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# Part 1. HIV-1 binding and entry events: Implications for vaccine strategies. Part 2. The effects of AZT on the relative mutation frequency of FIV

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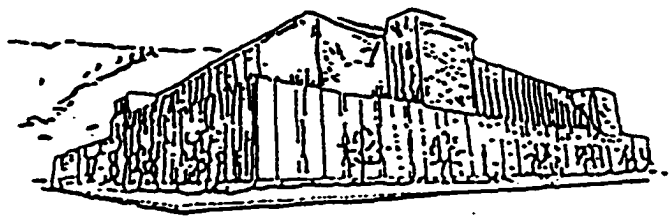

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**Part 1: HIV-1 Binding and Entry Events: Implications for Vaccine Strategies**

**Part 2: The Effects of AZT on the Relative Mutation Frequency of FIV**

by

Rachel A. LaCasse

B.S. University of Montana, 1993

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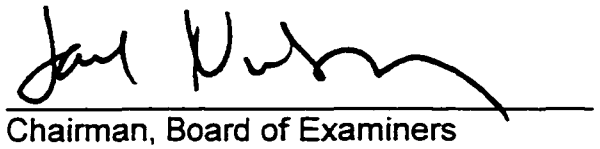

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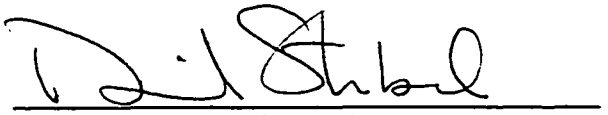

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Part 1: HIV-1 Binding and Entry Events: Implications for Vaccine Strategies
Part 2: The Effects of AZT on the Relative Mutation Frequency of FIV (121 pp.)

Director: Jack H. Nunberg

Part 1:

We have examined the relationship between coreceptor utilization and sensitivity to neutralization in a primary isolate (PI) of human immunodeficiency virus (HIV) and its T-cell line adapted (TCLA) derivative. We determined that adaptation of the PI virus 168P results in the loss of the unique capacity of PI viruses to utilize the CCR5 coreceptor, and in the acquisition by the TCLA 168C virus of sensitivity to neutralization by V3-directed monoclonal antibodies (MAbs). In experiments wherein infection by 168P is directed via either the CCR5 or CXCR4 pathway, we demonstrate that the virus, as well as pseudotyped virions bearing a molecularly cloned 168P envelope protein, remain refractory to neutralization by MAbs 257-D, 268-D, and 50.1 regardless of the coreceptor utilized. This study suggests that coreceptor utilization is not a primary determinant of differential neutralization sensitivity in PI and TCLA virus.

Current recombinant HIV gp120 protein vaccine candidates fail to elicit antibodies capable of neutralizing infectivity of primary patient isolates. Here, novel 'fusion-competent' HIV vaccine immunogens are discussed that capture the transient envelope-CD4-coreceptor structures which arise during HIV binding and fusion. In a transgenic mouse immunization model, these formaldehyde-fixed whole cell vaccines elicited antibodies capable of neutralizing infectivity of 23 of 24 primary HIV isolates form diverse geographical locations and genetic clades A-E. Development of these novel fusion-dependent immunogens may lead to a broadly effective HIV isolates.

Part 2:

We have developed a host range system to measure the mutation frequency of feline immunodeficiency virus (FIV), the feline homologue of HIV-1. When wild-type FIV was grown in the presence of a known mutagen, 5-bromo-2'-deoxyuridine (BUdR), a dose-dependent increase of host range mutants was detected. Using this system, we have evaluated the effects of antiviral drugs upon the mutation frequency of FIV. Subinhibitory concentrations of 3'-azido-3'-deoxythymidine (AZT), the most common antiviral drug used in AIDS chemotherapy, increased the mutation frequency of FIV in a dose-dependent manner. Two other antivirals, 2',3'-dideoxyinosine (ddI) and 2',3'-dideoxycytidine (ddC) did not show this effect.

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# Chapter 1

## Introduction

Human immunodeficiency virus type 1 (HIV-1) is the etiological agent of the acquired immune deficiency syndrome (AIDS) (1,2). This retrovirus has caused a worldwide epidemic and the WHO (World Health Organization) has estimated that 40 million people will be infected by the year 2000 (3). To date, there is no cure for HIV infection, limited effective therapy, and no protective vaccine. Current vaccine strategies have focused on the envelope glycoprotein of HIV, the viral protein that mediates the early binding and entry events that ultimately lead to infection. However, all vaccine candidates thus far have failed to elicit antibodies capable of neutralizing infection by primary isolates (PIs), i.e. viruses obtained from infected individuals (4-7).

### Synthesis and Processing of HIV Envelope Glycoprotein

The synthesis and processing of the HIV envelope glycoprotein occurs in the secretory pathway of the infected host cell. The envelope protein is originally synthesized as a precursor polyprotein, which is cotranslationally transferred into the lumen of the endoplasmic reticulum (ER). During this time, the precursor protein is modified by cellular enzymes by adding high-mannose units to the protein. This yields an HIV precursor glycoprotein with a molecular mass of 160 kd (gp160) (8). While in the ER lumen, intramolecular disulfide bonds are formed to produce a folded monomer of gp160 (9), which then associates into trimeric complexes (10). After oligomerization, the envelope complexes are

transferred to the Golgi apparatus where the carbohydrate moieties are terminally modified to produce complex/hybrid carbohydrate chains (11).

While in the Golgi apparatus, the gp160 precursors are cleaved into the noncovalently linked gp120 and gp41 subunits, forming the mature envelope glycoprotein (12). The cleavage is mediated by the cellular serine proteases furin and PC7 (13-15). These proteases act on each chain of the gp160 oligomer and cleave at a highly conserved basic region that defines the C-terminus of gp120 and the N-terminus of gp41. Cleavage of the precursor protein is thought to induce a conformational change that releases gp41 from the C-terminus of gp120 and buries the N-terminal hydrophobic fusion peptide of gp41 within the oligomer (16-18). After cleavage, the mature oligomers are transported from the Golgi apparatus to the plasma membrane of the host cell via a cellular vesicle transport system (19).

## The HIV-1 Envelope Glycoprotein

HIV envelope-mediated binding and entry is the initial step in the replication process of the virus. A series of molecular interactions between the host cell and virus is initiated by the binding of the viral envelope glycoprotein to the cellular CD4 molecule of CD4+ T lymphocytes and cells of the monocyte-macrophage lineage (20-25). The envelope protein consists of the surface subunit, gp120, which is noncovalently associated to the transmembrane subunit, gp41 (26,27). The gp120/gp41 heterodimer assembles into trimeric complexes to form the functional HIV envelope protein (11,28-31).

The gp120 subunit of the HIV envelope glycoprotein is a highly glycosylated, hydrophilic protein located on the surface of the virion and plasma membrane of infected cells. HIV gp120 has 24 potential sites for N-linked glycosylation (Asn-X-Ser/Thr), 13 of which are highly conserved among different HIV-1 viral isolates (32,33). The amino acid sequence comparison of gp120 from HIV-1, HIV-2, and SIV isolates also reveals that there are 18 highly conserved cysteine residues. The conservation of these amino acids suggests that disulfide bonds are essential to the structure and function of this protein. The secondary structure of the envelope glycoprotein and the nine putative disulfide bonds found in gp120 are depicted in Fig. 1 (32,34).

**Fig. 1:** The predicted folding patterns of HIV-1 envelope glycoprotein. Hypervariable domains of gp120 are designated V1-V5. Regions of gp41 are as follows: F, fusion peptide; TM, transmembrane domain.

Despite the conservation of the overall secondary structure of the envelope glycoprotein, all isolates of HIV-1, HIV-2, and SIV have extensive sequence heterogeneity throughout the envelope gene (35). There is up to 50% variation in the envelope amino acid sequence in HIV-1 strains, although other parts of the genome are relatively conserved (36). The gp120 subunit contains five variable loops, designated V1-V5, which are separated by conserved regions. It has been previously shown that the envelope protein contains the determinants for cell tropism, cytopathic effect, and coreceptor utilization. The variable V3 loop has been implicated as a key player in these determinants. Changes in the V3 loop can affect cell tropism by altering the ability of viral isolates to grow in primary monocyte-derived macrophages and T cell lines (5,37-42). This variable region is also a major determinant of cytopathic effects, such as syncytium formation in primary lymphocyte cultures (43-45), and has been implicated in differences in neutralization sensitivity of viral isolates to soluble CD4 (sCD4) (46,47) and to V3-directed monoclonal antibodies (mAbs) (48-50). Recently, the V3 loop has also been found to play a role in the differential utilization of coreceptors (51-54).

The gp120 subunit is anchored to the surface of virions or infected cells through noncovalent interaction with the transmembrane subunit, gp41. Gp41 traverses the lipid bilayer once and serves as the anchor of the envelope glycoprotein. Mutational analysis reveals that both the N-terminus and C-terminus of gp120 mediate the noncovalent contact between gp120 and gp41 (26,42,55,56). Although, the contacts mediated by gp41 are yet to be identified,

a disulfide loop in the N-terminus of gp41 is believed to form a knob that fits into a pocket in the gp120 subunit (57,58).

The N-terminus of gp41 has a hydrophobic region of approximately 20 amino acids which is required for fusion of the viral and cell membrane, and is termed the fusion peptide (26,59,60). Near the C-terminal end of gp41 resides an additional hydrophobic domain that spans the cell or viral membrane, serving as an anchor for the glycoprotein heterodimer (61). The domain between these two hydrophobic regions, the ectodomain, is highly conserved. The ectodomain is proposed to form a structure similar to a leucine zipper motif and is believed to form a coiled-coil structure, which facilitates the insertion of the fusion peptide into the target cell membrane upon activation (62).

## CD4 and Coreceptor Binding

It has long been known that the cellular CD4 molecule is the high affinity receptor to which the HIV envelope glycoprotein binds to initiate envelope-mediated membrane fusion (22,23). However, CD4 binding alone is not sufficient for HIV envelope-mediated fusion and infection of the host cell. When nonhuman cells were transfected to express human CD4, HIV-1 could not mediate envelope fusion and/or infection (24,63-65). This suggested that there was a coreceptor involved in HIV binding and entry.

Recently, HIV-1 coreceptors, cellular molecules that act in conjunction with the CD4 molecule to allow envelope-mediated fusion, have been elucidated (REF). The HIV-1 coreceptors are members of the chemokine receptor family

and are seven transmembrane-segment G-protein-coupled molecules that normally function as receptors to the α- and β-chemokines. Chemokines are small molecules involved in the chemotactic recruitment of selected leukocytes to sites of inflammation (66,67). With the discovery of these coreceptors has come the understanding that various isolates of HIV-1 utilize different coreceptors for binding and entry. The commonly used T-cell line-adapted (TCLA) isolates utilize the CXCR4 α-chemokine receptor exclusively (68,69), whereas PIs of HIV-1 that are monocytropic/non-syncytium-inducing (NSI) utilize only the β-chemokine receptor CCR5. HIV-1 PIs that are lymphocytropic/syncytium-inducing (SI) are able to utilize either CXCR4 or CCR5 (52,70-74).

## PI and TCLA Viruses

PIs of HIV are obtained by limited cultivation of patient peripheral blood mononuclear cells or patient plasma with uninfected cells (1,75). There is a spectrum of biological phenotypes of these PI viruses. SI isolates infect and induce syncytia formation in peripheral blood lymphocytes (PBLs). SI isolates can also replicate in the MT2 T-cell line, but are unable to replicate in less permissive T-cell lines, such as H9 cells (76,77). In contrast, NSI isolates are only able to replicate in primary cells, and unlike the SI isolates, are able to replicate in primary monocyte-derived macrophages (78). Transitional intermediates of these two biological phenotypes of the primary isolates have been detected *in vivo* (79). During active infection, NSI isolates predominate

during the early/asymptomatic stage while SI isolates slowly arise from the NSI population (79,80). The emergence of SI isolates during infection is associated with progression to AIDS (80). The spectrum of the biological phenotypes can also be extended to TCLA isolates (46), and the variation of these phenotypes may reflect the continuum of the multiple interactions between the virus and cell during the early binding and entry events of infection.

PI and TCLA viruses of HIV not only differ in coreceptor utilization and cell tropism, but also exhibit a marked difference in neutralization sensitivities. PI viruses exhibit decreased sensitivities to *in vitro* neutralization by sCD4 (5,7,81-83), sera from infected individuals (5,6,84,85), monoclonal antibodies (48-50), and HIV vaccine sera (4-7).

During adaptation of PI viruses to growth in established T-cell lines, the change in cell tropism is accompanied by changes in both neutralization sensitivity and cytopathic effect. The current generation of vaccines comprised of recombinant gp120 (rgp120) subunits elicits antibodies that potently neutralize infection of homologous TCLA viruses (86-89), however PI viruses are refractory to *in vitro* neutralization by the vaccine sera. The reason behind the universal failure of rgp120 vaccine sera to neutralize PI viruses remains elusive. However, the structural and/or functional differences between PI and TCLA viruses in the process of binding and entry may hold some of these answers.

**Conformational Changes Mediated by Binding and Entry Events**

During binding and entry, the HIV envelope protein undergoes a series of complex interactions and conformational changes that result in membrane fusion. Initially, the envelope complex binds to CD4 and undergoes a conformational change that results in an increased exposure of certain antibody epitopes, also known as CD4-induced (CD4i) epitopes (90-92). Monoclonal antibodies from HIV infected individuals that are specific for the CD4i epitopes have been isolated and been found to neutralize a range of PI and TCLA viruses (93,94). During the binding and entry process, the dissociation (or shedding) of the gp120 from the gp41 subunit of the envelope complex increases (95-99). Along with conformational changes occurring in the viral envelope complex upon CD4 binding, there also are conformational changes observed in the CD4 receptor (100,101). These changes in CD4 and envelope facilitate the subsequent interactions with the coreceptor, forming a trimolecular complex (53,54,102,103). The conformational changes in the envelope-CD4-coreceptor complex mediate the exposure and activation of the gp41 fusion peptide, and ultimately lead to the fusion of the opposed virus and cell membranes (26,104,105).

During the process of envelope-mediated fusion, a number of neutralizing epitopes are exposed, albeit transiently. As mentioned above, a number of human mAbs from infected individuals recognize CD4i epitopes, and are able to neutralize a diverse range of PI and TCLA viruses (93,94). It has also been

shown that sera from infected individuals typically neutralize PIs with frequencies of 30-50%, although the results vary for individual sera and individual viruses (106-108). This indicates that there is an inherent difference in the antibodies elicited from infected individuals as compared to the antibodies elicited from rgp120 vaccines.

## Proposed Work

The following studies explore the relationship between neutralization sensitivity and coreceptor utilization, especially in regards to the changes that accompany adaptation of PIs to growth in T-cell lines. We hypothesize that PIs are refractory to neutralization as a result of the unique ability to utilize the CCR5 coreceptor, rather than CXCR4. To test this hypothesis, we examine a well-characterized SI primary virus, 168P, under experimental conditions where infection is directed via the CXCR4 and CCR5 pathway to determine whether coreceptor pathway is a determinant of neutralization sensitivity.

