January 2006

Effects of Isolation of HIV-1 Envelope Clones from Patient 2044 on Tropism, Co-receptor Use, and Antibody Sensitivity

Christopher R. Dowdy
Worcester Polytechnic Institute

MQP-BIO-DSA-3672

EFFECTS OF ISOLATION OF HIV-1 ENVELOPE CLONES DERIVED FROM PATIENT 2044 ON TROPISM, CO-RECEPTOR USE, AND ANTIBODY SENSITIVITY

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:

_____

Christopher Dowdy

January 6, 2006

APPROVED:

_____	_____
Paul Chapham, Ph.D. Program in Molecular Medicine Umass Medical School Major Advisor	David Adams, Ph.D. WPI Project Advisor

ABSTRACT

Interaction with the CD4 co-receptor can change tropism in HIV-1. It has been shown that CCR5-using isolates cultured *in vitro* without the presence of neutralizing antibodies can be more efficient at infection, while at the same time becoming more sensitive to neutralization. This project studies CXCR4-using envelopes examining the effect of viral isolation on tropism, receptor use, and antibody sensitivity. The cultured CXCR4-using isolates did not evolve different properties from the uncultured CXCR4-using isolate.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

Firstly, I would like to express my gratitude towards Dr. Paul Clapham for allowing me to work in his lab, reading countless drafts of the project, and giving me this wonderful opportunity. I would also like to thank Dr. Paul Peters and Mr. William Matthew Sullivan for their help teaching me all the procedures of the lab. I would like to thank Dennis Burton of Scripps La Jolla, CA for providing the B6 and B12 antibodies, and the United Kingdom's CFAR for providing the mouse antibody Q4120. Finally, I would like to thank Dr. Dave Adams for setting up this project through UMass Medical School, and for all his help writing up the MQP.

BACKGROUND

Introduction to HIV

Human immunodeficiency virus (HIV) is a lentivirus and part of the retrovirus family. HIV is the only known lentivirus to infect humans (Knipe and Howley, 2001). HIV has many characteristics common to lentiviruses, such as a long and variable incubation period. An individual with HIV can be asymptomatic for a period of months or years before any complications present.

HIV is very prone to mutation. HIV mutates so often, that through the course of their infection, it is not uncommon to find different strains of HIV in the same patient. HIV's reverse transcriptase contains no system to check for errors. On average it will make a mistake every 1 in 100,000 bases, which translates to about one mistake per round of replication. With mistakes so frequent, any sample of virus will have a 'swarm' of variants, which are often called "quasi species." This rapid changing allows HIV to escape immunity and therapy. The ability to escape, coupled with the HIV's speed of replication make it very difficult to treat effectively.

HIV Classification and Affected Areas

There are two main types of HIV that can affect humans, HIV-1 and HIV-2. HIV-1 is more predominant throughout the world, with HIV-2 being found mainly in western Africa. It is believed that both forms of HIV resulted from zoonotic transmission to humans (Knipe and Howley, 2001). Both forms are closely related to simian

immunodeficiency virus (SIV). HIV-1 is closely related to SIV in chimpanzees, while HIV-2 is closely related to SIV in sooty mangabey monkeys (Knipe and Howley, 2001). HIV-1 is separated into 3 groups: M, the main group; O, the outlier; and the recently discovered N group (Knipe and Howley, 2001). The M group is further separated into subtypes A-J (Knipe and Howley, 2001). In North America, subtype B is the most predominant. Figure 1 shows a map of the world with the most predominant subtypes in each area. The larger circled letter indicates the predominant subtype and the smaller letters indicate the other subtypes present.

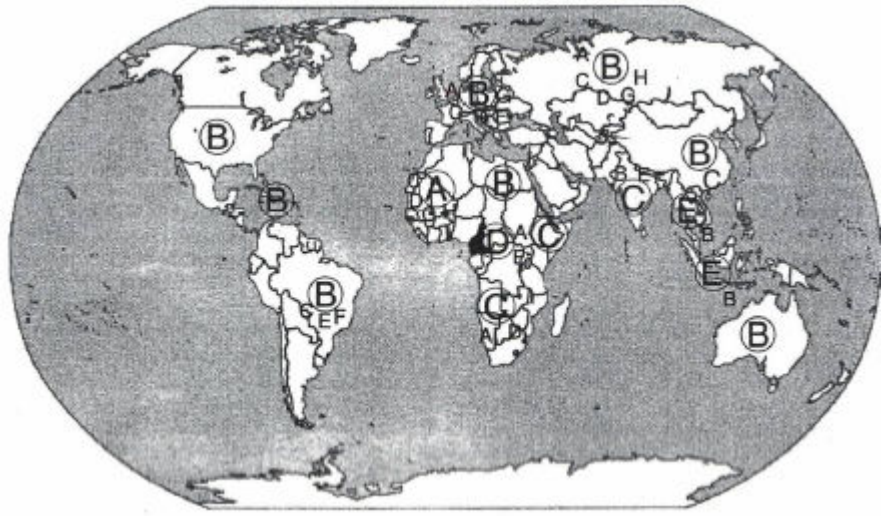

Figure 1. Worldwide Distribution of HIV-1 Sub-types (Knipe and Howley, 2001).

In humans, there are three major stages of disease progression, acute, asymptomatic, and symptomatic (Fauci, et al. 1996).

HIV Composition

HIV-1 has all of the standard retroviral structural genes including *gag*, *pol*, and *env* (Wiley, 2001). HIV also includes at least six more genes, including *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu* (Wiley, 2001). Table 1 lists each of these genes and the major proteins they encode.

Gene Name	Proteins Encoded
Group-specific antigen (*gag*)	Structural Protein; Precursor for the Matrix (MA), Capsid (CA), Nucleocapsid (NC), p6 proteins.
Polymerase (*pol*	Enzymes; Protease (PR), Reverse Transcriptase (RT), and Integrase (IN).
Envelope (*env*)	Structural Protein; Envelope glycoproteins
Trans-activator of viral transcription (*tat*)	Regulatory Protein; Enhances transcription.
Regulator of viral protein expression (*rev*)	Regulatory Protein; Regulates RNA splicing.
Negative factor (*nef*)	Accessory Protein; enhances viral replication. Down modulation and degradation on CD4 and MHC class I.
Virus Infectivity factor (*vif*)	Accessory Protein; Crucial for production of infectious virons.
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 Description of Encoded Proteins (Wiley, 2001; Knipe and Howley, 2001).

Figure 2 shows a map of the HIV genome, indicating where each protein is encoded.

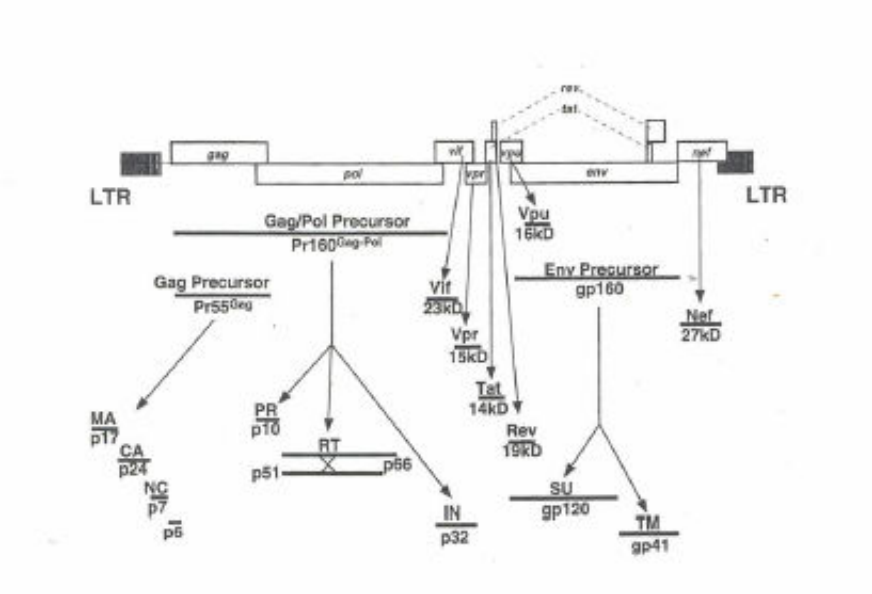

Figure 2. Map of the HIV Genome and Proteins Encoded. Listed size is in kilodaltons (Knipe and Howley, 2001).

Structural Proteins

HIV contains all of the structural proteins commonly found in retroviruses. The structural proteins are made from polyprotein precursors (Knipe and Howley, 2001). The *gag* precursor is cleaved into four different proteins: the matrix (MA), capsid (CA), nucleocapsid (NC), and p6 proteins. The MA protein is involved with the incorporation of envelope protein via interactions with the cytoplasmic tail of gp41 (Wiley, 2001). The CA protein forms the core of the HIV virus particle (Wiley, 2001). The NC protein encapsulates the viral genomic RNA and delivers it to the assembling viron (Wiley, 2001). The p6 protein has a role in late stages of viral release and has been shown to recruit cellular proteins needed for virus budding (Freed, 2002). Figure 3 is a model of the HIV-1 virus particle.

