博士（医学）論文

Doctor's Thesis

Role of Variable and Conserved Domains of Human Immunodeficiency Virus Type 1 (HIV-1) Envelope gp120 in CCR5 Coreceptor Utilization and Reduced Sensitivity to MIP-1α

（CCR5 利用性と MIP-1α抵抗性に関与する HIV-1 エンベロープ gp120 の可変領域および保存領域の役割）

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Preface

This thesis is the outcome of my four and half years of research as a postgraduate student at the Medical Virology department, Kumamoto University, School of Medicine, Japan. It is based on the following publications:

1) **Foda M, Harada S., and Maeda Y.** 2001. Role of V3 Independent Domains on a Dualtropic Human Immunodeficiency Virus Type 1 Envelope gp120 in CCR5 Coreceptor Utilization and Viral Infectivity. Microbiol Immunol. 45(7):521-30.

2) **Maeda Y, Foda M, Matsushita S, and Harada S.** 2000. Involvement of both the V2 and V3 regions of the CCR5-tropic human immunodeficiency virus type 1 (HIV-1) envelope in reduced sensitivity to macrophage inflammatory protein 1α. Journal of Virology. 74(4):1787-93

The aim of the studies presented herein is to define the role of different domains of the HIV-1 envelope in coreceptor utilization as well as in the reduced sensitivity to different C-C chemokines. The thesis is divided into four main sections. In the background and objectives section I reviewed the significance of the HIV epidemic; the structure, replication, tropism and coreceptor utilization of HIV-1; virus entry inhibitors and their escape pathways. In the materials and methods section, a detailed explanation of the various materials and procedures is described. The results and discussion sections both give a thorough description of the data we achieved as well as their interpretation.

I hope this work would highlight the importance of distinct domains other than the V3 domain in coreceptor utilization and viral infectivity. In addition, I believe that our discovery about the importance of V2 mutations in conjunction with V3 mutations for R5 escape mutants can give some clues to new pathways for HIV-1 escape *in vivo*.

Summary

The molecular mechanism of human immunodeficiency virus type 1 (HIV-1) entry into cells involves specific interactions between the viral envelope glycoprotein gp120 and two target cell proteins, CD4 and either CCR5 or CXCR4 chemokine receptors. Early in HIV infection, CCR5 utilizing strains (M-tropic or R5) predominate in the patient's serum and are sensitive to the the C-C chemokines (MIP-1α, MIP-1β, and RANTES) which are natural ligands for CCR5. As the disease progresses, CXCR4 utilizing strains (T-tropic or X4) take the upper hand and become resistant to C-C chemokines. Dualtropic (R5X4) strains are thought to be viral intermediates during the shift from R5 to X4 tropism and can use both CCR5 and CXCR4. However, little is known about envelope determinants of dualtropic strains for the usage of multiple chemokine receptors. Also found during disease progression is a selective pressure by the C-C chemokines that might lead to the evolution of X4 HIV-1 variants *in vivo*. In this study we aimed at (i) elucidating the role of HIV-1 envelope domains, from R5X4 and R5 isolates, in CCR5 coreceptor usage and resistance to the MIP-1α chemokine, respectively (ii) determining the role of the MIP-1α chemokine in phenotype switch of HIV-1 from CCR5 to CXCR4 usage.

To identify envelope determinants for CCR5 usage of the R5X4 HIV-1_{KMT}, a panel of chimeric viruses was used in which the V1/V2 and V3 domains of the envelope glycoprotein 120 (gp120) of HIV-1_{KMT} were introduced either alone or in combination into the X4 HIV-1_{NL4-3} envelope background. These chimeric constructs were employed in cell-cell fusion and cell-free virus infectivity assays using cell lines expressing CD4 and CCR5 chemokine receptor. In both assays, the V3 domain of HIV-1_{KMT} but not the V1/V2 domain proved to be the principle determinant of CCR5 coreceptor usage. However, in the cell-free viral infectivity

assay although a chimeric virus with a combined V1/V2 and V3 domains of HIV-1_{KMT} efficiently fused with coreceptor expressing cells, yet its infectivity was markedly diminished in CCR5 expressing cells. Restoring a comparable level of infection of such chimeric virus required the C3-V5 domain from HIV-1_{KMT} to be introduced and thus indicating an important role for the C3-V5 domain in viral infectivity.

To determine which envelope regions of the R5 HIV-1_{JR-FL} isolate were responsible for the reduced sensitivity to the C-C chemokines, we selected MIP-1α resistant variants by continuous passaging of HIV-1_{JR-FL} for three months in MOLT-4#8/CCR5 cells that were treated with increased concentrations of MIP-1α. Sequencing analysis for the V1-V2, V3, V4, C3, and C4 regions of the resistant variants' envelope revealed amino acid substitutions in V2 (valine 166 to methionine) and V3 (serine 303 to glycine) regions. A single-round replication assay using a luciferase reporter HIV-1 strain pseudotyped with mutant envelopes confirmed that mutations in both V2 and V3 were necessary to confer the reduced sensitivity to MIP-1α, as well as to MIP-1β and RANTES. However, the double mutant did not switch its chemokine receptor usage from CCR5 to CXCR4. This indicated for the first time that the V2 combined with the V3 region of the R5 HIV-1 envelope modulates the sensitivity of HIV-1 to C-C chemokines without altering the ability to use the CCR5 coreceptor.

We conclude that a complex structural interaction between the V1/V2, V3 and C3-V5 regions could determine the outcome of viral replication. Moreover, mutations in different variable and constant domains other than the V3 domain could give clues for the resistance of certain HIV-1 isolates to viral entry inhibitors *in vivo*. Collectively, this highlighted the importance of cooperative interaction between various envelope domains in determining viral infectivity and sensitivity to C-C chemokines.

Acknowledgements

I would like to express my deepest gratitude to Professor Shinji Harada for enabling me to conduct my research as a postgraduate student in his department of Medical Virology, School of Medicine, Kumamoto University, Japan. He generously gave me valuable advises, support and continuous encouragement throughout the whole period of my study which resulted in a fruitful worthy theme of research that I hope would contribute to the field of Basic HIV research. I also appreciate his broad and wise understanding of my middle-eastern culture which made life much easier for me in Japan.

I am enormously indebted to Dr. Yosuke Maeda, Associate Professor, Department of Medical Virology, School of Medicine, Kumamoto University. Without his intellectual insights, remarkable instructions and wise supervision, this work would not have been completed in the present form. I am sure his teachings in Molecular Virology will be everlasting and useful throughout my future career.

Special thanks to all members of the Medical Virology Department for their kind support and cooperation. Among them is Dr. Keisuke Yusa, Dr. Song Wei, Mrs Etsuko Kumagai, Mr Akira Tempaku, and Miss Aki Fujioka.

My warmest thanks would also go to Dr Isao Arita, Chairman of the Agency for Cooperation in International health (ACIH) for first introducing me to Japan as a JICA trainee in 1996, during which I started my HIV research with Dr. Shinji Harada. Nevertheless, my thanks would go as well to the Japanese government represented by MONBUSHO (Ministry of Education, Science and Culture, Japan) for providing me with their excellent generous scholarship to conduct my postgraduate studies.

I owe a great deal of respect and appreciation to all my former egyptian professors in the Department of Clinical Pathology, School of Medicine, Cairo University, as well as Professor Koka Saad-Eldin, Department of Microbiology, School of Medicine, Azhar University and Associate Professor Mohamed Elfarrash, Department of Microbiology, School of Medicine, Mansoura University.

Last but not least, my endless gratitude and love would go to my mother, father, parents in law, wife and children for their moral support and tolerance throughout my absence in Japan.

Abbreviations and Chemical Symbols

A	adenine or adenosine
AIDS	acquired immune deficiency syndrome
bp	base pair(s)
C	cytosine or cytidine
CA	capsid protein
$CaCl_2$	calcium chloride
cDNA	complementary DNA
CMV	cytomegalovirus
DMEM	dulbecco modified eagle medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
D-PEPTIDES	D-amino-acid-based peptides
Env gp	envelope glycoprotein
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
G	guanine or guanosine
GFP	green fluorescent protein
HBS	HEPES-buffered saline
HIV	human immunodeficiency virus
HOS	human osteosarcoma cell line
HRP	horseradish peroxidase
IC_{50}	50% inhibitory concentration
IN	integrase
kb	kilobase(s)
KCl	potassium chloride
KDa	kilo dalton
KH_2PO_4	potassium dihydrogenphosphate
LLP	lentivirus lytic peptides
LTR	long terminal repeat
Luc	luciferase
M	molar
MA	matrix protein
MAGI	multinuclear activation of a galactosidase indicator assay
MEM	minimum essential medium
$MgCl_2$	magnesium chloride

MIP	macrophage inflammatory protein
M-tropic	macrophage-tropic
Na_2HPO_4	disodium hydrogenphosphate
NaCl	sodium chloride
NC	nucleocapsid protein
PAGE	polyacrylamide gel electrophoresis
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PBS-T	PBS-tween
PCR	polymerase chain reaction
PR	protease
PVDF	polyvinylidene difluoride
RANTES	regulated upon activation, normal T-cell expressed and secreted
Rev	regulator of gene expression
RRE	rev responsive element
RT	reverse transcriptase
SD	standard deviation
SDF	stromal cell-derived factor
SDS	sodium dodecyl sulfate
SEAP	secreted form of the placental alkaline phosphatase
SIV	simian immunodeficiency virus
SU	surface subunit
T	thymine or thymidine
Taq LA	taq polymerase
Tat	transcriptional transactivator
TM	transmembrane subunit
T-tropic	T-lymphocyte-tropic
Δ	delta
α	alpha
β	beta
β-Gal	β-galactosidase
°C	degree celsius
7-TM	seven transmembrane domain

Background and Objectives

HUMAN IMMUNODEFICIENCY VIRUS (HIV), the subject of this thesis, was recognized relatively recently because of its association with the acquired immune deficiency syndrome (AIDS). This clinical syndrome, characterized by a marked reduction in the number of CD4-positive T-lymphocytes and the development of infections and cancers [103], results from the persistent replication and spread of HIV. AIDS constitutes one of the most serious crises currently facing human development, and threatens to reverse progress in the most severely affected countries by decades. Over the past 20 years since it was first identified, the HIV/AIDS epidemic has continued to exceed all expectations in the severity and scale of its impact. An estimated 40 million people worldwide are currently living with HIV, and some 20 million people have already died [74] (Fig. 1). The worst of the epidemic, and the region where the reality has most out-stripped the predictions, is sub-Saharan Africa where, at the end of 2001, there were an estimated 25.3 million people living with HIV. Moreover, if the number of new infections continues at the current rate, even the most devastating impact that can be anticipated from current levels of infection will seem minor compared with that of the future.

Much progress has been made over the past two decades in understanding the virus and two distinct HIV types have been described. HIV type one (HIV-1) refers to genetically related viruses found in several regions of Africa, Asia, Europe, and both North and South America [76, 120]; HIV type two (HIV-2) is prevalent in certain West African countries [125]. However, many of the previously established concepts about HIV have now been changed. These early concepts included the initial conclusions that the virus only uses the CD4 molecule as its receptor; that the clinical course would be short (not, as is currently known, 10 years for 50% of patients to develop AIDS); and that all HIV strains would be similar in their biologic, serologic, and molecular features. On this latter point,

beside the recognition of the two types, HIV-1 and HIV-2, the diverse and constantly changing properties of each strain are now recognized and present challenges to antiviral therapy and vaccines.

FIG. 1. Worldwide numbers of adults and children estimated to be living with HIV/AIDS at the end of 2001

Structure of HIV

Viewed by electron microscopy (Fig. 2), HIV-1 has the characteristics of a lentivirus. Virions are spherical in shape, about 110 nm in diameter, and consist of a lipid bilayer membrane (envelope), derived from the infected cell, that surrounds a cone-shaped nucleocapsid (Fig. 3). The lipid envelope is characteristically made up of 72 knobs or spikes [46, 57, 117]. Each knob is 9 to 10 nm long with an ovoid distal end, 14-15 nm in diameter, linked to the lipid membrane by a 7-8 nm stalk. The knob is thought to contain three or four heterodimers of the envelope

glycoprotein (Env gp). Biochemical and immunochemical analysis have demonstrated that the nucleocapsid within each mature virion is composed of two molecules of single-stranded RNA encoding the viral genome and encapsulated by proteins processed from the Gag precursor protein (Fig. 3, see next).

The genomic size of HIV-1 is about 9.8 kb long. The two single-stranded RNA molecules are converted after infection into double-stranded linear DNA by the process of reverse transcription. This process involves two strand-transfer steps to synthesize linear viral DNA with long terminal repeats (LTRs) flanking viral genes (Fig. 3). This linear DNA is integrated into the host cell genome to produce the provirus. Accordingly, HIV, like other retroviruses, has two genomic forms: single-stranded RNA in the extracellular phase of the viral life cycle (i.e. virions) and double-stranded DNA (i.e. provirus) within the cell. The genome encodes precursor polypeptides for virion proteins as well as several other open reading frames. The *gag* gene encodes the virion capsid proteins, the *pol* gene encodes virion enzymes, and the *env* gene encodes the Env gp. HIV-1 regulatory genes, transcriptional transactivator (*tat*) and regulator of gene expression (*rev*), are each encoded by two overlapping exons. HIV-1 also encodes genes that are nonessential or accessory for viral replication in tissue culture cells, including *vif, vpr, vpu,* and *nef.*

Products of the *gag* gene include the matrix protein (MA), which provides the matrix for viral structure and is vital for the integrity of the virion [57, 58]; the capsid protein (CA), which functions to package genomic viral RNA into virions and participates in uncoating and possibly other early steps of the viral replication cycle [70]; and the nucleocapsid protein (NC), which binds tightly to the viral RNA genome, and may also link the two viral genomes [133]. A transfer RNA^{lys} molecule is located near the 5' end of each RNA strand to serve as the primer for the initiation of negative strand viral DNA synthesis.

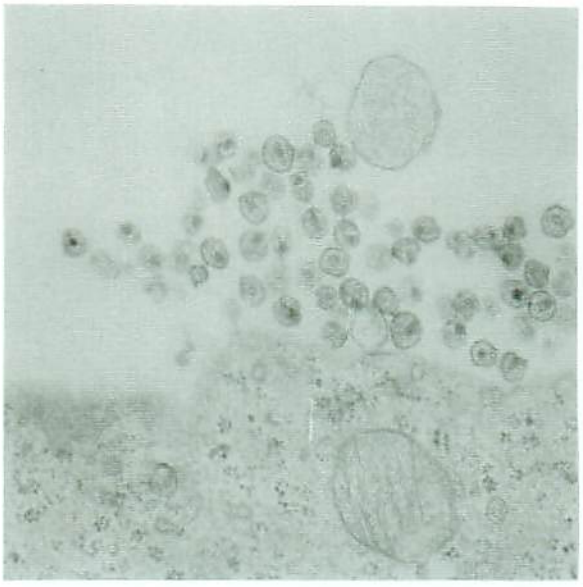


FIG. 2. HIV Virions. Scanning electron micrograph of budding and replicating HIV-1 particles on and inside a T-lymphocyte. Photomicrograph courtesy of Prof. Shinji Harada.

Viral enzymes derived from the *pol* gene precursor polypeptide are incorporated into virions and include protease (PR), that is involved in posttranslational processing of the viral proteins; reverse transcriptase (RT), that synthesizes DNA from RNA; and integrase (IN), that mediates covalent linkage of linear double-stranded viral DNA into the host genome. Closely associated with the core are viral accessory gene products Vif and Nef proteins [14, 92, 164]. Vif may help in proviral DNA synthesis and/or in virion assembly [134], while Nef can increase virus replication. Also found within the virion and most probably outside the core is the Vpr (or Vpx for HIV-2) accessory protein [94], which might play a role in an early step of the viral life cycle such as nuclear localization of the preintegration complex (see next). The Vpu accessory protein, which is not incorporated into virions, enhances virion release and destabilizes CD4 by disrupting the gp160-CD4 complexes [167]. HIV-1 Tat and Rev regulatory proteins are also not incorporated into virions and are translated early in infection from

multiply spliced transcripts. Tat augments levels of viral RNA by increasing transcriptional initiation and elongation [121], while Rev regulates splicing and transport of viral RNA from the nucleus to the cytoplasm [119].

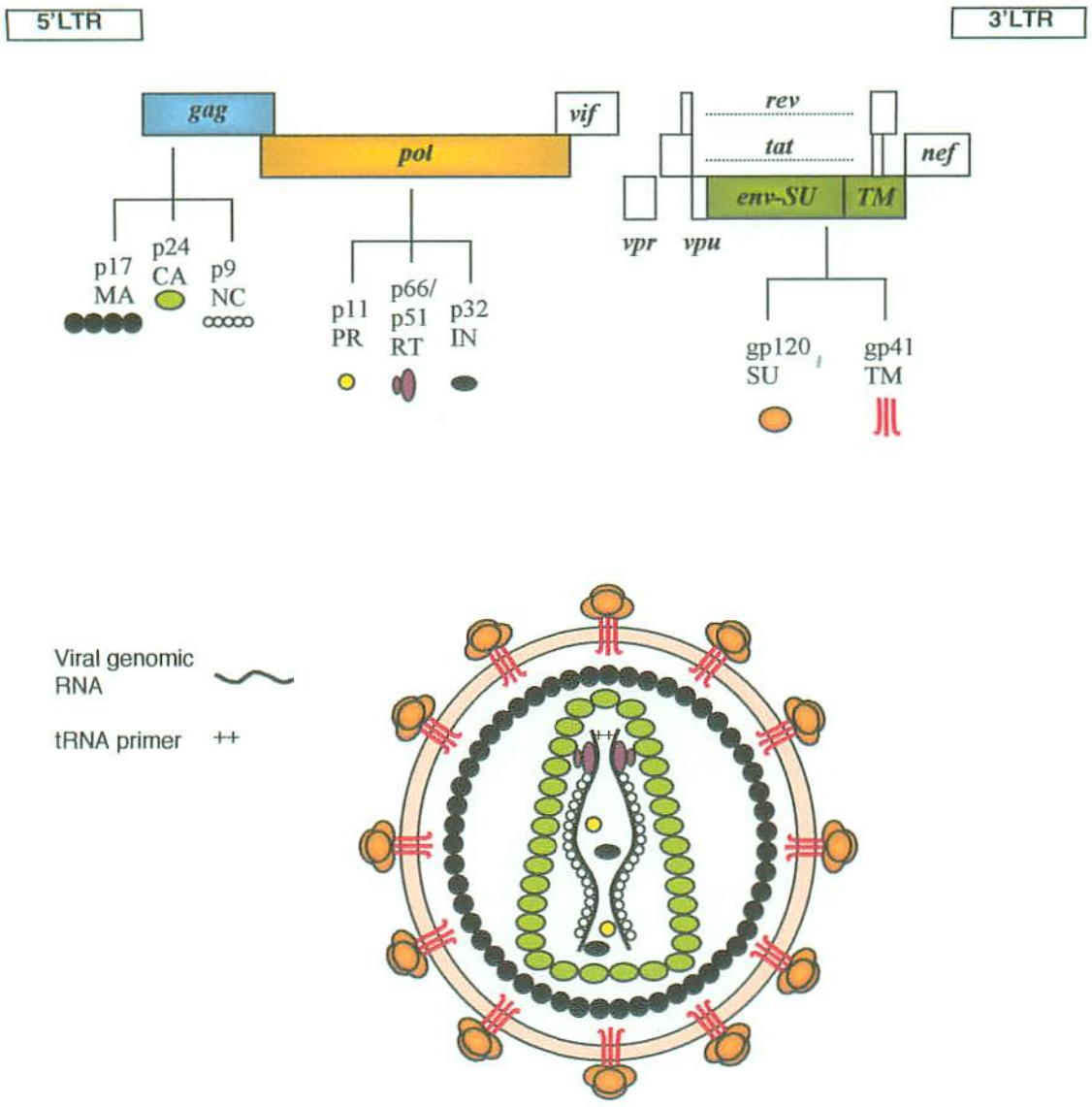


FIG. 3. Genomic map of HIV-1 and Virion Structure. Structural genes (*gag*, *pol*, and *env*) are heavily shaded. Accessory genes, including essential regulatory genes (*tat* and *rev*), and nonessential genes (*nef*, *vif*, *vpu*, and *vpr*), are white. The 5' and 3' LTRs flanking viral genes are shown as open boxes. Symbols representing the various virion proteins are indicated. Env gp are represented as tetramers. Exact positions of the proteins PR, RT, and IN in the viral core have not been elucidated.

Steps in Viral Replication

The replication cycle of HIV is divided into two phases; early and late. Each phase consists of sequential steps and many of these steps involve specific interactions of viral proteins and nucleic acids with host cell factors. The early phase begins with attachment of a virion to the cell surface receptors (CD4 and a coreceptor) and continues to formation of a provirus integrated into the host cell genome (Fig. 4). After binding of the Env gp in the HIV particle to the appropriate receptor on the cell surface, viral and cellular membranes fuse leading to release of the nucleocapsid into the cytoplasm while the viral enzymes RT and IN remain associated with it. The process of reverse transcription produces linear double-stranded viral DNA, which is maintained in a nucleoprotein complex (preintegration complex) that is subsequently transported into the nucleus. In a reaction mediated by IN, the ends of the linear double-stranded viral DNA are covalently linked to host cell DNA to produce the integrated provirus.

The late phase of replication begins with transcription and processing of viral RNA from the integrated proviral template and ends with release of progeny virions from the cell. In the provirus, the 5' LTR encodes *cis*-acting sequences for cellular factors that control initiation of viral transcription by RNA polymerase II, and the 3' LTR contains signals for processing the 3' ends of all viral transcripts (by attachment of poly-A tails). The viral transcriptional transactivator, Tat, acts through a signal at the 5' end of newly initiated viral transcripts to augment initiation and elongation by the host cell RNA polymerase II complex. Unspliced, singly spliced, and multiply spliced viral transcripts are transported to the cytoplasm and translated into various viral proteins. The viral transactivator, Rev, recognizes a *cis*-acting element in the full length viral transcript (rev responsive element, RRE) and controls the ratio of spliced and unspliced RNA; in addition, Rev may control both transport of viral RNA from the nucleus and translation on

cytoplasmic polysomes. A portion of the full-length viral transcripts interacts with virion precursor polypeptides (i.e. Gag and Gag/Pol) to produce immature nucleocapsids at the cell plasma membrane. These nucleocapsids acquire an envelope by budding through areas of the plasma membrane containing the Env gp. In newly released extracellular particles, the Gag and Gag/Pol polyproteins are proteotypically processed by the viral PR to yield fully infectious virions.

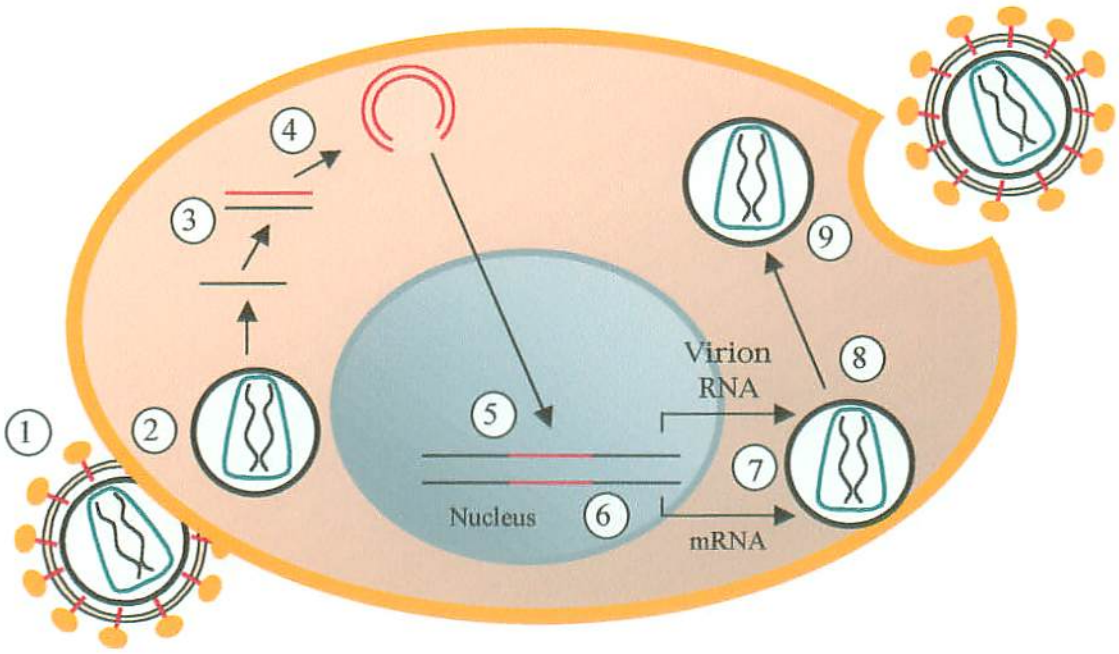


FIG. 4. Overview of HIV replication steps. 1, attachment; 2, uncoating; 3, reverse transcription; 4, circularization; 5, integration; 6, transcription; 7, translation; 8, Capsid assembly; 9, final assembly and budding.

Tropism of HIV-1

Isolates of HIV-1 display marked differences in cell tropism among CD4-positive cells *in vitro*. While all virus isolates replicate in primary CD4-positive T-cells, there tends to be differential ability to replicate in monocyte-derived macrophages and transformed T-cell lines. Macrophage-tropic (M-tropic) isolates are competent to replicate in macrophages but not in transformed T-cell lines, while T-lymphocyte-tropic (T-tropic) isolates replicate in transformed T-cell lines but not in macrophages. Dualtropic isolates, on the other hand, display an ability to grow in both cell types. These replicative designations have profound implications for HIV pathogenesis *in vivo*. M-tropic strains have been shown to be preferentially transmitted [129, 142, 179], while T-tropic strains have been shown to develop in a subset of patients during the course of disease. Emergence of these latter strains has been shown to correlate with progression to AIDS [30, 142, 155]. At the mean time, dualtropic strains may represent an important transitional phenotype between M- and T-tropic strains [29, 95]. Thus, an evolution during disease course from M- to T-tropic strains has been observed, with the emergence of T-tropic viruses being correlated with more rapid immune decline and disease progression.

The HIV-1 Envelope. Early studies showed that tropism was determined primarily by the *env* gene and that the restriction in viral replication in non-permissive CD4-positive target cells was at the level of virus entry [21, 80, 115]. The *env* gene of HIV-1 is responsible for fusion of the virus with host cell membranes, a process required for entry and infection of a target cell by all enveloped viruses [64, 91, 149]. The Env structures are derived from a 160-kDa precursor glycoprotein, gp160, which is cleaved inside the cell into a surface subunit (SU) that is about 550 amino acids long, and a transmembrane subunit (TM) that is about 350 amino acids long [104] (Figs. 3 and 5). SU and TM subunits

were designated gp120 and gp41, respectively, according to their electrophoretic mobility under denaturing conditions in polyacrylamide gels. Env gp120 is a highly glycosylated hydrophilic protein and has a binding domain for the CD4 receptor, located on the plasma membrane of CD4-positive T-lymphocytes, monocytes, macrophages, and dendritic cells, and thereby attach virions to cells. Env gp41 is relatively hydrophobic and traverses once the lipid bilayer of both virions and cells; thus, gp41 is classified as type one integral membrane protein. The gp120 and gp41 subunits remain noncovalently associated and form oligomeric trimers or tetramers on the mature virion surface [18, 162, 163]. Although, the gp120 subunit mediates attachment of the virion to the target cell surface through binding to the CD4 molecule, yet, this binding proved to be insufficient for the fusion reaction to occur. Conformational changes in gp120 induced by the initial binding event with CD4 allow subsequent interactions between gp120 and one of a number of seven transmembrane domain (7-TM) receptors present on the target cell surface (see next). Following the formation of a trimeric complex between gp120, CD4 and the 7-TM receptor, the fusion peptide of gp41 is inserted into the target cell membrane and a series of poorly understood events ensues, culminating in the fusion of the viral and host membranes.

