THE DEVELOPMENT OF MONOCLONAL ANTIBODIES AGAINST HUMAN IMMUNODEFICIENCY VIRUS-1 VIRAL PROTEIN R USING HYBRIDOMA TECHNOLOGY

By

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Submitted in Partial Fulfillment of the Requirements
for the Degree of
Masters of Science
in the
Biological Sciences
Program

YOUNGSTOWN STATE UNIVERSITY
August, 2015.

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ABSTRACT

Hybridoma technology allows the development of monoclonal antibodies that are specific to a target antigen. The goal of this project was to use hybridoma technology to develop monoclonal antibodies that would be able to recognize and bind both cell bound HIV-1 Viral protein r (Vpr) and cell free HIV-1 Vpr. A mouse was immunized with Vpr antigen and the spleen from the immunized mouse was isolated and teased in order to release antibody-secreting spleen cells. Isolated spleen cells were fused with myeloma cells using polyethylene glycol and then cultured in HAT medium in order to select for hybridomas. Hybridomas secreting anti-HIV-1 Vpr were screened by indirect ELISA. About 70 % of the hybridoma cell lines were able to recognize and bind to the Vpr antigen. Antibodies from DL.VPR.D1 had the best amount of binding to the Vpr antigen when compared to the positive control. The antibodies from DL.VPR.D1 were isotyped in order to determine what class of antibody they belong to. Non-specific binding of the negative control was noticed during isotyping. The use of casein blocking buffer helped in eliminating the non-specific binding. It was found that the antibodies from DL.VPR.D1 belong to the IgG2b class. DL.VPR.D1 was further subcloned by limiting dilution in order to develop monoclonal cells. Seven subclones were derived from DL.VPR.D1. Three of the seven subclones were positive with DL.VPR.D1.G6 having the highest amount of binding to Vpr antigen. Over time DL.VPR.D1.G6 antibodies lost its binding abilities of the HIV-1 Vpr antigen. Loss of reactivity to Vpr antigen was also noticeable in some of the subclones from cell lines (EH.VPR.C5.C6, DF.VPR.C4.D12) that were initially reactive to the Vpr. Amidst the shortcomings; this study demonstrates the importance of hybridoma technology in developing antibodies against a specific target.

ACKNOWLEDGEMENTS

I am grateful to God for giving me the strength, health and wisdom to complete my studies at Youngstown State University. I would like to express my deepest gratitude to my thesis advisor, Dr. Diana Fagan, for devoting her time and effort in making me a better person than I was 2 years ago. It was a privilege to be in her lab and I loved every seconds of it. I would also like to say a big thank you to my committee members, Dr. Jonathan Caguiat and Dr. Heather Lorimer for taking their time to and my thesis and for the good advice they gave to me during my thesis. I would also like to appreciate Dr. Dr. Ganesaratnam Balendiran for extracting and purifying the Viral Protein R, without him my thesis would be impossible. Thanks are also due to Esther Haung and Devon Archinal for her works on the purification of monoclonal antibodies; Daniel Lisko for his work on developing hybridomas.

I dedicate this thesis to my family, especially my parents Mr. Ogundiran Ogunwumi and Mrs. Olakitan Ogunwumi. Special thanks to my brothers Oluwatosin and Olaoluwa Ogunwumi; and my sister Oluwadamilola Ogunwumi for their love and words of encouragement. I am also grateful to my lab colleagues for their cooperation, exchange of research ideas and for providing excellent environment for research.

Lastly, I would like to thank YSU graduate school and the entire department of Biological science for funding my studies and for providing the equipment needed to complete my thesis.

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LIST OF ABBREVIATIONS

AIDS- Acquired Immunodeficiency Syndrome

ART- Antiretroviral Therapy

CA- Capsid Protein

CAF- Cell Antiviral Factor

CCL5- Chemokine (C-C motif) Ligand 5

CSF- Colony Stimulating Factor

ER- Endoplasmic Reticulum

FCS- Fetal Calf Serum

gRNA- Genomic RNA

HAART- Highly Active Antiretroviral Therapy

HIV- Human Immunodeficiency Virus

IFN-γ- Interferon Gamma

IL- Interleukin

IN- Integrase

LNP- Long Term Non-progressors

LTR- Long Terminal Repeat

MA- Matrix Protein

MAbs- Monoclonal Antibodies

MEM- Minimum Essential Medium

MIP-1α- Macrophage Inflammatory Protein 1 Alpha

MIP-1β- Macrophage Inflammatory Protein 1 Beta

MHC- Major Hisotcompatibility Complex

NC- Nucleocapsid Protein

Nef- Negative Regulatory Factor Protein

NK- Natural Killer Cells

PAbs-PM- Plasma Membrane

PBS- Phosphate Buffered Saline

PR- Protease

RBC's- Red Blood Cells

Rev- Regulation of the Expression of Virion Proteins

RT- Reverse Transcriptase

SDF-1- Stroma Cell Derived Factor-1

Tat- Trans-activator of Transcription protein

TMB- Tetramethylbenzidine Liquid Substrate system

TNF-α- Tumor Necrosis Factor

Vif- Viral Infectivity Factor Protein

Vpr- Viral Protein R

Vpu- Viral Protein U

Vpx- Virion-associate Protein X

CHAPTER I: INTRODUCTION

1.1 Human Immunodeficiency Virus

1.1.1 The History of HIV

The *morbidity and mortality weekly report* published by the U.S. Centers for Disease Control and Prevention (CDC) on June 5, 1981 brought the world's attention to an infection capable of rendering the immune system non-functional. This infection rendered the immune system of five homosexual men in Los Angeles, California, significantly less effective, leaving the men vulnerable to opportunistic infections (*Pneumocystis carnii* pneumonia), an unusually aggressive cancer (Kaposi's sarcoma) and autoimmune diseases. This mysterious infection was called Gay-related Immune Deficiency (GRID). In 1982, reported cases of GRID in Haitian immigrants, heroin users and hemophiliacs demonstrated that GRID was not just unique to homosexuals but could be spread at an alarming rate through the population. In July 1982, the name 4H disease (Homosexual, Haitian, Hemophiliac and Heroin user related disease) was adopted and it was later changed to Acquired Immunodeficiency Syndrome (AIDS) (Quagliarelllo, 1982).

In 1982 the causative agent of AIDS was still a mystery. The research group of Robert Charles Gallo and Jay Levy American Biomedical researchers, and Luc Antoine Montagnier a French virologist, successfully isolated the infectious agent causing AIDS in 1983. The isolated virus was named Human T-lymphotropic virus III (HTLV-III) by Gallo's group, AIDS associated virus II (ARV-2) by Levy's group (Levy et al., 1994) and Lymphadenopathy-associated virus (LAV) by Montagnier's group (Reviewed in Basavapathruni and Anderson, 2007). In 1986, further studies showed similarities among

isolates of HTLV-III, ARV-2 and LAV and they were jointly renamed as HIV (Basavapathruni and Anderson, 2007).

The emergence of HIV in the U.S. could be traced to an infected Haitian immigrant, but its birth place is the Sub-Saharan Africa. There is molecular phylogenetic evidence showing close similarities between the viral genomic sequences of Simian Immunodeficiency Virus (SIV), a virus predominant among the non-human primates from the Sub-Saharan Africa, and HIV. This evidence suggested that activities such as farming and hunting enhanced cross-species transmission of SIV from non-human primates to humans in the late 19^{th} century, with the evolution of SIV to HIV in humans (Korber et al., 2000).

Epidemiological statistics show that 1.6 million worldwide annual deaths are accounted to the HIV global pandemic. Of the approximately 7 billion population worldwide, about 35 million people live with HIV/AIDS and there are about 2.3 million new infections yearly (Myron et al., 2008). With the aim of curbing the HIV mortality rate, efforts have been geared toward developing treatments that would reduce the viral load and strengthen the immune system to fight opportunistic infections. The development of antiretroviral therapy (ART) has had little success and more advanced therapy known as HAART (highly active antiretroviral therapy, which is a combination of at least two classes of anti-HIV agents) have several problems associated with their use, such as the development of side effects in the patients, poor pharmacokinetic properties and, most importantly, development of resistance by HIV variants (Sierra et al., 2005).

The development of effective vaccines which exploit the immunologic vulnerabilities of HIV remains a dream millions of miles away due to the high mutation rate of HIV (Sabbah, 2014). However, due to the possibility of an increasing mortality rate resulting from HIV/AIDS, relentless efforts are focused on finding a lasting solution. Hence, further studies on HIV and a continuous search for effective anti-HIV agents is of great importance.

1.1.2 The Virology of HIV

1.1.2.1 The Structure and Genome of HIV

Based upon the Baltimore classification of viruses, HIV is a class VI virus, a member of the genus Lentivirus (a virus genus characterized by a long latency period) and belonging to the family *Retroviridae* (Baltimore, 1971). Structurally, it is about 120nm in diameter with an isometric, roughly spherical, nucleocapsid configuration. It is also enveloped with a lipid bilayer membrane. The genome of a mature HIV virion is made up of two identical copies of unspliced linear single stranded 9.2kb positive sense RNA (+ssRNA) enclosed in a cone shaped capsid. Unlike other positive sense RNA in which the RNA acts directly as mRNA during replication, the genome of retroviruses are unique because they serve as a template for the synthesis of linear double stranded DNA (dsDNA) through reverse transcription. The reverse transcriptase enzyme is encoded by the virus and packaged with the complete virion. Once synthesized, the viral dsDNA is integrated into the host DNA. At that point, the host cell biochemical machinery is used for further replication activities. This integration capability of HIV is one of the features

that makes it become latent and it enhances its ability to evade the immune system (Sierra et al., 2005).

The genome of a mature infectious HIV strain codes for at least nine genes, which result in the production of at least fifteen proteins. These encoded proteins have different functions with regards to the replication and viability of HIV. The proteins encoded by the HIV genome are generally classified as the structural proteins, the viral enzymes, the gene regulatory proteins and the accessory proteins (Sundquist and Kräusslich, 2012).

The group-specific antigen gene (*Gag*) and the envelope gene (*Env*) code for the structural proteins of HIV. The *Gag* encodes a 55KD Gag polyprotein, which is proteolytically cleaved by the action of an HIV protease into six structural proteins: the matrix protein (MA-p17), the capsid protein (CA-p24), the nucleocapsid protein (NC-p7), the phosphoprotein (p-6), the SP1 (spacer peptide 1, p2), and the SP2 (spacer peptide 2, p1) (Watts et al., 2009; Sundquist and Kräusslich, 2012). These six structural proteins are indispensable during the assembly and maturation stage of the HIV replication cycle. The matrix protein helps to recruit and concentrate the viral envelope proteins and to trigger the binding of packaged immature non-infectious virion to the plasma membrane of the host cell. The functions of the capsid proteins include the protection of the virus conformational integrity, facilitating the protein-protein interaction needed to assemble the virus genomic RNA and viral core proteins of the immature virion, and the formation of the cone shaped structure of the immature virion core during replication. The nucleocapsid protein plays a major role in the encapsidation of the viral genome during assembly and also enhances the reverse transcription of the viral genome during replication. The phosphoprotein (p-6) acts as a binding site for several accessory proteins

and cellular proteins of the endosomal sorting complexes required for transport. These accessory proteins and endosomal sorting complexes aid the release of the virions from infected cells through budding and also enhance HIV pathogenicity. The spacer peptide 1 and spacer peptide 2 help in the structural re-arrangement of HIV during maturation (Turner and Summers, 1999).

The *Env* gene codes for the envelope glycoprotein gp160 which is proteolytically cleaved during replication by furin, a cellular protease within the ER and Golgi apparatus of the host cell. Gp160 is cut into surface protein gp120 and transmembrane protein gp41 (Clapham and McKnight, 2002; Turner and Summers, 1999). Gp120 and gp41 are responsible for the spike-like projections at the surface of the HIV. Their main function is the attachment to the host cell and fusion of the virus to the host cell membrane.

The viral enzymes are encoded by the *Pol* gene. The *Pol* gene encodes the Pol polyprotein which serves as the precursor for the production of these viral enzymes: reverse transcriptase (RT), integrase (IN) and protease (PR, also known as aspartyl protease). The *Pol* gene is rarely expressed as a separate protein in most retroviruses but is usually expressed as a larger fusion protein such as Gag-Pol or Gag-Pro-Pol. The expression of the larger fusion protein is enhanced by the *Pol* open reading frame (Sundquist and Kräusslich, 2012). The RT is a heterodimer with two subunits- 66KDa and 55KDa subunit. The 66KDa (p66) subunit of 560 amino acids is made up of the polymerase active domain (RNA-dependent DNA polymerase) and the ribonuclease H (RNAse H) active domain. The 55KDA (p55) subunit of 440 amino acids is formed by the proteolytic cleavage of RNAse H domain from p66 (Wang et al., 1994). The RT catalyzes the reverse transcription of the +ssRNA viral genome into a linear dsDNA.