It has been previously shown that PI viruses are refractory to neutalization by rgp120 vaccine sera. This vaccine strategy incorporates a static envelope as the immunogen, i.e. an envelope that is not interacting with CD4 and coreceptor. In an infected individual, however, the immune system is responding to an envelope protein expressed in its native oligomeric conformation and is actively participating in the binding and entry process. An active infection, therefore, presents the immune system with the transient, fusion-dependent epitopes that arise during fusion. We hypothesize that the failure of current rgp120 vaccine

strategies is due to the fact that the fusion-dependent epitopes present during an active infection are not incorporated into the immunogen. We test this hypothesis by formulating a 'fusion-competent' vaccine that incorporates a functioning envelope interacting with CD4 and coreceptor into the immunogen, and determine if this new vaccine strategy can elicit antibodies capable of neutralizing primary isolates of HIV.

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# Chapter 2

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## Coreceptor Utilization by Human Immunodeficiency Virus Type 1 Is Not a Primary Determinant of Neutralization Sensitivity

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## Abstract

We have examined the relationship between coreceptor utilization and sensitivity to neutralization in a primary (PI) isolate of HIV and its T cell line adapted (TCLA) derivative. We determined that adaptation of the PI virus 168P results in the loss of the unique capacity of PI viruses to utilize the CCR5 coreceptor, and in the acquisition by the TCLA 168C virus of sensitivity to neutralization by V3-directed monoclonal antibodies (MAbs). In experiments wherein infection by 168P is directed via either the CCR5 or CXCR4 pathway, we demonstrate that the virus, as well as pseudotyped virions bearing a molecularly cloned 168P envelope protein, remain refractory to neutralization by MAbs 257-D, 268-D, and 50.1 regardless of the coreceptor utilized. This study suggests that coreceptor utilization is not a primary determinant of differential neutralization sensitivity in PI and TCLA viruses.

Although CD4 had long been recognized as the cellular receptor to which the human immunodeficiency virus type 1 (HIV) envelope protein binds (9, 21, 22), it had also been recognized that expression of CD4 alone is insufficient to render non-human cells susceptible to HIV infection (4, 5, 22). Similarly, different HIV isolates display different abilities to infect CD4-positive human macrophages, T lymphocytes, and established T cell lines (31, 32, 35), suggesting that additional molecules may be responsible for cell tropism specificity. During the past year, cellular molecules that act in conjunction with CD4 have been identified as required cofactors for HIV envelope protein-mediated binding and entry (1, 6, 10-12, 14). These HIV coreceptors are members of the superfamily of seven-transmembrane segment G-protein coupled receptors and act primarily as cellular receptors for chemokines.

The discovery of cellular coreceptors for HIV has provided new perspectives for understanding these early events in HIV infection (see review (2)). Thus, phenotypically distinct isolates of HIV utilize as coreceptors different chemokine receptor molecules. Although all primary isolates (PI) of HIV infect primary T lymphocytes, some also infect cells of the macrophage lineage (31, 32). These monocytropic isolates utilize the CCR5 chemokine receptor, whose natural ligands include the chemokines RANTES, MIP-1$\alpha$ and MIP-1$\beta$ (1, 6, 10-12). Monocytropic isolates do not induce syncytia in primary lymphocyte culture and do not infect established T cell lines (31). During the late course of HIV infection, syncytium-inducing (SI) primary viruses often arise from the population of monocytropic viruses (31, 32). These SI primary isolates no longer infect

macrophages, and utilize both CCR5 and another chemokine receptor, CXCR4 (7, 33, 38). CXCR4, whose natural chemokine ligand is SDF-1 (3, 27), was originally identified by Berger and colleagues as the cofactor used by laboratory-adapted viruses (14). In fact, the common laboratory viruses (IIIb/LAI, LAV, RF) are unable to utilize CCR5 coreceptor (1, 6, 10-12), presumably reflecting the lack of CCR5 expression in most established T cell lines (1, 13). Although some primary isolates utilize additional chemokine receptor molecules, notably CCR3 and CCR2b (6, 11, 18), the relationship between these coreceptors and viral phenotypes is less clear. The ability to utilize CCR5 coreceptor, however, is unique to PI viruses.

Paralleling these differences in coreceptor utilization and cell tropism are differences in sensitivity to virus neutralization. Although laboratory-adapted isolates of HIV can be potently neutralized by sera elicited by recombinant gp120 protein, primary isolates are largely refractory to neutralization by rgp120 vaccine sera (23, 37). Similarly, PI viruses are significantly more resistant than TCLA viruses to neutralization by gp120-directed monoclonal antibodies (MAbs) (25, 37) and to inhibition by soluble forms of CD4 (8). We and others have demonstrated that neutralization sensitivity develops concomitantly with adaptation of PI isolates to persistent growth in established T cell lines (24, 37). By studying pedigreed PI and TCLA viruses (168P and 168C, respectively), we have shown that adaptation renders the TCLA virus sensitive not only to rgp120 vaccine sera and CD4 immunoadhesin, but also to MAbs directed to the V3-loop

of gp120 (37). However, the basis for this increase in neutralization sensitivity remains unclear.

In this report, we explore the relationship between neutralization sensitivity and coreceptor utilization, especially with regard to changes that accompany adaptation. We examined neutralization sensitivity of the well characterized SI primary virus 168P under experimental conditions where infection can be directed via either the CXCR4 or CCR5 pathway. The pedigreed TCLA derivative 168C utilizes only CXCR4 and was sensitive to neutralization by the panel of V3-directed MAbs used in these studies. However, the primary 168P virus remained refractory to neutralization regardless of coreceptor pathway taken. Our findings suggest that envelope protein structure, and not coreceptor utilization, is the primary determinant of differential neutralization sensitivity in PI and TCLA viruses.

**Coreceptor utilization by pedigreed PI and TCLA viruses**

Cross-sectional surveys of coreceptor use have shown that primary SI isolates generally utilize CXCR4 and CCR5 coreceptors, whereas unrelated laboratory-adapted isolates utilize only CXCR4 (1, 6, 7, 10-12, 14, 33, 38). We wished to confirm this trend in a longitudinal study of adaptation. We previously described the adaptation of the primary SI virus 168P to persistent growth in the FDA/H9 T cell line, and the concomitant development of neutralization sensitivity in the resulting TCLA virus 168C (37). In the present study, the ability of these pedigreed viruses to utilize specific coreceptors was tested by infection of U87

human glioma cell lines expressing CD4 (U87-CD4) and the specific coreceptor (19). Figure 1 confirms the ability of the SI 168P virus to utilize both CXCR4 and CCR5, and the subsequent loss of this latter specificity in the 168C TCLA virus. Infection was dependent on coreceptor expression, and both PI and TCLA isolates could also utilize CCR3 (data not presented).

In keeping with the determined coreceptor specificity, infection could be blocked by addition of coreceptor-specific ligands. Thus, 168P virus infection of CCR5-expressing cells was blocked by the CCR5-specific ligands RANTES, MIP-1$\alpha$, and MIP-1$\beta$ (1, 6, 10-12) (Figure 1). Similarly, infection of CXCR4-expressing U87-CD4 cells by either virus could be blocked by the CXCR4-specific chemokine ligand SDF-1 (3, 27) (data not presented).

**Coreceptor pathway and neutralization sensitivity**

In previous work, we demonstrated that the PI 168P virus is refractory to neutralization by HIV MN gp120 vaccine sera and by several well-characterized V3-directed murine MAbs which strongly neutralize infectivity of the TCLA 168C virus (37). In the present study, we extended the panel of MAbs to include two V3-directed human MAbs: 257-D and 268-D (17). These well-characterized human MAbs recognize core epitopes at the crown of the V3 loop of gp120 (KRIHI and HIGPGR, respectively) - linear sequences known to be present in both 168P and 168C envelope proteins (37). These epitope predictions were confirmed by gp120-capture ELISA (26) which demonstrated equal binding to envelope protein in detergent-solubilized 168P and 168C virions (data not

presented). Sensitivity to neutralization by these human MAbs was determined in a standard assay using phytohemagglutinin (PHA)–activated peripheral blood lymphocytes (PBLs) (37). MAbs 257-D and 268-D were found to potently neutralize 168C, but failed to neutralize 168P (Figure 2). This pattern of neutralization sensitivity is similar to that previously described for the V3-directed murine MAb 50.1 (30, 36, 37).

To examine whether sensitivity to neutralization was affected by the coreceptor pathway utilized in infection of PBLs, we used inhibitory concentrations of CCR5-specific chemokine ligands RANTES, MIP-1α, and MIP-1β in order to restrict infection to the CXCR4 pathway. Addition of these chemokines to the PBL cultures did not affect virus growth, nor did it affect sensitivity to neutralization by the V3-directed human MAbs (Figure 2). To the extent that CCR5 blockade was complete, these results suggest that the simple availability of the CCR5 pathway is not a factor in the resistance of PI viruses to neutralization.

To strengthen this conclusion, we examined neutralization sensitivity in human U87-CD4 cell lines expressing only CXCR4 or CCR5. Using this method, we confirmed that the SI 168P virus remained refractory to neutralization by human MAbs 257-D and 268-D as well as by the murine MAb 50.1, regardless of whether infection occurred via CXCR4 or CCR5 (Figure 3). These results suggest that availability of the CCR5 pathway is not a primary determinant for the resistance of PI viruses to neutralization. The TCLA 168C virus utilized CXCR4 only and was sensitive to neutralization.

**Molecularly cloned PI and TCLA envelope genes**

In order to understand better the changes that accompany adaptation, and those that determine coreceptor utilization and neutralization sensitivity, we molecularly cloned the envelope genes of the 168P and 168C viruses. High-fidelity XL® PCR (rTth and VentR® DNA Polymerase; PE Applied Biosystems) and envA and envN primers (15) were used to amplify a 3.1 kb region of proviral DNA encoding the rev and envelope genes. PCR products were isolated by unidirectional T/A cloning in the eucaryotic expression vector pCR®3.1-Uni (Invitrogen). Expression in pCR®3.1-Uni is driven by the CMV immediate early promotor. Multiple clones were isolated from each virus, and transient transfection studies in COS-7 cells confirmed the surface expression and fusion competence of all clones tested (data not presented).

DNA sequence analysis demonstrated that all 168C molecular clones analyzed encoded the three adaptation-associated amino acid changes previously identified by PCR sequencing of the 168C virus population (V2: I166R, C2:I282N, V3:G318R) (37). Two molecular clones of each 168P and 168C envelope were subjected to complete DNA sequence analysis (GenBank accession numbers AF035532 - AF035534). Molecular clones 168C23 and 168C60 were identical throughout the envelope gene. Molecular clones 168P5 and 168P23 differed from each other, and from the previously determined sequence, at 4-5 positions distinct from those associated with adaptation. These scattered changes within the primary virus quasispecies are considered

inconsequential at the present level of analysis; the significance of the three adaptation-associated changes is under separate investigation.

Functional analysis of these molecularly cloned envelope genes was performed by incorporation of the molecularly cloned envelope protein into pseudotyped HIV virions. We utilized an envelope-defective provirus derived from the molecularly cloned NL4-3 provirus (kindly provided by I.S.Y Chen, UCLA). The pNLthyΔ*Bgl* provirus (28) contains a *Bgl*II-*Bgl*II deletion within the envelope gene and a substitution of the viral nef gene with a cDNA encoding the murine thy1.2 cell surface protein. The SV40 ori was subsequently introduced into the plasmid to generate pSVNLthyΔ*Bgl* (V. Planelles, unpublished). Cotransfection of COS-7 cells (16, 20) with pSVNLthyΔ*Bgl* provirus and the envelope expression plasmid resulted in the production of pseudotyped HIV virions. Culture supernatants were harvested 3 days post-transfection, filtered and used to infect U87-CD4 cell lines expressing coreceptor. Cells infected by virions bearing the complementing envelope protein were identified by immunostaining for murine thy1.2 or HIV proteins.

As anticipated, the molecularly cloned envelope proteins recapitulated the coreceptor specificity of the parental virus population (legend Figure 4). Pseudotyped virions containing 168C60 were only able to infect U87-CD4 cells expressing CXCR4, while virions containing 168P23 envelope were able to infect U87-CD4 cells expressing either CCR5 or CXCR4. Thus, the viral envelope protein appears to be the major, if not sole, determinant of viral coreceptor use. These findings also indicate that dual coreceptor use is a direct property of the

envelope protein complex, and not a result of a mixture of distinct envelope proteins in the SI virus population. This conclusion is corroborated by the failure of CCR5-specific chemokine ligands to diminish 168P virus production in PBL culture (Figure 2).

Finally, we wished to determine the neutralization sensitivity of pseudotyped virions containing the molecularly cloned 168P23 and 168C60 envelope proteins, and to confirm that coreceptor pathway is not a primary determinant of neutralization sensitivity. We found that infection of U87-CD4-CXCR4 cells by pseudotyped virions containing 168C60 envelope protein was sensitive to neutralization by MAbs 257-D, 268-D, and 50.1 at concentrations comparable to those determined using 168C virus (Figure 4). Pseudotyped virions containing 168P23 envelope protein remained refractory to neutralization by all three V3-directed MAbs, regardless of the coreceptor expressed by the U87-CD4 cell line.

In summary, we examined the relationship between coreceptor utilization and sensitivity to neutralization by V3-directed MAbs. The observed dichotomy in the sensitivity to neutralization of PI and TCLA viruses had suggested a discrete difference between these viruses, and we tested one hypothesis - that PI viruses are refractory to neutralization as a result of their unique ability to utilize the CCR5 coreceptor, rather than CXCR4. We examined neutralization sensitivity of a well-characterized SI primary isolate under experimental conditions wherein the virus was forced to utilize either CCR5 or CXCR4 for infection. We showed that coreceptor pathway is not a direct determinant of neutralization sensitivity.

The primary virus envelope protein remained refractory to neutralization by V3-directed MAbs regardless of the coreceptor pathway utilized. Similarly, coreceptor utilization did not affect neutralization sensitivity by soluble CD4 (34) or HIVIG (data not presented).

In discarding the otherwise attractive hypothesis that PI viruses escape neutralization through their unique ability to utilize CCR5, we are left to consider the as-yet undefined structural differences between the envelope protein complex of PI and TCLA viruses. Several studies have suggested that critical determinants in the envelope protein of PI viruses are less accessible than those of TCLA viruses and that it is this differential access that determines neutralization sensitivity (reviewed in 25). By contrast, our studies have indicated similar binding of V3-directed MAbs to PBLs infected with neutralization–resistant 168P or neutralization-sensitive 168C viruses (37). Thus the basis for the differential neutralization sensitivity of PI and TCLA viruses remains unresolved.

Our present studies also do not address whether changes in coreceptor utilization and/or neutralization sensitivity are necessarily linked as a consequence of adaptation. The analysis of independently-derived PI and TCLA viruses may allow further separation of these viral phenotypes. Subsequent dissection of the amino acid changes that distinguish pedigreed PI and TCLA envelope proteins will help to define the structural bases underlying the changes that accompany adaptation.