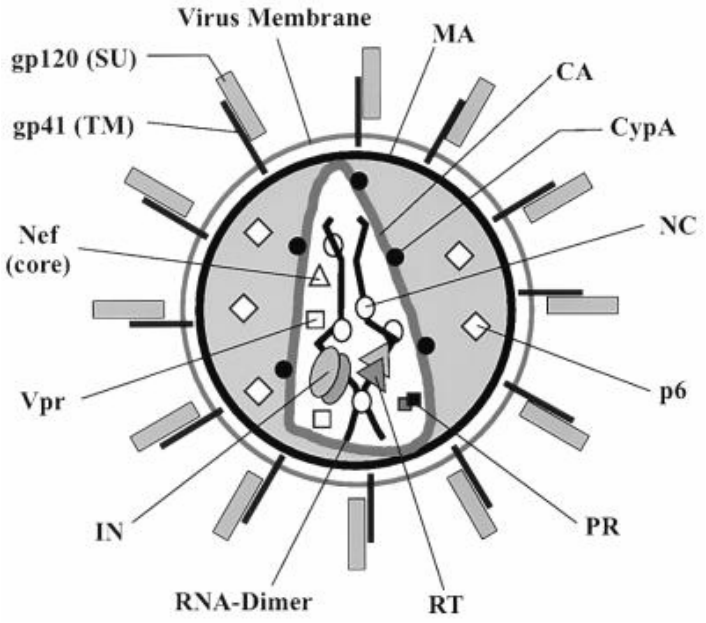

Figure 3. Model of HIV-1 Showing the Topology of Individual Proteins Within the Mature Virion (Welker, et al. 2000).

The *pol* precursor is cleaved into three proteins: viral protease (PR), reverse transcriptase (RT), and Integrase (IN) (Knipe and Howley, 2001). The PR protein cleaves gag and the gag-pol precursor proteins into constituents after the virus has budded from the host cell (Wiley, 2001). This process is called virion maturation. After infection of a new cell, the RT protein synthesizes viral RNA into double stranded DNA (Wiley, 2001). The IN protein assists in several reactions that involve integrating the viral DNA into the host genome (Wiley, 2001).

The *env* precursor is cleaved into two parts named for their size in kilodaltons: gp120 and gp41. The gp120 protein is the surface subunit of *env*, and offers protection against host antibodies (Losman, et al. 2001). The gp41 protein has a hydrophobic

domain that fuses the viral and host membranes. The envelope is described in more detail in the section titled HIV Co-Receptors and Fusion.

Regulatory Proteins

HIV has two regulatory proteins: the transactivator of viral transcription (*tat*) and the regulator of viral protein expression (*rev*). The *tat* protein enhances transcription activity by binding to the transactivating response element (TAR) on HIV mRNA molecules (Wiley, 2001). The *rev* protein regulates the transportation of unspliced viral mRNAs out of the nucleus.

Accessory Proteins

HIV has four accessory proteins: *nef*, *vif*, *vpr*, and *vpu*. These proteins are unique to lentiviruses and are not absolutely necessary for viral replication *in vitro* (Wiley, 2001). However, they are important for viral infection *in vivo* (Wiley, 2001). The *nef* protein is involved with CD4 and MHC class I down regulation, and modulation of cellular activation (Chowers, et al. 1994). The *vif* protein is involved with virus assembly, and degrades the antiviral cellular protein APOBEC3G (Rose, et al. 2004). The *vpr* protein increases virus expression and is important in early stages of HIV replication (Burkrinsky and Adzhubei, 1999). In SIV and HIV-2 the two *vpr* functions are carried out by two proteins, *vpr* and *vpx* (Fletcher, et al. 1996). The *vpu* protein

degrades CD4 within the endoplasmic reticulum and stimulates the release of virus (Willey, et al. 1992).

The Envelope Glycoprotein

HIV virus particles attach to receptors on the surface of the cell via glycoprotein spikes on the surface of the virus (Clapham and McKnight, 2002). The envelope is composed of two proteins, gp120 and gp41. A precursor molecule, gp160, is produced and then cleaved in the Golgi apparatus to form gp120 and gp41 (Clapham and McKnight, 2002). Each spike is trimeric and made up of three gp120 and three gp41 molecules (Clapham and McKnight, 2002). There are five variable loops (V1-V5) contained within gp120, with conserved regions between each loop, while gp41 is relatively conserved (Clapham and McKnight, 2002).

CD4 and Co-Receptors

The major receptor utilized by HIV-1 is the CD4 receptor. It is expressed on T-helper/inducer subset of lymphocytes, macrophages, and on some dendritic cells (Knipe and Howley, 2001). The presence of CD4 is a major determinant of HIV tropism. Normally, CD4 functions as an accessory receptor to increase the attraction between helper T cells and MHC class II antigen presenting cells (Clapham and McKnight, 2002). CD4 binds to gp120 causing a conformational change in the gp120 core (Myszka, et al, 2000). This change causes movement of the variable loops, exposing a binding site for a co-receptor. Co-receptors are the chemokine receptors CCR5 or CXCR4 (Clapham and

McKnight, 2002). Some viruses are able to use both CCR5 and CXCR4. Figure 4 is a model of HIV-1 co-receptor use by viral variants. CCR5-using viruses can infect memory T-cells and primary macrophages. CXCR4-using viruses can infect naïve T-cells and some macrophages.

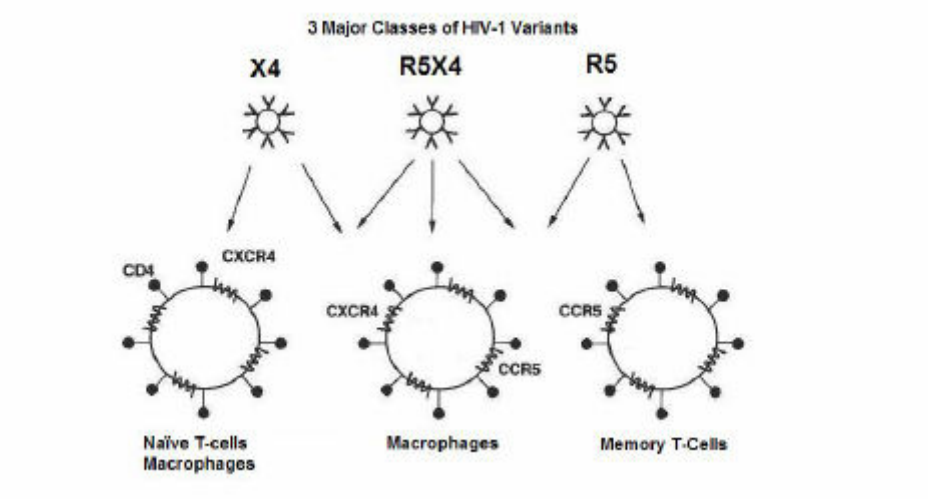

Figure 4. Model of HIV-1 Co-Receptor Usage (updated from Berger, et al 1999).

CCR5 and CXCR4 are both seven transmembrane (7TM) chemokine receptors. There is evidence that HIV-1 can use other 7TM chemokine receptors *in vitro* (e.g., CCR3, CCR8). However, there is little evidence that such alternative co-receptors are used *in vivo* (Clapham and McKnight, 2002).

HIV Co-Receptors and Fusion

It is thought that the binding of HIV to the co-receptor triggers membrane fusion (Doms and Moore, 2000). The most widely accepted Env mediated fusion model for HIV-1 proposes the extension of a coil in gp41 that protrudes the fusion peptide, which

then embeds in the cell membrane (Doms and Moore, 2000). Then gp41 folds back on itself to form a six helix bundle, or "hairpin," bringing the viral and cell membranes in close proximity to each other (Doms and Moore, 2000). The exact mechanisms that confer fusion after that point are still unclear. The observed transmembrane structures in HIV-1 are consistent with other unrelated enveloped viruses (e.g., Influenza, Ebola) (Clapham and McKnight, 2002). Figure 5 below shows the interactions of gp120 and gp41 for viral entry of HIV.

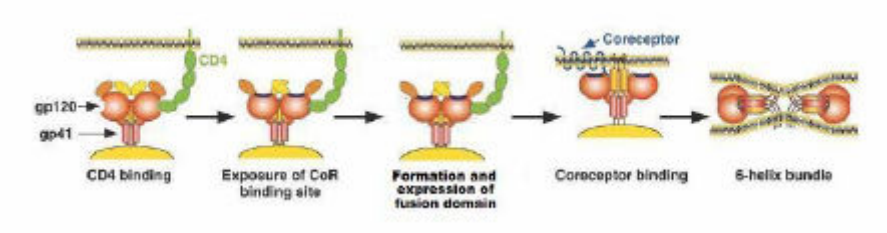

Figure 5. Model of Envelope Membrane Fusion in HIV-1 (Doms and Moore, 2000).

