dahlberg et al., 1974; Verma, 1977; Houts et al., 1979; Baltimore and Smoler, 1971; Cheng et al., 1987; Huber et al., 1989). In contrast to bacterial systems where the primer is synthesized during replication, retroviruses use preexisting host-encoded transfer RNA (tRNA) as primers (Weiss et al., 1985). Depending on the virus, different tRNA are used. The avian RT specifically recognize $tRNA^{Trp}$ as a primer; the murine RT, in contrast, use $tRNA^{Pro}$; for HIV RT, $tRNA^{Lys3}$ is used to prime DNA synthesis in HIV infection. The tRNA primers are encapsidated in virions through interactions with both viral RNA (Weiss et al., 1985) and reverse transcriptase (RT) (Panet et al., 1975; Barat et al., 1989). Retroviral RT is almost certainly responsible for the selective incorporation of tRNAs into the virion (Peters and Hu, 1980), and may be responsible for the annealing of the tRNA to the genomic RNA during virion assembly.

1.3 Retroviral reverse transcription

The mechanism by which reverse transcription occur is quite complex. Reverse transcription may proceed in the viral particle (Benz and Dina, 1979; Chen et al., 1980; Boone and Skalks, 1981; Li and Burrell, 1992; Trono, 1992). Viral DNA is reverse transcribed from viral genomic RNA in a multistep reaction (Gilboa et al., 1979; Panganiban and Fiore, 1988). Reverse transcription of genomic RNA is initiated by elongation of a primer with a free 3' OH end bound to a specific sequence in the viral RNA near the 5' end, referred to as the primer-binding site (PBS) (Baltimore and Smoler, 1971). As described in section 1.2., a base-paired primer used by all retroviruses is host-encoded transfer RNA molecule (tRNA) which is packaged along with RNA genome in virions (Verma et al., 1972). The interaction between the tRNA and PBS is complicated. The recognition and placement of the tRNA at the PBS is probably not due solely to base pair complementarity, as demonstrated that a virus could be recovered from transfection of a provirus containing a deletion of the last 12 nucleotides of the 18-nucleotide PBS (Rhim et al., 1991).

After initiating, the RT elongates from the tRNA primer to which deoxynucleotide are added to form a short DNA intermediate called minus-strand strong-stop DNA, the short product of copying from the primer to the 5' end of the genomic RNA. During DNA synthesis, the RNase H activity of RT removes the RNA from the RNA/DNA complex. Following removal of the 5' viral RNA, the minus-strand strong-stop DNA translocates or jumps to 3' end of viral genome (first template switch), using a short repeated sequence in the viral RNA to promote base pairing, before elongation of the minus strand continues (Coffin et al., 1978; Stoll et al., 1977).

After translocation, minus-strand DNA synthesis continues copying the RNA genome, resulting in long minus strand species. As elongation proceeds, the genomic RNA enters RNA/DNA complex which is subsequently degraded by RNase H activity of RT. However, degradation of the RNA genome is not complete; a polypurine tract near

the 3' end of RNA genome remains resistant to degradation. The remaining short ribonucleotides serve as primer for the synthesis of plus strand of DNA, yielding an initial part of second DNA strand called plus-strand strong-stop DNA (Peters et al., 1977; Luo et al., 1990 Resnick et al., 1984; Mitra et al., 1979). Synthesis of plus-strand strong-stop DNA is terminated when its extension results in coping of the tRNA still attached to the 5' end of the template. Subsequent removal of the terminal tRNA primer by the RNase H activity of RT allows complementarity between the minus- and plus-strand PBS to facilitate the second template-transfer step (second template switch).

In final steps, the minus- and plus-strand DNA are elongated, which result in the production of double-strand copy of the viral genome containing complete LTR at 5' and 3' ends. This form of the genome, referred to as the provirus, is the DNA substrate for integration into the host cell genome (Coffin, 1991). Retroviruses without a functional RNase H enzyme generate in reverse transcription only minus strong stop DNA which remains hybridized to viral RNA.

1.4. Retroviral recombination

Genetic recombination is a frequently observed feature of the retrovirus life cycle. High-frequency exchanges of genetic determinants between retrovirus genomes have been observed in retroviruses such as avian tumor viruses, murine leukemia virus and HIV (Blair et al., 1976; Kawai and Hanafusa, 1972; Wyke and Beamand, 1979; Faller and Hopkins, 1978; Wong and McCarter, 1973; Clavel et al., 1989). Moreover, exogenous viruses can recombine with endogenous retroviral sequences (Elder et al., 1977; Stephenson et al., 1974; Weiss et al., 1973), yielding viruses with expanded host range properties. It is also documented that retroviruses can recombine with cellular sequences to give rise to transforming viruses (Linial and Lair, 1984).

Several studies found that the frequency of genetic exchanges occurring during mixed infections with either avian or murine retroviruses is very high compared to other

RNA viruses (Blair et al., 1976; Kawai and Hanafusa, 1972; Wong and McCarter, 1973; Wyke and Beamand, 1979). The explanation for such frequency of genetic exchanges is that RNA rather than DNA molecules are the substrates for recombination between avian tumor viruses (Weiss et al., 1973). A significant feature of retroviral recombinations is that they do not occur within first round of infection, rather, they occur only after a second round of infection by the progeny of the initially infected cells (Hu et al., 1990; Weiss et al., 1973; Wyke et al., 1975). This feature may result from the nature of viral genome containing two RNA positive strands which are held together. It seems likely that virions produced initially after mixed infection contain RNA strand corresponding to each of the infecting viruses and that genetic exchanges occur only after a second round of infection by the heterozygous virions.

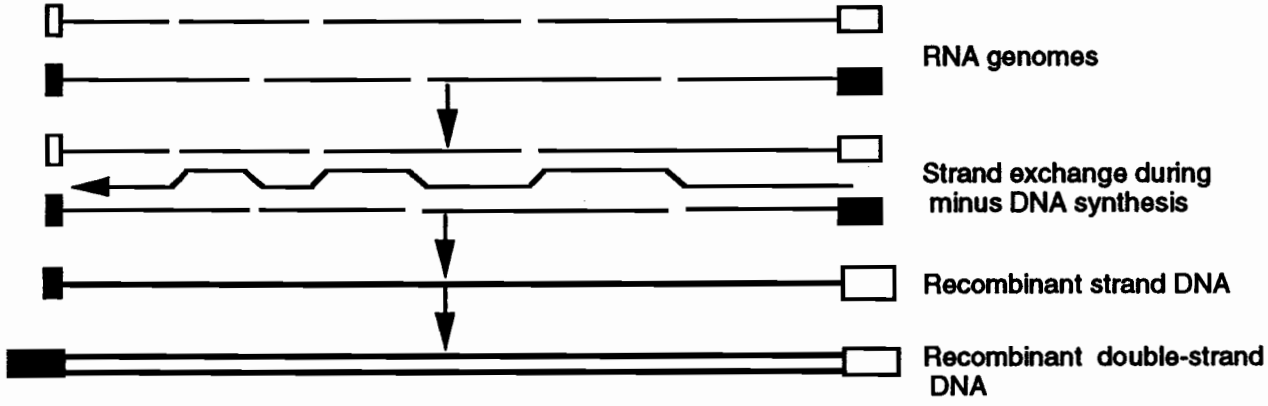
Several systems have been established for studying retroviral recombination in vitro. For example, a system of SNV-based retroviral vector that contain two selectable genes has been used for studying the frequency of retroviral recombination (Hu and Temin, 1990). Two vectors that contained inactivating mutations in either of the selectable markers were inserted into the same cell. Heterozygous particles were formed during first round of infection and recombination frequency observed in the subsequent provirus was determined to be 2% per kilobase per replication cycle (Hu and Temin, 1990).

Retroviral recombination between replicating viruses can be readily detected in tissue culture cells. However, it has been noted that infection and expression by one provirus results in resistance of cells to superinfection by a second virus with the same receptor specificity, presumably by blocking or down-regulating receptors due to the presence of viral env protein on the cell surface (Weiss, 1985).

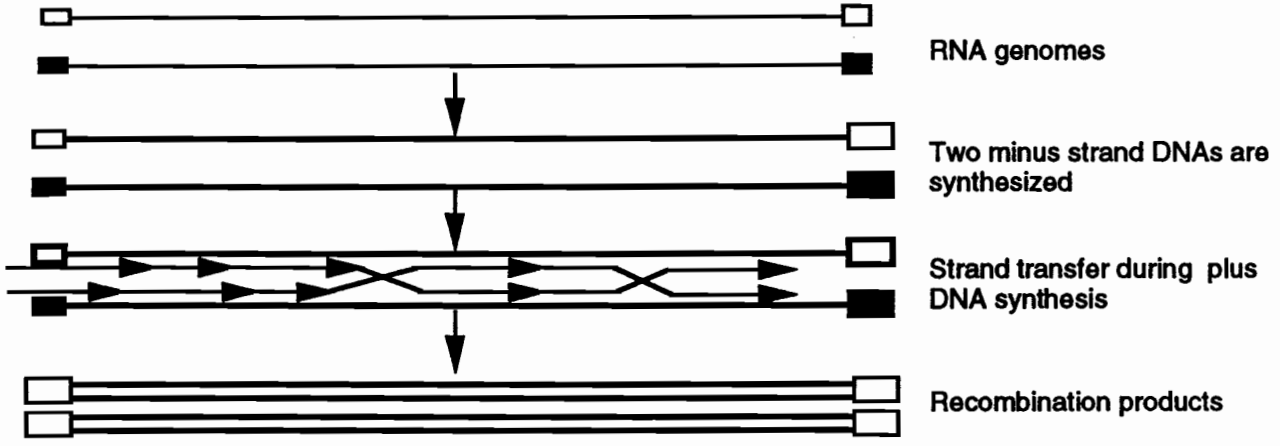
The precise mechanisms of retroviral recombination remain unclear. Two models, the forced copy-choice and the strand displacement-assimilation (Fig. 1), have been proposed to account for genetic exchanges during infection of cells with genetically

Figure 1. Models for retroviral recombination. A: Fored copy-choice model. B: Strand displacement-assimilation.

A


RNA genomes
Strand exchange during minus DNA synthesis
Recombinant strand DNA
Recombinant double-strand DNA

B


RNA genomes
Two minus strand DNAs are synthesized
Strand transfer during plus DNA synthesis
Recombination products

distinctive retroviruses; both require the formation of heterozygous virions in the initially infected cells and genetic exchanges during reverse transcription in subsequently infected cells. Two models differ in how and when the genetic exchanges are presumed to occur. The forced copy-choice model, described by Coffin (Coffin, 1979) (Fig. 1A), proposes that the genomic RNA in the virion is damaged. The synthesis of a minus strand is interrupted when reverse transcriptase encounters a break in the viral RNA, forcing it to switch to the other copy of genomic RNA to salvage the encoded genetic information. Thus, recombinant retroviral DNA molecules are formed by template switching during minus strand synthesis from the viral RNA template. This model was formulated, in part, to explain how retroviruses can survive the numerous breaks typically observed in virion RNA preparations and why one virus particle may yield only one DNA provirus (Hu and Temin., 1990; Panganiban and Fiore. 1988). The strand displacement-assimilation model (Junghans et al., 1982) (Fig. 1B) proposes that two complete minus strand DNAs are made from the viral RNA template during minus strands synthesis. Recombination subsequently occurs during plus strand viral DNA synthesis. This model is consistent with several biochemical features of retroviral replication.

Each model makes a specific prediction with regard to the nature of the product. The product of forced copy-choice must be a homoduplex, because recombination occurs during synthesis of minus strand. The strand-assimilation product would be a heteroduplex, because two minus strand DNA are involved during the process of recombination. The experimental evidence implicating heterozygous virions and reverse transcriptase is consistent with both models (Hu and Temin, 1990; Goodrich and Duesberg, 190; Stuhlmann et al., 1990). However, the evidence that reverse transcriptase can switch to a second RNA strand after reverse transcribing to the end of an RNA template in vitro is in favor of the forced copy-choice model (Luo and Taylor, 1990).

2. Human immunodeficiency virus (HIV)

2.1 Nature of HIV

HIV is the major etiologic agent of acquired immunodeficiency syndrome (AIDS), which was originally designated lymphoadenopathy-associated virus (LAV) (Barre-Sinoussi et al., 1983), human T lymphotropic virus (HTLV-III) (Gallo et al., 1984), or AIDS-associated retrovirus (ARV) (Levy et al., 1984). It is a RNA retrovirus which shares many features with other members of the nontransforming and cytopathic lentivirus family of retroviruses., such as visna virus of sheep, equine infectious anemia virus, and feline immunodeficiency virus (Pedersen et al., 1987). These viruses, including HIV in humans, induce a slowly progressive and inevitable fatal disease in their hosts. They all cause long latent infections as well as short term cytopathic effects. A persistent viremia associated with weak neutralizing humoral responses is observed. High mutational rates, cytolytic effects upon host cells and central nervous system (CNS) involvement are also common features among lentiviruses (Letvin, 1990).

As with other lentiviruses, which preferentially infect cells of the immune system in vivo, HIV has tropism for CD4+T lymphocytes as well as cell of monocytes/macrophages lineage. Infection with HIV results in a profound immunosuppression due predominantly to a selective depletion of helper/inducer T lymphocytes that express the receptor for HIV. HIV also has tropism for the brain leading to neuropsychiatric abnormalities

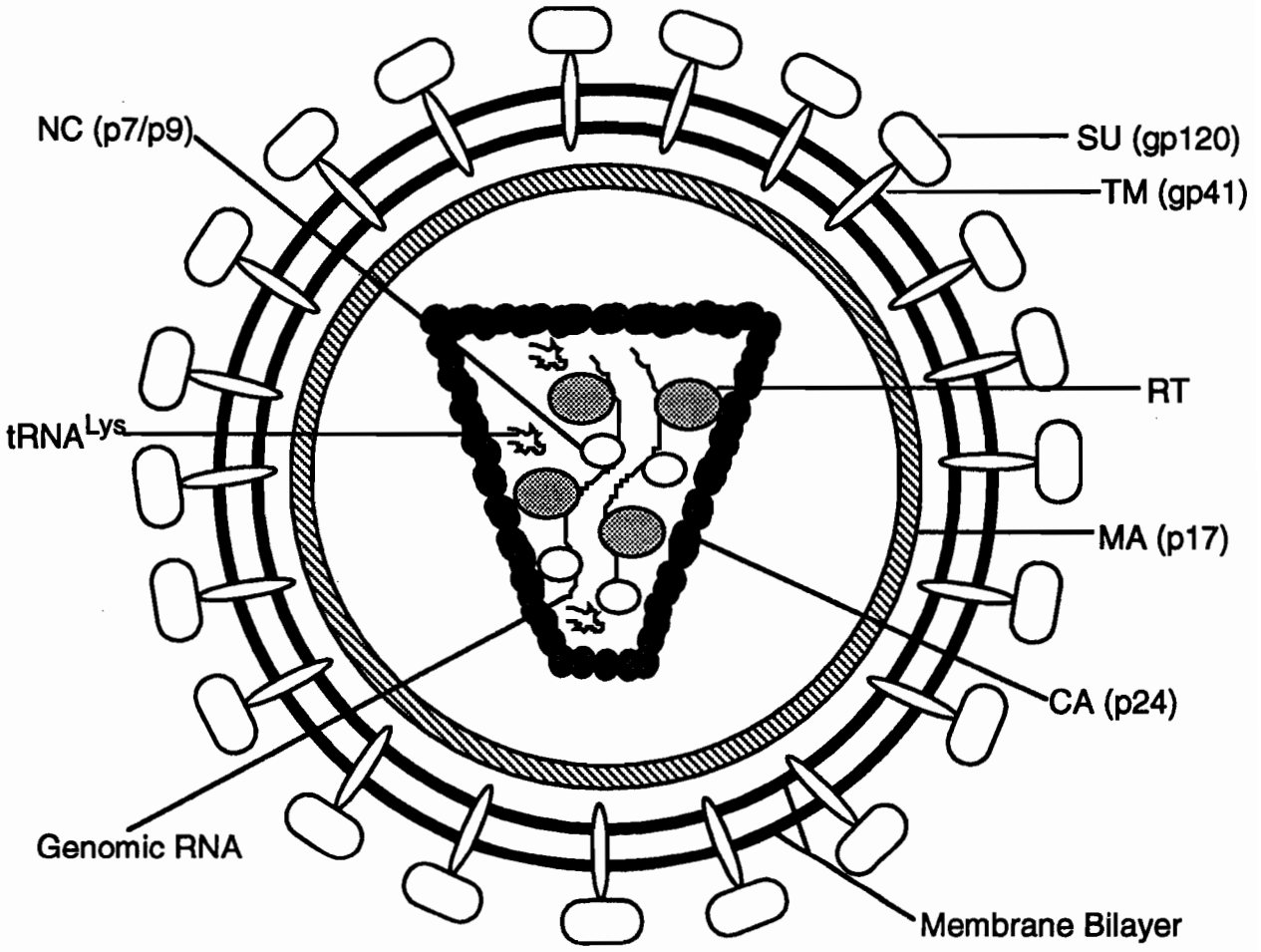
Two subtypes of human immunodeficiency virus, HIV-1 and HIV-2, have been identified (Coffin et al., 1986; Fauci, 1988). Both are the members of the lentiviruses family of retroviruses (Narayan & Clements, 1989), based on genomic sequence homologies, morphology and life cycle (Gonda et al., 1985). Phylogenetic reconstructions based on sequence analysis suggest that HIV-1 and HIV-2 diverged from a common ancestor roughly 40 years ago (Smith et al., 1988; Doolittle , 1989). Isolation and nucleoside sequencing of many variants of both HIV-1 and HIV-2 have indicated a significant degree of variation both within and between the HIV-1 and HIV-2 isolates,

particularly in the most antigenic regions of the viral surface glycoproteins. This observation complicates vaccine strategies based on using the typically immunogenic envelope glycoproteins. HIV-1 is mainly found in Central Africa, Europe and United States whereas HIV-2 is primarily isolated from AIDS patients in West Africa (Clavel et al., 1986; Kanki et al., 1987). HIV-2 is antigenically distinct from the HIV-1. Antisera from HIV-2 do not recognize the HIV-1 env antigen (Clavel et al., 1987) and there is no cross reactivity of antibody dependent cellular cytotoxicity (ADCC) observed between these two viruses (Ljunggren et al., 1988). HIV-2 shows the same tropism as HIV-1 for CD4+ cells, and results in a syndrome indistinguishable from HIV-1 (Brun-Vezinet et al., 1987).

2.2. Structure of HIV virion

HIV is an enveloped virus, which is surrounded by a cell-derived membrane (Aloia et al., 1988; Gelderblom, 1991; Gelderblom et al., 1987). Two viral proteins, the outer surface glycoprotein (SU) and the transmembrane glycoprotein (TM) are associated with the viral lipid membrane. The overall diameter of the spherical particle is approximately 100 nm. Electron microscopy studies have revealed the HIV virion is an icosahedral structure containing 72 external spikes (Gelderblom et al., 1987; Gonda et al., 1985) (Fig. 2). These spikes are formed by the viral-envelope proteins, SU and TM proteins. Like all retroviruses, the mature HIV virion contains a ribonucleoprotein core particle surrounded by an outer protein shell (Gonda, 1988). HIV virions have a characteristic dense protein core that encases the viral nucleic acid as well as enzymes required for efficient viral replication events. The club-shaped inner core particle of HIV-1 consists of the capsid (CA) and nucleocapsid (NC) proteins which associate with two copies of the plus stranded HIV genomic RNA. Within the core are molecules of the reverse transcriptase and integrase. This ribonucleoprotein assembly is surrounded by an

Figure 2. Model of the structure of the HIV virion.


NC (p7/p9)
SU (gp120)
TM (gp41)
RT
tRNA^{Lys}
MA (p17)
CA (p24)
Genomic RNA
Membrane Bilayer

icosadeltahedral outer protein shell consisting of the HIV-1 matrix (MA) protein (Gelderblom et at., 1987; Marx et al., 1988). This outer protein shell is, in turn, surrounded by a lipid bilayer envelope studded with external spikes. A single HIV-1 virion is estimated to contain 1200 CA molecules, 80 RT, and up to 280 molecules of viral SU protein (Hahn, 1994). In addition, HIV-1 virions contain $tRNA^{Lys3}$ molecules that are used for the initiation of reverse transcription.

2.3. Molecular organization of HIV

The HIV proviral genome has been well characterized (Fig. 3). The length of the HIV genome varies from 9.2 to 9.7 kilobases depending on the viral isolate (Alizon et al., 1984; Hahn et al., 1984). Like other retroviruses, HIV also contains three essential genes, gag, pol, and env, flanked by the long terminal repeat. HIV LTRs contain regulatory segments for viral replication such as the TATA promoter, polyadenylation signal sequences, cis-acting elements, negative regulatory elements, the NFkB enhancer region, as well as the transactivating responsive sequence (Starcich et al., 1985; Vaishnav & Wong-staal, 1991). The 5' LTR functions to promote proviral transcription whereas the 3' LTR is required for efficient polyadenylation of the resultant transcripts.

The gag gene of HIV-1 provides structural proteins required for virion assembly. The gag gene is translated from the full-length viral mRNA on membrane-free cytoplasmic ribosomes as polyprotein precursor (Pr55 gag) consisting of the matrix (MA) protein, capsid protein (CA), and nucleocapsid (NC) protein (Klug and Rhodes, 1987). Domains of this polyprotein play important roles in the assembly and release of virus particles. The phosphorylated p24 polypeptide forms the chief component of the inner shelf of the nucleocapsid, whereas the myristylated p17 protein is associated with the inner surface of the lipid bilayer and probably stabilizes the exterior and interior components of the virion. The p7 protein binds directly to the genomic RNA through a zinc-finger structural motif and together with p9 forms the nucleoid core (Gottlinger et

al., 1989; Bryant and Ratner, 1990; Haseltine, 1991).

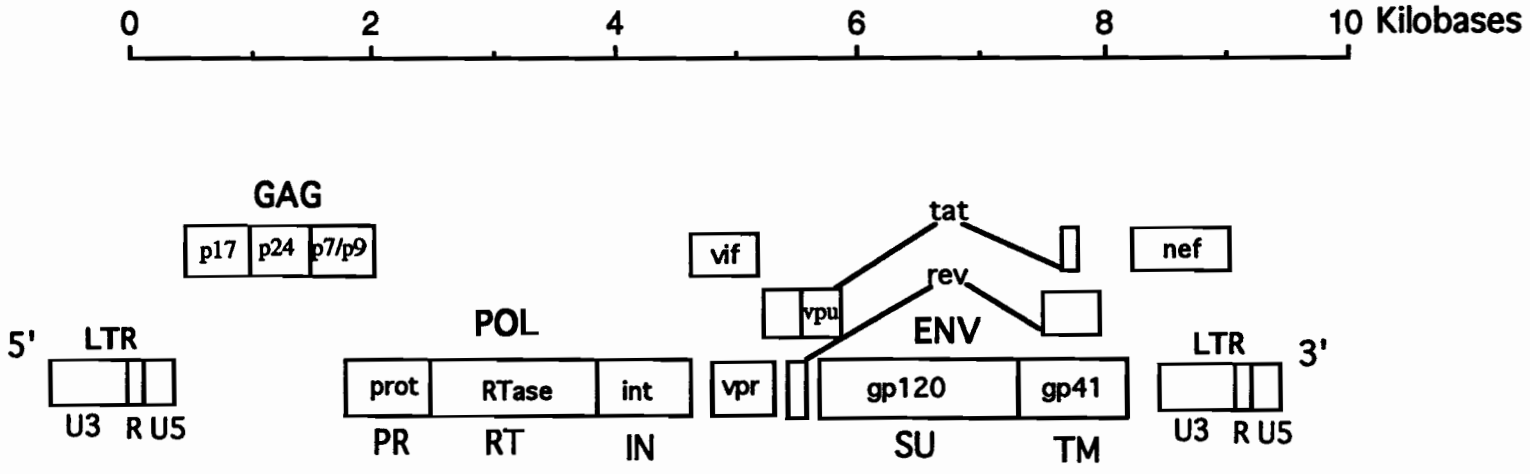
The pol gene codes for a polyprotein precursor with the order of NH_2-protease-reverse transcriptase (RT)-endonuclease-COOH which is cleaved to yield protease, RT, and integrase (Kohl, et al., 1988; Hansen et al., 1988; Sherman and Fyfe, 1990). As with the gag gene, the pol gene is also translated from the full-length mRNA. The open reading frames for gag and pol genes partially overlap, with pol in the -1 translational reading frame with respect to gag. The expression of the pol gene of HIV-1 occurs when the ribosome "slips back" one base during translation (ribosomal frameshifting), resulting in the synthesis of a fusion protein, $Pr160^{gag\text{-}pol}$ (Jacks et al., 1988; Wilson et al., 1988). Thus, ribosomal frameshifting provides an effective means of translational control for the synthesis of both Gag and Gag-Pol. The HIV-1 protease which is responsible for specific cleavage events leading to release from the polyprotein of the mature protease, RT and integrase, originates from $Pr160^{gag\text{-}pol}$ as result of autoprocessing events (Lillehoj et al., 1988; Mous et al., 1988; Park and Morrow, 1991; Peng et al., 1989). Replication of infectious HIV particles entirely is dependent on proteolytic processing by the viral protease. The HIV-1 RT is also translated as a component of the $Pr160^{gag\text{-}pol}$ polyprotein and subsequently processed by the pol-encoded protease to yield the active form of the enzyme which plays a crucial role in the viral replication (detailed review in section 3). The proteolytic processing by viral protease of the $Pr160^{gag\text{-}pol}$ also yields integrase, a 32-Kd protein which is essential for integration of retroviral DNA into host cell chromosome (Brown, 1990; Grandgenett and Mumm, 1990).

The HIV envelope glycoproteins are situated on the surface of virions and are involved in virus entry, cell fusion, and cell cytolysis. Unlike the gag gene, the envelope gene is translated initially as 88 Kd precursor from a single spliced mRNA (Allan et al., 1985). The precursor contains the hydrophobic amino-terminal signal sequence that directs the protein to secretory pathway of the cell. The N-terminal 30-60 amino acids constitute a signal peptide which is followed by the large gp120 that form the surface

"spike" and contains the receptor-binding site as well as the major sites recognized by neutralizing antibodies. As with other retroviruses, after folding, the envelope glycoproteins of HIV undergo oligomerization, which is important for intracellular transport and stability (Earl et al., 1990; Einfeld and hunter., 1988; Pinter et al., 1989). The glycosylated envelope precursor has a molecular mass of 160 Kd (gp160). As a fairly late step in maturation, the precursor gp160 is cleaved to separate the large gp120 from the smaller, C-terminal protein, gp41. The gp41 can be divided into three functional regions. An external region interacts with the gp120, which is thereby anchored to virion. A highly hydrophobic 23 amino acid or larger region spans the lipid membrane. The C-terminal cytoplasmic domain probably provides signals which help to direct the protein through the processing machinery of the host cell. In HIV-2, the env precursor can form dimers or trimers in the rough endoplasmic reticulum (Rey et al., 1989).

