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Investigation of HIV-1 Variants with Broader Tropism than Wild-type Virus

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Investigation of HIV-1 Variants with Broader Tropism than Wild-type Virus

A Major Qualifying Project Report

submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

in

Biology and Biotechnology

Submitted by:

__________________________________________

William Salomon

April 28th, 2005

APPROVED:

__________________________________________

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Abstract

HIV-1 predominantly uses CD4 and the chemokine receptor, CCR5 to gain entry into host cells. Different strains vary in ability to infect cells with low receptor concentrations. The MQP’s purpose was to analyze molecular clones of an HIV variant that could infect T-cells expressing low amounts of CCR5. Such variants may confer a broader tropism for immune cells. Envelope clones were tested for their ability to fuse with cells expressing different concentrations of CD4 and CCR5. Variant envelope clones that were more fusogenic for cells with low CCR5 or CD4 were identified. Amino acid substitutions in V2 and a region that binds CD4 were identified as potential determinants of the variant phenotype.
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Acknowledgements

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BACKGROUND

HIV Introduction

Virus Overview

The Human Immunodeficiency Virus (HIV) is in the genus of lentiviruses that is a part of the family Retroviridae. Characteristics presented by lentiviruses include long and variable incubation periods, persistent viral replication, neurological complications and destruction to particular types of hematologic or immunologic cells (Desrosiers and Letvin, 1987). There is extensive genetic and antigenic variability exhibited and a strong tropism for macrophages (Coffin, et al, 1997). Furthermore lentiviruses have a common morphology and morphogenesis along with specific regulatory genes not found in other groups of viruses in the Retroviridae family (Coffin, et al, 1997).

There are two different HIV virus groups, HIV-1 and HIV-2, for the only known lentiviruses affecting humans (Knipe and Howley, 2001). HIV-1 is more predominant than HIV-2 to which most cases are only found in Western Africa (Coffin, et al, 1997). HIV-1 is closely related to SIV_{cpz} in chimpanzees, while HIV-2 is closely related to SIV_{smm} in sooty mangabey monkeys. It is thought that both HIV-1 and HIV-2 emerged via zoonotic transmission to man (Knipe and Howley, 2001). HIV-1 has different types separated into three distinct groups. The first group is group M or main group, the second group is the O or outlier, and the third group is the recently discovered N group found to infect Cameroonianians (Knipe and Howley, 2001). The groups are further separated into genetic subtypes. The M type virus has can be separated into at least 8
different subtypes labeled A-J (Knipe and Howley, 2001). Figure 1 below shows the global prevalence of HIV-1.

![Figure 1: HIV-1 Subtypes and Their Worldwide Distribution as of 1998. The larger circles indicate the predominant subtype in the areas they are located (Knipe and Howley, 2001).](image)

Host and viral factors contribute to the pathogenicity of HIV-1 and HIV-2 diseases (Cohen et al, 1997). The factors include the tropism of the virus, co-receptor use, viral escape from the immune response, and the virus’s overall ‘fitness’ or effectiveness. The virus goes through 3 major stages of disease progression dictated by the CD4⁺ T-cell counts in an infected individual (Fauci et al, 1996) (Figure 2). The three stages are acute, asymptomatic, and symptomatic infection.
Viral Composition

The HIV-1 genome includes all of the typical retroviral structural genes including \textit{gag}, \textit{pol}, and \textit{env} (Wiley, 2001). The HIV genome consists of RNA and is more complex than other retroviruses with at least 6 more genes (Wiley, 2001) in its genome, including \textit{tat}, \textit{rev}, \textit{nef}, \textit{vif}, \textit{vpr}, \textit{vpu} (Wiley, 2001). Table 1 shows the names of the genes and the function of the proteins they encode.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Proteins Encoded</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-specific Antigen (\textit{gag})</td>
<td>Structural Protein; Precursor for the Matrix (MA), Capsid (CA), Nucleocapsid (NC), and p6 proteins.</td>
</tr>
<tr>
<td>Polymerase (\textit{pol})</td>
<td>Structural Protein; Protease (PR), Reverse Transcriptase (RT), and Integrase (IN).</td>
</tr>
<tr>
<td>Envelope (\textit{env})</td>
<td>Structural Protein; Envelope glycoproteins</td>
</tr>
<tr>
<td>Trans-activator of viral transcription (\textit{tat})</td>
<td>Regulatory Protein; Enhances transcription.</td>
</tr>
<tr>
<td>Regulator of Viral Protein Expression (\textit{rev})</td>
<td>Regulatory Protein; Regulates RNA splicing.</td>
</tr>
<tr>
<td>Negative Factor (\textit{nef})</td>
<td>Accessory Protein; Enhances viral replication. Down modulation and degradation of CD4 and MHC class I.</td>
</tr>
</tbody>
</table>
Virus Infectivity Factor (*vif*) | Accessory Protein; Crucial for infectious virions specific to cell type.
---|---
Viral Protein R (*vpr*) | Accessory Protein; Stimulates viral gene expression. Important for infection of non-dividing cells.
Viral protein U (*vpu*) | Accessory Protein; Enhances virus release and degrades CD4.

**Table 1: HIV Genes and a Brief Description of the Encoded Protein’s Function**
*(Wiley, 2001; Knipe and Howley, 2001).*

Additionally to the genes found within the 9.7 kb *(Knipe and Howley, 2001)* HIV genome there are two long terminal repeats (LTR). The LTR regions are divided into 3 domains U3, U5, and a central region, R *(Wiley, 2001).* The U3 domain is about 450 bp long and the U5 domain is around 80 bp which are untranslated regions *(Wiley, 2001).* The R region or central repeated region is approximately 100 bp long and it contains sequences vital to the control of transcription and encodes a binding site for the Tat protein *(Wiley, 2001).*