Upon successful transport of the viral dsDNA from the host cytoplasm into the nucleus of the host cell it is inserted into the host chromosome, becoming the proviral DNA. The RT is error prone during its transcriptional activities and this leads to the misincorporation of at least one nucleotide in every 2000-5000 polymerized nucleotides (Romani and Engelbrecht, 2009). The misincorporation of nucleotides by RT lead to a high number of HIV mutant strains during replication. The dsDNA has long terminal repeats (LTR) at both ends that are necessary for the integration of the ds viral DNA into the host chromosomal DNA (**Basavapathruni and Anderson, 2007)**. The integrase ensures successful integration of the proviral DNA into the host cell DNA. The integration of the viral DNA is a crucial step during viral replication because it ensures the evasion of the immune system. The main function of the protease is to cleave newly synthesized viral polyproteins in order to create an infectious HIV virion with the essential protein components (Mushahawar, 2006).

The gene regulatory proteins are encoded by the *Tat* and *Rev* genes. These genes are essential during HIV replication for the regulation of gene expression and also for productive infection in all cell types (Stark and Hay, 1998). The *Tat* gene encodes the Tat proteins, which binds to the promoter region of the proviral DNA (U3 region of the 5' LTR) and facilitates the transcription of the genes found in the integrated proviral DNA into nascent RNA transcripts. The Tat further acts by binding to the trans-activating response element (TAR) on the stem loop site of the nascent RNA transcript, leading to the recruitment of cellular proteins such as cyclin T, cyclin-dependent protein kinase-9 (Cdk9), and positive transcription elongation complex (P-TEFb). These recruitments lead to further transcription of HIV genes, transcription elongation, and production of a full

length viral RNA that serves as the genomic RNA (gRNA) (Frankel and Young 1998, Turner and Summers, 1999). The *Rev* gene encodes the Rev (regulation of expression of virion) proteins, which serve to shuttle the unspliced and incompletely spliced viral mRNA from the nucleus to the cytoplasm through its interaction with nuclear export factor. This shuttling mechanism is triggered after the attachment of the arginine-RNA binding motif of the Rev protein with the Rev-response element (RRE) located in the *Env* gene of the unspliced viral transcripts. The transport of the unspliced viral mRNA to the cytoplasm prevents the host RNA splicing machinery from acting upon the unspliced viral transcripts while in the nucleus. Thus, it ensures the production of structural proteins and a full length viral RNA genome of an infectious HIV virion (Strebel, 2003; Sierra et al., 2005).

The accessory proteins are encoded by the *Nef*, *Vif*, *Vpu*, *Vpx* and *Vpr* genes. The proteins encoded by these genes are called accessory proteins because their contribution to the establishment of productive HIV infections in proliferating T lymphocytes is negligible, but they are necessary for viral replication of HIV in non-dividing cell types (Stark and Hay, 1998). *Nef* encodes a 27KD Nef (negative regulatory factor) protein, which triggers immuno-modulation of the infected cells. Nef expression favors high viral replication, enhancement of HIV virulence, and disease progression to AIDS. The Nef protein triggers the reduction of cell-surface CD4 receptors on infected cells. This enhances the immune evasion mechanism of HIV and also prevents re-infection of infected cells by budding virions (Turner and Summers ael, 1999).

Vif encodes a 23KD highly insoluble Vif (viral infectivity factor) protein. The main function of Vif is to cause cellular degradation of APOBEC3G (apolipoprotein B

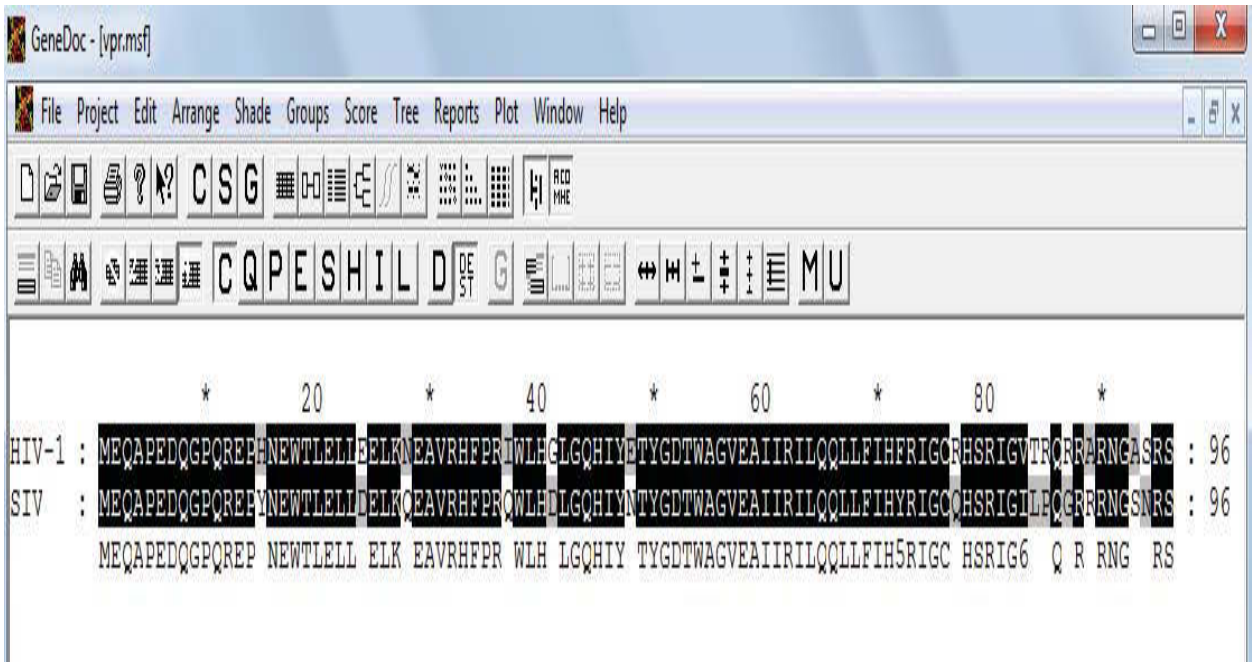
mRNA-editing, enzyme-catalytic, polypeptide-like 3G) or prevent the packaging of APOBEC3G into HIV particles. APOBEC3G is an antiviral host defense protein that induces deamination of viral cDNA, leading to hyper-mutation of newly synthesized HIV cDNA and the inhibition of viral replication (Strebel, 2003).

The *Vpu* gene encodes Vpu protein (viral protein u), a protein uniquely peculiar to the HIV-1 strain. Vpu has two major functions; the release of virions from the plasma membrane of infected cells, and the activation of the ubiquitin-proteasome pathway to aid in degradation of the HIV receptor CD4 protein in the ER of the infected cells before virion release. The endosomal degradation of the CD4 protein from HIV gp160 is done to enhance proteolytic cleavage of gp160 and to prevent the clumping together of HIV particles after its release from infected cells (Hussain et al. 2008; Turner and Summers, 1999).

The *Vpx* gene encodes Vpx protein (virion-associate protein x), a protein uniquely peculiar to the HIV-2 strain. The main function of Vpx is to increase the efficiency of viral replication in non-dividing cells (such as macrophage and dendritic cells) by counteracting the inhibitory effect of a host cellular protein known as SAMHD1 (SAM domain and HD domain-containing protein 1). SAMHD1 inhibits HIV replication by depleting the cytoplasmic pool of deoxynucleoside triphosphates available to reverse transcriptase to make viral cDNA (Laguette et al., 2012). In terms of genetic sequences, the *Vpx* gene shares great similarity with the *Vpr* gene. This similarity is suggestive of the possibility that *Vpr* gene underwent gene amplication, resulting in the molecular evolution of the *Vpx* gene (Ueno et al., 2003).

The *Vpr* gene encodes a 96 amino acid 14KDa protein usually expressed at the later stage of viral replication. This protein is known as Vpr (viral protein r) and it it well conserved among lentiviruses (Table 1). Due to its contribution to the pathogenicity of HIV and disease progression of HIV to AIDS through its numerous functions, it is imperative that the Vpr is incorporated into HIV-1 virions through its direct interaction with the p6 domain of the Gag protein (Romani and Engelbrecht, 2009).

Table 1: A pairwise sequence alignment of HIV-1 Vpr and SIV Vpr using the GeneDoc sequence alignment tool.



Amidst its numerous functions, Vpr is well known for its three major functions during viral replication, these are;

1. The establishment of a suitable environment which favors the persistent state of HIV infection at its chronic stage. This is achieved by preventing the clonal expansion of immunocompetent cells (T cells and other non-dividing cells) through the arrest of these proliferating cells at the G_2/M phase of cell cycle (Rogel et al., 1995). This function is

seen as being critical to the establishment of the immuno-suppressed state of the host due to the suppression of antibody production in arrested B lymphocytes and suppressed immune responses from antigen-specific CD8-mediated cytotoxic T-lymphocyte and T-helper type 1 (Romani and Engelbrecht, 2009). The molecular mechanisms that govern this arrest are initiated by the binding of the C-terminal part of Vpr and the C-terminal part of a host cellular protein known as 14-3-3 protein which forms a complex that interacts with Cdc25C phosphatase (M-phase inducer). This interaction inactivates Cdc25C phosphatase and hence stalls the mitotic phase of cell cycle (Kino et al., 2005). Further studies also showed that Vpr arrests infected cells at the G_2 phase of the cell cycle through the prevention of the dephosphorylation of the p34*cdc2*/cyclin B complex, resulting in its inactivation. This act delays the cell death of infected cells, which is believed to enhance high viral load (He et al., 1995).

2. The interaction of two distinct nuclear localization signals within Vpr's C and N termini with the nuclear transport pathways and machineries (e.g. importin α, importin β and nucleoporins) of the host cell facilitates the import of HIV pre-integration complex (PIC) from the cytoplasm of the host cell to the nucleus of host cell through the nuclear pore. The nuclear localization of pre-integration complex by Vpr enhances one of the properties of Lentiviruses, which is viral replication in non-dividing cells (Subbramanian et al., 1998). PIC is a nucleoprotein complex made up of the viral genetic material: dsDNA; viral proteins- Vpr, matrix proteins, integrase; and host cellular protein (such as barrier to autointegration factor 1 and high-mobility group protein I/Y) (Vodicka et al., 1998). Impaired nuclear localization of PIC by Vpr has been found in long term non-

progressors and this is an indicator that the impairment of Vpr could lead to the inability of HIV to undergo productive infection (Caly et al., 2008).

3. Enhancement of disease progression of HIV to AIDS through the depletion of infected $CD4^+$ T cells, infected non-dividing immunocompetent cells and non-infected or by-stander cells. This depletion is facilitated by the ability of the C-terminal peptides of Vpr to bind to host cell proteins, such as the permeability transition pore complex, adenine nucleotide translocator (ANT), proapoptotic members of the Bcl-2 family, and the voltage-dependent anion channel. These interactions induce pathways that result in mitochondrial membrane permeabilization and inward flow of sodium current. This membrane permeabilization leads to the release of cytochrome c, which results in the reduction of the mitochondrial membrane potential of the infected cells and ultimately results in programmed cell death or apoptosis of the cells (Le-Rouzic and Benichou, 2005; and Roumier et al., 2002).

Aside the above importance of Vpr to HIV replication, there are also other functions of Vpr that makes it important to the pathogenesis of HIV. These functions are as follows:

- **Modulation of gene expression:** The interaction of Vpr with host cellular proteins such as specificity protein 1, glucocorticoid receptor, transcription factor IIB, P300, nuclear factor κB (NF- κB), and activator protein 1 enhances the transactivation activities of the HIV-1 promoter at the LTR region. This transactivation activity induced by Vpr has been demonstrated to increase Nef expression prior to integration (Romani and Engelbrecht, 2009).

- **Suppression of immune cell activation and cytokine production: Vpr impairs the immunological functions of** macrophages and dendritic cells **by reducing the expression of immunologically important molecules (such as** CD40, CD80, CD83 and CD86) on these cells. Vpr's ability to stall the maturation of monocytes to dendritic cells has also been reported **(Muthumani, 2004). In addition, Vpr reduces the expression of** NF- κB, which is needed for the activation of T cells and the production of cytokines. A recent study has also suggested the ability of Vpr to impair NK cells activity and its ability to synthesize IFN-γ (**Majumder et al., 2008).**
- **Determinant of reverse transcription:** The reverse transcription of the viral genome is highly dependent on the presence of $tRNA^{Lys3}$-mediated priming. $tRNA^{Lys3}$ has to be deacetylated for effective priming. It has been demonstrated that Vpr binds to the Lys-tRNA synthetase (an enzyme that acetylates $tRNA^{Lys3}$) and inhibits the activities of this enzyme. Thus, Vpr promotes the priming of viral genome by deacetylated $tRNA^{Lys3}$ for reverse transcription to take place (Stark and Hay, 1998).
- **Ensuring the fidelity of reverse transcription:** The misincorporation of nucleotides during reverse transcription is seen as being beneficial to HIV, because it aids immune evasion and selection of multi-drug resistant variants. However, accumulation of these mutations could be detrimental to the HIV itself because it could lead to a non-functional HIV genome (Keele *et al.,* 2008). In order to avoid this, it has been demonstrated that the interaction of Vpr with a cellular protein, uracil–DNA glycosylase 2 (UNG2, which is a DNA-repair enzyme that prevents mutagenesis), leads to the incorporation of UNG2 into HIV particles. This ultimately prevents the excessive introduction of uracil into the viral genome. In the absence of Vpr, studies have shown that for every round of replication

there is a four- eighteen fold increase in the rate of G→A transition mutation (Chen *et al.,* 2002).