In addition to the genetic elements described above, HIV also has additional genes. At lease 6 additional genes, tat, rev, nef, vif, vpr and vpu, have been identified (Fig. 3). These genes have regulatory functions, and the expression of these genes almost certainly has an impact on pathogenic mechanisms exerted by HIV. The tat gene plays an important role in the amplification of virus replication by encoding a 14-15 Kd protein that binds the TAR region of the LTR (Rosen et al., 1985; Arya et al., 1985) and functions as a potent trans-activator of HIV gene expression (Rosen et al., 1985; Arya et al., 1985; Chen, 1986). Mutations and deletions in the gene abolish viral infectivity. Thus, the tat gene is essential for HIV replication (Dayton et al., 1986; Luciw et al., 1987; Fisher et al., 1986) The rev gene encodes a 20 Kd protein that is required for HIV replication and expression of long HIV transcripts which code for gag, pol, and env precursor polyproteins (Sadaie et al., 1988; Malim et al., 1988). In the absence of rev, viral structural proteins are not synthesized, but the regulatory proteins are not adversely affected. Analysis of mRNA species produced by the transfected mutant proviruses has revealed an altered mRNA profile. Rev- mutants fail to produce stable mRNA for gag-pol

Figure 3. HIV genome and gene products.


0
2
4
6
8
10 Kilobases
GAG
p17
p24
p7/p9
vif
tat
rev
nef
vpu
POL
ENV
5'
LTR
prot
RTase
int
vpr
gp120
gp41
LTR
3'
U3 R U5
PR
RT
IN
SU
TM
U3 R U5

(9.2 kb) and env (4.3 kb) and only multiply spliced 1.8-2 kb mRNA accumulates (Feinberg et al., 1986; Sadaie et al., 1988). Unlike the tat and the rev, the nef gene product is not required for HIV replication in culture. The nef gene encodes the 25-27 KDa protein (Arya & Gallo, 1986) that negatively regulates HIV replication and gene expression either by interfering with cellular signal or by activating trans-acting factors which interact with the negative regulatory elements (NRE) in the upstream portion of the LTR (Ahmad and Venkatesan, 1988; Niederman et al., 1989). Cloned proviruses with deletions or mutations in the nef gene give rise to viruses which replicate to high titers in tissue culture cells than the wild-type virus (Luciw et al., 1987). However, these negative effects of nef remain controversial as others have observed no effect of Nef protein on either viral replication or gene expression (Hammes et al., 1989; Kim et al., 1989). In addition, nef down regulates CD4 expression (Garcia and Miller, 1991) and thus may be important in maintaining latent infection. The vif gene encodes a 23 KDa protein that affects virus assembly and/or maturation and is important in the completion of viral DNA synthesis after entry (Lee et al., 1986; Arya & Gallo, 1986; Garrett et al. 1991; Von Schwedler et al., 1993). Vif predominantly affects cell-free virus transmission, with little effect on cell to cell viral transmission (Kan et al., 1986; Fisher et al., 1987). The vpu gene encodes a 15-20 KDa protein that is post-translationally modified by phosphorylation (Matsuda et al., 1988; Strebel et al., 1988; Cohen et al., 1988; Strebel et al., 1989). It is highly conserved among HIV-1 isolates but absent in HIV-2 (Cohen et al., 1988). The vpr encodes a 15 KDa protein that accelerates the replication and cytopathic effects of HIV in CD4+ T-cells, with the most pronounced effect exerted early in infection (Ogawa et al., 1989; Cohen et al., 1990).

2.4. Genetic heterogeneity of HIV

Genetic heterogeneity has been firmly established as prominent characteristic of the HIV (Shaw et al., 1984; Wong-Staal et al., 1985; Benn et al., 1985; Ratner et al., 1985;

Coffin, 1986; 1988; Fisher et al., 1988). No two isolates made from different patients were identical to each other. The pattern of nucleotide sequence variation is not consistent over the whole genome. The genes encoding gag and pol are considerably more conserved than env.

The extent of variation is also not constant in all regions within env. Studies of the sequencing of complete viral genomes, or parts of them, have revealed the existence of hypervariable regions in env that are interspersed with highly conserved sequences and regions of intermediate variability (Hahn et al., 1986; Alizon et al., 1986; Willey et al., 1986). Most diversity is observed in the region coding for outer membrane protein gp120, resulting in amino acid sequence diversity of higher than 25% (Hahn et al., 1985; Alizon et al., 1986). The overall predicted structure of gp120 does not appear to vary greatly among different isolates. However, the homology from one isolate to another is very small within the "hot spots" of the hypervariable regions. It is postulated that variability of the gp120-coding regions is responsible for significant antigenic variation of the virus. Somewhat less variation is also present in the transmembrane portion. All of the other genes, independently of whether their products have structural or regulatory function, have significant variation that results in protein diversity in the order of a few percent to more than 20% (Wong-Staal, 1985; Hahn et al., 1986). Considerable variation exists also in the LTR in which major form of any variation differed usually 1-4 residues (Alizon et al., 1986; Starcich et al., 1986; Meyerhans et al., 1989; Delassus et al., 1991).

Several kinds of virus population analyses have been applied directly to clinical sample, most commonly by PCR amplification of selected regions of viral RNA or DNA from infected tissue and by sequence analysis of a number of clones. Within some infected individuals, many different viruses have been detected (Saag et al., 1988; Fisher et al., 1988). One group found that in one patient, 13 highly related, yet genetically distinct, individual clones were detected in a total of 30 analyzed clones. In another patient the analysis of 16 clones yielded nine distinct viral genotypes (Saag et al., 1988).

Certain genotypes were observed in the more than one of the clones obtained and were evidently the predominant clones present in the patient, as determined by restriction analysis of viral DNA (Wong-Staal, 1985). Viruses isolated from one individual at different points of time also showed the evolution of genetic variants that were predominant at a certain time and were later replaced by other clones (Delassus et al., 1991; Hahn et al., 1986; Meyerhans et al., 1989). The rate of evolution of HIV-1 was estimated to be at least 10^{-3} nucleotide substitutions per site per year for the env gene and 10^{-4} for the gag gene (Hahn et al., 1986). Despite this relatively rapid rate of sequence divergence, virus isolates from any one patients were all much more related to each other than to viruses from other individuals (Hahn et al., 1986). The changes most frequently consisted of duplications or small deletions (Hahn et al., 1986). In contrast, long-term cultivation of virus isolates in vitro did result in any genomic changes, as determined by restriction analysis of viral DNA (Wong-Staal et al., 1985)

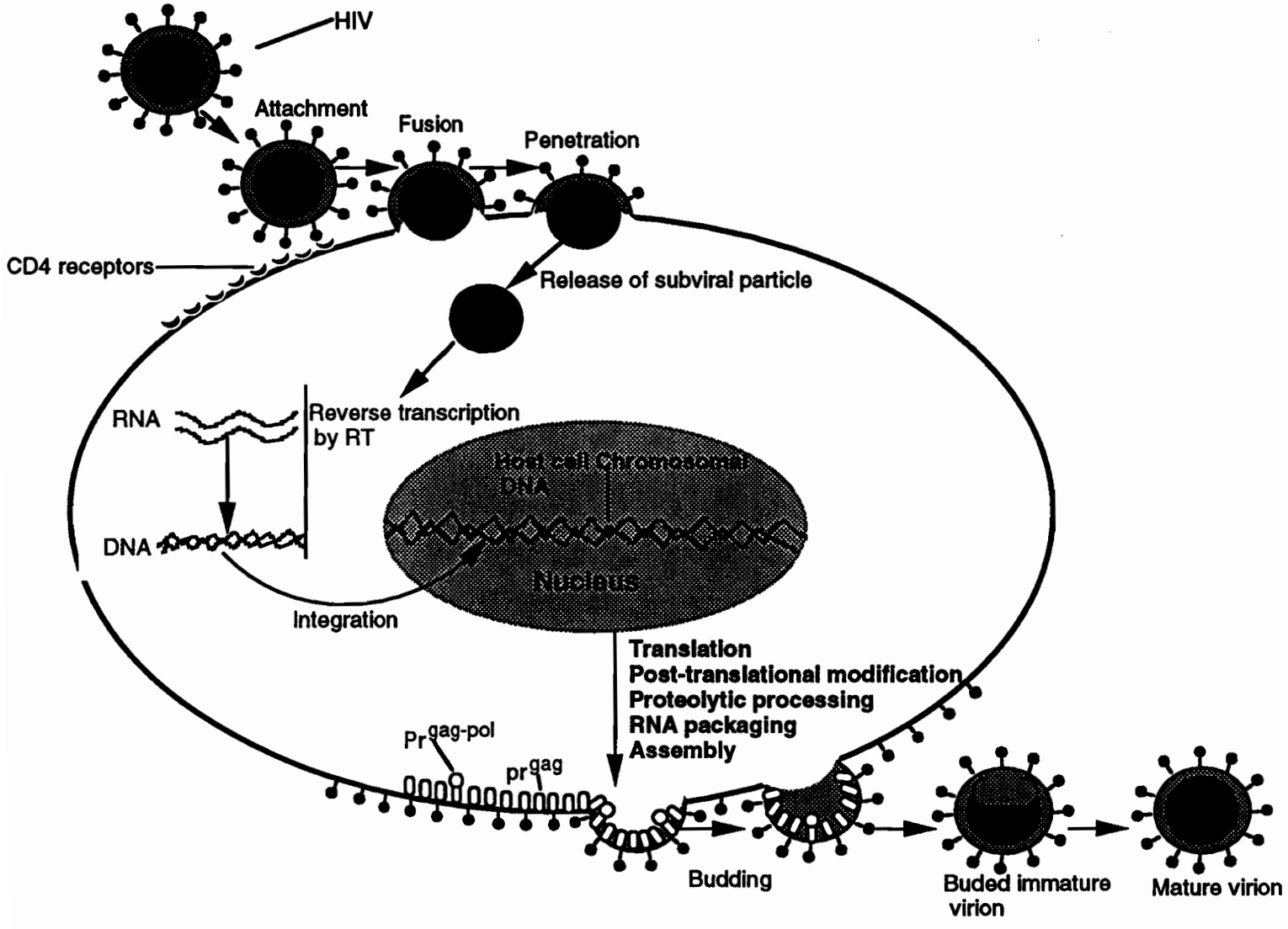
2.5. Life cycle of HIV

2.5.1. Adsorption of virion to host cell virus receptors

The initial steps in HIV infection are attachment to and penetration of cells bearing appropriate target receptors (Fig. 4). Interaction of HIV virion with a cell surface receptor molecule is an essential early event of the infection process, and the presence or absence of appropriate receptor can play a determining role in the host-range and tissue specificity of the HIV infection.

Thus far, HIV is the only retrovirus for which the nature of the cellular receptor has been identified (Dalgleish et al., 1984; Klatzmann et al., 1984; Maddon et al., 1986). Numerous studies have indicated that HIV use the CD4 as receptor to gain entry into the cell through the high-affinity binding of HIV envelope glycoprotein, gp120, to the external NH_2-terminal domain of the cell membrane CD4 receptor molecule (McDougal et al., 1986). The CD4 molecules is a 55-KDa plasma membrane-associated glycoprotein

Figure 4. Highly schematized representation of HIV infectious cycle.


HIV
Attachment
Fusion
Penetration
CD4 receptors
Release of subviral particle
RNA
Reverse transcription
by RT
DNA
Host cell chromosomal
DNA
Nucleus
Integration
Translation
Post-translational modification
Proteolytic processing
RNA packaging
Assembly
Pr gag-pol
pr gag
Budding
Buded immature
virion
Mature virion

that is found on 95% of thymocytes and 60% of peripheral blood T-cells and on macrophage/monocytes in humans (Shevach, 1989). Once bound to CD4, fusion of the cell membrane with an envelope glycoprotein, gp41, that is noncovalently associated with gp120 appears to take place. The entry of HIV into the cytoplasm of target cells occurs by a pH-independent mechanism (Stein et al., 1987), which suggests the fusion-mediated entry rather than receptor-mediated endocytosis (Evans and levy, 1989).

Although the CD4 molecule is the primary receptor for HIV, questions remain as to whether there are other low-affinity receptors for HIV-1 in neuronal tissue (Ezekowitz et al., 1989; Harouse et al., 1991; Koslowski et al., 1991). The in vitro infection of CD4-negative brain astrocytes (Cheng-Mayer et al., 1987) and human fibroblasts (Evans & Levy, 1989) and the detection of HIV in endothelial and epithelial cells of seropositive individuals (Wiley et al., 1986; Nelson et al., 1988) suggested that HIV infection could proceed in the absence of the CD4 receptor expression. One group recently report that the human CD26 molecule, cell surface protease, is an essential coreceptor for HIV entry into CD4+ cells (Callebaut et al., 1993), as demonstrated by inhibition of entry of HIV-1 or HIV-2 into T lymphoblastoid and monocytoid cell lines by monoclonal antibody or specific inhibitor against CD26 molecule. Coexpression of human CD4 and CD26 in murine NIH 3T3 cells rendered them permissive to infection by HIV-1 and HIV-2 (Callebaut et al., 1993). However, the function of CD26 as coreceptor for HIV remains controversial as subsequent studies failed to confirm that CD26 molecule is a cofactor for HIV infection (Broder et al., 1994; Patience et al., 1994; Camerini et al., 1994; Alizon and Dragic, 1994).

Interaction of HIV virion proteins with receptors may have important pathogenic consequences in addition to allowing virus infection. HIV infection is characterized by the formation of giant multinucleated cells which subsequently degenerate. This process involves fusion of infected with uninfected cells, mediated by the interaction of env proteins on the surface of the infected cell with receptors on the uninfected cell (Lifson et

al., 1986). This may be a major mechanism of cell killing in infected individuals.

2.5.2. Synthesis and integration of viral DNA

HIV behaves as a classical retroviruses with regard to many aspects of replication such as synthesis and integration of viral DNA. Following internalization, the HIV virion is rapidly uncoated and the enzymes within the nucleoprotein complex become activated in preparation for the replicative phase of its life cycle (Wong-Staal, 1991). Viral reverse transcriptase transcribes the viral RNA into a linear double-stranded DNA (Wong-Staal, 1991). A double-stranded free linear DNA molecule, which is created in the cytoplasm, is the precursor to the integrated viral DNA and to two circular forms that contain either one or two long terminal repeats (Brown et al., 1987 ; Brown et al., 1989). Studies of the kinetics of viral DNA synthesis in the cytoplasm of HIV-infected cells have shown that newly synthesized linear HIV DNA is generally detected in the cytoplasm within 4 h of initiation of infection (Kim et al., 1989). The time necessary for completion of synthesis of viral DNA depends on the physiological condition of the cell. In activated CD4+ cells, the synthesis and integration of viral DNA can occur within 12 h postinfection (Robinson and Zinkus, 1990; Stevenson et al., 1990; Zack et al., 1990). If virus infects a quiescent CD4+ cell, the process takes much longer up to several days, often resulting in incomplete synthesis of viral DNA (Stevenson et al., 1990; Zack et al., 1990). If the cell becomes activated during this time, the synthesis of viral DNA is completed, and the virus integrates into host cell chromosome (Stevenson et al., 1990).

The mechanism by which the HIV DNA translocates to the cell nucleus remains unclear, but nuclear targeting signals on viral core proteins have been identified (Bukrinskaya et al., 1992). The preintegration complex consisted of the integrase, matrix, and RT associated with the viral RNA and DNA. Interestingly, no capsid antigens were detected in this complex (Bukrinsky et al., 1993). One group has demonstrated that the preintegration complex of HIV-1 was rapidly transported to the nucleus of the host cell by

a process that required ATP, but was independent of cell division, and that functional HIV-1 integrase was not required for nuclear import of these complexes (Bukrinsky et al 1992).

After translocation to the nucleus, the viral DNA duplex is inserted into the host genome by the viral integrase. The sequence of the integrated provirus differs from the linear integrative DNA precursor at both ends. In general, 2 base pairs (bp) are removed from each end during the integration event (Bushman and Craigie, 1991). The loss of these bases is also common characteristic of retroviral integration, and the mechanism of loss is related to the actual process of integration (Bushman and Craigie, 1991).

Besides viral integrase, requirements for successful integration are the presence of intact LTR as well as certain cell-encoded functions (Wong-Staal, 1991; Haseltine, 1991). Integration may occur at many sites of host cell DNA. There appears to be no consensus site for integration of HIV proviruses in the host cell chromosome. It is generally believed that integration occurs at or near regions of the chromosome that are nucleosome free, that is, are not blocked by DNA binding proteins such as histones (Pryciak and Varmus, 1992). Some of the viral molecules remain in the nucleus in an unintegrated form. Persistence and accumulation of unintegrated viral DNA is usually associated with cytopathic effect and may contribute to cell killing (Ho et al., 1987). Like all other retroviruses, HIV, by integrating, become part of the genomic information of a cell that is passed on to all daughter cells. Once integrated, HIV might only be eradicated if all cells carrying the provirus were eliminated (Wong-Staal, 1991).

2.5.3. Expression of proviral DNA

The proviral genome is expressed in a way reminiscent of host cells genes. Viral RNA is synthesized by cellular RNA polymerase. The LTRs of proviruses are responsible for initiation and enhancement of expression of proviral DNA. RNA synthesis starts at the left end of the R sequences in the 5' LTR (Wong-Staal, 1991; Haseltine, 1991). Each

RNA transcript is polyadenylated post-transcriptionally near the right end of R in the 3' LTR. The initial transcription is promoted by the myriad of regulatory sequences found in the HIV-1 LTR (Wong-Staál, 1991; Haseltine, 1991). The binding of inducible and constitutive host transcription factors to sites in the LTR stimulates a low but important level of expression of the HIV genes (Cullen, 1991; Wong-Staal, 1991). The transcription of the HIV-1 genome during viral replication shows distinct kinetic phases (Feinberg et al., 1986; Kim et al., 1989; Pavlakis et al., 1991; Peterlin, 1991). The initial population of genomic-length viral messager RNA (mRNA) molecules reaches the cytoplasm exclusively as full-spliced, 2-kilobase viral transcripts (Kim et al., 1989), which encode the various regulatory proteins of HIV such as the HIV tat, rev, nef. Tat functions as a potent amplifier of viral-gene expression, leading to a high level of expression of all sequence linked to the HIV LTR. The HIV Rev protein then activates the expression of the HIV structural and enzymatic genes which simultaneously inhibit the production of regulatory proteins (Wong-Stall, 1991).

As levels of Rev increased, levels of unspliced mRNA encoding the Gag/Gag-Pol proteins and mRNAs encoding Env glycoprotein and other HIV gene products, including vif, vpu, and vpr, are increased. An interaction of Rev with Rev responsive element (RRE) is prerequisite for the transportation of the incompletely spliced mRNAs from the nucleus to the cytoplasm (Haseltine, 1991). Full-length RNA transcripts may be used in two different way. About half of these transcripts serve as genomic RNA subunits and packed into virion. The other half, after one or more events of splicing, serve as mRNA for the production of viral protein. The protein encoded by env are translated from a subgenomic 4.3-kb spliced mRNA, while the gag and gag-pol products are synthesized from the full-length mRNA. Usually, somewhat more gag than env mRNA is synthesized, and much more gag than pol. Gag, pol, and env all code for a polyprotein precursor that is cleaved into at least two final proteins (Wong-Stall, 1991; Haseltine, 1991).

2.5.4. Synthesis of viral proteins and assembly of virion

Following expression of proviral DNA, the final steps of the HIV life cycle, synthesis of viral proteins and their assembly into particles, occur. The HIV envelope glycoproteins are initially synthesized as a precursor from a single spliced mRNA on membrane-bound ribosomes (Allan et al., 1985; Dickson et al., 1984). Because of its amino-terminal signal sequence, the envelope glycoprotein is transported through the secretory pathway to the plasma membrane of the cell. The glycosylated HIV envelope precursor (gp160) is subsequently cleaved by a cellular protease to yield mature protein, gp120 and gp41. Similarly, Gag and Gag-Pol polyproteins synthesized as precursors from the full-length mRNA on free ribosomes are transported to the plasma membrane through an undefined cytoplasmic pathway (Dickson et al., 1984). The HIV gag proteins which, together with the pol products and the viral RNA, form the viral cores are derived from the cleavage of 55-kda precursor protein. The HIV-encoded protease mediates the cleavage yielding the p24, p17, p9, and p7 gag protein. The HIV pol protein is translated from the same transcript as the gag precursor by a novel ribosomal frame-shifting mechanism (Wong-Staal, 1991; Jacks et al., 1987). In general, approximately 20-fold more gag protein than pol protein is produced, reflecting the relative inefficiency of this frame-shifting process. Once translated, the pol-gene precursor is cleaved to produce several critical viral enzymes, including reverse transcriptase, integrase, ribonuclease and protease Haseltine, 1991).

Both the Gag and Gag-Pol polyproteins of HIV-1 bear a N-terminal myristic acid residue that is essential for virion assembly (Gottlinger et al., 1989). The HIV-1 genome contains a specific packaging signal within the RNA leader region, and recognition of this sequence requires the integrity of a zinc-finger motif within the nucleocapsid protein of Gag (Aldovini and Young, 1990; Gorelick et al., 1993; Lever et al., 1989). It is noted that assembly of the infectious HIV virion proceeds in a stepwise manner (Gottlinger et al.,

1989), initially involving aggregation of the ribonucleoprotein core in the cytoplasm which is composed of the HIV RNA, gag proteins and the viral enzymes encoded by pol gene (Haseltine, 1991). Once assembled, these cores move to the surface and bud through the plasma membrane, where they acquire their lipid membrane, complete with the two protein products of the HIV env gene. The cleavage events mediated by HIV-protease occur during this budding process. The assembly of HIV-1 requires that the activation of viral protease contained in the Gag-Pol polyprotein occurs until just before or immediately after release of virions, otherwise, premature activation of viral protease can lead to proteolytic processing of the precursors Gag or Gag-Pol polyprotein, precluding the assembly of infectious virus (Haseltine, 1991; Wong-Stall, 1991). Excessive amounts of viral protease can also results in alterations of the infected cell morphology, leading to cell death (Park and Morrow, 1991). In addition to the proteins which are components of HIV, the proteins functioning as regulatory proteins are translated from doubly spliced mRNAs.

Based on the extensive knowledge of HIV, including its structure and its replication, a number of antiviral strategies have been designed. It is believed that ideal strategies for antiviral intervention should target features of viral processes that are different from host cellular metabolism (Mitsuya et al., 1991; Wong-Staal., 1991). Any step in the biosynthesis of HIV is susceptible to attack by antiviral interaction. Thus, targeting at HIV life cycle for therapeutic intervention is a promising strategies for the treatment of HIV infection. The most prominent of these target is the virus-coded reverse transcriptase which catalyze reverse transcription because, unlike the cellular polymerases, RT operates in the cytoplasm and furthermore, because reverse transcription seems to be dispensable to cellular metabolism.

3. HIV RT

3.1. Functional features of HIV-1 RT

Like all retroviral RTs, HIV-1 RT are multifunctional proteins containing two major activities: a DNA polymerase activity capable of coping either RNA or DNA template and an RNase activity, termed RNase H, capable of degrading RNA only in RNA-DNA hybrid form (Jaeobo-Molina and Arnold, 1991). In addition, one study proposed that HIV-1 reverse transcriptase also possess a ribonuclease activity that specifically degrades double-stranded RNA (Ben-Artzi et al., 1992). However, the existence of such an activity remains controversial and other investigators have suggested that this nuclease activity can be correlated with traces of E. coli RNase III that contaminate these bacterially expressed preparation (Hostomsky et al., 1992).

It has been demonstrated that retroviral RTs used cellular-derived transfer RNA molecules (tRNA) to prime viral reverse transcription. For HIV-1 RT, the cellular tRNA serving as primer for DNA synthesis in HIV infection is $tRNA^{Lys3}$, as deduced by the sequence of primer-binding site (PBS) region of HIV-1 RT (Ratner et al., 1985). Indeed, using different experimental approaches including UV cross-linking, RNase footprinting, and gel retardation, a complex between mammalian $tRNA^{Lys3}$ and HIV-1 reverse transcriptase has been demonstrated. The regions of the primer tRNA in close contact with the enzyme have been identified as the anticodon region and the dihydrouridine loop (Barat et al., 1989; Sarih-Cottin et al., 1992). HIV-1 RT has been shown to preferentially bind $tRNA^{Lys3}$ even in the presence of a 100-fold excess of other tRNA (Barat et al., 1989)

HIV-1 RT is a very flexible protein that may be related to the several functions catalyzed by the enzyme. Studies on the interaction between HIV-1 RT and its primer tRNA demonstrated that important conformational changes of enzyme can be induced following binding of $tRNA^{Lys3}$ (Robert et al., 1990). These conformational changes induced by primer tRNA affect not only the DNA polymerase activity but also the RNase H activity of the retroviral polymerase (Andreola et al., 1992).

Several studies have indicated that the 3' end of the oligonucleotide makes a crucial

contribution to the binding of DNA polymerase with primers (Majumdar et al., 1988; Majumdar et al., 1989). Of all DNA polymerases studies, only HIV-1 RT showed higher affinity to riboprimers than to deoxyriboprimers (Nevinsky et al., 1992). Studies of localization of polynucleotide binding region in the HIV-1 RT was performed using UV-irradiation cross-linking by which a covalent link was created between the enzyme and primer (Sobol et al., 1991). Subsequent studies of covalently linking a primer with a 5' end modification to HIV-1 RT revealed that in the presence of the complementary template and labeled precursor, HIV-1 RT-linked primer was elongated by one radioactive nucleotide residue, as visualized by autoradiography (Andreola et al., 1993). This reaction, due to the "catalytic competence" of the enzyme, readily demonstrates that the 3' end of the primer is located at or near the catalytic center (Andreola et al., 1993). In addition, the observation that $tRNA^{Lys3}$ can compete for binding of covalent primer-template to the enzyme is in favor of the specificity of this primer binding reaction. For other DNA polymerases, no primer binding domain labeling was detected in the absence of a template having complementarity with primer analog (Mitina et al., 1990). These results suggest that competitive modification of the template is a unique feature of HIV-1 reverse transcriptase (Andreola et al., 1993).

The polymerase and RNase H domains of HIV RT reside within the same protein molecule. It can be assumed that both activities are tightly coupled (Gopalakrishnan et al., 1991). The RNase H acts primarily as endonuclease, which hydrolyzes the RNA to produce 3'-hydroxyls and 5'-phosphates. The resulting 3' termini are suitable as primers for DNA synthesis. The RNase H activity strictly requires that the substrate is in RNA-DNA hybrid form, and requires divalent cation. Studies with homopolymeric substrates show that most combinations of RNA-DNA hybrids are readily hydrolyzed by HIV-1 RNase H, although HIV-1 RNase H exhibits only weak activity against poly(rA).poly(dT) (Starnes and Cheng, 1989). The HIV-1 RNase H makes nicks in the RNA strand of long hybrids (Mizrahi, 1989), showing significant preferences for selected sequences. The

preferences detected for the HIV-1 RNase H are very different from those of the E. coli RNase H (Mizrahi, 1989; Taylor and Sharmeen, 1987).