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## Figure Legends

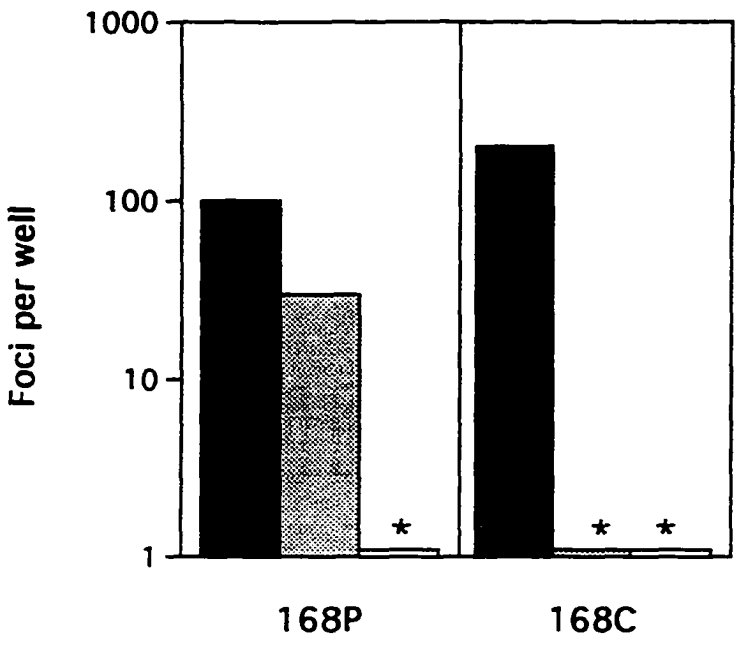
**Figure 1:** Coreceptor utilization by pedigreed PI and TCLA 168 viruses. U87-CD4 cell lines expressing CXCR4 (■) or CCR5 ( ) were used to define the ability of 168P and 168C viruses to utilize the respective coreceptor. CCR5 utilization was further tested by the addition to U87-CD4-CCR5 cells of CCR5-specific chemokines (RANTES, MIP-1α, and MIP-1β; R&D Systems) (□). Virus stocks were prepared from cell culture supernatants of PBLs (168P) or FDA/H9 cells (168C) and standardized to yield a submaximal number of foci of infection on U87-CD4-CXCR4 cells (approx 100-200 foci/96 well microplate culture). CCR5-specific chemokines (500 ng/ml each) were added to cells 1 hr prior to infection. After 2 days of incubation, cell monolayers were fixed with methanol:acetone and immunochemically stained using HIVIG (29), anti-human ABC kit (Biomeda Corp), and DAB substrate. * no foci were observed.

**Figure 2:** Neutralization sensitivity of 168 viruses in PBL culture. Virus neutralization assays in PHA-stimulated PBL culture were performed as previously described (37). 168P (○,●) and 168C (□,■) virus stocks were standardized to yield submaximal extents of virus spread during the 5 day infection. CCR5-specific chemokines (●,■) were added as described in Figure 1. The V3-directed MAbs are indicated. p24 antigen was determined using p24 antigen capture ELISA (SAIC Frederick) and was normalized to infected cell control values (168P - 190 ng/ml, 170 ng/ml with chemokines; 168C - 36 ng/ml, 33 ng/ml with chemokines).

**Figure 3:** Neutralization sensitivity of 168 viruses in U87-CD4 cell lines expressing CCR5 or CXCR4 coreceptor. 168P (○,●) and 168C (■) viruses were used to infect U87-CD4 cell lines expressing CXCR4 (●,■) or CCR5 (○) as described in Figure 1. The V3-directed MAbs were incubated with virus for 1 hr prior to infection.

**Figure 4:** Neutralization sensitivity of pseudotyped virions in U87-CD4 cell lines expressing CCR5 or CXCR4 coreceptor. Pseudotyped virions were derived by cotransfection of COS-7 cells with pSVNLthyΔ*BgI* provirus and plasmid expressing 168P23 (○,●) or 168C60 (■) envelope protein. Virion preparations were incubated with U87-CD4 cell lines expressing CXCR4 (●,■) or CCR5 (○) as described in Figure 1; V3-directed MAbs were added as indicated. The number of foci was normalized to control values (60-100 foci/well using U87-CD4-CXCR4 cells; 10 foci/well using U87-CD4-CCR5 cells). *, no foci were observed.

Fig. 1



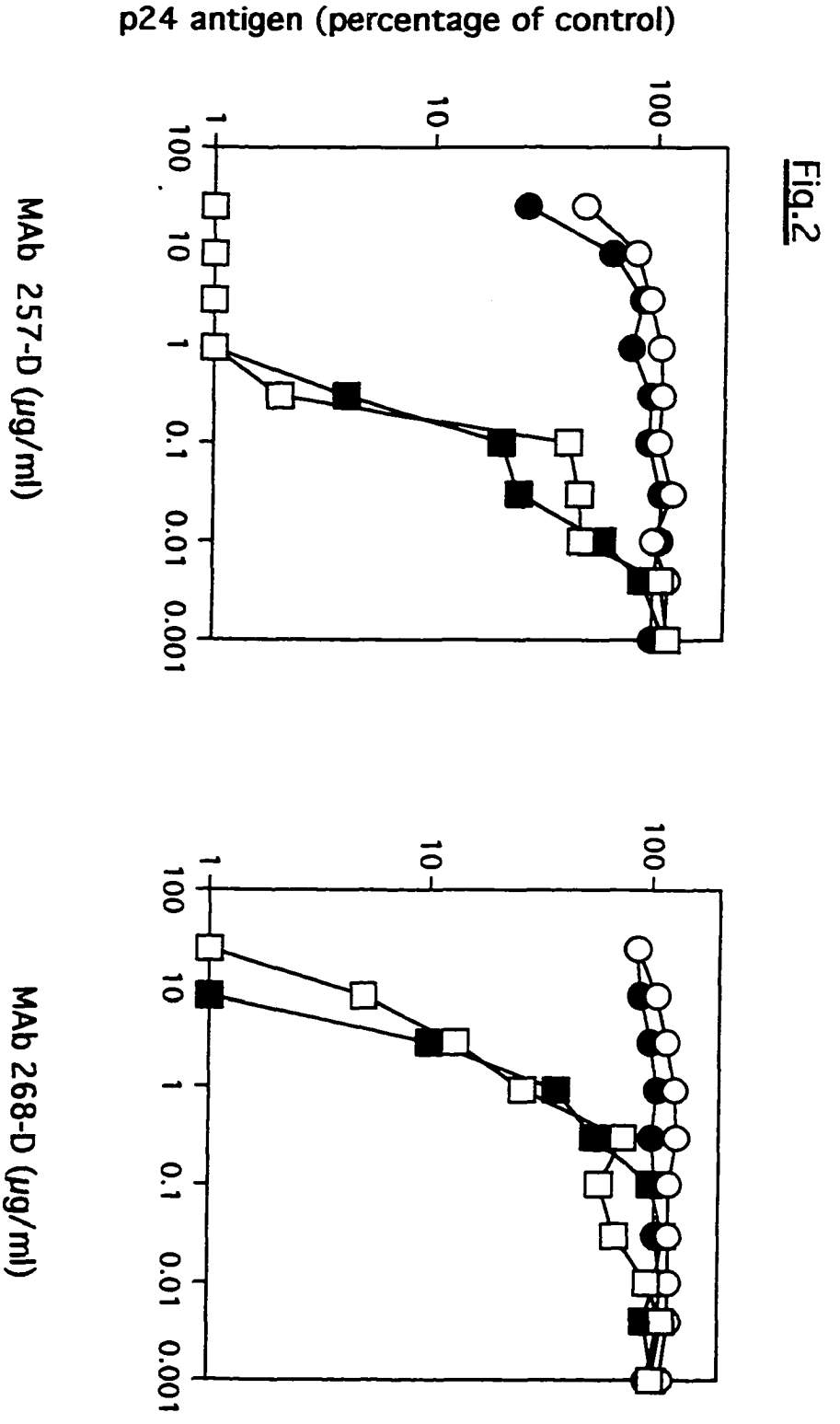



Fig.2

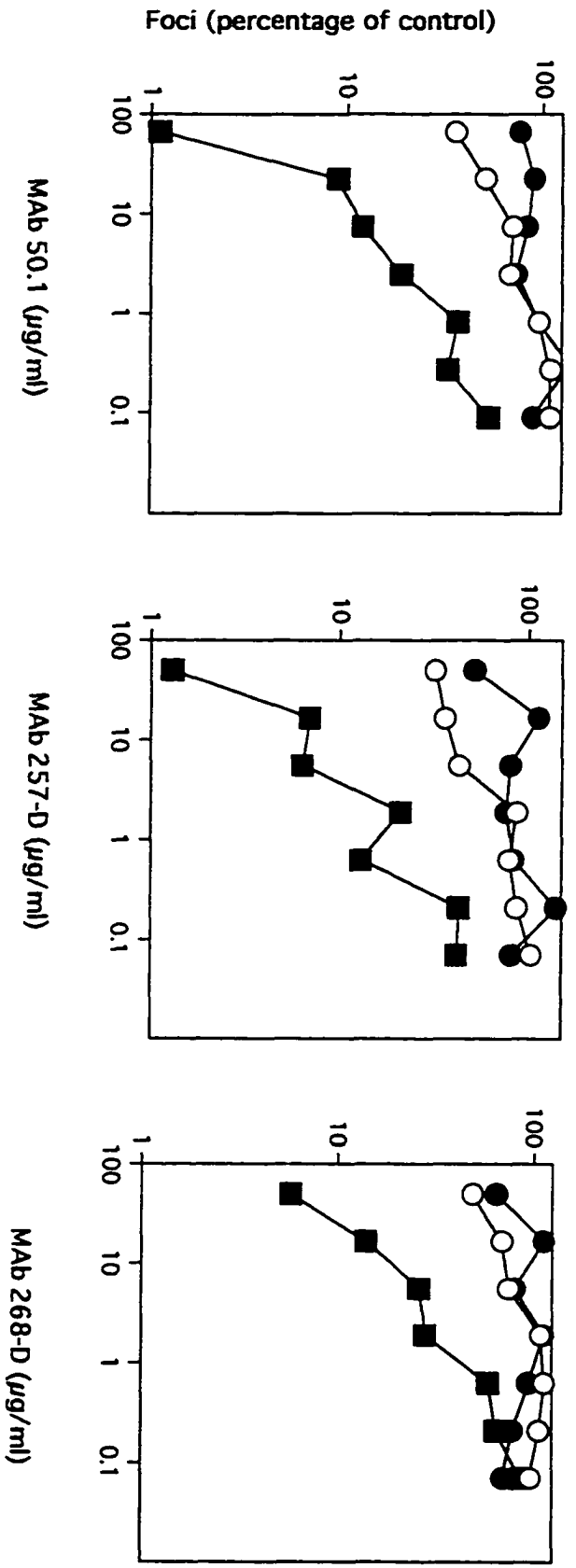



Fig. 3

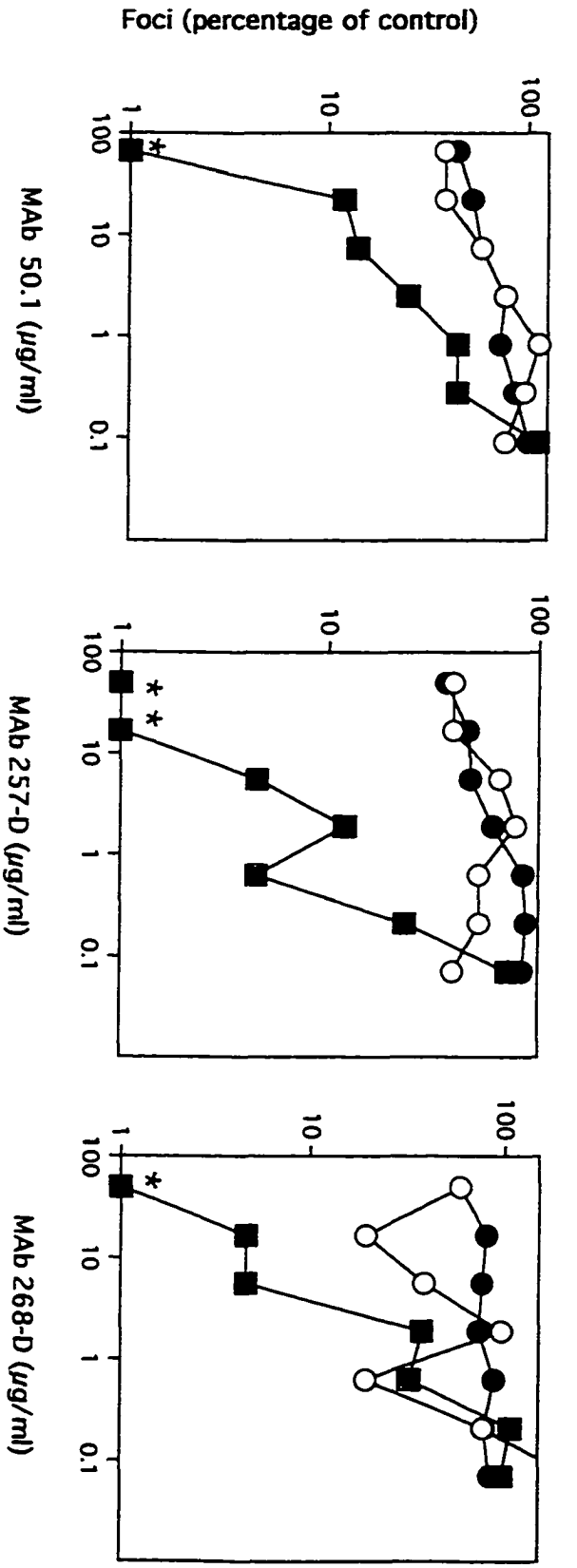



Fig.4

# Chapter 3

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## Fusion-competent Vaccine Immunogens Elicit Antibodies that Broadly Neutralize Primary Isolates of HIV

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**Abstract**

Current recombinant HIV gp120 protein vaccine candidates fail to elicit antibodies capable of neutralizing infectivity of primary patient isolates. Here, novel 'fusion-competent' HIV vaccine immunogens are discussed that capture the transient envelope-CD4-coreceptor structures which arise during HIV binding and fusion. In a transgenic mouse immunization model, these formaldehyde-fixed whole cell vaccines elicited antibodies capable of neutralizing infectivity of 23 of 24 primary HIV isolates from diverse geographic locations and genetic clades A-E. Development of these novel fusion-dependent immunogens may lead to a broadly effective HIV vaccine.

The expanding epidemic of HIV infection threatens to engulf more than 40 million persons worldwide by the year 2000 (1). The need for an effective HIV vaccine is urgent, but progress towards this goal has been slowed in part by the inability of any vaccine candidate to elicit antibodies capable of neutralizing infectivity of primary HIV isolates (PIs) from infected individuals (2). Because the HIV envelope protein mediates the early binding and entry steps in infection, many vaccine strategies have focused on this target. In 1993, two recombinant forms of the surface gp120 subunit of the HIV envelope protein (rgp120) were advanced as candidate vaccines for a large scale efficacy study sponsored by the National Institutes of Health (NIH). These rgp120 vaccines had been shown in clinical studies to be safe and to elicit antibodies capable of potently neutralizing related laboratory-adapted isolates of HIV (3). Progress was stalled, however, by findings that PI viruses, in contrast to their laboratory-adapted derivatives, were entirely refractory to neutralization by rgp120 vaccine sera (4). Although we now appreciate that laboratory-adapted isolates grown extensively in established T-cell lines differ in neutralization sensitivity from primary isolates taken from infected persons and subjected to limited growth in primary T lymphocytes, the basis for this difference remains unclear (5). More importantly, however, no HIV vaccine candidate has been shown to elicit potent and broad neutralization of biologically relevant primary isolates of HIV.