Env determinants of tropism. The *env* gene of HIV-1 isolates displays a remarkable sequence heterogeneity that tends to cluster in distinct regions resulting in five variable (V1-V5) and five constant regions/or domains (C1-C5) for the gp120 subunit [89, 108, 152] (Fig. 5). In contrast, the gp41 subunit shows less heterogeneity and is thus more highly conserved. The fully processed highly glycosylated gp120 molecule folds into a compact structure, which contains many conformationally sensitive and discontinuous epitopes for antibody binding [171].

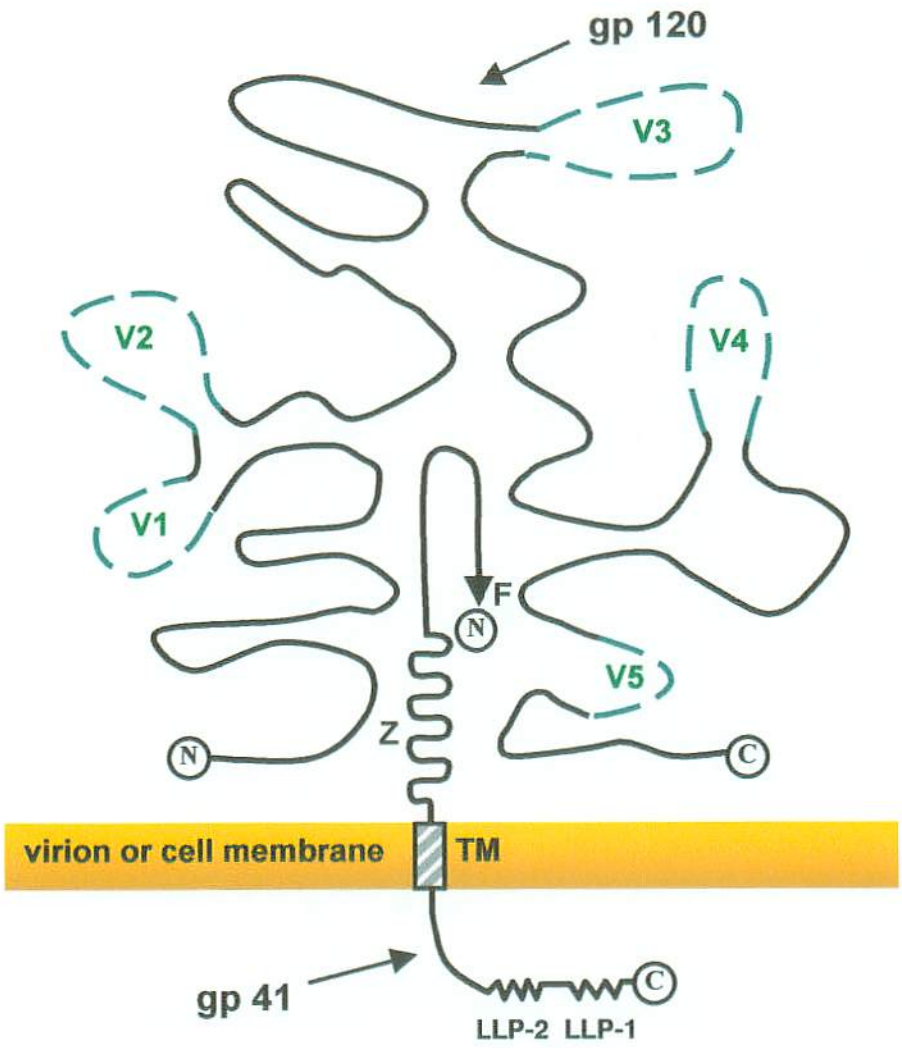


FIG. 5. Predicted folding pattern of HIV-1 Env gp. The predicted folding pattern of the gp120 subunit has been established by enzymatic digestion techniques combined with peptide mapping and sequencing [61]. Hypervariable domains are designated *V1* thru *V5* and are drawn as broken lines. Regions labeled in the gp41 subunit are: F, fusion peptide; Z, leucine zipper like region; TM, transmembrane domain; LLP-1 and LLP-2, lentivirus lytic peptides 1 and 2. Amino and carboxy termini are labeled N and C, respectively, for both subunits. (Adapted from Fields B, Knipe D, and Howley P (eds.): Fundamental Virology, third edition, chapter 27, Human Immunodeficiency Viruses and Their Replication, p859, 1996).

Chimeras between M- and T-tropic isolates have identified the third variable loop (V3-loop) of gp120 as one of the primary determinants of viral tropism [22, 71, 144, 154]. Even as little as a single amino acid change in this region is sufficient in certain isolates to alter cellular tropism [23, 154]. However, many of the mutations that have been identified in the V3-loop which alter tropism have strain-specific effects, and no single residue(s) have emerged as crucial for this phenomenon. The fact that numerous positions within the V3-loop have been implicated in cell tropism seems to imply that a certain degree of plasticity exists in the relationship between this area of Env and viral tropism. One generalization that can be made is that there is an association between the presence of basic residues in the V3-loop and T-tropism: an increase in the net positive charge in the V3-loop has been demonstrated in viral isolates from HIV-positive individuals over time and has been related to conversion to T-tropism [33, 53, 62, 144].

Despite the important role of the V3-loop, the V1/V2 region has also been shown to make important contributions to viral tropism in numerous virus strains. Many chimeric analyses indicated that the V1/V2 region modulates the efficiency of virus replication in a V3-dependent fashion, suggesting that V1/V2 and V3 interact in some way [16, 60, 83, 143, 156, 165]. Indeed, mutations in V1/V2 have been shown to alter binding of anti-V3-loop antibodies [54, 84]. The association between T-tropism and the accumulation of positive charges has also been noted in the V1/V2 region, again suggesting that the V1/V2 and V3 regions may play a similar and perhaps cooperative role in mediating virus tropism [60]. However, the V1/V2 region may not be as critical for Env function as the V3-loop that is highlighted by the fact that viral replicative ability can be maintained following deletion of the V1/V2 region [15, 172]. Removal of both regions still permits CD4 binding and does not seem to globally disrupt Env architecture based on antibody binding to other conformational structures [153, 172, 173]. Yet, there are indications that the V1/V2

region is important for Env function, given that mutations in V1/V2 are sufficient to change virus tropism under certain conditions [60, 84] and neutralizing antibodies exist to this region of Env [55, 59].

Coreceptors and HIV-1 infection

Since the identification of CD4 as the essential receptor for HIV more than a decade ago, it has been widely appreciated that additional cellular molecules are required for the entry of HIV into target cells. It was shown at an early stage that the expression of CD4 on the surface of human cells could render them susceptible to infection by HIV-1 virions and permitted envelope induced cell fusion [98]. Conversely, expression of human CD4 on the surface of murine cells did not confer susceptibility to infection, although CD4-positive murine cells are fully competent for binding the viral envelope glycoprotein gp120. In fact, almost all non-human cells remain refractory to HIV-1 infection even when engineered to express human CD4. In addition, it was shown that the resistance of CD4-positive murine fibroblasts to HIV-1 could be reversed by fusion with CD4-negative human cells, indicating that there is no dominant block to virus entry [44]. Taken together, these observations strongly implied the existence of additional human cell specific cell surface molecules that participate in virus entry. Until recently, the identity of these 'coreceptors' had remained elusive. However the identification of several chemokine receptors as molecules that, in conjunction with CD4, can mediate the entry of HIV and/or simian immunodeficiency virus (SIV) into target cells has led to an explosion in interest in this field, as well as a very rapid advance in our understanding of how primate lentiviruses infect target cells.

Chemokine receptors as coreceptors for HIV. The crucial advance in the path for the discovery of chemokine receptors as coreceptors for HIV was made by Feng *et al.* [51] who identified Lestr/fusin, a member of the 7-TM spanning,

G-protein coupled receptor family, as a fusion cofactor for T-tropic HIV-1 strains. The normal function of these receptors is to bind chemotactic cytokines (or chemokines), which signal the responsive cells to migrate towards or away from sites at which chemokines are released as part of a continuing immune response [7]. Thus, expression of CD4 and fusin on the surface of murine cells was sufficient to render them susceptible to infection by T-tropic HIV-1 strains. Furthermore, a CXC chemokine named stromal cell-derived factor-1 (SDF-1), has subsequently been identified as a ligand, which both binds fusin and specifically blocks infection by T-tropic but not M-tropic HIV-1 strains [10, 114]. Based on the identification of its chemokine ligand, fusin was renamed CXCR4. This discovery gave added significance to the earlier finding that the C-C chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β, and RANTES (regulated upon activation, normal T-cell expressed and secreted) are able to block infection of CD4-positive human T-cells by M-tropic but not T-tropic HIV-1 [27]. Soon after this, a number of groups independently showed that CCR5 served as a coreceptor for a wide range of M-tropic strains [1, 26, 35, 42, 45]. Moreover, disease progression in HIV-1-infected individuals was found to be associated with a gain of CXCR4 usage [31, 37] and with a loss of sensitivity to the C-C chemokines [72, 137], coinciding with the phenotypic change from M- to T-tropism. These results suggested that the change of chemokine receptor usage from CCR5 to CXCR4 may have a key role in the pathogenesis of HIV. However, it remains to be solved how HIV can acquire the ability to use CXCR4 during the course of infection.

In addition to the principal coreceptors CCR5 and CXCR4, other chemokine receptors including CCR3, CCR2b, CCR8, and CX_3CR1 can support fusion and entry by more restricted groups of strains [26, 42, 131]. Additionally, a growing number of orphan 7-TM receptors including STRL33, GPR1, GPR15, and APJ mediate entry by various HIV-1, HIV-2, or SIV isolates [25, 36, 47, 50, 90, 131]. US28, a chemokine

receptor encoded by cytomegalovirus (CMV), also supports fusion by several strains [123], which may be particularly relevant because HIV-infected people are frequently coinfected with CMV [101]. Nevertheless, there is good evidence that CCR3 can be used efficiently by a significant fraction of M-tropic HIV-1 isolates *in vitro* [26], and one particularly promiscuous strain (HIV-$1_{89.6}$) could make use of no less than four distinct coreceptors; CCR5, CXCR4, CCR3 and CCR2b [42].

The discovery of members of the chemokine receptor family as coreceptors for HIV has gone a long way towards explaining the differences in cell tropism by HIV-1. The majority of M-tropic strains utilize the chemokine receptor CCR5, which is present on macrophages and primary T-cells [1, 26, 35, 42, 45] (Fig. 6). T-tropic strains, on the other hand, utilize CXCR4, which is present on primary T-cells, transformed T-cell lines, and at lower levels on macrophages [51, 175]. The ability of dualtropic strains to use both CCR5 and CXCR4 explains the ability of these viruses to infect all three cell types [26, 42, 147] (Fig. 6). Therefore, M-, T-, and Dualtropic strains were designated R5, X4, and R5X4, respectively.

The importance of the HIV-1 coreceptors has also been highlighted by the fact that individuals who possess two alleles of a 32-base pair deletion in the CCR5 gene that abrogates cell surface expression of this molecule display near absolute resistance to HIV-1 transmission and infection by M-tropic strains [34, 93, 107, 126, 136]. Specifically, approximately 1% of the CCR5 alleles in Caucasian populations contain a 32-base pair deletion, which results in a frame shift and premature truncation of the receptor. These truncated receptors are not expressed on the cell surface and therefore do not function as HIV coreceptors. About 20% and 1% of Caucasians are heterozygous and homozygous, respectively, for the CCR5-delta 32 allele.

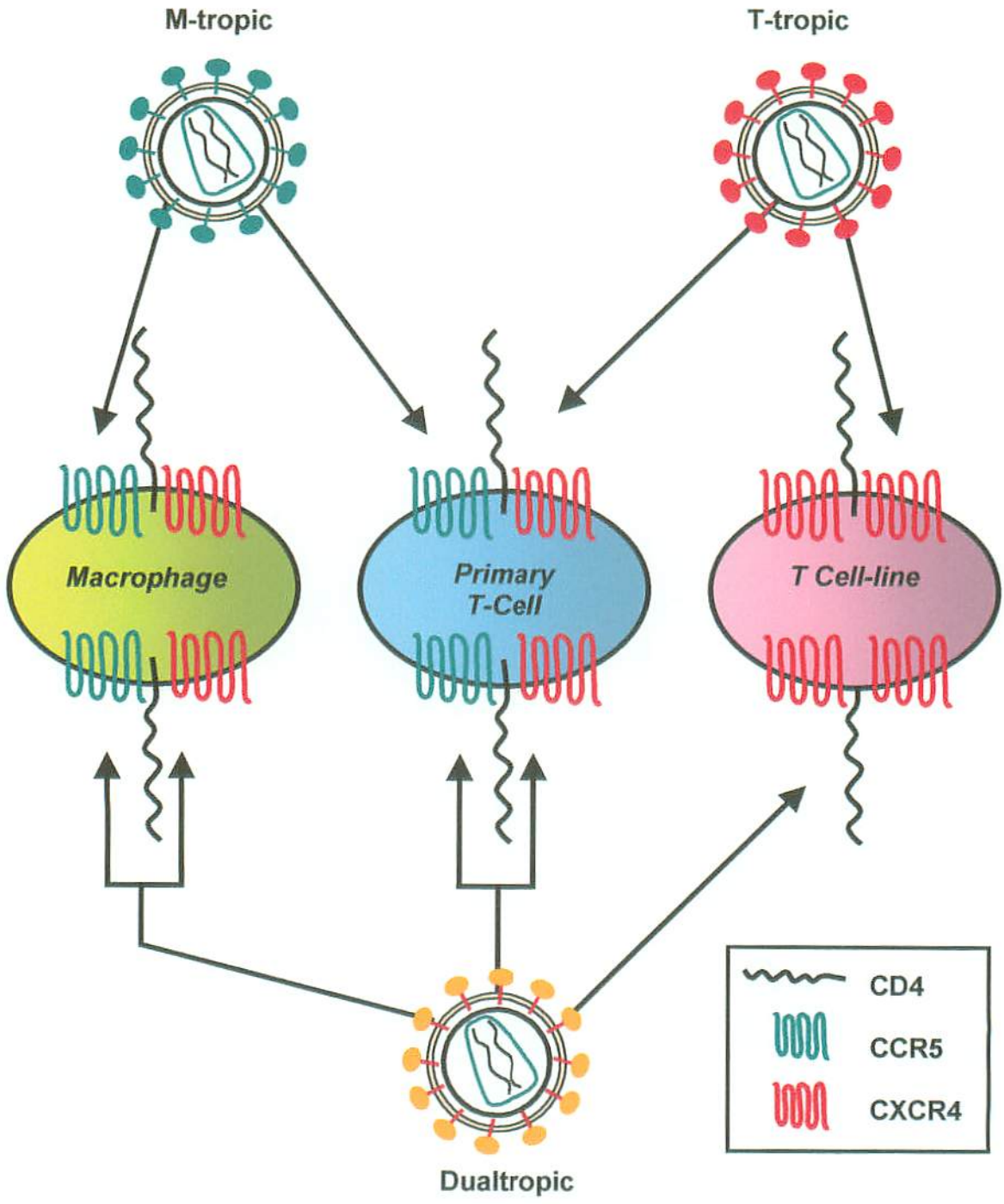


FIG. 6. Chemokine receptor usage and HIV-1 cell tropism. Three patterns of cellular tropism have been identified for isolates of HIV-1 on CD4-positive target cells *in vitro*. While all isolates of HIV-1 can replicate in primary T-cells, M-tropic (also called R5) strains replicate in macrophages but not transformed T-cell lines, while T-tropic (also called X4) strains replicate in transformed T-cell lines but not macrphages. Dualtropic strains (R5X4) are able to replicate in all three cell types.

Importantly, homozygosity for the CCR5-delta 32 allele is associated with a significant degree of infection resistance, and individuals who are heterozygous for the two major CCR5 alleles do not manifest a high degree of infection resistance [34, 136]. However, once infected, the progression of disease in heterozygotes appears to be somewhat retarded. This phenotype is associated with a measurably reduced virus load postseroconversion, and a decrease in frequency of symptomatic primary infection [106]. Taken together, these observations strongly imply that the major route of HIV transmission both between individuals and between cells within an individual (at least during the early stages of infection) is mediated by the CCR5 coreceptor.

Env determinants of chemokine receptor use. Previous work on mapping the regions of *env* involved in virus tropism allowed several groups to rapidly show that the V3 region is the primary determinant of coreceptor use [24, 26, 27, 130, 148, 150]. Subsequent work has shown that the specific nature of the V3 region (i.e. whether it is from an R5 or X4 strain) is the primary determinant of direct Env-chemokine receptor binding and of Env-mediated inhibition of chemokine and monoclonal antibody binding to chemokine receptors [27, 157, 169]. Due to drastic increases in the ability of Env to interact with the chemokine receptors in the presence of CD4, a model has been proposed where virus entry is mediated by an initial binding event between Env and CD4 which promotes subsequent interactions between Env and chemokine receptors [75, 87, 157, 169]. As noted above, the V3 region of many T-tropic isolates contains more basic residues than their M-tropic counterparts. Structure-function studies on CXCR4 indicate that the domains of this receptor most important for T-tropic virus entry are markedly acidic [8], suggesting that electrostatic interactions may be important between the acidic regions of CXCR4 and the basic V3 region of T-tropic isolates. However, structure-function studies on other chemokine receptors have indicated that there

are structural complexities involved in the interaction between Env and chemokine receptors that reach beyond the level of a simple charge-charge interaction [161].

As was the case for many of the studies on virus tropism, other regions of Env besides the V3 region may affect chemokine receptor use. Consistent with the modulatory effects of mutations in the V1/V2 region on virus tropism, this region also regulates the efficiency of chemokine receptor-mediated virus entry [58, 67]. The requirement of a cooperative interaction between V1/V2 and V3 for maximal usage of a chemokine receptor by Env has been demonstrated for numerous HIV-1 coreceptors, including CCR2b, CCR3, STRL33, and APJ [66, 150]. Thus, both studies on tropism and chemokine receptor use suggest that there is a critical cooperative effect of the V3 and V1/V2 regions in mediating interactions between Env and chemokine receptors on target cells.

Despite the apparent simplicity of the model that macrophage-tropism is mediated via CCR5 while T-tropism is mediated via CXCR4, there are some important exceptions to this model which suggest that understanding of virus tropism and Env-chemokine receptor interactions is incomplete. One observation that remains a mystery is the failure of most T-tropic isolates to utilize CXCR4 on macrophages for productive replication (but not other cell types such as primary T-cells and transformed T-cell lines) [126, 138, 175]. While the block in T-tropic virus infection of macrophages has been shown to be at the level of virus entry by some groups [148] and at post-entry level by others [68, 138], all studies agree that the prototype X4 strains fail to replicate in macrophages. However, most of these studies have been performed using virus strains, which have been extensively passaged *in vitro* and may not be representative of primary isolates, which also display an ability to grow in T-cell lines. In fact, some primary isolates have been shown to replicate in macrophages through a CXCR4-dependent pathway [175]. This suggests that CXCR4 present on macrophages is competent to support

replication under some circumstances, and that there are differences in the virus-coreceptor interaction in different cell types. Also studies using the dualtropic primary isolate HIV-$1_{89.6}$ [29], which is able to replicate in macrophages by CXCR4- and CCR5-dependent pathways [175], indicated that the *env* determinants of tropism and coreceptor use may comprise overlapping but distinct domains of *env*. While the *env*-determinants of coreceptor use for HIV-$1_{89.6}$ are present in V3-dependent and V3-indepenent domains of *env* [148], regions of gp120 outside V3-V5 are responsible for the ability of this virus to replicate in macrophages [79]. Thus, the V3 region of HIV-$1_{89.6}$ is able to confer CCR5 use to an X4 T-tropic virus (which cannot grow in macrophages), but a virus containing this *env* displays a post-entry block in macrophage replication [148].

Virus-entry inhibitors

Death rates due to HIV-1 infection have fallen considerably since specific inhibitors were developed to antagonize the viral reverse transcriptase and protease enzymes at least in those countries that can afford combination antiviral therapies (roughly $10,000 per year, per patient) [118]. Although over a dozen drugs are now on the market, they all target only these two viral enzymes. But up to 20% of patients cannot tolerate antiviral cocktails in the short term [96]; there is increasing concern about the long-term metabolic side-effects of protease inhibitors (notably, poorly understood problems with fat metabolism); and drug-resistant HIV-1 variants are emerging and spreading at an increasing rate [174]. Because antiviral therapy cannot eradicate HIV-1 from infected people [56], it became necessary to identify new classes of drugs suitable for long term use that can supplement, or partially replace, existing drug regimes.

Several stages of the viral life cycle are potentially vulnerable to specific inhibitors. These can be divided into the entry steps, which involve viral envelope

glycoproteins and their receptors, and the post-entry steps involving viral accessory gene products and the cellular proteins with which they interact. Although both viral and cellular factors can be targeted, it is usually less toxic to attack a viral factor than to disturb the function of a host protein. Therefore, I will try to highlight different approaches to target viral entry steps.

Targeting gp120–CD4 binding. The fusion process, which involves binding of the envelope glycoprotein complex to the cell-surface CD4 antigen, is vulnerable to agents that bind to either gp120 or CD4. The crystal structure of gp120 has revealed details of the CD4-binding site, including a recessed pocket that could accommodate a small-molecule inhibitor of CD4 attachment [86] (although no such inhibitor has yet been reported). It has been shown that polyanions including, sulphated polysaccharides such as dextran sulphate destabilize the functions of the envelope glycoprotein complex [12]. Negatively charged albumins also bind gp120 to prevent a stable association between HIV-1 and CD4-positive cells, and perhaps block gp120–CD4 attachment [13]. A larger, and more specific, inhibitor that binds to gp120 and acts as a soluble receptor decoy to prevent its association with CD4 is the soluble CD4 molecule. In its original incarnation as a monomeric protein, soluble CD4 showed no notable antiviral action *in vivo*. This was because, despite its efficient neutralization of cell-culture-adapted viruses *in vitro*, it had no effect against primary HIV-1 isolates of the type found in infected people. Peptides that mimic the gp120-binding region of CD4 domain also have an antiviral effect *in vitro* [160, 178], although their potency will probably need to be increased from the present micromolar range if they are to have antiviral activity *in vivo*.

The binding of gp120 to CD4 can also be blocked by targeting CD4. Monoclonal antibodies against CD4, such as Leu3a or OKT4A, do this efficiently *in vitro*. However, concerns about immunosuppression through depletion of

CD4-positive cells will probably prevent the clinical use of such antibodies. These concerns may be lessened for one anti-CD4 monoclonal, 5A8, which blocks virus-cell fusion after CD4 binding and seems not to cause depletion of CD4-positive cells in monkeys [127]. Whether 5A8 will have other immunosuppressive effects through its binding to CD4 is not clear. None of the above inhibitors of gp120–CD4 binding will make orally available drugs because they are protein-based compounds, although delivery by intravenous or subcutaneous injections is an alternative option.

Targeting gp120–coreceptor interactions. CCR5 and CXCR4 are the most important among coreceptors discovered as front-line pharmacological targets since they are the main coreceptors used by HIV-1 to enter primary CD4-positive T cells and macrophages. Blocking the function of CCR5 may have no negative side effects in patients because roughly 1% of Caucasians naturally lack this protein [34] (see before). In fact, *in vitro* experiments [170] and epidemiological studies [34] indicated that a reduced level of CCR5 can be beneficial by decreasing the ability of HIV-1 to infect cells. Inhibiting CXCR4 may be more problematic, as a CXCR4 deletion prevents fetal development in mice [180]. However, a CXCR4-specific inhibitor was not acutely toxic in adult mice [32].

The first inhibitors known to prevent coreceptor interactions of the viral envelope glycoproteins were the natural CC-chemokine ligands of CCR5 (MIP-1α, MIP-1β, and RANTES)[27]. Likewise, the CXC-chemokine (SDF-1α) inhibits entry through CXCR4 [10, 114]. Variants of these chemokines, usually amino-terminal modifications to RANTES or SDF-1α with increased potency and/or altered agonist properties *in vitro* were soon developed, as were short peptides with some antiviral activity, which were based on the amino-terminal chemokine domain [65, 146]. The chemokine-based inhibitors can interfere with HIV-1 replication in many ways. First, the chemokine and the gp120 may compete for binding to the

coreceptor; second, the coreceptor may be downregulated after chemokine binding and signal transduction; and third, signaling may alter the differentiation state of the target cell and affect HIV-1 replication later in the viral life cycle [2, 65, 97, 146, 159]. The signaling capacity of chemokines will probably affect their clinical use. Although it is not desirable to use agonists that affect the target cells for HIV-1 replication, non-agonists cannot cause coreceptor downregulation and therefore have reduced potency [2, 65, 97, 146]. *In vitro*, both CC- and CXC-chemokines can, under some conditions, enhance HIV-1 replication through their effects on target cells [82, 102, 158], and a modified RANTES derivative promoted the evolution of X4 viruses from one R5 strain when tested in a mouse model system [110].

Monoclonal antibodies targeted against CXCR4 and CCR5 can have considerable potency and breadth of action as inhibitors of HIV-1 replication *in vitro* , without being agonists [48, 116, 170]. As with chemokines, monoclonals will not be orally available, but they could be used as injectable agents. In principle, monoclonals might cause cellular depletion through immune targeting, which will need to be evaluated as suitable candidates for entering clinical trials.

Several small-molecule inhibitors of HIV-1 entry through CXCR4 and CCR5 are now known, all of which are receptor antagonists that have no signaling capacity themselves. CXCR4 inhibitors include highly cationic compounds such as T22, ALX40-4C, and AMD3100 (and their derivatives) [3, 40, 41, 111, 140], as well as cationic peptides derived from the V3 region of HIV-1 gp120 [135]. The only small-molecule CCR5 inhibitor whose structure has been described is TAK779, a non-agonist that blocks both virus entry and chemokine binding and likely binds to a hydrophobic pocket formed largely by the transmembrane domain helices of CCR5, a region that until now has not been directly implicated in coreceptor function [6]. With its single positive charge, TAK779 differs chemically from all the known CXCR4 inhibitors. This difference probably reflects the surface charges of

the two co-receptors, whereas the surface of CXCR4 is strongly anionic, CCR5 has an almost neutral surface, at least before posttranslational modifications. The difference in the physicochemical properties of the two coreceptors also, no doubt, affects the types of compounds that are identified as inhibitors in screens of compound libraries.