**Structural Proteins**

The structural proteins found in the HIV genome are common to all retroviruses. The structural proteins are synthesized from polyprotein precursors *(Knipe and Howley, 2001).* The Gag precursor (Pr55\(^{Gag}\)) is 55 kDa in size and is cleaved into 4 different proteins *(Knipe and Howley, 2001)* the matrix (MA, p17), capsid (CA, p24), nucleocapsid (NC, p7), and p6 proteins. The matrix protein is myristoylated and aggregates under the cell membrane. Its role is involved with the incorporation of the Env polyprotein via interactions with the long cytoplasmic tail of gp41 *(Wiley, 2001).* The capsid protein forms the central core of the HIV virus particle *(Wiley, 2001).* The CA protein also contains a region to which there is a dimerization domain; this allows for
the CA-CA interactions to form the core of the virus (Wiley, 2001). The NC protein’s major role is to encapsulate the viral genomic RNA and deliver it to the assembling viron (Wiley, 2001). At the C-terminus of the Gag polyprotein there is a proline rich, 6 kDa protein p6. This protein has a role in the late stages of viral release and it has been shown to recruit cellular proteins required for virus budding from the host cell (Freed, 2002).

The second structural polyprotein is Pol. It is translated as a part of a Gag-Pol product from a ribosomal frameshift during Gag translation which creates the p160 Gag-precursor (Wiley, 2001). The precursor is cleaved into three separate proteins: viral protease (PR, p10), reverse transcriptase (RT, p66), and Integrase (IN, p32) (Knipe and Howley, 2001). The PR protein is essential for viron maturation events following the budding from the host cell (Wiley, 2001). The protein is involved in cleaving the polyproteins from both the Gag and Gag-Pol polyproteins (Wiley, 2001). Reverse transcriptase (RT) is an enzyme that is a heterodimer as p51 and p66, its activity is involved with the synthesis of double stranded DNA from a single stranded RNA template (Wiley, 2001). The IN protein catalyzes a series of reactions that take place in the culmination of the integration of the viral DNA into the host genome (Wiley, 2001).

The third structural protein is Env. The Env protein is a glycoprotein that is a precursor (gp160) to two proteins, gp120 and gp41. The gp120 or surface subunit of Env is highly glycosylated and offers protection against host antibodies (Losman et al., 2001). The protein is also the first contact with target host cell receptors (Wiley, 2001). The transmembrane subunit of Env or gp41 has a hydrophobic domain that confers fusion of viral and host membranes (Wiley, 2001).
Regulatory Proteins

HIV has two regulatory proteins in its genome. The first one is transcribed from the *tat* or Transactivator of viral transcription. This gene encodes a protein of about 14 kDa in size (Knipe and Howley, 2001). The *tat* gene contains 2 exons and *in vivo* either a one exon or two exon protein is found (Rhim and Rice, 1994). The functional domain of the protein is located in the first exon and alone can enhance transcription activity by binding the transactivating response (TAR) element on newly initiated HIV mRNA molecules (Wiley, 2001).

The second regulatory protein is encoded by the *rev* gene to form the regulator of viral protein expression. The protein is 116 amino acids long and 19 kDa in size (Wiley, 2001). This protein’s function is to regulate the transport of unspliced viral mRNAs by binding to specific RNA sequences and structures (Wiley, 2001).

Accessory Proteins

The HIV genome also encodes four accessory proteins. The proteins are unique to lentiviruses and are given the name as accessory due to the fact that they are not absolutely necessary in viral replication at least *in vitro* (Wiley, 2001). However they perform important roles in the virus’s functions *in vivo* (Wiley, 2001). The first protein is encoded by the *nef* gene (Aiken and Trono, 1995). The 27 kDa *nef* protein is involved with CD4 and MHC class I down regulation and modulation of cellular activation pathways (Chowers *et al.*, 1994).

The second accessory protein is encoded by the *vif* gene and makes a 23 kDa (Knipe and Howley, 2001) protein called Virus Infectivity Factor. The protein is
involved with the stage of virus assembly and is important for the production of infectious virus particles (Gabuzda et al., 1994) by inducing degradation of an antiviral cellular protein, APOBEC3A (Rose et al., 2004).

The Vpr protein is encoded by the vpr gene to produce Viral Protein R. Vpr acts to increase virus expression and is involved with functions during early post-entry stages of the replication cycle of HIV (Bukrinsky and Adzhubei, 1999). In SIV and HIV-2 the two vpr functions are separated onto two proteins called vpr and vpx (Fletcher et al., 1996).

The gene vpu encodes a 16 kDa protein called viral protein U or p16. This protein is an integral membrane protein that has major roles in the degradation of CD4 within the ER and stimulating the release of virus (Willey et al., 1992). Figure 3 shows the genomic map of HIV and shows the proteins encoded by it.

**Viral Cell Entry**

**Structure of Virion Surface**

HIV virus particles attach to cell receptors via the glycoprotein spikes (Clapham and McKnight, 2002) on the surface of the virus. The envelope spikes are composed of two proteins gp120 and gp41. The proteins are also referred to as surface (SU) envelope protein (gp120) and transmembrane (TM) envelope protein (gp41). The envelope proteins are made from a gp160 precursor molecule as seen in Figure 3. The precursor molecule gp160 is cleaved in the Golgi apparatus by a cellular protease (Clapham and McKnight, 2002) to make the two glycoproteins that form the envelope spike. Each spike is made up of 3 gp120 and 3 gp41 proteins held together as a trimer (Clapham and McKnight, 2002).
The gp120 protein has 5 variable loops in its structure (V1-V5) interspersed with more conserved domains (Clapham and McKnight, 2002). The gp41 protein is relatively conserved in structure (Clapham and McKnight, 2002). It is thought that the variable loops in gp120 may help replicating virus escape antibody-mediated neutralization (Clapham and McKnight, 2002).