- **Cytopathogenicity determinant:** The extent of $CD4^+$ T cell depletion has been thought to be determined by the level of viremia. This notion was disproved when a study was carried on a long term non-progressor who had a normal CD4 count, similar to that seen in an uninfected person, even in the presence of an unusually high viral load. The result of this study showed that the insertions of both premature stop codons and an unusual Q3R polymorphism in Vpr genes led to the impairment of CD4 depletion. This result was an indicator of the important role of Vpr in viral cytopathogenicity (Somasundaran et al., 2002).
- **Neuronal dysfunction:** A recent study has shown the ability of Vpr to induce mitochondrial dysfunction in neurons. This leads to the distortion of neuronal communication and the inhibition of neuronal development (Rom *et al.*, 2009; Hiroko *et al.*, 2008).

1.1.2.2 The Genomic Differences in HIV Strains

The lack of proof-reading ability during HIV replication has resulted in numerous HIV mutant strains. However, based on some genetic similarities between these numerous strains, HIV can be broadly classified into two types- HIV-1 and HIV-2. Although HIV-1 and HIV-2 infections result in clinically undifferentiated AIDS, there are other differences between these viruses. HIV-1 group M is the most common and virulent strain of HIV, accounting for approximately 90% of reported AIDS cases. On the other hand, HIV-2 has a low incidence rate; it has no pandemic potential (rarely found outside the belt of West Africa and East Africa) and the period between initial infection

and manifestation of AIDS is longer when compared HIV-1 (Hemelaar et al., 2004). In addition, there are several genomic differences between HIV-1 and HIV-2. The *Vpu* gene is unique to HIV-1, with few sub-types of HIV-1 having a rare gene known as *Tev*. *Tev* is a regulatory gene that encodes a 28KDa protein known as p28tev. *Tev* is formed by the fusion of the functional domains of *Tat* and *Rev* with a small portion of *Env*. Thus, p28tev exhibits the regulatory properties of Tat and Rev proteins (Benko et al., 1990). On the other hand, the *Vpx* gene is unique to HIV-2 (Ueno et al., 2003). The genomic differences between HIV-1 and HIV-2 are a contributing factor in their differences in virulence (Hussain et al. 2008).

1.1.2.3 The Replication Cycle of HIV

The number of circulating $CD4^+$ T cells and their rate of depletion is a predictor of HIV disease progression to AIDS. The replication cycle of HIV-1 is initiated primarily by the attachment of the virus to the cell surface of $CD4^+$ T-helper cells. However, viral replication also takes place in some other suitable host cells. The binding of HIV-1 to the cell surface of $CD4^+$ cells is facilitated by the specific interaction of one or more of the extracellular domains of HIV-1 gp120 trimeric spikes with the amino-terminal immunoglobulin domain of the CD4 receptors on $CD4^+$ cells and also with one of the seven transmembrane chemokine co-receptors (notably CCR5 and CXCR4) on $CD4^+$ T cells (Turner and Summers, 1999; Sierra et al., 2005).

The type of co-receptor used as a co-entry factor is a marker for the cytopathogenicity of the HIV variants, the degree of replication kinetics of the HIV variants and its ability to form syncytia. Highly cytopathic, syncytia-inducing and high replicating HIV variants use CXCR4 as a co-receptor. Less cytopathic and non-syncytia-

inducing HIV variants use CCR5 as a co-receptor; while dual-tropic or X4R5 HIV variants use either of the co-receptors (Somasundaran et al., 2002). Use of CCR5 as a co-receptor for viral entry is a prominent feature of HIV-1 primary infection of the lymphoid cells, while use of CXCR4 as co-receptor is a characteristic of the more advanced stage of HIV infection (Llano et al., 2001). On the other hand, interactions of gp120 with CD4 or other cell surface receptors, such as lectin-like domains, glycolipid galactocerebroside and its sulphated derivatives, just to mention a few, facilitates binding to the surfaces of cells such as monocytes, macrophages, dendritic cells, colon epithelial cell lines, neurons and microglia in the brain (Clapham and McKnight, 2002).

The interaction of gp120 with one of the chemokine co-receptors leads to a conformational change in the gp120, which in turn leads to the exposure and unfolding of the fusion domain of the gp41. The exposed and unfolded fusion domain of gp41 inserts its hydrophic terminus into the host cell membrane, forming a fusion pore, and then folds back on itself to bring the viral envelope and host cell membrane into closer proximity. These events facilitate the fusion of both membranes, and the release of the viral core into the cytoplasm of the host cell. This cytoplasmic release is subsequently followed by the un-coating of the viral core to release the viral genome and the viral enzymes (Sierra et al., 2005; Turner and Summers, 1999).

While in the cytoplasm, the host $tRNA^{Lys3}$ serves as a primer by annealing to the 5' end of the viral ssRNA, which ultimately leads to the initiation of reverse transcription (Jones et al., 2013; Turner and Summers, 1999). The primed viral ssRNA is transcribed into a RNA-DNA double helix hybrid by the polymerase active domain of the RT. In addition to the primer, this RT-dependent DNA synthesis is also aided by the

nucleocapsid proteins. The primer and the RNA component of the RNA-DNA double helix hybrid are proteolytically cleaved by the RNAse H active domain of the RT to form a negative sense ss cDNA. Once the cleavage is successful, the DNA synthesis resumes once again to complete the formation of a dsDNA helix from the negative sense ss cDNA (Götte et al., 1999). The integrase then acts on the dsDNA by cleaving a dinucleotide from each 3' end of the DNA, creating two sticky ends, which enhances viral dsDNA integration into the host DNA. For productive infection, it is imperative that the Vpr aid the nuclear import of the pre integration complex from the cytoplasm to the nucleus of the host cell. Proviral DNA integration into the host DNA must also be aided by the integrase (Turner and Summers, 1999; Sierra et al., 2005). During this early phase of HIV replication, the Vif cleaves and prevents the expression of the inhibitory effects of host cellular proteins (APOBEC3G) on reverse transcription.

Once integration of the proviral DNA is achieved, transcription is aided by cellular RNA polymerase II to synthesize unspliced and spliced mRNA transcripts. The spliced mRNA transcripts are formed through a series of pre-mRNA splicing events, such as the recruitment of exon junction complex, capping at the 5' end, and cleavage and polyadenylation at 3' end. Specific interactions of the spliced mRNA transcripts with the transcription export complex (TREX) ensure nuclear export of the transcript to the cytoplasm (Swanson et al., 2010). Further splicing of some exported spliced mRNA transcripts to form sub-genomic mRNAs (fully spliced mRNA) are ensured once in the cytoplasm, while some are not further spliced (singly spliced mRNA containing functional introns). Cellular factors such as necrosis factor κB (NF-κB) are recruited to the long terminal repeat promoter region of the fully spliced mRNAs to trigger ribosomal

translation and to stimulate the production of Nef, Tat and Rev; the translational process of the singly spliced mRNAs leads to the expression of Env, Vif, Vpr and Vpu (Turner and Summers, 1999; Sierra et al., 2005; Swanson et al., 2010).

The nucleus bound unspliced-intron containing mRNA transcripts are of great importance to the HIV for the formation of gRNA of an infectious virion, expression of structural proteins (gag and gag-polymerase), expression of enzymatic proteins, and to promote virion assembly (Swanson et al., 2010). HIV employs a splicing-independent gene expression mechanism mediated by specific SR proteins- SRp40 and SRp55 (SR acronym is coined due to the presence of a domain with long repeats of serine and arginine amino acid residues in the proteins) and the Rev proteins to aid the recruitment of host nuclear export factors (such as nuclear shuttling protein exportin and Ran guanosine triphosphatase) to the unspliced transcripts in order ensure the expression of the aforementioned importance of the unspliced transcripts (Turner and Summers, 1999; Strebel, 2003). These recruitments facilitate nuclear export of the unspliced transcripts to the cytoplasm, which are then translated by polyribosomes into polyproteins. The proteolytic cleavage of the polyproteins by the viral protease ensures the formation of the viral core proteins which are needed for the formation of an infectious virion (Sierra et al., 2005).

The proteolytic products of the Gag polyprotein direct the assembly and packaging stage of the replication cycle of HIV at the plasma membrane of the host cell. In addition, these proteolytic products also facilitate the budding and maturation stage of the HIV replication cycle. The nucleocapsid proteins, two viral copies of ssRNA, host cellular $tRNA^{Lys3}$ and the three viral replicating enzymes assemble together; with the

capsomere proteins assembling around these particles to form the conical shaped capsid. The capsid enhances the packaging of all the virion components needed for infectivity. The release of the immature virion particles from the infected host cell is achieved through budding. As the virion particles bud off from the plasma membrane of the host cell, it acquires its lipid envelope from the membrane of the host cell along with the viral env proteins which forms the characteristic spike-like projections on the viral envelope (Sundquist and Kräusslich, 2012).

Subsequent to the release of the immature virion particles, a series of events occur in order to enhance the maturation and infectivity of the released immature virions. The viral protease encoded by the Gag-Pro-Pol is expressed, resulting in the proteolytic cleavage of this polyprotein to produce fully processed structural proteins and viral replicating enzymes. The processed proteins and enzymes re-arrange, with the spacer proteins aiding the transformational configuration of the virions, maturation of the virions and ultimately the ability of the mature virions to infect new host cells (Sundquist and Kräusslich, 2012).

1.1.3 The stages of HIV-1 disease

The CDC has classified HIV infection into three stages based upon the number of circulating $CD4^+$ T cells. The $CD4^+$ T cell count is an indicator of the required anti-retroviral therapy needed in order to ensure the proper management of an HIV infection, AIDS and the opportunistic infections associated with HIV/AIDS (Vajpayee et al., 2005).

1.1.3.1 Stage 1

The primary HIV infection is characterized by viral replication in activated $CD4^+$ T cells, flu-like non-specific symptoms, mononucleosis, lymphadenopathy, possible

systemic viral dissemination to the central nervous system, high viral load of non-syncitia inducing variants, development of elevated levels of HIV-specific cytotoxic $CD8^+$ T cells, progressive depletion of memory $CD4^+$ T cells and naive $CD4^+$ T cells (CD4 count $\geq$ 500 cells/µl) due to its destructive replication in the immature cells of the thymus, bone marrow, lymph nodes and gastrointenstinal epithelial cells of the host. (Llano et al., 2001).

1.1.3.2 Stage 2

The clinical latency (asymptomatic infection) can last an average of 8 years without anti-retroviral therapy and up to 20 years in long term nonprogressors without symptoms of infection. This stage of HIV infection is characterized by infection of resting memory cells of the immune system (viral reservoirs), fairly stable $CD8^+$ T cells total count, depletion of $CD8^+$ naive T cells, impairment of HIV-specific $CD8^+$ T cells effector functions, a high reservoir of HIV-specific $CD8^+$ memory T cells, a continuous decrease in $CD4^+$ T cells (CD4 count 200 to 500 cells/µl). In addition, this stage is also characterized by persistent and steady HIV replication with a low detectable viral load when compared to acute infection, extra-chromosomal viral DNA, and inhibition of reverse transcription, The clinical latency stage of HIV infection could also be induced if an HIV infected person is treated with highly active anti-retroviral therapy (Sierra et al., 2005; Ray et al., 2006).

1.1.3.3 Stage 3

The AIDS stage or advanced stage of HIV infection is characterized by the activation of the virus from latency, cachexia, the emergence of syncytium inducing variants, up-regulation of CXCR4 co-receptors, and lymphocytopenia (alarming

depletion rate of $CD4^+$ T cells population usually below 200cells/µl and depleted levels of $CD8^+$ T cells). This stage is also characterized by the inability of $CD8^+$ effector T cells to recognize the HIV genomic sequence and to exhibit its cytotoxic properties (secretion of antiviral factors), due to the high HIV mutational rate. As a result, there is high emergence of opportunistic infections (such as toxoplasmosis, pneumocystis pneumonia, tuberculosis, and chronic diarrhea) and opportunistic cancers (Kaposi's sarcoma, Burkitt's lymphoma) (Llano et al., 2001; Holmes et al., 2003; Ray et al., 2006).

The exploration of the complex inter-relationship between HIV pathogenesis and the host immune system is of importance in order to understand the intricacies of how HIV infections renders the immuno-competent cells defenseless to itseffects.

1.1.4 The Immunopathogenesis of HIV-1

The onset of the immunopathogenesis of HIV-1 can be attributed to viral replication in lymphoid tissues and its resulting depletion of lymphocytes (Metroka et al., 1983). The severity of HIV infection and the rate of disease progression, as determined by the rate of $CD4^+$ T cell depletion, is predominantly dependent upon virulence of the HIV or the type of HIV variant. Disease progression is also influenced by the presence of co-infections and the efficiency of the host defense mechanism (Sierra et al., 2005; Romani and Engelbrecht, 2009).