Interactions between gp120 with CD4 and co-receptors mainly determine virus tropism. The sites that dictate tropism and co-receptor binding include variable loops V1, V2, V3, and a conserved region of beta-strands (Clapham and McKnight, 2002). Variations in the loops allow HIV to interact with different co-receptors (Clapham and McKnight, 2002). This variability also likely helps the virus escape immune response (Clapham and McKnight, 2002). Variation in structure of the V3 loop can switch co-receptor use from CCR5 to CXCR4 (Clapham and McKnight, 2002).

V3 Loop Charge

There is a correlation between the sequence of the V3 loop in envelope, and the co-receptor it will use. Viruses that infect using CXCR4 as a co-receptor have significantly higher net charges in the V3 loop, while they seem to be unaffected by the charge in the V1 and V2 loops (Dong, et al. 2005). This association between charge of the V3 loop on envelope and co-receptor use is also seen in HIV-2 (Shi, et al. 2005).

Tropism Studies

Generally, CCR5-using viruses infect macrophages and CD4 positive T-Cells, while CXCR4-using viruses infect CD4 positive T-cells. CCR5-using viruses will infect memory T-cells which express CCR5, while CXCR4-using viruses evolve to target naïve T-cells, which express CXCR4 (Peters, et al. 2004).

It has been shown that CCR5-using viruses vary in their interactions with CD4 and CCR5, resulting in a range of tropism from macrophage-tropic to non-macrophage-tropic. More efficient use of CD4 and CCR5 can allow a virus to infect cells with low levels of these receptors (e.g., macrophages) (Peters, et al. 2004). It is believed that the changes in the variable loops of envelopes better expose the receptor binding sites on the virus envelope and allow more efficient interaction with cell surface receptors. However, while these changes increase the efficiency of interactions between the virus and cell surface receptors, increased exposure of receptor binding sites may confer increased sensitivity to neutralizing antibodies and receptor ligands. This is seen in the envelopes of virus amplified from brain tissue (Peters, et al. 2004). Most neutralizing antibodies are

too large to pass through the blood-brain barrier. The large variable loops that protect receptor binding sites from antibodies in other tissues are not necessary in the brain. Smaller loops allow for more efficient receptor interaction and the brain is a highly selective environment (Peters, et al. 2004).

Recent unpublished data confirm that CCR5-using envelopes amplified from brain tissue are more sensitive to neutralizing antibodies than envelopes amplified from other tissues (e.g., lymph node).

HIV-1 Isolation

HIV-1 is usually isolated by co-culturing HIV positive lymphocytes from patients with uninfected lymphocytes. HIV-1 isolates are thus amplified *in vitro* without the presence of neutralizing antibodies. These conditions may select for variants that interact more efficiently with CD4 and co-receptor and they are not representative of the predominant quasi species *in vivo*.

Isolate 2044

The patient from whom isolate 2044 was derived was from London, and the isolate was made at Addenbrooke's Hospital, Cambridge, England. This patient's CD4+ blood cell count was less than 190 cells per mm^3. The isolate is HIV-1 subtype B, and was cultured from phytohemagglutinin-interleukin-2-stimulated PBMCs derived from the peripheral blood from the affected individual (Simmons, et al. 1996).

The 2044.1 envelope clones were amplified directly from uncultured blood lymphocytes. The 2044 envelope clones were amplified from the primary isolate of 2044, which had been derived from phytohemagglutinin-interleukin-2-stimulated PBMCs *in vitro* (Simmons, et al. 1996). The uncultured envelopes from patient 2044 are mainly CCR5-using, while the cultured virus envelopes all use CXCR4. These envelopes provide an excellent case to study the effect of virus isolation on the sensitivity to neutralizing antibodies and receptor ligands.

PROJECT PURPOSE

The purpose of this project was three-fold: to investigate the tropism, receptor use, and antibody sensitivity of the cultured 2044 and uncultured 2044.1 envelope clones. CCR5-using viruses have been shown to expand tropism and become more sensitive to neutralizing antibodies when isolated in the absence of immunity, while less work has been done on viruses that use CXCR4. The 2044.1 clones are mainly CCR5-using while the 2044 clones are CXCR4-using. This project thus studies the differences between envelopes amplified directly from uncultured lymphocytes versus a virus isolate cultured *in vitro* on phytohemagglutinin-interleukin-2-stimulated peripheral blood mononuclear cells.

MATERIALS AND METHODS

Sub-Cloning Envelope DNA from pCR3.1uni Into pSVIIIenv

Preparation of DNA

HIV envelopes were amplified previously and supplied in the vector pCR3.1uni. The envelopes were sub-cloned into the mammalian expression vector pSVIIIenv via KpnI sites before further experiments were performed. TOP10F' cells transformed with the pCR3.1uni plasmid were cultured in 4 mL of LB Medium with ampicillin in a 37°C shaker overnight. STBL-2 cells with the pSVIIIenv plasmid were cultured in 4 mL of LB Medium with ampicillin in a 30°C shaker overnight. These cultures were "minipreped" using the Qiagen kit, and plasmid DNA was eluted with dH_20.

Gel Extraction

Envelope DNA was digested with the restriction enzyme KpnI. Vector pSVIIIenv DNA was digested with KpnI and then Antarctic Phosphatase. Both digests were electrophoresed on a 0.1% agar gel with crystal violet. The 5 kb band containing pSVIIIenv and the 3 kb bands containing env DNA were extracted using the Qiagen gel extraction kit. Isopropanol was added while extracting pSVIIIenv to increase yield.

Ligation

New England Biotech's Quick Ligase® was used to ligate each envelope clone into pSVIIIenv. Competent TOP10F' cells were transformed with 5 μL of the ligation mixture, and plated onto agar plates containing ampicillin. Envelope and vector only controls were plated with each ligation batch to provide background colony levels.

PCR Screening

Colonies were screened via PCR using the primers Vpu8 and Ltr1, which amplify a fragment from the 5' long terminal repeat (LTR) of pSVIIIenv with part of the envelope gene. Only plasmids with an envelope inserted will screen positive by PCR. Colonies that screened positive were cultured in 4 mL of LB Medium with ampicillin overnight in a 37°C shaker. The cultures were made into glycerol stocks by centrifuging at 4000 rpm for 15 min. The pellet was resuspended in 50% glycerol and stored at –80°C. DNA was then minipreped from this new stock.

Sequencing

DNA Sequencing was used to obtain the envelope nucleotide and amino acid sequences for 2044 and 2044.1 envelope clones. Analysis of the data was performed with MacVector, including the phylogenetic tree.

Cell Based Assays of Envelope Clones

Transfection of 293T Cells

293T cells were transfected with envelope and HIV backbone DNA using Promega's Profection® Mammalian Transfection System. The backbone DNA (NL4.3env-) is derived from NL4.3 type HIV and encodes all genes except envelope. On the day prior to transfection, 293T cells were plated into 6 well plates at $1x10^5$ cells/mL, 2 mL per well. Dulbecco's modified Eagle Medium (DMEM) with 4% fetal bovine serum (FBS) and gentamicin was used. The cells were incubated overnight at 37°C and 5% CO_2. The following day, cell media was changed 3 hours prior to transfection. The concentration of both envelope DNA and NL4.3env- was calculated to be 1.25 µg/µL. Transfection was performed using two 14mL snap-cap tubes for each clone. Tube A contained envelope DNA, NL4.3env-, DNase free water, and calcium chloride reaching a final volume of 83.4 µL. Tube B contained 83.4 µL of 2X Herpes Buffers Saline (HBS). Tube A was then added to Tube B dropwise while vortexing. The mixture was incubated at room temperature for 30 min. After the incubation period, all 166.8 µL of mixture was added to one well of 293T cells dropwise with gentle swirling. The media was changed on the 293Ts the following day, approximately 24 hours after transfection.

Fusion Assays with GHOST CCR5 and GHOST CXCR4

The GHOST cell fusion assays were done by mixing GHOST cells with 293T cells transfected with (and expressing) the HIV envelope. For envelope expression, 293T

cells were transfected with pSVIIIenv envelope and backbone DNA. Control envelopes included were from subject NA20; B59 and LN3, NL4.3 (a standard HIV-1 envelope). JRCSF and JRFL envelopes were also from one individual. All control envelopes use CCR5, except for NL4.3 which uses CXCR4. One day prior to the assay GHOST cells of both lines were set up in 48-well plates. Cells were plated at $8x10^4$ cells/mL with 500 µL per well. DMEM with 4% FBS and gentamicin was the media used. GHOST cells were incubated overnight so that they would attach to the bottom of the wells. The following day, 100 µL and 50 µL of 293T cells (transfected 48 hours previously) were added to separate wells at a concentration of $2x10^5$ cells/mL. The plates were incubated overnight. GHOST cells carry a reporter gene controlled by an HIV promoter. Green fluorescent protein (GFP) is expressed when GHOST cells become fused to 293T cells expressing the HIV tat protein. After 24 hours of incubation with the 293T cells, the plates were viewed under ultra-violet (UV) light. The wells were scored from 0-5 on the extent of green fluorescence and photographed.