3.2. Structural features of HIV RT

The HIV-1 RT has been identified in viral particles as a heterodimer consisting of two tightly associated chains of 66 and 51 kDa (Veronese et al., 1986; Wondrak et al., 1986; Lightfoote et al ., 1986). The two chains of the HIV-1 RT p66/p51 heterodimer are identical at their amino termini which contain the DNA polymerase domain as characterized by sequences comparison and site-directed mutagenesis. The p51 subunit of the heterodimer lacks the C-terminal end spanning the RNase H domain (Hansen et al., 1988), which is apparently derived by proteolytic processing of the p66 chain by the HIV protease. Several studies have identified the carboxyl-terminal residue of the p51 chain in the heterodimer derived by proteolysis with the HIV-1 protease as Phe-440/Try-441 (Mizrahi et al. 1989; Graves et al. 1990; Schulze et al., 1991). Enzymatic treatment of the p66/p66 homodimer with purified HIV-1 protease yields products that resemble the p66/p51 heterodimer (Ferris et al., 1990).

Both of p66 and p51 subunits contain the polymerase domain, but the p51 subunit either as monomer or dimer has very low polymerase activity when compared with p66/p66 homodimer or p66/p51 heterodimer (Johnson et al., 1986; Starnes et al., 1988; Tisdale et al., 1988; Muller et al., 1989; Hansen et al., 1988). The p66 subunit can exist as both a monomer and a dimer. The dimeric form of the p66 subunit appears to have a high level of polymerase activity than does the monomeric form (Muller et al., 1989; Restle et al., 1990). The C-terminal 15 kDa protein, generated during the cleavage of p66 into p51, has no detectable ribonuclease activity on its own (Davies et al., 1991; Le Grice et al., 1991). The addition of purified p51 subunit to p15 leads to the reconstitution of ribonuclease activity (Hostaomsky et al., 1991). An interaction between the p51 and the p66 subunits is required for optimal polymerase activity. Evidence that these subunits are

indeed associated in vivo came from immunoprecipitation studies with HIV-1. Treatment of virion extracts with a monoclonal antibody specific for an epitope located in the carboxyl terminal of p66 resulted in recovery of both the 66- and 51-Kd polypeptides in a 1:1 subunit stoichiometry, indicating that heterodimer complex of p66/p51 exists in vivo (Lowe et al. 1988; Tisdale et al. 1988).

Modeling studies of HIV RT enzyme suggest that the HIV RT may comprise three major functional domains, the N-terminal polymerization domain, the tether domain, and the RNase H domain (Barber et al., 1990; Narasimhan and Maggiora, 1992). The N-terminal two-third of the RT, in the case of M-MuLV, contains the RNA-dependent and DNA-dependent DNA polymerase activity, while the C-terminal one-third contains the RNase H activity (Tanese and Goff, 1988; Coffin, 1991). For HIV-1 RT, it has been established that the polymerization and RNase H activities of HIV-1 RT map to the N- and C-terminal portion of the p66, respectively, and that the p51 chain lacks the RNase H domain. A high degree of structural relatedness may exist between functional domains of retroviral RTs, as deduced by the sequences of RT polymerases and RNase H domains with property of high similarity. For HIV-1 RT, linker insertion mutations at the N-terminus can affect RNase H activity while such mutations at the C-terminus can disrupt polymerization, suggesting a functional interdependence of two domains (Hizi et al., 1989; Prasad and Goff, 1989; Hizi et al., 1990).

As with other polymerases, retroviral RTs contain a highly conserved region or motif, Tyr-X-Asp-Asp (YXDD) (Argos, 1988). This motif is necessary for polymerase activity. Site-directed mutagenesis experiments have verified that this characteristic amino acid sequences is required for polymerization activity of HIV-1 RT (Larder et al., 1987; Prasad and Goff, 1989; Boyer et al., 1992). For HIV-1, both of p66 and p55 subunits contain this motif represented by amino acids 183-186. Mutation of this motif in the p51 subunit of the heterodimer does not impair polymerase activity (Le Grice et al., 1991), whereas the same mutation in the p66 subunit results in a disappearance of RNA-

dependent DNA polymerase activity, indicating that the p51 subunit does not directly contribute to the polymerase activity in the heterodimer. (Le Grice et al, 1991). Substitutions of Leucine or Tyrosine for methionine at codon 184 resulted in polymerase activity reduced by more than 90% (Larder et al., 1987; Prasad and Goff, 1989). Change of the two aspartic acid residue into asparagine, histidine or glutamine abolishes all polymerase activity (Larder et al., 1987; Larder et al., 1989; Le Grice et al., 1991; Boyer et al., 1992).

Crystallographical studies have resolved the probable structure of RT and ribonuclease H (RNase H) (Kohlstaedt et al 1992; Davies et al., 1991; Arnold et al., 1992). The structure of HIV-1 RT-inhibitor complex demonstrated that p66/p51 heterodimer has an unprecedented degree of asymmetry (Kohlstaedt et al 1992). The p66 polymerase domain forms a large cleft capable of containing a template. This structure is similar to that of the DNA-polymerase-containing (Klenow) fragment of E. coli DNA polymerase I. The p51 subunit, which supports the p66 subunit, has a different structure and no cleft. The p66 subunit is folded into five separated subdomains: four polymerase subdomains and a RNase H domain (Kohlstaedt et al 1992). Three polymerase subdomains forming structure reminiscent of right hand are named the subdomain "fingers", "palm" and "thumb". The fourth polymerase subdomain situates between the remainder of the polymerase and RNase H domain, and so is called the "connection" domain. The subdomain "fingers", together with subdomain "thumb" and "palm", form a cleft capable of containing a template. The "connection" subdomain contacts the RNase H domain as well as the "thumb" and "connection" subdomain of p51. Unlike the conformation of p66 subunit, the "conformation" subdomain of the p51 subunit contacts all three of the other subdomains (Kohlstaedt et al 1992).

3.3. The fidelity of HIV RT

The HIV-1, as described in section 2.4, exhibits extensive genomic heterogeneity

that gives the virus properties of a quasispecies (Steinhauer and Holland, 1986; Meyerhans et al., 1989). It is now well accepted that the high mutation rate of the HIV-1 is due to the low fidelity of the HIV-1 RT. Like other retroviral RTs, HIV-1 RT has no 3'→5' exonuclease activity (proofreading) to remove misincorporated nucleotide during polymerization (Battlula and Loeb; 1976; Roberts et al., 1988). A number of studies have revealed an unusually high error rate of HIV-1 RT in coping DNA or RNA template, equal to or greater than those of RTs from other retroviruses (Takeuchi et al., 1988; Roberts et al., 1988; Preston et al., 1988 Bebenek et al., 1989; Weber and Grosse, 1989), demonstrating error-prone nature of HIV-1 RT may involve more than just the absence of a proofreading exonuclease activity. In these studies, HIV-1 RTs purified from both virions and E. coli expressing the recombinant enzyme were used. There were no differences found between the two sources of HIV-1 RT, indicating the authenticity of the recombinant RT and the lack of any posttranslation modification that might affect the fidelity of DNA synthesis.

Early studies, using the synthetic polymer template assay, showed that the fidelity of HIV-1 RT was lower than AMV or MuLV during DNA synthesis. The misincorporation rate for HIV-1 RT was 1 per 32600 nucleotide compared to 1 per 112000 nucleotide for MuLV and to 1 per 93500 nucleotide for AMV (Takeuchi et al., 1988). Similar results were demonstrated through a genetic assay in subsequent studies in which a single-stranded bacteriophage DNA was used as template for DNA synthesis by HIV-1 RT (Preston et al., 1988; Robert et al., 1988). Several studies have also investigated the fidelity of RNA template-directed polymerization by HIV-1 RT. As expected, high error rate of HIV-1 RT with RNA template was detected. However, the error rate with RNA and DNA template was unequal, suggesting that mistake during minus- and plus-strand DNA synthesis may not contribute equally to mutation rate of HIV-1 (Boyer et al., 1992; Hubner et al., 1992).

The misincorporation of HIV-1 RT mainly results from base substitution and

frameshifts errors during DNA synthesis. HIV-1 RT showed base substitution errors at average rate of 1.6 x 10^{-4} during DNA template-directed polymerization. At specific sites, located at boundaries of homopolymeric nucleotide runs, HIV-1 RT is capable of generating substitution errors at extraordinarily high rate (10^{-2} to 10^{-3}) during DNA synthesis (Roberts et al., 1988; Bebenek et al., 1989). HIV-1 RT, for frameshifts, also displays high error rate of 2.3 x10^{-4} (Bebenek and Kunkel, 1993). The error rate for frameshift is higher at homopolymeric sequence than at other location.

The mechanisms for the error specificity of HIV-1 RT remain unclear. It has been proposed that one base frameshifts at template may have resulted from template-primer slippage during synthesis. For the base substitution, the initiating event may also be template-primer slippage, followed by correct incorporation of the next correct nucleotide. However, unlike simple slippage, the template-primer the realigns prior to continued synthesis, creating a mispaired intermediate that leads to the observed base substitution error at the end of homopolymeric run (Bebenek et al., 1989; Bebenek et al., 1993).

4. Inhibitors of HIV RT

4.1. Nucleoside analogs

The antiviral drugs prominent to date in the therapy of HIV diseases and AIDS fall broadly into HIV RT inhibitors. These drug can be divided into two groups, depending on whether they interact with the substrate or nonsubstrate binding site (De Clercq, 1992). The most potent of these are a family of nucleosides with a 2',3'-dideoxyribose moiety, which lack a 3'-hydroxyl group and thus share a common mode of antiviral action that involves chain termination of viral DNA syntheses. These antiviral drugs need to be converted to their triphosphate form before they can act as substrate mimics and inhibitors of HIV-1 RT. A number of studies have demonstrated that these antiviral drugs, such as AZT, ddI, ddC, and 3TC, can inhibit the infectivity and replication of divergent HIV

strains in vitro or in vivo (Mitsuya et al., 1985; Mitsuya and Broders, 1986; Hamamoto et al., 1987; Herdewijn et al., 1987; Schinazi et al., 1992).

These 2',3'-dideoxynucleosides were originally synthesized in the 1960s or earlier. Among them, AZT was initially used as a potential anti-cancer drug (Horwitz et al., 1969). These nucleoside analogs were shown to block HIV replication either in T cell or in monocytes and macrophages in vitro (Mitsuya et al., 1985; Hamamoto et al., 1987; Herdewijn et al., 1987). In appropriate target cells, the dideoxynucleoside analogs are sequentially phosphorylated in the cytoplasm to yield dideoxynucleoside 5'-triphosphates which can be incorporated into the growing DNA chain to bring about termination of viral DNA synthesis or can compete with natural substrates for binding to HIV RT (Mitsuya and Broder, 1986; Cooney et al., 1986; Johnson et al., 1988). Each of these dideoxynucleoside analogs may take two or three phosphorylation steps to its active form. Following entry into cell, for example, AZT is initially converted by a cellular thymidine kinase to a monophosphate (AZT-MP). Subsequent phosphorylation of AZT-MP by a cellular thymidylate kinase give rise to a diphosphate. Finally, other cell enzymes convert this diphosphate to active triphosphate form (AZT-TP) which behaves as an alternative substrate and competitive inhibitor of HIV RT with respect to TTP binding (Cheng et al., 1987; St. Clair et al., 1987). These dideoxynucleoside analogs have higher affinities for HIV RT than for cellular DNA polymerase. However, at higher concentrations than those required to block RT activity, these dideoxynucleoside analogs can also competitively inhibit the function of cellular DNA polymerase (Cheng et al., 1987; Vazquez-Padua et al., 1990). The activity against cellular DNA polymerase might explain certain side effects, such as a toxic mitochondrial myopathy in AIDS patients receiving long-term AZT therapy (Fischl et al., 1987; Dalakas et al., 1990).

4.2. Nonnucleosides

The second group of HIV RT inhibitors are nonnucleosides with diverse molecular

structures such as TIBO compounds, nevirapine, pyridinones and piperazines, which have been reported to be active against HIV replication in vitro .(Baba et al., 1989; Miyasaka et al., 1989; Pauwels et al., 1990; Merluzzi et al., 1990; Debyser et al., 199; De Clercq, 1992). These HIV RT specific inhibitors seem to share a common mode of action. They do not require any intracellular conversion, but are able to interact directly with their target enzyme, HIV-1 RT, at an allosteric (non-substrate binding) site (De Clercq, 1992). These HIV-1 specific NNRT inhibitor are highly potent inhibitor of HIV-1 but not of HIV-2 or any other retroviruses (Pauwels et al., 1990; Goldman et al., 1991). It is noted that they are not inhibitory to HIV-2 RT or any other DNA polymerase even at concentration of 500 μM, as has been demonstrated repeatedly with these drugs (Pauwels et al., 1990; Baba et al., 1991; Merluzzi et al., 1990; White et al., 1991; Goldman et al., 1991). Most members of these NNRT inhibitors are active at concentrations in the nanomolar range to inhibit the spread of HIV-1 infection in the cell culture. However, the potency of inhibition of HIV-1 RT by such inhibitors varies depending on the primer-template used to assay activity. For TIBO derivatives, they are more active when poly(rC).oligo(dG) rather than poly (rA).oligo(dT) is used (Debyser et al., 1991). They appear most active, as demonstrated with IC_{50} values between 6 and 15 nM, when ribosomal RNA is used as a template (White et al., 1991).

Nevirapine is another member of potent and specific NNRT inhibitors extensively documented with IC_{50} values between 40-84 nM, as determined by in situ hybridization, inhibition of viral p24 antigen production, and lack of syncytia formation in cultured human T cell lines and freshly isolated human peripheral blood lymphocytes (Merluzzi et al., 1990; Koup et al., 1991). It inhibits mutant isolates of HIV-1 resistant to AZT and displays synergy with AZT in inhibiting AZT-sensitive virus (Richman et al., 1991a). Several studies revealed that nevirapine is noncompetitive with respect to template, primer, and nucleoside triphosphates, indicating that nevirapine does not act directly at catalytic site (Wu et al., 1990; Cohen et al., 1991). Several groups, employing either

photoaffinity labeling of HIV-1 RT or structural studies, investigated the binding site for this inhibitor (Wu et al., 1990; Cohen et al., 1991; Kohlstaedt et al., 1992). The non-substrate binding site for this inhibitor in HIV-1 RT has been identified as a pocket flanked by the tyrosine residues at positions 181 and 188 (Cohen et al., 1991; Kohlstaedt et al., 1992), suggesting HIV-1 RT amino acid residues Y 181 and Y 188 are critical for the activity of NNRT inhibitors.

It appears that this inhibitor binds at a site of the subdomains "palm" and "thumb" distal to the substrate-binding pocket on the enzyme and makes contact with the side chains of Y181 and Y188, which is close to the putative polymerase catalytic site of RT containing the highly conserved "YMDD" residues. Nevirapine only binds to p66 subunit of HIV-1 RT, but not to p51 subunit because the binding pocket does not exist in smaller p51 subunit, due to a confirmation different from that of p66 (Kohlstaedt et al., 1992). Thus, it is possible that nevirapine indirectly affects the confirmation of the critical residues, Y181 and Y188, on p66 subunit of HIV-1 RT and thereby inhibits HIV-1 RT activity.

5. HIV resistance to reverse transcriptase inhibitors

5.1. HIV clinical isolates resistant to nucleoside analogs

Antiviral therapy of HIV infection by nucleoside analogs seems to be compromised by the emergence of drug-resistant virus strains. Resistance of HIV to nucleoside analogs develops following prolonged therapy of HIV-infected individuals with these drugs, as first shown for AZT (Larder et al., 1989; Rooke et al., 1989), and later with ddI (St. Clair et al., 1991) and ddC (Richman, 1993). The initial descriptions of AZT resistance were based on analysis of patients with advanced disease, who received AZT therapy for more than three months (Larder et al., 1989; Rooke et al., 1989). Isolates from patients not treated with AZT displayed a narrow range of susceptibility to AZT, with 50% inhibitory concentration (IC_{50}) ranging from 0.01 to 0.05 μM, by using a syncytia focus reduction

assay in a CD4-expressing HeLa cell line. In contrast, HIV isolates from patients who had received AZT for 6 months or more possessed more than 100 fold increases in IC_{50}.

AZT-resistant isolates appeared to be cross-resistant to other nucleoside analogs that contain a 3'-azido group (e. g. AZdU) (Larder et al., 1989a; Larder et al.,1990). However, they remained sensitive to a wide range of other nucleoside analogs, including ddI and ddC, in addition to nonnucleoside analog inhibitors of HIV-1 (Larder et al., 1989). Subsequent studies, employing different procedures in which viruses from patients receiving prolonged AZT therapy were directly assessed for ability to replicate in cocultured PBMC or cord blood lymphocytes (CBL) in the presence of AZT, confirmed the emergence of AZT-resistant isolates with prolonged AZT therapy. Further studies of detection of HIV drug resistance indicated that at least 6 months of AZT therapy are generally necessary in order for AZT-resistant variants of HIV-1 to be detected in phenotype-based detection systems (Wainberg et al., 1993). Following initiation of therapy, patients with relatively advanced disease, e. g. AIDS-related complex (ARC) of AIDS, generally yield drug-resistant variants of HIV-1 more rapidly than asymptomatic patients (Richman et al., 1990). Several studies have reported that approximately 60-75% of patients with advanced disease, in comparison with 20% of asymptomatic individuals, are likely to possess drug-resistant variants after 1 year of therapy with AZT (Rooke et al., 1989; Richman et al., 1990; Richman, 1991b). Furthermore, the degree of AZT resistance increases over time. In is commonly seen that patients who have received AZT therapy for 6-8 months possess viruses that are about 10 fold resistance to AZT. This degree of resistance can frequently increase to greater than 100 fold in patients maintained on AZT monotherapy for longer than 18 months (Wainberg et al., 1993).

With the discovery of AZT resistance, the possibility that HIV-1 variants resistant to other nucleoside analogs would emerge following use of nucleoside analogs other than AZT during antiviral therapy was raised. In some individuals who have become intolerant to AZT or appear to be deteriorating clinically, therapy has been changed to ddI or ddC.

One group has examined the relative sensitivity of HIV isolates to AZT and ddI prior to and after individuals were switched to ddI (St. Clair et al., 1991). After 12 months of ddI treatment, HIV isolates possess 6-26 fold resistance to ddI in comparison with isolates obtained before therapy. Interestingly, HIV isolates from patients who had been treated with AZT and from whom AZT-resistant viruses could be isolated reversed susceptible to AZT when HIV isolates develop resistance to ddI (St. Clair et al., 1991). It is also found that in some individual, who were switched from AZT therapy to ddI therapy, HIV isolates possess resistance to ddI but not reversed sensitivity to AZT (Eron et al., 1993). In general, the degree of resistance to ddI ranges between 5-20 fold, and is not as high as seen with AZT (St. Clair et al., 1991; Eron et al., 1993). Moreover, one group reported that the percentage of patients who develop resistance to ddI is not likely to be as high as that documented for AZT (Wainberg et al., 1993). This study has been carried out on viruses isolated from each of 25 patients with CD4 cell counts <300/mm^3 who received ddI for at least 1 year after having previously been treated with AZT. After 12 months, resistance to ddI was seen in only 25% of subjects in comparison to 75-85 of similarly immunocompromised individuals who would be expected to have some degree of AZT resistance after therapy with that drug. Furthermore, the extent of resistance observed was generally far less than that seen with viruses isolated from patients receiving AZT.

5.2. Molecular basis of HIV resistance

That mutations within the viral pol gene, which encodes reverse transcriptase, is a likely basis for HIV resistance to RT inhibitors was first documented in a genetic study on the RT enzyme (Larder et al., 1989b). The function of RT HIV-1 was examined by using site-directed mutagenesis, followed by expression of active RT with altered amino acid residues in E. coli. Two resulting infectious viral mutants which expressed altered RT activity possessed significant resistance to Foscarnet, an RT inhibitor (Larder et al. 1989b). Thus, specific mutations in HIV RT can lead to decreased viral sensitivity to RT

inhibitors.

Subsequent studies, by analyzing nucleotide sequences of the reverse transcriptase coding region of paired sensitive and resistant isolates from AIDS patients (Larder and Kemp, 1989), revealed a number of specific common amino acid substitutions in the RT-coding region of resistant viruses. The most resistant isolates had either four mutations at codons 67 (Asp→Asn), 70 (Lys→Arg), 215 (Thr→Phe/Tyr) and 219 (Lys→Glu) or the first three of these. Introduction by site-directed mutagenesis of these mutations into the susceptible infectious molecular clone pHXB2 showed that these amino acid changes resulted in mutant viruses with decreased AZT sensitivity, indicating that these mutations are sufficient to make viruses resistant to AZT (Larder and Kemp, 1989). Further studies revealed that the sequential, cumulative acquisition of these mutations could be demonstrated in the isolates of patients undergoing prolonged AZT therapy. In addition, the presence of high-level resistance was often associated with the combination of at least three of these mutations in RT-coding region of HIV pol gene.

Following the initial report describing the four mutations responsible for AZT resistance, an additional mutation at codon 41 (Met→Leu) contributing to AZT resistance was also identified in the clinical isolates from patients treated with AZT (Kellam et al. 1992). A mutation at codon 41 alone only conferred a moderate increase in AZT resistance. When this mutation was introduced into cloned viral DNA with mutations at codons 67, 70, and 215, the resulting virus possessed higher resistance to AZT. Thus, a mutation at codon 41 (Met→Leu) in RT accounts for the additional resistance seen in the clinical isolates (Kellam et al., 1992). Studies of cloning and sequencing AZT-resistant variants selected in vitro also revealed that the same mutations at codons 67, 70, and 215 were associated with AZT resistant phenotype observed in vitro selection (Larder et al. 1991a; Gao et al. 1992).

It is noted that HIV-1 resistance to AZT would seem to develop in an orderly pattern (Boucher et al. 1990; Boucher et al., 1992) (Fig. 5). A mutation at codon 70

commonly occurs first during AZT treatment of HIV-1 positive asymptomatic individuals, but is then replaced by a more stable mutation at codon 215. Upon prolonged treatment mutations at codons 41, 67, and again 70 join in so that the virus acquires increased resistance to AZT. This seem to occur only after progression to disease, as no highly resistant virus could be isolated from asymptomatic individuals (Boucher et al. 1992).

A mutation at codon 74 (Leu→Val) was detected in ddI-resistant isolates from patients who had received prolonged AZT therapy before switching to ddI therapy (St. Clair et al., 1991). The biological significance of this mutation was confirmed by site-directed mutagenesis with use of the infectious molecular clone pHXB2. The resulting virus was demonstrated to possess considerable resistance to ddI over wild-type virus. Furthermore, a single mutation at codon 74 could also result in cross-resistance to ddC and partially reverse AZT resistance (St. Clair et al., 1991). One group, however, observed that AZT resistance coexisted with ddI resistance following acquisition of a mutation at codon 74 (Leu→Val) in clinical isolates, suggesting that the suppressive effect of mutation at codon 74 on the AZT resistance did not occur in all genetic contexts (Eron et al., 1993). Moreover, both ddI and ddC resistance caused by the mutation at codon 74 could be augmented by certain AZT resistance mutations (Eron et al., 1993). Studies on selection of drug-resistant variants of HIV-1 in cell culture revealed that a mutation at codon 184 (Met→Val/Ile) could also be detected in ddI-resistant variants as well as ddC-resistant or 3TC-resistant variants selected in cell culture (Gu et al., 1992; Gao et al., 1993; Boucher et al., 1993). Mutant virus, resulting from site-directed mutagenesis of this single mutation at codon 184 into the wild-type genetic background of HIV-1 pol gene, possessed apparent resistance to each of ddI, ddC, and 3TC (Gu et al., 1992; Gao et al., 1993; Boucher et al., 1993). However, the degree of resistance to these drugs, conferred by this mutation at codon 184, differed significantly. The virus containing this mutation had 5-10 fold resistance to ddC and ddI and more than 1000 fold

Figure 5. Model for the sequential development of HIV-1 resistance to AZT.

resistance to 3TC over wild-type viruses.

One group found that HIV clinical isolates from patients who have received prolonged ddC therapy contained a mutation at codon 69 (Thr→Asp) in RT (Fitzgibbon et al., 1992). Substitution of aspartic acid for the wild-type threonine at this codon was introduced into the RT of a molecular clone of HIV by site-directed mutagenesis. The resulting mutant virus showed a reduction of about 5 fold in susceptibility to ddC. Unlike the mutation at codon 74 (Leu→Val), mutant virus with the codon 69 mutation did not confer cross-resistance to ddI or AZT (Fitzgibbon et al., 1992). Recently, two groups have independently identified a mutation at codon 65 (Lys→Arg) in ddC-resistant variants selected in cell culture. HXB2-derived site-directed mutant viruses with this codon 65 mutation possessed 4-15 fold decrease in ddC susceptibility. Furthermore, mutant viruses showed a moderate cross-resistance to both ddI and 3TC, but remained sensitive to AZT (Gu et al., 1994; Zhang et al., 1994).

A summary of the RT mutations to date that have been identified to be responsible for HIV drug resistance is listed in Table 1.

5.3. Evaluation of mutant genotype of clinical isolates by polymerase chain reaction (PCR)

Following identification of resistance conferring mutations, several groups have used the specific primer pairs that can distinguish the wild-type residues from mutated residues in HIV-1 RT from patients to directly identify resistance conferring viral genotype by the polymerase chain reaction technique (Boucher et al., 1990; Gingeros et al., 1991; Richman et al., 1991). A number of studies have documented the 215 mutation directly in the PBMC of asymptomatic patients receiving chronic therapy with AZT (Boucher et al., 1992; Larder and Kemp, 1989; Gingeras et al., 1991). The gradual appearance of mutations during 2 years of therapy correlated with the resistance phenotype. By using PCR technique, one group detected the genotype of 304 independent

Table 1. Mutations in the HIV-1 RT gene conferring resistance to nucleoside analog

Codon	Mutation	Compound(s)	References
41	Met→Leu	AZT	Kellam et al., 1992
65	Lys→Arg	ddC, ddI, 3TC	Gu et al., 1994 Zhang et al., 1994
67	Asp→Asn	AZT	Larder etal., 1989
69	Thr→Asp	ddC	Fitzgibbon et al., 1992
70	Lys→Arg	AZT	Larder et al., 1989
74	Leu→Val	ddI, ddC	St. Clair et al., 199
184	Met→Val	ddI, ddC, 3TC	Gu et al., 1992 Gao et al., 1993
215	Thr→Phe/Tyr	AZT	Larder et al., 1989
219	Lys→Gln	AZT	Larder et al., 1989

isolates from 168 patients (Richman, 1991b). As expected, all of 67 isolates obtained before AZT therapy displayed the wild-type genotype. Sixty isolates from patients receiving AZT therapy were detected containing mutation which were previously identified conferring resistance to AZT. Most isolates had a mutant sequence at a single residue: 5 isolates at residue 67, 23 at residue 70, 31 at residue 215, and 1 at residue 219. Others, by using PCR technique, identified the presence of resistance-conferring mutation at time prior to the isolation of viruses possessing a drug-resistance phenotype from patients (Larder et al., 1991b). In some cases, however, the existence of multiple variants of HIV in patients complicates determination of the genetic composition at residues associated with resistance and clinical relevance of resistance.