Highly polar compounds such as T22, ALX40-4C and AMD3100 often have limited bioavailability, which restricts their potential as drugs. This problem will be exacerbated for ALX40-4C and T22 (and its derivatives T134 and T140) because they are peptides [3, 41, 111]. Although AMD3100 has negligible oral bioavailability, when delivered intravenously or intraperitoneally by infusion pumps it showed antiviral activity in a mouse model, without acute toxicity [32]. AMD3100 is now being evaluated in human clinical trials (using non-oral delivery mechanisms). A distamycin analogue, NSC 651016, apparently blocks the interactions of chemokines with many receptors (including both CXCR4 and CCR5) and has anti-HIV-1 activity *in vitro* [67]. How it achieves this is not yet known.

Targeting gp41-mediated fusion. The final stage of fusion is mediated by conformational changes in the trimeric gp41 glycoprotein, which force the amino-terminal fusion peptides into the target cell membrane. The potential of this process as a drug target was first revealed when peptides derived from gp41 were found to inhibit HIV-1 infection *in vitro* [166]. These peptides substitute for one or more components of the gp41 trimer, thereby inhibiting the conformational changes and preventing the intermolecular interactions necessary for fusion [75]. One such peptide, T20, reduces viral load significantly in clinical trials (the first demonstration that a fusion inhibitor works in humans) [78]. Because it is a peptide, T20 was initially developed as an injectable drug and it is now being delivered by the subcutaneous route. A related peptide with increased potency *in vitro*, T1249, is now in clinical trials.

A possible target in gp41 is a cavity of a suitable size and in an appropriate location to act as the binding site for a small-molecule fusion inhibitor [52]. Protease-resistant, and therefore relatively stable, D-amino-acid-based peptides (D-PEPTIDES) that fit into this pocket have been identified, and inhibit membrane fusion in the 10–100 μM range [52]. Improvements to these peptides seem possible, and they can be used in assays to screen conventional chemical libraries for small molecules able to recognize the gp41 pocket [52].

Escape pathways. When entry inhibitors are used *in vivo*, HIV-1 will escape from them. But which pathways will it use? For the gp41 peptides, such as T20, the most efficient escape pathway has been defined [124]. Sequence changes in two amino-acid residues, within the T20-binding region of gp41, are enough to confer resistance *in vitro* by reducing the binding affinity of gp41 for the peptide [124]. Negatively charged albumins that bind to gp120 to prevent its association with CD4 can be evaded by mutant viruses with sequence changes in and around the V3 region, which in turn lower the strength of the albumin–gp120 interaction [13].

Many coreceptor-targeted inhibitors have been studied *in vitro*. Several mechanisms for escape are possible, depending on how the experiment is designed. For instance, the escape mutant may continue to use the same coreceptor in an inhibitor-insensitive manner; coreceptor switching may occur (so an R5 virus becomes able to use CXCR4, or vice versa); or an entirely different coreceptor may be used by the escape mutant. So far, the first mechanism is the most common, whereas the third has not been found *in vitro*. Any structural alterations in the viral envelope glycoproteins that create a coreceptor inhibitor escape mutant must, of course, allow these proteins to retain their natural resistance to the action of neutralizing antibodies. Otherwise, the atypical sensitivity of the escape virus to the host's humoral immune response would compromise its ability to persist.

Escape-mutant studies have been done on several CXCR4-specific inhibitors. Initial studies with AMD3100 showed that, after 63 passages with increasing doses, the mutant virus had 15 amino-acid changes scattered throughout gp120, indicating that escape from AMD3100 is not easy [168]. Follow-up studies confirmed that the escape mutant still used CXCR4, but in an AMD3100-insensitive manner [49, 139]. So there may be more than one way for gp120 to contact CXCR4, while still permitting fusion. A similar experiment was done with SDF-1α, with a similar outcome [139]. There was partial cross-resistance between the AMD3100- and SDF-1α-resistant viruses, with about half the amino-acid substitutions in gp120 common to the two escape mutants [139].

Coreceptor switching was not possible in the above experiments because a CCR5-negative cell line was used. When AMD3100 resistance was selected for isolates that could use either CCR5 or CXCR4 to enter peripheral blood mononuclear cells (PBMCs) expressing both coreceptors, R5 viruses rapidly dominated the cultures [49]. But when viruses able to use only CXCR4 were used, they mutated under drug-selection pressure to acquire CCR5 usage [49]. So far, escape-mutant studies with CCR5-specific inhibitors have been more limited. An initial report using RANTES derivatives in an animal model concluded that an R5 virus switched to use CXCR4 [110]. But this may not happen with other CCR5-targeted compounds, and hence, additional studies still are required to address the role of other different CCR5 ligands (MIP-1α, MIP-1β, and RANTES).

Objectives of the current study

- Since few dualtropic strains have been tested so far and little is known about determinants of dualtropic envelope glycoproteins that enable them to use multiple chemokine receptors for cellular entry. We therefore, attempted to shed more light on the envelope determinants of tropism and cofactor selectivity for dualtropic isolates by widening the spectrum of strains being tested. HIV-1_{KMT} is a recently isolated dualtropic strain [109] that has been formerly used in a chimeric study to determine the role of its V1/V2 region in modulating the sensitivity to neutralization by soluble CD4 and cellular tropism. In the current study, we wanted to identify which envelope domains of HIV-1_{KMT} are necessary for CCR5 usage? This could be accomplished by testing the ability of recombinant viruses, constructed between HIV-1_{KMT} and the T-tropic HIV-1_{NL4-3} isolate, to fuse with and infect different CD4-positive cell lines expressing the CCR5 coreceptor.

- Although the C-C chemokine production level varies in different clinical stages of HIV-1-infected individuals [5, 105, 177], we hypothesized that the selective pressure by the natural ligands for CCR5, including MIP-1α, MIP-1β, and RANTES, may lead to the evolution of HIV-1 variants *in vivo*. Several studies which have indicated that these C-C chemokines inhibit R5 virus but enhance X4 virus [38, 82] also supported our hypothesis. However, escape-mutant studies with CCR5-specific inhibitors have been limited. Therefore, we sought to determine (i) which envelope regions of the R5 HIV-1_{JR-FL} are responsible for the reduced sensitivity to the C-C chemokines. (ii) whether X4 or R5X4 viruses emerge from R5 HIV-1_{JR-FL} by selective pressure of ligands for CCR5.

Materials and Methods

Cells and culture conditions

- **NP-2/CD4** [73], a CD4-expressing human glioma cell line, was provided H. Hoshino, maintained in Eagle's minimum essential medium (MEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco BRL), 2mM L-glutamine, 100 µg/ml penicillin and streptomycin. CXCR4 or CCR5 expressing NP-2/CD4 cells (NP-2/CXCR4 and NP-2/CCR5), were maintained in MEM supplemented with 10% FCS, 100 µg/ml of G418 and 1 µg/ml puromycin.
- **COS-7 and 293T**, are monkey kidney and human embryonic kidney cell lines, respectively. They were maintained in Dulbecco modified Eagle medium (DMEM) (ICN, Costa Meda, CA, USA) supplemented with 10% FCS and 100 µg/ml of penicillin and streptomycin.
- **GHOST**, a human osteosarcoma cell line (HOS), was provided by V. KewalRamani and D. Littman through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), USA. Maintenance of cells was carried out in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 µg/ml penicillin and streptomycin, 200 µg/ml of G418, 25 µg/ml of hygromycin, and 1 µg/ml of puromycin [17, 77]. These cells were stably transfected with human CD4, green fluorescent protein (GFP) reporter gene under control of the HIV-2 long terminal repeat (LTR), and one of the coreceptors (CXCR4, CCR5, CCR2b, CCR3, CCR8, BOB, Bonzo, and V28).
- **MOLT-4#8, CEM, MT-4, and MT-2**, are CD4-positive T cell leukemia cell lines grown in RPMI 1640 (Gibco BRL) medium supplemented with 10% heat-inactivated FCS, 2mM L-glutamine, and 100 µg/ml of penicillin plus streptomycin. MOLT-4#8 and CEM are acute lymphoblastic leukemia cell lines, while MT-4 and MT-2 are adult T cell leukemia cell lines.
- **HeLa-CD4/LTR/β-galactosidase (MAGI)**[81], a cervical carcinoma cell line that was kindly provided by M. Emerman through the AIDS Research and Reference Reagent Program (NIAID, NIH) and maintained in DMEM supplemented with

10% FCS, 2 mM L-glutamine, 100 μg/ml of penicillin plus streptomycin, 100 μg/ml of G418, and 50 μg/ml of hygromycin B (Wako, Osaka, Japan).

Reporter gene plasmids

- **pNL-Luc-ΔBgl**, is a luciferase-expressing HIV-1 plasmid (the HIV-1_{NL4-3} provirus backbone with a *luciferase reporter* gene insertion instead of *nef* gene) that was provided by ISY. Chen.
- **pLTR-SEAP**, is a plasmid with an HIV-1/LTR driven secreted form of the placental alkaline phosphatase (SEAP). It was constructed by the insertion of HIV-1 LTR from pU3R-III CAT (provided by J. Sodroski through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) into pSEAP2-basic (Clonetech, Palo Alto, CA, USA) using *Xho* I and *Hind* III restriction enzyme sites.

Construction of chimeric envelopes and infectious molecular clones

The PCR fragment from the primary isolate HIV-1_{KMT} and the infectious chimeric molecular clones were constructed as described previously [109]. For the preparation of the HIV-1_{NL4-3} and HIV-1_{JR-FL} envelope expression vectors (pCXN/NLenv and pCXN/FLenv), the HIV-1_{NL4-3} and HIV-1_{JR-FL} *env* regions were cloned by PCR and ligated into a TA cloning vector, pCR2.1 (Invitrogen, NV Leek, Netherlands), generating pCR2/NLenv and pCR2/FLenv. The sequence of the amplified *env* region of HIV-1_{NL4-3} and HIV-1_{JR-FL} was confirmed by using an ABI Prism 377 automated DNA sequencer (Applied Biosystems, Foster city, CA). The *Eco*R I fragment of pCR2/NLenv and pCR2/FLenv containing the entire *env* region was ligated into pCXN2 expression vector with the chicken β-actin promoter to give pCXN/NLenv and pCXN/FLenv. The pCXN/chimeric envelope expression vectors were constructed by the substitution of the *Dra* III – *Xho* I fragment from the pKS/chimeric infectious molecular clones into pCXN/NLenv.

HIV-$1_{KMT/L}$ *env* was cloned by PCR from virus recovered after infection of the MT-2 cell line with the primary isolate HIV-1_{KMT} as in the following protocol:

Protocol 1.

A. Extraction of viral DNA from infected MT-2 cells:

1. Pellet 200 µl of MT-2 cell suspension in a microfuge tube. Spin at 10,000 rpm for 1 minute and remove the supernatant.
2. Resuspend the cells in 1 ml of 1X PBS and centrifuge for 1 minute at 10,000 rpm.
3. Resuspend cells in autoclaved water at 20-30 cells/ml.
4. Add 20 µl of the cell suspension to 200 µl of InstaGene matrix (Instagene, Bio-Rad, CA, USA). Incubate at 56°C for 20 minutes.
5. Vortex at high speed for 10 seconds. Place the tube in a 100°C heat block for 8 minutes.
6. Vortex at high speed for 10 seconds. Spin at 10,000 rpm for 3 minutes.
7. Use 20 µl of the resulting supernatant per 50 µl PCR reaction. Store the remainder in –20°C.

B. Design of primer sets:

Forward: 5'-**GTGGTCCATAGTAATCATAGAATAT**-3' (nt 6123-47 of HIV-1_{NL4-3})
Reverse: 5'-**TTCTAGGTCTCGAGATACTGCT**-3' (nt 8879-900 of HIV-1_{NL4-3})

C. PCR reaction mixture: Volume is adjusted to 50 µl in total.

Template (DNA from cellular lysate) (1µg)	5µl
Forward primer (100 picomoles)	1µl
Reverse primer (100 picomoles)	1µl
Taq LA (Takara, Tokyo, Japan) (2.5 units)	0.5µl
10X Buffer	5µl
dNTP (0.2 mM)	8µl
$MgCl_2$ (1.5 mM)	5µl
Distilled Water	24.5µl

D. PCR cycles:

Temperature	Time	Number of cycles
94°C	2 minutes	1
98°C	10 seconds	35
55°C	1 minute	
72°C	4 minutes	
72°C	10 minutes	1
4°C	Indefinite	0

The amplified product was then cloned into a pCR2.1 cloning vector and DNA sequences were verified using an automated sequencer, ABI 310 (Applied Biosystems). The 2.7 kb *env* coding fragment was then ligated into pCXN2 using the *Eco*RI site to produce pCXN/KMT/Lenv. To generate recombinants between envelopes of HIV-1_{NL4-3} and HIV-$1_{KMT/L}$, both *env* fragments were first subcloned into pUC18 (Pharmacia) using the *Eco*R I site to generate pUC/NLenv and pUC/KMT/Lenv. Chimeric molecules were generated by using shared restriction sites (*Dra* III *and Stu* I sites for V1/V2 and two *Bgl* II sites for V3-V5) and standard subcloning techniques. For the substitution of the C3-V5 region, the *Nhe* I restriction site was introduced into the pUC/KMT/Lenv by point mutation using recombination PCR. Mutagenesis is achieved by PCR with the use of specially designed primers that include in their sequence the desired changes for the *Nhe* I restriction site that is to be incorporated in the gene construct (Fig. 7). Because the mutagenizing primers also have terminal complementarity, two overlapping fragments can be fused together in a subsequent extension reaction. The inclusion of outside primers in the extension reaction amplifies the fused product by PCR. Thus, the region of the gene containing the introduced mutations is lengthened in a single reaction.

Primer sets:

Forward (KMBG-F1): 5'-**GTAGTAATTAGATCTG**-3'

Reverse (KMBG-R2): 5'-**GGTCTAAAGATCTCGG**-3'

Forward (KMNH-F2): 5'-**CAGGTAGCTAGCAAATTAAGAGAAC**-3'

Reverse (KMNH-R1): 5'-**AATTTGCTAGCTACCTGTTTTAAAG**-3'

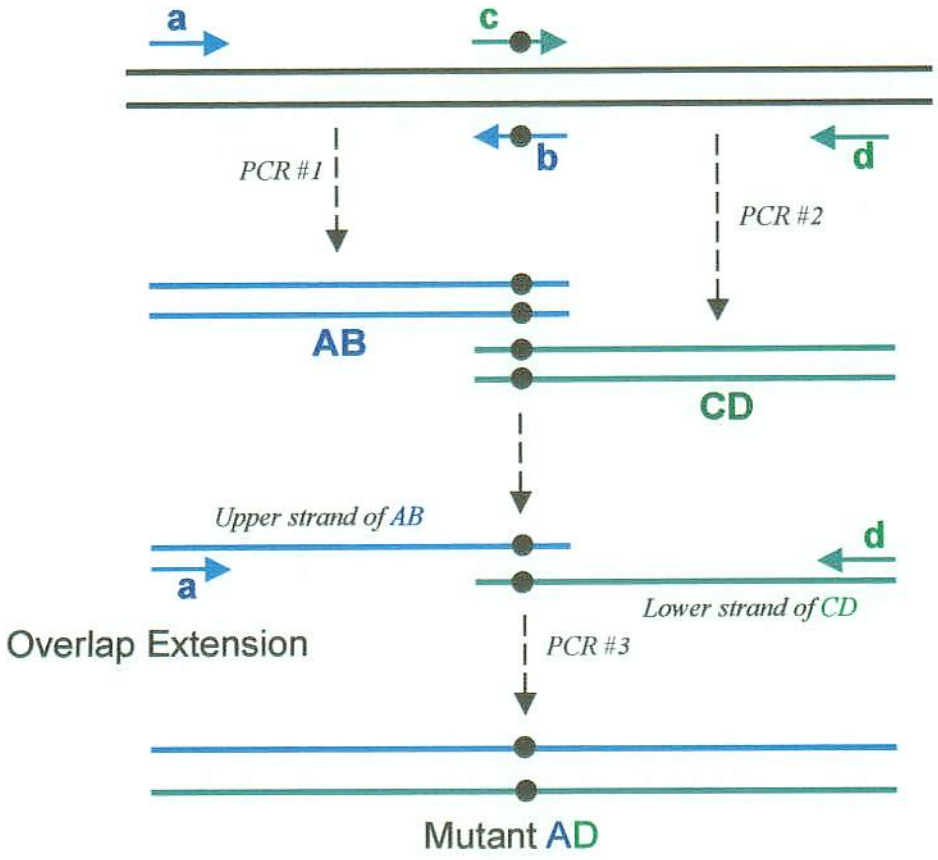


FIG. 7. Mutagenesis by overlap extension. Two segments of the pUC/KMT/L*env* were PCR-amplified independently (PCR #1 and PCR #2) and then fused together in a subsequent reaction (PCR #3). Mutations are introduced into a targeted region with the use of specially designed mutagenizing primers (b and c representing KMNH-R1 and KMNH-F2, respectively), which contain nucleotide mismatches (represented by solid circles or red nucleotides in the sequence) in the center of the primers. Because these primers are complemntary, strands of PCR products generated independently with these primers will have an overlap that can be extended in a subsequent reaction (PCR #3) to form the mutant product containing the *Nhe* I site. When outside primers (a and d representing KMBG-F1 and KMBG-R2, respectively) are added to this latter reaction, the fused mutant product is amplified as soon as it is formed.

The pUC/KMT/Lenv was amplified and mutated in two separate PCR amplifications using two sets of primers (KMBG-F1/KMNH-R1 and KMNH-F2/ KMBG-R2). Each of the PCR products was purified and eluted after running on 0.8% agarose gel using GlassMax DNA Isolation Matrix System (GIBCO BRL). Both PCR products were then included in a second PCR reaction as templates for primers KMBG-F1 and KMBG-R2. The amplified product was then run on 0.8% agarose gel, purified, digested with the *Bgl* II restriction enzyme and ligated by T4 DNA Ligase into a dephosphorylated pUC/KMTenv with a deleted fragment between its two *Bgl* II restriction sites. Each recombinant *env* was verified by restriction analysis.

Construction of the CCR5 and CXCR4 expression vectors

The complementary DNA (cDNA) clones encoding human CCR5 and CXCR4 were obtained by PCR using a human primary lymphocyte cDNA as the template. Description of the PCR reaction is shown as follows:

Protocol 2.

A. Design of primer sets:

For the CCR5 gene

Forward: 5'-**TGCACAGGGTGGAACAAGATGGATTATC**-3'
Reverse: 5'-**TAAGCCATGTGCACAACTCTGACTGGGTCA**-3'

For CXCR4 gene

Forward: 5'-**CCATGGAGGGGATCAGTATAT**-3'
Reverse: 5'-**CTGTGTTAGCTGGAGTGAAAACT** -3'

B. PCR reaction mixture: Volume is adjusted to 50 µl in total

Template (DNA from cellular lysate) (1µg)	1 µl
Forward primer (100 picomoles)	1 µl
Reverse primer (100 picomoles)	1 µl
Taq LA (Takara, Tokyo, Japan) (2.5 units)	0.5 µl
10X Buffer	5 µl
dNTP (0.2 mM)	8 µl
$MgCl_2$ (1.5 mM)	5 µl
Distilled water	28.5 µl

C. PCR cycles:

Temperature	Time	Number of cycles
95°C	2 minutes	1
98°C	10 seconds	30
60°C	1 minute	
72°C	1 minute	
72°C	10 minutes	1
4°C	Indefinite	0

The amplified products were then ligated into pCR2.1 cloning vector and designated pCR2-CCR5 and pCR2-CXCR4. The complete sequence of each product was verified with an automated DNA sequencer (ABI Prism 377; Applied Biosystems). The CCR5- or CXCR4-encoding fragment was then ligated into a pZeoSV2 expression vector (Invitrogen) using the *Hind* III and *Xho* I sites to give pZeoSV-CCR5 or pZeoSV-CXCR4, respectively.

Construction of mutant envelope expression vectors

For the construction of mutant envelope expression vectors, we used pCXN2, which has a chicken β-actin promoter. Briefly, the HIV-1_{JR-FL} *env* region was cloned by PCR and ligated into pCR2.1, generating pCR2-FLenv wild type.

The sequence of the amplified *env* region of HIV-1_{JR-FL} was confirmed using the DNA sequencer. The *Eco*R I fragment of pCR2-FLenv containing the entire *env* region was ligated into pCXN2 to give pCXN-FLenv wild type. For the cloning of the V2 region of *env*, an *Nde* I – *Stu* I fragment cloned by PCR with a valine-to-methionine substitution at codon 166 (V166M) was ligated into pCXN-FLenv, generating pCXN-FLenv V166M. For the cloning of the V3 region, a *Bgl* II – *Bgl* II fragment with a serine-to-glycine substitution at codon 303 (S303G) in the *env* region cloned by PCR was ligated into pCXN-FLenv, generating pCXN-FLenv S303G. A mutant envelope expression vector, which contains both V166M and S303G, was also constructed in the same manner, generating pCXN-FLenv V166M/S303G. The HIV-1_{NL4-3} *env* region was also amplified and ligated into pCXN2, generating pCXN-NLenv in the same manner as pCXN-FLenv.

Transduction of cell lines with the CCR5 and CXCR4 genes

The cDNA encoding the CCR5 gene cloned in the pCR2.1 vector pCR2-CCR5 was ligated into pBluescript II $KS^{(+)}$ (Stratagene, La Jolla, CA, USA) using the *Hind* III and *Xho* I sites to yield pKS-CCR5, and the CCR5 gene carrying the *Not*I-*Eco*RV fragment of pKS-CCR5 was then transferred into the *Not* I and *Sna*B I sites of the retrovirus vector pG1TKNeo to produce pG1TKNeo-CCR5. pG1TKNeo-CCR5 was transfected into the murine retrovirus packaging cell line PA317 by the calcium phosphate method (protocol 3) with a 'Profection kit' (Promega, Madison, WI, USA), and a retrovirus-producing cell clone was chosen in the presence of G418 (0.8 mg/ml).

Protocol 3.

A. Reagents required:

- **2M $CaCl_2$**
- **Nuclease-free water**

- **HBS (HEPES-Buffered Saline)**, 2X, pH 7.1

50 mM	HEPES, pH 7.1
280 mM	NaCl
1.5 mM	Na_2HPO_4

- **DMEM** culture medium
- **PBS (Phosphate Buffered Saline)**, 1X, pH 7.4

0.2 g	KCl
8.0 g	NaCl
1.15 g	Na_2HPO_4
0.2 g	KH_2PO_4

Add the components above one at a time to 900 ml of room temperature deionized water and stir until completely dissolved. Adjust the pH by using 1N NaOH or 1N HCl if necessary and bring the final volume to 1 litre. Autoclave the solution at 121°C for 20 minutes and store in tightly-capped sterile bottle.

B. Applied procedure:

1. Plate the cells one day before transfection as shown in table 1. The cells should be approximately 80% confluent on the day of transfection.

TABLE 1. Area of culture plates for Cell Growth

Size of Plate	Relative area[a]
96 well	0.2X
24 well	1X
12 well	2X
6 well	5X
35 mm	4.2X
60 mm	11X
100 mm	29X

[a] Relative area is expressed as a factor of the growth area of the 24 well plate which is recommended for optimization studies. To determine the proper plating density, multiply 5.5 x 10^4 cells by this factor.

2. Three hours prior to transfection, remove the culture medium from the cells and replace it with fresh growth DMEM medium.
3. Thaw all system components and warm them to room temperature. Mix each component thoroughly by swirling the container or vortexing.
4. For each transfection, prepare the DNA and 2X HBS solutions in separate sterile tubes (Table 2). Add the DNA and water to the first tube, mix well, then add the 2M $CaCl_2$ and mix again. Add the specified amount of 2X HBS to the second tube.

TABLE 2. Specified amounts of DNA and solutions for each transfection

	Per 60mm dish	Per 100mm dish
DNA	6-12 μg	10-20 μg
2M $CaCl_2$	37 μl	62 μl
Nuclease-free water	To 0.3 ml final	To 0.5 ml final
2XHBS	0.3 ml	0.5 ml

5. Working in a tissue culture hood, gently vortex the tube containing the 2X HBS solution while dropwise adding the prepared DNA solution. Incubate the solution at room temperature for 30 minutes.
6. Vortex the transfection solution again just prior to adding it to the cells. Add the solution dropwise to the plates. Swirl the plates to distribute the precipitate evenly over the cells. Incubate the plates at 37°C in a CO_2 incubator.
7. Culture medium is changed 4-16 hours after transfection and culture supernatant may be harvested or selective media applied 48-72 hours after the transfection.

The MOLT-4#8 cell line was transduced with the CCR5 gene by coculturing with retrovirus-producing cells. Briefly, MOLT-4#8 cells were cocultured with irradiated retrovirus-producing cells for 2 days and then suspension cells were cultured in the presence of G418 (0.8 mg/ml). The expression level of CCR5 in

transduced cells was confirmed using the anti-CCR5 monoclonal antibody 2D7 (Pharmingen, San Diego, CA, USA) with a fluorescence activated cell sorter (FACS) (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). To clone CCR5-expressing cells that are highly sensitive to R5 HIV infection, limiting dilution was performed, and a clone that was able to induce ballooning after infection with the R5 HIV-1_{JR-FL} was selected. This clone was designated MOLT-4#8/CCR5 and used for propagation and selection of the resistant mutant.

HeLa-CD4/LTR-β-Gal cells were transfected with pZeoSV-CCR5 using the calcium phosphate method. An R5 HIV-1-sensitive stable transfectant was cloned in the presence of 1.0 mg/ml of G418, 0.5 mg/ml of hygromycin, and 0.5 mg/ml of zeomycin (Invitrogen). These cells were designated MAGI/CCR5 and their CCR5 expression level was confirmed by FACS analysis using the 2D7 anti-CCR5 monoclonal antibody. The susceptibility of these cells to R5 HIV was determined by infecting them with HIV-1_{JR-FL}.

CCR5- and CXCR4-expressing NP-2/CD4 cells were also established by the transfection of expression vectors pZeoSV-CCR5 and pZeoSV-CXCR4, respectively. Stable transfectants were selected in the presence of 0.5 mg/ml of zeomycin. The expression level of CCR5 and CXCR4 of the transfectants was confirmed by FACS analysis using the 2D7 and 12G5 monoclonal antibodies against CCR5 and CXCR4 (R&D Systems, Minneapolis, MN, USA), respectively.

Titration of virus stocks

The M-tropic virus infectious clone pJR-FL was provided by Y. Koyanagi (unpublished data). The virus was recovered after transfection of the plasmid into COS-7 cells using the Lipofectamine method (Gibco BRL) (protocol 4).

Protocol 4.