Fusion Assays with NP2 cells

NP2 fusion assays were performed as described for the GHOST assays. GHOST cells express low levels of endogenous CXCR4, while NP2 cells do not. The NP2 lines used were NP2/CD4, NP2/CCR5, NP2/CD4/CCR5, and NP2/CD4/CXCR4. They were plated at $1x10^5$ cells/mL the day before co-culture, and incubated for 24 hours so the NP2 cells would attach to the bottom of the wells. The following day, 100 µL and 50 µL of transfected 293T cells were added to separate wells at a concentration of $2x10^5$ cells/mL.

After 24 hours of incubation, plates were fixed and stained for syncytia. Media was aspirated off and the wells were washed once with 1X Phosphate Buffer Solution (PBS). Then syncytia stain (1% methylene blue, 0.2% basic fuchsin in methanol) was added and left on the cells for 10 minutes. The solution fixed the cells and stained syncytia blue. After 10 minutes, the stain was removed and the plates were washed twice with 1X PBS. Finally, 1X PBS/ 0.05% Azide was added to preserve the cells and protect against bacterial growth. The wells were scored on a scale from 0-5 according to the amount of syncytia and photographed.

Pseudotype Virus Stocks

Transfection of 293T cells was performed, as described above with pSVIIIenv and pNL4.3env-. 24 hours later, the media was changed. 48 hours after transfection pseudotype viruses were harvested. The media was removed and placed in a 15mL centrifuge tube. The tubes were centrifuged for 10 minutes at 1,200 rpm to remove cell debris. Supernatant from each vial was added to cryovials in 500 µL aliquots. The vials were then frozen rapidly in liquid nitrogen, and were transferred to –152°C for storage. Pseudotype viruses carry the RNA genome of pNL4.3env- and assemble the envelope of choice expressed from pSVIIIenv. Pseudotypes cannot synthesize new envelope protein, and are therefore only capable of one round of replication.

Titers of Virus Stocks

Pseudotype virus stocks were titered on TZM-BL cells, GHOST CCR5, and GHOST CXCR4 cells. TZM-BL cells are Hela cells that express CD4, CCR5, and CXCR4. They carry a beta-galactosidase reporter gene controlled by an HIV promoter. Beta-galactosidase is therefore induced if HIV infects and expresses tat. 24 hours prior to infection, the target cells were plated on 48 well plates at $4x10^4$ cells/mL with 500 µL per well.

DMEM with 4% FBS and gentamycin was used. On the day of infection, virus stocks were thawed and diluted in a 48 well plate. Undiluted virus and three 10-fold dilutions were used. The media of each well was aspirated off and replaced with 100 µL of diluted virus. After 3 hours, media was added so that each well contained approximately 500 µL. 72 hours after infection, the TZM-BL cells were fixed with glutaraldehyde and stained with X-gal (40 mg/mL in N,N,-dimethyl formamide, diluted to 0.5 mg/mL in 1X PBS with 3 mM potassium ferrocyanide, 3 mM potassium ferricyanide, and 1 mM magnesium chloride). Stained cells were viewed under microscope and blue focus forming units (FFU) were counted. 72 hours after infection, GHOST CCR5 and CXCR4 cells were viewed under UV light, and green foci of infection were counted.

Cell Surface Receptor Inhibition assays

Q4120 is a mouse antibody against CD4. AMD3100 is a small organic molecule that binds to CXCR4. Envelope positive pseudotypes were tested for sensitivity to both reagents. Infection inhibition assays were performed on TZM-BL cells. TZM-BL cells

were plated at $4x20^4$ cells/mL with 200 µL per well into 96 well luminometer plates 24 hours prior to infection. DMEM with 4% FBS and gentamicin was used. On the day of infection, Q4120 and AMD3100 were serially diluted in two-fold steps. Media on the TZM-BL cells was aspirated and replaced with 50 µL of diluted inhibitor. The plates were then incubated at 37°C for 1 hour. 4000 FFU of each virus was then added. 3 hours after infection, the virus containing media was aspirated off and replaced with media including diluted inhibitor. 48 hours after infection the media on the plates was replaced with a 1:1 mixture of DMEM without phenol red and Beta-Glo. Beta-Glo provides a luminescent readout for beta-galactosidase. The plates were incubated at room temperature for at least 30 minutes and then read in a luminometer.

Human Monoclonal Antibody Inhibition Assays

The human monoclonal antibodies B6 and B12 bind to the receptors on virus envelope. These assays were carried out as described for cell surface receptor inhibitions with two exceptions: dilutions of antibody were incubated with virus at room temperature for one hour, with the virus and antibody mixture being used to infect cells. After 3 hours of infection, the mixture was aspirated off and replaced with DMEM with 4% FBS and gentamicin.

RESULTS

Cloning and Expression

Sub-cloning envelopes into expression vector pSVIIIenv

Cultured 2044 and uncultured 2044.1 envelope genes were sub-cloned into the mammalian expression vector pSVIIIenv via KpnI sites. TOP10F' cells were transformed with the plasmid and then grown on LB agar plates containing ampicillin. Colonies were screened by PCR. Amplification of a 600 bp band by the Ltr1 and Vpu8 primers confirmed correct insertion of the envelope gene. Figure 6 is an example of PCR screening. The center lanes on the top and bottom are both 10 kb DNA ladder. Lanes with a band approximately 600 bp in size are positive (lanes 1, 4, 5, 10, 24, 25, 29, 30, 32, 33, and 34).

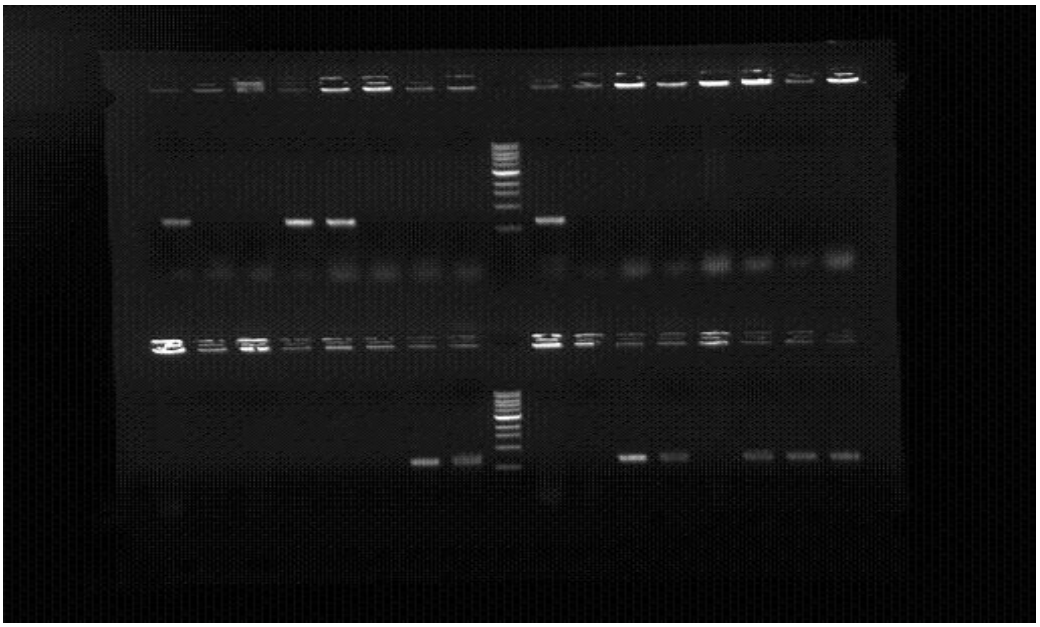

Figure 6. Sample PCR Screening Gel.

Sequencing

The V3 loop in the 2044 clones and 2044.1 cl 2 has a different charge than the 2044.1 clones 102 and 105. The net charge of +5, compared to +3, is responsible for the shift in use of CCR5 to CXCR4. Figure 7 is an alignment of the primary structure of envelope proteins from the 2044 and 2044.1 clones. The variable loops V1-V4 are noted for reference. The nucleotide sequences of the 2044 envelopes were analyzed and compared to NA20 B59 as a reference. Figure 8 is the generated phylogenetic tree. The tree shows the 2044.1 clones 102 and 105 that are likely to be CCR5-using (by V3 loop charge) are distinct from clones likely to be CXCR4-using. 2044.1 clone 2 clustered closely with clones from the virus isolate, all of which are likely to be CXCR4-using.