The CD4 Receptor

The HIV-1 virus utilizes the CD4 receptor as the major receptor. CD4 is most commonly found on T-helper/inducer subset of lymphocytes, certain lineages of macrophages, and in some types of dendritic cells (Knipe and Howley, 2001). Thus, CD4 expression is a major determinant of HIV cell tropism. CD4 functions primarily as an accessory receptor in the cellular immune response to increase the attraction between helper T cells and MHC class II$^+$ antigen presenting cells (Clapham and McKnight,
CD4 binds gp120 causing rearrangement of the gp120 core (Myszka et al., 2000). This conformational change in gp120 causes movement of the variable loops of the protein which exposes a binding site for a co-receptor, usually CCR5 or CXCR4 (Clapham and McKnight, 2002). CCR5 and CXCR4 are a part of the seven transmembrane (7TM) chemokine receptor family (Clapham and McKnight, 2002). There are also other types of co-receptors in the same family as CCR5 and CXCR4 but they are used less extensively than CCR5 or CXCR4 (Clapham and McKnight, 2002).

**HIV Co-Receptors and Fusion**

The binding of HIV to the co-receptor is thought to be the trigger for membrane fusion, see Figure 4 (Doms and Moore, 2000). The HIV-1 Env mediated fusion model that is most widely accepted proposes the extension a coiled-coil in gp41 that protrudes the fusion peptide which embeds in the cell membrane (Doms and Moore, 2000). The protein gp41 then folds back on itself to form a six helix bundle that brings viral and cell membranes in close proximity to each other (Doms and Moore, 2004). The exact mechanisms that confer fusion of viral and cell membranes following these events are less clear. The observed transmembrane structures in HIV-1 for fusion are consistent with other unrelated enveloped viruses (Clapham and McKnight, 2002). Figure 4 below shows the viral entry step of HIV and the interactions of gp120 and gp41.

![Figure 4: Model of HIV-1 Env Membrane Fusion (Doms and Moore, 2000).](image-url)
The interactions between gp120 and CD4 co-receptors determine HIV’s tropism (i.e. which host cells it enters). The sites on gp120 that dictate tropism and co-receptor binding include the Variable loops V1/V2, V3, and also a conserved region of $\beta$-strands (Clapham and McKnight, 2002). Variations in the variable loops are thought to enable HIV to interact differently with co-receptors (Clapham and McKnight, 2002). This variability may help the virus escape immune response and/or expand its tropism toward other co-receptor sites (Clapham and McKnight, 2002). Figure 5 shows a ribbon diagram of gp120 illustrating the bridging sheet located between the V1/V2 and V3 loop. Variation in structure in the V3 loop switches co-receptor use from CCR5 to CXCR4 (Clapham and McKnight, 2002).

Chemokine receptors are rod like structures in the membrane that have a center pore surrounded by 7 transmembrane regions (Clapham and McKnight, 2002).

**Figure 5: Structure of gp120 After CD4 Is Bound.** The bridging sheet and sites on the V3 loop contact the co-receptor. Arrows show discontinuous regions that bind CD4 (Kwong et al, 1998).

Chemokine receptors have four domains exposed from the surface of the cell; the domains are the N-terminus and 3 extracellular loops E1, E2, and E3 (Clapham and McKnight, 2002). The N-terminus of CCR5 is important for HIV entry (Hill et al, 1998).
Mutagenesis to this domain of the CCR5 receptor has shown to eliminate entry of HIV-1 R5 type viruses (Hill et al, 1998).

Individuals who are homozygous for a genetic defect that eliminates functional CCR5 are strongly protected from HIV-1 infection (Moore et al, 2004). Individuals who are heterozygous for this genetic defect have slow disease progression (Moore et al, 2004). Individuals who lack the CCR5 receptor can be infected with HIV-1 strains that utilize the CXCR4 but this occurs only rarely (Moore et al, 2004). The importance of co-receptors and co-receptor usage for HIV-1 replication and cell tropism is very significant. The HIV-1 virus can use several different cellular receptors from the chemokine family as co-receptors for infection in vitro. However, there is little evidence to support use of co-receptors other than the major receptors CCR5 and CXCR4. Figure 6 shows a model of HIV-1 variants and the different types of co-receptors the virus utilizes.

Figure 6: Currently Accepted Model for HIV-1 Co-Receptor Usage. R5 tropic strains are specific for the CCR5 receptor on memory T-cells and can also infect primary macrophages. X4 tropic viruses or viruses utilizing the CXCR4 receptor can infect CD4+ Naïve T-cell lines as well as certain macrophages. R5X4 strains, also known as dual tropic can infect using either the CCR5 or CXCR4 receptors (Figure updated from Berger et al, 1999).
Theory of Expanded Tropism of HIV-1

HIV, like other retroviruses, exhibits genetic diversity and variation through nucleotide substitutions, deletions, insertions, and homologous and non-homologous recombination (Coffin, et al, 1997). HIV significantly exhibits genetic diversity where the virus changes over time within an infected individual (Kitrinos et al, 2003). The factors that contribute to HIV’s variability include the high rate of replication, an error prone reverse transcriptase (HIV’s DNA polymerase), and efficient recombination (Kitrinos et al, 2003). The ability of HIV to change allows it to escape immune response and also to expand its tropism to different cell types. Genetic variability is not equal along the HIV genome (Kitrinos et al, 2003). The env gene exhibits the most genetic variation (Kitrinos et al, 2003). The env gene encodes two proteins gp120 and gp41. Gp120 is most variable with 5 variable loops. The V3 loop predominantly defines the virus’s tropism as R5 or X4 (Kitrinos et al, 2003), although other env regions (e.g. V1V2) also contribute (Hoffman et al, 2002). R5 viruses are usually present at all stages of infection. However X4 and/or X4R5 usually emerge in late stages of disease progression marked by the decline of the immune responses (Labrosse et al, 2001). Most HIV+ individuals are infected initially with an R5 virus. The R5 virus can lead an individual to progress to AIDS in the absence of X4 or R5X4 viruses. It is unclear whether the R5 tropism broadens during the course of infection without utilizing another type of receptor. It has been observed that in 50% of people infected with HIV the viral tropism switches to X4 and/or R5X4 late in disease. This switch has been rarer in clade C infections in
Africa. The broader tropism of this late stage switch allows HIV to colonize new T-cell populations.