On primary exposure to HIV, the humoral immune system could either be over-stimulated or under-stimulated. Hyperactivation of the humoral immune system leads to immediate elicitation of immune response with secretion of antibodies characterized with having very weak neutralizing potential against HIV due to mutations in some specific sites on the HIV epitopes (Wei et al., 2003). The hyperactivation of the humoral immune

system can lead to B-cell lymphomas, polyclonal hyperglobulinemia, and autoimmune disease due to secretion of high plasma levels of auto-reactive antibodies. On the other hand, leukopenia or hyporesponsiveness (under-stimulation) of the protective antibody response of the humoral immune system leads to immunosuppression (Conge et al., 1998).

The effectiveness of these neutralizing antibodies is dependent upon the HIV variant. Although, the presence of non-syncitium inducing HIV variants during primary infection does not have much effect upon $CD4^+$ T cells depletion, the rate of $CD4^+$ T cells depletion increases with the emergence of the syncytium inducing HIV variant at a more advanced stage of HIV infection. Neutralizing antibodies produced by the humoral immune system have little or no effect in fighting against HIV infection and in the prevention of T cell depletion. (Llano et al., 2001).

The continuous increase in viral load following the primary infection triggers cell-mediated immune response (i.e. proliferation, maturation and expansion of $CD8^+$ T cells). $CD8^+$ T cells bind to the peptides presented by MHC-1 on infected cells and exert their cytolytic activity by the secretion of perforin, which creates a portal of entry for cellular proteases, such as granzyme, secreted by $CD8^+$ T cells. The T cell's cytolytic activities help to inhibit the production of progeny virions and lower the plasma level of HIV (Hersperger et al., 2010). In addition, the interaction of the Fas ligand on $CD8^+$ T cells and Fas molecules on infected cells results in the lysis of the cell by apoptosis (Hadida et al., 1999). Activated $CD8^+$ T cells are known to secrete some antiviral cytokines, such as IFN-γ, which inhibit the infection of neighboring cells, and the secretion of IL-2 which helps to increase $CD4^+$ T cells by impairing the replication competency of HIV through

the integration of the host restriction factor (APOBEC3G) during replication (Oguariri et al., 2013). Other $CD8^+$ cytokines include CCL5, MIP-1α and MIP-1β, which inhibit HIV binding to the CCR5 co-receptor (Cocchi et al., 1995). CAF(CD8 antiviral factor) inhibits viral transcriptional activities during HIV replication (Mackewicz et al., 1995), while SDF-1 prevents HIV entry by blocking the CXCR4 co-receptor (Oberlin et al., 1996). IL-2 has been proposed to be a contributing factor in the development of long term non-progressors, as it curtails viral replication. However, the antiviral ability of IL-2 is still under investigation.

The induced activation of $CD8^+$ T cells by HIV-1 results in the expression of high levels of CD38 on $CD8^+$ T cells. CD38 is a molecule involved in cell adhesion, signal transduction and calcium signaling by $CD8^+$ T cells. Successful highly active antiretroviral therapy has been characterized with low expression of CD38. Thus, the expression levels of CD38 is directly proportional to HIV disease progression and the expression levels of CD38 can be adopted as one of the predictors of HIV disease progression, (Savarino et al., 2000). The production of high number of HIV variants due to lack of proof-reading during replication and the induction of programmed cell death of immune cells has enhanced HIV's ability evade the immune system and cause immunosuppression. This mutational rate remains one of the greatest challenges in the immune response to HIV and the treatment of this disease.

There are several virulence factors that contribute to HIV disease progression. Some of these virulence factors include impairment of post binding lytic events in NK cells by HIV (Grassi et al., 1999), and impairment of antigen presenting activities to T cells in macrophage infected cells (Ennen et al., 1990). HIV's oversaturation of the entire

surface of dendritic cells also leads to a decrease in the number and functionality of dendritic cells. As a result of deficiencies in T cell function, a concomitant decrease is also seen in the activation of B cells. . In addition to those virulence factors, host cellular immune dysfunction and dysregulation are also contributing factors that could favor HIV disease progression. Some of these host factors include; inefficient T-cell proliferation, the inability of immunocompetent cells to produce cytokines with antiviral activities, or the production of cytokines that enhance virus replication and HIV survival (Clerici et al., 1989).

In order to compensate for the depletion of $CD4^+$ T cells, IL-7 is synthesized to enhance rapid proliferation of CD4+ cells, and differentiation and maturation of immature thymocytes into T cells. IL-7 unfortunately also increases viral production due to the availability of more $CD4^+$ T cells needed for more virus replication. IL-7 also induces the expression of CXCR4. Hence, IL-7 is not a good therapeutic option during suppressive anti-retroviral therapy, due to its ability to promote survival, persistence and progression of HIV- infection by increasing the rapid proliferation of resting memory $CD4^+$ T cells (reservoirs) that harbors replication-competent HIV-1 (Vandergeeten et al., 2013). In addition, cytokines such as IL-4, IL-6, IL-12, TNF-α and colony stimulating factor enhance viral replication (Oguariri et al., 2013; Dezube et al., 1997). The synthesis of IL-4 during HIV- infection favorably select for the replication of highly virulent HIV variants by decreasing the expression of CCR5 co-receptor and enhancing the expression of CXCR4 co-receptor (Llano et al., 2001). The inability of the host immune system to effectively fight HIV infection and put a stop to its spread indicates a need for more potent therapeutic agents.

1.2 Anti-HIV and The Emergence of Resistance

Anti-retroviral drugs exert their antiviral effects by targeting a particular phase of HIV replication cycle. There are six classes of anti-HIV drugs presently known, and they are listed below with examples (Sierra et al., 2005; Mathieu et al., 2013; Kondru et al., 2008);

1. Nucleoside reverse transcriptase inhibitors (NRTI): Zidovudine and Tenofovir.
2. Non- nucleoside reverse transcriptase inhibitors (NNRTI): Nevirapine and Delaviridine.
3. Protease inhibitors (PI): Lopinavir and Indinavir.
4. Fusion inhibitors (FI): Maraviroc and Enfuvirtide.
5. Integrase inhibitors: Elvitegravir and Dolutegravir.
6. CCR5 antagonists or Entry inhibitors (EI): Aplaviroc and Maraviroc.

The discoveries of these drugs have had little effect on our ability to solve the menace of HIV. The hyper-variability of HIV, patient non-compliance to medication regimens, and poor management of medication therapy are some of the reasons for the emergence of multi-drug resistant HIV, even when these drugs are administered as combinations of drugs (HAART) (Kiertiburanakul and Sungkanuparph; 2009). Much effort are still focused on the research for more potent novel drugs such as ViroStatics, Tat inhibitors, nucleocapsid inhibitors, CXCR4 co-receptor inhibitors, just to mention a few (Sierra et al., 2005; Lori et al., 2005). The development of HIV vaccines (genetically engineered HIV genes in viral vectors) has had little success, primarily due to the hyper-variability of HIV. In addition, the effectiveness of the new potential HIV vaccine

candidates are also been threatened by this same problem (Sabbah, 2014; Thomas and Michael, 2014).

In response to the adverse effects seen with HARRT, the use of a more advanced combination therapy, known as antiretroviral salvage therapy (new antiretroviral drugs against novel targets) seems to be a plausible solution to the emergence of multi-drug resistant HIV (Imaz et al., 2011). However, determination of the aggressiveness of the HIV variant by early or proper diagnosis could enable a better medication therapy management and could ultimately be a good preventive measure that would reduce the risk of selecting for multi-drug resistant HIV resulting from bad therapy.

In view of this, as much as new drugs and vaccines are being researched, there should also be a focus on how to improve the diagnosis of HIV. Of all the Lentiviral proteins (HIV inclusive), Vpr is one of the most highly conserved, indicating that it is indispensable to the pathogenesis of the Lentiviruses (Table. 1) (Stark and Hay, 1998). This makes Vpr as one of the best candidates for use during HIV screening.

1.3 Genetic Modification of Viral Protein r and Its Potential Advantages

Multi-drug resistant HIV has been a huge problem in the fight against HIV disease progression. The inefficiency of antiretroviral therapy to fight multi-drug resistant HIV and the difficulties associated with the development of an HIV vaccine have led to exploring other means of finding a more efficient novel anti-HIV agent. A research group led by Serio explored the possibility of trying to disrupt the normal events involved in viral maturation. Due to the numerous functions of Vpr in HIV pathogenesis, a genetically engineered dysfunctional Vpr was developed in order to alter the normal sequence of events associated with viral maturation. This dysfunctional Vpr is known as

chimeric Vpr (Vpr-C). Vpr-C has specific HIV-1 genetic sequences added to its C-terminus. These genetic sequences are similar to the nine cleavage sites of Gag and the Gag-pol precursors of HIV-1. In virions with Vpr-C containing the cleavage sequences from the junction of p24 and p2, the production of incompletely processed non-infectious virion particles was noticed. This was due to the ability of the protease cleavage signal on Vpr-C interfering with the normal activities of the viral protease (Serio et al., 1997). This data emphasizes how vital the Vpr is to the HIV.

In view of this, it would be worthwhile if hybridoma technology could be exploited to develop monoclonal antibodies against Vpr with the core focus of bettering HIV diagnosis and ultimately contributing to the fight against multi-drug resistant HIV.

1.4 Hybridoma Technology and Monoclonal Antibodies (MAbs)

1.4.1 Overview

For effective elimination of antigens, the humoral immune response is characterized by the production of pools of polyclonal antibodies from activated B cells (Plasma cells). These polyclonal antibodies react to many different molecules on the surface of the pathogen. Although the effectiveness of polyclonal Abs in terms of antigen clearance is unquestionable, its application in therapeutics and medical research is limited by lack of specificity, inconsistent production and short life span (Lipman et al., 2005).

In order to produce an unlimited supply of antibodies specific for one molecule, César Milstein and Georges J. F. Köhler in 1975 fused murine myeloma cells and murine plasma cells to form hybrid cell lines (hybridomas) (Köhler and Milstein 1975). The experiment was based on the exploitation of the intrinsic ability of murine myeloma cells to grow indefinitely and the ability of murine plasma cells to produce antibodies. The

myeloma cells used were unable to produce the HGPRT (hypoxanthine-guanine phosphoribosyltransferase) and thymidine kinase enzymes needed for cell division and nucleotide synthesis by the salvage pathway when grown in the presence of HAT (hypoxanthine-aminopterin-thymidine) medium (Schlosser et al., 1981; Köhler and Milstein, 1975). In order to grow in the presence of HAT, the myeloma cells had to be fused to the plasma cells which provided the HGPRT or TK enzyme. This allowed the investigators to eliminate unfused myeloma cells from the cultures. Plasma cells that were unfused to myeloma cells would die naturally within a week. The successful fusion of these cells (myeloma and plasma cells), made the dream of continuously producing antibodies of predefined specificity or the continuous production of monoclonal antibodies also known as MAbs a reality. The success of this experiment demonstrated a new immunological technique with many applications known as Hybridoma technology.

1.4.2 Therapeutic and Diagnostic Applications of MAbs in HIV

Murine MAbs are immunogenic in man, with the possibility of its recipients developing allergic reactions or developing a human anti-mouse antibody (HAMA) response, due to the difference between the murine and human immune systems. In view of this, the HAMA response reduces the half-life of the administered murine MAbs and limits the use of murine MAbs during immunotherapy. However, better tolerable MAbs (Chimeric, Humanized and Human MAbs) have been engineered to address this problem (Mitchell and Cheryl, 2001). In addition, during therapeutic applications, these engineered MAbs could either be used as naked MAbs or as conjugated MAbs (linked to a radioactive substance, drug-activated enzymes or a cytotoxin) (Nabi and Doerr, 1992).

The therapeutic and diagnostic application of MAbs has impacted most of the diseases known to man, such as cardiovascular diseases, respiratory diseases, cancer, malaria, hormonal disorders, autoimmune diseases, treatment of septic shock, just to mention a few. Therapeutically, recent studies on MAbs have revolutionized anti-HIV research. The MAb VRC01 is seen as a potential candidate to reduce the risk of mother to child HIV transmission (Voronin 2014). A milestone was attained in anti-HIV research when a research group drastically reduced the viral load of rhesus monkeys that were chronically infected with the pathogenic simian–human immunodeficiency virus SHIV-SF162P3 after been treated with a cocktail of N332 glycan-dependent MAb (PGT121) and CD4-binding-site-specific MAbs (3BNC117 and b12) (Barouch et al., 2013)

In terms of HIV diagnostics, one of the early works on the use of MAbs for the identification of HIV-1 and 2 was the generation of A4F6 (anti-capsid antibody), R5C4 (anti-matrix antibody) and R5F6 (anti-matrix antibody) that were reactive against HIV-2 isolates. RIC7 (anti-capsid antibody); MAbs were also generated that were cross reactive with HIV-1, HIV-2 and SIV isolates (Minassian et al., 1988). Recent studies have developed anti-CCR5 MAbs for the diagnosis of HIV-1 (William and Jeffrey, 2009). In addition, two MAbs (3B3 and 16H3) were able to cross react with 8 subtypes of Env proteins, proving the ability of MAbs to retain its binding affinity even with the hyper variations in HIV proteins (Gao et al., 2009).