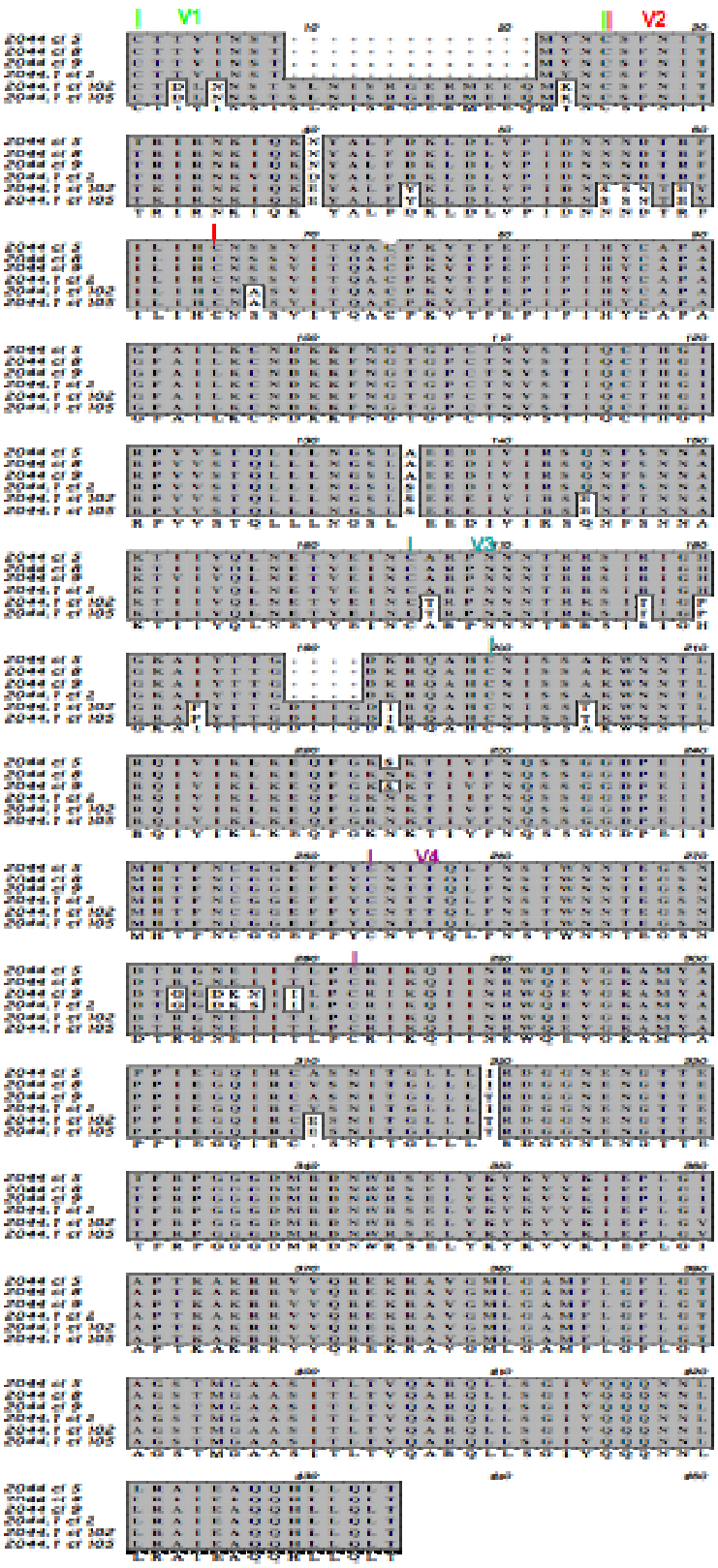

Figure 7. Alignment of Envelope Protein Primary Structure. The V1-V4 regions are noted.

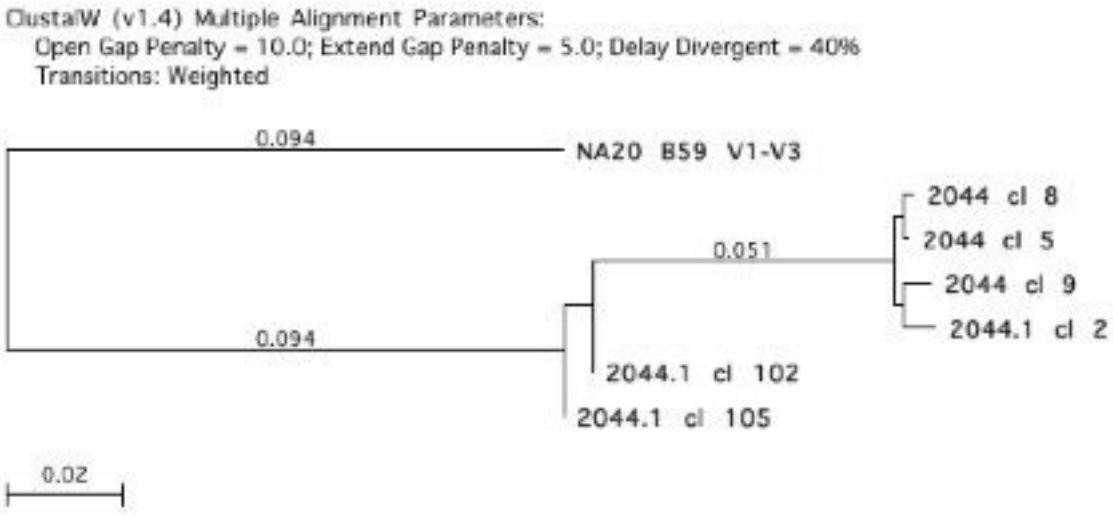

Figure 8. Phylogenetic Tree of 2044 Clones Compared to NA20 B59.

Fusion Assays

Fusion assays were performed with GHOST CCR5 and GHOST CXCR4 cells to determine the functional viability of cloned 2044 and 2044.1 envelopes. Two different assays were performed to monitor cell fusion. The first assay used GHOST cells. Green fluorescent protein (GFP) is expressed when GHOST cells (transfected with a gene encoding an HIV co-receptor) become fused to 293T cells expressing a compatible HIV env protein and the HIV tat protein. An example of the GFP green glow is shown in Figure 9.

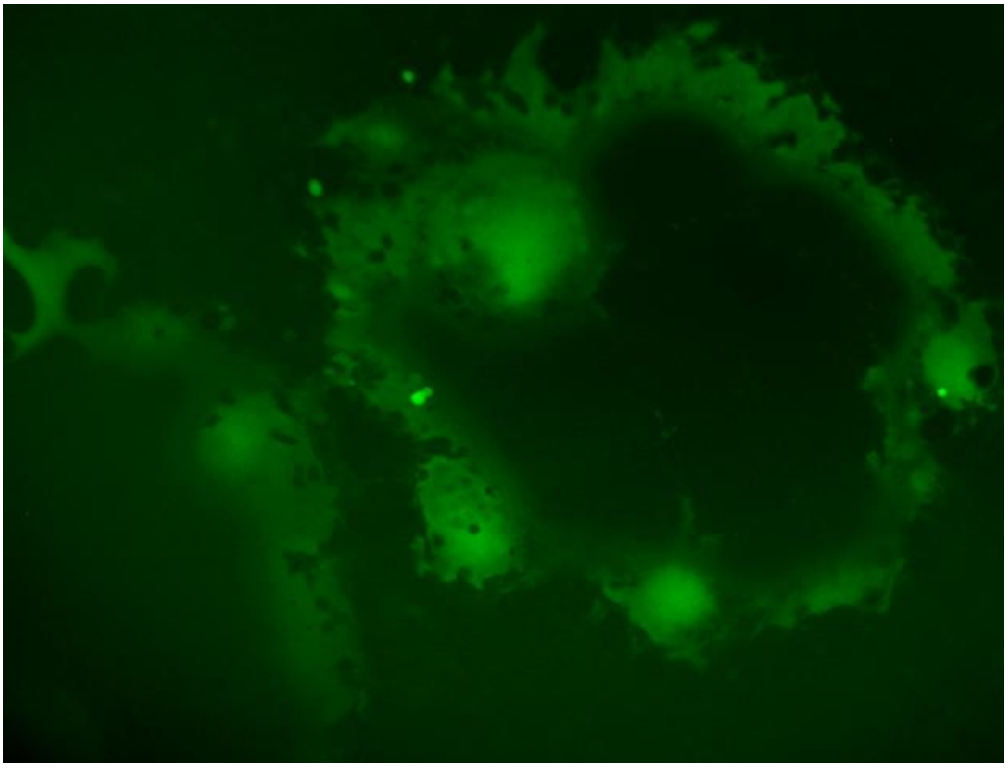

Figure 9. An Example of GFP Fluorescence in GHOST Cell Syncytia. Visualized under UV light.

To clarify the fusion results, the cloned envelopes were also assayed for fusion with NP2 cells, which have no background expression of CXCR4, using a blue dye (methylene blue/fuscin) for syncytia formation (see Figure 10 for an example).