The existence of mixed populations of virus with different AZT susceptibility has been documented by PCR analysis (Boucher et al., 1990). Patients who simultaneously harbored HIV variants with both wild-type and mutant sequences at residue 215 were detected (Boucher et al., 1990). The simultaneous presence in same patients of mixtures of mutation at residue 215 was also confirmed by cloning and sequencing the RT region of isolates (Lopez-Galindez et al., 1991; Mayers et al., 1992). Thus, mixtures of mutation in patients receiving AZT therapy may account for phenotypic mixtures in patients observed by in vitro drug susceptibility assay.

5.4. HIV resistance to nonnucleoside analogs

Shortly after the first HIV-1-specific NNRTs inhibitors were described, it became evident that these drugs can promptly lead to the emergence of drug-resistant variants upon passage of HIV-1 in the presence of drugs. Unlike the development of variants resistant to nucleoside analogs, variants of HIV-1 resistant to NNRT inhibitors could be rapidly selected in cell culture (Nunberg et al. 1991; Richman et al. 1991c; Mellors et al. 1992). As with 3TC resistant variants, the degree of resistance to NNRT inhibitor was seen as high as up to 1000 fold increase over parental wild-type HIV-1 (Nunberg et al.

1991). Furthermore, extensive cross-resistance was seen between the different class of NNRT inhibitor (Nunberg et al. 1991; Richman et al. 1991), suggesting that these RT inhibitors bind to the same site on the enzyme.

A cloning and sequencing analysis of RT-coding region of resistant variants selected with NNRT inhibitors showed a common mutation at codon 181 (Tyr→Cys) (Nunberg et al., 1991; Richman et al., 1991; Larder 1992; Mellors et al., 1992). One group has documented that HIV-1 mutant resistant to pyridinone containing RT inhibitors contained second mutation at codon 103 (Lys→Asn) in addition to mutation at codon 181 (Nunberg et al. 1991). Both mutations together rendered a virus 10 fold more pyridinone resistant than viruses with mutation at codon 181 (Tyr→Cys) alone (Nunberg et al., 1991). Subsequent studies revealed that HIV-1 mutants resistant to a TIBO derivative or derivatives of dipyridodiazepinones contained the mutation at codon 100 (Leu→Ile) for the TIBO compound and codon 106 (Val→Ala) for dipyridodiazepinones (Richman et al., 1993).

It was suggested that drug-resistant variants emerging upon passage of HIV-1 in the presence of NNRT inhibitors in cell culture may seem predictive of variants with resistance to the NNRT inhibitors that could arise in patients treated with these drugs. Indeed, clinical studies with NNRT inhibitors have documented that resistant variants of HIV can readily emerge, in some cases in a matter of weeks (Richman et al. 1993). The prediction of likely HIV-1 resistant mutants occurring during therapy was substantiated by HIV-1 mutant isolates that contained mutations identical to those previously identified in resistant mutant selected in cell culture. With the emergence of drug resistance phenotypes, however, acquisition of one or more mutations at codons 100 (Leu→Ile), 106 (Val→Ala), 108 (Val→Ile), 181 (Tyr→Cys) and 188 (Tyr→Cys) has also been found in clinical isolates of HIV-1 after therapy with BI-RG-587 or L-697661 (Richman, 1993).

Chapter 2

In Vitro Selection of Drug-Resistant Variants of the Human Immunodeficiency Virus Type 1

ABSTRACT

Drug-resistant variants of HIV-1 have been isolated by *in vitro* selection. MT-4 cells were infected with either a laboratory strain (HIV-III_B) or a clinical isolate (187) of HIV-1 and maintained in medium containing sub-effective concentrations of the drugs AZT or ddI. By gradually increasing the drug concentration in the culture medium during propagation of the virus on fresh MT-4 cells, we were able to isolate variants of HIV-III_B and clinical isolate 187, which showed up to 100-fold increases in resistance to the drugs. The drug-resistant phenotypes remained stable after propagation of the variants in the absence of drug pressure for over 2 months. However, variants resistant to one drug showed little or no cross-resistance to the other, suggesting that the genetic basis for resistance to each of the compounds differed in each case. Genotypic analysis of these nucleoside-resistant variants, using polymerase chain reaction (PCR) and primer pairs previously shown to correspond to mutations responsible for resistance to AZT, was also carried out. A heterogeneity of genotypes was observed to be present, with known mutations at *pol* codons 70 and 215 occurring in most of the AZT-resistant variants generated from either HIV-III_B or clinical strain 187. However, mutations in codons 67 and 219 were less frequently detected, and none of these changes were observed in each of four variants resistant to ddI. Cloning and sequencing studies of the reverse transcriptase coding region of two of the isolates were also performed, and confirmed the PCR data that had been obtained. In addition to previously described mutation sites responsible for resistance to AZT, a HIV-III_B-resistant variant was shown to be mutated at positions 108 (val _ ala) and 135 (ile _ thr) while a resistant variant of strain 187 was mutated at positions 50 (ile _ arg) and 135 (ile _ val).

INTRODUCTION

A variety of antiviral agents are able to interfere *in vitro* in the life cycle of the human immunodeficiency virus (HIV-1), the etiological agent of acquired immunodeficiency syndrome (AIDS) (5,21,22).

Considerable emphasis has been placed on nucleoside analogues which function as chain terminators of viral reverse transcriptase activity. Certain analogues, such as 3'-azido-3'-deoxythymidine (AZT), have been used clinically, and, despite side effects (8,9), have been found to improve both the quality and the length of life of patients infected with HIV-1 (8,9,27,40,41).

The reverse transcriptase of HIV-1 (39) exhibits a considerable degree of infidelity during the replication of viral RNA (25,28,36). Therefore, genetic heterogeneity is characteristic of HIV-1 (6) and viral mutations with reduced sensitivity might be expected to be selected under drug pressure. Indeed, several groups have reported the isolation and partial characterization of AZT-resistant HIV-1 from patients on prolonged drug therapy (12,14,29). Specific mutations have been described in the *pol* sequences of certain of these AZT-resistant HIV-1 variants, isolated from patients (12,16).

The molecular characterization of resistance to anti-viral nucleosides would be enhanced if strains of HIV-1 with reduced drug sensitivity could be generated *in vitro*, and if such procedures could be utilized to give rise to HIV-1 variants resistant to compounds other than AZT. We now report the generation and characterization of several such variants, resistant to either AZT or 2',3'-dideoxyinosine (ddI). These variants were generated through *in vitro* selection procedures using both the III_B laboratory strain of HIV-1 as well as a clinical isolate.

MATERIALS AND METHODS

Cells and viruses

MT-4 cells were provided by Dr. N. Yamamoto, Yamaguchi University, School of Medicine, Ube, Japan (23). Cells were mycoplasma-negative, as assessed by fluorescence microscopy using the dye 4',6-diamine-2-phenylindole (DAPI). Cells were maintained in suspension culture (3-5 x 10^5 cells/ml) in RPMI 1640 medium (Gibco Laboratories, Mississauga, Ontario) supplemented with 10% fetal bovine serum (Flow Laboratories, Toronto, Ontario), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The HIV-III_B laboratory strain was a gift of Dr. R.C. Gallo, NIH, Bethesda, MD. Viral strain 187 was a clinical isolate obtained by coculture of cord blood lymphocytes with peripheral blood lymphocytes from an infected patient who had not been treated with antiviral agents. Both strains were propagated on MT-4 cells. Stock virus was prepared from clarified culture media by ultracentrifugation and stored at -70°C. In addition, peripheral blood mononuclear cells (PBMC) were obtained from healthy donors and infected with HIV-1 as previously described (30). A CD4(+) line of CEM cells was also employed in some studies, and was replicated and infected by HIV-1 as described (38).

Drugs: 3'-azido-3'-deoxythymidine (AZT) and 2'-3'-dideoxyinosine (ddI) were gifts of Burroughs-Wellcome Inc. (Research Triangle Park, N.C.) and Bristol-Myers-Squibb Inc. (Wallingford, CT), respectively. Stock solutions of these drugs were prepared in medium RPMI 1640 and stored at -20°C until use.

Selection process

MT-4 cells (3 x 10^5 cells/ml) were preincubated for 30 min with the appropriate concentration of drug, then infected with HIV-III_B or clinical isolate 187 at a multiplicity of infection of 0.01 plaque-forming units per cell. Virus had first been titrated by plaque assay on MT-4 cells as described (10). After a 3 h incubation at 37°C, the cells were washed twice to remove excess virus, then maintained in medium containing drug concentrations equivalent to those used in the preincubation. The initial concentrations of

AZT or ddI in the cell culture medium were slightly below the minimum effective concentrations able to inhibit viral replication in the cells. Culture fluids were replaced twice weekly with fresh medium containing an appropriate drug concentration, which was gradually increased during propagation of the virus on fresh MT-4 cells in the following manner. During each cycle of infection, undiluted clarified culture supernatants (0.5 ml) obtained from HIV-infected MT-4 cells, cultured in the presence of drug, were used to infect fresh MT-4 cells (6×10^5 cells). Half of these newly-infected cells were cultured in medium containing drug concentrations identical to those of the previous cycle of infection. The remaining cells were cultured in medium containing a higher drug concentration. Clarified culture fluids from infected cells grown in the presence of the higher drug concentrations were used as the source of virus for each subsequent cycle of infection.

Selection of drug-resistant variants of HIV-1

Preliminary studies to isolate drug-resistant variants using HIV-1-infected MT-4 cells cultured in the presence of high concentrations of drug were unsuccessful. We therefore determined the minimum effective concentration of each of the drugs that was able to prevent the appearance of infectious virus over 14 days in culture supernatants of MT-4 cells infected with either $HIV\text{-}III_B$ or our clinical isolate 187. We then chose concentrations of drug slightly below these minimum effective concentrations for use in the initial selection process. These concentrations were AZT, 0.015 μM and ddI, 17 μM. Infectious supernatants were obtained from HIV-1 infected MT-4 cells at 4 days post-infection during the initial selection at these low drug concentrations. As drug concentrations were subsequently increased during the selection process, the appearance of virus was delayed to 6 days post-infection, as assessed by both indirect immunofluorescence for p24 Ag and reverse transcriptase activity. Details of the *in vitro* selection process are summarized in Table 1.

Immunoblot analysis of viral proteins

Virus in clarified culture supernatants from infected MT-4 cells was concentrated by ultracentrifugation (100,000 x g, 1 h), resuspended in 400 µl TNE buffer (10 mM Tris, pH 7.4/100 mM NaCl/1 mM EDTA), further purified by sucrose density gradient centrifugation, recentrifuged and resuspended in viral lysis buffer (TNE buffer containing 0.5% NP-40, 0.5% Triton X-100, aprotinin (2 µg/ml), leupeptin (2 µg/ml), pepstatin (1 µg/ml) and phenyl methyl sulfonyl fluoride (20 µg/ml), as previously described (2,30). Viral proteins were subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted with a 1:100 dilution of serum obtained from an HIV-1 positive patient (2,37). The blots were washed, incubated at room temperature for 1 h with [^{125}I]-labelled Protein A (Amersham), re-washed, dried and exposed to Kodak X-OMAT film for 16 h at -70°C using an intensifying screen.

Assays of viral replication

Viral reverse transcriptase assays and indirect immunofluorescence assays (IFA) for detection of p24 Ag were performed as previously described (2,30). Antigen capture tests for determination of p24 levels in culture fluids were carried out using kits purchased for this purpose from Abbott Laboratories (North Chicago, IL).

Polymerase chain reaction (PCR) detection of relevant DNA sequences

For this purpose, DNA was extracted according to published procedures (2,32) from either 10^6 uninfected MT-4 cells or from MT-4 cells that had been infected with either parental-type HIV-1 (clinical isolate 187 or HIV-III$_B$) or AZT-resistant variants that had been selected and amplified using the procedures described above. Using a double amplification procedure, 1 µg of extracted DNA was initially used per PCR sample (100 µl), containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 2.5 U Taq polymerase, 0.2 mM each of dATP, dGTP, dCTP and dTTP, and 1 µM of each of two previously described oligonucleotide primers, A and NEI, chosen to selectively amplify the region of the *pol* gene in which relevant mutations would be most likely to occur (16,18). Samples were overlaid with 100 µl of light mineral

oil and heated to 94°C for 5 min, prior to being subjected to 30 thermal cycles of 1 min at 94°C for denaturation, 22 sec at 45°C for annealing, and 1.5 min at 72°C for extension in a Perkin-Elmer Cetus Thermal Cycler. DNA thus generated (5 µl) was then subjected to further specific amplification, using primers which recognize previously described mutations which account for AZT resistance in the viral *pol* gene, i.e. primer A paired with 2 W or 2 M or primer B paired with 3 W or 3 M (1 min at 94°C, 22 sec at 45°C, 30 sec at 72°C for 30 cycles), to detect either wild-type (W) or mutated (M) sequences at codons 70 and 215, respectively (16). Similar procedures were employed in the detection of mutated sequences corresponding to codons 67 and 219 (16). DNA was extracted with chloriform, precipitated with ethanol, electrophoresed on agarose gels, and visualized by ethidium bromide staining.

Cloning and sequencing

Cellular DNA was extracted as described above from MT-4 cells infected with AZT-resistant variants of either HIV-III_B or clinical isolate 187. A 1742 base segment containing the complete reverse transcriptase (RT) coding region, 34 bases of the 3' end of the integrase coding sequence and 28 bases of the 5' end of the proteinase coding sequence was amplified by PCR as described above, using the primers 5 ' G T A G A A T T C T G T T G A C T C A G A T T G G 3 ' and 5'GATAAGCTTGGGCCTTATCTATTCCAT3' for the 5' and 3' ends, respectively. The PCR-amplified segments were purified from agarose gels, digested with *Hind*III and *Eco*RI (Pharmacia Fine Chemicals, Montreal, Canada), and ligated with digested M13mp19 (32). TG1 cells were transfected with the recombinants (32) and screened by digesting double-stranded DNA with restriction endonucleases. Single-stranded DNA was prepared from recombinant M13 clones, and nucleotide sequences were determined using a TaqTrack sequencing kit (Promega Inc., Madison, WI).

RESULTS

Variants of both HIV-III_B and clinical isolate 187 with significant drug resistance were noted approximately 8 weeks after initiation of the selection process. These variants showed up to 100-fold resistance to the drugs as compared with the parental drug-sensitive strains (Table 2).

Stability of the drug-resistant phenotype

The drug-resistant variants described in the first portion of Table 2 were, in some cases, further propagated on MT-4 cells in the absence of drug for 2 months. The subsequent replication of these viruses in the presence of AZT or ddI showed that these variants retained their resistance phenotype and exhibited a level of drug-susceptibility virtually identical to that of virus which had been propagated in the continuous presence of drug (Table 2). No evidence of decreased sensitivity to AZT on the part of ddI-resistant isolates was detected in these studies (Figure 1).

Infectiousness of drug-resistant variants of HIV-1

As illustrated in Figures 2 and 3, the kinetics of infection of MT-4 cells in the absence of drug, using a moi of 0.01, by the *in vitro* selected drug-resistant variants were virtually identical to those of the parental drug-sensitive strains. A peak of virus production, as measured by RT activity in culture fluids, was observed at 4 days post-infection. In the presence of high drug concentrations (up to 100-fold greater than normally required to inhibit viral replication), maximal virus production was delayed to about 7 days post-infection. Significant virus-induced cytopathology was seen in each case after three days; this was delayed to five days in the presence of high drug concentrations (not shown).

We further investigated the ability of our drug-resistant variants to infect each of CEM cells, MT-4 cells, and PBMC, as monitored by IFA, in the

Table 1. Details of procedure for in vitro selection of drug-resistant variants of HIV-1

Virus	Cycle of infection	Duration of infection cycle (days) in presence of:		Concn (μM) of drug employed in infection cycle	
		AZT	ddI	AZT	ddI
HIV-III_B	1	4	4	0.015	17
	2	5	5	0.0225	34
	3	5	6	0.0375	85
	4	6	7	0.375	169.5
	5	7	7	0.75	254
	6	7	7	1.5	339
	7	7	7	1.9	424
Clinical isolate 187	1	4	4	0.015	17
	2	5	5	0.0225	34
	3	7	7	0.0375	85
	4	7	7	0.375	169.5
	5	7	7	0.75	212
	6	7	7	1.5	254
	7	7	7	1.9	339
	8		7		424

Table 2. Sensitivity of drug-resistant variants of HIV-1 to antiviral agents[a]

Variants	ID_{50} (μM) after:			
	Initial selection[b]		Additional propagation in absence of drug[c]	
	AZT	ddI	AZT	ddI
$HIV\text{-}III_B$	0.009 ±0.0006	10.6 ± 0.85	ND	ND
AZT-resistant HIV-III	0.9 ± 0.1	10.6 ± 0.85	0.76 ± 0.075	ND
ddI-resistant HIV-III	0.009 ± 0.0007	230 ± 9	ND	220 ± 10
ddI-resistant HIV-III	0.007 ± 0.0004	140 ± 16	0.0008 ± 0.0004	ND
Clinical isolate 187	0.01 ± 0.0006	10.6 ± 0.2	ND	ND
AZT-resistant 187	0.86 ± 0.075	11. ± 0.2	0.86 ± 0.075	ND
ddI-resistant 187	0.012 ± 0.0004	220 ± 12	ND	210 ± 11
ddI-resistant 187	0.012 ± 0.0001	170 ± 18	0.0092 ± 0.0006	ND

[a] Virus production was assessed by measurement of RT activity in clarified culture supernatants of infected MT-4 cells and by indirect immunofluorescence analysis for HIV-1 p24; the two methods gave identical results. Results were obtained 7 days postinfection. ID_{50} were determined by curve-fitting analysis and are reported as means ± standard deviations of three independent determinations.
[b] Data are from variants obtained after 8 weeks of our selection protocol, as described in Materials and Methods and in Table 1.
[c] Data are from the same resistant variants which had been further propagated on MT-4 cells for 2 months in the absence of drug following completion of the selection protocol. ND, not determined.

Figure 1. Sensitivity of drug-sensitive and drug-resistant variants of HIV-III_B (A) and clinical isolate 187 (B) to AZT. MT-4 cells were infected with HIV and cultured in the absence or the presence of the indicated concentrations of AZT for 7 days. Virus production was then assessed by measurement of reverse transcriptase activity in aliquots of clarified culture supernatants. (O——O), drug-sensitive parental virus; (O——O), AZT-resistant virus; (Δ----Δ), ddI-resistant virus; (Δ----Δ), second isolate of ddI-resistant virus.

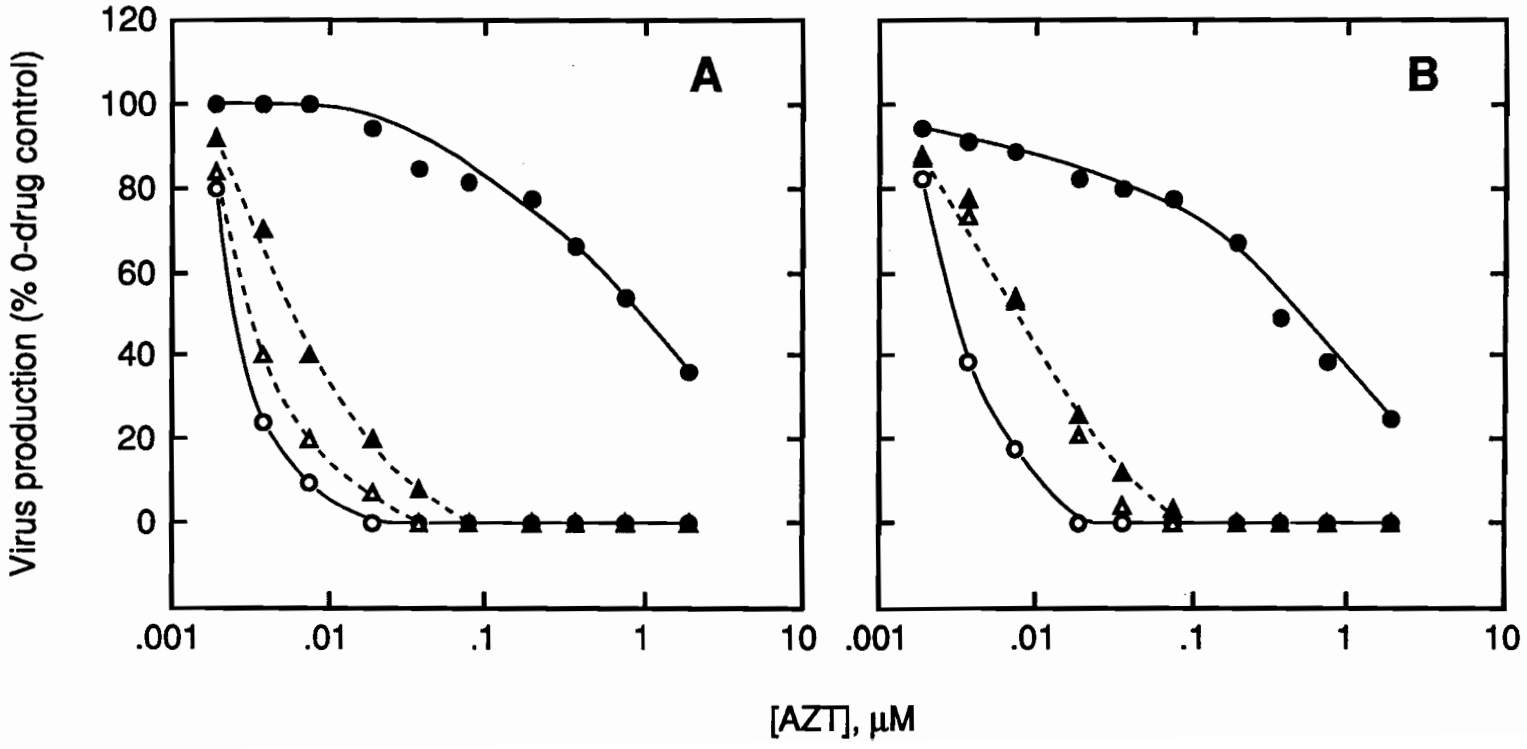

Virus production (% 0-drug control)
120
100
80
60
40
20
0
A
B
.001
.01
.1
1
10
[AZT], μM

Figure 2. Time course of virus production in MT-4 cells infected with variants of HIV-III_B and cultured in the absence (——) or the presence (---) of 1.9 μM AZT (A), 0.4 mM ddI (B), or 0.8 mM ddI (C). Virus production was assessed by measurement of reverse transcriptase activity in aliquots of clarified culture supernatants, (O), drug-sensitive parental HIV-III_B; (O), AZT-resistant HIV-III_B; (Δ), ddI-resistant HIV-III_B; (Δ), second isolate of ddI-resistant HIV-III_B.

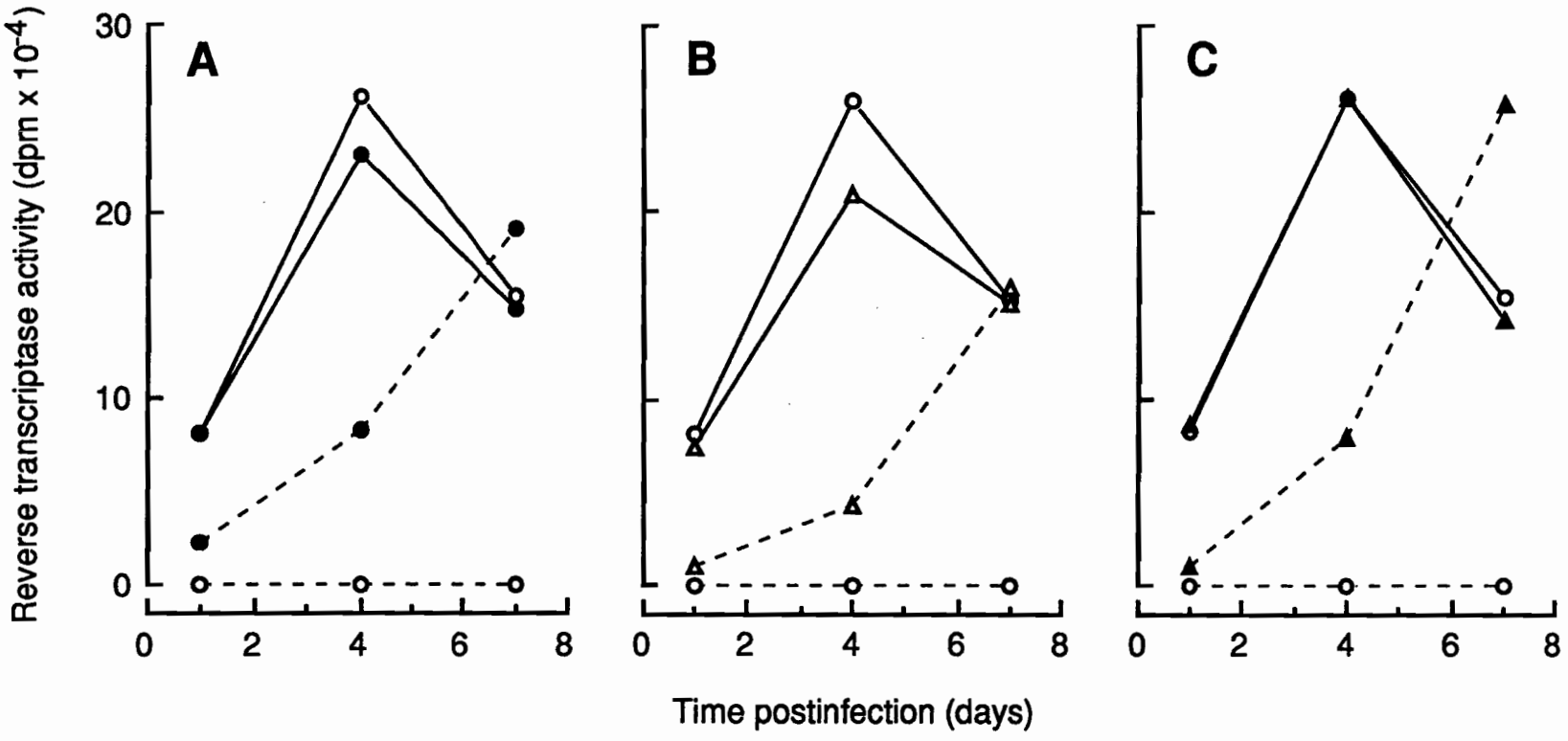

A
B
C
30
20
10
0
0
2
4
6
8
Reverse transcriptase activity (dpm x 10⁻⁴)
Time postinfection (days)

Figure 3. Time course of virus production in MT-4 cells infected with variants of chemical isolate 187 and cultured in the absence (——) or the presence (---) of 1.9 μM AZT (A), 0.4 mM ddI (B), or 0.8 mM ddI (C). Virus production was assessed by measurement of reverse transcriptase activity in aliquots of clarified culture supernatants, (O), drug-sensitive parental chemical isolate 187; (O), AZT-resistant chemical isolate 187; (Δ), ddI-resistant chemical isolate 187; (Δ), second isolate of ddI-resistant strain 187.