HIV Infection in Brain

HIV predominantly infects microglia and perivascular macrophages in the brain. In the perivascular region of the brain, cells come in contact with infected cells in the blood (González-Scarano et al., 2005). The brain macrophages and the microglia cells are the brain’s resident immune cells that respond to all types of stimuli (González-Scarano et al., 2005). The cells have similar receptors to other phagocytic cells in the blood due to their derivation from the bone marrow in development (González-Scarano et al., 2005).

Patient JR-CSF Strain of HIV

The JR-CSF strain of HIV was reported in 1987 in *Science* by Yoshio Koyanagi et al. They studied virus from brain tissue and cerebrospinal fluid from several HIV+ individuals. All of the patients they studied had AIDS encephalopathy (Koyanagi et al., 1987). The patient J.R. died with Kaposi’s sarcoma and severe AIDS encephalopathy (Koyanagi et al., 1987). The brain of the patient exhibited many giant cell syncytia as seen from frontal lobe autopsy (Koyanagi et al., 1987). Analysis of the patient samples indicated there were two genotypically different but related strains present (Koyanagi et al., 1987). Thus JRFL from the brain was shown to be macrophage tropic (M-tropic), while JR-CSF was not, even though both strains use CCR5 (Koyanagi et al., 1987).

JR-CSF and Expanded Tropism

The JR-CSF strain was studied by Boyd et al who isolated a variant virus that could replicate in CD4+ T-cell lines (Boyd et al., 1993). Studies have shown that changes
in the V3 loop of the gp120 env protein can determine tropism (Hoffman et al., 2002), and a single amino acid substitution in V3 is sometimes sufficient to change the tropism of HIV to either X4 or R5 (Hoffman et al., 2002). In the case of JR-CSF Boyd et al reported that the capacity of JR-CSF to replicate in \( \text{CD4}^+ \) T-cell lines was due to a single base substitution in the V1 loop (Boyd et al., 1993). Subsequently, Dejucq et al showed that the JR-CSF variant still used CCR5 and had not switched to using CXCR4 or other potential co-receptors (Dejucq et al., 1999). Thus the T-cell line adopted variant of JR-CSF, may provide information on how R5 viruses expand tropism \textit{in vivo} without co-receptor switch.
PROJECT PURPOSE

It has been reported that single base substitutions in the V3 and V1/V2 loops of gp120 can affect the tropism of HIV-1. The strain JR-CSF of HIV-1 can infect primary cells but not CD4+ T-cell lines in vitro. Boyd et al in 1993 published that the JR-CSF C3 variant could infect the MOLT4 T-cell line that had low levels of CCR5. The infection of the MOLT4 cells was said to be the result of a single amino acid substitution in the V1 loop of gp120. However the methodology required amplification of mutagenized virus in culture, which could have introduced of further mutations. The MQP project first aimed to confirm these results utilizing a pseudotype virus that utilized a JR-CSF envelope with the specific V1 amino acid substitution. The project also explored JR-CSF clones created from virus that was passaged 4 times through PBMCs. The importance of this research is to explore the observation of HIV-1’s ability to switch tropism in late stages of infection. The function of the variable loops is not completely understood but observations show they determine the co-receptor usage. These variable loops could be a target of therapy to prevent entry of the virus into host cells.
MATERIALS AND METHODS

Construction of Variant Clones

Construction of JR-CSF Clone with Single Point Mutation

The creation of the JR-CSF clone with a single mutation utilized several cloning steps. The envelope expression vector (pSVIII) carrying the JR-CSF envelope was used to create the mutation. Mutagenesis of JR-CSF pSVIII env with a Guanine to Alanine base substitution involved a PCR procedure. Round 1 with tubes 1 and 2 used pairs of primers. Tube 1 used C3 alpha (5’- AAA ATT AAC CCC ACT CTG TGT TAC -3’) and C3 kappa (5’- CAT CAT TCC CTC ACT ATT ACT AGT -3’) and tube 2 used primers C3 beta (5’- ACT AGT AAT AGT GAG GGA ATG ATG -3’) and C3 lambda (5’- ATT ACA ATT TCT GGA TCC CCT CCT -3’). Each tube contained a total volume of 50 µl that contained: 2 µl of 100 µM dNTPs, 4 µl of PCR Buffer (Clontech), 0.5 µl of HiFi Taq Polymerase (Clontech), and 41 µl of PCR H2O, 1 µl of Sense Primer (C3 alpha or C3 beta), 1 µl Anti-sense Primer (C3 kappa or C3 lambda) and 0.5 µl of JR-CSF pSVIII env [SalI]. The PCR reaction ran for 25 cycles of 94°C for 1 minute, 48°C for 1 minute, and 72°C for 5 minutes. The tubes were stored at 4°C.

Round 2 of mutagenesis had a total volume of 50 µl and consisted of: 1 µl of C3 alpha, 1 µl of C3 lambda, 2 µl of 100 µM dNTPs, 4 µl of PCR Buffer (Clontech), 1 µl of Tube 1 from round 1, 1 µl of Tube 2 from round 1, 0.5 µl of HiFi Taq enzyme (Clontech), 39.5 µl of PCR H2O. The PCR reaction ran for 25 cycles at 94°C for 1 minute, 48°C for 1 min, and 72°C for 2 minutes. The PCR reaction was stored at 4°C.
The PCR reaction was then purified and cloned using an Invitrogen® PCR XL-TOPO kit. The colonies (23) were PCR screened using Qiagen Taq polymerase and the C3 alpha and C3 lambda primers to detect inserts. The Kan'\textsuperscript{r} positively screened colonies were then grown in 3 mL of liquid LB + Km. Plasmid DNA from the colonies was mini-prepped using the Qiagen Mini-prep kit.

Plasmid DNA containing the mutated PCR product was digested using BsmI (NEB) and BamHI (NEB) in a dual digest and cloned into a pGEM construct that contained a SalI fragment from the pSVIII env JR-CSF. Figure 7 shows a diagram illustrating the construction of the clone. Restriction digests were carried out at 37°C using NEB Buffer. The plasmid containing the vector was also treated with antartic phosphatase to prevent self-ligation. PCR screening, test digestion and sequencing verified that the fragment was successfully cloned.