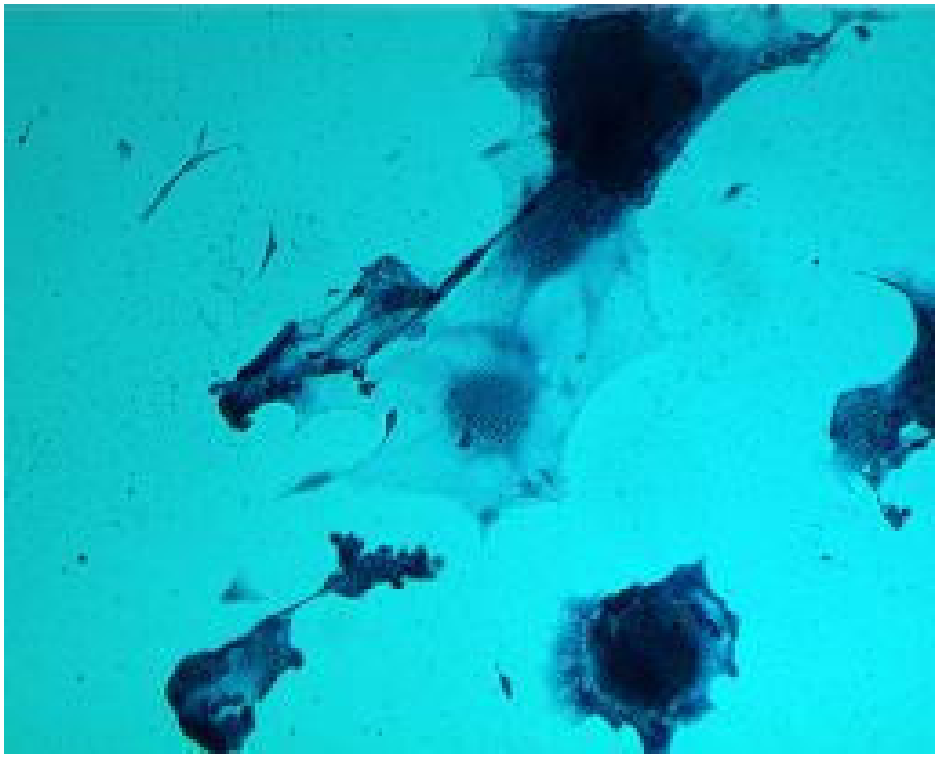

Figure 10. An Example of Fused NP2 Cells, With Syncytia Stained Blue.

Table 2 is a summary of data from both GHOST and NP2 Fusion assays. Fusion was evaluated on a scale of 0 to 5, with 5 being the highest level of fusion. GHOST cells were scored by GFP production under UV light. NP2 cells were scored for methylene blue-stained syncytia. The results indicate that all 2044 envelope clones use CXCR4, as does 2044.1 clone 2, while 2044.1 clones 102 and 105 use CCR5. As expected, the control envelopes NA20 B59, NA20 LN3, JRCSF, and JRFL use CCR5, while the remaining control envelope, NL4.3, uses CXCR4.

Clone	GHOST CCR5	GHOST CXCR4	NP2/CD4	NP2/CCR5	NP2/CD4/ CCR5	NP2/CD4/ CXCR4
2044 cl 5	1	5	0	0	1	4
2044 cl 8	1	5	0	0	1	4
2044 cl 9	1	5	0	0	1	4
2044.1 cl 2	1	5	0	0	1	4

2044.1 cl 102	5	0	0	0	3	0
2044.1 cl 105	5	0	0	0	3	1
NA20 B59	2	1	0	0	3	1
NA20 LN3	3	0	0	0	3	0
NL4.3	1	5	0	0	1	4
JRCSF	5	1	0	0	3	0
JRFL	5	1	0	0	3	0

Table 2 GHOST and NP2 fusion results.

Sensitivity to Inhibition

Sensitivity to a CD4 specific monoclonal antibody, Q4120

Q4120 is a mouse antibody against CD4. It bound the CD4 on the surface of the target cells and inhibited infection conferred by all 2044 and 2044.1 envelopes, as shown in Figure 11. The macrophage-tropic controls (JRFL and NA20 B59) are more resistant to Q4120 than the non-macrophage-tropic controls (JRCSF and NA20 LN3). Note that the CCR5-using clones (2044.1 clones 102, 105) are more sensitive, while all CXCR4-using envelopes are more resistant.

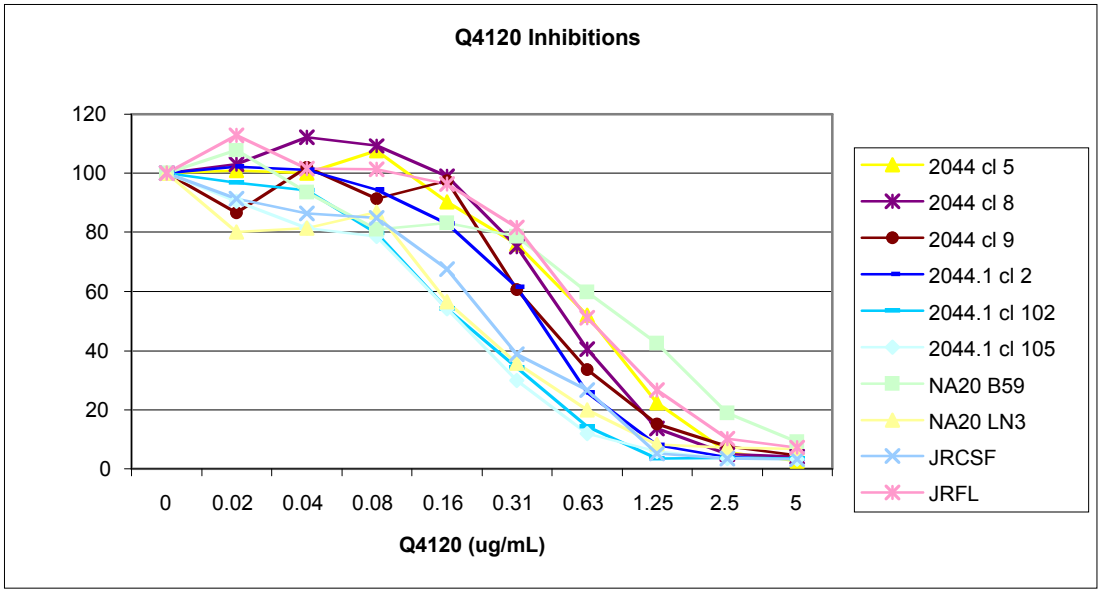

Figure 11. Virus Inhibition With CD4 Antibody Q4120.

Sensitivity to a small molecule CXCR4 inhibitor, AMD3100

AMD3100 is a small organic molecule that binds to CXCR4 molecules on the cell surface. As seen in Figure 12, as expected, all CCR5-using control viruses were completely resistant to AMD3100, while infection of CXCR4-using control viruses was knocked out at very low drug concentrations. All of the CXCR4-using clones (2044 clones 5, 8, 9, and 2044.1 clone 2) (as determined in the fusion assays) showed similar sensitivity to AMD3100. The control envelope NL4.3 was by far more sensitive than any other envelope. It was reduced to almost no residual infection with the smallest dose of inhibitor.