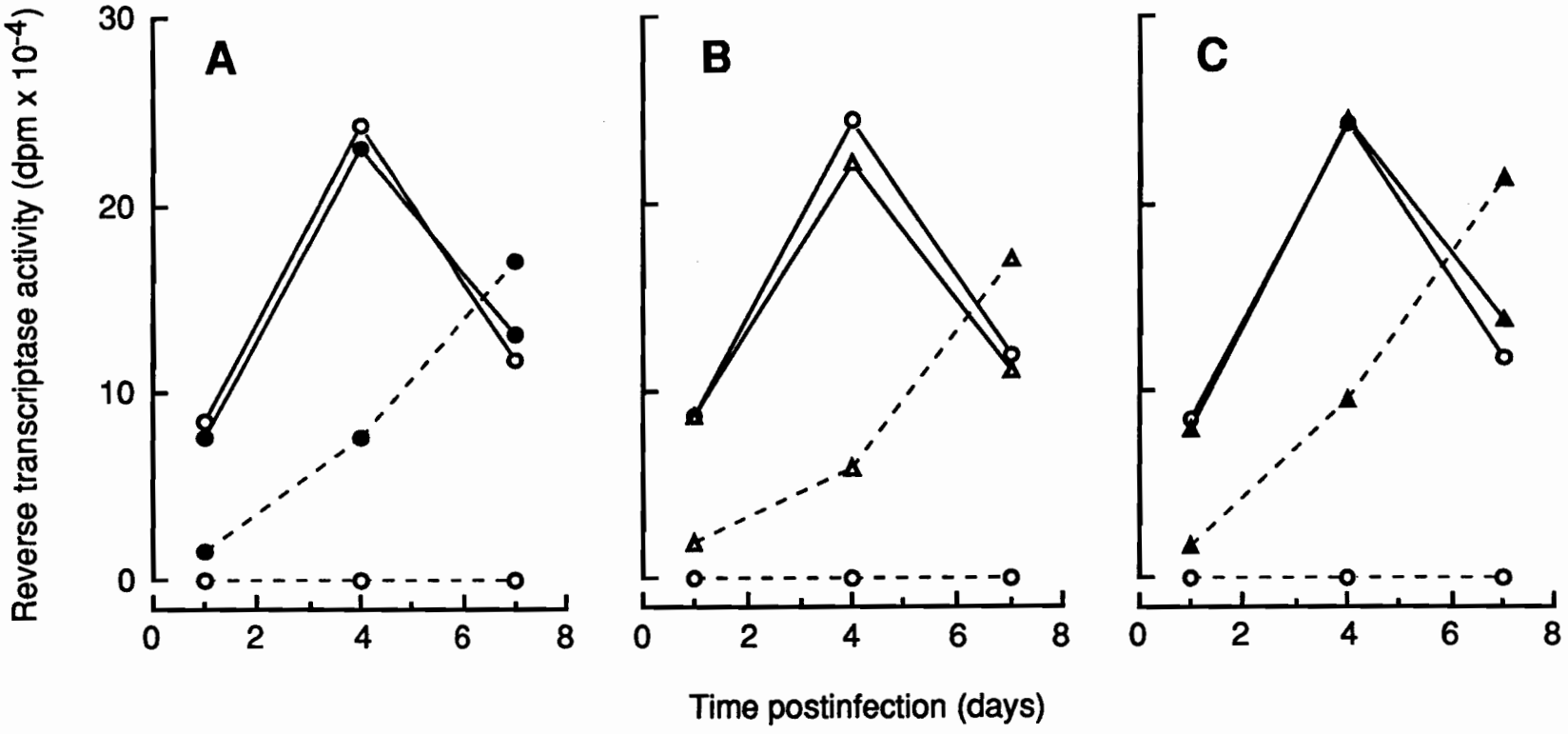

A
B
C
30
20
10
0
0
2
4
6
8
Reverse transcriptase activity (dpm x 10^{-4})
Time postinfection (days)

Table 3. Infectiousness of drug-resistant HIV-1 on different cell types in the presence of drug

Cell type and HIV-1	%p24(+) cells[a] with			
	AZT		ddI	
	0.037 μM	0.19 μM	21.2 μM	106 μM
MT-4				
AZT-resistant HIV-IIIB	100	100	5	0
AZT-resistant 187	100	100	0	0
ddI-resistant HIV-IIIB	0	0	100	100
ddI-resistant 187	0	0	100	100
CEM				
AZT-resistant HIV-IIIB	17	0	0	0
AZT-resistant 187	10	0	0	0
ddI-resistant HIV-IIIB	0	0	16	0
ddI-resistant 187	0	0	14	0
PBMC				
AZT-resistant HIV-IIIB	17	0	0	0
AZT-ressistant 187	12	0	0	0
ddI-resistant HIV-IIIB	0	0	43	0
ddI-resistant 187	0	0	40	0

[a] Virus production was assessed 7 days postinfection by indirect immunofluorescence assay for HIV-1 p24. Values are reported as percentagfe of that noted with the same drug-resistant isolate propagated in the absence of drug, which was generally close to 100% of actual cell numbers present for MT-4 cells, 75% for CEM cells, and 10% for PBMC. No virus production was noted in any of the cell types in the presence of indicated concentrations of drug when parental drug-sensitive HIV-IIIB or clinical isolate 187 was used.

Table 4. Detection of specific mutated sequences in individual nucleoside-resistant variants of HIV-1[a]

HIV-1 variant	ID_{50} (μM) of :			Mutation at codon			
	AZT	ddI	ddC	60	70	215	219
$HIV\text{-}III_B$	0.05	5.52	0.35	—	—	—	—
$HIV\text{-}III_B$-AZT-1	1.4	1.65	0.55	—	±	±	—
$HIV\text{-}III_B$-AZT-2	2.7	1.45	0.6	—	±	—	—
$HIV\text{-}III_B$-ddI-1	0.06	45.7	0.75	—	—	—	—
$HIV\text{-}III_B$-ddI-2	0.04	53.8	0.66	—	—	—	—
187	0.06	2.75	0.4	—	—	—	—
187-AZT-1	5.2	1.65	0.65	±	±	±	—
187-AZT-2	4.8	3.75	0.75	—	±	±	—
187-ddI-1	0.05	26.3	0.45	—	—	—	—
187-ddI-2	0.06	38.2	0.65	—	—	—	—

[a] ddC, dideoxycytidine.

presence of drug, using a moi of 0.1. Each of these viruses was able to cause infection in the presence of the same drug that had been employed in its selection (Table 3). Although these variants showed increased sensitivity to the drugs in PBMC and CEM cells, as compared with MT-4 cells, viral replication nonetheless occurred in the presence of concentrations significantly higher than those which abolished replication of the parental, drug-sensitive strains.

HIV-1 viral proteins

To rule out that phenotypic mixing between HTLV-I, known to be present in MT-4 cells, and HIV-1 might have contributed to the observed drug resistance, drug-sensitive and -resistant viruses were concentrated from the culture fluids of drug-treated, infected MT-4 cells and analyzed by immunoblot, using serum from a HIV-1 seropositive individual. No differences were observed between the patterns of viral proteins detectable in the different viral strains and the only proteins consistently detected were those of HIV-1 (Figure 4). Nor were differences observed in the patterns of viral proteins, after these strains had been propagated in the absence of drug in MT-4 cells (data not shown).

PCR detection of relevant DNA sequences

Figure 5 illustrates a typical experiment on the detection of previously described mutations in HIV-1 variants selected *in vitro* for resistance to AZT. Results obtained with *in vitro*-generated AZT-resistant variants, derived from each of clinical strain 187 and the III_B laboratory strain of HIV-1 are shown. We found that mutated codon 70, associated with AZT drug resistance (R), was present in proviral DNA obtained from MT-4 cells that had been infected with either of these viral variants. As expected, the non-mutated codon 70, associated with sensitivity (S) to AZT, was not detected in either of these cases. In contrast, studies on codon 215 revealed that the mutated form, previously shown to be associated with AZT resistance (R), was present in the proviral DNA of amplified, resistant strain 187 but not of AZT-resistant HIV-III_B. Proviral DNA from cells infected

Figure 4. Immunoblots of viral preparations harvested from uninfected or infected MT-4 cells. Lane designations are: A, uninfected cells; B, cells infected by HIV-III_B; C, clinical isolate 187; D, AZT-resistant HIV-III_B; E, AZT-resistant strain 187; F, ddI-resistant HIV-III_B; G, ddI-resistant strain 187; H, second strain of ddI-resistant HIV-III_B; I, second strain of ddI-resistant strain 187.

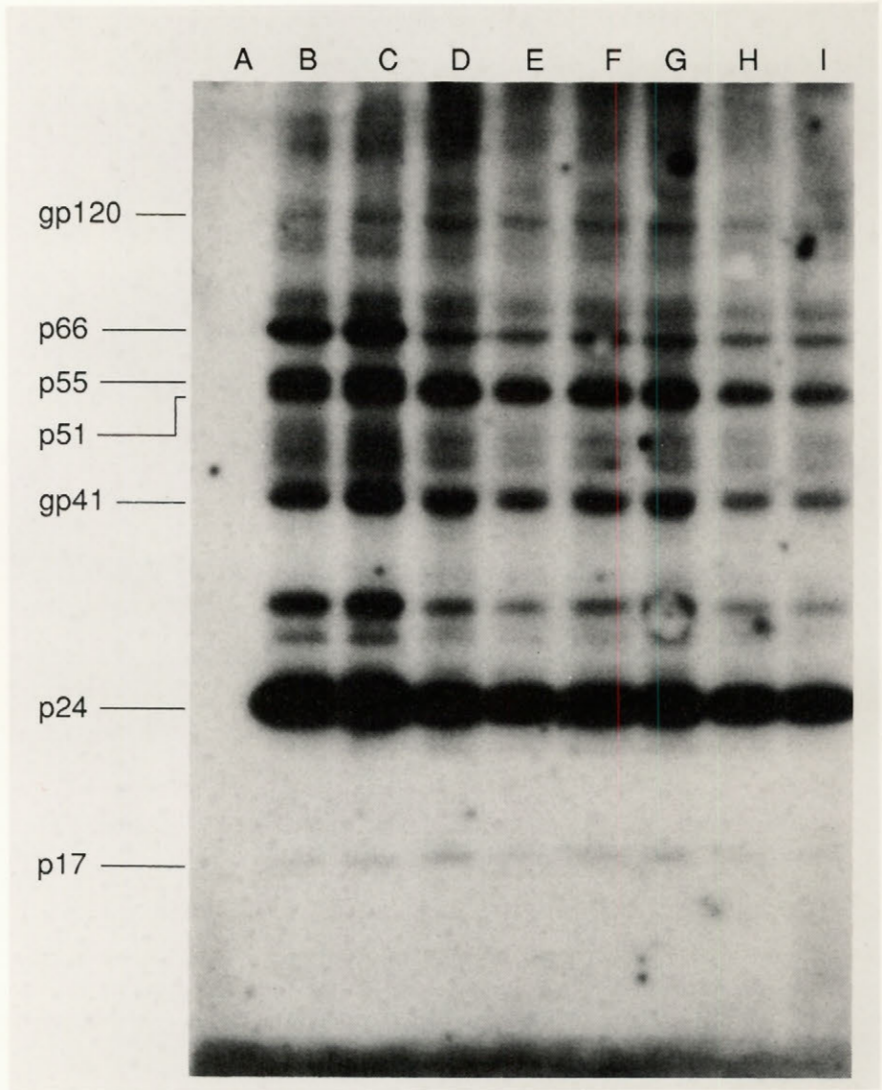
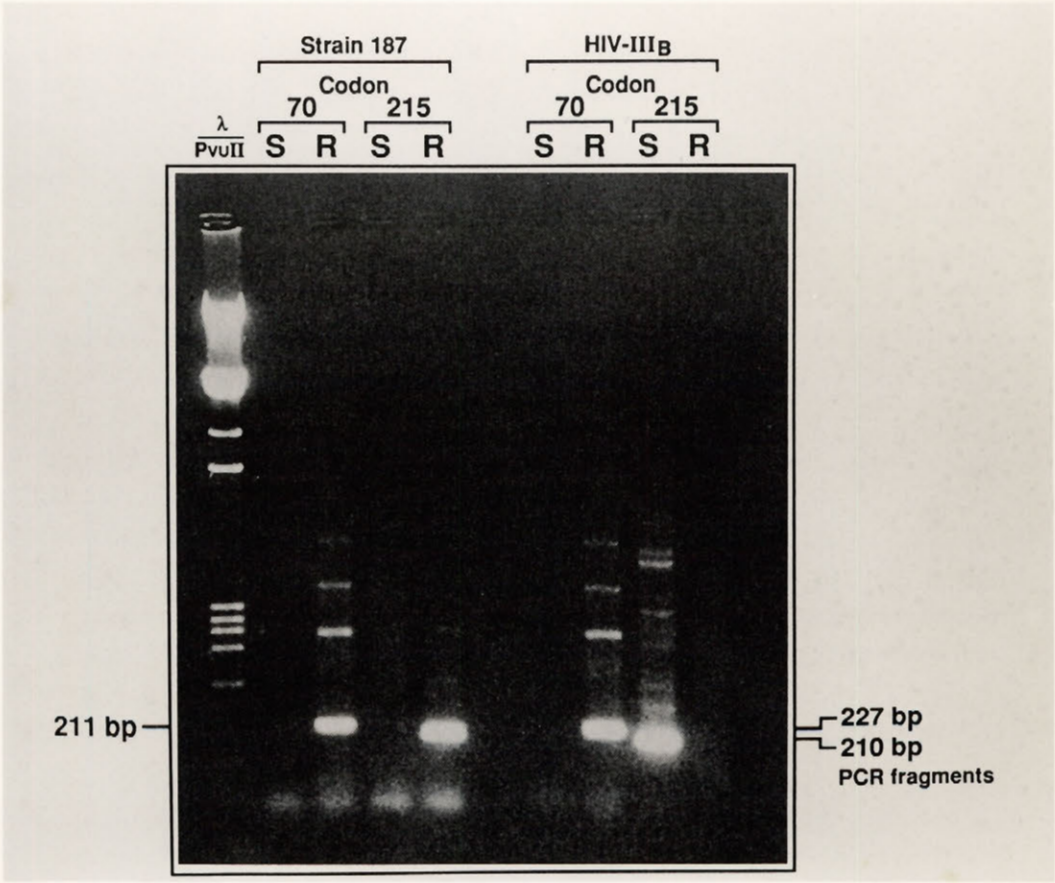

A
B
C
D
E
F
G
H
I
gp120
p66
p55
p51
gp41
p24
p17

Figure 5. Detection of sensitive, wild-type (S) or resistant, mutated (R) sequences corresponding to codons 70 and 215 of the HIV-1 *pol* gene by PCR in AZT-resistant variants of each of clinical strain 187 and HIV-III_B, generated by *in vitro* selection. These are the same AZT-resistant viruses described in Table 2.


Strain 187
HIV-III$_B$
Codon
70
215
λ
PvuII
S R S R
S R S R
211 bp
227 bp
210 bp
PCR fragments

with the latter variant continued to express wild-type or sensitive type codon 215.

The data of Table 4 provide further details with regard to a variety of nucleoside-resistant variants of HIV-1 that have been generated *in vitro*. Although a mutation at position 70 was detected in each case of AZT resistance studied, changes at codons 67, 215 and 219 were found in only some of the AZT-resistant isolates tested, and never in the case of viruses resistant to ddI. No correlation was apparent between degree of resistance to AZT (as measured by ID_{50}) and the presence or absence of mutated codons 67, 70, 215 and 219. These results indicate the heterogeneity of genotypes, which may account for resistance to AZT, and make clear that not all AZT-resistant strains of HIV-1 can be expected to have all or most of the previously described mutations. These PCR analyses are not quantitative, and thus may not adequately reflect the broad range of quasispecies that may be present in each of the isolates chosen for examination.

Cloning and sequencing

To confirm and extend the data obtained by PCR analysis, using primer pairs previously shown to correspond to mutation sites responsible for resistance to AZT, we amplified a 1742 base segment of the HIV-1 genome, containing the entire RT coding region, from the same AZT-resistant variants of each of the HIV-III_B and 187 strains of HIV-1 described in Figure 4. Cloning into TG1 cells was performed as described above. Sequencing results revealed that the HIV-III_B variant was mutated at each of positions 67 (asp _ his), 70 (lys _ arg), 108 (val _ ala) and 135 (ile _ thr). Strain 187 was found to be mutated at each of positions 50 (ile _ arg), 70 (lys _ arg), 135 (ile _ val) and 215 (thr _ tyr). Parental drug-sensitive strains of HIV-III_B and clinical isolate 187 were not mutated at any of these positions, when compared with previously published sequences of the RT coding region (15,16,18).

patients on prolonged AZT therapy was carried out directly on MT-2 cells, a procedure successful in the isolation of HIV-1 only about 30% of the time (15,16). This process may have contributed to selection of a relatively small sub-population of AZT-resistant variants, possessing relevant mutations only at positions 67, 70, 215 and 219. Of these latter, mutations at codons 70 and 215 are presumably of greatest importance, since when inserted singly into drug-sensitive strains of HIV-1 they can induce AZT resistance to develop (15). In contrast, insertion of mutated codons 67 and 219 did not yield similar results. This is the reason that the presence of previously described mutations at codons 70 and 215 were analyzed by PCR in the present study.

It had been hoped that PCR amplification of known mutated sequences, responsible for resistance to AZT or other drugs, might be performed on blood samples from patients on prolonged anti-HIV therapy. The detection of such sequences might be possible long before the isolation of viruses possessing a resistance phenotype from these same individuals. Thus, recommendations for alternative therapy could be considered at times prior to the emergence of clinically significant burdens of resistant viruses. The fact that the number of mutation sites, associated with AZT resistance, is larger than first believed may complicate attempts to establish the molecular diagnosis of HIV drug resistance by PCR and similar techniques. Of course, the new mutation sites described here may not occur under clinical conditions. Efforts to validate the existence of these mutated codons in clinical material is now being conducted by PCR, using newly generated primer pairs.

It is important that the PCR analyses shown here not be over-interpreted. For one thing, they are not quantitative and may not reflect the broad range of quasispecies potentially present in the samples which were amplified. The failure to find a given change could be due to the presence of nonmutated viral subpopulations which were amplified, while the presence of a given change may not have biological significance, if it was due to the presence of a subpopulation that did not contribute to resistance.

An important characteristic of HIV-1 is its genomic diversity, due in part to the infidelity of viral RT in transcription of viral nucleic acid (25,28,36). We are uncertain whether the drug-resistant viral strains identified in this study pre-existed among the heterogeneity of viral quasi-species, prior to *in vitro* selection, or whether relevant mutations occurred during replication under drug pressure. In view of the shortness of the period (i.e. <8 weeks) required for drug-resistant variants to appear in our study, it is not unlikely that these variants, in fact, pre-existed, and that the gradient of drug pressure employed resulted in the elimination of drug-sensitive strains. However, in previous studies, we failed to isolate drug-resistant forms of either HIV-III_B or clinical isolate 187 *in vitro*, when we began with moderate to high initial concentrations of AZT (30). In the current study, the resistant variants generated possessed up to 100-fold increased resistance to this compound.

The current studies were performed with MT-4 cells, due to the ability of HIV-1 to rapidly propagate in this cell line. However, the MT-4 line is also infected with HTLV-I (10,23). The possibility that phenotypic mixing between HTLV-I and HIV-1 might have contributed to the observed drug resistance in this study was ruled out by immunoblot analysis showing no differences between drug-sensitive and -resistant viral isolates. This does not rule out that interactions between HTLV-I and HIV-1 might have contributed to enhanced HIV-1 replication and *pol* gene mutations in this study, e.g. through the effects of the HTLV-I encoded transactivator protein (tat-1) on the HIV-1 long terminal repeat (LTR) sequence (33). Nor can a role for other co-infecting viruses be excluded in the development of clinical resistance to AZT on the part of HIV-1 (20,34). In this context it is important to note that the drug-resistant variants of HIV-1 described in this study were able to replicate in the presence of high drug concentrations in cells other than MT-4. Of course, it would be useful to know whether drug-resistant strains of HIV-1 could be selected in PBMC or other types of primary cell culture.

Viral drug resistance has, of course, been described in a variety of other systems,

including herpes viruses and acyclovir (3,4,13,17), cytomegalovirus and ganciclovir (1,7), influenza virus and amantadine (11,19). In each case, it has been possible to both isolate viruses which possess a resistance phenotype from subjects receiving prolonged anti-viral therapy as well as to generate such viruses through the types of *in vitro* selection procedures utilized in our study. AZT and similar drugs have also been used to antagonize the replication of murine (31) and feline immunodeficiency-inducing viruses (24) in both tissue culture and animals; in some cases it has been possible to demonstrate drug resistance in virus-infected animals that have been treated with anti-viral drugs (26).

It is therefore not surprising that *in vitro* selection of nucleoside-resistant variants of HIV-1 should now have been demonstrated. However, it is noteworthy that a number of groups, including our own, have previously attempted to demonstrate the *in vitro* selection of such variants without success (14,18,30,35). The use in our study of a cell line that replicates HIV-1 more efficiently than those used previously (30), coupled with the tedious schedule of increasing drug concentrations described in Table 1, probably both played important roles in our success.

The current studies indicate that no single drug may be sufficient to attenuate viral replication in infected cells over protracted periods. The technique of *in vitro* selection of nucleoside-resistant variants of HIV-1, described here, may be useful in predicting the likelihood of emergence of HIV-1 drug resistance in clinical trials. Current efforts are aimed at determining whether combinations of different anti-viral drugs, used either simultaneously or sequentially, will also yield drug-resistant variants of HIV-1 under tissue culture conditions.

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Chapter 3

In the preceding chapter we have described a system to select for HIV resistance in cell culture. Using such a selection system , variants of both the HIV-III_B strain and wild-type clinical strain of HIV-1 which showed resistance to either AZT or ddI were selected in vitro. These variants behaved similarly to wild-type viruses with regard to replication rate. The drug resistance phenotype remained stable after propagation of these variants in the absence of drug pressure. Since other members of this nucleoside family may be used in the treatment of HIV-infected individuals, it is likely that HIV-1 variants resistant to such compounds may emerge as AZT-resistant variants do during therapy. To explore this possibility, we further investigated whether HIV-1 variants that display resistance to other compounds can be also selected using our selection system. The work presented in the next chapter describes selection for HIV resistance with several other compounds, including dimers in which two compounds are linked through phosphodiester linkages.

Generation of Drug-Resistant Variants of the Human Immunodeficiency Virus Type 1 by In Vitro Passage in Increasing Concentrations of 2',3'-Dideoxycytidine and 2',3'-Dideoxy-3'-Thiacytidine (BCH-189)

ABSTRACT

We have selected HIV-1 variants *in vitro* that are resistant to each of 2',3'-dideoxycytidine (ddC) and the racemic mixture of 2'3'-dideoxy-3'-thiacytidine (BCH-189). The EC_{50} values obtained for the resistant viruses ranged between 10-50 times above those of parental wild-type strains for both ddC and BCH-189, and extensive cross-resistance was observed against 2',3'-dideoxyinosine (ddI) but not 3'-azido-3'-deoxythymidine (AZT). Two dimer compounds, in which either AZT and ddI or AZT and BCH-189 were linked through phosphodiester linkages, did not permit the emergence of variants resistant to either BCH-189, ddI, or AZT but were ineffective at inhibiting the replication of AZT-resistant viruses.

INTRODUCTION

The clinical relevance of HIV resistance to 3'-azido-3'-deoxythymidine (AZT) and other antiviral agents is an area of active study (1,8,11). We and others have demonstrated the emergence, under cell culture conditions, of HIV-1 variants that display resistance to each of AZT and 2',3'-dideoxyinosine (ddI) (5,7). It has been reported as well that HIV-1 resistance against non-nucleoside inhibitors of reverse transcriptase (RT) can be selected for under conditions of *in vitro* viral replication (10).

Because it is apparent that other members of the nucleoside family may be used in the therapy of HIV-associated disease, it is important to determine whether variants of HIV-1, that show diminished resistance to other compounds such as 2',3'-dideoxycytidine (ddC) and the (-)enantiomer of 2',3'-dideoxy-3'-thiacytidine (3TC) (3,4,14) will be likely to emerge as well. The racemic mixture, from which the latter is derived, known as BCH-189, is a cytosine analogue that possesses anti-HIV activity in both (+) and (-) enantiomeric forms (3,16). We now report that HIV-1 drug resistance against each of ddC and BCH-189 may be selected for in cell culture as efficiently as previously shown for AZT and ddI (5). However, the use of dimer compounds in which either AZT and ddI (3'-azido-3'-deoxythymidilyl-(5',5')-2',3'-dideoxy-5'-inosine; AZT-P-ddI) or AZT and BCH-189 (3'-azido-3'-deoxythymidilyl-(5',5')-2',3'-dideoxy-3'-thiacytidine; AZT-P-BCH-189) were linked through phosphodiester linkages (15) did not permit the development of drug resistance in our *in vitro* assay, in spite of the fact that these drugs could not efficiently block the replication of AZT-resistant variants of HIV-1.

MATERIALS AND METHODS

Cells and viruses

The MT-4 line of CD4+ lymphocytes was utilized in these experiments. These cells were maintained as suspension cultures (3-5 x 10^5 cells/ml) in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine and antibiotics as described (5). Dr. Robert Gallo, NIH, Bethesda, MD, kindly provided the HIV-III_B laboratory strain of HIV-1. In addition, we worked with a number of clinical isolates which had been obtained by co-culture of peripheral blood lymphocytes of infected individuals with cord blood lymphocytes provided by our Hospital's Department of Obstetrics, as described (11). In particular, we extensively studied HIV-1 isolates from patients 174 and 278; these viruses were obtained at times both prior to and twelve months following initiation of AZT therapy (500 mg/day). Viruses which were recovered were propagated on MT-4 cells as described (5) and stored at -70°C.

Drugs

AZT, ddI, and ddC were obtained from Wellcome Inc. (Research Triangle Park, N.C), Bristol-Myers Squibb (Wallingford, CT) and Sigma Chemicals Corp (St. Louis, MO), respectively. BCH-189 was a gift of BioChem Pharma Inc., Montreal, Canada. AZT-P-ddI and AZT-P-BCH-189 dimer compounds were gifts of IVAX Corp., Miami, FL.