The pGEM construct now contained the single mutation in a pSVIII envelope SalI fragment. The construct was digested using SalI (NEB) and ligated back into pSVIII JR-CSF (Figure 7). The final pSVIII JR-CSF with 1 point mutation (G to A) was verified by sequencing, test digests, and PCR. All clones had glycerol stocks prepared and were stored at -80°C.
Figure 7: Cloning Strategy Used To Construct Single Amino Acid Substituted pSVIII JR-CSF envelope.
Construction of JR-CSF RT Fragment Clones

The JR-CSF RT clones were made from viral RNA reverse transcribed from JR-CSF passaged 4 times through PBMCs. The Invitrogen kit SuperScript First-Strand Synthesis System for RT-PCR was used. The primer used was envN (5’- CTG CCA ATC AGG GAA GTA GCC TTG TGT -3’). After first-strand synthesis, amplification of the cDNA (PCR forward) was performed using the Invitrogen kit. The only exception to the protocol was that Roche Expand HiFidelity Taq was used. Primers envA (5’- GGC TTA GGC ATC TCC TAT GGC AGG AAG AA -3’) and envN were used. In round two of RT-PCR envB (5’- AGA AAG AGC AGA AGA CAG TGG CAA TGA -3’) and envM (5’- TAG CCC TTC CAG TCC CCC CTT TTC TTT TA -3’) primers were used. An EtBr 0.8% agarose gel was run to verify positive bands. Six samples were selected to be purified using a crystal violet gel and the Invitrogen® PCR XL-TOPO kit for cloning. After the fragment was inserted in the TOPO vector a PCR screen was done to verify the transformation and identify clones that carried a PCRed fragment as an insert. The positive colonies were grown in 3 mL of LB + Km and then mini-prepped using the Qiagen Mini-prep Kit. The fragments were then cut out of the TOPO vector utilizing a KpnI (NEB) restriction site. The pSVIII JR-CSF was also digested with KpnI (NEB) and Antarctic Phosphatase to prepare for ligating to the RT fragment. The pSVIII JR-CSF [KpnI] was ligated with the RT fragment from the TOPO vector after being cut with KpnI (see Figure 8). The ligation and transformation was verified by PCR screen and test digestion to check orientation of fragments. All clones (7) had glycerol stocks prepared and stored at -80°C. The Qiagen Mini-prep kit was used to prepare the DNA from the 7 clones.
Testing Variant Clones using Cell based Assays

Transfection of 293T cells

Transfections of the clone DNA and an HIV backbone were done using the Profection® Mammalian Transfection System from Promega. The HIV backbone is derived from the NL4.3 standard of HIV-1 and encodes all HIV-1 genes except envelope. One day before transfection 293T cells were plated in a 6 well plate at $1 \times 10^5$ cells/mL, 2 mL in each well. Dulbecco’s modified Eagle Medium (DMEM) + 4% Fetal Bovine Serum (FBS) + Gentamicin media was used. The cells were incubated at 37°C in 5% CO₂ cabinets overnight. On the day of transfection the cells had their media changed at least 3 hours prior to transfection. The transfection was calculated by determining the concentration of the Env clone’s DNA and the L4-1-1 Backbone’s DNA. The concentration used for each of the DNAs was 1.25 µg/µl. The preparation of each clone to be transfected was done by using two 14 mL snap cap tubes (poly-propylene). Tube A
had the clone Env DNA, backbone DNA, 2M CaCl₂ (10.34 µl), and Nuclease-free H₂O to bring the final volume to 83.40 µl. Tube B had 83.40 µl of 2X HEPES buffered saline. Tube A was added to Tube B dropwise while vortexing. The mixed tubes were incubated at room temperature for 30 minutes. After 30 minutes the transfection (166.8 µl) was added to a well of 293T cells. The transfection was added dropwise while slowly swirling to distribute DNA evenly.

Figure 9: HIV-1 Backbone Derived from NL4.3. The backbone has a premature stop codon in the env gene.

**HeLa Cell Fusion Assay**

The HeLa cell fusion assay was done using 293T cells that had been transfected with pSVIII env and backbone DNA and were therefore expressing envelope on the cell surface. Control envelopes typically used were pSVIII: P20 B59, P20 LN8, P6 LN40, P353 B27 and SF162. The wild-type JR-CSF was also used as a control. On the same day that the 293T cells were transfected, HeLa cells (8 lines) were plated in 48-well trays. The cells were plated at 8 x 10⁴ cells/mL with 500 µl per well. The medium used was DMEM + 4% FBS + Gentamicin. The cells were incubated overnight so they could attach to the well. The cell types used were a RC line that has low CD4 presentation, and a JC line that has a high CD4 presentation. The lines each had varying amounts of CCR5 and are shown in Table 2.
<table>
<thead>
<tr>
<th>Level of CCR5</th>
<th>High CD4</th>
<th>Low CD4</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>JC53</td>
<td>RC49</td>
</tr>
<tr>
<td>Medium</td>
<td>JC37</td>
<td>RC23</td>
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<tr>
<td>Low</td>
<td>JC10</td>
<td>RC33</td>
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<tr>
<td>None</td>
<td>HIJ</td>
<td>HIR</td>
</tr>
</tbody>
</table>

Table 2: Chart of Different HeLa Cell Types and Their Receptor Concentration the CD4 and CCR5 receptors.

The 293T cells were added to each of the wells usually in different concentrations. The cells were plated at \(2 \times 10^5\) cells/mL in 50 \(\mu\)l and 100 \(\mu\)l amounts. After addition of the HeLa cells the plates were incubated for 4 hours. After 4 hours the plates were fixed and stained for syncytia. The plates had all the media aspirated off and then were washed twice with 1X Phosphate Buffer Solution (PBS). Addition of the syncytia stain (1% methylene blue, 0.28% basic fuchsin in methanol) was added and allowed to sit for 10 minutes. After 10 minutes had passed the stain was removed and the wells were rinsed using \(dH_2O\). The wells then had some 1X PBS/0.05% Azide added as a preservative to prevent bacterial growth. The wells were scored and photographed using an inverted light microscope.