In contrast to antibodies elicited by rgp120 vaccines, antibodies from persons actively infected with HIV are able to neutralize infectivity of PI viruses,

albeit incompletely (6). Surveys of patient sera typically report low-level neutralization of 30-50% of primary isolates, although breadth and titers vary. We speculated that this ability to neutralize PI viruses might be related to the presentation of functioning envelope protein in active infection, as compared to the static, non-functioning presentation of the envelope protein in rgp120 vaccines.

It has long been known that the oligomeric envelope protein complex binds the cell surface CD4 molecule (7). However, with the recent discovery of HIV coreceptors (8) has come a new vision of the dynamics of HIV envelope protein function. We now recognize that the HIV envelope protein orchestrates a complex series of protein-protein interactions and structural changes that ultimately results in fusion of the virus and cell membranes, and infection of the cell. Upon binding to CD4, the envelope protein undergoes conformational change that facilitates subsequent interaction with one of several coreceptor molecules (9). These 7-transmembrane domain G-protein-coupled cellular proteins normally function as receptors for chemokines, small proteins that mediate a variety of cell-cell interactions including those in inflammation. HIV utilizes these coreceptors as necessary components for membrane fusion. In general, non-syncytium-inducing (NSI)/monocytropic PI viruses use the CC chemokine receptor 5 (CCR5), whereas SI/T-lymphocytropic PI viruses use both CCR5 and the CXC chemokine receptor 4 (CXCR4). Interaction with either coreceptor induces further conformational change in the envelope protein and exposure of the hydrophobic fusion domain of the transmembrane gp41 subunit,

which then mediates fusion of the apposed cell and virus membranes (10). On the basis of this dynamic model of HIV binding and entry, we undertook to develop HIV vaccine immunogens that explicitly incorporate these functional intermediate structures.

One measure of envelope protein function is the ability to mediate cell-cell fusion. When cells expressing envelope protein are cocultured with cells expressing CD4 and coreceptor, multinucleated syncytia form over the course of 6-24 hr (Fig 1). For our vaccine studies, we asked whether this process of binding and fusion might be captured in progress by formaldehyde-crosslinking prior to extensive syncytium-formation. In these studies, the functioning envelope protein was derived from a T-lymphocytropic PI virus obtained from the Amsterdam Cohort (ACH168.10; 168P) (11). The molecularly cloned envelope protein, as well as the parental SI virus, utilizes both CCR5 and CXCR4 coreceptors. COS-7 cells were transfected to express the envelope protein (COS-env) and subsequently cocultured with human U87 glioma cells that express CD4 and CCR5 coreceptor (U87-CD4-CCR5) (12). To capture transitional intermediates during the process of binding and fusion, cocultures were fixed in 0.2% formaldehyde after 5 hrs (13) when few if any multinucleate cells were evident (Fig 1). This inactivated whole cell preparation is the 'fusion-competent' immunogen.

In order to meaningfully test the ability of these complex immunogens to elicit neutralizing antibodies, it was necessary to restrict the immune response to viral and virus-induced epitopes. Otherwise, antibodies to CD4 and CCR5 would

be generated that would themselves block infectivity. Therefore, it was essential to use an animal model that was immunologically tolerant to the human (hu) CD4 and CCR5 components of the vaccine, as would also be the case in human immunization. Thus, immunogenicity studies were performed using transgenic mice that express hu CD4 and hu CCR5 coreceptor.

These transgenic mice were originally developed as a potential small animal model for HIV infection (14). Expression of hu CD4 is regulated by a murine enhancer linked to a human CD4 minigene and is active in thymocytes, T helper lymphocytes, and monocyte lineage cells. Expression of the hu CCR5 cDNA is under the control of a murine CD4
expression cassette and is active in thymocytes and T helper lymphocytes. Although post-entry blocks to HIV replication have to date limited the utility of this transgenic model in infectivity studies, these animals were expected to be immunologically tolerant to the expressed human transgenes. Mice expressing all three relevant markers (hu CD4, hu CCR5, and mouse CD4) were used in the immunogenicity studies reported here (15). Formaldehyde-fixed immunogens were formulated with Ribi Adjuvant and mice were immunized subcutaneously at 3 week intervals (16).

In pilot studies, mice were immunized with either 'fusion-competent' immunogen or with cell controls (U87-CD4-CCR5 cells, alone or cocultured with mock-transfected COS cells). Sensitivity of the homologous 168P virus to neutralization by vaccine sera was determined using U87-CD4 cells expressing either CCR5 or CXCR4 coreceptor (17). No inhibition of infectivity was observed

in sera from mice immunized with cell controls, suggesting that the transgenic mice were in fact tolerant to hu CD4 and CCR5 and that other adventitious cellular reactivities did not interfere with the virus infectivity assay (Fig 2A).

More importantly, however, sera from mice immunized with 'fusion-competent' immunogens were able to neutralize the homologous 168P PI virus. Additional studies demonstrated that this neutralization activity was antibody-mediated and could be adsorbed to, and subsequently eluted from, a solid support containing Protein-A and Protein-G (18). In our experience, neutralization of this PI virus by vaccine antiserum was unprecedented.

Furthermore, neutralization of the 168P virus by 'fusion-competent' serum was observed regardless of the coreceptor used in the U87-CD4 cell infection assay (Fig 2A). Several reports have demonstrated that in general neutralization sensitivity is independent of specific coreceptor use (19). The fact that neutralization is observed in this study using CXCR4, a coreceptor to which the animal had not been exposed, argues that neutralization does not directly target the CCR5 component of the vaccine.

To explore the role of fusion-dependent determinants in the induction of PI virus neutralization, we expanded our studies to include 'fusion-incompetent' immunogens - cocultures that do not undergo cell-cell fusion. 'Fusion-incompetent' immunogens tested include: COS-env cocultured with U87 cells (no CD4 or CCR5 coreceptor), COS-env cocultured with U87-CD4 cells, and COS-env cells to which soluble CD4 (sCD4) was complexed (20). These immunogens were fixed with formaldehyde and harvested (12, 13). An additional 'fusion-

incompetent' immunogen comprised COS-env and U87-CD4-CCR5 cells that were separately fixed with formaldehyde prior to mixing during the formulation of the vaccine. Results from neutralization studies of 'fusion-competent' and 'fusion-incompetent' vaccine sera are summarized in Fig 2B.

In marked contrast to 'fusion-competent' immunogens, all 'fusion-incompetent' immunogens were unable to elicit significant neutralization of the homologous PI virus. These results are consistent with the well-documented failure of rgp120 vaccines to elicit PI virus neutralization. The striking distinction in neutralization by 'fusion-competent' and 'fusion-incompetent' vaccine sera was also observed in assays utilizing human primary blood lymphocytes (PBLs) (Fig 3).

As with conventional rgp120 vaccines, 'fusion-incompetent' vaccines were able to elicit neutralization of a related laboratory-adapted isolate, in this case the T-cell line adapted derivative of 168P, 168C (21) (Fig 4). Neutralization titers of the TCLA 168C virus were comparable among 'fusion-competent' or 'fusion-incompetent' vaccine sera, suggesting a similar degree of inherent immunogenicity among the vaccines.

The failure of 'fusion-incompetent' vaccines to elicit PI virus neutralization in the transgenic mouse model highlights the specificity of the neutralization elicited by 'fusion-competent' vaccines (22). Furthermore, the consistent failure of 'fusion-incompetent' vaccine sera to inhibit PI virus infectivity argues strongly that the immune response is not directed to adventitious human cellular targets, such as those that confounded early studies of inactivated SIV vaccines (23).

Rather, we suggest that 'fusion-competent' immunogens present unique fusion-dependent determinants that mediate neutralization of PI viruses.

Although the potent neutralization demonstrated against the homologous PI virus is unprecedented in HIV vaccine experience, it is understood that the 'homologous' virus will never be encountered in nature. HIV is an enormously variable virus that exists as a swarm of related, rapidly-evolving genotypes that cluster into phylogenetically-defined clades (24). Thus, a critical issue in HIV vaccine development centers on the ability of vaccine antisera to neutralize a broad range of diverse PI viruses.

To determine the breadth of PI virus neutralization elicited by 'fusion-competent' immunogens, we examined the sensitivity of a panel of representative PI viruses from five prevalent and geographically-diverse genetic clades (25). As depicted in Fig 5, 'fusion-competent' sera elicited by a functioning clade B envelope protein were able to neutralize 23 of 24 PI viruses tested - monocytropic/NSI and T-lymphocytropic/SI viruses from North America/Europe (clade B), Africa (clades A and D), Thailand (clades B and E), and India (clade C). Despite the sequence diversity among these isolates, most were similarly sensitive to neutralization by 'fusion-competent' vaccine sera. One clade A isolate 92RW008 failed to attain >50% neutralization; this exception to the otherwise broad pattern of neutralization further argues that 'fusion-competent' immunogens target primarily viral, rather than cellular, determinants. 'Fusion-incompetent' sera were uniformly unable to neutralize these heterologous PI viruses, in keeping with the historic failure of rgp120 immunogens. The broad

and uniform neutralization of diverse PI viruses suggests that the critical determinants presented by 'fusion-competent' immunogens are highly conserved, and may be intimately tied to the basic functioning of the envelope protein in binding and fusion.

We then sought to define the molecular target for PI virus neutralization by 'fusion-competent' vaccines. We had shown that the elicitation of PI virus-neutralizing antibodies required as immunogen a functional envelope-CD4-CCR5 interaction. Nonetheless, these neutralizing antibodies might recognize native, non-functioning envelope protein as antigen. A similar instance has been reported for monoclonal antibody (mAb) 17b (26) which targets a CD4-induced epitope on the gp120-CD4 complex but which also binds isolated gp120. Alternatively, another gp120-CD4 specific mAb CG10 (27) does not recognize isolated gp120 or CD4. Thus, we tested whether neutralizing antibodies could be removed from 'fusion-competent' vaccine sera on incubation with envelope protein complex expressed on the surface of transfected COS cells. Formaldehyde-fixed COS cells expressing 168P envelope protein were incubated with 'fusion-competent' serum and the recovered serum was then tested for PI virus neutralization (28). Neutralization activity in 'fusion-competent' vaccine serum was removed by incubation with envelope-expressing cells, but only minimally reduced by incubation with COS cell controls (Fig 6). Although the static form of the envelope protein does not function as an effective immunogen, we found that the critical fusion-dependent epitopes are sufficiently represented on the static protein to allow binding. These data argue that PI virus neutralizing

antibodies target, at least in part, the HIV envelope protein. Studies in progress will define the role of specific envelope protein sequences in binding, as well as the possible contribution of virus-induced CD4 or CCR5 determinants. Importantly, these data also provide independent support to our argument that neutralizing antibodies do not target adventitious cellular targets; any such targets would be equally recognized (or not recognized) on control COS cells.

Further delineation of the critical PI virus neutralizing determinant(s) captured by 'fusion-competent' immunogens will be facilitated by the development of PI virus neutralizing mAbs to 'fusion-competent' immunogens. Based on the requirement for interaction with coreceptor, we speculate that neutralization may target a late event in virus binding and entry. Upon binding to coreceptor, the envelope protein must mediate fusion of the viral and cell membranes. Cryptic but highly conserved determinants may be exposed during this process. One possible target for neutralization might involve the triple-stranded coiled-coil structure that mediates membrane fusion and is highly conserved among Orthomyxoviridae (Influenza), Filoviridae (Ebola), and Retroviridae (HIV) as well as in fusion of cellular membranes (29). The fusion-active structure of the HIV envelope protein is believed to form subsequent to CD4 and coreceptor binding by the collapse of two helical coils within each gp41 monomer to form the trimeric coiled-coil core. This structure is thought to drive membrane insertion of the hydrophobic fusion domain of gp41 and to initiate membrane fusion. Synthetic peptides that comprise either of the gp41 helical coils are able to bind the cognate helical region and broadly inhibit viral infectivity

(30). We speculate that broadly-neutralizing antibodies to 'fusion-competent' immunogens may likewise target structures involved in the activation of fusion.

The potency and breadth of neutralization by 'fusion-competent' immunogens appears to surpass that observed in sera from infected individuals. Perhaps formaldehyde-fixation traps critical fusion-dependent structures that are only transiently presented during active infection. It remains to be determined whether 'fusion-competent' immunogens target the same neutralizing determinants as active infection.

The ability of antibodies induced by 'fusion-competent' immunogens to neutralize a broad range of PI viruses necessitates a shift in our thinking of HIV vaccines. Whereas previous discussions regarding the possible number of HIV serotypes were moot in the absence of any vaccine-induced neutralization, it had been widely accepted that multiple envelope protein immunogens might be needed to span the range of HIV sequence diversity. We now show that an appropriately-presented clade B envelope protein can elicit potent neutralization against most PI viruses, from multiple HIV clades. We show that not only is PI virus neutralization achievable, we suggest that broad vaccine protection may not require an unlimited number HIV serotypes.

Although the immunological basis for HIV vaccine efficacy is presently unknown and controversial, there is ample reason to believe that pre-existing neutralizing antibody may offer protection against infection and/or disease. Passively administered neutralizing antibodies can exert potent antiviral effects in several experimental models of HIV infection, as manifested by complete or

partial protection (31). Antibody-mediated protection is, of course, well-accepted in other viral infections and vaccines (32). With the ability to elicit potent HIV neutralization will come the opportunity to determine the role of pre-existing neutralizing antibody in HIV prophylaxis. Concerns regarding the diversity of HIV populations are lessened by the breadth of neutralization elicited by 'fusion-competent' immunogens. Still, one may raise the concern that antibodies in HIV-infected persons do not protect against disease progression. Whether the ultimate disease course is modified by the ongoing antibody response is difficult to assess. In any case, an established chronic infection presents special challenges in terms of host competence and virus load and diversity, challenges that are distinct from those envisioned in prophylactic immunization. The effect of pre-existing antibodies in exposure to a minimal infectious dose of virus in an immunocompetent host remains to be determined in preclinical and clinical studies.

We recognize that in its current form an inactivated whole cell 'fusion-competent' vaccine is not practical for clinical development. Nonetheless, 'fusion-competent' formulations that incorporate critical fusion-dependent determinants of PI virus neutralization can be envisioned. For example, recombinant viral vectors that respectively express envelope and CD4 with coreceptor could be coadministered to drive critical fusion events in vivo. Alternatively, purified fusion-active complexes could be developed as an inactivated subunit vaccine. This novel concept of 'fusion-competent' immunogens may also be applicable to other

enveloped viruses where protection has been difficult to generate other than by live attenuated virus immunization.

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15. For immunogenicity studies, pups were screened for expression of hu CD4, hu CCR5, and mouse CD4 by flow cytometry using a Coulter EPICS ELITE flow cytometer in collaboration with Dr Edwin Walker (Ribi ImmunoChem Research,

Inc.). The following antibody reagents were used: mouse α-human CD4/CyChrome (Pharmingen), mouse α-human CCR5 MAB 180 (R&D Systems) with goat α-mouse Ig/FITC (Caltag), and rat α-mouse CD4 L3T4/PE.