In the light of the above achievements of MAbs in anti-HIV research, the use of MAbs appears to open windows of opportunities to fighting the menace of HIV. Hence, improvement in HIV diagnosis will result from developing MAbs against a highly conserved HIV protein (Vpr). Measurement of the amount of Vpr present could be an

indicator of the aggressiveness of the HIV strain and could help in tailoring a better ART in the bid to reduce the risk of selecting for multi-drug resistant HIV.

CHAPTER II: MATERIALS

A hybridoma starter pack, Hypoxanthine-Aminopterin-Thymidine (HAT) 50X, Hypoxanthine-Thymidine (HT) 50X, Aminopterin 50X, 8-Azaguanine 50X, Polyethylene Glycol (PEG) MW 3000-3700; OPI 100X (1mM oxaloacetate, 0.45 mM pyruvate, 0.2 U/ml insulin); L-glutamine 200 mM (100X) stock solution were purchased from Sigma-Aldrich, Co. (St. Louis, MO).

Qualified Fetal Calf Serum was purchased from GibcoBRL Life Technologies (Grand Island, NY).

Murine myeloma cell line P3x63-AG8.653; human fetal lung adherent fibroblast MRC-5 cells were purchased from American Type Culture Collection (Manassas, VA).

All hybridomas tested were produced in the Fagan laboratory. Hybridomas produced prior to this study include DF.VPR (produced by Diana Fagan), EH.VPR (produced and subcloned by Esther Huang and purified by Devon Archinal) and Dl.VPR (produced by Dan Lisko).

RPMI medium 1640, MEM with Earle's salt, were purchased from GibcoBRL life technologies (Grand Island, NY).

BalbC mice were purchased from Charles River laboratories (Spencerville, OH)

Casein block was purchased from Thermo Fisher Scientific Inc. (Salineville, OH).

CHAPTER III: REAGENTS AND SOLUTIONS

Blocking buffer : PBS containing 1% BSA or Casein block.

MRC-5 media: Minimal essential media containing Earle's salts and non-essential amino acids, 1.5 g sodium bicarbonate (tissue culture tested) per liter, pH 7.2-7.4. Other additions to the media were 2 mM L-glutamine (1ml of 100x/100ml), 10umM Sodium pyruvate (1 ml of 100x/100ml), 10% FCS (previously heat inactivated at 55-60^{0}C for 45 min).

P3X media: RPMI 1640 media, 2 g sodium bicarbonate (tissue culture tested) per liter, pH 7.2-7.4. Other additions to the media were 2 mM L-glutamine (1ml of 100x/100ml), 20% Fetal calf serum (previously heat inactivated at 55-60^{o}C for 45 min).

Poly ethylene glycol (PEG): was prepared by warming it at 60^{0}C and then diluted to 50% (.5 g PEG + .5 ml media + 50 ul DMSO) with RPMI without FCS. After the addition of media, the PEG was maintained at 37^{0}C until the fusion was performed.

Phosphate buffered saline (PBS) working solution: 10X Stock solution of PBS was diluted 1:10 in MilliQ H_2O to a final concentrations of 0.01M phosphate and 0.15 M NaCl.

Sample buffer (PBS, 0.1% BSA and 0.05% Tween-20): 50 ml of PBS, 0.05 g BSA,and 25 μL of Tween-20; or PBS, 0.05% Tween-20 and 10% casein block.

Tris-buffered ammonium chloride: mix 90 ml of 0.16 M NH_4CL and 10 ml of 0.17 M Tris. The pH of the buffer was adjusted from7.65 to 7.2 by adding HCl.

Wash buffer (PBSand 0.05% Tween-20): 500 ml of PBS and 250 μL of Tween-20.

CHAPTER IV: METHODS

4.1 Fusion of murine myeloma cell line with murine spleen cells

The first immunization was done subcutaneously at the neck region of the mouse with 50 µg of Vpr in complete Freund's adjuvant (10 µg/ml of protein from *Mycobacterium tuberculosis* and incomplete Freund's adjuvant at 1:10). After three weeks, the second immunization was done intra-peritoneally with 50 µg of Vpr in incomplete Freund's adjuvant. Three weeks after the second immunization and three days prior to cell fusion, the Vpr immunized mouse was given a final i.v. boost of 50 ug of Vpr antigen in PBS. The final immunization was done by suspending 50 µg of Vpr in saline (0.85% Nacl). 100 µl of this antigen preparation was injected intravenously and 100 µl of this antigen preparation was injected intra-peritoneally (Harlow and Lane, 1998).

MRC5 feeder cells (2 x 10^4/well) were added to a 24 well plate the day prior to fusions. Prior to cell fusion, all media were pre-warmed to 37^0C. P3X murine myeloma cells werel sub-cultured 24 hours prior to fusion. The spleen from the Vpr immunized mouse was teased in a petri-dish containing RPMI to release antibody-secreting spleen cells. The cells were pelleted and resuspended in Tris-NH4Cl (0.1 ml packed cells/ml Tris-NH_4Cl) to lyse contaminating red blood cells. The resuspended cells were held at room temperature for 2 mins. After 2 mins, the resuspended cells were underlaid with FCS and centrifuged at 300 x*g* for 10 mins at room temperature. The supernatant was discarded; the cell pellets were resuspended in 10 ml of RPMI (Harlow and Lane, 1998).

In a 50 ml conical tube, spleen cells and myeloma cells were placed together at a ratio of 5 spleen cells (approximately 1 x 10^8) to 1 myeloma cell (approximately 2 x 10^7)

and were washed twice in serum-free RPMI by centrifuging at 200 x*g* for 8 mins. The supernatant was discarded; 1 ml of 50% PEG was added to the cell pellet over a period of 1 min while shaking the conical tube. Subsequently, 1 ml of serum-free RPMI was added over a period of 1 min while shaking. Serum-free RPMI (8 ml) was then added over 5 mins. The tube was then filled with serum-free RPMI, centrifuged at 200 x*g* for 8 mins, and the resulting supernatant was discarded and the cell pellet was resuspended in 24ml of RPMI containing 20% FCS, L-glutamine and OPI. MRC-5 media was aspirated from the previously seeded 24 well plate, 1 ml of the reuspended cell pellet was dispensed in each well of a 24 well plate containing MRC-5 feeder cells and incubated at 37^0C in a water jacketed 5% CO_2 incubator (Harlow and Lane, 1998).

At 24 hrs, 1 ml of 2X HAT media (100X HAT diluted 1:50 in RPMI + FCS) was added to each well and at 4-5 days following fusion, 1 ml of 2X HAT media was removed and 1 ml of fresh 1X HAT media (100X HAT diluted 1:100 in RPMI + FCS) was added. OPI was also used in addition to the 1X HAT media until the wells in the 24 well plate were two thirds confluent. The acidity level of the media was checked every two days and if the media turns yellow or the cells were rapidly growing, the cells were fed with fresh 1X HAT media. At day 14, the cells were fed with 1X HT media every two days for a week and subsequent feedings were done with P3X media, until cells per well of the 24 well plate were 2/3 confluent. For each well that was 2/3 confluent, 300 µl of supernatant was collected in a microfuge tube and then the cells were transferred to a six well plate. When the cells were two thirds confluent in the six well plate, they were transferred to a T25 flask containing 5 ml of P3X media. When cells were approximately 1/2 confluent in the T25 flask, the cells were frozen in cell culture media containing 10%

DMSO and stored in liquid nitrogen. For future use of the frozen monoclonal cells, Cells were thawed quickly in 5 ml of P3X media. The cells were then centrifuged to remove the DMSO and resuspended in P3X media (Harlow and Lane, 1998).

4.2 ELISA

Wells of a polyvinyl chloride plate were coated with 100 µl Vpr (1ug/ml Vpr in 50 mM sodium carbonate buffer, pH 9.6) and incubated for 2 hrs at room temperature. After 2 hrs, each well was aspirated off and washed with 200 µl of wash buffer. Wash buffer was aspirated off and the wells were incubated with 200 µl of blocking buffer (PBS + 0.05% Tween-20 + 10% casein block) at 37^0C for 30 mins. Blocking buffer was aspirated off and the wells were coated with100µl antibody in sample buffer (positive control diluted 1:1000 in sample buffer), 100µl sample buffer alone (negative control), or 100 µl hybridoma supernatant (test) and incubated for 30 mins at 37^0C. The plates were washed with wash buffer 3 times. Goat anti-mouse Ig-peroxidase conjugate (100 µl of 1:1000 in sample buffer) was added to all wells and incubated 30mins at 37^0C. After 30 mins, the plates were washed with wash buffer 3 times, and then 100 µl TMB substrate was added to each well and incubated at room temperature until a color change was seen. Subsequently, 50 µl stop solution (2 N $H_2SO_{4)}$ was added as soon as color change was noticed in any of the wells. Absorbance was read at 450 nm with the aid of a microplate absorbance reader (Sinha et al., 1999).

4.3 MRC-5 Trypsinization

MRC-5 media was aspirated off the MRC-5 cells and the cells were washed with PBS. Trypsin-EDTA (1 ml) was added to the adherent MRC-5 cells and incubated for 5

mins at 37^0C. Cells were checked under a phase contrast microscope. As soon as cells rounded up, the T25 flask was tapped in order to dislodge the cells. The dislodged cells were resuspended cells in 5 ml RPMI (Harlow and Lane, 1998).

4.4 Subloning of cells by limiting dilution

100 µl of trypsinized feeder cells (MRC-5 in MRC-5 media, 5 x 10^4/ml) were added to each well in a 96 well plate and incubated overnight. MRC-5 media was aspirated off and 100 µl P3X media containing OPI was added to every well. Hybridoma cells (100 □l of rapidly growing cells) were added to the top left hand well (A1) and 1:2 serial dilutions were performed down the left hand row (row 1) of the 96 well plate. P3X media (100 ul) was then added to row 1. Serial 1:2 dilutions across were then performed across the plate using an 8 well multipipettor. The number of clones per well was examined after 7 days of incubation. The wells with one clone were selected and the cells were transferred to the next size container when they were 2/3 confluent. When the cells were nearly confluent in a T25 flask they were cryopreserved. The process of subcloning was repeated for the previously subcloned cells in order to ensure purity (Harlow and Lane, 1998).

4.5 Mouse monoclonal antibody isotyping (Indirect ELISA method)

The purified mouse antibody was diluted to 1µg/ml 50 mM sodium carbonate buffer, pH 9.6 and 100 □l was placed 96 well plate. Goat serum and mouse serum were diluted in sodium carbonate at a dilution factor of 1:1000 and 100 µl added to the control wells. The goat serum served as a positive control for the rabbit anti-goat IgG peroxidase while the mouse serum served as the positive control for the goat anti-mouse polyvalent Ig peroxidase. Subsequently, the plate was incubated at 37^0C for an hour (*Goding, 1980)*.

At the end of incubation, the wells were washed three times with 200 µl of wash buffer. Subsequently, 200 µl of casein block (Thermo Scientific, Prod# 37532) was added to all wells and then incubated at room temperature for 30 mins. During the incubation, the six isotype specific reagents (goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA and IgM) were prepared by diluting each reagent in sample buffer at a dilution factor of 1:1000. In addition, goat anti-mouse IgG was diluted in sample buffer at a dilution factor of 1:1000, and also normal goat serum was diluted in sample buffer at a dilution factor of 1:1000. At 30 mins, the casein block was removed and 100 µl of each diluted isotype specific reagents were placed in the test wells in triplicates, 100µl of wash buffer were placed in the blank wells (no primary antibody), 100 µl of diluted goat anti-mouse IgG were placed in the 3 wells of the first negative control (no secondary antibodies) and finally 100µl of diluted normal goat serum were placed in the 3 wells of the final negative control wells (non-specific primary antibody). Then, incubation was done at room temperature for 30mins (Goding, 1983).

During incubation, rabbit anti-goat IgG peroxidase and goat anti-mouse polyvalent Ig peroxidase (G, A and M) were separately diluted in sample buffer at a dilution factor of 1:5000. At the end of 30 mins, each well was washed with 200 µl of wash buffer three times. 100 µl of diluted rabbit anti-goat IgG peroxidase were added to all wells except the negative control wells with no secondary antibody and the positive control wells that were coated with mouse serum. 100 µl of diluted goat anti-mouse polyvalent Ig peroxidase to each wells of the positive control wells coated with mouse serum, while 100 µl of wash buffer was added to negative control wells with no secondary antibody. Afterwards, the plate was incubated at room temperature for 15

mins, each well was washed 3 times with wash buffer, and 100 µl of TMB was added to each well. The TMB was incubated at room temperature until blue color develops. The color reaction was stopped by the addition of 50 µl of 2 N H_2SO_4, and optical density was measured at 450 nm with the aid of the spectrophotometer (Goding, 1983) as shown in Table 2.