**MOLT 4 Clone 8 Fusion Assay**

MOLT 4 Clone 8 (MOLT4/8) cells are a suspension cell line that was grown in RPMI medium + 10% FBS + Gentamicin. The cell line was derived from a young adult with acute lymphoblastic leukemia. The cells express CD4 and CXCR4 in equal concentrations and a very low amount of CCR5. 293T cells were transfected the day before infection. The cells were then were added to 48 well plate wells at \(5 \times 10^4\)
cells/mL at 400 µl per well. The MOLT4/8 cells were added to each well at 100 µl in 3 different concentrations 5 x 10⁵/mL, 2.5 x 10⁵/mL, and 1.25 x 10⁵/mL. The cells were checked after 24 and 48 hours. Cells were scored for syncytia formation and photographs were taken.

Production of Pseudotype Virus Stocks

Transfection of 293T was performed as described on page 24. After 24 hours of incubation media was changed. After another 24 hours (48 hours after transfection) the media was pipetted off each well and added to 15 mL centrifuge tubes. The tubes were centrifuged for 10 minutes at 1,200 rpm to remove cell debris. The supernatant from each vial was added to cryovials in 0.5 mL amounts. The vials were then "snap" frozen in liquid nitrogen. After at least one hour had passed the vials were stored in a -152 °C freezer.
RESULTS

The main goal of the project was to further examine the determinants of the HIV-1 envelope that broaden tropism of R5 viruses without a co-receptor. The project first investigated a single amino acid change in the V1 loop reported to broaden tropism. The project further investigates changes observed in the variant JR-CSF after a short passage. The project aims are to correlate the phenotypic differences of the variant clones when compared to wild-type. Genotypic differences are identified as possible causes of the phenotypic differences between variant clones.

Construction of Variant Clones

There were two different types env of clones constructed. The first clone was the JR-CSF with a single amino acid substitution. The second type of clones constructed was reverse-transcribed from JR-CSF virus passage 4 times in PBMCs.

JR-CSF Variant with Point Mutation

JR-CSF S141N was reported by Boyd et al in 1993. The same mutation was put into the pSVIII envelope encoding the JR-CSF envelope using PCR mutagenesis. The protocol that Boyd et al used to produce JR-CSF S141N involved amplification of the mutant virus in PBMCs. Such conditions may allow the introduction of additional mutations. The method I used to verify Boyd’s mutation using a system that involves only 1 round of virus replication does not provide opportunity for further culture introduced mutations in envelope. Sequence analyses confirmed the Serine to Asparagine substitution (S141N). This change does not introduce or eliminate an N-
linked glycosylation site. Figure 10 shows pictures of gels from the test digestions that were done to check orientation of the ligated fragment.

![Figure 10: Test Restriction Digest of JR-CSF point mutated clones. XhoI (NEB) and MfeI (NEB) were used to cut the clones. Gel A has the following in its lanes: 1) 10 kb DNA Marker, 2-5) clones 2-6, 6) JR-CSF wild-type. Gel B has the following: 1) 10 kb DNA Marker, 2-10) clones 7-15.](image)

Amino Acid sequence of the V1V2 loop of \textit{env} gene where the one point amino acid substitution was introduced is seen in Figure 11. The mutation takes place at position 141 and is a Serine to Asparagine switch.

![Figure 11: Amino Acid Sequence of JR-CSF and the JR-CSF S141N clone. The location of the substitution is at amino acid 141 where a serine (blue) residue is substituted with an asparagine residue (red).](image)

**JR-CSF Variants from Passage through PBMCs**

JR-CSF viral RNA was obtained from JR-CSF virus that had been passaged 4 times through PBMCs. The RNA was reverse transcribed and the \textit{env} fragment was obtained. The \textit{env} fragment was purified and digested using the KpnI restriction site.
The KpnI fragment of the JR-CSF P4 was ligated into pSVIII JR-CSF ΔKpnI expression vector. After transformation the clones were screened by PCR. Positives clones had an XhoI restriction digest performed to check the orientation of the fragment in pSVIII JR-CSF ΔKpnI. Figure 12 shows a gel run of the restriction digest with XhoI. The envelope pSVIII JR-CSF was used as a positive control.

![Figure 12: Test Digest to Check Orientation of Inserts Using XhoI Restriction Site with JR-CSF P4 pSVIII Clones. Lane are as followed: 1) 10 kb DNA marker, 2) positive control (pSVIII JR-CSF), 3-10) positively PCR screened clones. Lane 9 is a clone that has the fragment in the wrong way.]

**Cell Based Assays**

**HeLa Cell Fusion Assays — JR-CSF Single Amino Acid Substitution Clone**

The pSVIII env carrying JR-CSF envelope clones with the correct single amino acid substitution at residue 141 (JR-CSF S141N) were tested for functionality on several cell lines. Seven clones were selected that were positive for the single point substitution and were transfected into 293T cells. HeLa cells were co-cultivated with the 293T cells. After 24 hours the cells were fixed and stained and observed for fusion (data not shown). Two of the clones (3 and 8) were selected for further fusion assays.
The clones were transfected into 293T cells along with a panel of control envelopes (Table 3). The 293T cells were incubated overnight and then co-cultivated on 8x10^4 HeLa Cells. The HeLa cell lines used were RC (Low CD4) and JC (High CD4) with varying concentrations of CCR5 (see Table 2, page 26).

<table>
<thead>
<tr>
<th>Control Envelope</th>
<th>Macrophage Infection</th>
<th>Infection via Low CD4/Low CCR5</th>
</tr>
</thead>
<tbody>
<tr>
<td>P20 B59</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P20 LN8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P353 B27</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P6 LN40</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SF162</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>JR-CSF wild-type</td>
<td>-</td>
<td>+/-</td>
</tr>
</tbody>
</table>

Table 3: Controls Used In HeLa Cell Fusion Assay. HeLa cell assays were performed using pSVIII envelope controls. Positive (+) envelopes will cause the cells to fuse and can infect.