16. Vaccines comprised formaldehyde-fixed whole cells ($3x10^6$ cells/0.1 ml) formulated with an equal volume of Ribi adjuvant(R-700); in some experiments, the initial immunization was with adjuvant containing cell wall material (R-730). Mice received 0.05 ml vaccine in four subcutaneous sites. Booster immunizations were at three week intervals, and mice were bled at 10-28 days post-immunizations from the tail. Animal care was in accordance with institutional guidelines. Serum antibodies directed to gp120 were quantitated by ELISA (J. Moore, L. Wallace, E. Follett, J. McKeating, *AIDS* **3**, 155-163 (1989)).

17. This rapid PI virus neutralization assay has been validated relative to our standard neutralization assay in PBL culture (R. A. LaCasse, et al., *ibid* **72**, 2491-2495 (1998); K. E. Follis, M. Trahey, R. A. LaCasse, J. H. Nunberg, *Journal of Virology* **72**, 7603-7608 (1998)) and performs well in the presence of mouse serum. All sera were heat-inactivated prior to use in neutralization assays.

18. Serum was adsorbed sequentially to Protein-A Sepharose (Sigma) and Protein-G agarose (Sigma) at 4°. Adsorption of antibody was confirmed by anti-gp120 ELISA. The solid supports were combined and antibodies were eluted

using 100 mM glycine pH 2.5. The eluate was neutralized and dialyzed by centrifugal ultrafiltration (Microcon 100; Amicon).

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20. Envelope-expressing cultures were incubated with sCD4 (E. A. Berger, T. R. Fuerst, B. Moss, *Proceedings of the National Academy of Sciences USA* **85**, 2357-2361 (1988)) (5 μg/ml; 1 hr at 37°) and subsequently washed to remove unbound sCD4.

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22. A statistical comparison was performed on the combined data sets. A simple model for virus-antibody binding was used to calculate a 'binding constant' K for each assay, and a mean K value was determined for each mouse. Analysis of variance with follow-up tests demonstrated a significant difference in mean neutralization between 'fusion-competent' and 'fusion-incompetent' immunogens ($p < 0.01$).

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28. 'Fusion-competent' vaccine serum was sequentially adsorbed four times with approximately $10^6$ formaldehyde-fixed COS cells expressing 168P envelope. Incubations were for 1 hr at 4° with rocking. Controls included prebleed serum and formaldehyde-fixed mock-transfected COS cells. Final sera were tested for neutralization of HIV 168P using U87-CD4-CXCR4 cells.

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## Figure Legends

**Figure 1**. Cell-cell fusion in cocultures of COS-env and U87-CD4-CCR5 cells (12). Both fields are at 200x magnification.

**Figure 2**. Neutralization of the homologous 168P PI virus by 'fusion-competent' and 'fusion-incompetent' vaccine sera. **(A)** Transgenic mice (hu CD4+, hu CCR5+, mouse CD4+) in Studies 1-3 were immunized with 'fusion-competent' immunogen (COS-env with U87-CD4-CCR5) (■, ❑; n = 3 mice) or with cell controls (U87-CD4-CCR5 cells alone or cocultured with mock-transfected COS cells) (●, ○; n = 3 mice). Unimmunized mice were also used (▲, Δ; n = 2 mice). Sera were tested for neutralization of 168P using U87-CD4 cells expressing either CXCR4 (■,●,▲) or CCR5 (❑,○,Δ). Data represent averages of 3-6 neutralization assays using serum obtained 2 weeks following second and third immunization. **(B)** Transgenic mice in Study 4 were immunized with 'fusion-competent' immunogen (■, n = 4), 'fusion-incompetent' immunogens (COS-env with U87 cells, n = 4; COS-env with U87-CD4 cells, n = 3; COS-env with sCD4, ◇n = 2; COS-env with U87-CD4-CCR5 cells, each fixed separately prior to mixing for immunization, n = 2). Mock-transfected cell controls (○; n = 2) and unimmunized mice (Δ; n = 2) were also used. Neutralization was independent of specific coreceptor use (Fig 1A) and data here represent averages of 3-6 neutralization assays in U87-CD4-CXCR4 or -CCR5 cells. In some cases individual animals were pooled to conserve serum.

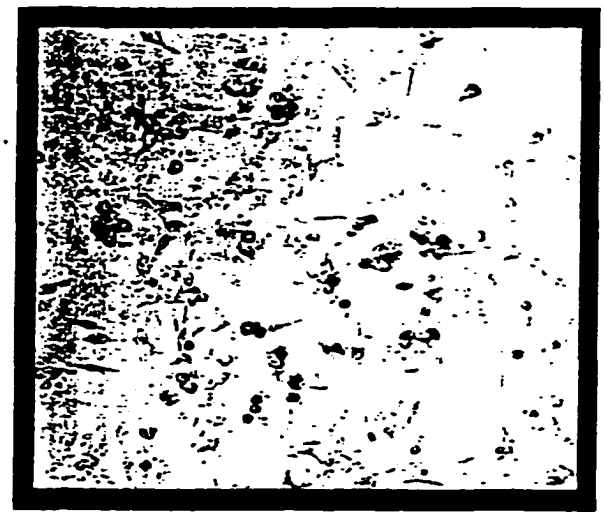
**Figure 3.** Neutralization of the homologous 168P PI virus in human PBL culture. Lymphocytes were isolated, stimulated with phytohemagglutinin, and grown in the presence of interleukin-2; neutralization was determined as described (17). HIV p24 antigen was determined after 5 days of culture by ELISA (Coulter Corporation). Vaccine groups are as defined in Fig 2B, and sera from individual animals were pooled for this assay.

**Figure 4.** Neutralization of TCLA 168C virus by 'fusion-incompetent' vaccine sera. Neutralization sensitivity of the 168P PI virus and its TCLA derivative 168C were tested in U87-CD4-CXCR4 cells. Vaccine groups and symbols are as defined in Fig 2B; sera from individual animals were pooled for this assay.

**Figure 5.** Neutralization of diverse PI viruses from clades A-E. Primary isolates were expanded in human PBLs and neutralization was determined in permissive U87-CD4-CCR5 (or -CXCR4) cells. Viral biotype is indicated if known. Vaccine groups and symbols are as defined in Fig 2B; sera from individual animals were pooled for this assay.

**Figure 6**. Adsorption of PI virus neutralization activity by formaldehyde-fixed COS-env cells. 'Fusion-competent' vaccine serum (■) was repeatedly incubated with formaldehyde-fixed COS-env ( ) or control COS (❑) cells and tested for

residual neutralization of 168P using U87-CD4-CXCR4 cells (27). Prebleed serum was similarly adsorbed (●, ○, respectively).



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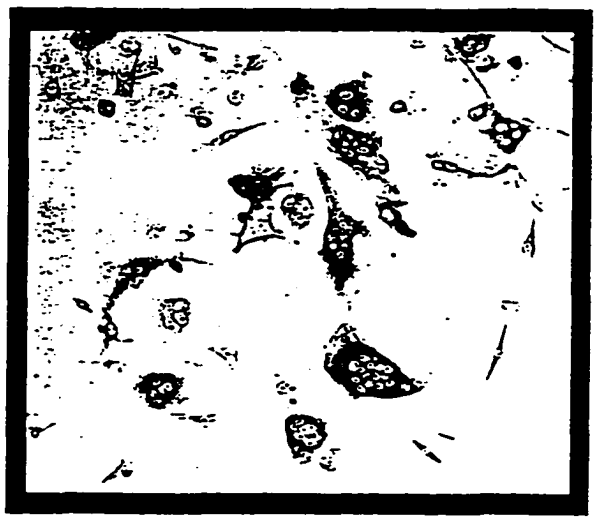

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Fig. 2

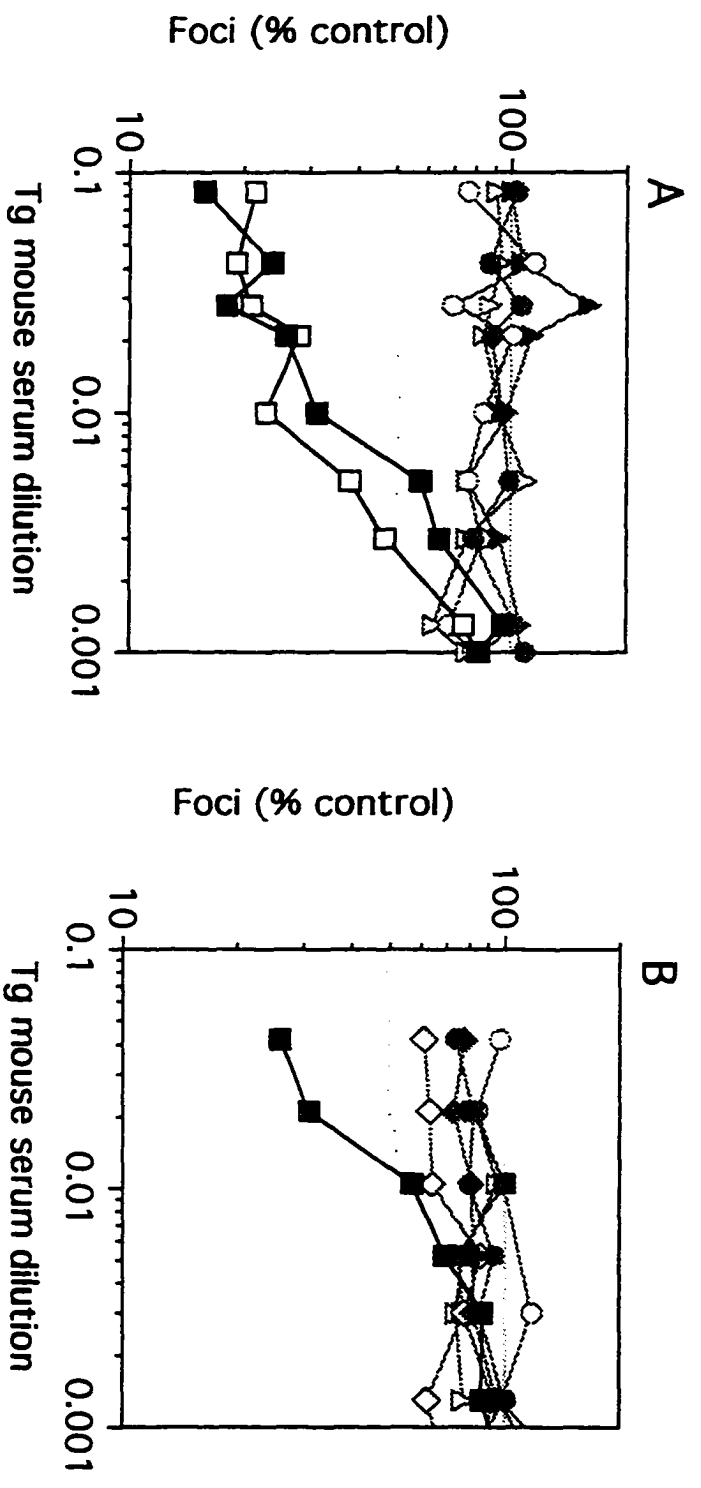
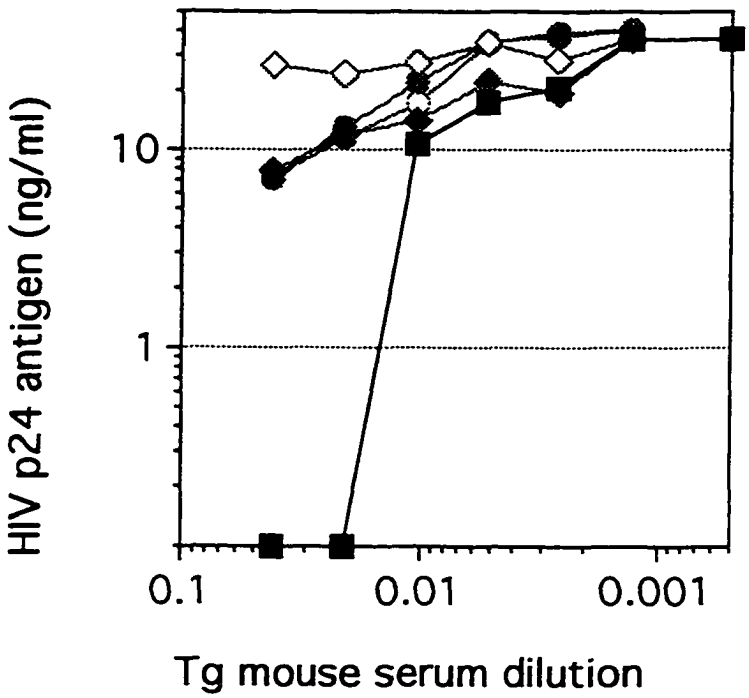