Table 2: Mouse monoclonal antibody isotyping experimental design.

	Test wells	**Positive control**	**Positive control**	**Blank with no primary antibody**	**Negative control with no secondary antibody**	**Negative control with non-specific primary antibody**
Coating of wells	Pure mouse Ig	Goat serum diluted in sodium carbonate	Mouse serum diluted in sodium carbonate	Pure mouse Ig	Pure mouse Ig	Pure mouse Ig
Reagents added	Isotype specific reagents	Wash buffer	Wash buffer	Casein block	Diluted goat anti-mouse IgG	Normal goat serum
Enzyme labeled antibody added	Diluted rabbit anti-goat IgG peroxidase	Diluted rabbit anti-goat IgG peroxidase	Diluted goat anti-mouse polyvalent Ig peroxidase	Diluted rabbit anti-goat IgG peroxidase	Wash buffer	Diluted rabbit anti-goat IgG peroxidase

CHAPTER V: RESULTS

Investigating the amount of binding of the Vpr antigen to different pools of hybridoma supernatants

Specific binding to the Vpr antigen was tested for different pools of hybridoma supernatants using indirect ELISA, as shown in Figure 1. The purpose of Figure 1 was to select the best pool of hybridoma cells that has the highest amount of soluble antibody specific to the Vpr antigen in its supernatant prior to developing hybridoma clones for further analysis. Using the indirect ELISA method (Sinha et al., 1999), 96 well plates of poly vinyl chloride were coated with Vpr antigen. Supernatant from different pools of hybridoma cells were introduced into the wells and incubated for 30 minutes. Goat anti-mouse Ig-peroxidase conjugate and TMB substrate were added into the wells in order to detect the amount of positive binding between the hybridoma supernatant and the Vpr antigen. The wells with the highest absorbance at 450nm indicated the highest amount of antibody bound to the antigen.

Hybridoma supernatants from DL.VPR.A3, DL.VPR.A6, DL.VPR.C1, DL.VPR.C2, DL.VPR.C4, DL.VPR.D1, DL.VPR.D2, DL.VPR.D4, and DL.VPR.D5 had antibodies with a high amount of binding. Hybridoma supernatants from DL.VPR.A1, DL.VPR.B1 DL.VPR.B3, and DL.VPR.D6 had a low amount of antibody binding. Finally, hybridoma supernatants from DL.VPR.A5, DL.VPR.B2, DL.VPR.B5, DL.VPR.C3 DL.VPR.C5, and DL.VPR.D3 had no detectable Vpr specific antibody.

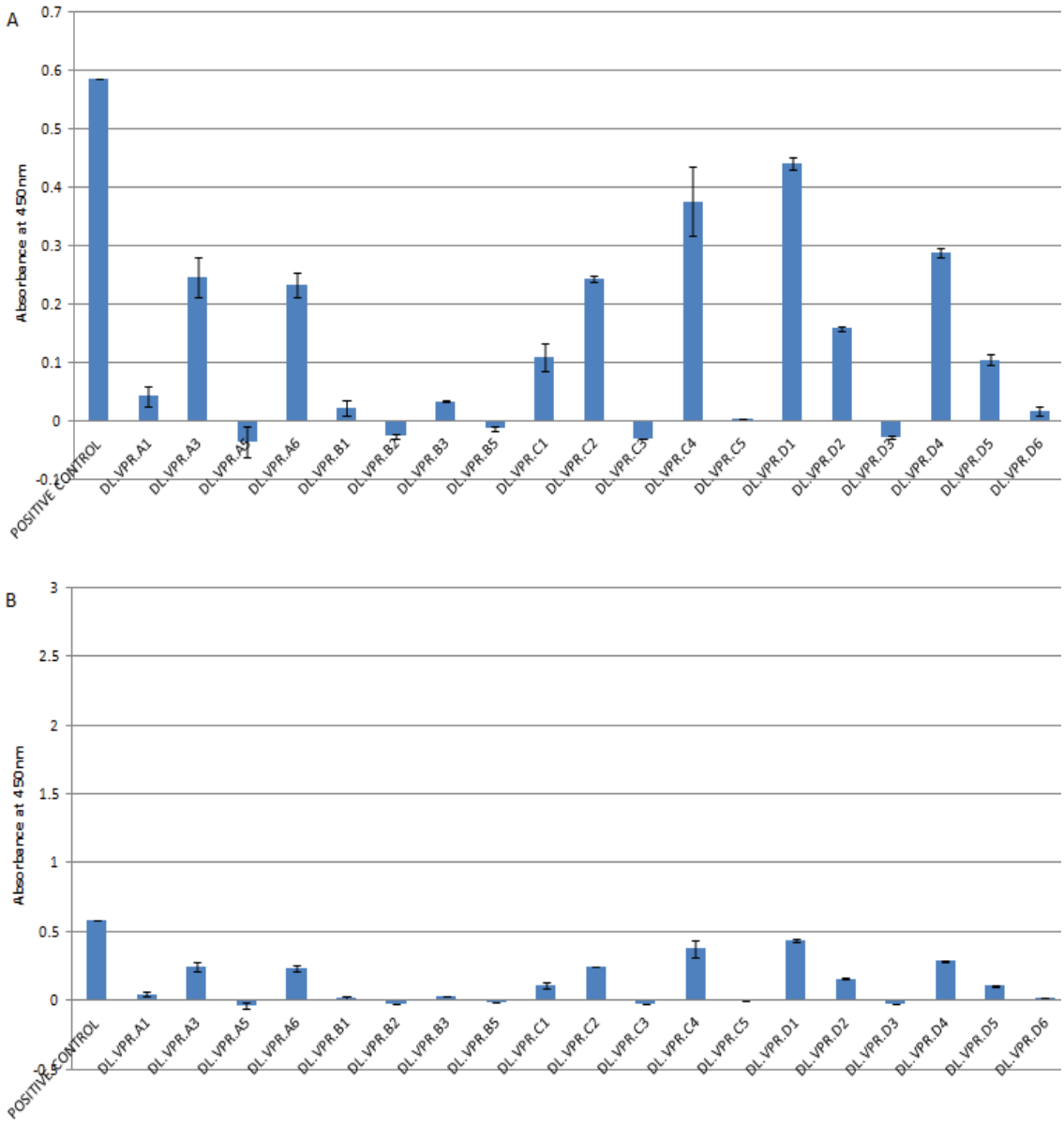


FIGURE 1: ELISA demonstrating binding of anti-Vpr polyclonal antibodies to Vpr antigen.

The hybridoma supernatants from the different pools of fused cells were used to determine the amount of binding of the soluble anti-Vpr polyclonal antibodies to the Vpr antigen. The mouse was immunized with Vpr and antisera collected to serve as a positive control. Cells from the spleen from the immunized mouse were isolated and fused to myeloma cells using 50% polyethylene glycol containing 10% DMSO in RPMI. The

cells were cultured in 1X HAT for two weeks. For each well that was two-thirds confluent, supernatants were collected, as shown on the Y axis, and screened by ELISA for antibody binding to Vpr. The absorbance was read at 450nm, as shown on the Y axis. Data represents the mean +/- SD for two replicas. The two graphs are identical, except that the Y axis in Figure 1A was expanded to allow better visualization of the error bars.

Limiting dilution of DL.VPR.D1

The pool of wells labeled DL.VPR.D1 was then subcloned using limiting dilution. The importance of this step is the generation of monoclonal cells that are specific to a single epitope found on Vpr. Selection of a single clone of hybridoma cells was done (Harlow and Lane, 1998) by adding hybridoma cells (100 µl of rapidly growing DL.VPR.D1 cells) to the top left hand well (A1) and 1:2 serial dilutions were performed down the left hand row (row 1) of the 96 well plate. P3X media (100ul) was then added to row 1. Serial 1:2 dilutions across were then performed across the plate using an 8 well multipipettor. At the end of this procedure, the numbers of uncontaminated wells containing single clones were quite few. The monoclonal cells were; DL.VPR.D1.D10, DL.VPR.D1.E12, and DL.VPR.D1.G6 (Figure 2). The amount of binding of the soluble antibodies from these monoclonal cells to the Vpr antigen was investigated using the indirect ELISA method. The result shows that DL.VPR.D1.G6 had the highest amount of monoclonal antibody specific to HIV-1 Vpr in its hybridoma supernatant. However, the positive control (anti-Vpr mouse antisera) had a low amount of binding to the Vpr antigen. The cause of this low amount of binding in the positive control needed to be investigated. As we anticipated a possible problem with the positive control, DL.VPR.D4 (an hybridoma cell of known amount of binding from Figure 1) was introduced into the experimental set up to make objective comparison.

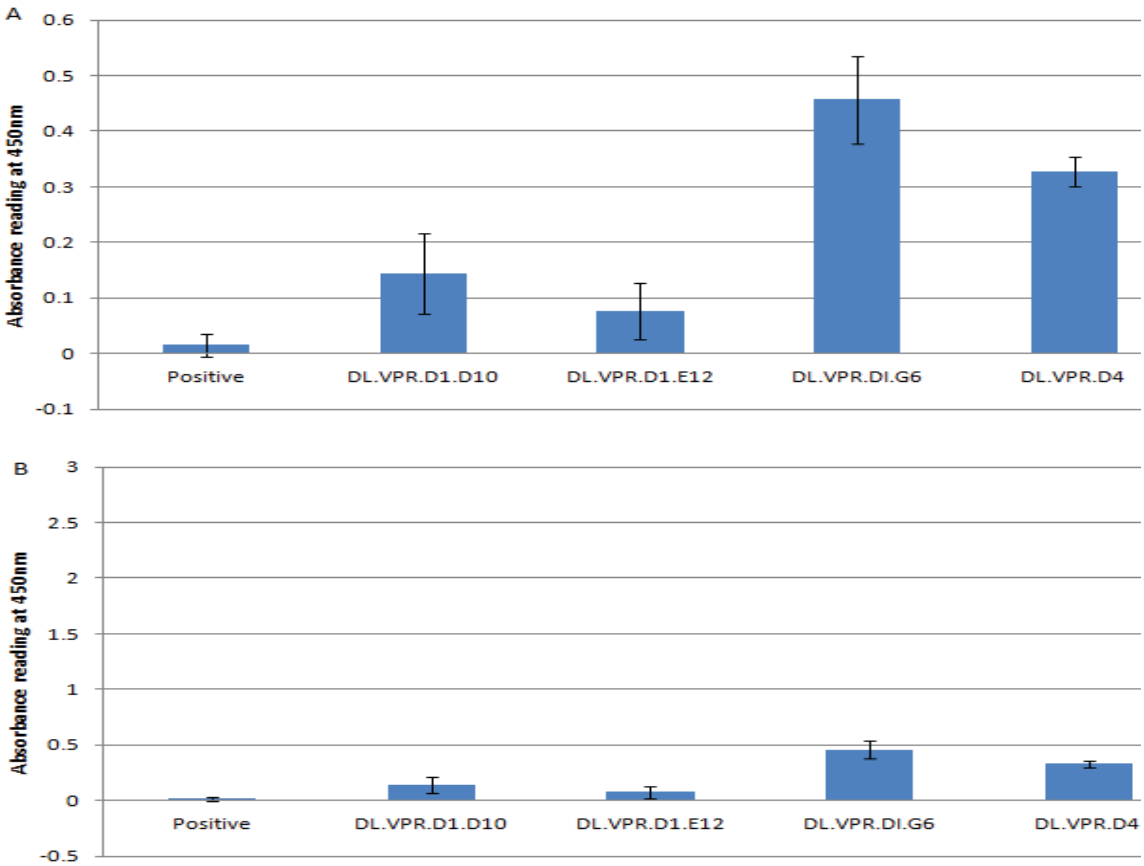


FIGURE 2: ELISA demonstrating the binding of anti-Vpr monoclonal antibodies to Vpr antigen following sub-cloning of DL.VPR.D1.

DL.VPR.D1 was cloned using limiting dilution in order to generate monoclonal antibodies. The clones were DL.VPR.D1.E12, DL.VPR.D1.D10, and DL.VPR.D1.G6, as shown on the X axis. As described in Fig. 1, the hybridoma supernatant was tested for binding to the Vpr antigen using an indirect ELISA. Absorbance is shown on the Y axis. Mouse Vpr antiserum was used as the positive control. DL.VPR.D4 was added as an alternate positive control to the mouse antisera control. Data represents the mean +/- SD for two replicas. The two graphs are identical, except that the Y axis in Figure 2A was expanded to allow better visualization of the error bars.

Isotyping of purified monoclonal antibody EH.VPR.C5.C6

The essence of this experiment was to determine the class of antibody purified EH.VPR.C5.C6 belongs to, to determine if the isotyping reagent were still viable, and to determine the effect of purification on the viability of the antibody. EH.VPR.C5.C6 is a monoclonal antibody to Vpr which had been previously purified by hydroxyapatite column chromatography. Six isotypic reagents were used to carry out an isotyping analysis (Goding, 1983) on purified EH.VPR.C5.C6 monoclonal antibody. The isotypic reagents were goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA and IgM.