The extent of syncytium formation was scored by eye (Table 4). There was no significant difference between JR-CSF and the clones. The scores were given on a scale that was based on the P20 B59 envelope that is able to exploit low CD4 and CCR5 for infection and on P20 LN8, an envelope cloned from the same patient as B59, but it requires high amounts of CD4 for fusion.
Table 4: Scores of the HeLa Cell Fusion of S141N Clones. The P20 B59 virus is the positive control. Cell lines HIR and HIJ are negative controls. The fusion assay was fixed and stained 4 hours post co-cultivation.

<table>
<thead>
<tr>
<th>Virus</th>
<th>RC33</th>
<th>RC23</th>
<th>RC49</th>
<th>HIR</th>
<th>JC10</th>
<th>JC37</th>
<th>JC53</th>
<th>HIJ</th>
</tr>
</thead>
<tbody>
<tr>
<td>P20 B59</td>
<td>+</td>
<td>++++</td>
<td>++++</td>
<td>-</td>
<td>-</td>
<td>++++</td>
<td>++++</td>
<td>-</td>
</tr>
<tr>
<td>P20 LN8</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>++++</td>
<td>++++</td>
<td>-</td>
</tr>
<tr>
<td>P6 LN40</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++++</td>
<td>++++</td>
<td>-</td>
</tr>
<tr>
<td>P353 B27</td>
<td>-</td>
<td>++++</td>
<td>++++</td>
<td>-</td>
<td>+</td>
<td>++++</td>
<td>++++</td>
<td>-</td>
</tr>
<tr>
<td>JR-CSF S141N 3</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>+++</td>
<td>++++</td>
<td>-</td>
</tr>
<tr>
<td>JR-CSF S141N 8</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>+++</td>
<td>++++</td>
<td>-</td>
</tr>
</tbody>
</table>

Pictures of the HeLa cells were taken at 200X magnification. Syncytia formation is due to cell: cell fusion events that occur between env+ and receptor- cells. Figure 13 shows no significant difference when comparing JR-CSF wild-type versus the S141N clone. In this assay, the P6 LN40 envelope that only fuses on high CD4 cell lines and was used as a control.
Figure 13: HeLa Cell Fusion Assay Panel. P20 B59 (positive control), P6 LN40, JR-CSF wild-type, and JR-CSF S141N clone 8. The cell lines photographed are: RC23 (Low CD4, Med CCR5), RC49 (Low CD4, High CCR5), JC53 (High CD4, High CCR5).

HeLa Cell Fusion Assays — JR-CSF Passage 4 RT Clones

Seven JR-CSF clones that were reverse transcribed from viral RNA after four passages were tested in similar fashion to that of the JR-CSF S141N clones. The clones
were tested for their ability to fuse on various HeLa cell types. The clones were scored just as the JR-CSF S141N clones. The fusion assay was performed with two different concentration of 293T cells applied to each well, 50 and 100 µl.

Table 5 shows the scores from the fusion assay. The broad tropic control envelopes; and P353 B27 fused RC23 as expected. Some of the clones were non-functional (7A1-4 and 7B1-2). At least two clones (5A4-2 and 7B4-1) conferred higher amounts of fusion on RC49 when compared to the wild-type JR-CSF.

<table>
<thead>
<tr>
<th>Transfection #</th>
<th>Virus</th>
<th>Conc. x10^5</th>
<th>Low CD4</th>
<th>High CD4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cells/mL</td>
<td>RC33</td>
<td>RC23</td>
</tr>
<tr>
<td>1</td>
<td>P20 B9</td>
<td>1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>2</td>
<td>P20 LN8</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>P6 LN40 / B33</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>P3E3 B27</td>
<td>1</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>5</td>
<td>JRCSF</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>JRCSF 5A1-2</td>
<td>1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>JRCSF 5A4-2</td>
<td>1</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>8</td>
<td>JRCSF 5B3-3</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>JRCSF 6A8-3</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>JRCSF 7A1-4</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>JRCSF 7B1-2</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
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<td>-</td>
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<tr>
<td>12</td>
<td>JRCSF 7B4-1</td>
<td>1</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>-</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 5: HeLa Fusion Score for the JR-CSF Passage 4 RT Clones. The scoring is based on the amounts of syncytia formation observed. The concentration is the amount of 293T cells co-cultivated with the HeLa cells. The wells were scored four hours post co-cultivation.

The differences observed between the clones and JR-CSF are shown in Figure 14. Many more syncytia were observed in RC49 co-cultivation for envelopes 5B4-2 and 7B4-1.
Figure 14: HeLa Cell Fusion Assay of JR-CSF P4 RT Clones. Pictures were taken 4 hours post co-cultivation. The magnification was at 100X.

MOLT4 Clone 8 Cell Fusion Assay

The MOLT4 Clone 8 (MOLT4/8) cell line is derived from a young adult with acute lymphoblastic leukemia. These cells express the CD4 and CXCR4 in equal concentrations on the cell surface, but express CCR5 in only trace amounts on the cell surface. Molt4/8 cells cultures also contain a low level of small background syncytia, which has to be taken into account in syncytial assays. The assay was performed by co-cultivating transfected 293T cells along with MOLT4/8 cells. The MOLT4/8 cells are plated in three concentrations of cells: 5 x 10^5/mL, 2.5 x 10^5/mL, and 1.25 x 10^5/mL.
Table 6 shows the scores seen 1 day after the cells were co-cultivated. The JR-CSF wild-
type and JR-CSF S141N had almost identical amounts of fusion observed which appeared to be background. The two JR-CSF RT P4 clones have a significant amount of fusion observed when comparing to the JR-CSF wild-type. This can be seen in Figure 15 where there are larger and more syncytia are present. Syncytia are seen by large circular formations illustrated by arrows.