1. In a six-well tissue culture plate, seed 2 x 10^5 COS-7 cells/well in DMEM medium. Incubate the cells at 37°C in a CO_2 incubator until they are 50-80% confluent within 18-24 hours.
2. Prepare the following solutions in two eppendorf tubes:

   Solution A: 100 µl of 1µg of pJR-FL plus Opti-MEM medium

   Solution B: 100 µl of 8µl lipofectamine reagent plus 92 µl Opti-MEM medium
3. Combine the two solutions, mix gently, and incubate at 37°C for 45 minutes.
4. Rinse the cells twice with 2 ml of Opti-MEM.
5. Add 0.8 ml of Opti-MEM to tubes complex.
6. Overlay the diluted complex solution onto the rinsed cells and incubate for 2-24 hours (usually 5-8 hours are enough) at 37°C in CO_2 incubator.
7. Add 1 ml of 20% FCS/DMEM with and incubate overnight.
8. Change the medium with 2 ml of fresh 10% FCS/DMEM and incubate for 2-3 days.
9. Harvest the supernatant and filter through a 0.22 µm filter.
10. Add the supernatant onto MOLT-4#8/CCR5 cells and the virus in the cell culture supernatant was later recovered within 7 to 10 days.

Viral titers were determined by the Multinuclear Activation of a Galactosidase Indicator Assay (MAGI assay) (Fig. 8) (Protocol 5). This assay requires that the virus encoded Tat protein transactivates an integrated copy of an HIV-1 LTR-driven modified *lacZ* gene present in the indicator (HeLa derived) MAGI/CCR5 cell line. Infected cells are identified by the blue color that results from the hydrolysis and staining of the substrate X-gal by β-galactosidase. To improve the visualization of infected cells, the *β-galactosidase* gene has been modified to include the nuclear localization signal of the SV40 T antigen, thereby concentrating the blue color into the nucleus.

Protocol 5.

A. Reagents required:

- **Culture Medium**
  DMEM with 10% FCS, 0.1 mg/ml G418, 0.05 mg/ml Hygromycin B, and 0.05 mg/ml Zeomycin.
- **Fixing Solution**
  1% formaldehyde and 0.2% glutaraldehyde in PBS.
- **X-Gal Stock** (5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside)
  Dissolve 20 mg of X-gal in 1.0 ml of dimethyl-formamide. Store in the dark at -20°C for 1 month.
- **Staining Solution**
  For preparing 1.0 ml, combine 950 µl PBS, 20 µl 0.2 M potassium-ferrocyanide, 20 µl 0.2 M potassium ferri-cyanide, 1.0 µl 2.0 M $MgCl_2$, and 10 µl X-gal stock.

B. Applied procedure:

1. MAGI/CCR5 cells were seeded in 48 well plates at 1.5 x 10^4 cells per well in a 37°C, 5% CO_2 incubator.
2. One day after plating the cells, dilutions of the test virus in a 150 µl volume were added to each well. Virus is left to adsorb for 2 hours.
3. Add 150 µl of fresh culture medium to each well and incubate the cells for 48 hours until they become confluent.
4. Remove the culture medium and apply 500 µl of fixing solution to each well. Incubate exactly for 5 minutes at room temperature.
5. Remove the fixing solution and wash the cells twice with PBS. Add enough staining solution just to cover the cells and incubate for exactly 50 minutes at 37°C.
6. Wash the wells twice with PBS and count the number of blue-stained cells under the microscope. Plates are stored away from sunlight in PBS with sodium azide for permanent record. Titration values are expressed as the number of stained cells multiplied by viral dilution.

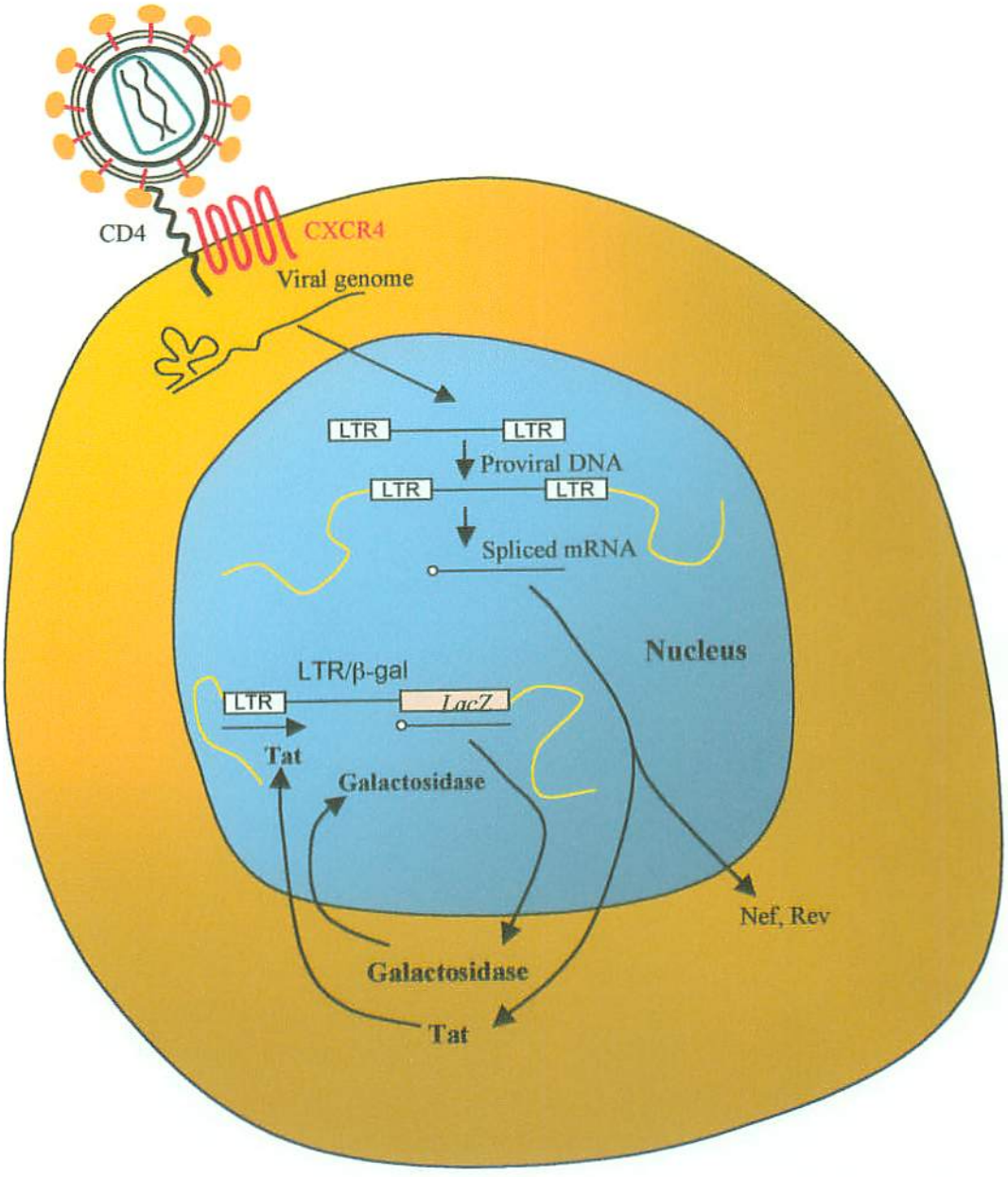


FIG. 8. Multinuclear Activation of a Galactosidase Indicator Assay (MAGI assay).

Chemokines

Saccharomyces cerevisiae-derived recombinant MIP-1α [yLD78β [112,113]], kindly supplied by the Chemo-Sero-Therapeutics Research Institute (Kumamoto, Japan), was used for the selection of MIP-1α-resistant virus. Other chemokines, MIP-1β and RANTES, were purchased from R&D Systems.

Flow cytometric analysis for coreceptor utilization

Determination of coreceptor utilization using GHOST cells expressing different chemokine receptors was carried out as previously described [17, 150]. Cells were seeded in 24-well plates (Iwaki, Tokyo, Japan) at 6 x 10^4 cells per well. On the following day, cells were infected with equal amounts of HIV-1 isolates in the presence of 10 μg/ml of hexadimethrine bromide (Sigma, St. Louis, MO, USA) to enhance infectivity. On day 2 postinfection, cells were harvested, washed once with phosphate-buffered saline (PBS), and fixed in paraformaldehyde at a final concentration of 2%. Infected cells were then analyzed by FACS for GFP expression.

Analysis of Env-mediated cell-cell fusion

This approach employs effector 293T cells that express infectious chimeric clones, and target 293T cells transiently expressing CD4 and CCR5 or CXCR4 coreceptors. 293T target cells were also transfected with a plasmid encoding the SEAP reporter gene under control of an HIV-1/LTR promoter [9]. Fusion between these cells results in Tat-mediated activation of the HIV-1/LTR promoter, and hence in enhanced SEAP expression in coculture supernatant. Effector cells were generated by transfection of 293T cells (2.5 x 10^5 per well in 6-well plate) using calcium phosphate method with 2.4 μg of the infectious chimeric molecular clones as well as plasmids encoding HIV-1_{NL4-3} and HIV-1_{JR-FL} as positive controls for

CXCR4 and CCR5 utilization, respectively. Target cells were generated by cotransfection of 293T cells with 800 ng of pEneo-CD4, 800 ng of pLTR-SEAP, and 800 ng of pZeoSV-CXCR4 or CCR5 [9]. Two days later, effector and target cells were harvested by trypsinization, and equal numbers of cells (5×10^4) were cocultured in 48-well plates for 48 hours. SEAP activity in coculture supernatant was measured with a Phospha-Light substrate (Tropix, Bedford, MA, USA) using a luminometer (Lumat LB 9501/16, Berthold, Germany) according to the manufacturer's protocol as relative light units per second. To determine background SEAP activity, 293T cells were cotransfected with only pEneo-CD4 and pLTR-SEAP then cocultured with various effector cells.

Infection assay with luciferase readout

An assay based on single-cycle infection was used to assess coreceptor usage of different chimeric envelopes [19, 30, 45, 99]. In this assay, infection of cells with env-pseudotyped virions results in a single-cycle of viral replication and produces readily detectable luciferase activity quantified by measuring chemiluminescence.

First, recombinant luciferase reporter virus stocks pseudotyped with various HIV-1 envelopes were generated by cotransfection of 293T cells with 10 µg of HIV-1NL/LucΔBgl and 5 µg of pCXN2 plasmids expressing envelopes from either HIV-1_{NL4-3}, HIV-1_{JR-FL}, HIV-1_{KMT}, HIV-$1_{KMT/L}$, or various chimeric envelopes. Calcium phosphate method was used for the cotransfection and at day three posttransfection, the cell culture supernatant containing the pseudotyped luciferase reporter virus was collected, filtered through a 0.22-µm pore size filter and stored until use for infection at -80°C.

For the determination of chemokine receptor usage, target NP-2 cells expressing CD4 along with either CCR5 or CXCR4 coreceptors were seeded one

day before infection in 48-well plates (Iwaki, Tokyo, Japan) at 1 x 10^4 cells per well and infected with 2 ng (p24 antigen) of luciferase-reporter viruses in a total volume of 0.2 ml medium in the presence of 1 μg/ml of polybrene. Seventy-two hours postinfection, infected target cells were washed once with PBS and lysed with 100 μl of 1X reporter lyses buffer as instructed for the luciferase assay system (Promega, Madison, WI, USA). Luciferase activity was quantified by adding 50 μl of luciferase substrate to 10 μl of lysate in 75 x 12 mm tubes (Rohren, Sarstedt, Germany) and measuring light activity by luminometer. In all experiments, light activity is reported as relative light units per second. All values are within the linear range of luciferase detection.

For the determination of the sensitivities to chemokines, MAGI/CCR5 cells (10^4/well) were seeded in 48-well plates and incubated with various concentrations of chemokines for 1 h at 37°C followed by infection with the HIV-1_{NL4-3} or HIV-1_{JR-FL} luciferase reporter viruses for two days and then measuring luciferase activity. The sensitivity of a chemokine was determined from the 50% inhibitory concentration (IC_{50}) of the virus.

Immunoblotting

Expression level of Env of different chimeric clones from 293T-transfected cells was detected as follows:

Protocol 6.

1. Cotransfected 293T effector cells were lysed at a concentration of 2 x 10^7 cells/ml in buffer containing 1% NP40, 150 mM NaCl, 10 mM Tris-Cl, pH 7.4.
2. After 30 minutes of incubation on ice, the cellular lysates were centrifuged (13,000*g*) for 5 minutes at 4°C to remove the insoluble fractions.
3. Samples were then boiled with an equal volume of 2X Laemmli sample buffer containing 2% sodium dodecyl sulfate (SDS) and 0.5% mercaptoethanol.

4. Samples corresponding to 2 x 10^5 cells were separated by 7.5% SDS-polyacrylamide gel (SDS-PAGE), and then electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane (Atto, Tokyo, Japan).
5. PVDF membrane was blocked overnight by 5% non-fat skimmed milk followed by incubation with serum from an HIV-1 infected individual.
6. An hour later, membrane was washed twice for 5 minutes each with 20 ml of PBS-Tween buffer (PBS-T) and incubated with horseradish peroxidase (HRP)-conjugated antibody against human immunoglobulin (Cappel, Aurora, OH, USA) for another hour.
7. Membrane was washed again twice for 5 minutes each with 20 ml of PBS-T and equal amounts of the detection chemiluminescent solutions 1 and 2 (ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK) were added to the protein side of the membrane for precisely one minute at room temperature without agitation.
8. Excess detection reagent was then drained off and membrane was wrapped in Saran wrap with gentle smoothing out of air pockets.
9. In a dark room membrane was rapidly placed with protein side up in a film cassette and a sheet of autoradiography film (Hyperfilm ECL, Amersham Pharmacia) was placed on top of the membrane.
10. Cassette was closed for approximately 30 seconds to 5 minutes and then the film was removed for development by autoradiography (Fujifilm, FPM800A, Japan).

Isolation of the R5 HIV-1_{JR-FL} MIP-1α-resistant mutant in *vitro*

For the selection of the MIP-1α-resistant HIV-1_{JR-FL}, MOLT-4#8/CCR5 cells were treated with various concentrations of MIP-1α and then infected with HIV-1_{JR-FL}. After the virus was passaged in MOLT-4#8/CCR5 cells, MIP-1α was

removed from the virus-infected cells and then the virus was recovered from the cell culture supernatant. The sensitivity of the virus resistant to MIP-1α was determined using MAGI/CCR5 cells as previously described [100]. Briefly, MAGI/CCR5 cells (10^4/well) were seeded in 48-well flat-bottom plates (Iwaki, Chiba, Japan). The following day, the cells were incubated with various concentrations of C-C chemokines for 1 hour and then the previously titrated virus that gave 200 blue cells/well was added. 48-hours after exposure to virus, cells were fixed and stained with X-Gal substrate (see above). Blue cells in each well were then counted under microscope. All experiments were done in duplicate. $HIV\text{-}1_{JR\text{-}FL}$ was also passaged in MOLT-4#8/CCR5 cells in the absence of MIP-1α for 3 months to exclude the effect of long-term culture.

Extraction of DNA from virus-infected cells was done by using the Instagene (Bio-Rad) protocol mentioned above and then subjected to PCR using *Taq* polymerase. The amplified products were cloned into pCR2.1 (see above), and then the *env* regions in both the passaged and selected virus were sequenced using the ABI PRISM 377 automated DNA sequencer.

Measurement of p24 antigen in culture supernatant

An enzyme linked immunosorbant assay (ELISA) (RETRO-TEK, Buffalo, NY, USA) was used to detect p24 antigen in culture supernatant of transfected 293T cells as well as infected MOLT-4#8, CEM, MT-2, and MT-4 cells.

Protocol 7.

A. Reagents required:

- **HIV-1 p24 antibody coated microplate for 96 determinations.**
- **HIV-1 p24 detector antibody.**
- **HIV-1 p24 antigen standard.** Prepare a series of six standards (125, 62.5, 31.3, 15.6, 7.8, 0 pg/ml).

- **Streptavidin-peroxidase and substrate.**
- **Plate wash buffer.**
- **Lysing buffer.**
- **Stop solution.**

B. Applied procedure:

1. Treat specimens of cell culture supernatant in a test tube by pipetting 50 µl of lysing buffer into 450 µl specimen and mix well.
2. Wash each well of microplate 6 times with 1X plate wash buffer.
3. Leave one well empty during the assay for a substrate blank.
4. Pipet 200 µl of standards #1-6 into wells into duplicate wells.
5. Pipet 200 µl of each specimen into duplicate wells.
6. Cover microplate with a plate sealer and incubate for 2 hours at 37°C.
7. Aspirate and wash plate as described in step 2.
8. Pipet 100 µl of HIV-1 p24 detector antibody into each well, except substrate blank. Cover microplate with sealer and incubate for 1 hour at 37°C.
9. Aspirate and wash plate as described in step 2.
10. Pipet 100 µl of streptavidin-peroxidase working solution into each well except substrate blank. Cover microplate with sealer and incubate for 1 hour at 37°C.
11. Aspirate and wash plate as described in step 2.
12. Pipet 100 µl of substrate working solution into each well and incubate uncovered for 30 minutes at room temperature. A blue colour will develop in wells containing viral antigen.
13. Stop the reaction by pipetting 100 µl of stop solution into each well. A colour change from blue into yellow will result.
14. Within 15 minutes, read the optical density of each well at 450 nm using a microplate reader (Titetek Mutiscan MCC/340, Labsystems, Finland).

C. Test validity and calculation:

Determine the mean optical density values for each standard and specimen. For the test to be valid, it must meet the following criteria:

- The mean optical density of the 0 pg/ml standard and the substrate blank must be less than 0.100.
- The mean optical density of the 62.5 pg/ml standard must be greater than or equal to 0.500

The concentration of p24 antigen was then calculated by a software computer program (*Delta3* SOFT), using a point-to-point algorithm, after inputting the different mean absobance values for the diluted specimens.

Results

Part 1

Coreceptor utilization by the HIV-1_{KMT} isolate

In a previous study, the primary dualtropic HIV-1_{KMT} isolate from an HIV-1-infected individual was grown in a T lymphoid cell line [109]. To determine which coreceptors are utilized by HIV-1_{KMT}, individual GHOST cell lines expressing CD4 and one of a variety of chemokine receptors including CXCR4, CCR5, CCR2b, CCR3, CCR8, BOB, Bonzo, and V28 [20, 35] were infected with the HIV-1_{KMT} isolate. Two days later, the level of GFP expression in cells was measured by flow cytometry. High level of GFP was detected in cells expressing the CXCR4 and CCR5 major coreceptors (Fig. 9), indicating that HIV-1_{KMT} is an R5/X4 HIV-1 isolate.

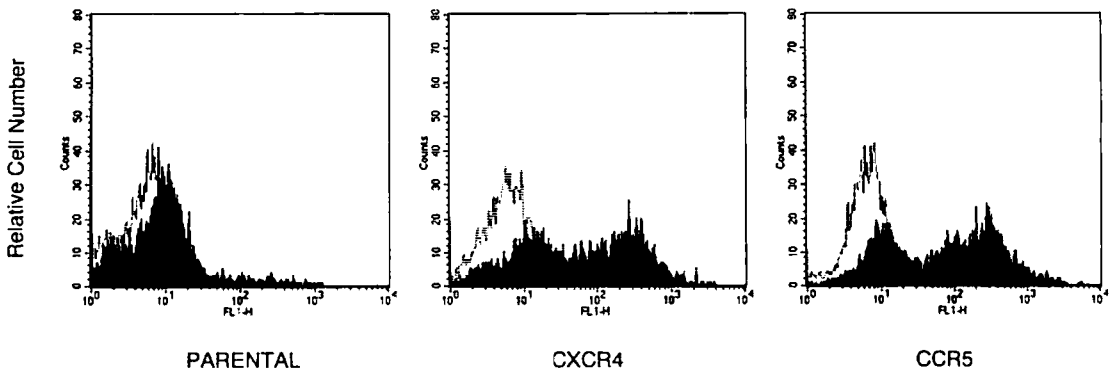


FIG. 9. Flow cytometric analysis of chemokine receptor usage by the primary HIV-1_{KMT} isolate in GHOST cell line. GHOST cells stably expressing CD4 with CXCR4 or CCR5 were infected with the primary isolate HIV-1_{KMT} or mock infected. Two days postinfection, cells were recovered and GFP fluorescence intensity was analyzed by a flow cytometer. The dotted line indicates mock-infected cells as a negative control. Each histogram is a representative of at least two separate experiments.

Domains on HIV-1_{KMT} envelope involved in CCR5 utilization

To specifically address which domains within the HIV-1_{KMT} envelope glycoprotein could determine the CCR5 coreceptor usage, we used a previously constructed panel of chimeric viruses [109] in which two different V1/V2 (NAI-V1/2 and DNT-V1/2) and V3 (E-V3 and Q-V3) sequences cloned from HIV-1_{KMT} (Fig. 10) were transferred to the (X4) HIV-1_{NL4-3} backbone (Fig. 11). A highly sensitive cell-cell fusion assay, utilizing a secreted alkaline phosphatase (SEAP)-chemiluminescence detection system, was used to monitor the envelope/coreceptor interaction.

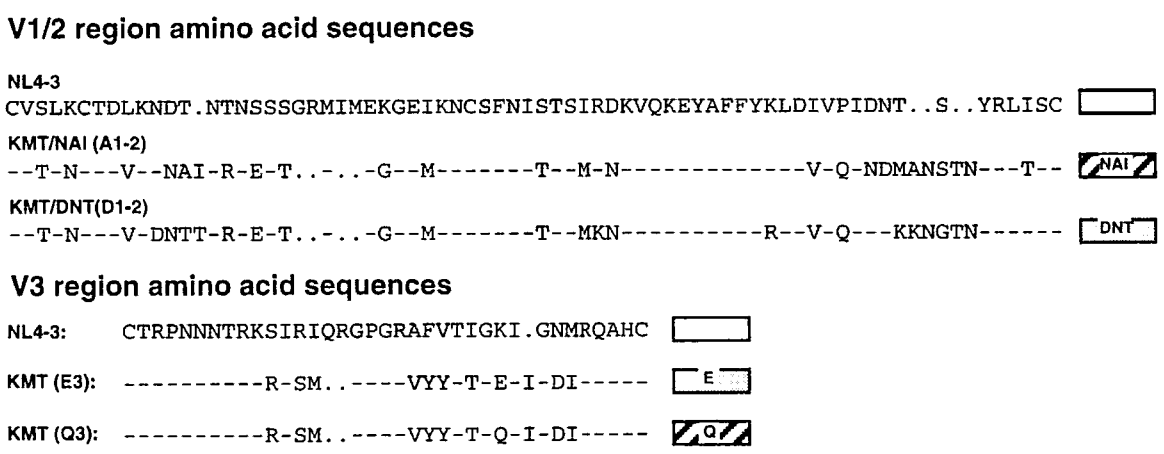


FIG. 10. Amino acid sequences of the V1/2 and V3 regions used to generate chimeric molecular clones. The predicted amino acid sequence is represented by the single-letter amino acid code. Dashes denote amino acid identity. Gaps are indicated as points.

As shown in Fig. 12, effector 293T cells expressing chimeric envelopes with the V3 domain of HIV-1_{KMT} (E3/N and Q3/N) successfully fused with target 293T cells expressing CD4 and CCR5. In contrast, chimeric envelopes with the

V1/V2 domain of HIV-1_{KMT} (A1-2/N and D1-2/N), like their HIV-1_{NL4-3} parent, failed to confer fusion with CCR5 expressing cells (albeit at a minimal level in case of D1-2/N) and maintained fusion with CXCR4 expressing cells. Moreover, the V1/V2 domain when combined with the V3 domain had no effect on (A1-2E3/N and D1-2E3/N) and even significantly reduced (A1-2Q3/N and D1-2Q3/N) fusion activity with coreceptor expressing cells. These results indicated that the V3 domain of HIV-1_{KMT} is the major determinant of fusion activity with CCR5 expressing cells, without any evident contribution from the V1/V2 domain.

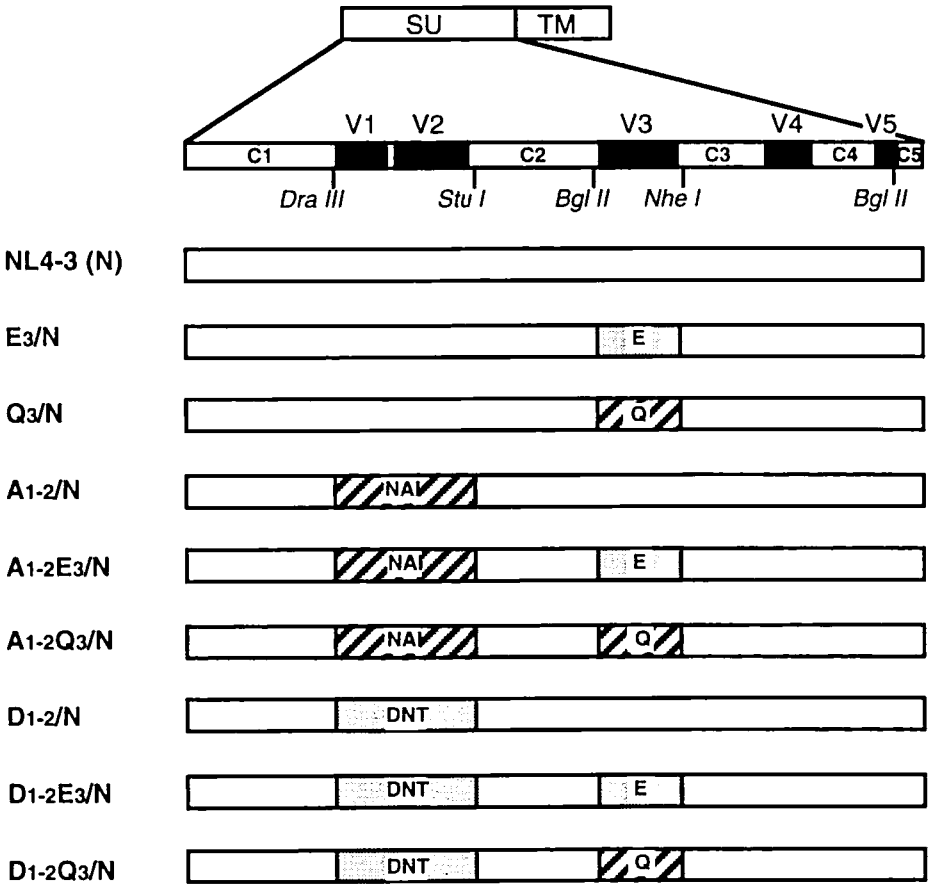


FIG. 11. Schematic diagram of a panel of chimeric envelope proteins. Chimeras were generated through the exchange of restriction fragments between HIV-1_{NL4-3} and HIV-1_{KMT} isolates. Open bars are DNA sequences from HIV-1_{NL4-3}, hatched and grey bars from HIV-1_{KMT} isolates.