Selection of drug-resistant variants of HIV-1

MT-4 cells were pre-incubated for 30 min with sub-effective concentrations of drug and subsequently infected with either clinical isolates or the HIV-III_B laboratory strain at a multiplicity of infection of 0.01. Virus had first been titrated by plaque assay on MT-4 cells, as described elsewhere (5). After 3 hr, the cells were washed and maintained in culture medium containing the same sub-effective concentration of drug used during pre-incubation. The medium was changed twice weekly and at each replacement contained a gradually increasing drug concentration, according to the schedule described

in Table 1. Culture fluids (0.5 ml) from each round of HIV infection were employed to infect fresh MT-4 cells each time, as described (5). Cells were monitored for the presence of viral p24 antigen and reverse transcriptase activity by indirect immunofluorescence assay and enzyme assay, respectively, as described (2,12). In addition, viral p24 antigen was monitored by antigen capture assay, using commercially available kits purchased from Abbott Laboratories (North Chicago, Il).

Drug susceptibility assays

MT-4 cells were pretreated with test drugs and subsequently incubated with viruses at a multiplicity of infection (MOI) of 0.001 $TCID_{50}$/cell. After 2 hours at 37^0C. the culture was washed and resuspended. The cell suspensions were plated into 24-well plates containing serial dilutions of test drugs in a volume of 1.5 ml ($3x10^5$/cell). Virus production was assessed 7 days postinfection by measurement of RT activity in clarified culture supernatants of infected MT-4 cells. EC_{50} values were determined by curve-fitting analysis.

RESULTS

Generation of drug-resistant variants of HIV-1

The results of Table 2 show that significant levels of viral RT activity and p24 antigen could be generated in cell culture as soon as two weeks after initial selection for resistance in the presence of AZT. Similar results were obtained with each of ddI, ddC and BCH-189. After four weeks of augmentation of drug concentration, we were often able to grow drug-resistant variants of each of two clinical isolates (174 and 278) and of the HIV-III_B laboratory strain in concentrations of AZT as high as 2.5 μM, concentrations of ddI as high as 655 μM, and in 25 μM of each of ddC and BCH-189. Table 3 contains summary data on the ability of each of AZT, ddI, ddC and BCH-189 to inhibit the replication of both wild-type HIV-1 as well as variants that were selected to display resistance against each of these compounds. Median effective concentrations (EC_{50}) for the various drugs studied were calculated by curve fitting analysis on the basis of three independent determinations of RT values in culture fluids, as previously described (6), using viruses that had been passaged in MT-4 cells about eight times.These values are in excess of 50-fold usual inhibitory concentrations of AZT and 10-30 fold those for ddI, ddC and BCH-189.

Cross-resistance of drug-resistant variants selected in in vitro

Viruses that were resistant to AZT were sensitive to each of ddI, ddC and BCH-189, when studied in MT-4 cells. Similarly, viruses selected for resistance against any of ddI, ddC and BCH-189 all retained sensitivity to AZT. In contrast, however, extensive cross-resistance was documented among each of ddI, ddC and BCH-189. Interestingly, viruses selected in the presence of increasing concentrations of ddC displayed much higher levels of resistance against BCH-189 than against ddC itself.

Use of AZT dimer compounds

Dimer compounds, in which AZT and other nucleosides have been linked through phosphodiester linkages have been reported to inhibit replication of HIV-1 (15). The

results of Table 2 show that viruses resistant to either AZT or ddI could not be detected after 8 weeks in culture in the presence of either AZT-P-ddI or AZT-P-BCH-189, using procedures identical to those followed above for selection of drug resistance to either of the parental nucleosides. The results of Table 3 also show that AZT-resistant variants of HIV-1 were able to replicate efficiently in the presence of normally inhibitory concentrations of either of the two dimers, AZT-P-ddI and AZT-P-BCH-189. In contrast, variants of HIV-1 that were resistant to ddI were not able to replicate in the presence of either dimer.

TABLE 1. Procedure used to select drug-resistant variants of HIV-1

Cycle of infection	Duration of infection cycle (days)	Drug concn (μM)			
		AZT	ddI	ddC	BCH-189
1	6	0.0018	19	0.75	0.75
2	6	0.0225	34	1.1	1.1
3	6	0.0325	85	5.2	5.2
4	6	0.0327	169.5	11.5	11.5
5	7	0.75	254	16.4	16.4
6	7	1.5	339	25	25
7	7	1.9	424		
8	7	2.5	655		

TABLE 2. Generation of drug-resistant variants of HIV-1 by passage in MT-4 cells in the presence of nucleoside analogs

Virus	Drug (initial concn [μM])[a]	RT activity (cpm/ml [10^4]) at the following time after infection[b]:			% Fluorescent cells at the following time after infection[c]:		
		2 wk	4 wk	6 wk	2 wk	4 wk	6 wk
Clinical isolate from patient 174	None	23.5	26.0	29.2	88	82	85
	AZT (0.018)	4.2	17.5	25.7	28	55	79
	ddI (19)	9.3	21.6	27.8	30	46	58
	ddC (0.75)	8.2	20.3	29.5	28	53	76
	BCH-189 (0.75)	7.8	23.5	27.2	32	48	79
Clinical isolate from patient 278	None	19.4	29.2	26.8	91	84	86
	AZT (0.018)	3.8	19.7	27.5	19	63	70
	ddI (19)	10.8	18.4	29.3	28	52	75
	ddC (0.75)	6.3	14.2	24.0	18	51	69
	BCH-189 (0.75)	9.0	15.6	27.4	24	59	62
$HIV\text{-}III_B$	None	17.3	29.8	27.1	79	94	88
	AZT (0.018)	3.9	16.2	29.3	35	62	84
	ddI (19)	8.1	23.5	33.1	28	49	91
	ddC (0.75)	5.6	19.4	27.6	19	61	80
	BCH-189 (0.75)	6.3	22.5	31.7	23	58	89
Clinical isolate from patient 174	None	27.6	19.8	30.8	71	85	82
	AZT (0.018)	5.1	20.3	26.4	19	61	75
	ddI (19)	3.4	16.2	28.0	20	49	83
	AZT-P-ddI (0.018)	1.6	1.4	1.9	0	0	0
	AZT-P-BCH-189 (0.018)	3.5	1.7	2.5	0	0	0
$HIV\text{-}III_B$	None	19.7	18.5	23.8	85	92	88
	AZT (0.018)	2.3	16.7	27.2	22	52	73
	ddI (19)	1.7	12.8	25.6	18	38	67
	AZT-P-ddI (0.018)	2.5	3.4	2.1	0	0	0
	AZT-P-BCH-189 (0.018)	2.8	1.7	3.0	0	0	0

[a] All cultures were maintained at the original concentration of drug for at least 6 days. In cases in which cultures became positive, higher drug concentrations were used, following the schedule described in Table 1.

[b] Data are means of three replicate samples.

[c] Data are means of three replicate samples.

TABLE 3. Effect of nucleoside analogs and dimer compounds on replication of drug-resistant variants of HIV-1

Selection pressure	Origin of virus used	EC_{50} (μM)[a]					
		AZT	ddI	ddC	BCH-189	AZT-P-ddI	AZT-P-BCH-189
None	$HIV\text{-}III_B$	0.03 ± 0.002	4.2 ± 0.5	0.45 ± 0.08	0.55 ± 0.7	ND[b]	ND
None	Patient 263	0.02 ± 0.001	3.6 ± 0.4	0.35 ± 0.04	0.65 ± 0.3	ND	ND
None	$HIV\text{-}III_B$	0.04 ± 0.002	10.6 ± 0.9	0.65 ± 0.08	ND	0.08 ± 0.009	0.05 ± 0.007
None	$HIV\text{-}III_B$	0.06 ± 0.008	10.4 ± 1.2	0.48 ± 0.02	ND	0.02 ± 0.001	0.10 ± 0.008
AZT	$HIV\text{-}III_B$	1.6 ± 0.008	3.9 ± 0.5	0.40 ± 0.06	0.50 ± 0.05	ND	ND
AZT	Patient 263	2.5 ± 0.02	5.2 ± 0.3	0.45 ± 0.05	0.65 ± 0.04	ND	ND
AZT	$HIV\text{-}III_B$	2.2 ± 0.03	10.6 ± 1.4	0.35 ± 0.02	ND	0.95 ± 0.06	0.65 ± 0.05
AZT	$HIV\text{-}III_B$	1.3 ± 0.01	10.5 ± 1.8	0.73 ± 0.11	ND	0.85 ± 0.03	0.31 ± 0.04
ddI	$HIV\text{-}III_B$	0.02 ± 0.003	54.5 ± 3.2	5.2 ± 0.05	6.1 ± 0.4	ND	ND
ddI	Patient 263	0.05 ± 0.002	47.8 ± 6.7	4.3 ± 0.6	4.9 ± 0.5	ND	ND
ddI	$HIV\text{-}III_B$	0.08 ± 0.003	236.4 ± 19.0	0.78 ± 0.05	ND	0.08 ± 0.002	0.05 ± 0.004
ddI	$HIV\text{-}III_B$	0.06 ± 0.004	134.9 ± 12.8	0.55 ± 0.03	ND	0.06	0.03 ± 0.005
ddC	$HIV\text{-}III_B$	0.04 ± 0.001	25.3 ± 3.2	3.2 ± 0.4	6.8 ± 0.4	ND	ND
ddC	Patient 263	0.07 ± 0.004	15.8 ± 2.1	4.8 ± 0.6	10.4 ± 1.3	ND	ND
ddC	$HIV\text{-}III_B$	0.08 ± 0.010	10.76 ± 1.6	8.2 ± 0.6	ND	0.07 ± 0.006	0.06 ± 0.009
BCH-189	$HIV\text{-}III_B$	0.05 ± 0.002	3.7 ± 0.7	5.9 ± 0.3	7.2 ± 0.7	ND	ND
BCH-189	Patient 263	0.02 ± 0.003	16.0 ± 1.1	7.5 ± 0.5	9.0 ± 0.5	ND	ND

[a] Data are means ± standard deviations for three replicate samples.
[b] ND, not done.

DISCUSSION

The major findings of this paper relate to our ability to have generated strains of HIV-1 resistant to each of ddC and BCH-189 through *in vitro* culture and drug pressure procedures. Such resistance against individual nucleosides can develop in culture over periods ranging between 2 to 8 weeks. In our studies, the AZT-resistant variants of HIV-1 that have been selected for in culture possess similar genotypes to those that have been isolated from patients under prolonged therapy with this drug (5). However, this does not necessarily prove that the mechanisms responsible are the same, although other workers have noted that the same order of *pol* gene mutations is likely to occur both *in vivo* and *in vitro* (9).

What is the basis for the differential results obtained when the dimer compounds were used to inhibit replication of drug-resistant variants of HIV-1? Each component of the dimers was present at equimolar concentrations. The dimers are probably only active following intra-cellular hydrolysis to individual nucleosides, which are then phosphorylated through the usual pathways to yield products with direct antiviral activity. The AZT representation in the dimers is sufficient to impede replication of wild-type HIV isolates, when these compounds are present at concentrations of at least 0.1 μM, since the usual EC_{50} of AZT for HIV in MT-4 cells ranges between 0.01 and 0.1 μM. Accordingly, it is not surprising that the dimers were able to impede the replication of ddI-resistant viruses, even when used at concentrations as low as 0.05 μM, since a sufficient level of AZT was present to affect those variants which retained a AZT-sensitive phenotype.

In contrast, the levels of ddI and BCH-189 necessary to impede HIV replication are much higher, i.e. 2-5 μM. Thus, when used at low concentrations, i.e. 0.1 μM, all of the antiviral activity of these dimers is attributable to the AZT component rather than to ddI or BCH-189. In the case of variants resistant to AZT, there is insufficient ddI or BCH-189 in the dimers, when used at 0.1-1 μM to impact on viral replication.

Understandably, these compounds were able to impede replication of AZT-resistant variants when employed at concentrations within the usual IC_{50} inhibitory range, i.e. 2-5 μM for ddI and BCH-189. Although the relatively low concentrations of ddI and BCH-189, present in the AZT dimers, was presumably not able to antagonize replication of AZT-resistant variants of HIV-1, they might nonetheless have acted synergistically with AZT to prevent emergence of resistance against either AZT or ddI (Table 2).

Cross-resistance among ddI and ddC has previously been described (6,13). It is therefore not surprising that this should extend as well to BCH-189, which is structurally similar to ddC. It will be of interest to determine whether resistance against 3TC will develop as rapidly in MT-4 culture as that seen for BCH-189.

Finally, we succeeded in selecting for HIV drug resistance by *in vitro* passage only when we used the MT-4 cell line. Similar attempts which employed either human cord blood lymphocytes or human peripheral blood mononuclear cells ended in failure. Clinical trials with the drugs described here will be necessary to determine whether drug resistance will also emerge under conditions of prolonged anti-viral chemotherapy.

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Chapter 4

Studies presented in chapter 2 and 3 showed that HIV-1 variants resistant to either AZT, ddI, ddC, or BCH-189 can be generated in vitro in the presence of increasing concentration of drugs. Viruses selected for resistance to BCH-189 showed cross resistance against ddC. In the case of ddC-resistant variants cross resistance to BCH-189 was observed. Viruses selected for resistance against AZT remained sensitive to each of ddI, ddC, and BCH-189. To investigate whether multiply drug-resistant variants resistant to each of AZT, ddI, and ddC could be generated in cell culture, we carried out a study for the selection for multiple drug resistance in vitro. This study will be described in the following chapter.

Generation of Multiple Drug resistance by Sequential in Vitro Passage of the Human Immunodeficiency Virus Type 1

ABSTRACT

We have sequentially passaged both laboratory and clinical isolates of the human immunodeficiency virus type 1 (HIV-1) in MT-4 cells in the presence of increasing concentrations of different drugs to derive viral variants that are multiply resistant to each of ddC, ddI, d4T and AZT. The EC_{50} values obtained for the viruses thus generated varied between 50-100 times above those of parental wild-type strains in the case of AZT, 20-30 times for d4T, but only 10-15 times for ddI and ddC. Cultivation of AZT-resistant viruses in the presence of increasing concentrations of ddI yielded viruses that were resistant to the latter compound, with no apparent decrease in susceptibility to AZT. Sometimes, viruses selected for resistance against ddI were cross-resistant as well against ddC, although most viruses selected for resistance to ddC were not cross-resistant to ddI. Combinations of two or three of these compounds inhibited replication of HIV variants that displayed resistance to the same drugs when tested individually. No emergence of drug resistance was demonstrable when combinations of drugs were employed simultaneously in these selection protocols or when single drugs were used in concert with interferon-2α or high dilutions of virus-neutralizing antisera. Cloning and sequencing of some viruses resistant to each of AZT, ddI, and ddC revealed the simultaneous presence of mutations at sites 41, 74, 184 and 215 within the HIV pol gene open reading frame.

INTRODUCTION

The use of 3'-azido-3'-deoxythymidine to treat HIV-1 infected individuals has resulted in both improved survival and quality of life [4,24,25]. The use of anti-HIV nucleosides has furthermore been shown to both stimulate numbers of CD4 positive lymphocytes and to cause diminutions in levels of circulating viral p24 antigen [9,25]. Nonetheless, prolonged therapy with AZT and other drugs has commonly resulted in ultimate treatment failure. Reasons for progression of HIV-associated disease in the face of antiviral therapy include the fact that nucleoside compounds act by antagonizing the HIV reverse transcriptase and not by affecting postintegrational events in the viral life cycle. Thus, continued viral production by cells that already harbour integrated proviral DNA in their nuclei can occur, resulting in each of direct virus-induced cytopathicity, production of potentially immunosuppressive viral proteins, involvement of viral proteins in antibody-dependent cellular cytoxicity reactions, and induction of cytokines that may, under some circumstances, cause further immunosuppression through direct or indirect mechanisms.

In addition, a decline in specific immune responsiveness against HIV-associated antigens could give rise to higher levels of overall viral replication. The correspondingly higher number of reverse transcription events, now involved in infection of new cells, could give rise to mutations in the viral polymerase, with the potential to encode HIV drug resistance. Thus, it is not surprising that important correlations between diminutions in CD4 counts and development of viral drug resistance have been reported by several groups [1,16,26].

An alternative explanation, however, is that HIV drug resistance may develop under conditions of selective pressure exerted by the very drugs used in antiviral chemotherapy. This possibility must be considered seriously, in view of the error-prone nature of the viral RT and the likelihood that mutations with the potential to encode viral drug resistance constantly occur. Previous research has shown that viruses which possess

MATERIALS AND METHODS

Cells and Viruses

We grew the MT4 line of CD4 positive lymphocytes in suspension culture (3-5 x 10^6 cells per ml) in RPMI-1640 medium supplemented with 10% fetal calf serum, 2mM glutamine and antibiotics as described) [5]. We worked with a number of clinical isolates, obtained from patients prior to antiviral therapy by co-culture of patient peripheral blood lymphocytes with cord blood lymphocytes [2] and with the HIV-III_B laboratory strain of HIV-1, kindly provided by Dr. Robert C. Gallo, National Institutes of Health, Bethesda, MD. Viruses were propagated on MT4 cells and stored at -70° C [5]. Serum samples were obtained from HIV-infected asymptomatic donors and uninfected healthy controls.

Drugs

2'-3'-dideoxyinosine (ddI) and 2',3'-dideoxy-3'-didehydrothymidine (d4T) were obtained from Bristol-Myers-Squibb (Wallingford, CT). 3'-azido-3'-deoxythimidine (AZT; zidovudine) was obtained from Burroughs-Wellcome Inc., Research Triangle Park, NC. 2',3'-dideoxicytidine (ddC) was purchased from Sigma Chemicals Inc., St. Louis, MO. The racemic mixture of the (-) and (+) enantiomers of 2'deoxy-3'thiacytidine, known as BCH-189 [22] was provided by BioChem Pharma Inc., Montreal, Canada, which also provided the (-) enantiomer, known as 3TC [20]. Interferon-2a (IFN-2a) was a gift of Schering Inc., Pointe-Claire, Que, Canada.

Selection of Drug-Resistant Variants of HIV-1 by Culture Passage

MT-4 cells were pre-incubated for 30 minutes with sub-effective doses of compound (at or below the usual EC_{50} in each case) and were subsequently infected with HIV-1, using a multiplicity of infection of 0.01 tissue culture infectious doses-50% ($TCID_{50}$) per cell. Following a three hour absorbtion, the cells were washed and maintained in tissue culture medium at the same concentration of drug as used during both pre-incubation and infection. Medium changes were carried out twice weekly; each medium replacement contained gradually increasing drug concentrations as described [5].

Culture fluids (0.5ml) from each round of viral replication were used to infect fresh MT4 cells [5]. Cultures were monitored on a regular basis for production of both viral reverse transcriptase activity and viral p24 antigen as described [2]. Polymerase chain reaction (PCR) methodology was used to detect previously described RT mutations that account for HIV drug resistance as previously described [7,8,12,14,20,23].

RESULTS

In vitro selection of drug-resistant HIV variants

MT-4 cells infected with either HIV-III_B or clinical isolate 187 were cultured in the presence of increasing concentrations of each of AZT, d4T, ddI, and ddC as previously described [5]. Initial drug concentrations were 0.018 μM for AZT, 0.025 μM for d4T, 19 μM for ddI and 0.75 μM for ddC. HIV variants present after 8 passages (4 weeks) demonstrated nearly 100-fold resistance to AZT, 30-fold resistance to d4T, and 10-20 fold resistance for each of ddI and ddC, in comparison to parental strains (Table 1). Step-wise increases in levels of drug resistance are shown for each of passage levels 4, 6 and 8, at which times such assessments were carried out.

The results of Table 2 present data on nucleoside susceptibilities of viruses selected for resistance in AZT, ddI, or ddC, and include replication studies performed with each of the above compounds as well as d4T and 3TC. The viruses that were resistant to ddI were resistant as well to ddC, although the reverse was not true. AZT-resistant viruses remained fully susceptible to each of ddI and ddC, although some cross-resistance against d4T was demonstrated, confirming previous findings [19]. Variants selected for resistance to either ddI or ddC remained susceptible to AZT, but were cross-resistant to 3TC.

In vitro selection of multiple HIV drug resistance

We next investigated whether multiple drug resistance could also be generated by in vitro selection. We began with AZT resistant viruses, which were propagated on MT-4 cells in the presence of increasing concentrations of ddI or ddC, respectively, as described above and in Table 1. Fresh MT-4 cells were included at each passage, as in the case of selection for resistance against a single drug, and viruses were assessed for susceptibility to each of AZT, ddI, and ddC. Through 10 such passages, we were able to generate variants that retained 90-fold resistance to AZT and also possessed 10-15 fold resistance to either ddI or ddC (Table 3). It is interesting that no diminution in levels of resistance

Table 1. Susceptibility of HIV variants at different passage levels to anti-viral nucleosides in single drug selection protocols [a]

Passage No.	Drug used in selection (μM)				EC_{50} (μM)[b]							
					$HIV\text{-}III_B$				Clinical isolate187			
	AZT	d4T	ddI	ddC	AZT	d4T	ddI	ddC	AZT	d4T	ddI	ddC
1	0.018	0.25	19	0.75	0.01	0.45	10.2	0.41	0.015	0.41	10.5	0.45
2	0.025	0.5	34	1.1								
3	0.0325	1.8	85	2.5								
4	0.0375	3.7	169.5	5.2	0.25	3.5	96.5	2.3	0.32	4.0	104.2	2.8
5	0.75	7.5	254	11.5								
6	1.5	15	339	11.5	0.71	11	170.4	4.8	0.85	14	188.2	5.8
7	1.9	19	424	25								
8	1.9	19	424	25	0.92	15	215.4	5.8	1.2	17	227.2	7.2

a. HIV variants were generated in vitro selection. using $HIV\text{-}III_B$ laboratory strain and clinical isolate 187 were obtained from a patient prior to nucleosides therapy.
b. EC_{50} values were determined by measurement of p24 Ag levels in culture fluids.

against AZT was detected during this selection procedure. Furthermore, we observed that no further resistance against ddI could be generated after 8 weeks of selection, while resistance against ddC increased marginally between 8 and 10 weeks.

To investigate whether triple drug resistance could be independently generated, we began with AZT-resistant viruses, which were propagated on MT-4 cells in the presence of increasing concentrations of ddI or BCH-189, respectively. This was followed by a subsequent selection in the presence of d4T. The data of Table 4 indicate that multiple drug resistance could indeed be demonstrated using this approach.

We also examined these multiply resistant HIV variants by polymerase chain reaction (PCR) to determine whether they contained mutation sites previously identified as responsible for diminished sensitivity to AZT [12,14], ddI [23], and BCH-189 [7,20]. The results of Table 4 show that viruses resistant to AZT contained codon alterations at sites 41 and 215; ddI-resistant viruses possessed mutation 74 and BCH-189 resistant viruses contained mutation 184. Additional passage in nucleoside analogs could be shown to result in acquisition of new mutations in the case of both ddI and BCH-189.

Stability of the multiple drug resistance phenotype

Multiply resistant HIV variants that had been generated in tissue culture were further propagated on MT-4 cells in the absence of drug for 2 months. After this time, viruses were assessed for ability to replicate in the presence of anti-viral nucleosides, as assessed by p24 Ag levels in culture fluids. No significant loss of drug resistance was detected (Table 5).

Use of combinations of nucleosides and neutralizing antisera or interferon-2α

We further determined whether drug resistance would emerge when a single compound was used in our selection protocol together with high dilutions of virus-neutralizing antisera. As controls, we employed combinations of various nucleosides simultaneously or combinations of single nucleosides with interferon-2a. The results of Figure 1 demonstrate that the addition of sub-titer concentrations of virus-neutralizing

Table 2. Patterns of drug resistance using HIV variants selected for resistance to AZT, ddI or ddC

Variant	EC_{50} (μM)				
	AZT	ddI	ddC	d4T	3TC
HIV-III_B	0.01	10.2	0.41	0.45	0.47
AZT-resistant HIV-III_B	0.9	10.4	0.43	2.1	0.61
ddI-resistant HIV-III_B	0.018	215.2	0.51	0.39	2.1
ddc-resistant HIV-III_B	0.015	18.4	5.8	0.47	4.9
Clinical isolate 187	0.013	10.5	0.45	0.41	5.4
AZT-resistant 187	1.4	10.2	0.45	3.5	0.49
ddI-resistant 187	0.02	237.2	1.3	0.43	2.9
ddc-resistant 187	0.018	15.2	8.2	0.41	5.7

EC_{50} values were calculated on the basis of viral reverse transcriptase (RT) activity in culture fluids. Care was taken to ensure that peak levels of RT activity were being monitored in each case

Table 3. Susceptibility of AZT-resistant variants at different passage levels in the presence of ddI and ddC [a]

Variant	Drug pressure during passage	EC_{50} (μM) at passage														
		0			4			6			8			10		
		AZT	ddI	ddC	AZT	ddI	ddC	AZT	ddI	ddC	AZT	ddI	ddC	AZT	ddI	ddC
AZT-resistant $HIV\text{-}III_B$	ddI	0.9	10.4	0.41	0.88	52.4	0.63	0.82	88.5	0.84	0.79	131.8	1.7	0.85	120.1	1.9
AZT-resistant $HIV\text{-}III_B$	ddC				0.86	13.4	1.3	0.84	12.2	2.3	0.88	13.1	5.4	0.91	11.5	6.1
AZT-resistant strain 187	ddI	1.4	10.2	0.42	9.8	61.5	0.81	1.2	92.4	1.4	1.1	109.7	2.6	1.2	105.6	2.9
AZT-resistant strain 187	ddC				1.1	10.1	1.5	9.7	12.5	4.1	1.4	11.7	6.6	1.1	10.8	7.3

a. AZT-resistant variants were selected over 10 passages for subsequent resistance to ddI or ddC as described in Materials and Methods. EC_{50} values were calculated from RT activity in culture supernatants.

Table 4. Drug susceptibilities of AZT-resistant and other resistant variants after passage in the presence of other nucleosides [a]

Variant	Initial mutation at site	Drug pressure during second passage series	Subsequent mutation	Drug pressure during third passage series	EC_{50} (μM) after third passage series				
					AZT	ddI	ddC	d4T	BCH-189
AZT-resistant HIV-III_B	41, 215	ddI	74	–	0.9	120	0.5	0.5	0.8
AZT-resistant HIV-III_B	41, 215	ddI	74	d4T	1.2	134	1.6	8.5	2.9
AZT-resistant HIV-III_B	41, 215	BCH-189	184	–	0.85	15.6	3.8	0.6	187
AZT-resistant HIV-III_B	41, 215	BCH-189	184	d4T	1.1	12.8	7.1	12.5	50.2
ddI-resistant HIV-III_B	74	d4T	na[b]	–	0.2	114	1.4	6.9	2.7
BCH-189-resistant HIV-III_B	184	–	–	–	0.03	47	2.7	0.8	480

[a]AZT-resistant pr ddI-resistant variants were selected over 10 passages for subsequent resistance to other compounds as described in Materials and Methods. EC_{50} values were calculated from RT activity in culture supernatants

[b]na Not analyzed

Table 5. Susceptibility of multiply drug-resistant HIV variants to nucleosides after propagation in the absence of drug pressure [a]

Variant	EC_{50} (μM) after propagation in the absence of drug for					
	1 months			2 months		
	AZT	ddI	ddC	AZT	ddI	ddC
Multiply-resistant HIV-III_B	0.81	123.4	1.4	0.84	115.7	1.4
Multiply-resistant HIV-III_B	0.94	15.2	6.3	0.91	12.8	5.5
Multiply-resistant 187	0.98	120.5	1.9	1.1	119.2	2.3
Multiply-resistant 187	1.2	11.4	6.8	9.2	14.5	6.2

[a]Multiply drug-resistant variants of HIV-1 were [propagated on MT-4 cells in the absence of drug. Suscptibility assays were performed after 1 and 2 months, respectiviely, in the absence drug pressure.