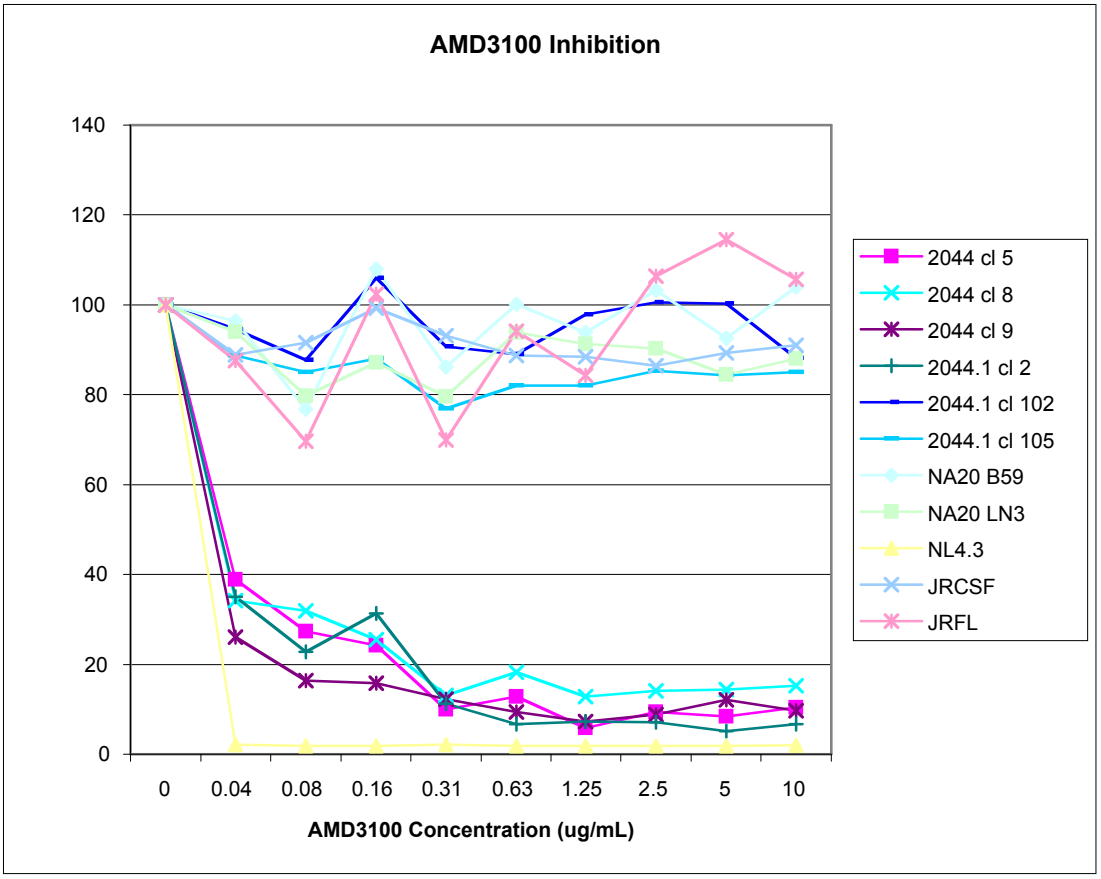

Figure 12. Virus Inhibition with CXCR4-Binding Drug AMD3100.

Sensitivity to Human Monoclonal Antibodies

B6

B6 is an antibody that recognizes the CD4-binding site on gp120. This site is usually protected on primary isolates but is accessible on some highly passaged lab strains (e. g., NL4.3). Only the positive control envelope, NL4.3 (a highly passaged lab isolate) (yellow in the figure), was sensitive to B6, as seen in Figure 13.

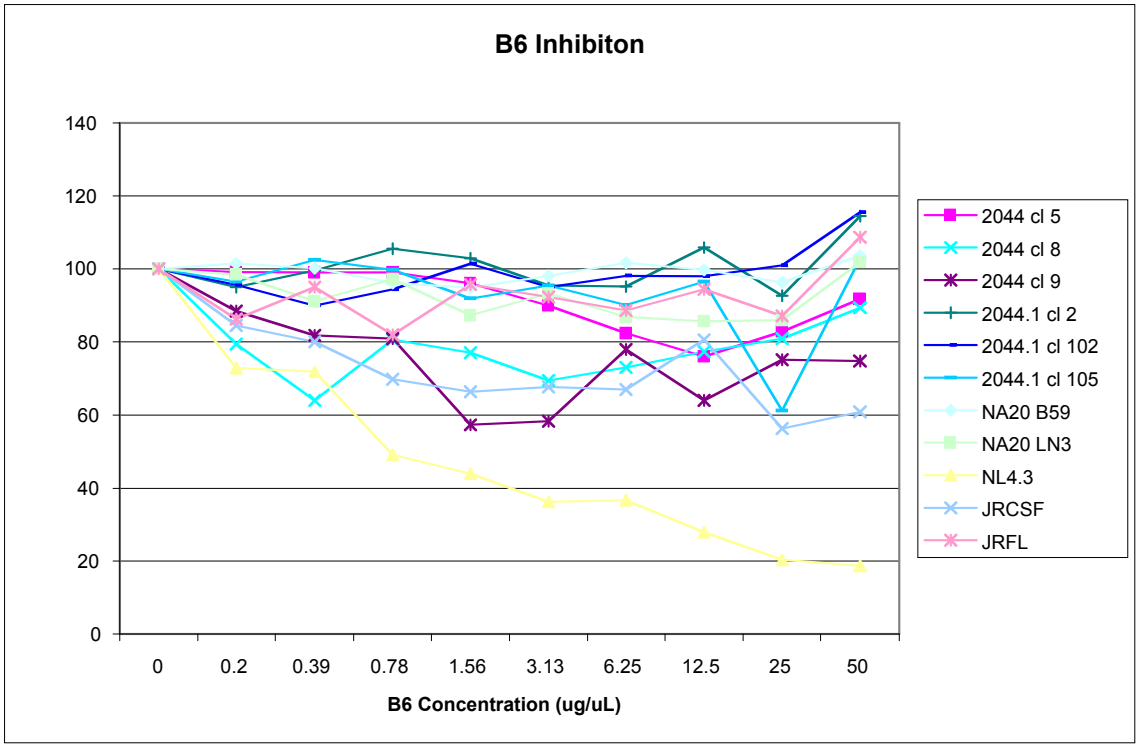

Figure 13. Virus Inhibition With B6.

B12

B12 is another antibody that binds to the CD4-binding site on the virus envelope. B12 has an unusual structure with long complimentarily determining loops that allow it to access the CD4-binding site on some primary HIV-1 isolates. As seen in Figure 14, NL4.3 was sensitive to B12, in addition to JRCSF and JRFL. NA20 B59 showed some reduction. All cultured 2044 envelopes were resistant.

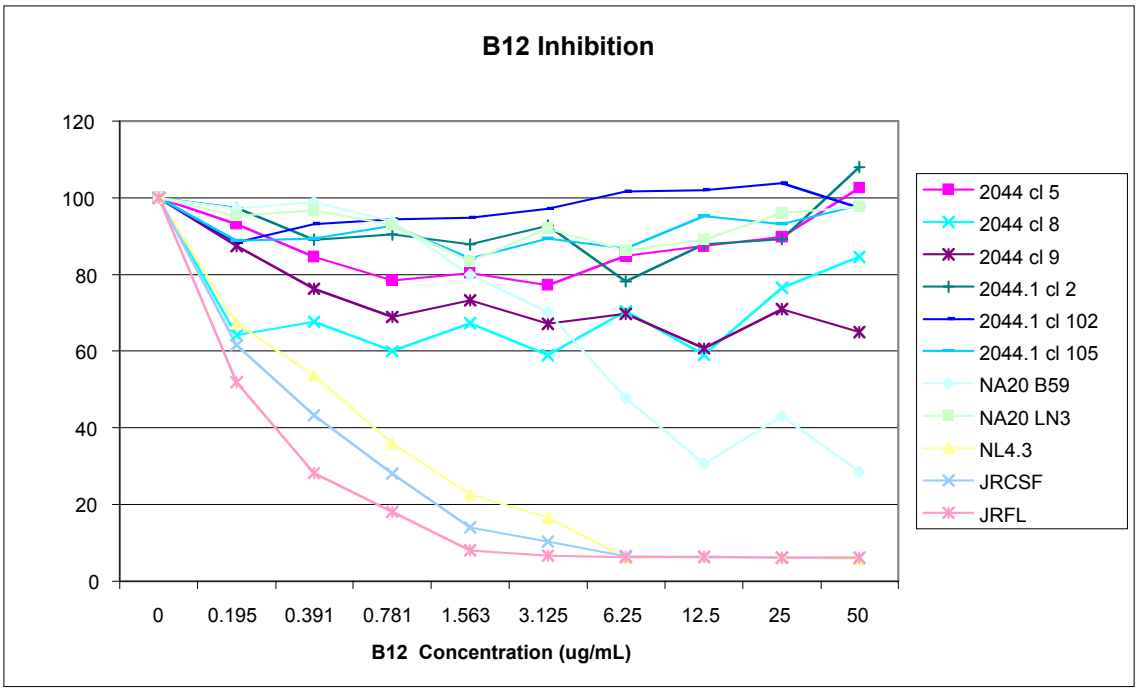

Figure 14. Virus Inhibition With B12.

Macrophage Infection

Clones were titered on macrophages, and the macrophage titer was compared to that virus's TZM-BL titer. The resulting ratios are shown in Table 3, and are also plotted in Figure 15. Focus forming units per mL were compared.

Envelope	Macrophage Titer (FFU per mL)	TZM-BL Titer (FFU per mL)	Percent TZM-BL Titer
2044 cl 5	25	2,400	1.04
2044 cl 8	35	11,750	0.30
2044 cl 9	55	21,000	0.26
2044.1 cl 2	5	2,256	0.22
2044.1 cl 102	5	1,400	0.36
2044.1 cl 105	10	1,800	0.56
NA20 B59	19,800	38,000	52.11
NA20 LN3	0	3,850	0.01

NL4.3	5	5,400	0.09
JRCSF	25	7,700	0.32
JRFL	23,400	39,500	59.24
Isolate 2044	15,550	228,000	6.82

Table 3. Percent Macrophage Infection.