Fig. 3



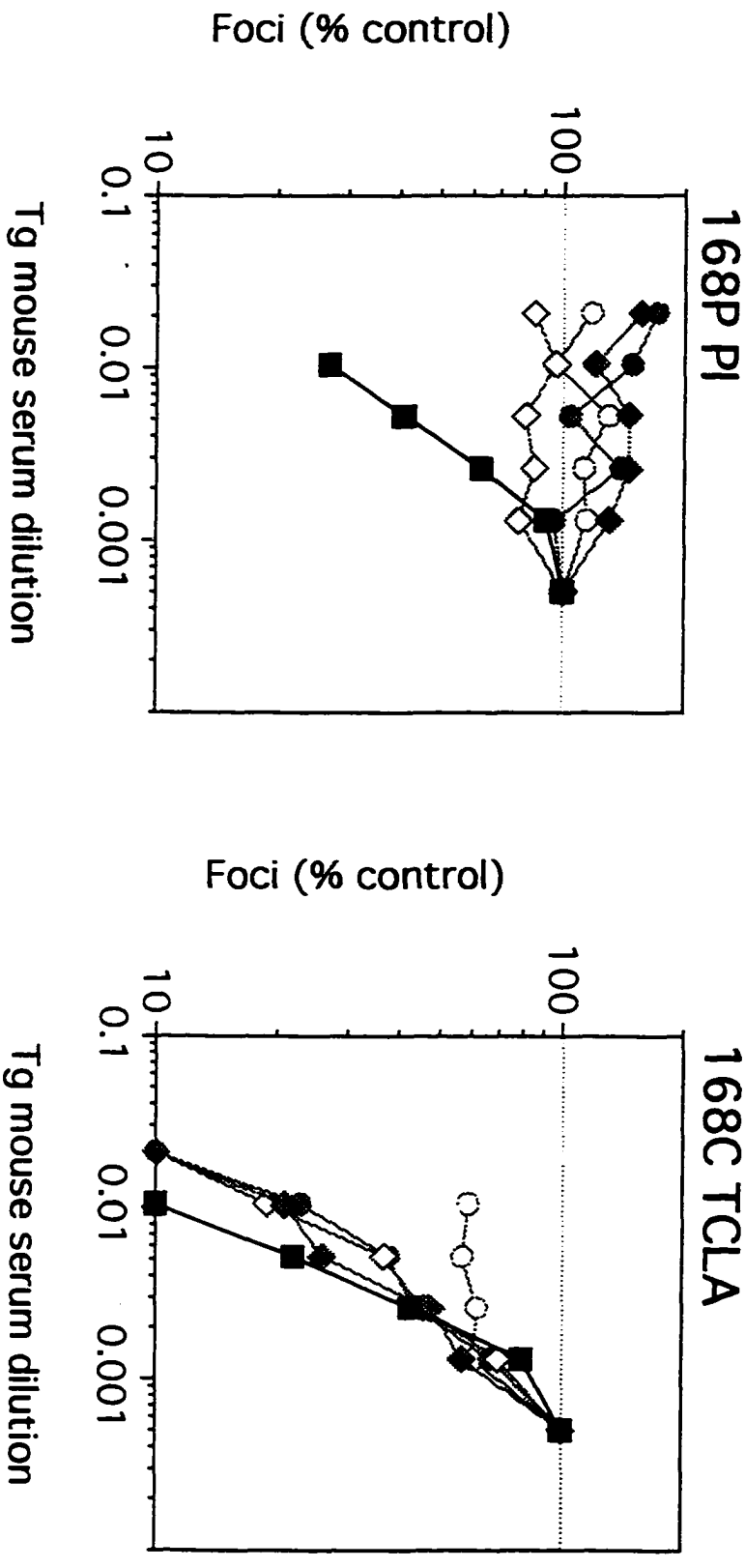



Fig. 4

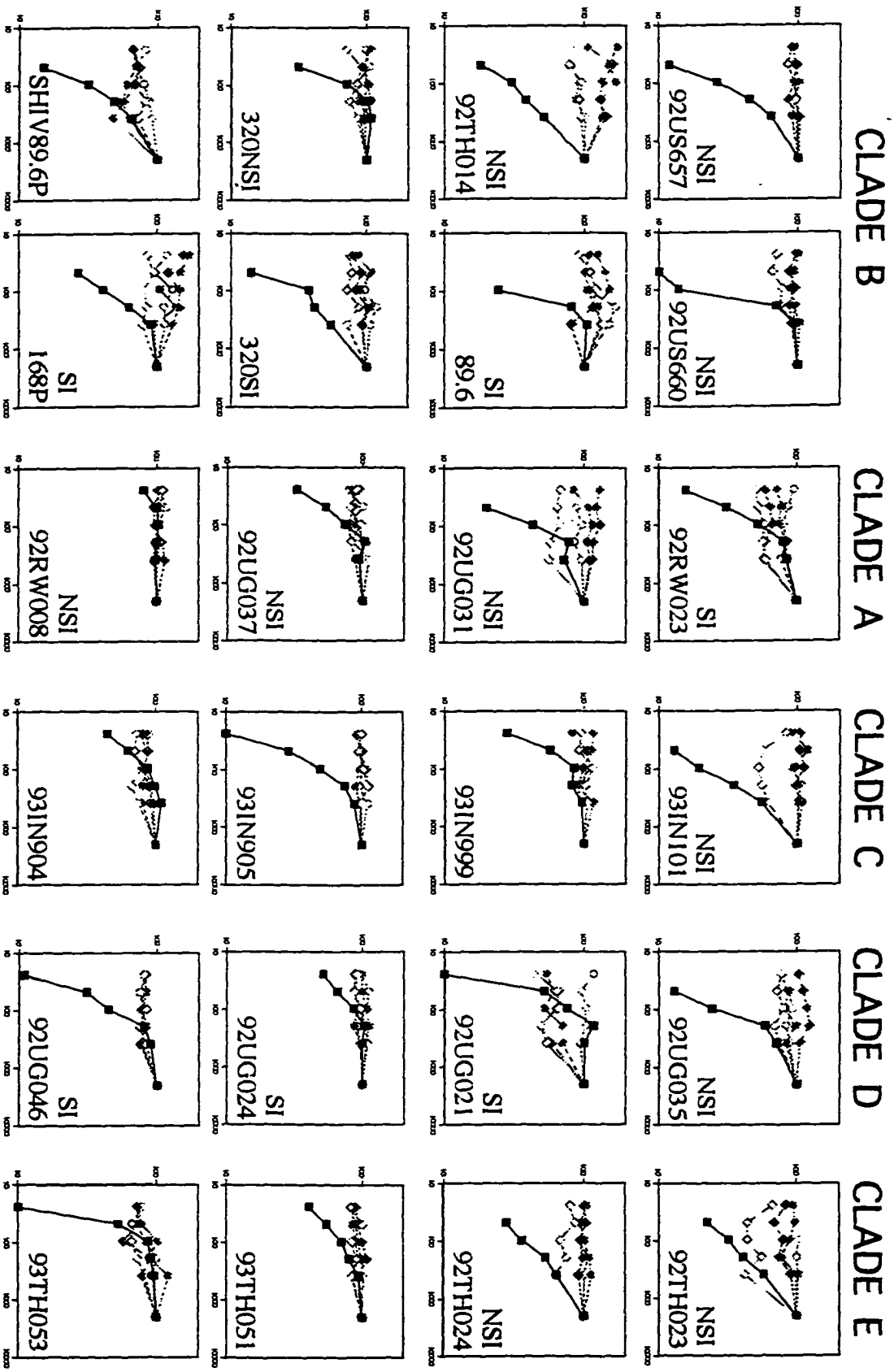
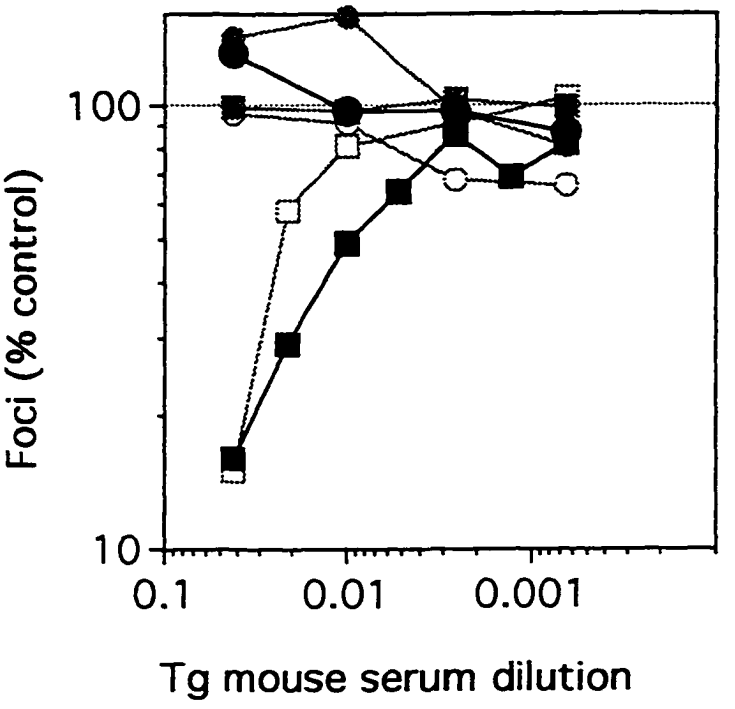


Fig. 6



# Chapter 4

## Discussion

The HIV envelope protein initiates a series of complex protein-protein interactions that ultimately lead to the fusion of the virus and cell membranes. This process of envelope-mediated fusion has lead to the vision of a protein that undergoes a series of complex conformational changes that expose potential neutralizing epitopes that are conserved throughout the different clades of HIV. We have developed a vaccine strategy that incorporates these fusion-dependent epitopes of the functioning envelope into the immunogen, in contrast to the current vaccine candidates which are composed of a static, nonfunctioning envelope. We propose that it is the incorporation of the functioning envelope interacting with CD4 and coreceptor that has made this vaccine strategy successful in eliciting antibodies capable of neutralizing PIs of HIV *in vitro*.

There are numerous studies that will stem from this project. First and foremost, spleens from the 'fusion-compenent' vaccinated mice will be harvested to produce hybridomas for monoclonal antibody production. These monoclonal antibodies will be essential for the elucidation of the epitope(s) responsible for the production of neutralizing antibodies within this vaccine. Interestingly, it has recently been shown that a peptide (DP-178) corresponding to the helical regions of the gp41 core (the coiled-coiled core that consists of the leucine zipper motif) is a potent inhibitor of HIV infection (1-4). This peptide is able to bind to the envelope protein and inhibit conformational changes that are necessary for envelope-mediated fusion (4-6). It was found that this peptide is

only able to bind to the envelope complex and inhibit fusion after it interacts with the cellular receptors, suggesting the coiled-coil domain of gp41 may become accessible during the fusion process (7). It could be hypothesized that this region is transiently accessible during binding and entry events and could be a potential epitope for neutralization. Future studies include evaluation of our vaccine sera to determine if the neutralizing antibodies target this region of gp41, as well as define other epitopes present in both gp41 and/or gp120 responsible for eliciting neutralizing antibodies of PI viruses.

It is recognized that the current form of the inactivated whole cell 'fusion-competent' vaccine is not practical for clinical development. Therefore, vaccine strategies incorporating the critical fusion-dependent determinants of neutralization will be developed. One approach being taken is to utilize recombinant viral vectors (e.g. vaccinia) that would respectively express envelope and CD4 with coreceptor. These recombinant vectors would then be coadministered to drive the fusion events *in vivo*. An additional approach to be pursued is to purify the fusion-active complexes comprised of envelope, CD4, and coreceptor, and incorporate these complexes into an inactivated subunit vaccine. The development of these vaccine strategies to elicit neutralizing antibodies to PI viruses will hopefully lead to a pratical formulation of the 'fusion-competent' vaccine for application in humans.

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# Part 2

## Chapter 5

### General Introduction

Feline immunodeficiency virus (FIV) is a lentivirus that causes a natural immunosuppressive disease in cats that is very similar to AIDS in humans (1-4). FIV is similar to HIV-1 not only in disease progression, but also in sensitivities to many of the antiviral drugs used in AIDS chemotherapy and in its ability to develop resistance to these drugs. In the past, the FIV model system had been developed to study the phenomenon of drug resistance (5-7). However, it has now been expanded to evaluate the mutation frequency of the virus and how it is affected by antiviral therapy.

Antiviral agents usually target a viral component that is required for replication, such as a receptor needed for entry or an enzyme that is essential to the replication of the viral genome. One such target is the viral RNA-directed DNA polymerase, or reverse transcriptase (RT) of retroviruses. This enzyme is required for the synthesis of a double-stranded DNA copy of the single-stranded RNA viral genome. A number of reverse transcriptase inhibitors have been approved and used for AIDS chemotherapy, including 3'-azido-3'-deoxythymidine (AZT), 2',3'-dideoxycytidine (ddC), 2',3'-dideoxyinosine (ddI), and 2'-deoxy-3'-thiacytidine (3TC).

The RT of FIV has been shown to be similar to HIV-1 RT in physical properties, catalytic activities, and sensitivities to several important antiviral

nucleosides (9-12). Both of these enzymes have similar $Mg^{++}$ requirements and similar template specificities. The sensitivities to various RT inhibitors are also very comparable. The competitive inhibitors 2',3'-dideoxythymidine 5'-triphosphate (ddTTP), 2',3'-dideoxycytidine 5'-triphosphate (ddCTP), 3'-fluoro-3'-deoxthymidine 5'-triphosphate (3'-F-dTTP), 2',3'-dideoxy-2',3'-didehydrothymidine 5'-triphosphate (d4TTP), and 3'-amino-3'-deoxythymidine 5'-triphosphate (3'-$NH_2$-dTTP) were determined to have similar inhibition constants for both HIV-1 and FIV RT. This also held true for phosphonoformate (PFA) and phosphonoacetate (PAA), two noncompetitive inhibitors of RT (9,11). In addition, it has been found that the FIV RT has a low fidelilty, similar to that described of HIV-1 RT (Garvey et al., unpublished observations). These features have made the FIV model highly relevant to HIV.

The RT inhibitor AZT is a nucleoside analogue that has been found to have potent antiviral activity against HIV-1, and was the first nucleoside analogue to be approved for use in AIDS chemotherapy (12). AZT requires activation by cellular enzymes to its active form, AZT triphosphate (AZTTP). This activated form is a dTTP analogue and competes with the natural substrate in the cell, dTTP, for incorporation into the growing chain of DNA by RT. Once AZT has been incorporated, the 3'-azido group blocks the formation of the 3',5'-phosphodiester bond that is necessary for chain elongation, hence, DNA synthesis is terminated. (12). This drug is widely used for treatment of HIV infection.

The following study utilizes the FIV system to evaluate the mutation frequency of this retrovirus. The high mutation rate of HIV is believed to be a critical factor in the ability of the virus to evade the immune system (13). It also may contribute to the emergence of drug-resistant variants seen in patients undergoing antiviral chemotherapy (13-15). Therefore, it is important to evaluate the effect antiviral drugs have on the mutation frequency of the virus during chemotherapy.

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# Chapter 6

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## The Mutation Frequency of Feline Immunodeficiency Virus Enhanced by 3'-Azido-3'-deoxythymidine

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## Summary

We have developed a host range system to measure the mutation frequency of feline immunodeficiency virus (FIV), the feline homologue of human immunodeficiency virus type 1 (HIV-1). When wild-type FIV was grown in the presence of a known mutagen, 5-bromo-2'-deoxyuridine (BUdR), a dose-dependent increase of host range mutants was detected. Using this system, we have evaluated the effects of antiviral drugs upon the mutation frequency of FIV. Subinhibitory concentrations of 3'-azido-3'-deoxythymidine (AZT), the most common antiviral drug used in AIDS chemotherapy, increased the mutation frequency of FIV in a dose-dependent manner. Two other antivirals, 2',3'-dideoxyinosine (ddI) and 2',3'-dideoxycytidine (ddC) did not show this effect.

**Key words:** Antiviral drugs-Feline immunodeficiency virus-Lentivirus mutation frequency.

## Introduction

The high mutation frequency of human immunodeficiency virus type 1 (HIV-1) (1-3) is a major barrier to development of vaccines and chemotherapy and may also be important in the pathogenesis of the acquired immune deficiency syndrome (AIDS) (4). The high misincorporation rate of HIV-1 reverse transcriptase (RT), estimated to be 2.5-5 x $10^{-4}$ with either DNA or RNA templates, is believed to play a major role in this. RT infidelity would result in five to ten errors each time the genome is replicated (2,3), although the actual mutation rates may be substantially lower than would be predicted by fidelity of RT in vitro (5). This high variability is believed to be a critical factor in the ability of the virus to evade the immune system, and it is a substantial limitation to development of a vaccine (4). The high mutation frequency of HIV-1 may also contribute to the emergence of drug-resistant variants, a serious problem faced in the treatment of AIDS patients (4,6,7). High variability is also seen from the genotypic differences between HIV-1 isolates obtained from AIDS patients versus the isolates obtained early after infection. It has been suggested that the accumulation of mutations affecting tropism, replication rates, and so on may contribute to the pathogenesis of AIDS (4,8,9). Since the high mutation frequency of the virus appears to be important in terms of pathogenesis and therapy, the effects of antiviral drugs upon mutation frequency need to be considered. Therefore, we have developed a system to evaluate the effects of antiviral drugs on mutation frequency of a lentivirus, and we report here that the most widely used anti-HIV-1 drug, 3'-azido-

3'-deoxythymidine (AZT), increases the mutation frequency of feline immunodeficiency virus (FIV).