Figure 1. Virus production assessed by RT activity in MT-4 cells infected with HIV-III_B (A) and clinical isolate 187 (B) in the presence of 1:1000 diluted patient serum (❑), 1:200 diluted patient serum (■), 0.01 μM AZT plus 1:1000 diluted patient serum (Δ), 19 μM ddI plus 1:1000 diluted patient serum (▲), 0.75 μM ddC plus 1:1000 diluted patient serum (○), 0.01 μM AZT plus 1:200 diluted patient serum (●), 19 μM ddI plus 1:200 diluted patient serum (×), 0.75 μM ddC plus 1:200 diluted patient serum (◆).

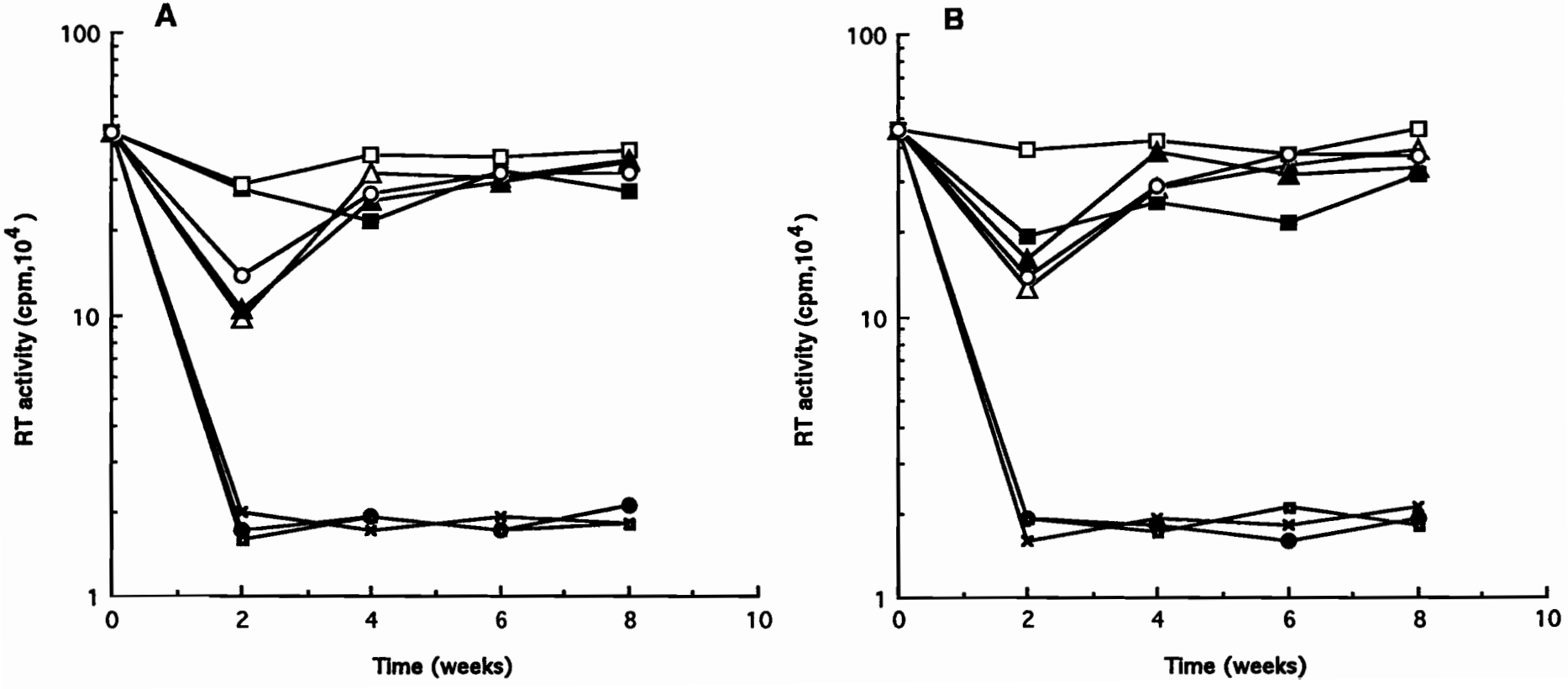

A
B
100
10
1
RT activity (cpm,10^4)
0
2
4
6
8
10
Time (weeks)

Figure 2. Virus production assessed by RT activity in MT-4 cells infected with HIV-III_B (A) and clinical isolate 187 (B) in the presence of 10 IU IFN-α2 (□), 50 IU IFN-α2 (■), 0.01 μM AZT plus 10 IU IFN-α2 (Δ), 19 μM ddI plus 10 IU IFN-α2 (▲), 0.75 μM ddC plus 10 IU IFN-α2 (○), 0.01 μM AZT plus 50 IU IFN-α2 (●), 19 μM ddI plus 50 IU IFN-α2 (×), and 0.75 μM ddC plus 50 IU IFN-α2 (◆).

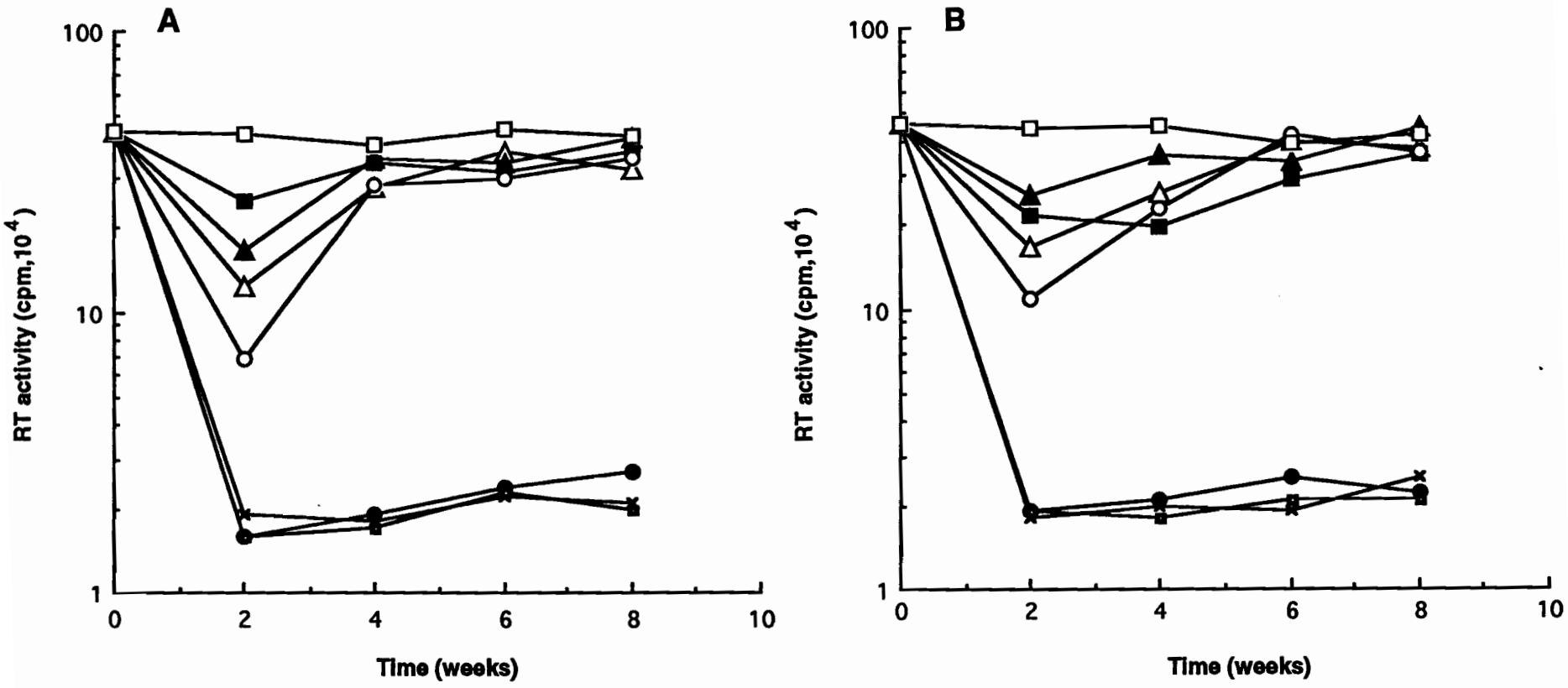
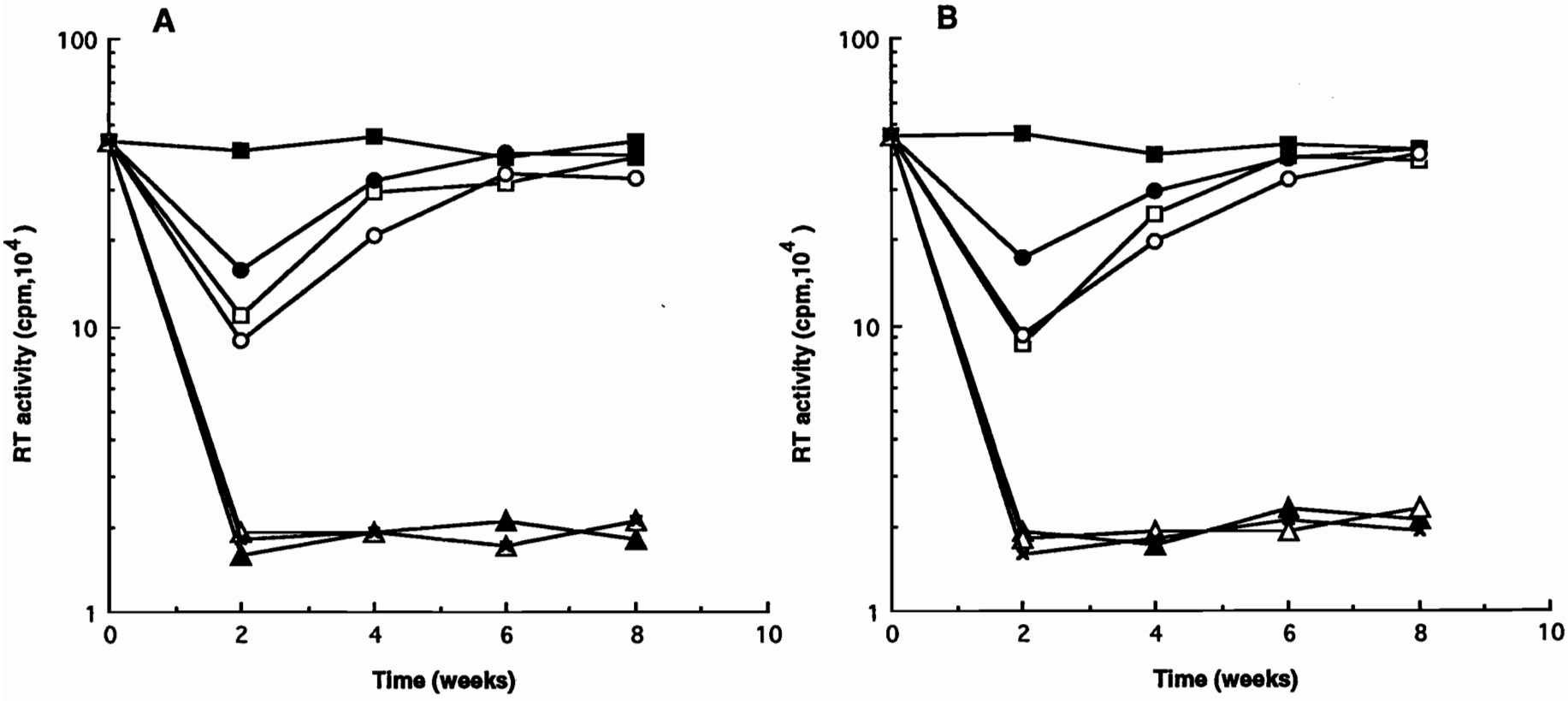

A
B
100
10
1
0
2
4
6
8
10
RT activity (cpm,10^4)
Time (weeks)

Figure 3. Viral replication assessed by RT activity in MT-4 cells infected with HIV-III_B (A) and clinical isolate 187 (B). Infected MT-4 cells were cultured in the absence of compound (■) or in the presence of 0.01 μM AZT (❑), 19 μM ddI (●), 0.75 μM ddC (○), 0.01 μM AZT plus 19 μM ddI (▲), 0.01 μM AZT plus 0.75 μM ddC (Δ), 19 μM ddI plus 0.75 μM ddC (×).


A
B
100
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10
RT activity (cpm,10^4)
Time (weeks)

sera to the AZT and ddI concentration gradients described in Table 1 was non-permissive for emergence of HIV variants resistant to either of these drugs. This effect could be further diluted through the use of even lower serum concentrations to permit the emergence of drug-resistant viruses.

The development of HIV drug resistance was also reduced when combinations of IFN-2a were used together with each of AZT, ddI and ddC in our selection protocols (Figure 2). The synergistic effects obtained with regard to non-emergence of resistance to each of AZT, ddI and ddC could no longer be documented when very low concentrations of IFN-2a were utilized, indicating that this effect was concentration-dependent. However, the concentrations of IFN-2a that effected synergy with either AZT or ddI in terms of non-emergence of drug resistance were below those able to achieve a significant anti-viral effect in the absence of nucleoside antagonists of viral RT activity [6].

Combinations of various sub-threshold concentrations of nucleosides, including AZT plus ddI, AZT plus ddC, and ddI plus ddC, were likewise non-permissive for emergence of drug-resistant variants (Figure 3). Of course, these data indicate that a first round of viral replication could not be achieved when certain drug combinations were present. Since some level of viral multiplication would be necessary for selection of drug resistance, these data do not exclude the possibility that other conditions might permit the emergence of resistant variants.

DISCUSSION

The major findings of this paper relate to our ability to have generated strains of HIV-1 that are multiply resistant to AZT, ddI and ddC through in vitro culture and drug pressure procedures. Previous work showed that such resistance against individual nucleosides can develop in tissue culture over periods between 2-8 weeks. Combinations of nucleosides, even at sub-effective concentrations, or use of IFN-2α in concert with nucleosides, did not permit the emergence of resistant variants, presumably due to synergy [6]. Of course, generalization with regard to patterns of cross-resistance among different isolates cannot be made on the basis of the limited numbers of samples reported here. Others have also shown that the use of triple drug combinations can yield multiple resistance, using protocols similar to those described here [13].

The drug-resistant variants derived in the current study possessed 50- 100-fold resistance against AZT and 10- 25-fold resistance against either ddI or ddC. These viruses displayed a stable multiple drug resistance phenotype, when grown in the absence of drug for 1-2 months. Non-reversion to a drug-sensitive phenotype probably reflects the stability of the mutations at specific sites in the HIV-1 pol gene that are responsible for drug resistance. Extensive viral passage in the absence of drug did not yield progeny of diminished drug sensitivity. Mutations at codons 41 and 215, among other sites, confer resistance to AZT, while mutations at codons 74 and 184 are associated with resistance to both ddI and ddC [8, 23]. Our PCR analyses showed that the multiply resistant viruses studied (AZT, ddI, ddC) contained mutations at codons 74 and 184 as well as at codons 41 and 215. The mutations at positions 74 and 184 probably resulted from passage of AZT-resistant species in ddI and ddC.

The AZT-resistant variants selected possessed similar genotypes to those isolated from patients under prolonged therapy with this drug [5,15]. Other workers have noted that the same order of pol gene mutations seen in patients is likely to occur in vivo [1,14].

We used relatively fresh isolates of HIV-1, selected after fewer than 10 culture passages, to minimize the extent of heterogeneity in reverse transcriptase.

We also demonstrated that no emergence of drug resistance occurred when single drugs were used in concert with either IFN-2α or high dilutions of virus-neutralizing antisera. Significantly, the concentrations of IFN-2α employed were as low as 10 IU/ml, far below those previously shown to possess an anti-viral effect. Previous studies demonstrated synergy between AZT and IFN-2α with regard to inhibition of acute HIV replication in tissue culture, but viral breakthrough was ultimately observed even when IFN concentrations of 50 IU were employed in the presence of AZT. The viruses which emerged from such investigations were wild-type and could be inhibited by both AZT and IFN-2α during subsequent replication cycles. In vitro selection for resistance may be a more sensitive method than acute infection for demonstrating cooperativity between interferon and nucleosides. Similar cooperative effects were demonstrated between nucleosides and neutralizing antibodies, which were shown to be effective in dose-dependent fashion.

HIV-1 variants that display multiple drug resistance have been isolated from patients sequentially treated with different drugs [23]. Our study demonstrates that such viruses can be selected more easily in vitro under conditions of sequential rather than simultaneous drug exposure. This finding is consistent with reports of the persistence of AZT resistance-conferring mutations in plasma, even after treatment with ddI and appearance of resistance to the latter drug [21]. The concept of combination simultaneous therapy should be further investigated.

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Chapter 5

In the preceding chapter we demonstrated that combinations of antiviral drugs can not only provide synergy for inhibition of viral replication but can also delay or prevent the emergence of drug-resistant variants. The sequential passage of AZT-resistant variants in cell culture in the presence of increasing concentrations of either ddI or ddC, however, gave rise to variants that showed multiple resistance to each of AZT, ddI, or ddC. With the generation of multiple drug resistance by passage of HIV-1 variants in cell culture, we asked whether syncytium formation among cells infected with distinct single drug-resistant variants could lead to the generation of multiple drug resistance through the possibility of genetic recombination. The work presented in the following chapter addresses this issue.

Recovery of Human Immunodeficiency virus type 1 Recombinants that Possess Resistance to Two Drugs Following Fusion of Cells Infected with Distinct Single Drug-Resistant Variants

ABSTRACT

We have attempted to relate the occurrence of the syncytium-inducing (SI) phenotype of the human immunodeficiency virus type 1 (HIV-1) to HIV drug resistance. We used polyethylene glycol to induce fusion among sub-clones of U-937 cells that carried HIV recombinants resistant to either 3'-deoxy-3'-deoxythymidine (AZT) or the (-)enantiomer of 2',3'-dideoxy-3'-thiacytidine (3TC). The parental viruses employed contained well-defined mutations in the pol gene at positions 70 and 215, associated with resistance to AZT, or position 184, responsible for resistance to 3TC. Fusion of cells, followed by co-culture with the MT-4 lymphocyte cell line for purposes of viral amplification, yielded viral progeny that, in some cases, possessed different patterns of drug resistance than parental viruses. In two instances, the viruses recovered possessed diminished sensitivity to both drugs and contained relevant mutation sites associated with resistance to these agents. A third variant possessed both a wild-type phenotype and genotype with regard to these characteristics. These mutational analyses were performed by polymerase chain reaction for identification of resistance-conferring mutations and confirmed by direct sequencing of single strands of amplified DNA segments, indicating that viral recombination had occurred. These findings establish a theoretical basis on which to conclude that the acquisition of multiple drug resistance on the part of HIV-1 may be related to its ability to promote cell fusion.

INTRODUCTION

Mutations in the HIV pol gene can affect viral reverse transcriptase (RT) activity and give rise to resistance against antiviral drugs (Larder and Kemp, 1989). HIV variants that possess a drug-resistance phenotype can be isolated from patients undergoing prolonged antiviral chemotherapy (Larder et al., 1989; Rooke et al., 1989) and can be selected in vitro by culture passage in the presence of increasing concentrations of drugs (Gao et al., 1992; Larder et al., 1991). HIV resistance against a wide array of drugs has been identified (Eron et al., 1993; St. Clair et al., 1991).

The possibility that genetic recombination may occur in vivo, leading to the generation of viruses resistant to several drugs, should be considered. In this context, retroviral recombination has been shown to occur in a variety of systems (originally reviewed by Coffin, 1979; Hunter, 1979). Sequence data from HIV isolates suggests that recombination among env gene loci can occur in vivo (Groenink et al., 1992). Infectious virus has been generated following transfection of two exogenous segments of HIV DNA into HIV-susceptible cells (Srinivasan et al., 1989). Fusion of cells carrying envelope-defective non-infectious particles with cells unable to generate RT activity also yielded infectious progeny as a consequence of recombination (Boulerice et al., 1991).

The presence of distinct drug-resistance-conferring mutations in the HIV pol gene may represent a useful model for analysis of this question. Although HIV variability is more extensive in the env gene than in pol, the rapid mutability of env sequences may not propvide sufficient genetic stability to assess whether viral recombination can occur with a high degree of frequency. In contrast, the pol gene mutations that confer drug resistance are stable and do not appreciably impact on viral replication (Gu et al., 1992; Gu et al., 1994; Larder and Kemp, 1989; Tisdale et al., 1993). Furthermore, patients who receive anti-viral chemotherapy are subjected to a selection pressure for resistance-conferring viral mutations that presumably extends to all cells in which HIV replication can occur. As an example, such drugs as AZT have been shown to cross the blood brain barrier, and

are able to inhibit viral multiplication in cultured brain tissue as well as in cultured peripheral blood lymphocytes and monocytes.

The present work began with attempts to fuse HIV-infected T lymphocytes that carried HIV recombinant viruses resistant to different drugs, in the hope of demonstrating viral recombination and establishment of dual drug resistance. These efforts were unsuccessful due to non-viability of the hybrid cells generated. HIV can also infect CD4+ cells of monocyte/macrophage lineage. The latter are thought to be an important reservoir for HIV persistence and dissemination (McInnes and Rennick, 1988; Roy and Wainberg, 1988). The possibility of such interactions in vivo leading to viral recombination cannot be excluded, particularly since interleukin-4 (IL-4) and interferon-α2 (IFN-α2) can both induce monocytic giant cell formation (McInnes and Rennick, 1988).

Syncytium formation mediated by interactions between human immunodeficiency virus type 1 (HIV-1) envelope proteins (gp41 and gp120/160) on infected cells and CD4 receptors on uninfected cells are commonly observed in HIV-infected cultures (Tersmette et al., 1988). Although such syncytia are not generally found in the blood of HIV-infected individuals, new studies indicate that they may exist in lymph nodes and other lymphoid tissues in which HIV infection is extensive (Pantaleo et al., 1993). Cell fusion induced by HIV usually leads to cell death in culture, may contribute to the depletion of CD4+ T lymphocytes in AIDS patients (Lifson et al., 1986; Sodroski et al., 1986), and may constitute an important means of virus transmission within the host. In addition, fusion between HIV-infected cells may also occur, creating a milieu in which genetic mixing permits the emergence of molecular viral hybrids with novel properties.

The current study was designed to assess the possibility that acquisition by HIV variants of a syncytium-inducing (SI) phenotype, usually seen at late stages of HIV infection (Boucher et al., 1992; Montaner et al., 1993), might be related to genetic recombination and the development of multiple drug resistance.

MATERIALS AND METHODS

Cells and viruses

MT-4 cells were provided by N. Yamamoto, Yamaguchi University School of Medicine, Ube, Japan. U-937 cells were obtained from the American Type Culture Collection. Both cell lines were maintained in suspension cultures (3×10^5 to 5×10^5 cells/well) in RPMI 1640 medium as described (Bour et al., 1991). The HIV-III_B laboratory strain of HIV was kindly provided by Dr. Robert C. Gallo, National Institutes of Health, Bethesda, MD. Viruses were propagated on MT-4 cells and stored at -70°C as described (Gu et al., 1992). AZT was obtained from Burroughs-Wllcome Inc., Research Triangle Park, NC and 3TC was provided by Glaxo Group Research, Greenford, United Kingdom. Methods used to infect MT-4 cells with wild-type and recombinant viruses have been described (Gu et al., 1992; Gu et al., 1994).

Generation of recombinant viral clones

Procedures used in our lab to generate HIV variants resistant to AZT, 3TC, and other drugs have been described (Gao et al., 1992), as have methods used to generate recombinant viruses that display drug resistance (Gu et al., 1992; Gu et al., 1994). This study was performed with recombinant HIV variants that displayed resistance to either AZT or 3TC but not both. The former virus contained previously described mutations at positions 70 (lys _ arg) and 215 (thr _ tyr) in the HIV pol gene (Larder and Kemp, 1989), while the latter contained a meth _ val substitution at codon 184 (Gu et al., 1992; Tisdale et al., 1993).

Infection of U-937 cells

U-937 cells (3×10^5) were co-cultivated with MT-4 cells (6×10^5), that had been previously infected with either wild-type HIV or HIV variants that were resistant to AZT or 3TC at a ratio of 1:2. The mixtures were maintained as suspensions (5×10^5 cells/ml). After two weeks, the MT-4 cells had disappeared due to virus-induced cell death. This was confirmed by the absence of cells reactive with OKT3 monoclonal antibodies, that

recognize the CD3 marker present on all lymphocytes [as determined by indirect immunofluorescence assay (IFA) and flow cytometry]. After 5 weeks, over 95% of the surviving U-937 cells were positive for viral p24 antigen by IFA.

Fusion of infected U-937 cells

Briefly, U-937 cells (total of 10^6) infected with wild-type HIV-III_B or with either AZT- or 3TC-resistant viruses were co-incubated at a ratio of 1:1. The mixture was washed twice by centrifugation in serum-free RPMI 1640 medium. The cell pellets were then gently resuspended in 0.7 ml of prewarmed polyethylene glycol (PEG 1500) and diluted 1:1 into medium for 60 sec using large-bore transfer pipettes. A double volume of medium was then added into the mixture to dilute the PEG 1500. The cells were then washed by centrifugation, resuspended, and aliquoted in fresh medium into a 6-well plate. The appearance of large polymorphonuclear cells indicated that fusion had occurred. Giant cells were harvested by micromanipulation, under an inverted microscope, using extra-small-bore Pasteur pipettes and seeded individually into a 96-well plate to which fresh uninfected MT-4 cells (3×10^4 cells/well) had been placed. Once syncytia appeared in these MT-4 co-cultures, cell suspensions were transferred to culture flasks (5 ml medium; 5×10^5 cells/ml) for virus amplification. Clarified culture fluids were used as a source of virus for drug sensitivity assays. Similar procedures were utilized in attempts to establish viable hybrids of MT-4 cells that had been infected with drug-resistant viruses of HIV. However, these efforts were unsuccessful, and no viable giant cells were obtained.

Viral genomic analysis

DNA was extracted from cells as described (Gu et al., 1994), and a double-amplification procedure using nested primers was used to amplify such material by polymerase chain reaction (PCR) (1 μg DNA/sample) (Gu et al., 1994). The DNA products thus generated were subjected to further specific amplification using selective oligonucleotide primers paired with common primers, allowing discrimination within the

RT coding region of wild-type from specific mutations at codons 70, 215 and 184. The former two mutations are associated with resistance to AZT (Larder and Kemp, 1989), while the latter encodes resistance against 3TC (Gu et al., 1992; Tisdale et al., 1993). Table 1 lists the primers used in this study. DNA products of PCR were electrophoresed on agarose gels and visualized by ethidium bromide staining.