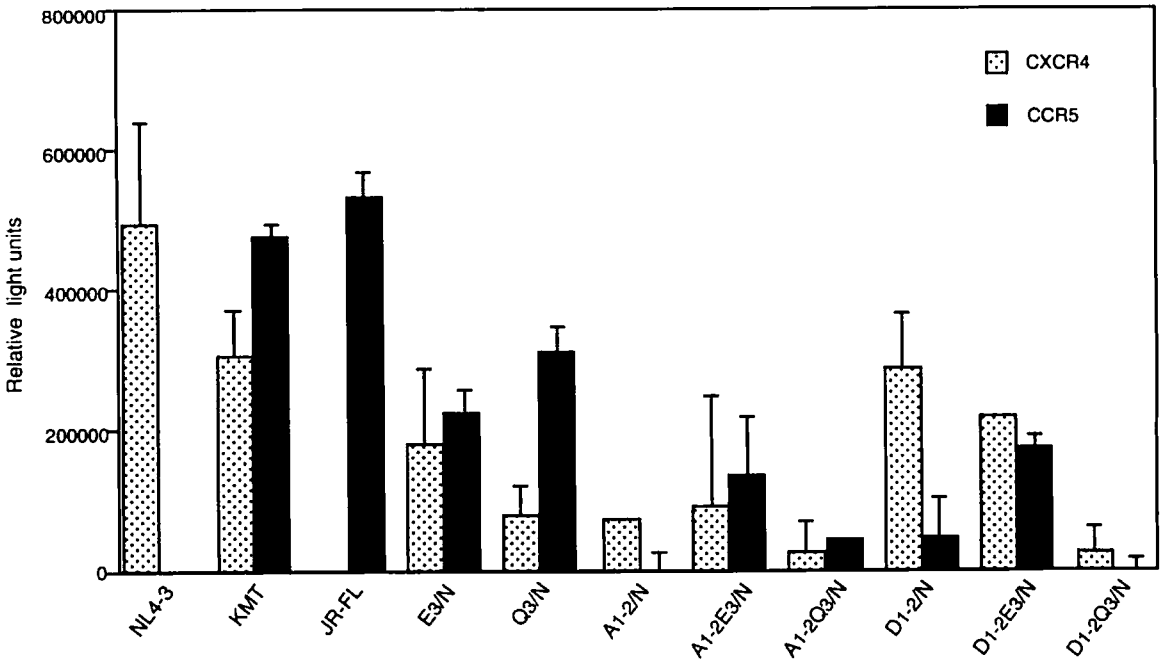


FIG. 12. Use of CCR5 in a cell-cell fusion assay by chimeric molecular clones. Cell-cell fusion between effector 293T cells expressing infectious chimeric molecular clones and target 293T cells expressing CD4 with either CCR5 or CXCR4 results in expression of the SEAP reporter gene by target cells and is monitored by chemiluminescence (relative light units). Envelopes of HIV-1_{NL4-3} and HIV-1_{JR-FL} strains were regarded as positive controls for the expression of CXCR4 and CCR5 coreceptors, respectively. Background SEAP activity was subtracted from all results. This experiment is representative of two to three assays performed with these envelopes. Error bars indicate standard deviation (SD).

Env-pseudotyped virus infection of coreceptor expressing cells

To determine whether viral replication was correlating to fusion activity, we employed a single-cycle virus infection assay [35, 131] with the luciferase reporter gene. Target cells were from a CD4-expressing human glioma cell line, NP-2/CD4, since this cell line does not allow replication of any HIV strain as described previously by Jinno *et al.* [73]. First, we established the CCR5- or CXCR4-expressing NP-2/CD4 cells; designated NP-2/CCR5 or NP-2/CXCR4 cells, respectively (see methods). The expression level of each chemokine receptor in these cells was verified using the 2D7 and 12G5 monoclonal antibodies for CCR5 and CXCR4 coreceptors, respectively. Cells were then infected with equal amounts of env-pseudotyped luciferase-expressing chimeric viruses that was prepared by cotransfection of 293T cells. In parallel to our observations in the cell-cell fusion assay, the V3 domain of HIV-1_{KMT} conferred significant CCR5 utilization to HIV-1_{NL4-3} (Fig. 13). Chimeric viruses with only the V1/V2 domain of HIV-1_{KMT} did not replicate in NP-2/CCR5 cells and maintained their ability to replicate in NP-2/CXCR4 cells. However, despite that the $D_{1-2}E_3/N$ chimera with the V1/V2 and V3 domains from HIV-1_{KMT} successfully fused with CCR5 and CXCR4 expressing cells, yet it failed to successfully infect NP-2/CCR5 and NP-2/CXCR4 cells. For further confirmation, we infected the GHOST cell line expressing CD4 along with one of the major (CXCR4 or CCR5) or minor coreceptors (CCR2b, CCR3, CCR8, BOB, BONZO, and V28) with our various env-pseudotyped chimeras. Comparable levels of infection were observed for CXCR4 and CCR5 expressing cells. In case of the CCR8 minor coreceptor expressing cells, we were able to detect a minimal support of infection with few chimeras as well as with the HIV-1_{NL4-3} isolate (Table 2).

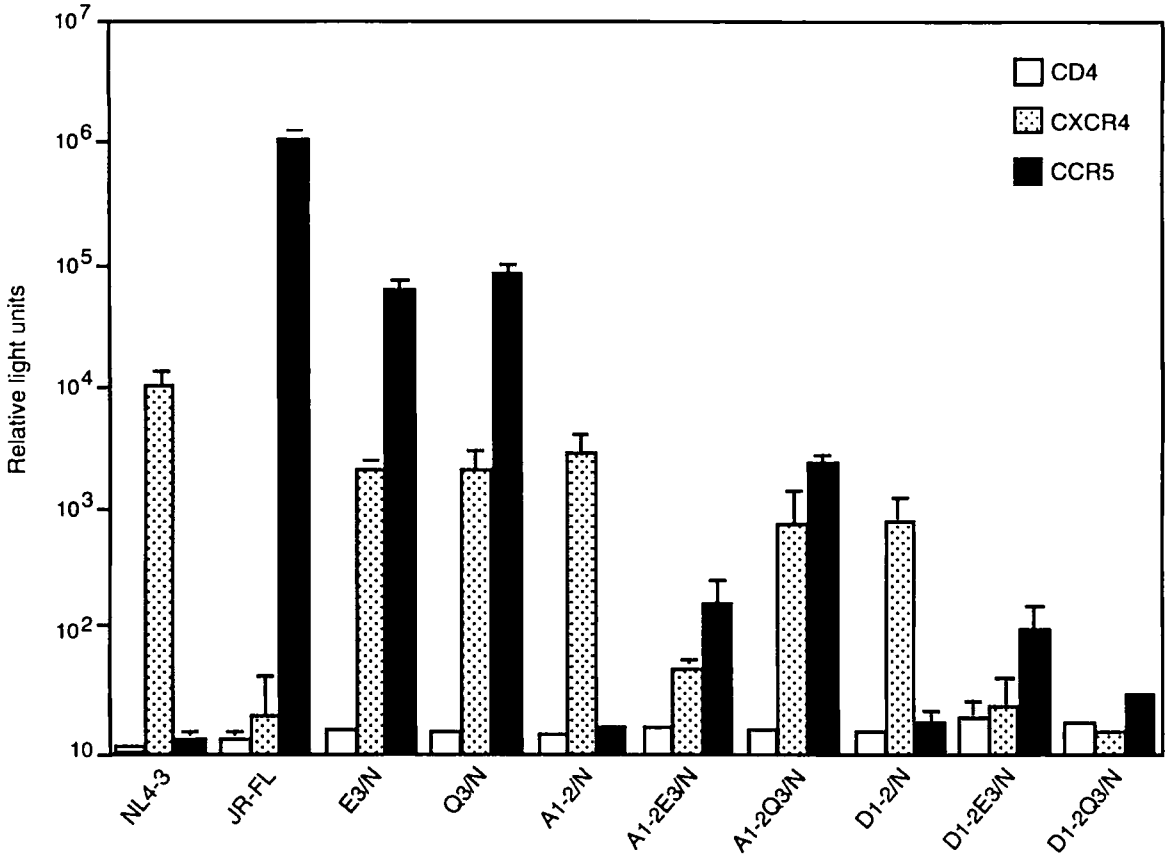


FIG. 13. Infectivity of chimeric HIV-1 in CCR5 expressing cells. Stocks of the control and chimeric HIV-1 were prepared as described in Materials and Methods. NP-2/CD4 cells expressing CCR5 or CXCR4 were seeded one day prior to infection with luciferase reporter pseudotyped viruses expressing various chimeric and parental envelopes. Infection of target cells leads to integration of the reporter genome and long terminal repeat-mediated production of luciferase. Luciferase activity was quantified in cell lysates 2-3 days after infection. Experiments were performed more than 3 times using at least two independently derived virus stocks. HIV-1_{NL4-3} and HIV-1_{JR-FL} are shown as controls for CXCR4 and CCR5 expression, respectively.

TABLE 3. Coreceptor usage of *env* clones pseudotyped with luciferase expressing virus using GHOST cells expressing CD4 with different coreceptors [a]

	CD4	CCR5	CXCR4	CCR3	CCR8	V28	BOB	BONZO	CCR2b
HIV-1_{NL4-3} [b]	1,392	1,555	85,161	363	**14,672**	905	145	332	1,398
HIV-1_{JR-FL}	209	4,567,434	280	499	502	398	157	178	1,598
E3/N	180	335,322	245,436	418	718	129	173	256	145
Q3/N	175	166,862	176,398	177	664	156	130	460	276
A1-2/N [b]	1,119	457	38,436	142	**16,006**	2,252	145	310	807
A1-2E3/N	236	2,340	5,214	172	1,698	162	127	145	1,549
A1-2Q3/N	543	13,660	10,967	152	**3,852**	663	185	129	174
D1-2/N [b]	1,748	608	16,339	923	**5,601**	1,369	306	480	1,036
D1-2E3/N	153	547	615	157	340	141	160	126	139
D1-2Q3/N	173	702	2,714	159	733	186	128	132	134

[a] Infection was determined by measurement of luciferase activity as relative light units per second.

[b] This isolate or chimera replicated in GHOST/CD4 cells with no coreceptor, probably due to utilization of endogenous CXCR4.

Env Expression and Processing

Given that we noticed an equal p24 antigen level determined in culture media of transfected 293T cells used to produce chimeric viral pseudotypes, we anticipated that the chimeric envelopes would be expressed and processed normally. However, because the D1-2E3/N pseudotyped chimeric virus failed to support infection although it successfully fused with coreceptor expressing cells, we analyzed the expression and processing of such chimeric virus. Media and cell lysates from 293T cells used to make viral pseudotypes were analyzed for Env by SDS/PAGE and Western blot using serum from an HIV-1 infected individual. Interstingly, the D1-2E3/N chimeric Env was successfully processed as judged by generation of gp120 from gp160 (Fig. 14).

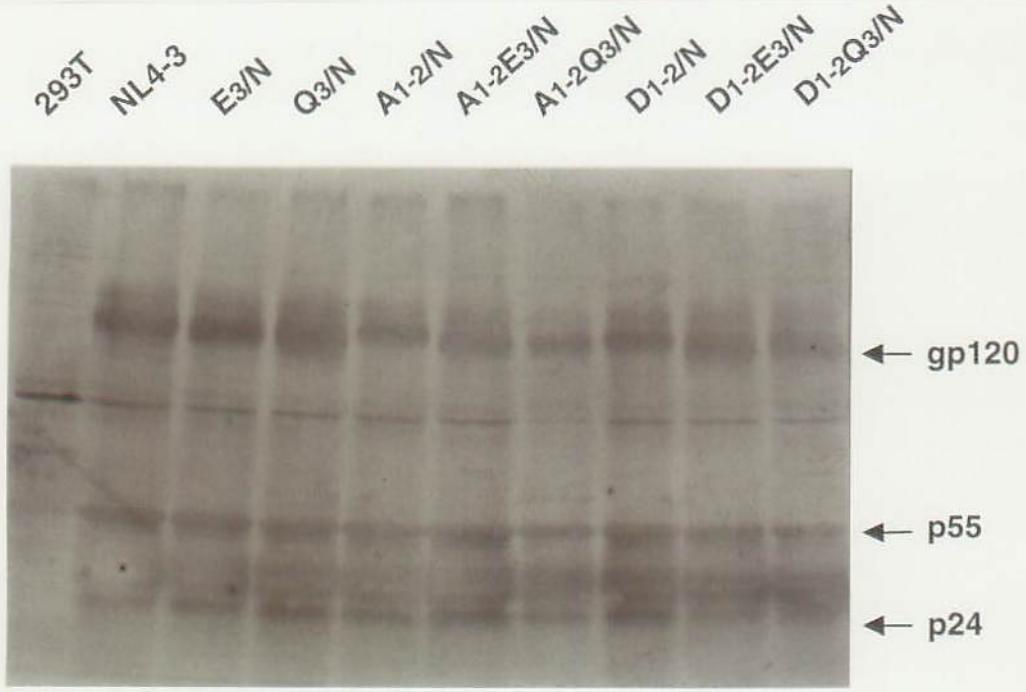


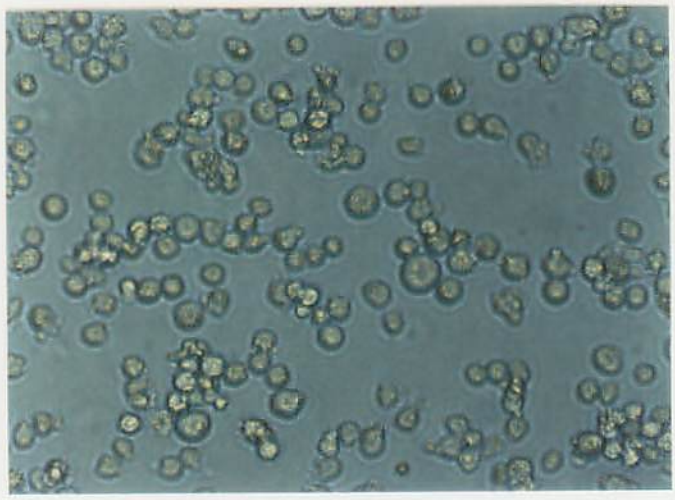
FIG. 14. Western blot lysates of 293T cells transfected to produce infectious chimeric molecular clones. Viral gp120, p55, and p24 are indicated by pointing arrows.

Env sequence and coreceptor utilization of a highly replicative HIV-1_{KMT} isolate

The apparent loss in infectivity of the D1-2E3/N chimeric virus suggested that either additional HIV-1_{KMT} domains are required for the efficient utilization of CCR5 and CXCR4, or that different HIV-1_{KMT} clones other than those presently used to construct our chimeric pseudotyped viruses are capable of infection. To exclude the latter possibility, we cloned and sequenced an HIV-1_{KMT} isolate that efficiently replicated in the CD4-positive MT-2 cell line. Viral replication was monitored by the induction of syncytium formation and ballooning of cells (a phenomena characteristic of HIV infection, most probably resulting from changes in membrane permeability) (Fig. 15) along with a consistently rising p24 antigen level in culture supernatant (from 5 ng/ml on day 2 to 57 ng/ml on day 7). The recovered clone was designated HIV-$1_{KMT/L}$ and its amino acid sequence analysis revealed that it had a V1/V2 and V3 sequence identical to that of the D1-2E3/N chimeric *env* (Fig. 16) which failed to replicate previously. Moreover, considerable amino acid sequence differences between the C-terminal *env* domain (C3-V5) of HIV-$1_{KMT/L}$ and that of HIV-1_{NL4-3} were elicited.

We also checked whether the entire gp120 Env of HIV-$1_{KMT/L}$ could support the infection of NP-2/CXCR4 and NP-2/CCR5 cells. As expected, HIV-$1_{KMT/L}$ env-pseudotyped luciferase-expressing virus efficiently infected both CXCR4 and CCR5 expressing cells with a luciferase activity at comparable levels to those of control cells infected with the HIV-1_{NL4-3} and HIV-1_{JR-FL} pseudotyped viruses, respectively (Fig. 17B). This raised the possibility that domains other than the V1/V2 and V3 play a role in HIV-1_{KMT} replication and we anticipated that inclusion of those domains into the D1-2E3/N chimeric *env* could be detrimental in restoring its infectivity to coreceptor expressing cells.

A



B

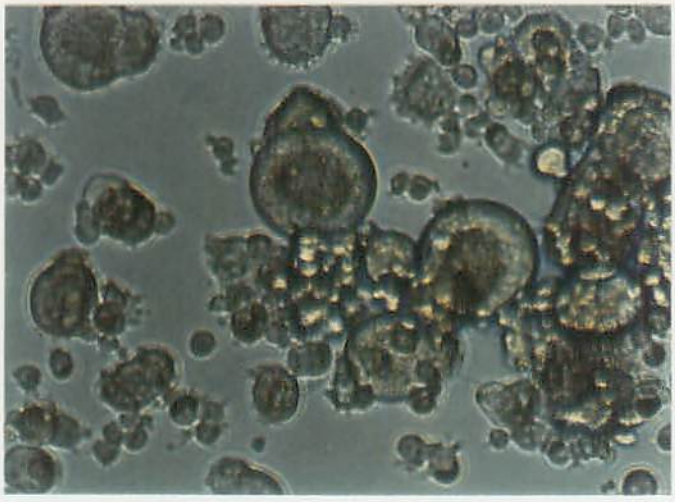


FIG. 15. Syncytium formation induced by a highly replicative HIV-1_{KMT} in MT-2 cell line. (A) MT-2 cells at day one postinfection with 5 ng p24 antigen of HIV-1_{KMT}. (B) Prominent syncytia formation and ballooning of infected MT-2 cells at day four postinfection

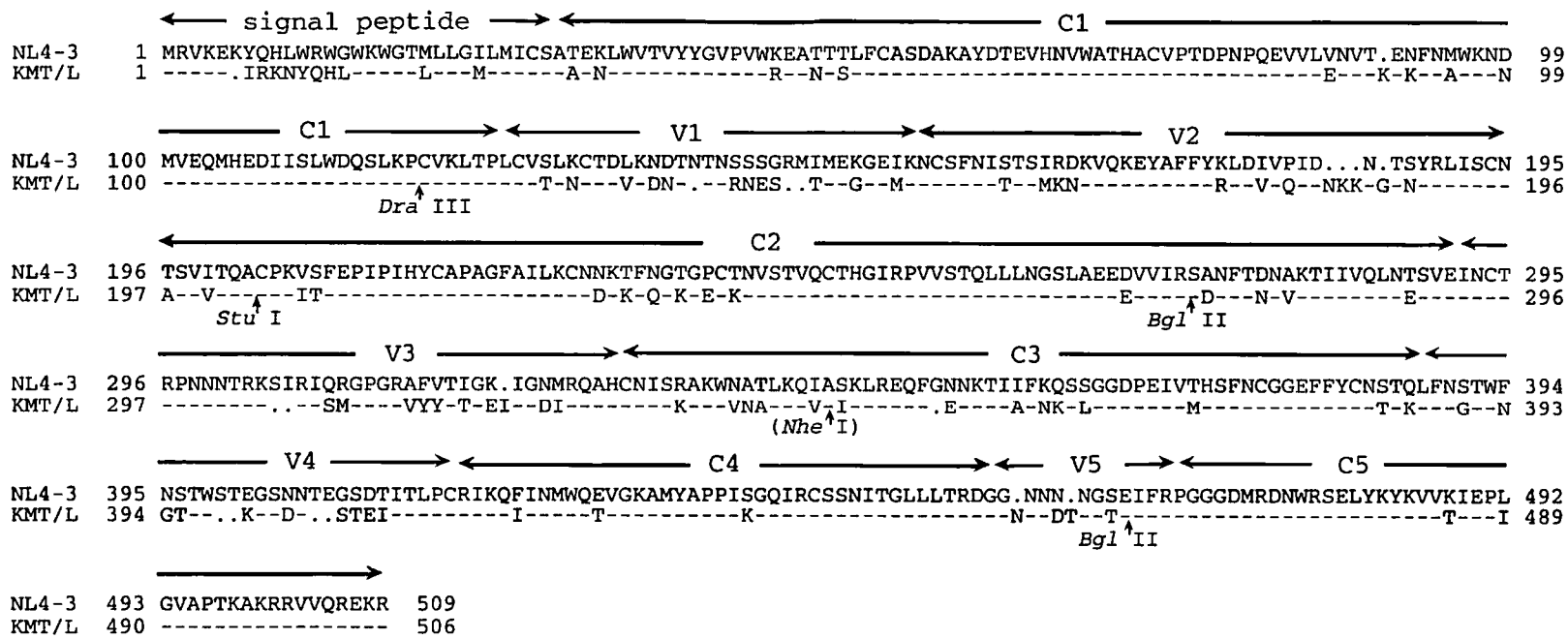


FIG. 16. Comparison of deduced amino acid sequences in the envelope regions of HIV-1_{NL4-3} and HIV-$1_{KMT/L}$. HIV-1_{NL4-3} is used as the reference sequence. A dash indicates amino acid identity, and a dot indicates a gap created to maximize the alignment. Sites of the restriction enzymes used in the construction of recombinant molecular clones are indicated.

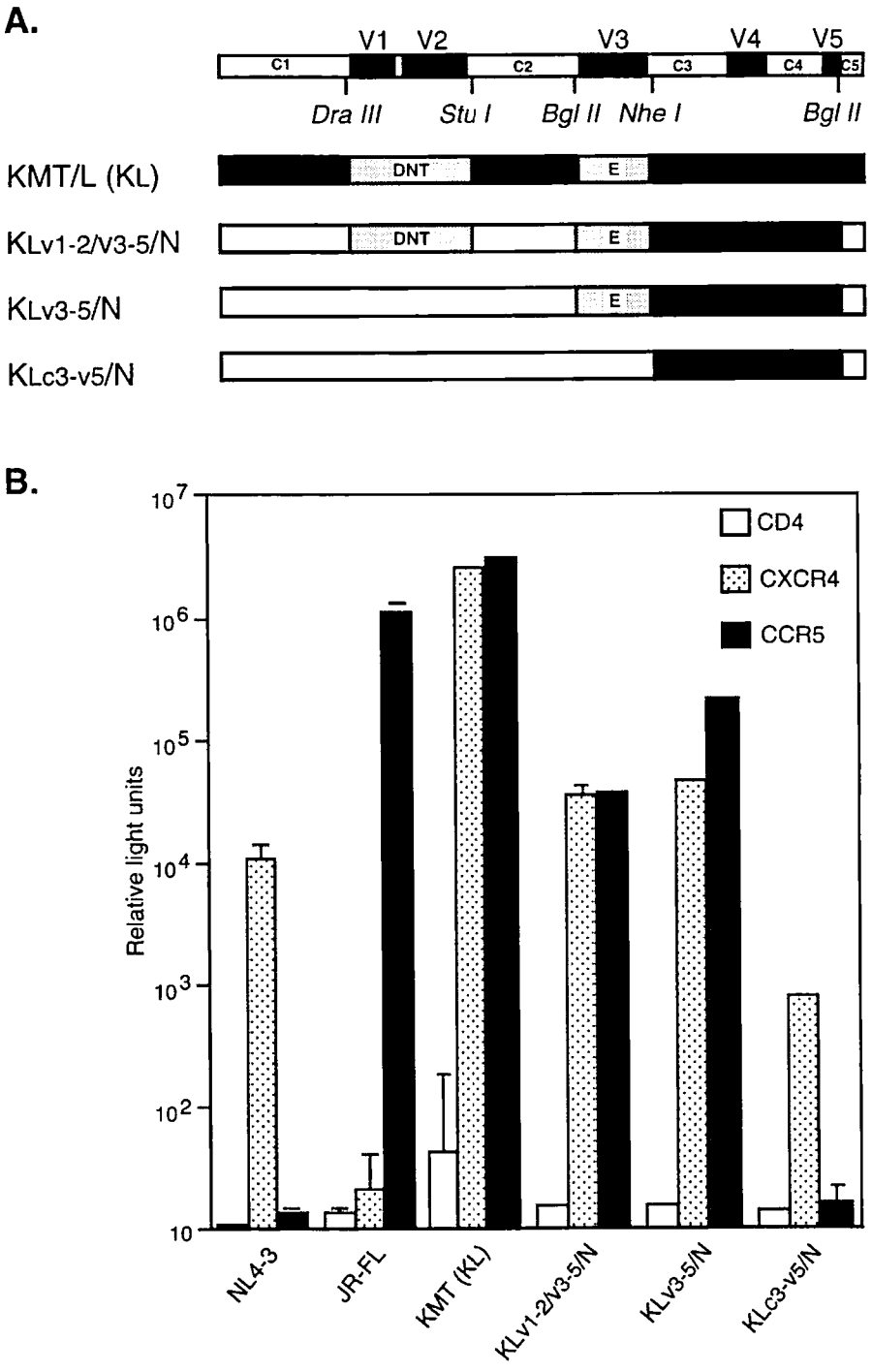


FIG. 17. Chimeric Constructs from HIV-$1_{KMT/L}$ and their infectivity. (A) Schematic diagram of chimeric envelope proteins generated through the exchange of restriction fragments between HIV-1_{NL4-3} and HIV-$1_{KMT/L}$ isolate. Open bars are DNA sequences from HIV-1_{NL4-3}, while black bars are from HIV-$1_{KMT/L}$ isolate. Grey bars denote the similar amino acid sequence to D1-2E3/N chimera. (B) Infectivity of HIV-$1_{KMT/L}$ chimera in NP-2/CCR5 cell line using luciferase assay method.

Role of C3-V5 domain of HIV-$1_{KMT/L}$ envelope in viral replication

Based on the above findings, an additional panel of env-pseudotyped chimeric luciferase reporter viruses was constructed between HIV-$1_{KMT/L}$ and HIV-1_{NL4-3} in order to determine whether the C3-V5 domain from HIV-$1_{KMT/L}$ could enable the D1-2E3/N chimeric virus to efficiently infect the NP-2/CCR5 and NP-2/CXCR4 cells (Fig. 17A). Interestingly, the KLv1-2/v3-5/N chimeric virus with a combination of the C3-V5 domain from HIV-$1_{KMT/L}$ together with the V1/V2 and V3 domains of D1-2E3/N efficiently infected both CCR5 and CXCR4 expressing cells (Fig. 17B). This clearly demonstrated a significant role played by the C3-V5 domain in restoring the infectivity of the D1-2E3/N chimeric virus. In addition, the KLv3-5/N chimeric virus that included the V3 and C3-V5 domains from the E3/N chimera and HIV-$1_{KMT/L}$, respectively, had a higher level of infectivity than that of the E3/N chimera (Figs. 13 and 17B). However, a chimeric virus with only the C3-V5 domain from HIV-$1_{KMT/L}$ (KLc3-5/N) was unable to infect the NP-2/CCR5 cells. Collectively, these data clarify an important role played by the C3-V5 domain in CCR5 utilization upon combination with the V1/V2 and V3 domains of HIV-1_{KMT}, and highlights the importance of such domain in HIV-1 infectivity.

Part 2

Selection of a MIP-1α-resistant HIV from the R5 HIV-1_{JR-FL}

For the isolation of a MIP-1α-resistant mutant from the R5 HIV in vitro, CCR5-expressing MOLT-4#8 cells, designated MOLT-4#8/CCR5 cells, were established (see methods) by stable trandsduction. This cell line expressed both CXCR4 and CCR5 coreceptors and could produce prominent syncytia upon infection with either X4 or R5 HIVs. This cell line was, therefore, expected to enable a possible shift in coreceptor usage from CCR5 to CXCR4. The R5 HIV-1_{JR-FL} strain was used for the selection of the MIP-1α-resistant virus. In order to determine the sensitivity of HIV-1_{JR-FL} to MIP-1α, we established the CCR5-expressing MAGI cell line, designated MAGI/CCR5 (expressing the *β-galactosidase* reporter gene). MAGI cells already express the CXCR4 coreceptor and therefore MAGI/CCR5 cells could support infection of both X4 and R5 HIVs.