Although the high error rate of HIV-1 RT is well documented, assessment of the mutation rate at the level of virus replication has been difficult. Point mutation rates of a murine retrovirus, murine leukemia virus, have been determined (10), as have the mutation rates of the spleen necrosis virus containing a reporter gene (11). However, no data are available on mutation rates or frequencies using endogenous genes of a replicating virus, and no data are available for a lentivirus.

Our main interest in lentivirus mutation frequencies is in the rate of emergence of drug-resistant variants. We have previously developed a model system using FIV for studies of resistance to AZT and other antiviral drugs (12-14). With this system, we reported the first in vitro selected AZT-resistant mutants (12), which are phenotypically similar to the AZT-resistant clinical isolates of HIV-1 reported by Larder et al. (15,16). FIV is a lentivirus that causes an immune deficiency in domestic cats that is clinically similar to human AIDS (17-20), and this immunosuppressive disease can be induced experimentally in specific-pathogen-free cats(21,22). We have shown that the purified FIV RT is similar to the HIV-1 RT in physical properties, catalytic activities, and sensitivities to several important antiviral nucleotides (23-26). The RT of FIV also has low fidelity, similar to that of HIV-1 (Garvey et al., unpublished observations).

These features make FIV an attractive model to study mutation frequency and how it is affected by antiviral drugs or by mutations conferring drug-

resistance. The FIV model is also highly relevant to HIV. For these studies, we developed a host-range system that allows measurement of mutation frequencies in a manner analogous to classic genetic studies with bacteriophage systems (27). Using this system we can evaluate the effects of antiviral drugs on the mutation frequency of a lentivirus. We have presented a preliminary report that the most widely used anti-HIV-1 drug, AZT, increases the mutation frequency of FIV (28). We report here the details of the FIV host-range system and the effects of antiviral nucleosides on the mutation frequency of FIV.

# MATERIALS AND METHODS

## Chemicals

5-bromo-2'-deoxyuridine (BUdR) and 2',3'-dideoxycytidine (ddC) were purchased form Sigma Chemicals Co. (St. Louis, MO, U.S.A.). AZT was provided by Phillip A. Furman of Burroughs Wellcome Co. (Research Triangle Park, NC, U.S.A.). 2',3'-Dideoxyinosine (ddI) was provided by the Developmental Therapeutics Branch, Division of AIDS, National Institute of Allergy and Infectious Diseases.

## Virus and Cells

The virus strains used in this study were FIV Petaluma (17,19) and FIV 34TF10, a molecular clone derived from the Petaluma strain (29). Virus was grown and maintained in Crandell feline kidney (CrFK) cells, which were obtained from American Type Culture Collection (ATCC) and were cultured under condition previously described (26). An FIV-resistant variant of CrFK cells, CrFK-13, is described in Results. When cells reached confluency, they were passaged by trypsinization, diluted approximately 100-fold, and reseeded in 25-$cm^2$ flasks. A single passage includes the period between trypsinization, dilution, and reseeding the cells, until they become confluent and are ready for trypsinization again. Cells were passaged approximately once a week. They were routinely tested with a Gen Probe *Mycoplasma* detection kit (Gen Prob

Inc., San Diego, CA, U.S.A.) and were found to be free of any *Mycoplasma* contamination.

### Focal Infectivity Assay

Infectious FIV was quantified by the focal infectivity assay (FIA) as previously described (12). Briefly, uninfected CrFK cells were infected with ~20-80 focus forming units of FIV. After 5 days, media were removed, and the cells were fixed with methanol. Infectious foci were detected by reacting with polyclonal antiserum obtained from FIV-infected, specific-pathogen-free cats (generously provided by N.C. Pedersen, School of Veterinary Medicine, University of California, CA, U.S.A.) and then reacted with peroxidase-conjugated anti-cat immunoglobulin (Organon Teknika). The peroxidase stain was developed with $H_20_2$ and amino-ethyl-carbazole. Infectious foci appeared as red cells against a background of uninfected (unstained) cells. Plaque purification of virus from a single foci was performed as previously described (13).

### Determination of Mutation Frequencies

To determine mutation frequencies, cell-free virus was differentially plated on CrFK-13 (FIV-restrictive) and CrFK (FIV-permissive) cells. For these experiments, virus grown in the absence or presence of a mutagen or inhibitor was differentially plated on CrFK-13 cells and FIV-permissive CrFK cells. Before infection, cells were pretreated with 8 μg/ml diethylaminoethyl dextran for 20 min

to facilitate adsorption of the virus. The values reported represent the number of foci formed on CrFK-13 cells divided by the number of foci formed on FIV-permissive CrFK cells, as determined by FIA.

# RESULTS

## Development of the Host-Range System

The system we developed uses a strain of Crandell feline kidney (CrFK) cells that is resistant to infection by wild-type FIV Petaluma or the molecular clone, FIV 34TF10. As described herein, these cells are used to measure the proportion of host-range mutant in a population of virus. The FIV-resistant cells were isolated after we observed that with prolonged passage of FIV-permissive CrFK cells, we obtained cells that were highly restrictive to FIV infection. Wild-type FIV Petaluma infected these restrictive cells ~1,000 times less efficiently than permissive CrFK cells, as determined by FIA. As shown in Fig. 1, the susceptibility of CrFK cells to infection with FIV decreased dramatically as a function of passage number. We are not certain of the reason for the change in the cells, but it is presumably due to different growth conditions. ATCC had grown the CrFK cells in minimum essential medium (Delbecco's MEM) supplemented with 10% horse serum, which is different from the medium (L & M medium supplemented with 10% fetal bovine serum) (26) that is optimal for FIV replication.

Because the population of FIV-restrictive CrFK cells was expected to be a heterogeneous mixture, we isolated 19 single-cell clones from this population. The susceptibilities of these clones to FIV infection are shown in Table 1. Several of the clones were highly resistant to FIV, whereas others, such as CrFK-3, were less restrictive, confirming that the original population was a heterogeneous mixture. We chose one of the clonal isolates, CrFK-13, as a

highly restrictive host cell for further characterization. This choice was made based upon the ability to detect a few (potentially mutant) foci of FIV with these cells.

From these early studies, it was determined that wild-type FIV Petaluma infected CrFK-13 cells, $1.4\pm0.4 \times 10^{-3}$, as efficiently as permissive CrFK cells. FIV-permissive CrFK cells are CrFK cells that have been passaged four or fewer times after they were obtained from ATCC. Experiments were performed to determine whether the occasional foci observed upon infection of CrFK-13 by FIV were host-range mutants that were present in the swarm population of the virus, rather than simply wild-type virus able to form foci owing to leakiness. We plaque-purified several potential mutants by isolating single foci of FIV Petaluma on CrFK-13 cells. The resulting host-range (HR) mutants remained infectious to the FIV-permissive CrFK cells and also infected CrFK-13 cells with much higher efficiency that the potential wild-type FIV (Table 2).

In order to ascertain whether these viruses are truly mutants, we assessed their phenotypic stability by passing one of the HR mutants, HR-3, back into the FIV-permissive CrFK cells. This was done to test the possibility that HR mutants were the result of a biochemical adaptation rather than a stable phenotype. When HR-3 was passaged back into the FIV-permissive cells, progeny virus retained the ability to infect CrFK-13 cells 100 fold more efficiently that wild-type FIV (data not shown). This finding confirmed the phenotypic stability of HR-3. These data suggest that the CrFK/CrFK-13 HR system could be useful for studies to evaluate the mutation frequency of FIV.

With this assay, HR mutants are detectable in a normal population at a frequency of 0.14%. However, the actual mutation frequency is somewhat higher when corrected for efficiency of plating of HR mutants on CrFK-13 cells (Table 2). Based upon the six HR mutants we have analyzed, the average plating efficiencies for HR mutants range from 2.7-18%. This efficiency results in an actual mutation frequency somewhere between 0.8 and 5.6%. It is apparent that these variants represent a substantial fraction of the swarm population, but are at a significant disadvantage relative to wild-type FIV.

To test this system, we examine the effects of a known mutagen, BUdR (30), to determine whether it increases the frequency of HR mutant in a population. As shown in Fig. 2A, passage of wild-type FIV Petaluma in the presence of BUdR increased the mutation frequency in a dose-dependent manner, up to a level where cytotoxicity was observed (>0.7-1.0 μM). This finding confirms that our system can detect an increase in the mutation frequency due to replication of the virus in the presence of a known mutagen. The maximum increase in mutation frequency that we observed with BUdR is only 2.5- to three-fold. We believe that the extremely high basal mutation rate of FIV limits the extent of mutagenesis and that higher mutation rates would result in too many lethal mutations. We believe that the apparent leveling off of the mutation frequency seen with higher concentrations of BUdR is not due to an actual leveling off of the mutation frequency, but rather to a limitation in the sensitivity of the system when the inhibition of virus replication results in titers too low for accurate scoring of mutants.

The experiments with BUdR was also performed with FIV obtained from the molecular clone FIV 34TF10, which represents a more homogenous population than the swarm population of FIV Petaluma. The mutation frequency of FIV 34TF10 also increased in the presence of BUdR. This result indicates that replication in the presence of the mutagen increased the mutation frequency of the virus regardless of the degree of heterogeneity of the starting population.

## Effects of Antiviral Drugs on the Mutation Frequency of FIV

With the confirmation that our HR system is able to detect an increase in the mutation frequency due to the presence of a mutagen, we evaluated the effects of antiviral drugs upon the mutation frequency of FIV. As shown in Fig. 2B, when wild-type FIV Petaluma replicated in the presence of AZT, a dose-dependent increase of the mutation frequency was detected. Similar results were obtained in experiments with the molecular clone FIV 34TF10. We also evaluated two other antiviral nucleosides approved for AIDS chemotherapy. Replication of wild-type FIV Petaluma in the presence of ddI and ddC resulted in little or no detectable increase in the mutation frequency (fig 2C and 2D, respectively).

## DISCUSSION

AZT is negative in the Ames test, only weakly mutagenic in assays with cultured mammalian cells, and weakly carcinogenic in rodents (31). However, the mutagenicity of AZT to FIV cannot be considered weak; AZT enhances the

mutation frequency of FIV by nearly as much as BUdR, a well-characterized mutagen. This ability of AZT to enhance the mutation frequency of a lentivirus may have important implications for AIDS chemotherapy, but it is not clear whether this enhanced mutagenesis will be beneficial or detrimental. If AZT produces an increased frequency of lethal mutations among the population of virus escaping drug, it may contribute to antiviral activity. However, it is likely that an enhanced mutation rate will also contribute to mutations involved in antigenic variation, evasion of the immune system, and changes in cell tropism. If the rate of pathogenesis is in fact dependent upon accumulation of multiple mutations, then AZT therapy may alter the rate of pathogenesis.

The genetic system we have developed quantitates the proportion of virus with a particular phenotype, in this case the number of HR mutants present within a population of viable virus. The baseline frequencies we report are not mutation rates, but rather the proportion of mutants in an equilibrium population. The mutation frequency of a retrovirus is dependent upon forward mutation rate, reverse mutation rate, replication rate, and number of replication cycles (32. 33). AZT lowers the replication rate and number of replication cycles, and so these two factors are not important to the AZT-induced enhancement of mutation frequency. The reverse mutation rate will be considerably lower than the forward rate, owing to the fact that HR mutants are a minor fraction of the population; thus it will contribute little. Therefore, the AZT-induced increase in mutation frequency is most likely due to an enhancement of the mutation rate by AZT. We cannot be certain that the other drugs tested, ddI and ddC, are not

mutagenic, since it is possible that they exert and effect that is below our level of detection. Nevertheless, it is clear that AZT is much more mutagenic than these other antivirals to FIV.

The mutagenicity of AZT was at subinhibitory concentrations of AZT. We believe there would be even more mutagenicity at higher levels. We also believe that studies performed at these low drug levels are relevant to the clinical use of AZT. It is clear that patients are exposed to concentrations that inhibit HIV-1 replication only partly, or at subtherapeutic levels, during periods of therapy. The dose of AZT used in humans is three- to six-fold lower than was suggested from early pharmacokinetic studies, and trough levels of AZT are considerably below the minimum inhibitory concentration of the drug (34,35). Virus that is replicating, albeit at a reduced rate, during these periods of exposure to low AZT concentrations may have an enhance mutation rate.

We do not know the mechanism of this AZT-induced mutagenesis. AZT has been shown to cause alterations in levels of DNA precursors (dNTPs) (36,37), presumably due to effects of AZTMP on thymidylate kinase (38). It is well established that dNTP pool imbalances can enhance mutation rates of cultured cells (39). It is possible that the reverse transcriptase-catalyzed replication of retroviruses is particularly sensitive to pool imbalances owing to the lack of an associated exonuclease to carry out an editing function. It is also possible that the mutagenicity of AZT results from an effect of AZTTP directly on RT or is an indirect result of the incorporation of this analog into DNA (and attempted repair). It is also possible that the apparent mutagenic effects of

BUdR and AZT are due to selection of HR variants rather than mutagenic effects. However, we believe it is unlikely in view of the well-known mutagenic properties of BUdR (30). Moreover, AZT-resistant mutants of FIV do not display an HR phenotype (data not shown).

The HR system we have developed with FIV is conceptually similar to phage genetic systems, and we believe that it will be important for studies on variability and fidelity of replication of a lentivirus. Our initial goal in development of this system was to obtain a genetic marker independent of the reverse transcriptase-encoding region of the *pol* gene. We would like to use this system to identify *pol* mutants of FIV that have mutator or antimutator phenotypes. We have already determined that the FIV RT has a low fidelity, similar to that reported for the HIV-1 RT (Garvey et al., unpublished observations). We also have a large number of FIV variants containing *pol* mutations that were selected as drug-resistant mutants. The systems we have developed will be used to determine how alterations of fidelity at the enzyme level correlate with mutation rates during virus replication.

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## FIGURE LEGENDS

**FIG. 1.** Effects of passage of CrFK cells on susceptibility to FIV infection. Susceptibility of these cells to infection by FIV was determined by FIA. The same cell-free virus stock was used to infect all of the preparations of CrFK cells. The infectivities of FIV Petaluma (▲) and 34TF10 (■) were determined as a function of increasing passage number. Each value represents an average of at least four determinations.

**FIG. 2.** Mutation frequency of FIV grown in the presence of a mutagen or antiviral drugs. CrFK cells were infected with wild-type FIV Petaluma (▲) or FIV 34TF10 (■) in the presence of indicated concentrations of BUdR **(A)**, AZT **(B)**, ddI **(C)**, and ddC **(D)**. Mutation frequencies in the absence of drugs were 0.0050±0.0003 for FIV Petaluma and 0.0057±0.0003 for FIV 34TF10. The mutation frequency value seen here for FIV Petaluma is slightly different from what is seen in Table 1, owing t the fact that these assays were performed using a different stock of virus. It has been found that different stocks of virus have slightly different background levels of HR mutants. Each value represents two or more experiments with at least four determinations per experiment. The error bars represent the standard error of the mean.