Cloning and sequencing

The PCR-amplified segments were purified from agarose gels, digested with Hind III and EcoRI (Pharmacia Fine Chemicals, Montreal, Canada), and ligated with digested M13mp19 (Gu et al., 1992; Gu et al., 1994). TG1 cells were transfected with cloned viral DNA recombinants and screened by digesting double-stranded DNA with restriction endonucleases. Single-stranded DNA was prepared from recombinant M13 clones and nucleotide sequences were determined using a TaqTrack sequencing kit (Promega Inc., Madison, WI) as described (Gu et al., 1994).

Table 1. Oligonucleotide primers used in PCR detection of relevant DNA sequences

Primer	Sequence	nt position
70W	5'-GAAATCTACTAATTTTCTCCATT-3'	2779—2757
70G	5'-GAAATCTACTAATTTTCTCCATC-3'	2779—2757
184U	5'-TACAATGTGCTTCCACAGGG-3'	2984—3003
184D	5'-CCATCCAAAGGAATGGAGG-3'	3221—3239
184W	5'-TCCTACATACAAATCATCCAT-3'	3098—3117
184E	5'-GATCCTACATACAAATCATCT-3'	3098—3117
215W	5'-GATGTTTTTTGTCTGGTGTGG-3'	3212—3192
215T	5'-GATGTTTTTTGTCTGGTGTGT-3'	3212—3192

RESULTS

Generation of hybrid cells

We performed fusions of U-937 cells that had been infected with distinct drug-resistant variants of HIV-1, as described in Materials and Methods. Large polymorphonuclear cells were seen within 1-2 min of addition of PEG 1500 to infected U-937 cells. Approximately 200 such cells were selected for co-culture with MT-4 cells. Of these 200 co-cultures, about 190 produced viral progeny of which 15 produced virus that seemed doubly resistant or doubly sensitive to both AZT and 3TC on initial screen. Eight of the latter were chosen for genetic analysis, and produced detectable virus within 4-6 days, as demonstrated by appearance of syncytia in the MT-4 cultures, RT activity in culture fluids and viral p24 Ag by IFA (Table 2). These viruses were plaque purified on MT-4 cells prior to phenotypic testing (Gao et al., 1992).

Drug sensitivities of viruses recovered from hybrid cells

To determine drug-resistance phenotypes of these variants, sensitivity to antiviral drugs was assessed on MT-4 cells by monitoring production of viral p24 antigen in culture fluids. The results of Table 3 show that the viruses generated in five of eight cases by this method possessed resistance to either AZT or 3TC but not both. In one instance (progeny of co-culture 3), the viruses were 40-50 fold resistant against AZT and 90-120 fold resistant against 3TC, in comparison with wild-type HIV-III_B. In the case of hybrid 5, that contained both the 184 and 215 mutations, a renewed sensitivity to AZT was seen against a background of sensitivity to 3TC. The progeny of hybrid cell 8 were apparently sensitive to both AZT and 3TC. The parental viruses employed in this work possessed resistance phenotypes of about this same magnitude for these drugs. As an additional control, we also studied the profiles of these viruses to d4T, and found that all of the variants tested were sensitive to this drug. We also observed that the viruses that were doubly-resistant were as infectious for each of MT-4 and PBMC as wild-type viruses or those possessing resistance to a single drug only, as indicated by levels of reverse

Table 2. Viral expression by hybrid cells

Hybrid cell	Parameters of HIV-1 infection [a]		
	Induction of syncytia	RT activity ($cpm/mlx10^4$)	Fluorescent cells (%)
1	+	10	10
2	+	8	6
3	+	8	6
4	+	11	7
5	+	13	11
6	+	9	7
7	+	11	9
8	+	9	10

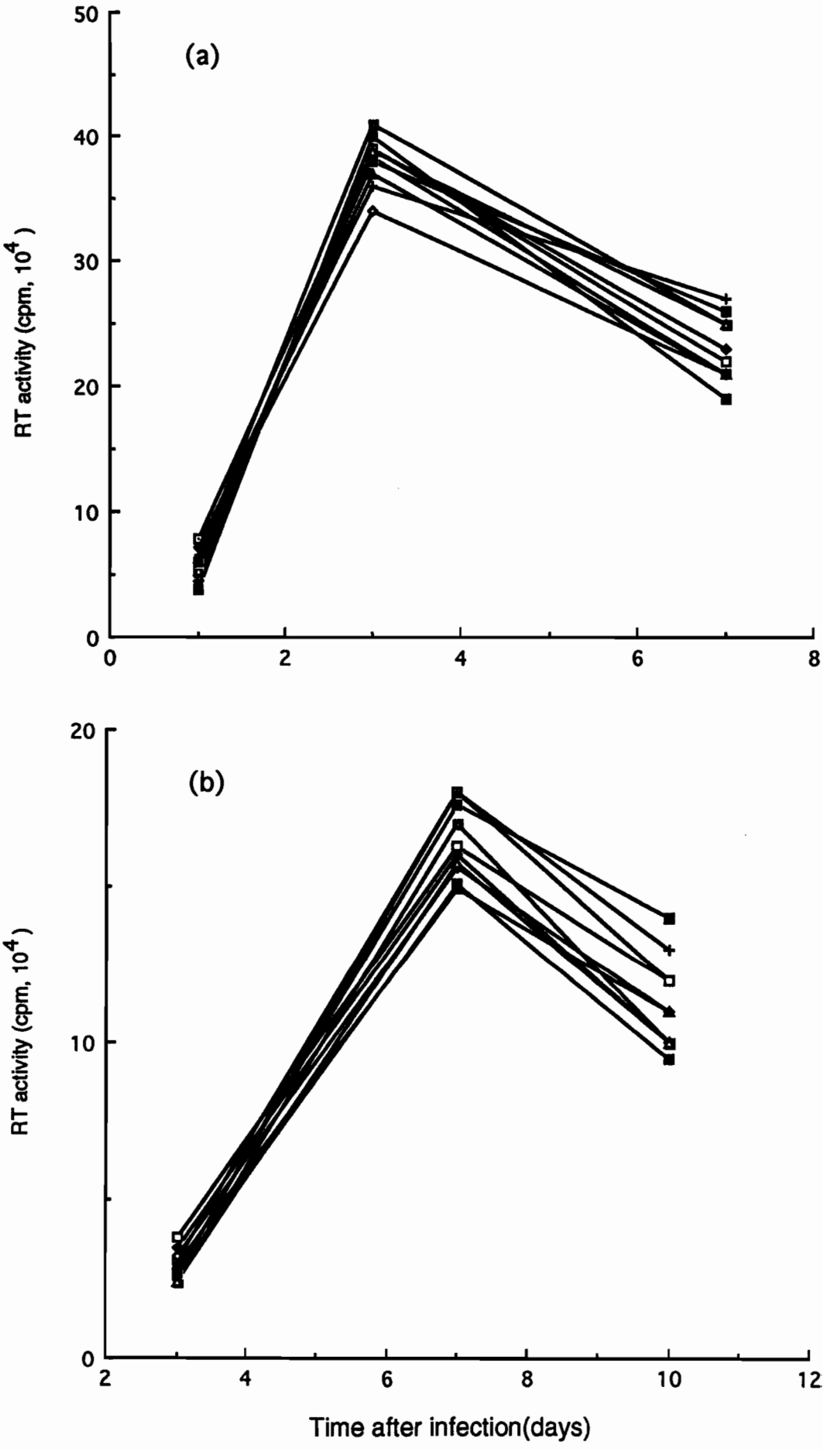
[a] After appearance of syncytia, RT activity in clarified coculture supernatants was measured and cells were assessed for expression of viral p24 antigen by IFA.

Table 3. Drug sensitivities of HIV variants recovered from hybrid cells

HIV-1 variant	EC_{50} (μm)[a]		
	AZT	3TC	d4T
HIV-III_B	0.03 ± 0.002	0.7 ± 0.08	0.5 ± 0.06
AZT resistant/III_B	2.5 ± 0.03	0.6 ± 0.12	0.6 ± 0.16
3TC resistant/III_B	0.04 ± 0.001	910 ± 81	0.45 ± 0.06
Hybrid cell 1	0.08 ± 0.004	752 ± 62	0.7 ± 0.09
Hybrid cell 2	1.2 ± 0.006	1.1 ± 0.3	0.4 ± 0.15
Hybrid cell 3	1.5 ± 0.005	852 ± 94	0.5 ± 0.09
Hybrid cell 4	0.05 ± 0.002	547 ± 55	0.6 ± 0.04
Hybrid cell 5	0.1± 0.005	659 ± 52	0.9 ± 0.12
Hybrid cell 6	2.2 ± 0.04	1.5 ± 0.4	0.5 ± 0.05
Hybrid cell 7	2.5 ± 0.03	0.8 ± 0.2	0.2 ± 0.18
Hybrid cell 8	0.04 ± 0.006	1.4 ± 0.1	0.6 ± 0.05

[a] Results were calculated on the basis of p24 Ag levels in clarified culture fluids of infected MT-4 cells, using three replicate samples. Data are means ± standard deviations.

Figure 1. Growth of parental viruses and viral products of fused U-937 monocytes on each of MT-4 cells (a) and peripheral blood mononuclear cells (b). Symbol designations are : HIV-IIIB (■); progeny of hybrid cells 1 (❑); 2 (▲); 3 (Δ); 4 (✚); 5 (◆); 6 (●); 7 (○); 8 (◘).


(a)
RT activity (cpm, 10^4)
50
40
30
20
10
0
0
2
4
6
8
(b)
RT activity (cpm, 10^4)
20
10
0
2
4
6
8
10
12
Time after infection(days)

transcriptase activity in culture fluids (Fig. 1). In some cases, viruses from several plaques of the same co-cultures were analyzed, with similar drug sensitivity results in each instance.

PCR detection of mutation sites and sequencing analysis

To determine whether mutations associated with resistance against AZT or 3TC could be detected in viruses derived from our hybrid cells, viruses grown in MT-4 cells after co-culture were plaque-purified in the MT-4 cell line. Cellular DNA extracted from MT-4 cells infected with these plaque-purified viruses was amplified for the presence or absence of mutation sites corresponding to codons 70, 215 and 184, using oligonucleotide primers that have been previously described (Gu et al., 1992; Gu et al., 1994; Larder and Kemp, 1989). The results of Table 4 show that the mutated forms of codons 70 and 215 were present in variants derived from hybrid cells 2, 6 and 7 and were present as well in the parental AZT-resistant virus preparation used in this work. The mutated form of codon 184 was detected in virus derived from hybrid cells 1 and 4, in addition to being present in parental 3TC-resistant virus. The variant produced by hybrid 5 contained mutations at both codons 215 and 184, and mutations at all three codons i.e. 70, 215 and 184, were detected in the case of virus derived from hybrid cell 3. Virus from hybrid cell 8 was sensitive to both AZT and 3TC and contained wild-type codons at each of these positions. Two of our other co-cultures (see above) also appeared to have generated virus that was fully drug-sensitive. However, these were not further analyzed, nor were six others that appeared to have produced only parental-type viruses.

To confirm the results of our PCR analysis, and to rule out the possibility that we had merely detected mutations along separate proviral DNA strands, a 1742 base segment of the pol gene of certain of these viruses, containing the complete RT coding region, was cloned as described above. Extracted cloned DNA was then amplified. Sequencing studies confirmed that the parental AZT-resistant variant contained mutations at codons 70 (Lys _ Arg) and 215 (Thr _ Tyr), while the parental 3TC-resistant virus was mutated at

Table 4. Detection of specific mutations in pol genes of HIV variants by PCR

HIV-1 variant	Resistance to		Mutations at codon		
	AZT	3TC	70	184	215
HIV-III_B	–	–	–	–	–
AZT resistant /III_B	+	–	+	–	+
3TC resistant/III_B	–	+	–	+	–
Hybrid cell 1	–	+	–	+	–
Hybrid cell 2	+	–	+	–	+
Hybrid cell 3	+	+	+	+	+
Hybrid cell 4	–	+	–	+	–
Hybrid cell 5	+	+	–	+	+
Hybrid cell 6	+	–	+	–	+
Hybrid cell 7	+	–	+	–	+
Hybrid cell 8	–	–	–	–	–

codon 184 (Met _ Val). Mutations at codons 70 and 215 were also detected in samples derived from hybrid cells 2, 6 and 7, while variants derived from hybrid cells 1 and 4 were mutated at codon 184 only. In the case of hybrid cell 3, all three mutations were present. While samples derived from hybrid cell 5 contained the 215 and 184 mutations only, virus derived from hybrid cell 8 possessed a wild-type genotype and phenotype, suggesting that two recombination events may have occurred in this instance as well. In some cases, viruses from different plaques of the same cultures were sequenced, with the same results.

DISCUSSION

In this study, we generated HIV variants that contained three distinct mutations associated with resistance to AZT (codons 70 and 215) and 3TC (codon 184). This is not the same combination of mutation sites as that recently shown to contribute to diminished resistance to AZT (i.e. codons 184 and 215 only) (Tisdale et al., 1993). Indeed, virus derived from hybrid cell 5 possessed mutations at codons 184 and 215 and appeared to possess renewed sensitivity to AZT against a background of 3TC resistance, consistent with previous results (Tisdale et al., 1993). The progeny of hybrid 8 possessed wild-type genotype and phenotype. The period between PEG 1500-induced cell fusion and generation of doubly-resistant progeny was relatively short, possibly arguing for a two-stage process. Initially, particles produced by fused cells may have contained different RNA molecules within the same virion. This complementation may have been followed by viral replication; a more detailed mechanistic analysis will require knowing how many copies of proviral DNA were present in each of the cell clones.

A previous report showed that two different retroviral RNA molecules could be packaged into a single virion, after transfection of target cells with spleen necrosis virus constructs that contained marker genes that conferred resistance to either neomycin or hygromycin (Hu and Temin, 1990). The resultant viruses contained both genes as a probable consequence of non-homologous recombination (Hu and Temin, 1990). As a control for the current studies, we co-infected MT-4 cells with mixtures of AZT- and 3TC-resistant viruses , but were unable to recover viruses that were resistant to both drugs, following plaque purification of individual isolates. Possibly, the rapid initiation of reverse transcription in the protocols utilized are permissive only for the use of a single genomic template. In this study, we utilized U-937 cells as target cells for fusion, because of their relative stability and because attempts to fuse MT-4 cells and other lymphocytes with PEG-1500 were unsuccessful due to toxicity. Furthermore, virus-mediated fusion in culture among different types of HIV-infected lymphocytes may not be feasible, due to

the down-modulation of cell surface CD4 that occurs in productively-infected cells (Bour et al., 1991; Sattentau and Weiss, 1988).

A key issue in these experiments is to rule out that mixed virus populations may have been produced as opposed to recombinants. First, the degree of resistance generated against each of 3TC and AZT by the presumed hybrid cells 3 and 5 (Table 3) is consistent with the notion that individual mutations along a single proviral DNA chain were responsible. This result is substantiated by our sequencing data obtained on individual cloned segments of proviral DNA. Although a reversion to a wild-type codon by an error-prone reverse transcriptase could account for some of these data, the fact is that all of the mutations described in this manuscript have been previously shown to be stable by other investigators (Gu et al., 1992; Gu et al., 1994; Larder and Kemp, 1989; Tisdale et al., 1993).

It remains to be explained what mechanisms may account for genetic recombination in these experiments. Possibly, some degree of premature template switching may have occurred after the generation of full-length (-)strong-stop DNA, i.e. between what are commonly referred to as the first and second template switch events. Similar findings have previously been obtained in other systems; the potential significance of such occurrences for genetic recombination has previously been noted (Hu and Temin, 1990). Although viral recombination similarly occurred with a higher degree of frequency in our experiments than in those of other investigators, it should be noted that our infections were allowed to proceed over multiple rounds of viral replication, while other investigators employed only a single multiplication cycle (Ho and Temin, 1990). Similarly, our failure to have detected viral recombinants in the mixed infection protocol described above (Table 1), may likewise be attributable to the fact that this protocol could only have detected viral recombinants that emerged during a single cycle of virus replication.

In view of the fact that the development of each of HIV drug resistance (Larder et

al., 1989; Rooke et al., 1989) and the SI phenotype (Boucher et al., 1992; Tersmette et al., 1988) are associated with clinical progression, it is tempting to hypothesize that the latter may contribute to the former in either of two ways. First, syncytium formation and viral recombination could result in the acquisition of double or multiple resistance, as hypothesized here. Second, such events may play a role in the acquisition by a single viral quasi-species of multiple mutations that act in synergy to confer high level resistance to drugs such as AZT (Larder and Kemp, 1989).

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Chapter 6

General discussion

Selection for drug resistance in culture has been shown to be a valuable tool in viral drug susceptibility studies. In the case of certain viruses, drug-resistant variants can be generated in cell culture after only a few passages in the presence of drug (Larder et al 1985; Field, 1989). Laboratory studies can be used to predict the likely nature and frequency of resistant viruses that may be observed clinically during antiviral therapy. Unexpectedly, selection for HIV drug-resistant mutants in culture proved difficult, with the exception of that against nonnucleosides inhibitors of RT (Smith et al., 1987; Larder et al., 1989; Numberg et al., 1991; Richman et al., 1991).

An important characteristic of HIV is its genetic diversity (Wong-Staal et al., 1985; Robson et al., 1988; Fisher et al., 1988). Thus, HIV variants with reduced drug sensitivity may occur and be amplified over wild-type viruses under drug pressure. The finding that AZT-resistant variants can be isolated from treated individuals (Larder et al., 1989; Rooke et al., 1989), raises several important questions: 1) is HIV able to acquire resistance to drugs other than AZT? 2) can selection for drug-resistant viruses be performed in cell culture? 3) what are the properties of such drug-resistant viruses? With these considerations in mind, we attempted to develop a system to select for HIV resistance in cell culture.

Our selection system is indeed useful for generation of drug-resistant HIV variants. Initially, we failed when wild-type virus was used to infect cells in the presence inhibitory concentrations of AZT. Subsequent work began with subinhibitory concentrations that were gradually increased, and yielded HIV resistant to either AZT, ddI, ddC, or BCH-189. This work was performed with both the HIV-1 III_B laboratory stain as well as clinical samples.

The high error rate of reverse transcriptase, especially during transcription from genomic RNA to proviral DNA, is the major factor contributing to the generation of

mutations in the HIV-1 genome. The error rate of HIV-1 is estimated to give rise to five to ten errors per genome per replication cycle; this is believed to contribute to observed mutations within the viral genome (Preston et al., 1988; Roberts et al., 1988). Previous studies have described mutations located within either the HIV env or pol gene (Javaherian et al., 1990; LaRosa et al., 1990; Larder et al., 1989a). Mutations in env can give rise to extensive variability in antigenicity of vial gp120 (Desai et al., 1986; Javaherian et al., 1990; LaRosa et al., 1990; Laman et al., 1992). Mutations in pol gene have resulted in resistance to AZT and other drugs that target the viral reverse transcriptase, itself a product of the pol gene (Larder et al., 1989a; Larder et al., 1989).

The overall probability of a mutation occurring at a specific position is governed by both the error rate for that position and the number of replication cycles. Some mutations may be incompatible with viral replication and will therefore not be detected. Tolerated mutations that permit viral replication but render the virus resistant to drug may also go undetected, unless they can be amplified under selective pressure against a background of wild-type viruses. Under appropriate drug pressure, viruses containing mutations in the pol gene that confer drug resistance will possess replication advantage. Such variants will be amplified over drug-sensitive wild-type viruses and detected. Although inhibitory concentrations of drug also provide such selective pressure, the probability of selectable mutations is decreased due to reduced numbers of replication cycles of virus populations.

The success of our in vitro selection for HIV resistance may be attributed to the subinhibitory concentrations of drug used that allowed marginally resistant virus to be amplified over wild-type in initial passage; step-wise increasing drug concentrations were employed at subsequent passages. Thus, mutated viruses were initially amplified, while the number of replication cycles of wild-type viruses were not significantly reduced.

A number of resistance studies have described the virulence and pathogenic potential of drug-resistant viruses. Most acyclovir-resistant herpesviruses appear to have reduced virulence (Field and Darby, 1980; Darby et al., 1981). Rimantadine-resistant

influenza A virus strains, on the other hand, are as virulent as their wild-type counterparts (Sweet et al., 1991; Hayden et al., 1989). With the generation of HIV-1 resistance in vitro, we investigated the kinetics of viral replication, as determined by RT activity. Our results showed that HIV mutants selected in vitro resembled their parental wild-type viruses in viral replication, although maximum production was delayed in the presence of drug. These results suggested that drug-resistant viruses are probably as infectious as wild-type viruses. Thus, drug-resistant viruses may play an important role in HIV disease. Delayed virus production in the presence of drug may result from mixed populations. In the presence of high drug concentration, variants with moderate levels of resistance may still be inhibited. Further studies, using plaque-purified virus that is subsequently amplified in the presence of drugs, will facilitate study of this issue.

To investigate whether the observed HIV resistance was cell type-dependent, we further studied the ability of our drug-resistant variants to infect cell types other than MT-4. Each of the viruses in question were able to infect other cells in the presence of the same drug used in selection. To some cells, increased drug sensitivity was noted compared with MT-4 cells. This is probably due to the fact that intracellular phosphorylation systems which convert nucleoside analogs to their active forms differ from one cell type to another (Balzarini et al., 1988).

Previous studies have documented the nature of the relationship between the drug resistance phenotype and genotype (Larder et al., 1989). HIV-1 resistance is associated with alterations in the HIV genome, as demonstrated by identification of mutations conferring resistance to AZT in the RT-coding region of the HIV-1 pol gene (Larder et al., 1989). Our resistant variants displayed a stable drug resistance phenotype, when grown in the absence of drug for two months, indicating that the mutations in question do not confer any replication disadvantage. A genetic analysis of these variants was performed using polymerase chain reaction and cloning and sequencing. Mutations at codons 70 and 215, previously shown to be responsible for resistance to AZT, were

detected in AZT-resistant variants derived from either wild-type HIV-IIIB or clinical isolates. This work employed primer pairs that can discriminate mutated from wild-type sequences. These findings are consistent with previously described genomic alterations in AZT-resistant variants isolated from treated individuals (Larder et al., 1989). Our cloning and sequencing analysis not only confirmed the existence of mutations at codons 70 and 215 in the pol gene of AZT-resistant variants but also revealed that additional mutations at codons 50, 108, and 135 may be also associated with resistance to AZT. This indicates that patterns of genomic alterations associated with HIV resistance are likely to be complex.

AZT-resistant viruses display cross-resistance to nucleoside analogs that contain a 3'-azido moiety but not to ddI or ddC (Larder al., 1989). Neither did our AZT-resistant variants possess cross-resistance against either ddI, ddC, or BCH-189, suggesting that these drugs may be useful as alternative therapy in patients who have documented resistance to AZT. However, our cross resistance studies showed that ddC-resistant variants possessed reduced sensitivity to BCH-189 and that BCH-189-resistant variants displayed cross-resistance to ddC. It is interesting to note that both compounds resemble each other in molecular structure. This cross-resistance is likely due to genomic alterations that confer resistance to both drugs. Work in our laboratory showed that both ddI- and BCH-189-resistant variants contained the same mutations in their pol gene (Gu et al., 1994).

An antiviral strategy of choice is the combination of various antiviral drugs that act synergistically to inhibit HIV-1 replication (De Clercq, 1992; Johnson, 1994). Synergy between AZT and ddI as well as between AZT and each of recombinant soluble CD4 and recombinant interferon-a-A have been described in vitro (Johnson et al., 1990; Dornsife et al., 1991; Johnson et al., 1991). In our studies, the sequential passage of AZT-resistant variants in increasing concentrations of either ddI or ddC yielded variants that displayed resistance to both AZT and ddI or ddC. The acquisition of such multidrug resistance was

associated with genomic alterations in the viral pol gene. Consistent with our results, others have also reported the generation of multiply drug-resistant variants by using protocols similar to those described here (Larder et al., 1993). Combinations of various drugs can not only provide synergy for inhibition of viral replication but can also delay or prevent the development of drug resistance. Switching antiviral therapy in patients who already harbor drug-resistant viruses may lead to multidrug resistance. Combinations of antiviral drugs should be considered early in therapy to prevent or delay multidrug resistance.

Syncytium formation among HIV-infected cells, commonly observed in culture, may create a milieu in which genetic mixing can occur. We speculated that this could lead to genetic recombination and the generation of viruses resistant to several drugs. To explore this possibility, we performed fusion of monocytic cells that had been infected with viruses resistant to either AZT or 3TC. Our results showed that viruses recovered from the hybrid cells possessed resistance to both drugs in a limited number of cases. Genetic analysis revealed that resistance-conferring mutations relevant to both drugs were present in the HIV pol gene. These findings suggest that syncytium formation in vivo may be a means whereby viral recombination can occur, giving rise to multidrug resistance. Although such syncytia are not generally found in the blood of HIV-1 infected individuals, they may exist in lymph node tissue in which HIV infection is extensive (Pantaleo et al., 1993).

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Contributions to Original Knowledge*

The following novel findings have been demonstrated in this thesis:

A system to select for HIV resistance in cell culture has been developed. Variants of both HIV-1 IIIB and clinical isolate 187 which displayed high levels of resistance to either AZT, ddI, ddC, or 3TC were selected by passage in tissue culture in the presence of increasing concentration of drugs. These variants were able to infect CEM-T4 cells and PBMC as well as MT-4 cells in the presence of drugs. The drug resistance phenotype remained stable after propagation of these variants in the absence of the drug pressure for over two months. Study of the kinetics of viral replication by RT activity assay revealed that drug resistant variants behaved similarly to parental wild-type viruses, suggesting that drug-resistant variants are possibly as infectious as wild-types. Cross resistance studies showed that ddC-resistant variants possessed a moderate degree of resistance against 3TC and that 3TC-resistant variants were also cross-resistant to ddC.

The combination of subinhibitory concentration of two drugs prevented the development of HIV-1 resistance over eight weeks. However, multiply drug-resistant variants were generated when different drugs were sequentially but not simultaneously used in in vitro selection procedures. The multiple drug resistance phenotype also remained stable when viruses were propagated in the absence of drug pressure. Genotypic analysis of multiply drug-resistant variants revealed that acquisition of such multiple resistance was associated with mutations occurring in the RT-coding region of the HIV-1 pol gene.

Fusion of cells infected with distinct single drug-resistant variants by using PEG led to the emergence of molecular viral hybrids with novel properties. Viruses recovered from these hybrid cells possessed different patterns of drug resistance than parental viruses. Fusion of U937 cells infected by AZT-resistant variants with U937 cells infected by 3TC-resistant variants gave rise to viruses that possessed resistance to both drugs,

suggesting that genetic recombination might have occurred. Cloning and sequencing of the RT-coding region of these variants showed that they contained mutations associated with resistance to each of AZT and 3TC. These findings constitute a theoretical basis for the generation of multiple HIV drug resistance, possibly related to the ability of HIV to promote cell fusion.

* This section is a mandatory requirement of Ph. D. thesis submitted to the Faculty of Graduates Studies and Research, McGill University , Montreal.