The cell culture supernatant of HIV-1_{JR-FL}-infected MOLT-4#8/CCR5 cells was first used for the selection of the virus. The initial concentration of yeast-derived recombinant MIP-1α (yLD78β) for the selection process was 10 ng/ml. This concentration can inhibit HIV-1_{JR-FL} infection by 50% as determined by the MAGI assay (see methods). For the selection of a MIP-1α-resistant mutant, MOLT-4#8/CCR5 cells were first treated with MIP-1α and then infected with HIV-1_{JR-FL}. After observing the syncytium formation, virus was recovered for the next infection with increasing amounts of MIP-1α. HIV-1_{JR-FL} was also passaged in MOLT-4#8/CCR5 cells in the absence of MIP-1α to exclude the effect of long-term culture. After three months of passaging with increasing concentrations of MIP-1α (up to 200 ng/ml), the virus was subjected to MAGI assay using MAGI/CCR5 cells. After removing the residual MIP-1α from the culture by passaging infected cells for

three days without MIP-1α, MAGI/CCR5 cells were infected with the supernatant from the selected culture after treatment with various concentrations of MIP-1α followed by counting blue cells two days postinfection (Fig. 18). The selected virus displayed reduced sensitivity (4-fold) to MIP-1α (Fig. 18A). The IC_{50} of the selected virus for MIP-1α was 45 ng/ml, while that of wild-type HIV-1_{JR-FL} was 11 ng/ml. This resistant mutant also displayed a reduced sensitivity to MIP-1β (>4-fold) and RANTES (6-fold) (Fig. 18B and C).

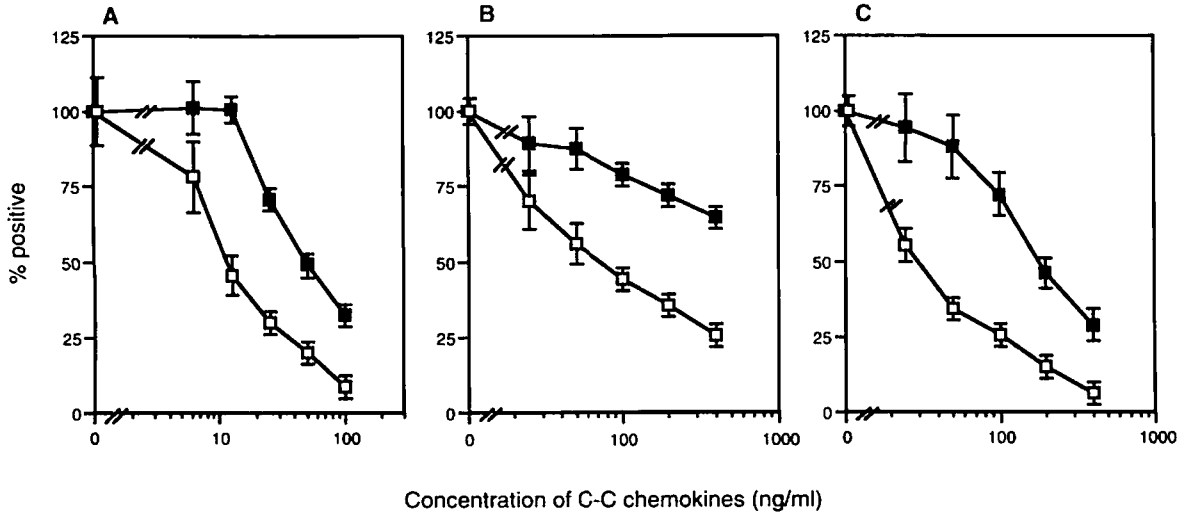


FIG. 18. Sensitivity of a selected mutant to C-C chemokines. MAGI/CCR5 cells were treated with various concentrations of MIP-1α **(A)**, MIP-1β **(B)**, and RANTES **(C)**, followed by an inoculation of wild-type HIV-1_{JR-FL} (□) and virus selected for 3 months in the presence of increasing concentrations of MIP-1α (■). Blue cells were counted 2 days after infection. The *y* axis represents the percentages of positive blue cells counted. The *x* axis represents the concentrations of each C-C chemokine. All experiments were performed in duplicate. The data are expressed as means ± standard deviations.

Sequence analysis of the *env* region of the MIP-1α-resistant mutant

To determine which *env* region is responsible for the reduced sensitivity of the MIP-1α-resistant mutant to C-C chemokines, the V1-V2, V3, V4, C3, and C4 regions of the envelope were sequenced after cloning the PCR product of each region (Fig. 19) using DNA from infected cells as template.

A) V2 amino acid sequences

JR-FL	CSFNITTSIRDEVQKEYALFYKLDVVPIDNNNTSYRLISC	clones with sequence/ total tested
passaged 2 wks.	----------	9/9
passaged 3 mo.	----------	5/8
	----------V----------	1/8
	----------P----------	1/8
	----------M----------	1/8
selected by MIP-1 α	----------M----------	9/10
3 mo	----------	1/10

B) V3 amino acid sequences

JR-FL	CTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHC	clones with sequence/ total tested
passaged 2 wks.	----------G----------	8/9
	----------	1/9
passaged 3 mo.	----------G----------	10/11
	----------	1/11
selected by MIP-1 α	----------G----------	21/22
3 mo	----------G----------N------	1/22

FIG. 19. V2 and V3 amino acid sequences from HIV-1_{JR-FL}-infected cells passaged and selected in MIP-1α for 3 months. Amplified products from infected MOLT-4#8/CCR5 cells passaged for 2 weeks and 3 months and selected by MIP-1α were cloned, and 8 to 22 clones from each sample were sequenced. The wild-type HIV-1_{JR-FL} amino acid sequences of V2 and V3 are shown in the top line. Numbers on the right are the numbers of clones with the sequence over the total number of clones tested. In each set of clones, the deduced amino acid sequences of the V2 (A) and V3 (B) regions were aligned by the single-amino-acid code. Dashes denote sequence identity.

Ten to 22 clones from each PCR product were isolated and sequenced. Analyses of *env* sequences from the resistant isolate using DNA from selected virus-infected cells revealed that the selected virus had a valine-to-methionine substitution at codon 166 (V166M) in the V2 region of the envelope (9 out of 10 clones) as well as a serine-to-glycine substitution at codon 303 (S303G) in the V3 region (Fig. 19) (22 out of 22 clones). Surprisingly, the virus passaged in MOLT-4#8/CCR5 cells for both 2 weeks and 3 months without MIP-1α showed the substitution at codon 303 (S303G) (10 out of 11 clones) but not the V166M substitution (none of 10 clones). The passaged virus without MIP-1α showed almost the same sensitivity to MIP-1α, suggesting that the V3 region substitution in selected viruses was not due to a selective pressure of MIP-1α but that it was probably due to an adaptation in MOLT-4#8/CCR5 cells. Other regions, including V1, V4, C3, and C4, had no remarkable changes in the resistant mutant envelope.

Determination of chemokine sensitivity for each mutant clone by single-round replication assay

To confirm whether V2 and V3 region mutations in the envelope were responsible for the reduced sensitivity to MIP-1α, an envelope complementation assay was performed. First, the envelope expression vectors with the intended mutations were constructed using the pCXN2 vector. Luciferase reporter HIV stocks pseudotyped with HIV envelopes were generated by cotransfecting 293T cells with HIV envelope expression vectors and a luciferase reporter HIV plasmid. Luciferase-reporter viruses were pseudotyped with the wild-type HIV-1_{JR-FL}, wild-type HIV-1_{NL4-3} or with mutated HIV-1_{JR-FL} (singly mutated with V166M in the V2 region, singly mutated with S303G in the V3 region, and doubly mutated with V166M and S303G) envelopes. As expected, the luciferase reporter HIV strain pseudotyped with the HIV-1_{NL4-3} envelope was totally resistant to MIP-1α (Fig. 20).

On the other hand, neither the strain with a single mutation at codon 166 (V166M) nor that with a single mutation at codon 303 (S303G) displayed a reduced sensitivity to MIP-1α (Fig. 20). Only the strain with a double mutation of both V166M and S303G (V166M/S303G) displayed a reduced sensitivity to MIP-1α (fourfold) to a level similar to that in the selected virus. This double mutant envelope was also responsible for reduced sensitivities to both MIP-1β and RANTES in the same assay (Table 3). The level of resistance to MIP-1α was similar to that of the selected variant (fivefold).

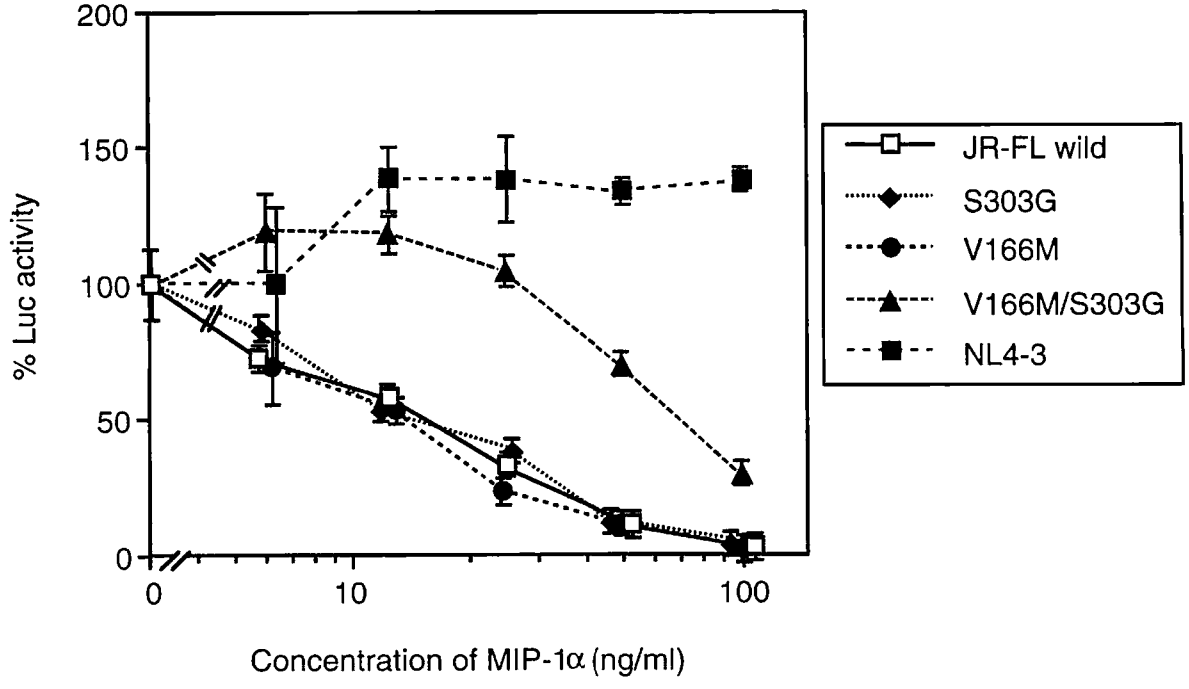


FIG. 20. Sensitivities of luciferase reporter HIV strains pseudotyped with mutant envelope to MIP-1α. MAGI/CCR5 cells were infected with luciferase reporter HIVs pseudotyped with the HIV-1_{JR-FL} envelope wild type (□), an S303G mutant (♦), a V166M mutant (●), an S303G/V166M mutant (▲), and the HIV-1_{NL4-3} envelope (■). Luciferase (Luc) activity was measured 3 days after infection. The *y* axis represents the relative luciferase activity of each virus. All experiments were performed in duplicate. The data are expressed as means ± standard deviations.

TABLE 4. Sensitivities of luciferase reporter HIV strains pseudotyped with mutant envelope to C-C chemokines

Envelope	*IC_{50} (ng/ml)* [a]		
	MIP-1α	**MIP-1β**	**RANTES**
JR-FL			
Wild type	16 ± 0.5	260 ± 7.1	130 ± 9.2
S303G	14 ± 1.3	290 ± 42	160 ± 6.4
V166M	13 ± 1.2	220 ± 7.1	110 ± 0.7
V166M/S303G	69 ± 1.4	>400	320 ± 35
NL4-3	>100	ND	ND

[a] All assays were performed in duplicate and the mean IC_{50} values (± standard deviations) are shown. ND, not done.

Determination of the chemokine receptor usage of each mutant clone and cellular tropism

We used MOLT-4#8/CCR5 and MAGI/CCR5 cells for determination of sensitivity to C-C chemokines and the selection of a MIP-1α-resistant mutant, respectively. If the resistant mutant has acquired the ability to use another major coreceptor like CXCR4, which was expressed on both cell lines, then the selected variant would display resistance to C-C chemokines because MIP-1α, MIP-1β, and RANTES are not able to interact with CXCR4. To determine whether these resistant mutants acquired the ability to use CXCR4, we infected the NP-2/CCR5 or NP-2/CXCR4 cell lines with luciferase reporter viruses pseudotyped with the wild type or mutant HIV-1_{JR-FL} envelope or HIV-1_{NL4-3} envelope. Luciferase activity after the infection of pseudotyped viruses with mutant HIV-1_{JR-FL} envelope clones, including S303G, V166M, and S303G/V166M mutants, showed that none of the mutants changed their chemokine receptor phenotype (Fig. 21). We also attempted to infect MOLT-4#8, CEM, MT-2, and MT-4 cells, which express only the CXCR4 and not the CCR5 coreceptor needed for HIV entry. Infection was

determined from both p24 antigen production in the cell culture supernatant and an indirect immunofluorescence assay using an anti-p24 antigen monoclonal antibody. The cell lines used were not infected with the S303G/V166M mutant, confirming that this resistant mutant does not display acquisition of other coreceptor usage.

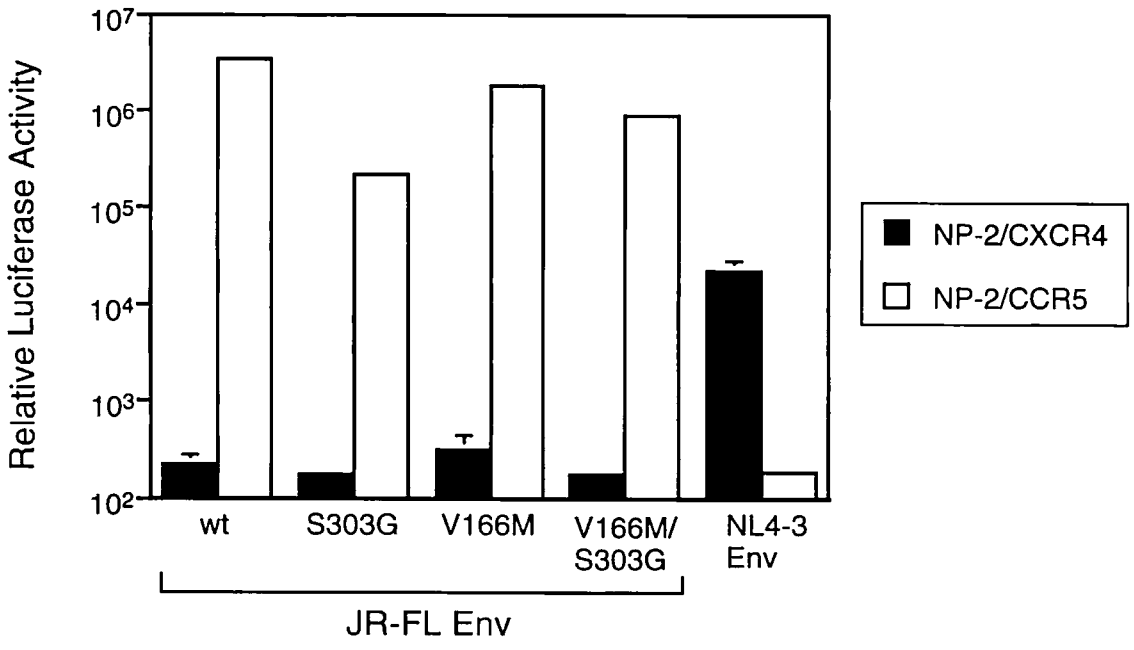


FIG. 21. Chemokine receptor usage of luciferase reporter HIVs pseudotyped with mutant envelope. NP-2/CD4 cells expressing CXCR4 or CCR5 were infected with luciferase reporter HIVs pseudotyped with the HIV-1_{JR-FL} envelope with the indicated mutations or the HIV-1_{NL4-3} envelope. Luciferase activity was measured 3 days after infection. The data presented are geometric means ± standard deviations of duplicate determinations.

Discussion

Utilization of chemokine receptors in conjunction with CD4 by HIV-1 strains largely accounts for the mechanisms underlying viral tropism at the level of virus entry [1, 26, 51]. A greater understanding about determinants of tropism and coreceptor selectivity for dualtropic strains could provide insights on the envelope regions or domains that mediate and modulate interactions with chemokine receptors and ultimately govern viral entry.

In the first part of our present study we sought to characterize the envelope regions of the dualtropic HIV-1_{KMT} isolate responsible for coreceptor utilization and efficient infection of CCR5 expressing cells. To do this, we employed two assay systems: i) cell-cell fusion assay with the SEAP reporter gene to detect fusion between envelope expressing cells and CD4/coreceptor expressing cells ii) cell-free env-pseudotyped virus infection assay with the luciferase reporter gene to assess viral replication. The former assay represents only the CD4-dependent viral fusion to the target cell, whereas the latter assay detects viral replication until Tat translation including viral adsorption and fusion. Findings in this part together with those of Cho *et al.* [24] revealed assay-dependent discrepancies between both systems that could influence conclusions about envelope determinants of coreceptor usage. These inconsistencies also hint at some of the complexities on the cell surface required for productive viral infection, and hence cannot be predicted by one system of assay. However, our findings confirmed by using both systems that the V3 region played the major role in determining fusion activity and viral infection to CCR5 expressing cells (Figs. 12 and 13). This is in accordance with previous studies which stated that the V3 configuration is crucial to cellular tropism and coreceptor usage of HIV-1 isolates [23, 33, 39, 63, 71, 143, 144, 165]. Other groups suggested that the V1/V2 configuration is also involved in cellular tropism by proving that several amino acid changes in the V2 region were able to alter cellular tropism [11, 60, 83, 84]. Additionally, Morikita *et al.* [109] observed that the V1/V2

configuration was important to cellular tropism and soluble CD4 sensitivity in combination with the V3 configuration. More recently, the V1/V2 and other V3 independent variable regions have been shown to influence or alter the usage [66, 130] of coreceptors. It has been shown that the V1/V2 region appears to play a crucial role in CXCR4 utilization of the dualtropic HIV-1_{DH12} and HIV-$1_{89.6}$ isolates [88, 148]. Our findings about the role of the V1/V2 region have been contradictory to the above reports, since we found that the V1/V2 region played no role in conferring CCR5 utilization to HIV-1_{NL4-3} (chimeras A1-2/N and D1-2/N) (Figs. 12 and 13). Therefore, we suggested that the role of V1/V2 could be dispensable for CCR5 utilization and hence in viral replication. In support of our observation are other reports [15, 151] showing that the deletion of V1 or V2 from the M or T-tropic primary HIV-1 isolates did not abrogate the ability of these viruses to replicate in peripheral blood mononuclear cells, macrophages or transformed T cell lines. Moreover, they indicated that binding of the 17b monoclonal antibody to the CD4-induced chemokine receptor-binding epitope was not influenced by deletion of the V1/V2 domain. Thus, it remains to be evaluated whether the V1/V2 region has any significant role in the infectivity of different HIV-1 strains.

In the cell-free infection assay, it was unexpected to find that viral infection became significantly diminished in CCR5 and CXCR4 expressing cells upon the introduction of both the V1/V2 and V3 domains from HIV-1_{KMT} into the HIV-1_{NL4-3} backbone (D1-2E3/N). This is because the same construct successfully fused with 293T cells expressing CD4 and either CCR5 or CXCR4 (Fig. 12). This finding demonstrated that fusion activity of HIV-1 is distinct from its infectivity and is in agreement with a previous study by Kato *et al.*, which mentioned that cellular tropism is not dictated solely by viral coreceptor utilization [77]. This also could be attributed either to differences in the binding affinity of gp120 to CD4; the involvement of gp120 in a postentry step necessary for viral replication; or a

different gp41 fusion activity among different chimeras. We considered it to be unlikely that the diminished infectivity was due to differences in the chemokine receptor interaction or an altered expression of gp120 by the virion since the same envelope successfully induced cell-cell fusion activity and was normally processed (Fig. 14). Thus, it became more likely that the D1-2E3/N chimeric Env protein lacked the necessary determinants for the efficient infection of coreceptor expressing cells.

The crystallographic resolution of a gp120 core structure of an X4 isolate bound to CD4 has revealed an intervening *"bridging sheet"* consisting of conserved residues between the inner and outer domains of gp120 that may serve as a major contact site for the chemokine receptor [128, 171] (Fig. 22). Amino acids from the C4 domain and the V1/V2 stem make up most of the *bridging sheet.* It is also important to note that the chemokine receptor-binding region is located between the bases of the V1/V2 and V3 loops [128]. On the other hand, the CD4 binding site is distributed among more than 6 domains including V1/V2 stem, C3 and C4 [86]. Crystallization studies revealed that CD4 binding to gp120 induces conformational changes that result in movement of the V1/V2 loops away from the *bridging sheet* leading to the exposure and/or formation of a binding site for specific chemokine receptors. However, in previous studies it was shown that the affinity of antibodies directed against the CD4 binding region for the multimeric envelope glycoprotein was altered by removal of the V1/V2 or V3 regions [151, 173]. This indicated that the V1/V2 or V3 domains may play a role in the attachment of virions to target cells before the conformational change takes place in its envelope glycoprotein. As we have shown, the D1-2E3/N chimeric virus with V1/V2 together with the V3 domain from HIV-1_{KMT} could not efficiently infect coreceptor expressing cells unless the C3-V5 domain from the same isolate was included in order to endorse the proper conformation of the CD4 binding epitope. Thus, it is

likely that the entire conformation of some of our chimeric envelopes, required for CD4 binding or fusion, became improperly folded when the C3-V5 domain was from a distinct isolate.

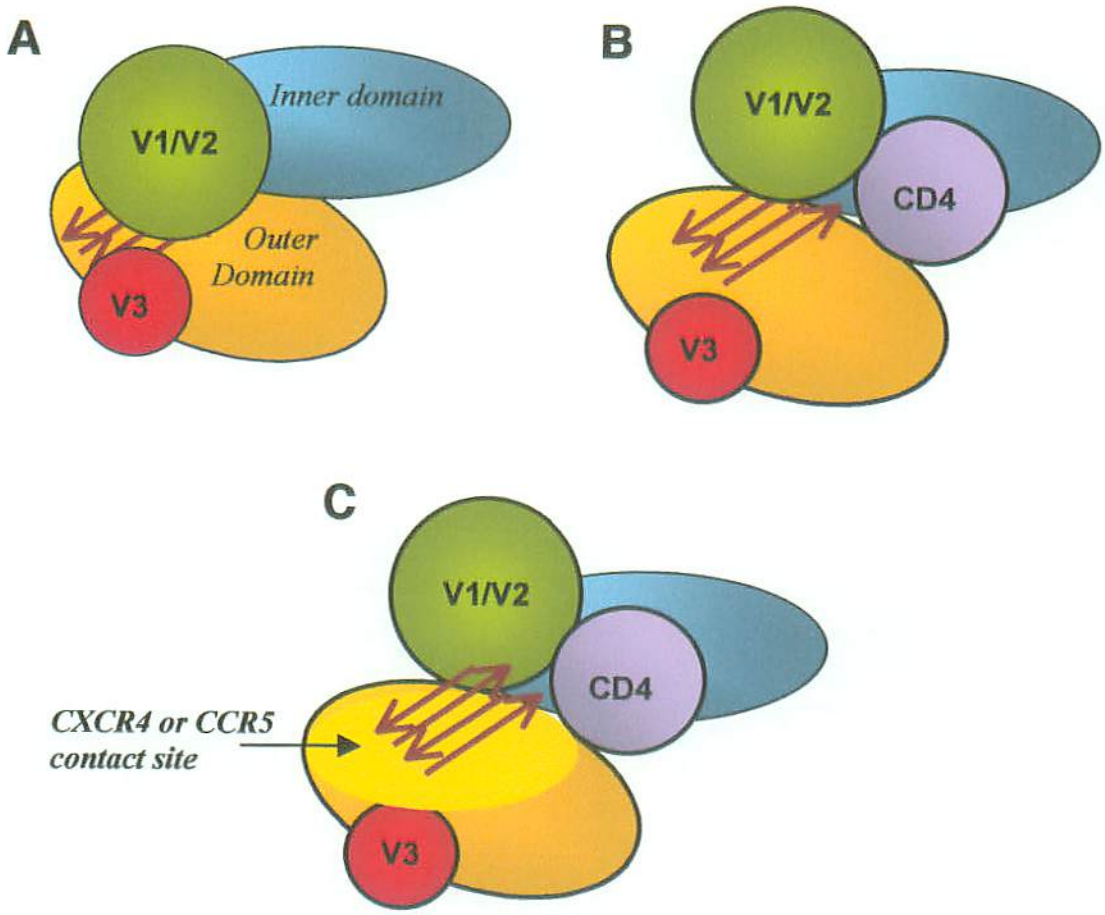


FIG. 22. Proposed conformational changes during binding of gp120 to its receptor (CD4) and coreceptor (CXCR4 or CCR5). A) The bridging sheet (hidden arrows), which spans the outer and inner gp120 domains, is made of residues from the stem of the V1/V2 loop and the CD4 binding region. **B)** CD4 binds to a region that forms a crevice in gp120, which may move the inner and outer domains relative to one another, exposing the bridging sheet. **C)** The flexibility of CD4 allows viral envelope to approach the target cell membrane, where CXCR4 or CCR5 interact with the bridging sheet. The charge or conformation of the V3 loop may determine which coreceptor will be used. (Moore JP and Binley J, Nature 393:630)

Until now, it is unknown whether the envelope glycoprotein has a role in viral replication after the CD4-induced conformational changes and viral fusion take place. Therefore, it could be a speculation that the discrepancy between cell-cell fusion and cell-free infection is due to the involvement of the envelope glycoprotein in any of the postentry steps necessary for viral replication. Taken together, both optimal utilization of coreceptors and viral replication may require all variable domains of certain HIV-1 isolates. Therefore, crystallization of the dualtropic Env including all variable regions appears necessary to resolve the above discrepancies.

Despite our finding that the HIV-$1_{KMT/L}$ pseudotyped virus efficiently utilized both CXCR4 and CCR5 coreceptors on NP-2/CD4 cells (Fig. 17B) and that dualtropic isolates could utilize CXCR4 expressed by primary macrophages [175], yet HIV-1_{KMT} was unable to replicate in macrophages (unpublished observation). This is consistent with other reports [20, 69, 77] which stated that CCR5 is not the single cofactor that determines entry and efficient replication of R5 and R5X4 isolates in these primary cells. Altogether, our findings emphasize that other functions of the Env protein, in addition to the cofactor-mediated entry, are necessary for productive infection, which would possibly be through additional cofactor mediated interactions.