The result shown in Figure 3 indicates that there is a high chance that the purified EH.VPR.C5.C6 monoclonal antibody belongs to IgM class of antibody clones. However, the absorbance readings of the other isotypes were slightly higher than that of the negative controls, indicating a possible non-specific binding of the primary or secondary antibodies.

Effect of blocking buffer on the isotyping of EH.VPR.C5.C6

In order to trouble shoot the possible reasons for the high non-specific binidng during the isotyping of purified monoclonal antibody EH.VPR.C5.C6, we tested changing the blocking buffer from PBS + 1 % BSA to casein block. In addition, two different sample buffers (used to dilute the antibodies) were also used during the analysis- PBS only and PBS + 0.1 % BSA + 0.05 % Tween-20 in order to be able to ascertain what might have resulted in the non-specific binding seen in Figure 3.

From Figure 4, one could conclude the sample buffer had little effect on the negative controls, but the use of PBS as sample buffer decreased binding of antibody to the positive controls. Most importantly, the use of casein block seems to help reduce the

absorbance reading noticed with the negative controls from Figure 3. Thus, the use of casein block as the blocking buffer would be of great importance going forward.

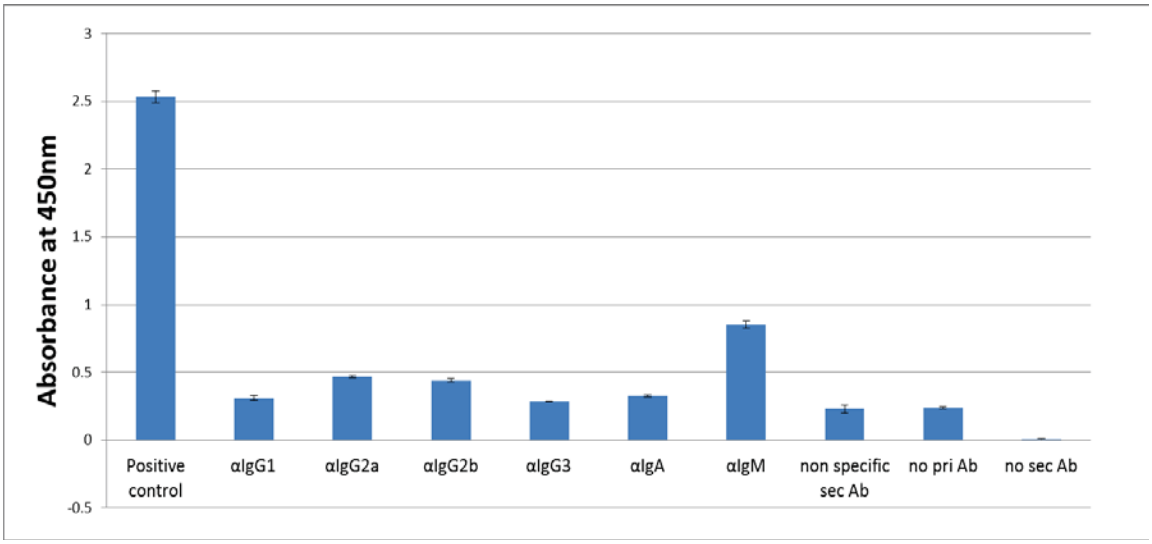


FIGURE 3: Isotyping of purified monoclonal antibody EH.VPR.C5.C6 using an indirect ELISA method.

EH.VPR.C5.C6 had been previously purified by hydroxyapatite column chromatography. The monoclonal antibody was tested for binding to goat primary antibodies against mouse IgG1, IgG2a, IgG2b, IgA and IgM (isotypic antibodies), using an indirect ELISA. The positive control was goat serum bound to the plate and detected with the secondary antibody, peroxidase conjugated rabbit anti-goat antisera. Instead of the isotypic antibodies, diluted mouse serum was placed in the first negative control wells (designated non-specific secondary antibody), 100 µl of PBS + 1 % BSA were placed in the second negative control wells (designated no primary antibody), and finally, 100 µl of diluted goat anti-mouse IgG was placed in the third negative control wells designated no secondary antibody. Results from a blank (no primary or secondary antibody) which had pure EH.VPR.C5.C6 + Na_2CO_3, PBS + 1 % BSA, wash buffer was subtracted from all samples. Data represents the mean +/- SD for triplicate replicas.

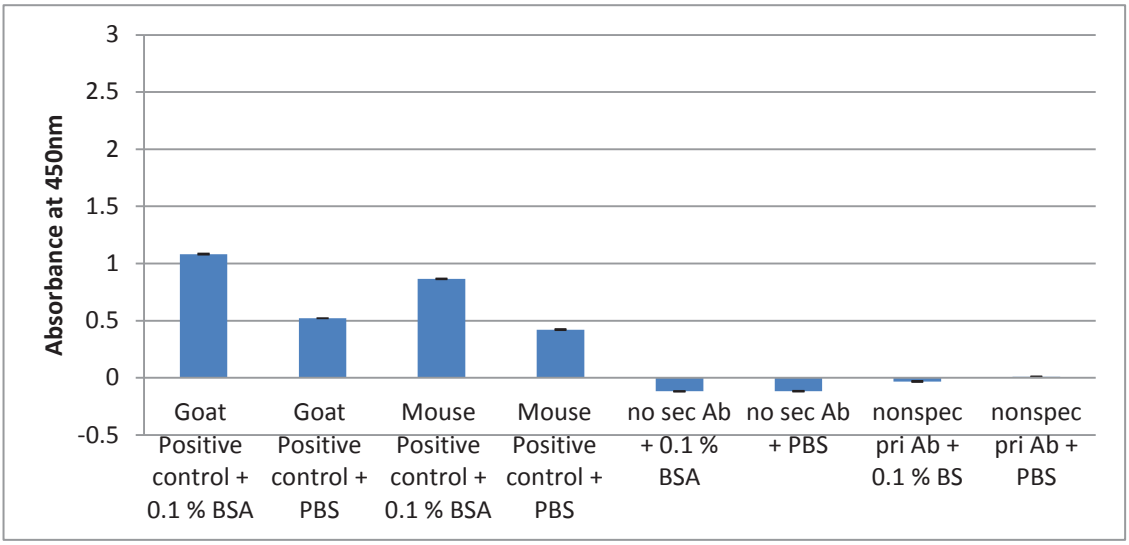


FIGURE 4: Testing of casein block on isotype controls.

The isotyping method used was identical to Fig. 3, with the exception that only the controls were used. In addition, casein block was used in place of PBS + 1 % BSA. Two different sample buffers were also used during the analysis- PBS only and PBS + 0.1 % BSA + 0.05 % Tween-20. Results for a blank (no primary or secondary antibody) which had pure EH.VPR.C5.C6 + Na_2CO_3, PBS + 1 % BSA, wash buffer were subtracted from all sample data. Data represents the mean +/- SD for three replicas.

The use of casein sample buffer in EH.VPR.C5.C6 isotyping

Since the casein block decreased non-specific binding in the iostype controls (Figure 4), casein sample buffer was also tested. Purified EH.VPR.C5.C6 was retested for isotype using casein block and casein sample buffer (PBS + 10 % casein + 0.05 % Tween-20) (Figure 5). In this experiment, we did see decreased binding in negative controls, but the amount of binding of the pure EH.VPR.C5.C6 to the isotypic reagents was also extremely low. Thus, the modification of the sample buffer did not give an overall beneficial result.

Isotyping of EH.VPR.C5.C6 cell culture hybridoma supernatant

The type of blocking buffer used during the isotyping experiment seems to be the most important parameter for achieving specificity and sensitivity. Lack of results with the purified antibody EH.VPR.C5.C6 may have resulted from the degradation of the antibody during the hybridoma procedure or from the use of casein sample buffer. The isotyping was repeated using the original cell culture supernatant from the isolated clone EH.VPR.C5.C6 (Figure 6). Even though some degree of binding of the cell culture supernatant of EH.VPR.C5.C6 to isotypic reagent of IgG2a, IgG2b, IgG3 and IgM were noticeable, the results shows that the antibodies in the original cell culture supernatant of EH.VPR.C5.C6 has the highest probability of being an IgA class of antibody (Figure 6).

Isotyping of DL.VPR.D1 cell culture hybridoma supernatant

This experiment was run side by side with the isotyping of EH.VPR.C5.C6 cell culture hybridoma supernatant (Figure 7). The main purpose of this experiment was to ensure the isotypic reagent were viable and reactive to soluble antibodies in hybridoma supernatants.

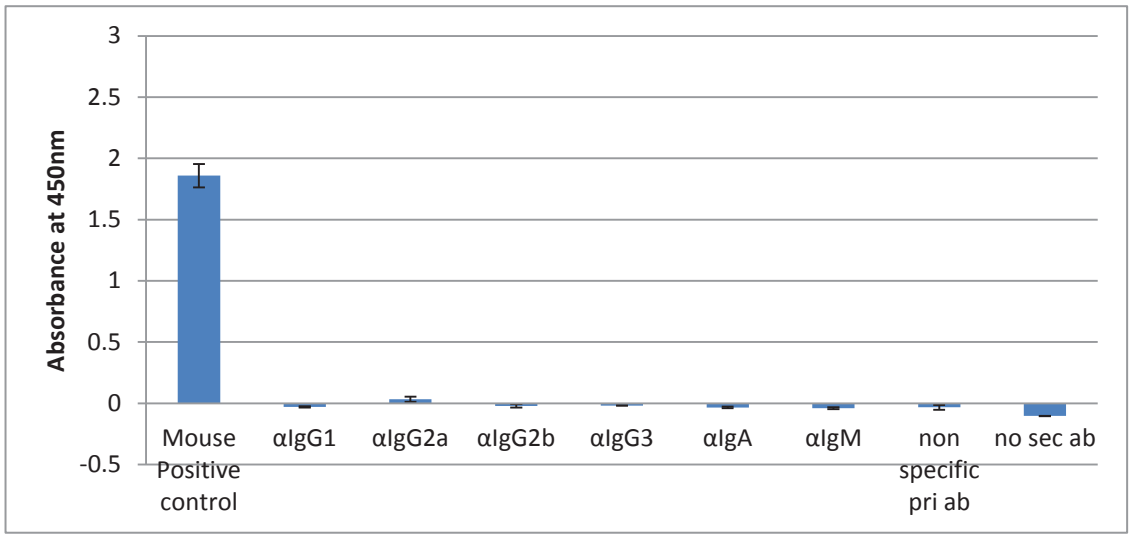


FIGURE 5: Testing of casein block and casein sample buffer on purified EH.VPR.C5.C6 iostyping.

The isotyping method used was identical to Fig. 3 but with a few modifications. The sample buffer was modified from PBS + 0.1 % BSA + 0.05% Tween-20 to casein sample buffer (PBS + 10% casein + 0.05 % Tween- 20). The results from a blank (no primary antibody) which had pure EH.VPR.C5.C6 + Na_2CO_3, PBS + 1 % BSA, rabbit anti-goat IgG peroxidase was subtracted from the data for all samples. Data represents the mean +/- SD for two replicas.

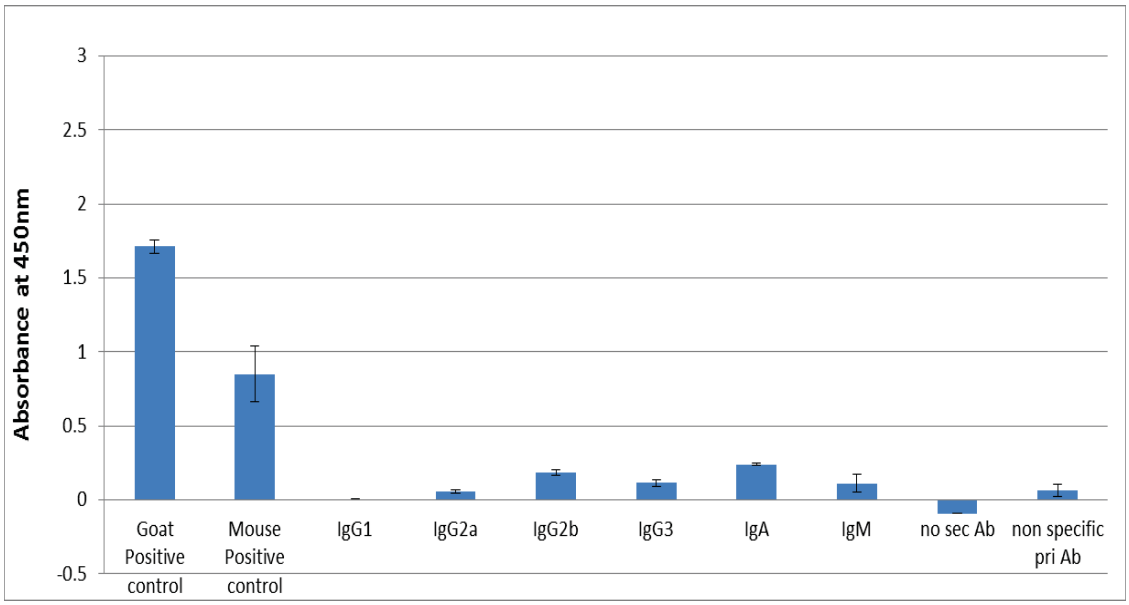


FIGURE 6: Isotyping of EH.VPR.C5.C6 hybridoma supernatants.