<table>
<thead>
<tr>
<th>Virus</th>
<th>293T Cell Conc.</th>
<th>P20 B59</th>
<th>P353 B27</th>
<th>JR-CSF</th>
<th>JR-CSF</th>
<th>JR-CSF P4</th>
<th>JR-CSF P4</th>
<th>Neg Ctrl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$5 \times 10^5$</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>$2.5 \times 10^5$</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>$1.25 \times 10^5$</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 6: Scores of MOLT4 Clone 8 Fusion Assay. Cells were scored 1 day after co-cultivation of 293T cells and the MOLT4/8 cells. The assay was performed in 3 different concentrations. P20 B59 is the positive control and JR-CSF is the wild-type control.

Figure 15: Picture of MOLT4 Clone 8 Fusion Assay. Pictures taken at 100X magnification one day after co-cultivation with 293T cells. Negative control had no 293T cells added; P20 B59 was the positive control. The arrows indicate a syncytia formation.
Sequence Analysis— JR-CSF RT Clones

Sequence analysis of the JR-CSF clones was performed on the V1/V2 loop and V3 loop using specific env primers. The primers used were specific for V1/V2 and V3/a portion up stream that includes the CD4 binding site. Figure 16 shows the alignment of the clones with JR-CSF wild-type. There are two differences observed, one in each clone. Clone 5A4-2 has a 1 pt substitution in the CD4 binding site of gp120 where an aspartic acid residue is substituted for an asparagine residue. Figure 17 show the sites a CD4 that bind on gp120, the yellow arrow indicates the location to where the point mutation is observed. Clone 7B4-1 has a 1 point substitution in the V2 loop of gp120 where an aspartic acid residue is substituted with an asparagine residue.

Figure 16: Sequence Analysis of the V1V2 Loop, CD4 Binding Site, and V3 Loop of gp120. Analysis performed on two of the RT JR-CSF P4 and aligned with JR-CSF wild-type. Conserved amino acids are in blue, red amino acids are substitutions.

Figure 17: Structure Of gp120 Protein. The arrows indicate the points to which CD4 binds. The yellow arrow indicates the location to where clone 5A4-2 has a substituted Asparagine residue.
DISCUSSION

HIV-1 predominately uses CD4 and a chemokine receptor to gain entry into host cells. The class of HIV-1 that is most commonly transmitted uses the chemokine receptor CCR5 (R5). It is observed in 50% of AIDS patients in late stage of disease that the virus switched to use CXCR4 as a co-receptor. CXCR4 using viruses have widely expressed an expanded tropism among T-cell populations. Patients that lack X4-using viruses also develop AIDS and may evolve R5 viruses with a broader cell tropism. Broad tropic R5 viruses were discovered in the brain of AIDS patients. Broader tropism was conferred by the capacity to infect cells via low CD4 and low CCR5 levels. The project aimed to further elucidate the envelope determinants of R5-using HIV-1 that broaden in tropism and may increase cell death of T-cells late in disease.

Most HIV research is performed on virus isolates. The isolation of HIV strains involves culture in the absence of immune response (in vitro). This allows for the potential to select for variants with different properties than would be seen in an immune responsive environment (in vivo). A possibility that is theorized about the virus and its response to immune environments is that the variables loops (e.g. V1V2) sit over the CD4 co-receptor binding site to protect it from neutralizing antibodies. Culture in vitro may allow more exposure of env proteins to receptors and increase its interaction. Thus, variants may evolve that can infect cells with lower receptor levels and allow the tropism to broaden. The environment that brain is apart of is one that is immuneprivileged and coincides with the type of environment the virus is exposed with in vitro since there is no presence of neutralizing antibodies.
The first variant the project looks at is the JR-CSF mutant that Boyd et al reported in 1993 as changing tropism. The mutant of JR-CSF (S141N) had a single amino acid substitution at residue 141 of gp120’s V1 loop from Ser-Asn. The observation of the JR-CSF S141N was that it expressed a greater amount of infection on MOLT4/8 cells. Boyd’s results had a clear and significant difference between the mutant and JR-CSF wild-type. The project results show very little or no difference in cell fusion between the wild-type and the JR-CSF S141N mutant. Dejucq et al reported in 1999 that CCR5 inhibitors sensitively blocked infection by JR-CSF S141N. This confirmed that the mutant still requires the CCR5 receptor for infection. The CCR5 receptor is present in trace amounts on MOLT4/8 cells. As discussed above, Boyd et al results could possibly be explained by the method to how the virus was amplified. The virus was passaged through PBMCs before the infectivity assays which could have introduced other mutations. The project demonstrates through several cell fusion assays involving HeLa and MOLT4 cell lines expressing known concentrations of the CCR5 receptor that there is no significant difference between the JR-CSF wild-type and JR-CSF S141N. Infectivity assays that will be needed to confirm results seen by the cell: cell fusion assays have been initiated.

In contrast to S141N mutant, two variant envelopes cloned from JR-CSF passaged just 4 times through PBMCs showed distinct differences in capacity to induce HeLa and MOLT4/8 fusion. The differences observed in the phenotype when compared to S141N and JR-CSF wild-type could be examples of the virus having a broadening of tropism toward either low levels of CCR5 or utilizing the CXCR4 receptor that is present in MOLT4/8 T-cells. Sequence analysis of the two variant clones identified single point
mutations in both clones. A mutation in the 5A4-2 clone is located in the CD4 binding site, an Aspartic Acid – Asparagine residue substitution. This mutation might show a stronger interaction with CD4 helping the virus utilize the low levels of CCR5. The other mutation observed in the 7B4-1 clone is a change in the V2 loop where an Aspartic Acid residue is substituted for an Asparagine. Further sequencing of the envelope genome is needed to determine if these substitutions are the only observed changes from a short passage of 4 times. Further confirmatory studies of creating mutants with these observed substitutions should be performed to elucidate the broaden tropism.

The project exemplifies HIV-1’s ability to rapidly evolve an altered phenotype during *in vitro* and *in vivo*. Here, I studied how limited genetic changes acquired *in vitro* can affect virus phenotype. Phenotypic variations in R5-using viruses that alter tropism have implication for viral transmission and death of CD4+ cells in disease progression, as well as research on HIV-1 isolated by *in vitro* culture.