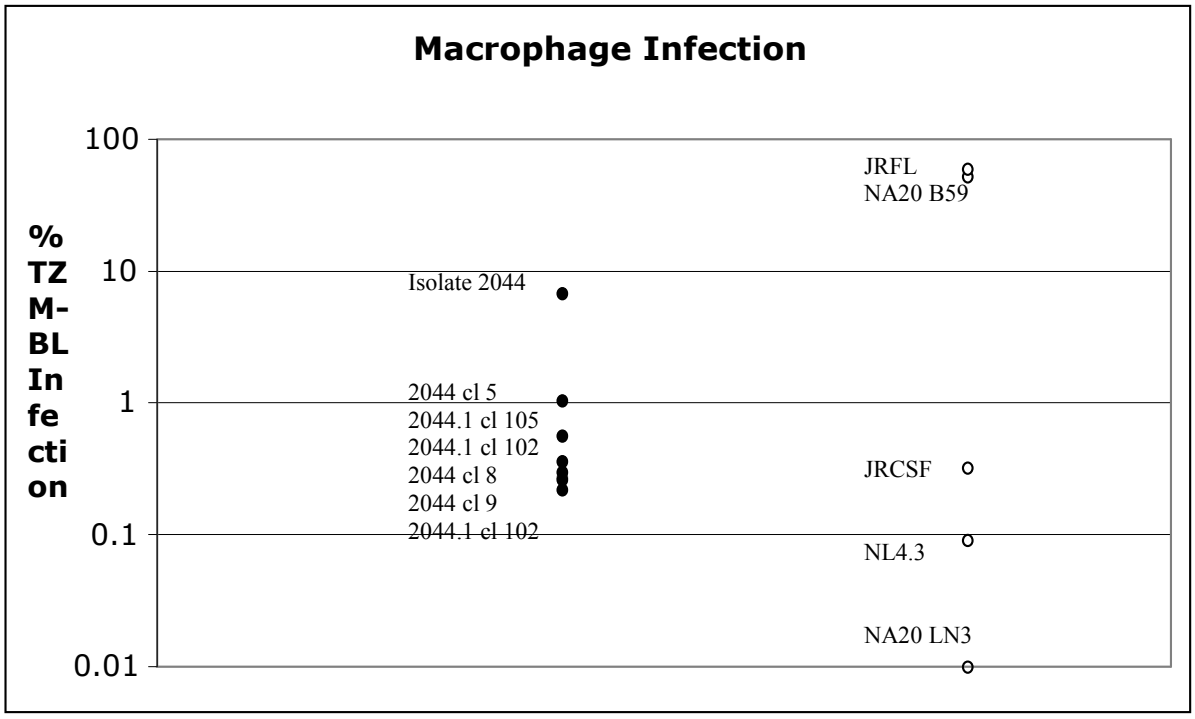

Figure 15. Macrophage Infection Percentages.

DISCUSSION

The culture of HIV-1 in PBMCs in the absence of neutralizing antibodies may select for variants that are not representative of quasi species *in vivo*. This project studied HIV-1 envelopes derived from patient 2044. Envelopes were amplified from a primary isolate cultured in PBMCs, and from uncultured PBMCs. The uncultured PBMCs yielded three envelopes, two apparently CCR5-using (2044.1 clones 102 and 105) and one CXCR4-using (2044.1 clone 2) as determined by cell fusion assays. Cultured PBMCs yielded only CXCR4-using envelopes (2044 clones 5, 8, and 9).

All of the CXCR4-using envelopes, both cultured and uncultured, displayed similar properties. They all had similar sensitivity to receptor ligand and neutralizing antibodies. The CXCR4 receptor ligand AMD3100 was able to block infection of both uncultured and cultured CXCR4-using envelopes at low concentrations. The cultured envelopes did not become sensitive to anti-CD4 antibodies B6 or B12. All CXCR4-using envelopes were more resistant to the mouse anti-CD4 antibody Q4120 than CCR5-using envelopes. This is consistent with an increased affinity for CD4 compared to CCR5-using envelops.

The original 2044 isolate infects macrophages at 6% TZM-BL infection. However, all of the clones, both CCR5-using and CXCR4-using, were much less efficient for macrophage infection. 2044 clone 5 infected macrophages the best, reaching 1% of TZM-BL infection. Note that 2044 infection of macrophages was previously shown to be sensitive to CXCR4 ligands and not CCR5 ligands (Simmons, et al. 1996). All of the

envelope clones contain only one version of envelope, while the 2044 isolate presumably contains envelope quasispecies that were not represented by the amplified clones.

The 2044 envelopes amplified from the primary isolate have not evolved different properties from the CXCR4-using envelope amplified from uncultured PBMCs. None of the amplified envelopes, including all of the CXCR4-using and the two CCR5-using envelopes from uncultured PBMCs, were macrophage-tropic.

The macrophage-tropic isolate 2044 is not representative of the envelopes amplified from uncultured PBMCs. However, it is unclear whether the envelopes amplified are representative of the viral population *in vivo* or even in the virus isolate. PCR of a larger number of envelopes by endpoint dilution is required to address these issues.

Safety issues prevented the use of infectious 2044 isolates until late in the project. Comparison of the isolate with amplified envelopes for sensitivity to receptor ligand and neutralizing antibodies would have been helpful.

Works Cited

Berger EA, Murphy PM, Farber JM (1999) Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. *Annual Review of Immunology* 17: 657-700.

Bukrinsky M, Adzhubei A (1999) Viral protein R of HIV-1. *Reviews of Medical Virology* 9: 39-49.

Clapham PR, McKnight A (2002) Cell surface receptors, virus entry and tropism of primate lentiviruses. *Journal of General Virology* 83: 1809-1829.

Chowers MY, Spina CA, Kwoh TJ, Fitch NJ, Richman DD, Guatelli JC (1994) Optimal infectivity in vitro of human immunodeficiency virus type 1 requires an intact nef gene. *Journal of Virology* 68: 2906-2914.

Doms R, Moore J (2000) HIV-1 membrane fusion: targets of opportunity. *The Journal of Cell Biology* 151: F9-F13.

Dong XN, Chen X, Chen Y, Ablimit A, Ye Z, Wu Y, Chen YH (2005) Short Communication: HIV type 1 phenotype, tropism, and sequence patterns: association and preference. *AIDS Research and Human Retroviruses* 21(3): 234-238.

Fauci AS, Pantaleo G, Stanley S, Weissman D (1996) Immunopathogenic mechanisms of HIV infection. *Annals of Internal Medicine* 124: 654-663.

Fletcher TM 3rd, Brichacek B, Sharova N, Newman MA, Stivahtis G, Sharp PM, Emerman M, Hahn BH, Stevenson M (1996) Nuclear import and cell cycle arrest functions of the HIV-1 Vpr protein are encoded by two separate genes in HIV-2/SIV(SM). *The EMBO Journal* 15(22): 6155-6165.

Freed EO (2002) Minireview: Viral Late Domains. *Journal of Virology* 76: 4679-4687.

Knipe DM, Howley PM (2001) *Fundamental Virology*, 4th edition. Philidelphia, PA: Lippincott Williams & Wilkins, 913-922.

Losman B, Bolmstedt A, Schonning K, Bjorndal A, Westin C, Fenyo EM, Olofsson S (2001) Protection of neutralization epitopes in the V3 loop of oligometric human immunodeficiency virus type 1 glycoprotein 120 by N-linked oligosaccharides in the V1 region. *AIDS Research and Human Retroviruses* 20: 111-126.

Myszka DG, Sweet RW, Hensley P, Brigham-Burke M, Kwong PD, Hendrickson WA, Wyatt R, Sodroski J, Doyle ML (2000) Energetics of the HIV gp120-CD4 binding reaction. *Proceedings of National Academy of Sciences* 97: 9026-9031.

Peters PJ, Bhattacharya J, Hibbitts S, Dittmar MT, Simmons G, Bell J, Simmonds P, Clapham PR (2004) Biological analysis of human immunodeficiency virus type 1 R5 envelopes amplifies from brain and lymph node tissues of AIDS patients with neuropathology reveals two distinct tropism phenotypes and identifies envelopes in the brain that confer an enhanced tropism and fusigenicity for macrophages. *Journal of Virology* 78: 6915-6926.

Rose KM, Marin, Kozak SL, Kabat D (2004) The viral infectivity factor (Vif) of HIV-1 unveiled. *TRENDS in Molecular Medicine* 10: 291-297.

Shi Y, Brandin E, Vincic E, Jansson M, Blaxhult A, Gyllensten K, Moberg L, Brostrom C, Fenyo EM, Albert J (2005) Evolution of human immunodeficiency virus type 2 co-receptor usage, autologous neutralization, envelope sequence and glycosylation. *Journal of General Virology* 86(Pt 12): 3385-3396.

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

Welker R, Hohenb H, Tessmer U, Huckhagel C, Kräusslich H (2000) Biochemical and Structural Analysis of Isolated Mature Cores of Human Immunodeficiency Virus Type 1. *Journal of Virology* 74(3): 1168–1177.

Wiley S (2001) Ph.D. Dissertation. *UMASS Medical School Program in Molecular Medicine*.

Willey RL, Maldarelli F, Martin MA, Strebel K (1992) Human immunodeficiency virus type 1 Vpu protein induces rapid degradation of CD4. *Journal of Virology* 66: 7193-7200.