In the second part of our study we conducted an *in vitro* dose-escalation experiment using MIP-1α C-C chemokine to isolate a chemokine resistant R5 HIV-1_{JR-FL} isolate. We determined that such resistant isolate displayed amino acid substitutions in both V2 (V166M) and V3 (S303G) regions. Importantly, the amino acid change in V3 (S303G) also occurred in long-term culture without MIP-1α, indicating that this substitution might be necessary for its adaptation to replicate in MOLT-4#8/CCR5 cells and not for its resistance. However, we confirmed that both mutations in V2 and V3 were crucial for the reduced sensitivity to MIP-1α using

luciferase reporter HIV pseudotyped with molecularly cloned mutant envelopes. Neither a single mutation in V2 nor a single mutation in V3 displayed reduced sensitivity to C-C chemokines, suggesting that a single amino acid change in the envelope region is not sufficient enough to obtain mutants resistant to C-C chemokines.

We anticipated that the resistant variant would have the ability to use CXCR4 as a coreceptor for HIV-1 entry. However, it became unlikely that this mutant could utilize CXCR4, since it was already unable to replicate in parental MOLT-4#8 cells. In addition to that, our chemokine receptor usage experiment with pseudotyped HIV confirmed that the double mutant clone (V166M/S303G), which displayed reduced sensitivity to MIP-1α, did not acquire the ability to utilize CXCR4. In support of this observation is a study done by Schols *et al.* [42], which showed an SDF-1α-resistant virus isolated from X4 virus that did not switch coreceptors. The mechanism(s) by which the coreceptor usage changes is still poorly understood.

Recent studies using chimeric chemokine receptors showed that multiple epitopes on the extracellular domains were involved in the interaction of HIV-1 but that the chemokine binding site was limited to only the N-terminal domain of the receptor [4, 9, 42, 95, 122, 132]. Thus, it is possible that the double mutant interacts with another portion(s) of CCR5. Alternatively, the affinity of the resistant mutant envelope for CCR5 after CD4 binding may compete the binding of MIP-1α to CCR5 even if it uses the same portion of CCR5.

Cocchi *et al.* [27] reported that the blockade of R5 HIV-1 by C-C chemokines was determined by the V3 region. Jansson *et al.* [72] further showed that a serine-to-glycine substitution in the V3 region, which was also found in our mutant (S303G), was associated with a loss of sensitivity to C-C chemokines together

with an additional amino acid substitution (glutamic acid to arginine) in the V3 region of some HIV-1-infected individuals during disease progression. Other studies showed that positively charged amino acid substitutions in the V3 region were correlating with the syncytium-inducing phenotype of HIV-1 isolates [33, 53]. Our resistant mutant, however, did not acquire positively charged amino acid changes in V3 during the selection, suggesting that it displays an intermediate preference for the CCR5 and CXCR4 chemokine receptors. This serine-to-glycine substitution might increase the replicative ability of HIV since it occurred in passaged virus in MOLT-4#8/CCR5 cells. Further, several amino acid changes, especially of positively charged amino acids in the V3 region, may be necessary to change coreceptor usage. On the other hand, our chimeric envelope experiments revealed that the sensitivity of HIV to C-C chemokines was dependent on the cooperative interaction of CCR5 with both V2 and V3. Previous studies have shown that the V2 configuration is associated with disease progression in combination with the V3 configuration [60, 141, 145, 176], suggesting that the V166M amino acid change in V2 has a role in the evolution of HIV. It is also of note that some primary isolates in the Los Alamos database [85] have methionine at position 166 in the V2 region, although, the sensitivity of those isolates to C-C chemokines is not known. Thus, both the V2 and V3 regions could be associated with a loss of sensitivity to C-C chemokines and a phenotype switch from CCR5 to CXCR4 usage during the evolution of HIV and disease progression *in vivo*.

The recent crystallization of the gp120 core which defined the intervening *bridging sheet* might also give insights on how our R5 mutant became insensitive to MIP-1α. As already mentioned the *bridging sheet* is composed mostly of the V1/V2 stem and C4. CD4 binding to gp120 distorts the V1/V2 loop, leading to exposure of the *bridging sheet* and further allowing for CCR5 binding. The mutations in the V1/V2 stem combined with V3 mutations might affect the

formation of the *bridging sheet*, allowing this mutant to interact with CCR5 differently after CD4 binding. Alternatively, some residues in the V2 stem may be directly involved in chemokine receptor interaction, as previously suggested [128, 171]. It is also conceivable that this V2 mutation combined with the V3 mutation alters the binding affinity of gp120 for CD4, resulting in conformational changes in the *bridging sheet*. Our resistant mutant did not alter cellular tropism probably because of its low level of resistance, suggesting that another factor is necessary for the phenotype switch from CCR5 to CXCR4 usage.

In conclusion, we have shown that a complex structural interaction between the V1/V2, V3 and C3-V5 regions could determine the outcome of viral replication for certain dualtropic HIV-1 isolates like our HIV-1_{KMT}. Moreover, we indicated that a combination of mutations in both the V2 and V3 regions (and not of each region alone) of the CCR5-tropic HIV-1_{JR-FL} envelope modulates the sensitivity of HIV-1 to C-C chemokines without altering the ability to use chemokine receptors. Collectively this highlights the importance of cooperative interaction between various envelope domains in determining viral infectivity and sensitivity to chemokines. Therefore, further studies appear to be necessary in order to elucidate the role of envelope glycoproteins particularly from dualtropic isolates in productive viral infection as well as virus-cell interactions. In addition to that, further selection of mutants resistant to C-C chemokines might elucidate the role of C-C chemokines in the ability to switch chemokine receptors and in cellular tropism during the course of HIV infection. We believe these future attempts together with those presented herein might offer a clearer understanding about HIV-1 tropism *in vivo*.

References

1) Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM, Berger EA. 1996. CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science.* **272**:1955-1958.

2) Amara A, Gall SL, Schwartz O, Salamero J, Montes M, Loetscher P, Baggiolini M, Virelizier JL, Arenzana-Seisdedos F. 1997. HIV coreceptor downregulation as antiviral principle: SDF-1alpha-dependent internalization of the chemokine receptor CXCR4 contributes to inhibition of HIV replication. *J Exp Med.* **186**:139-146.

3) Arakaki R, Tamamura H, Premanathan M, Kanbara K, Ramanan S, Mochizuki K, Baba M, Fujii N, Nakashima H. 1999. T134, a small-molecule CXCR4 inhibitor, has no cross-drug resistance with AMD3100, a CXCR4 antagonist with a different structure. *J Virol.* **73**:1719-1723.

4) Atchison RE, Gosling J, Monteclaro FS, Franci C, Digilio L, Charo IF, Goldsmith MA. 1996. Multiple extracellular elements of CCR5 and HIV-1 entry: dissociation from response to chemokines. *Science.* **274**:1924-1926.

5) Aukrust P, Muller F, Froland SS. 1998. Circulating levels of RANTES in human immunodeficiency virus type 1 infection: effect of potent antiretroviral therapy. *J Infect Dis.* **177**:1091-6.

6) Baba M, Nishimura O, Kanzaki N, Okamoto M, Sawada H, Iizawa Y, Shiraishi M, Aramaki Y, Okonogi K, Ogawa Y, Meguro K, Fujino M. 1999. A small-molecule, nonpeptide CCR5 antagonist with highly potent and selective anti-HIV-1 activity. *Proc Natl Acad Sci U S A.* **96**:5698-5703.

7) Berger EA. 1997. HIV entry and tropism: the chemokine receptor connection. AIDS. **11**:S3-16.

8) Berson JF, Doms RW. 1998. Structure-function studies of the HIV-1 coreceptors. *Semin Immunol.* **10**:237-248.

9) Bieniasz PD, Fridell RA, Aramori I, Ferguson SS, Caron MG, Cullen BR. 1997. HIV-1-induced cell fusion is mediated by multiple regions within both the viral envelope and the CCR5 coreceptor. *EMBO J.* **16**:2599-2609.

10) Bleul CC, Farzan M, Choe H, Parolin C, Clark-Lewis I, Sodroski J, Springer TA. 1996. The lymphocyte chemo-attractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. *Nature.* **382**:829-833.

11) Boyd MT, Simpson GR, Cann AJ, Johnson MA, Weiss RA. 1993. A single amino acid substitution in the V1 loop of human immunodeficiency virus type 1 gp120 alters cellular tropism. *J Virol.* **67**:3649-3652.

12) Bugelski, P. J., Ellens, H., Hart, T. K. & Kirsh, R. L. 1991. Soluble CD4 and dextran sulphate mediate release of gp120 from HIV-1: Implications for clinical trials. *J Acq Immun Def Synd.* **4**:923-924.

13) Cabrera C, Witvrouw M, Gutierrez A, Clotet B, Kuipers ME, Swart PJ, Meijer DK, Desmyter J, De Clercq E, Este JA. 1999. Resistance of the human immunodeficiency virus to the inhibitory action of negatively charged albumins on virus binding to CD4. *AIDS Res Hum Retroviruses.* **15**:1535-1543.

14) Camaur D, Trono D. 1996. Characterization of human immunodeficiency virus type 1 Vif particle. *J Virol.* **70**:6106-6111.

15) Cao J, Sullivan N, Desjardin E, Parolin C, Robinson J, Wyatt R, Sodroski J. 1997. Replication and neutralization of human immunodeficiency virus type 1 lacking the V1 and V2 variable loops of the gp120 envelope glycoprotein. *J Virol.* **71**:9808-9812.

16) Carrillo A, Ratner L. 1996. Cooperative effects of the human immunodeficiency virus type 1 envelope variable loops V1 and V3 in mediating infectivity for T cells. *J Virol.* **70**:1310-1316.

17) Cecilia D, KewalRamani VN, O'Leary J, Volsky B, Nyambi P, Burda S, Xu S, Littman DR, Zolla-Pazner S. 1998. Neutralization profiles of primary human immunodeficiency virus type 1 isolates in the context of coreceptor usage. *J Virol.* **72**:6988-6996.

18) Chan DC, Fass D, Berger JM, Kim PS. 1997. Core structure of gp41 from the HIV envelope glycoprotein. *Cell.* **89**:263-273.

19) Chen BK, Saksela K, Andino R, Baltimore D. 1994. Distinct modes of human immunodeficiency virus type 1 proviral latency revealed by superinfection of

nonproductively infected cell lines with recombinant luciferase-encoding viruses. *J Virol.* **68**:654-660.

20) Cheng-Mayer C, Liu R, Landau NR, Stamatatos L. 1997. Macrophage tropism of human immunodeficiency virus type 1 and utilization of the CC-CKR5 coreceptor. *J Virol.* **71**:1657-1661.

21) Cheng-Mayer C, Quiroga M, Tung JW, Dina D, Levy JA. 1990. Viral determinants of human immunodeficiency virus type 1 T-cell or macrophage tropism, cytopathogenicity, and CD4 antigen modulation. *J Virol.* **64**:4390-4398.

22) Chesebro B, Nishio J, Perryman S, Cann A, O'Brien W, Chen IS, Wehrly K. 1991. Identification of human immunodeficiency virus envelope gene sequences influencing viral entry into CD4-positive HeLa cells, T-leukemia cells, and macrophages. *J Virol.* **65**:5782-5789.

23) Chesebro B, Wehrly K, Nishio J, Perryman S. 1996. Mapping of independent V3 envelope determinants of human immunodeficiency virus type 1 macrophage tropism and syncytium formation in lymphocytes. *J Virol.* **70**:9055-9059.

24) Cho MW, Lee MK, Carney MC, Berson JF, Doms RW, Martin MA. 1998. Identification of determinants on a dualtropic human immunodeficiency virus type 1 envelope glycoprotein that confer usage of CXCR4. *J Virol.* **72**:2509-2515.

25) Choe H, Farzan M, Konkel M, Martin K, Sun Y, Marcon L, Cayabyab M, Berman M, Dorf ME, Gerard N, Gerard C, Sodroski J. 1998. The orphan seven-transmembrane receptor apj supports the entry of primary T-cell-line-tropic and dualtropic human immunodeficiency virus type 1. *J Virol.* **72**:6113-6118.

26) Choe H, Farzan M, Sun Y, Sullivan N, Rollins B, Ponath PD, Wu L, Mackay CR, LaRosa G, Newman W, Gerard N, Gerard C, Sodroski J. 1996. The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell.* **85**:1135-1148.

27) Cocchi F, DeVico AL, Garzino-Demo A, Arya SK, Gallo RC, Lusso P. 1995. Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. *Science.* **270**:1811-1815.

28) Cocchi F, DeVico AL, Garzino-Demo A, Cara A, Gallo RC, Lusso P. 1996. The V3 domain of the HIV-1 gp120 envelope glycoprotein is critical for chemokine-mediated blockade of infection. *Nat Med.* **2**:1244-1247.

29) Collman R, Balliet JW, Gregory SA, Friedman H, Kolson DL, Nathanson N, Srinivasan A. 1992. An infectious molecular clone of an unusual macrophage-tropic and highly cytopathic strain of human immunodeficiency virus type 1. *J Virol.* **66**:7517-7521.

30) Connor RI, Mohri H, Cao Y, Ho DD. 1993. Increased viral burden and cytopathicity correlate temporally with CD4+ T-lymphocyte decline and clinical progression in human immunodeficiency virus type 1-infected individuals. *J Virol.* **67**:1772-1777.

31) Connor RI, Sheridan KE, Ceradini D, Choe S, Landau NR. 1997. Change in coreceptor use coreceptor use correlates with disease progression in HIV-1-infected individuals. *J Exp Med.* **185**:621-628.

32) Datema R, Rabin L, Hincenbergs M, Moreno MB, Warren S, Linquist V, Rosenwirth B, Seifert J, McCune JM. 1996. Antiviral efficacy in vivo of the anti-human immunodeficiency virus bicyclam SDZ SID 791 (JM 3100), an inhibitor of infectious cell entry. *Antimicrob Agents Chemother.* **40**:750-754.

33) De Jong JJ, De Ronde A, Keulen W, Tersmette M, Goudsmit J. 1992. Minimal requirements for the human immunodeficiency virus type 1 V3 domain to support the syncytium-inducing phenotype: analysis by single amino acid substitution. *J Virol.* **66**:6777-6780.

34) Dean M, Carrington M, Winkler C, Huttley GA, Smith MW, Allikmets R, Goedert JJ, Buchbinder SP, Vittinghoff E, Gomperts E, Donfield S, Vlahov D, Kaslow R, Saah A, Rinaldo C, Detels R, O'Brien SJ. 1996. Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. *Science.* **273**:1856-1862.

35) Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, Di Marzio P, Marmon S, Sutton RE, Hill CM, Davis CB, Peiper SC, Schall TJ, Littman DR, Landau NR.

1996. Identification of a major co-receptor for primary isolates of HIV-1. *Nature.* **381**:661-666.

36) Deng HK, Unutmaz D, KewalRamani VN, Littman DR. 1997. Expression cloning of new receptors used by simian and human immunodeficiency viruses. *Nature.* **388**:296-300.

37) Dittmar MT, McKnight A, Simmons G, Clapham PR, Weiss RA, Simmonds P. 1997. HIV-1 tropism and co-receptor use. *Nature.* **385**:495-496.

38) Dolei A, Biolchini A, Serra C, Curreli S, Gomes E, Dianzani F. 1998. Increased replication of T-cell-tropic HIV strains and CXC-chemokine receptor-4 induction in T cells treated with macrophage inflammatory protein (MIP)-1alpha, MIP-1beta and RANTES beta-chemokines. *AIDS.* **12**:183-190.

39) Donaldson YK, Bell JE, Holmes EC, Hughes ES, Brown HK, Simmonds P. 1994. In vivo distribution and cytopathology of variants of human immunodeficiency virus type 1 showing restricted sequence variability in the V3 loop. *J Virol.* **68**:5991-6005.

40) Donzella GA, Schols D, Lin SW, Este JA, Nagashima KA, Maddon PJ, Allaway GP, Sakmar TP, Henson G, De Clercq E, Moore JP. 1998. AMD3100, a small molecule inhibitor of HIV-1 entry via the CXCR4 co-receptor. *Nat Med.* **4**:72-77.

41) Doranz BJ, Grovit-Ferbas K, Sharron MP, Mao SH, Goetz MB, Daar ES, Doms RW, O'Brien WA. 1997. A small-molecule inhibitor directed against the chemokine receptor CXCR4 prevents its use as an HIV-1 coreceptor. *J Exp Med.* **186**:1395-1400.

42) Doranz BJ, Lu ZH, Rucker J, Zhang TY, Sharron M, Cen YH, Wang ZX, Guo HH, Du JG, Accavitti MA, Doms RW, Peiper SC. 1997. Two distinct CCR5 domains can mediate coreceptor usage by human immunodeficiency virus type 1. *J Virol.* **71**:6305-6314.

43) Doranz BJ, Rucker J, Yi Y, Smyth RJ, Samson M, Peiper SC, Parmentier M, Collman RG, Doms RW. 1996. A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell.* **85**:1149-1158.

44) Dragic T, Charneau P, Clavel F, Alizon M. 1992. Complementation of murine cells for human immunodeficiency virus envelope/CD4-mediated fusion in human/murine heterokaryons. *J Virol.* **66**:4794-4802.

45) Dragic T, Litwin V, Allaway GP, Martin SR, Huang Y, Nagashima KA, Cayanan C, Maddon PJ, Koup RA, Moore JP, Paxton WA. 1996. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature.* **381**:667-673.

46) Earl PL, Doms RW, Moss B. 1990. Oligomeric structure of the human immunodeficiency virus type 1 envelope glycoprotein. *Proc Natl Acad Sci U S A.* **87**:648-652.

47) Edinger AL, Hoffman TL, Sharron M, Lee B, Yi Y, Choe W, Kolson DL, Mitrovic B, Zhou Y, Faulds D, Collman RG, Hesselgesser J, Horuk R, Doms RW. 1998. An orphan seven-transmembrane domain receptor expressed widely in the brain functions as a coreceptor for human immunodeficiency virus type 1 and simian immunodeficiency virus. *J Virol.* **72**:7934-7940.

48) Endres MJ, Clapham PR, Marsh M, Ahuja M, Turner JD, McKnight A, Thomas JF, Stoebenau-Haggarty B, Choe S, Vance PJ, Wells TN, Power CA, Sutterwala SS, Doms RW, Landau NR, Hoxie JA. 1996. CD4-independent infection by HIV-2 is mediated by fusin/CXCR4. *Cell.* **87**:745-756.

49) Este JA, Cabrera C, Blanco J, Gutierrez A, Bridger G, Henson G, Clotet B, Schols D, De Clercq E. 1999. Shift of clinical human immunodeficiency virus type 1 isolates from X4 to R5 and prevention of emergence of the syncytium-inducing phenotype by blockade of CXCR4. *J Virol.* **73**:5577-5585.

50) Farzan M, Choe H, Martin K, Marcon L, Hofmann W, Karlsson G, Sun Y, Barrett P, Marchand N, Sullivan N, Gerard N, Gerard C, Sodroski J. 1997. Two orphan seven-transmembrane segment receptors which are expressed in CD4-positive cells support simian immunodeficiency virus infection. *J Exp Med.* **186**:405-411.

51) Feng Y, Broder CC, Kennedy PE, Berger EA. 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science.* **272**:872-877.

52) Ferrer M, Kapoor TM, Strassmaier T, Weissenhorn W, Skehel JJ, Oprian D, Schreiber SL, Wiley DC, Harrison SC. 1999. Selection of gp41-mediated HIV-1 cell entry inhibition from biased combinational libraries of non-natural binding elements. *Nature Struct Biol.* **6**:953-959.

53) Fouchier RA, Groenink M, Kootstra NA, Tersmette M, Huisman HG, Miedema F, Schuitemaker H. 1992. Phenotype-associated sequence variation in the third variable domain of the human immunodeficiency virus type 1 gp120 molecule. *J Virol.* **66**:3183-3187.

54) Fox DG, Balfe P, Palmer CP, May JC, Arnold C, McKeating JA. 1997. Length polymorphism within the second variable region of the human immunodeficiency virus type 1 envelope glycoprotein affects accessibility of the receptor binding site. *J Virol.* **71**:759-765.

55) Fung MS, Sun CR, Gordon WL, Liou RS, Chang TW, Sun WN, Daar ES, Ho DD. 1992. Identification and characterization of a neutralization site within the second variable region of human immunodeficiency virus type 1 gp120. *J Virol.* **66**:848-856.

56) Furtado MR, Callaway DS, Phair JP, Kunstman KJ, Stanton JL, Macken CA, Perelson AS, Wolinsky SM. 1999. Persistence of HIV-1 transcription in peripheral-blood mononuclear cells in patients receiving potent antiretroviral therapy. *N Engl J Med.* **340**:1614-1622.

57) Gelderblom HR, Hausmann EH, Ozel M, Pauli G, Koch MA. 1987. Fine structure of human immunodeficiency virus (HIV) and immunolocalization of structural proteins. *Virology.* **156**:171-176.

58) Gelderblom HR, Ozel M, Pauli G. Morphogenesis and morphology of HIV. 1989. Structure-function relations. *Arch Virol.* **106**:1-13.

59) Gorny MK, Moore JP, Conley AJ, Karwowska S, Sodroski J, Williams C, Burda S, Boots LJ, Zolla-Pazner S. 1994. Human anti-V2 monoclonal antibody that neutralizes primary but not laboratory isolates of human immunodeficiency virus type 1. *J Virol.* **68**:8312-8320.

60) Groenink M, Fouchier RA, Broersen S, Baker CH, Koot M, van't Wout AB, Huisman HG, Miedema F, Tersmette M, Schuitemaker H. 1993. Relation of phenotype evolution of HIV-1 to envelope V2 configuration. *Science.* **260**:1513-1516.

61) Harrison SC. CD4: the receptor for HIV. In: Wimmer E, ed. Cellular receptors for animal viruses. Cold Spring Harbor, NY: Cold Spring Harbor Press; 1994:33-48.

62) Harrowe G, Cheng-Mayer C. 1995. Amino acid substitutions in the V3 loop are responsible for adaptation to growth in transformed T-cell lines of a primary human immunodeficiency virus type 1. *Virology.* **210**:490-494.

63) Henkel T, Westervelt P, Ratner L. 1995. HIV-1 V3 envelope sequences required for macrophage infection. *AIDS.* **9**:399-401.

64) Hernandez LD, Hoffman LR, Wolfsberg TG, White JM. 1996. Virus-cell and cell-cell fusion. *Annu Rev Cell Dev Biol.* **12**:627-661.

65) Heveker N, Montes M, Germeroth L, Amara A, Trautmann A, Alizon M, Schneider-Mergener J. 1998. Dissociation of the signalling and antiviral properties of SDF-1-derived small peptides. *Curr Biol.* **8**:369-376.

66) Hoffman TL, Stephens EB, Narayan O, Doms RW. 1998. HIV type I envelope determinants for use of the CCR2b, CCR3, STRL33, and APJ coreceptors. *Proc Natl Acad Sci U S A.* **95**:11360-11365.

67) Howard OM, Oppenheim JJ, Hollingshead MG, Covey JM, Bigelow J, McCormack JJ, Buckheit RW Jr, Clanton DJ, Turpin JA, Rice WG. 1998. Inhibition of in vitro and in vivo HIV replication by a distamycin analogue that interferes with chemokine receptor function: a candidate for chemotherapeutic and microbicidal application. *J Med Chem.* **41**:2184-2193.

68) Huang ZB, Potash MJ, Simm M, Shahabuddin M, Chao W, Gendelman HE, Eden E, Volsky DJ. 1993. Infection of macrophages with lymphotropic human immunodeficiency virus type 1 can be arrested after viral DNA synthesis. *J Virol.* **67**:6893-6896.

69) Hung CS, Vander Heyden N, Ratner L. 1999. Analysis of the critical domain in the V3 loop of human immunodeficiency virus type 1 gp120 involved in CCR5 utilization. *J Virol.* **73**:8216-8226.

70) Hunter E. 1994. Macromolecular interactions in the assembly of HIV and other retroviruses. *Sem Virol.* **5**:71-83.

71) Hwang SS, Boyle TJ, Lyerly HK, Cullen BR. 1991. Identification of the envelope V3 loop as the primary determinant of cell tropism in HIV-1. *Science.* **253**:71-74.

72) Jansson M, Popovic M, Karlsson A, Cocchi F, Rossi P, Albert J, Wigzell H. 1996. Sensitivity to inhibition by beta-chemokines correlates with biological phenotypes of primary HIV-1 isolates. *Proc Natl Acad Sci U S A.* **93**:15382-15387.

73) Jinno A, Shimizu N, Soda Y, Haraguchi Y, Kitamura T, Hoshino H. 1998. Identification of the chemokine receptor TER1/CCR8 expressed in brain-derived cells and T cells as a new coreceptor for HIV-1 infection. *Biochem Biophys Res Commun.* **243**:497-502.

74) Joint United Nations Programme on HIV/AIDS and World Health Organization. 2001. *AIDS Epidemic Update:UNAIDS*, Durban.

75) Jones PL, Korte T, Blumenthal R. 1998. Conformational changes in cell surface HIV-1 envelope glycoproteins are triggered by cooperation between cell surface CD4 and co-receptors. *J Biol Chem.* **273**:404-409.

76) Kaminchik J, Margalit R, Yaish S, Drummer H, Amit B, Sarver N, Gorecki M, Panet A. 1994. Cellular distribution of HIV type 1 Nef protein: identification of domains in Nef required for association with membrane and detergent-insoluble cellular matrix. *AIDS Res Hum Retroviruses.* **10**:1003-1010.

77) Kato K, Sato H, Takebe Y. 1999. Role of naturally occurring basic amino acid substitutions in the human immunodeficiency virus type 1 subtype E envelope V3 loop on viral coreceptor usage and cell tropism. *J Virol.* **73**:5520-5526.

78) Kilby JM, Hopkins S, Venetta TM, DiMassimo B, Cloud GA, Lee JY, Alldredge L, Hunter E, Lambert D, Bolognesi D, Matthews T, Johnson MR, Nowak MA, Shaw GM, Saag MS. 1998. Potent suppression of HIV-1 replication in humans by T-20, a peptide inhibitor of gp41-mediated virus entry. *Nat Med.* **4**:1302-1307.

79) Kim FM, Kolson DL, Balliet JW, Srinivasan A, Collman RG. 1995. V3-independent determinants of macrophage tropism in a primary human immunodeficiency virus type 1 isolate. *J Virol.* **69**:1755-1761.

80) Kim S, Ikeuchi K, Groopman J, Baltimore D. 1990. Factors affecting cellular tropism of human immunodeficiency virus. *J Virol.* **64**:5600-5604.

81) Kimpton J, Emerman M. 1992. Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated beta-galactosidase gene. *J Virol.* **66**:2232-2239.

82) Kinter A, Catanzaro A, Monaco J, Ruiz M, Justement J, Moir S, Arthos J, Oliva A, Ehler L, Mizell S, Jackson R, Ostrowski M, Hoxie J, Offord R, Fauci AS. 1998. CC-chemokines enhance the replication of T-tropic strains of HIV-1 in CD4(+) T cells: role of signal transduction. *Proc Natl Acad Sci U S A.* **95**:11880-11885.