TABLE 1. PLATING EFFICIENCY OF WILD-TYPE FIV ON SINGLE-CELL CLONES OF FIV-RESTRICTIVE CrFK CELLS*

| CrFK CLONE† | PLATING EFFICIENCY‡ |
|---|---|
| 1 | $7.8 \times 10^{-3}$ |
| 2 | $<4.4 \times 10^{-4}$ |
| 3 | $4.3 \times 10^{-2}$ |
| 4 | $2.6 \times 10^{-3}$ |
| 5 | $2.6 \times 10^{-3}$ |
| 6 | $<4.4 \times 10^{-4}$ |
| 7 | $8.7 \times 10^{-3}$ |
| 9 | $2.1 \times 10^{-3}$ |
| 10 | $<4.4 \times 10^{-4}$ |
| 11 | $<4.4 \times 10^{-4}$ |
| 12 | $<4.4 \times 10^{-4}$ |
| 13§ | $1.4 \pm 0.4 \times 10^{-3}$ |
| 14 | $<4.4 \times 10^{-4}$ |
| 15 | $4.4 \times 10^{-4}$ |
| 16 | $<4.4 \times 10^{-4}$ |
| 17 | $1.4 \times 10^{-2}$ |
| 18 | $<4.4 \times 10^{-4}$ |
| 19 | $1.7 \times 10^{-3}$ |
| 20 | $1.9 \times 10^{-2}$ |

* This table represents the initial screening of all of the clones.
†Limited dilutions of a population of FIV-restrictive CrFK cells were used to isolate single cell clones.
‡By differentially plating cell-free FIV Petaluma on the restrictive clones and on the FIV-permissive CrFK cells, plating efficiency of the virus was obtained by dividing the number of foci detected on restrictive cells by the number of foci detected on the FIV-permissive CrFK cells.
§Clone CrFK-13 was selected for further experiments and used for the development of the host range system. The standard error of the mean from more than five experiments is indicated.

TABLE 2. RELATIVE PLATING EFFICIENCIES OF HOST-RANGE (HR) MUTANTS

| Virus* | PLAT. EFFIC.† | FOLD INCREASE§ |
|---|---|---|
| Petaluma | 0.0014 ± 0.0004‡ | 1X |
| HR1 | 0.039 ± 0.007 | 28X |
| HR2 | 0.027 ± 0.010 | 19X |
| HR3 | 0.18 ± 0.034 | 129X |
| HR4 | 0.072 ± 0.014 | 51X |
| HR5 | 0.16 ± 0.059 | 114X |
| HR6 | 0.042 ± 0.008 | 30X |

* Each potential mutant was isolated from a single focus formed on CrFK-13 cells.

† Plating efficiencies of the HR mutants were determined by dividing the number of foci seen on CRFK-13 cells by the number of foci seen on FIV-permissive CrFK cells. Each value represents the results of two or more experiments with at least four determinations per experiment.

‡ Standard error of the mean.

§ Increased efficiency of infection of HR mutants on FIV-restrictive cells as compared to the control, FIV Petaluma.

**Figure 1**

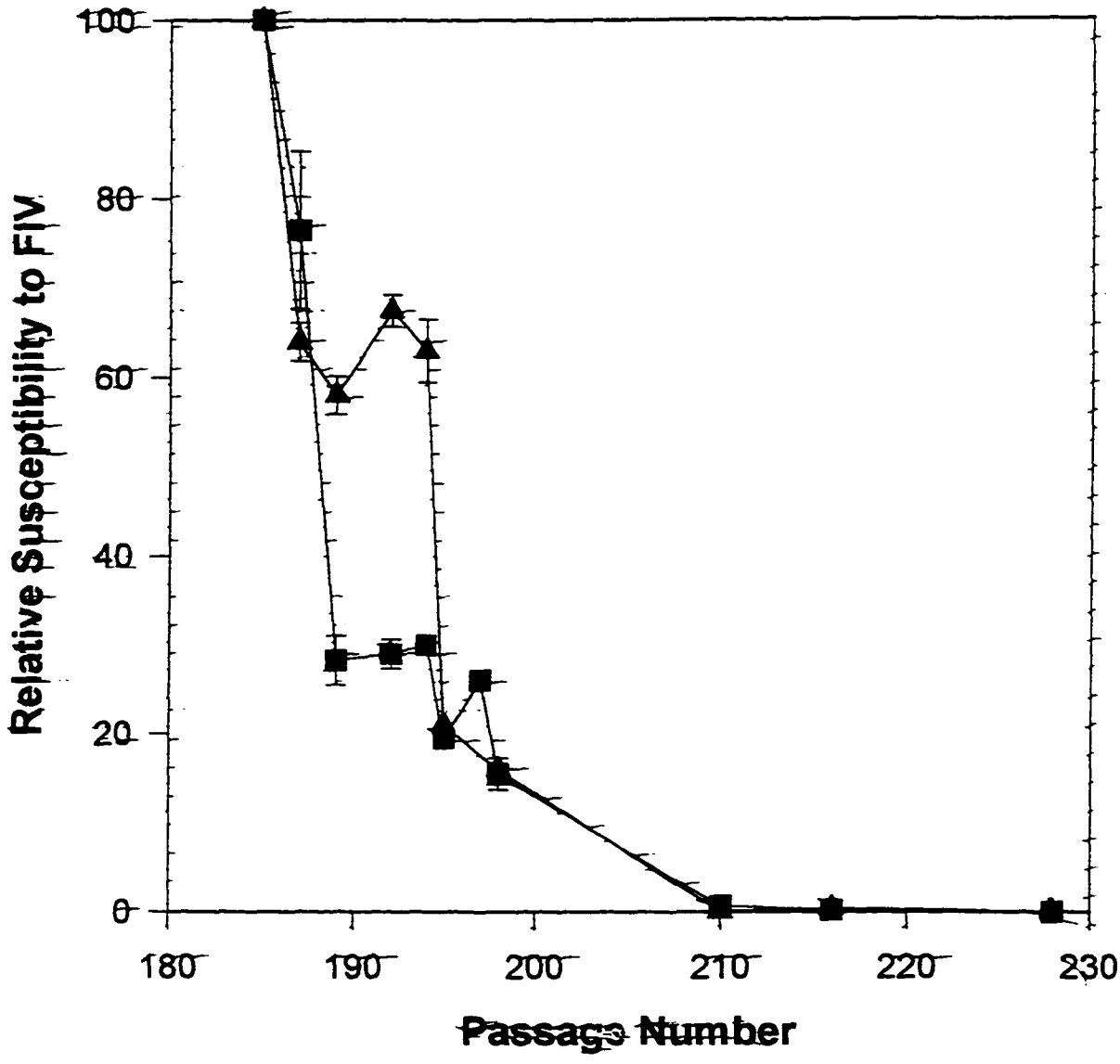
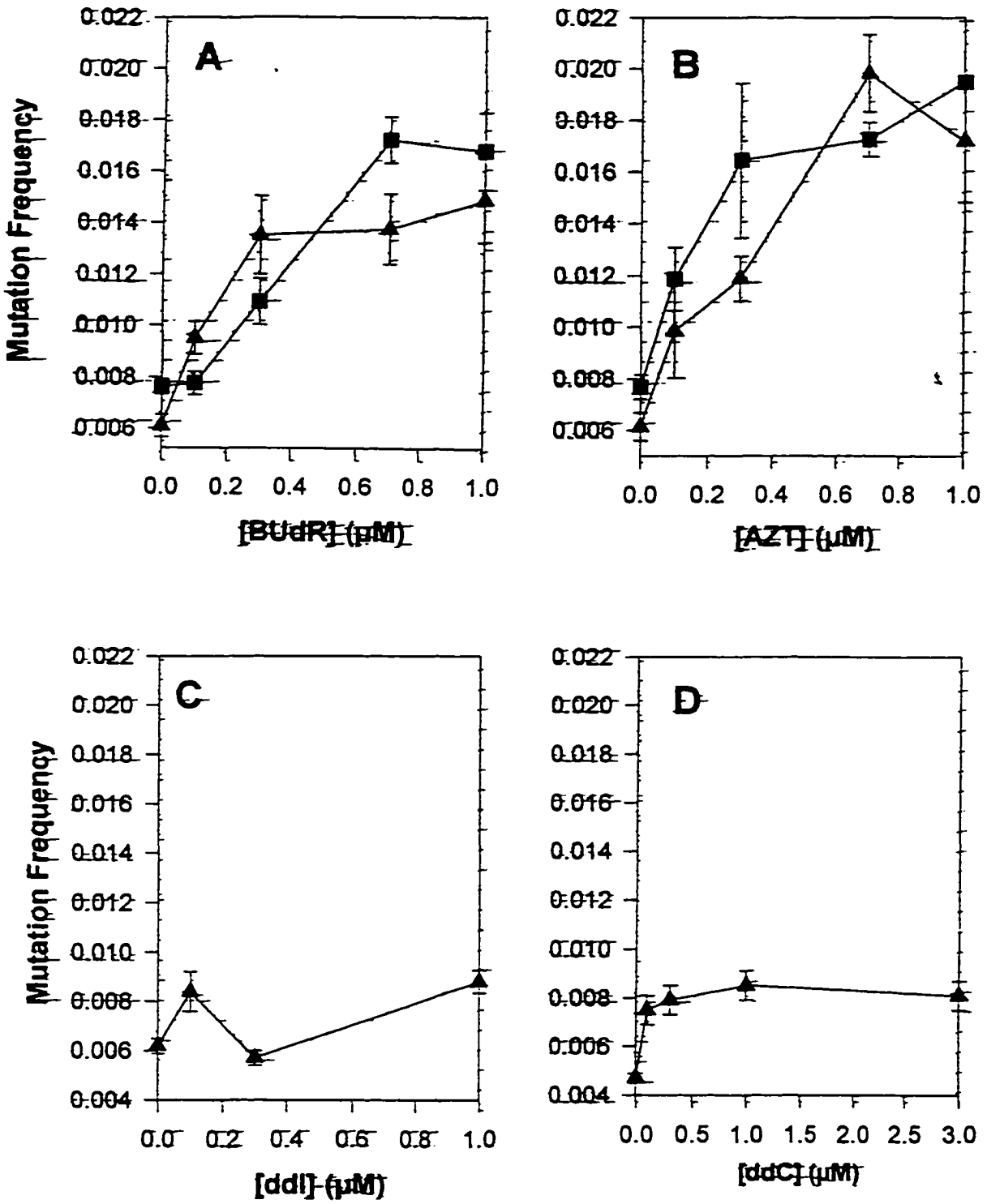


Figure 2



# Chapter 7

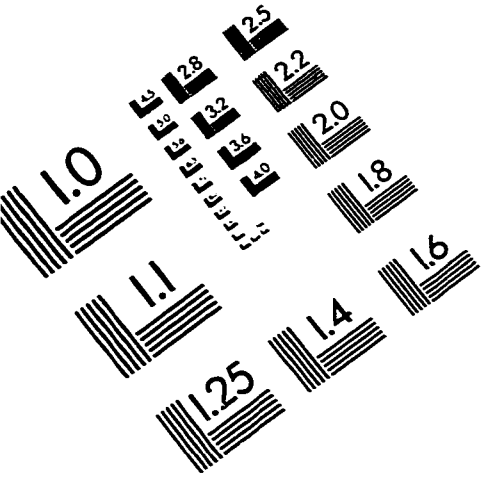
## Conclusions

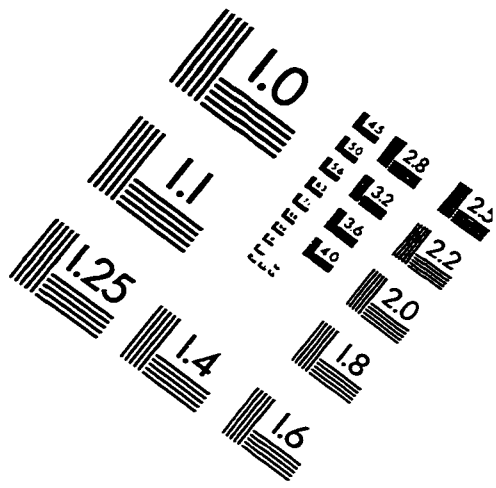
By utilizing the host range system of the FIV model, it has been determined that AZT is mutagenic to FIV. The mutagenicity of AZT to FIV is nearly as high as the well-characterized mutagen, BUdR. However, two other RT inhibitors, ddI and ddC, tested negative for mutagenicity to FIV with this system. The mutagenicity of AZT could have major implications for AIDS chemotherapy, although it is not clear whether an increase in the mutation frequency of the virus would be beneficial or detrimental. If there was an increase in the frequency of lethal mutations during replication, it may contribute to the antiviral activity of the drug. However, it is likely that the enhanced mutation rate would contribute to mutations involved in genetic variation, evasion of the immune system, and changes in cell tropism. If pathogenesis is dependent upon accumulation of multiple mutations, AZT therapy could potentially alter the rate of pathogenesis.

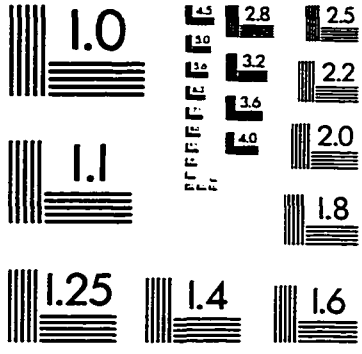
The FIV host range system will be important for studying the variability and fidelity of the replication of the lentivirus. It can be utilized to identify *pol* mutations with antimutator and mutator phenotypes. There are a number of drug resistant mutants with known *pol* mutations available and can be evaluated for these phenotypes. This system can also be used to determine how alterations of fidelity at the enzyme level correlate with the mutation rates during viral replication. In addition, this system can also be used to evaluate the mutagenic

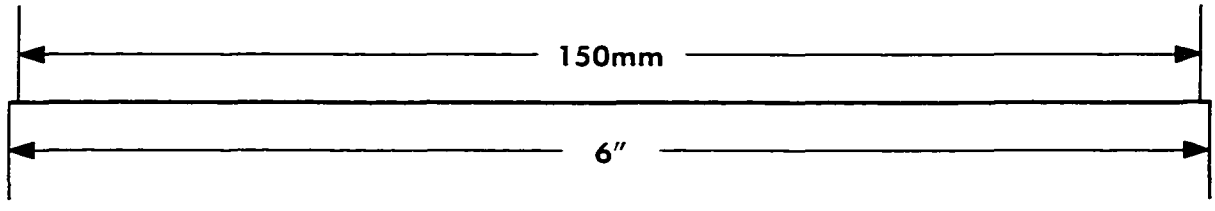
effect of new antiviral drugs being developed (or drug combination) for AIDS chemotherapy.

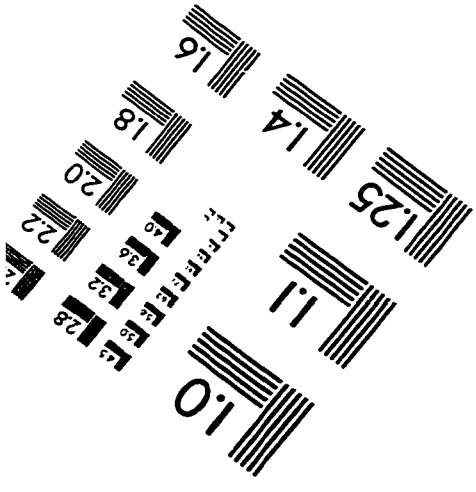
# IMAGE EVALUATION TEST TARGET (QA-3)

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