83) Koito A, Harrowe G, Levy JA, Cheng-Mayer C. 1994. Functional role of the V1/V2 region of human immunodeficiency virus type 1 envelope glycoprotein gp120 in infection of primary macrophages and soluble CD4 neutralization. *J Virol.* **68**:2253-2259.

84) Koito A, Stamatatos L, Cheng-Mayer C. 1995. Small amino acid sequence changes within the V2 domain can affect the function of a T-cell line-tropic human immunodeficiency virus type 1 envelope gp120. *Virology.* **206**:878-884.

85) Korber, B., B. Hahn, B. Foley, J. W. Mellors, T. Leitner, G. Myers, F. McCutchan, and C. L. Kuiken. 1997. Human retroviruses and AIDS: a compilation and analysis of nucleic acid and amino acid sequences. Los Alamos National Laboratory, Los Alamos, N. Mex.

86) Kwong PD, Wyatt R, Robinson J, Sweet RW, Sodroski J, Hendrickson WA. 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature.* **393**:648-659.

87) Lapham CK, Ouyang J, Chandrasekhar B, Nguyen NY, Dimitrov DS, Golding H. 1996. Evidence for cell-surface association between fusin and the CD4-gp120 complex in human cell lines. *Science.* **274**:602-605.

88) Lee MK, Heaton J, Cho MW. 1999. Identification of determinants of interaction between CXCR4 and gp120 of a dual-tropic HIV-1DH12 isolate. *Virology.* **257**:290-296.

89) Leonard CK, Spellman MW, Riddle L, Harris RJ, Thomas JN, Gregory TJ. 1990. Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. *J Biol Chem.* **265**:10373-10382.

90) Liao F, Alkhatib G, Peden KW, Sharma G, Berger EA, Farber JM. 1997. STRL33, A novel chemokine receptor-like protein, functions as a fusion cofactor for both macrophage-tropic and T cell line-tropic HIV-1. *J Exp Med.* **185**:2015-2023.

91) Lifson JD, Feinberg MB, Reyes GR, Rabin L, Banapour B, Chakrabarti S, Moss B, Wong-Staal F, Steimer KS, Engleman EG. 1986. Induction of CD4-dependent cell fusion by the HTLV-III/LAV envelope glycoprotein. *Nature.* **323**:725-728.

92) Liu H, Wu X, Newman M, Shaw GM, Hahn BH, Kappes JC. 1995. The Vif protein of human and simian immunodeficiency viruses is packaged into virions and associates with viral core structures. *J Virol.* **69**:7630-7638.

93) Liu R, Paxton WA, Choe S, Ceradini D, Martin SR, Horuk R, MacDonald ME, Stuhlmann H, Koup RA, Landau NR. 1996. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell.* **86**:367-377.

94) Lu YL, Spearman P, Ratner L. 1993. Human immunodeficiency virus type 1 viral protein R localization in infected cells and virions. *J Virol.* **67**:6542-6550.

95) Lu Z, Berson JF, Chen Y, Turner JD, Zhang T, Sharron M, Jenks MH, Wang Z, Kim J, Rucker J, Hoxie JA, Peiper SC, Doms RW. 1997. Evolution of HIV-1 coreceptor usage through interactions with distinct CCR5 and CXCR4 domains. *Proc Natl Acad Sci U S A.* **94**:6426-6431.

96) Lucas GM, Chaisson RE, Moore RD. 1999. Highly active antiretroviral therapy in a large urban clinic: risk factors for virologic failure and adverse drug reactions. *Ann Intern Med.* **131**:81-87.

97) Mack M, Luckow B, Nelson PJ, Cihak J, Simmons G, Clapham PR, Signoret N, Marsh M, Stangassinger M, Borlat F, Wells TN, Schlondorff D, Proudfoot AE. 1998.

Aminooxypentane-RANTES induces CCR5 internalization but inhibits recycling: a novel inhibitory mechanism of HIV infectivity. *J Exp Med.* **187**:1215-1224.

98) Maddon PJ, Dalgleish AG, McDougal JS, Clapham PR, Weiss RA, Axel R. 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. *Cell.* **47**:333-348.

99) Maeda Y, Foda M, Matsushita S, Harada S. 2000. Involvement of both the V2 and V3 regions of the CCR5-tropic human immunodeficiency virus type 1 envelope in reduced sensitivity to macrophage inflammatory protein 1alpha. *J Virol.* **74**:1787-1793.

100) Maeda Y, Venzon DJ, Mitsuya H. 1998. Altered drug sensitivity, fitness, and evolution of human immunodeficiency virus type 1 with pol gene mutations conferring multi-dideoxynucleoside resistance. *J Infect Dis.* **177**:1207-1213.

101) Mann M, Shelhamer JH, Masur H, Gill VJ, Travis W, Solomon D, Manischewitz J, Stock F, Lane HC, Ognibene FP. 1997. Lack of clinical utility of bronchoalveolar lavage cultures for cytomegalovirus in HIV infection. *Am J Respir Crit Care Med.* **155**:1723-1728.

102) Marechal V, Arenzana-Seisdedos F, Heard JM, Schwartz O. 1999. Opposite effects of SDF-1 on human immunodeficiency virus type 1 replication. *J Virol.* **73**:3608-3615.

103) Marriott D, McMurchie M. 1996. Managing HIV. Part 2: Phases of disease. 2.4 HIV and advanced immune deficiency. *Med J Aust.* **164**:111-112.

104) McCune JM, Rabin LB, Feinberg MB, Lieberman M, Kosek JC, Reyes GR, Weissman IL. 1988. Endoproteolytic cleavage of gp160 is required for the activation of human immunodeficiency virus. *Cell.* **53**:55-67.

105) McKenzie SW, Dallalio G, North M, Frame P, Means RT Jr. 1996. Serum chemokine levels in patients with non-progressing HIV infection. *AIDS.* **10**:29-33.

106) Meyer L, Magierowska M, Hubert JB, Rouzioux C, Deveau C, Sanson F, Debre P, Delfraissy JF, Theodorou I. 1997. Early protective effect of CCR-5 delta 32 heterozygosity on HIV-1 disease progression: relationship with viral load. The SEROCO Study Group. *AIDS.* **11**:73-78.

107) Michael NL, Louie LG, Rohrbaugh AL, Schultz KA, Dayhoff DE, Wang CE, Sheppard HW. 1997. The role of CCR5 and CCR2 polymorphisms in HIV-1 transmission and disease progression. *Nat Med.* **3**:1160-1162.

108) Modrow S, Hahn BH, Shaw GM, Gallo RC, Wong-Staal F, Wolf H. 1987. Computer-assisted analysis of envelope protein sequences of seven human immunodeficiency virus isolates: prediction of antigenic epitopes in conserved and variable regions. *J Virol.* **61**:570-578.

109) Morikita T, Maeda Y, Fujii S, Matsushita S, Obaru K, Takatsuki K. 1997. The V1/V2 region of human immunodeficiency virus type 1 modulates the sensitivity to neutralization by soluble CD4 and cellular tropism. *AIDS Res Hum Retroviruses.* **13**:1291-1299.

110) Mosier DE, Picchio GR, Gulizia RJ, Sabbe R, Poignard P, Picard L, Offord RE, Thompson DA, Wilken J. 1999. Highly potent RANTES analogues either prevent CCR5-using human immunodeficiency virus type 1 infection in vivo or rapidly select for CXCR4-using variants. *J Virol.* **73**:3544-3550.

111) Murakami T, Nakajima T, Koyanagi Y, Tachibana K, Fujii N, Tamamura H, Yoshida N, Waki M, Matsumoto A, Yoshie O, Kishimoto T, Yamamoto N, Nagasawa T. 1997. A small molecule CXCR4 inhibitor that blocks T cell line-tropic HIV-1 infection. *J Exp Med.* **186**:1389-1393.

112) Obaru K, Fukuda M, Maeda S, Shimada K. 1986. A cDNA clone used to study mRNA inducible in human tonsillar lymphocytes by a tumor promoter. *J Biochem. (Tokyo)* **99**:885-894.

113) Obaru K, Hattori T, Yamamura Y, Takatsuki K, Nomiyama H, Maeda S, Shimada K. 1989. A cDNA clone inducible in human tonsillar lymphocytes by a tumor promoter codes for a novel protein of the beta-thromboglobulin superfamily. *Mol Immunol.* **26**:423-426.

114) Oberlin E, Amara A, Bachelerie F, Bessia C, Virelizier JL, Arenzana-Seisdedos F, Schwartz O, Heard JM, Clark-Lewis I, Legler DF, Loetscher M, Baggiolini M, Moser B. 1996. The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. *Nature.* **382**:833-835.

115) O'Brien WA, Koyanagi Y, Namazie A, Zhao JQ, Diagne A, Idler K, Zack JA, Chen IS. 1990. HIV-1 tropism for mononuclear phagocytes can be determined by regions of gp120 outside the CD4-binding domain. *Nature.* **348**:69-73.

116) Olson WC, Rabut GE, Nagashima KA, Tran DN, Anselma DJ, Monard SP, Segal JP, Thompson DA, Kajumo F, Guo Y, Moore JP, Maddon PJ, Dragic T. 1999. Differential inhibition of human immunodeficiency virus type 1 fusion, gp120 binding, and CC-chemokine activity by monoclonal antibodies to CCR5. *J Virol.* **73**:4145-4155.

117) Ozel M, Pauli G, Gelderblom HR. 1988. The organization of the envelope projections on the surface of HIV. *Arch Virol.* **100**:255-266.

118) Palella FJ Jr, Delaney KM, Moorman AC, Loveless MO, Fuhrer J, Satten GA, Aschman DJ, Holmberg SD. 1998. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. *N Engl J Med.* **338**:853-860.

119) Parslow TG. 1993. Post-transcriptional regulation of human retroviral gene expression. In: Cullen BR, ed. Human retroviruses. Oxford:IRL Press;101-136.

120) Peliska JA, Benkovic SJ. 1992. Mechanism of DNA strand transfer reactions catalyzed by HIV-1 reverse transcriptase. *Science.* **258**:1112-1118.

121) Peterlin BM, Adams M, Alonso A, Baur A, Ghosh S, Lu X, Luo L. 1993. Tat trans-activator. In: Cullen BR, ed. Human retroviruses. Oxford:IRL Press;75-100.

122) Picard L, Simmons G, Power CA, Meyer A, Weiss RA, Clapham PR. 1997. Multiple extracellular domains of CCR-5 contribute to human immunodeficiency virus type 1 entry and fusion. *J Virol.* **71**:5003-5011.

123) Pleskoff O, Treboute C, Brelot A, Heveker N, Seman M, Alizon M. 1997. Identification of a chemokine receptor encoded by human cytomegalovirus as a cofactor for HIV-1 entry. *Science.* **276**:1874-1878.

124) Pritsker M, Jones P, Blumenthal R, Shai Y. 1998. A synthetic all D-amino acid peptide corresponding to the N-terminal sequence of HIV-1 gp41 recognizes the wild-type fusion peptide in the membrane and inhibits HIV-1 envelope glycoprotein-mediated cell fusion. *Proc Natl Acad Sci U S A.* **95**:7287-7292.

125) Quinn TC. 1994. Population migration and the spread of types 1 and 2 human immunodeficiency viruses. *Proc Natl Acad Sci U S A.* **91**:2407-2414.

126) Rana S, Besson G, Cook DG, Rucker J, Smyth RJ, Yi Y, Turner JD, Guo HH, Du JG, Peiper SC, Lavi E, Samson M, Libert F, Liesnard C, Vassart G, Doms RW, Parmentier M, Collman RG. 1997. Role of CCR5 in infection of primary macrophages and lymphocytes by macrophage-tropic strains of human immunodeficiency virus: resistance to patient-derived and prototype isolates resulting from the delta ccr5 mutation. *J Virol.* **71**:3219-3227.

127) Reimann KA, Lin W, Bixler S, Browning B, Ehrenfels BN, Lucci J, Miatkowski K, Olson D, Parish TH, Rosa MD, Oleson FB, Hsu YM, Padlan EA, Letvin NL, Burkly LC. 1997. A humanized form of a CD4-specific monoclonal antibody exhibits decreased antigenicity and prolonged plasma half-life in rhesus monkeys while retaining its unique biological and antiviral properties. *AIDS Res Hum Retroviruses.* **13**:933-943.

128) Rizzuto CD, Wyatt R, Hernandez-Ramos N, Sun Y, Kwong PD, Hendrickson WA, Sodroski J. 1998. A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding. *Science.* **280**:1949-1953.

129) Roos MT, Lange JM, de Goede RE, Coutinho RA, Schellekens PT, Miedema F, Tersmette M. 1992. Viral phenotype and immune response in primary human immunodeficiency virus type 1 infection. *J Infect Dis.* **165**:427-432.

130) Ross TM, Cullen BR. 1998. The ability of HIV type 1 to use CCR-3 as a coreceptor is controlled by envelope V1/V2 sequences acting in conjunction with a CCR-5 tropic V3 loop. *Proc Natl Acad Sci U S A.* **95**:7682-7686.

131) Rucker J, Edinger AL, Sharron M, Samson M, Lee B, Berson JF, Yi Y, Margulies B, Collman RG, Doranz BJ, Parmentier M, Doms RW. 1997. Utilization of chemokine receptors, orphan receptors, and herpesvirus-encoded receptors by diverse human and simian immunodeficiency viruses. *J Virol.* **71**:8999-9007.

132) Rucker J, Samson M, Doranz BJ, Libert F, Berson JF, Yi Y, Smyth RJ, Collman RG, Broder CC, Vassart G, Doms RW, Parmentier M. 1996. Regions in beta-chemokine

receptors CCR5 and CCR2b that determine HIV-1 cofactor specificity. *Cell.* **87**:437-446.

133) Sakaguchi K, Zambrano N, Baldwin ET, Shapiro BA, Erickson JW, Omichinski JG, Clore GM, Gronenborn AM, Appella E. 1993. Identification of a binding site for the human immunodeficiency virus type 1 nucleocapsid protein. *Proc Natl Acad Sci U S A.* **90**:5219-5223.

134) Sakai K, Ma XY, Gordienko I, Volsky DJ. 1991. Recombinational analysis of a natural noncytopathic human immunodeficiency virus type 1 (HIV-1) isolate: role of the vif gene in HIV-1 infection kinetics and cytopathicity. *J Virol.* **65**:5765-5773.

135) Sakaida H, Hori T, Yonezawa A, Sato A, Isaka Y, Yoshie O, Hattori T, Uchiyama T. 1998. T-tropic human immunodeficiency virus type 1 (HIV-1)-derived V3 loop peptides directly bind to CXCR-4 and inhibit T-tropic HIV-1 infection. *J Virol.* **72**:9763-9770.

136) Samson M, Libert F, Doranz BJ, Rucker J, Liesnard C, Farber CM, Saragosti S, Lapoumeroulie C, Cognaux J, Forceille C, Muyldermans G, Verhofstede C, Burtonboy G, Georges M, Imai T, Rana S, Yi Y, Smyth RJ, Collman RG, Doms RW, Vassart G, Parmentier M. 1996. Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature.* **382**:722-725.

137) Scarlatti G, Tresoldi E, Bjorndal A, Fredriksson R, Colognesi C, Deng HK, Malnati MS, Plebani A, Siccardi AG, Littman DR, Fenyo EM, Lusso P. 1997. In vivo evolution of HIV-1 co-receptor usage and sensitivity to chemokine-mediated suppression. *Nat Med.* **3**:1259-1265.

138) Schmidtmayerova H, Alfano M, Nuovo G, Bukrinsky M. 1998. Human immunodeficiency virus type 1 T-lymphotropic strains enter macrophages via a CD4- and CXCR4-mediated pathway: replication is restricted at a postentry level. *J Virol.* **72**:4633-4642.

139) Schols D, Este JA, Cabrera C, De Clercq E. 1998. T-cell-line-tropic human immunodeficiency virus type 1 that is made resistant to stromal cell-derived factor

1alpha contains mutations in the envelope gp120 but does not show a switch in coreceptor use. *J Virol.* **72**:4032-4037.

140) Schols D, Struyf S, Van Damme J, Este JA, Henson G, De Clercq E. 1997. Inhibition of T-tropic HIV strains by selective antagonization of the chemokine receptor CXCR4. *J Exp Med.* **186**:1383-1388.

141) Schuitemaker H, Fouchier RA, Broersen S, Groenink M, Koot M, van 't Wout AB, Huisman HG, Tersmette M, Miedema F. 1995. Envelope V2 configuration and HIV-1 phenotype: clarification. *Science.* **268**:115.

142) Schuitemaker H, Koot M, Kootstra NA, Dercksen MW, de Goede RE, van Steenwijk RP, Lange JM, Schattenkerk JK, Miedema F, Tersmette M. 1992. Biological phenotype of human immunodeficiency virus type 1 clones at different stages of infection: progression of disease is associated with a shift from monocytotropic to T-cell-tropic virus population. *J Virol.* **66**:1354-1360.

143) Shioda T, Levy JA, Cheng-Mayer C. 1991. Macrophage and T cell-line tropisms of HIV-1 are determined by specific regions of the envelope gp120 gene. *Nature.* **349**:167-169.

144) Shioda T, Levy JA, Cheng-Mayer C. 1992. Small amino acid changes in the V3 hypervariable region of gp120 can affect the T-cell-line and macrophage tropism of human immunodeficiency virus type 1. *Proc Natl Acad Sci U S A.* **89**:9434-9438.

145) Shioda T, Oka S, Xin X, Liu H, Harukuni R, Kurotani A, Fukushima M, Hasan MK, Shiino T, Takebe Y, Iwamoto A, Nagai Y. 1997. In vivo sequence variability of human immunodeficiency virus type 1 envelope gp120: association of V2 extension with slow disease progression. *J Virol.* **71**:4871-4881.

146) Simmons G, Clapham PR, Picard L, Offord RE, Rosenkilde MM, Schwartz TW, Buser R, Wells TN, Proudfoot AE. 1997. Potent inhibition of HIV-1 infectivity in macrophages and lymphocytes by a novel CCR5 antagonist. *Science.* **276**:276-279.

147) Simmons G, Wilkinson D, Reeves JD, Dittmar MT, Beddows S, Weber J, Carnegie G, Desselberger U, Gray PW, Weiss RA, Clapham PR. 1996. Primary, syncytium-inducing human immunodeficiency virus type 1 isolates are dual-tropic and most can use either Lestr or CCR5 as coreceptors for virus entry. *J Virol.* **70**:8355-8360.

148) Smyth RJ, Yi Y, Singh A, Collman RG. 1998. Determinants of entry cofactor utilization and tropism in a dualtropic human immunodeficiency virus type 1 primary isolate. *J Virol.* **72**:4478-4484.

149) Sodroski J, Goh WC, Rosen C, Campbell K, Haseltine WA. 1986. Role of the HTLV-III/LAV envelope in syncytium formation and cytopathicity. *Nature.* **322**:470-474.

150) Speck RF, Wehrly K, Platt EJ, Atchison RE, Charo IF, Kabat D, Chesebro B, Goldsmith MA. 1997. Selective employment of chemokine receptors as human immunodeficiency virus type 1 coreceptors determined by individual amino acids within the envelope V3 loop. *J Virol.* **71**:7136-7139.

151) Stamatatos L, Wiskerchen M, Cheng-Mayer C. 1998. Effect of major deletions in the V1 and V2 loops of a macrophage-tropic HIV type 1 isolate on viral envelope structure, cell entry, and replication. *AIDS Res Hum Retroviruses.* **14**:1129-1139.

152) Starcich BR, Hahn BH, Shaw GM, McNeely PD, Modrow S, Wolf H, Parks ES, Parks WP, Josephs SF, Gallo RC. 1986. Identification and characterization of conserved and variable regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS. *Cell.* **45**:637-648.

153) Sullivan N, Thali M, Furman C, Ho DD, Sodroski J. 1993. Effect of amino acid changes in the V1/V2 region of the human immunodeficiency virus type 1 gp120 glycoprotein on subunit association, syncytium formation, and recognition by a neutralizing antibody. *J Virol.* **67**:3674-3679.

154) Takeuchi Y, Akutsu M, Murayama K, Shimizu N, Hoshino H. 1991. Host range mutant of human immunodeficiency virus type 1: modification of cell tropism by a single point mutation at the neutralization epitope in the env gene. *J Virol.* **65**:1710-1718.

155) Tersmette M, de Goede RE, Al BJ, Winkel IN, Gruters RA, Cuypers HT, Huisman HG, Miedema F. 1988. Differential syncytium-inducing capacity of human immunodeficiency virus isolates: frequent detection of syncytium-inducing isolates in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex. *J Virol.* **62**:2026-32.

156) Toohey K, Wehrly K, Nishio J, Perryman S, Chesebro B. 1995. Human immunodeficiency virus envelope V1 and V2 regions influence replication efficiency in macrophages by affecting virus spread. *Virology.* **213**:70-79.

157) Trkola A, Dragic T, Arthos J, Binley JM, Olson WC, Allaway GP, Cheng-Mayer C, Robinson J, Maddon PJ, Moore JP. 1996. CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR-5. *Nature.* **384**:184-187.

158) Trkola A, Gordon C, Matthews J, Maxwell E, Ketas T, Czaplewski L, Proudfoot AE, Moore JP. 1999. The CC-chemokine RANTES increases the attachment of human immunodeficiency virus type 1 to target cells via glycosaminoglycans and also activates a signal transduction pathway that enhances viral infectivity. *J Virol.* **73**:6370-6379.

159) Trkola A, Paxton WA, Monard SP, Hoxie JA, Siani MA, Thompson DA, Wu L, Mackay CR, Horuk R, Moore JP. 1998. Genetic subtype-independent inhibition of human immunodeficiency virus type 1 replication by CC and CXC chemokines. *J Virol.* **72**:396-404.

160) Vita C, Drakopoulou E, Vizzavona J, Rochette S, Martin L, Menez A, Roumestand C, Yang YS, Ylisastigui L, Benjouad A, Gluckman JC. 1999. Rational engineering of a miniprotein that reproduces the core of the CD4 site interacting with HIV-1 envelope glycoprotein. *Proc Natl Acad Sci U S A.* **96**:13091-13096.

161) Wang ZX, Berson JF, Zhang TY, Cen YH, Sun Y, Sharron M, Lu ZH, Peiper SC. 1998. CXCR4 sequences involved in coreceptor determination of human immunodeficiency virus type-1 tropism. Unmasking of activity with M-tropic Env glycoproteins. *J Biol Chem.* **273**:15007-15015.

162) Weiss CD, Levy JA, White JM. 1990. Oligomeric organization of gp120 on infectious human immunodeficiency virus type 1 particles. *J Virol.* **64**:5674-5677.

163) Weissenhorn W, Dessen A, Harrison SC, Skehel JJ, Wiley DC. 1997. Atomic structure of the ectodomain from HIV-1 gp41. *Nature.* **387**:346-348.

164) Welker R, Kottler H, Kalbitzer HR, Krausslich HG. 1996. Human immunodeficiency virus type 1 Nef protein is incorporated into virus particles and specifically cleaved by the viral proteinase. *Virology.* **219**:228-236.

165) Westervelt P, Trowbridge DB, Epstein LG, Blumberg BM, Li Y, Hahn BH, Shaw GM, Price RW, Ratner L. 1992. Macrophage tropism determinants of human immunodeficiency virus type 1 in vivo. *J Virol.* **66**:2577-2582.

166) Wild C, Oas T, McDanal C, Bolognesi D, Matthews T. 1992. A synthetic peptide inhibitor of human immunodeficiency virus replication: correlation between solution structure and viral inhibition. *Proc Natl Acad Sci U S A.* **89**:10537-10541.

167) Willey RL, Ross EK, Buckler-White AJ, Theodore TS, Martin MA. 1989. Functional interaction of constant and variable domains of human immunodeficiency virus type 1 gp120. *J Virol.* **63**:3595-3600.

168) Wilson C, Reitz MS Jr, Aldrich K, Klasse PJ, Blomberg J, Gallo RC, Robert-Guroff M. 1990. The site of an immune-selected point mutation in the transmembrane protein of human immunodeficiency virus type 1 does not constitute the neutralization epitope. *J Virol.* **64**:3240-3248.

169) Wu L, Gerard NP, Wyatt R, Choe H, Parolin C, Ruffing N, Borsetti A, Cardoso AA, Desjardin E, Newman W, Gerard C, Sodroski J. 1996. CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5. *Nature.* **384**:179-183.

170) Wu L, Paxton WA, Kassam N, Ruffing N, Rottman JB, Sullivan N, Choe H, Sodroski J, Newman W, Koup RA, Mackay CR. 1997. CCR5 levels and expression pattern correlate with infectability by macrophage-tropic HIV-1, in vitro. *J Exp Med.* 185:1681-1691.

171) Wyatt R, Kwong PD, Desjardins E, Sweet RW, Robinson J, Hendrickson WA, Sodroski JG. 1998. The antigenic structure of the HIV gp120 envelope glycoprotein. *Nature.* **393**:705-11.

172) Wyatt R, Moore J, Accola M, Desjardin E, Robinson J, Sodroski J. 1995. Involvement of the V1/V2 variable loop structure in the exposure of human immunodeficiency virus type 1 gp120 epitopes induced by receptor binding. *J Virol.* **69**:5723-5733.

173) Wyatt R, Sullivan N, Thali M, Repke H, Ho D, Robinson J, Posner M, Sodroski J. 1993. Functional and immunologic characterization of human immunodeficiency

virus type 1 envelope glycoproteins containing deletions of the major variable regions. *J Virol.* **67**:4557-4565.

174) Yerly S, Kaiser L, Race E, Bru JP, Clavel F, Perrin L. 1999. Transmission of antiretroviral-drug-resistant HIV-1 variants. *Lancet.* **354**:729-733.

175) Yi Y, Rana S, Turner JD, Gaddis N, Collman RG. 1998. CXCR-4 is expressed by primary macrophages and supports CCR5-independent infection by dual-tropic but not T-tropic isolates of human immunodeficiency virus type 1. *J Virol.* **72**:772-777.

176) Yoshimura K, Matsushita S, Hayashi A, Takatsuki K. 1996. Relationship of HIV-1 envelope V2 and V3 sequences of the primary isolates to the viral phenotype. *Microbiol Immunol.* **40**:277-287.

177) Zanussi S, D'Andrea M, Simonelli C, Tirelli U, De Paoli P. 1996. Serum levels of RANTES and MIP-1 alpha in HIV-positive long-term survivors and progressor patients. *AIDS.* **10**:1431-1432.

178) Zhang W, Canziani G, Plugariu C, Wyatt R, Sodroski J, Sweet R, Kwong P, Hendrickson W, Chaiken I. 1999. Conformational changes of gp120 in epitopes near the CCR5 binding site are induced by CD4 and a CD4 miniprotein mimetic. *Biochemistry.* **38**:9405-9416.

179) Zhu T, Mo H, Wang N, Nam DS, Cao Y, Koup RA, Ho DD. 1993 Genotypic and phenotypic characterization of HIV-1 patients with primary infection. *Science.* **261**:1179-81.

180) Zou YR, Kottmann AH, Kuroda M, Taniuchi I, Littman DR. 1998. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature.* **393**:595-599.