The method used is identical to Fig. 3 but with few modifications. First, the goat serum was added to serve as a positive control for the rabbit anti-goat IgG peroxidase. Second, the sample buffer was changed from casein sample buffer (PBS + 10% casein + 0.05 % Tween- 20) to PBS + 0.1 % BSA + 0.05% Tween-20. Third, hybridoma supernatant from EH.VPR.C5.C6 was used for testing rather than purified EH.VPR.C5.C6. The blocking buffer used was casein block. The results from a blank (no primary Ab) which comprises EH.VPR.C5.C6 cell culture supernatant + Na_2CO_3, casein block, rabbit anti-goat IgG peroxidase was subtracted from the data for all samples. Data represents the mean +/- SD for two replicas.

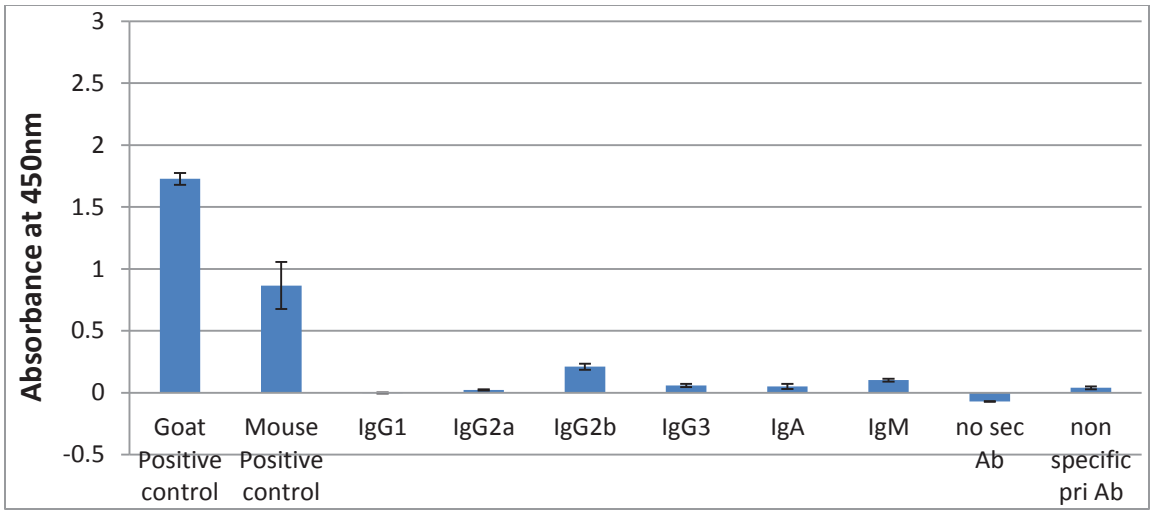


FIGURE 7: Isotyping of DL.VPR.DI hybridoma supernatants.

The method used is identical to Fig. 6 The results from a blank (no primary Ab) which contains DL.VPR.DI cell culture supernatant + Na_2CO_3, casein block, rabbit anti-goat IgG peroxidase was subtracted from the data for all other samples. Data represents the mean +/- SD for two replicas.

The result obtained from this experiment further shows that lack of reactivity between purified antibody EH.VPR.C5.C6 (Figure 5) and the isotypic reagent might be due to antibody degradation and not because of non-viability of the isotypic reagent. The result shows that the soluble antibodies of DL.VPR.D1belong to the IgG2b class of antibody (Figure 7).

Selection of the best clones for further subcloning

In order to ascertain the viability of the hybridoma cells that would be selected for limiting dilution and further analysis, an indirect ELISA experiment was conducted on hybridoma supernatants from DF.VPR.C4.D12, DL.VPR.C2, DL.VPR.D4, EH.VPR.C5.C6, and EH.VPR.C5.G6 (Figure 8). Unfortunately, while it appear that DL.VPR.D4 would be a good clone to study, no data can be derived from this figure due to lack of interaction between the positive control and the Vpr antigen.

Troubleshooting the reason for the lack of reactivity between the positive control and the Vpr antigen

The effectiveness of the positive control (anti-Vpr mouse anti sera) was retested by the indirect ELISA method. Anti-FENO anti mouse sera binding to FENO antigen was introduced into the experimental set up in order to make comparison with the amount of binding of the anti-Vpr mouse anti sera to the Vpr antigen. FENO antigen (1 μg/ml) was introduced into wells that were tested against anti-FENO mouse antisera. In addition, two previously positive clone supernatants (DF.VPR.A5.C12 and DF.VPR.C3) were tested as possible alternate positive controls. Anti Vpr antisera to the Vpr antigen was again not positive. A very low amount of binding was noticed between the hybridoma supernatant

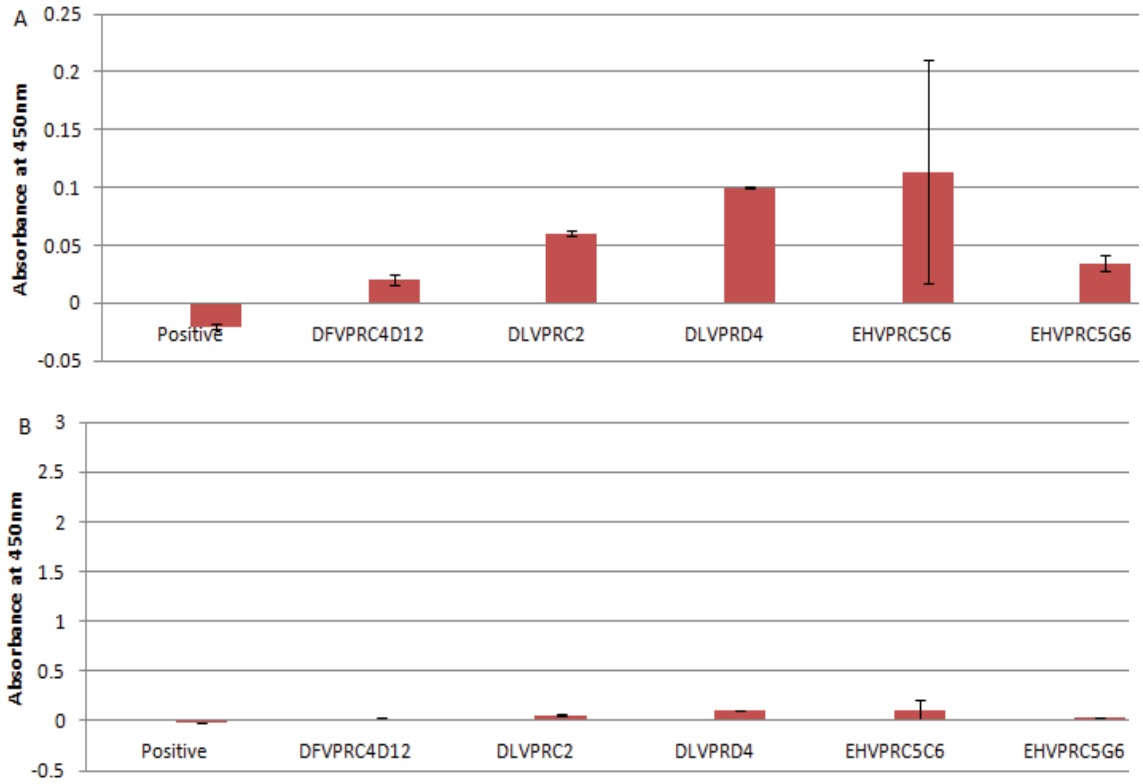


FIGURE 8: ELISA demonstrating the amount of binding of hybridoma supernatants from EH.VPR.C5.C6, EH.VPR.C5.G6, DF.VPR.C4.D12 monoclonal cells; and DL.VPR.C2, DL.VPR.D4 polyclonal cells to Vpr antigen.

Method used is identical to that described in Fig. 1. Data represents the mean +/- SD for two replicas. The two graphs are identical, except that the Y axis in Figure 8A was expanded to allow better visualization of the error bars.

from DF.VPR.A5.C12 and the Vpr antigen. Thus, soluble antibodies in the Vpr antisera and in DF.VPR.A5.C12 appear to have been degraded. However, a positive result was seen with DF.VPR.C3 (Figure 9). The cell supernatant from DF.VPR.C3 was used in the remaining experiments as the positive control.

Indirect ELISA of the subclones of EH.VPR.C5.C6 and DF.VPR.C4.D12

The purpose of this experiment was to determine if the frequency of culturing or subcloning contributes to antibody degradation. For this experiment, DF.VPR.C4.D12 and EH.VPR.C5.C6 were selected because they have been subcloned twice. Aside from the low amount of binding seen in EH.VPR.C5.C6.H4, every other subclones showed no reaction or specificity with the HIV-1 Vpr (Figure 10). This is also an indication that as the cells divide they probably lose their specificity due to some mutation that occurs as the cells divide.

Retesting the specificity of all frozen clones to the Vpr antigen

Due to the worrisome trend of lost of specificity of hybridoma cells to Vpr antigen and due to the low amount of binding of clones that were initially known to have high amount of binding to Vpr antigen, all frozen cell lines were thawed and new cell culture supernatants collected to be retested for the presence of antibody. Supernatants from DF.VPR.C4.D12 and EH.VPR.C5.C6 cells showed no positive result even from the thawed cell lines (Figure 11). All thawed cell lines in Figure 12 and 13 displayed no specificity of binding to the Vpr antigen. Worthy of mention is the lack of binding between DL.VPR.DI and Vpr antigen (Figure 12), and the lack of binding between

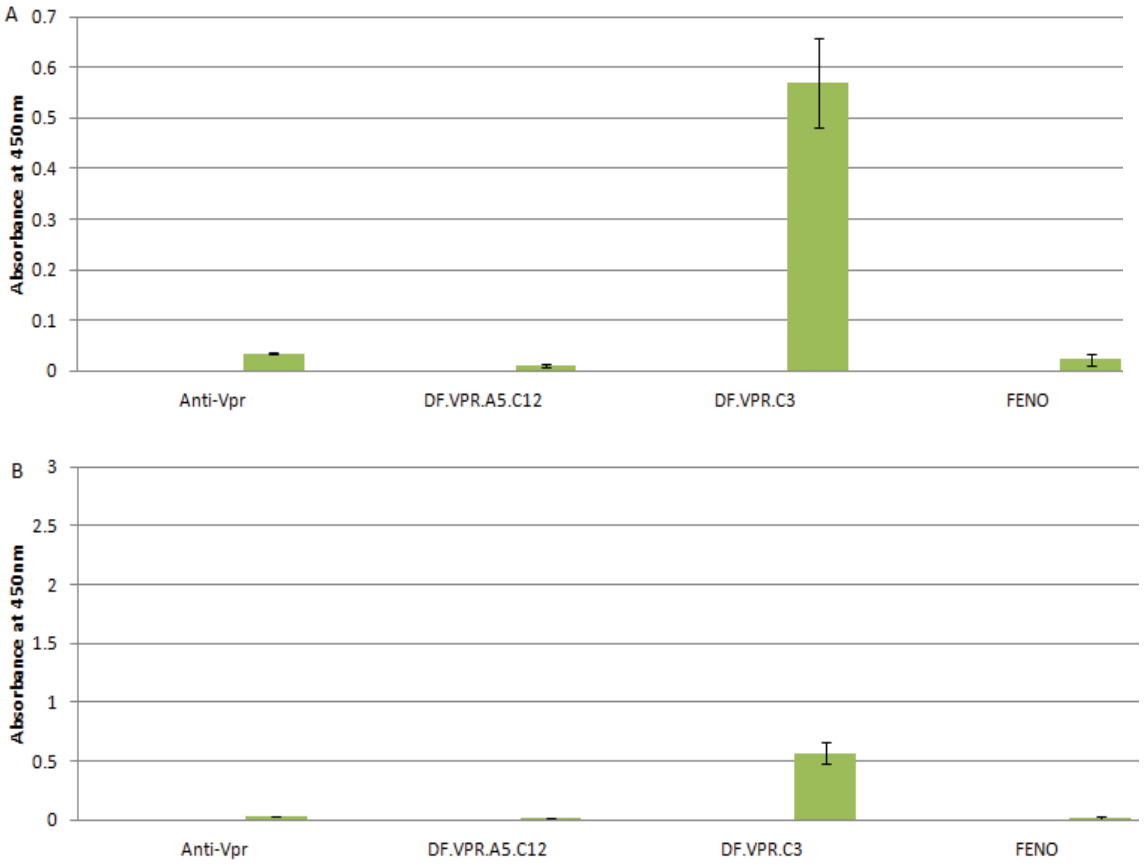


FIGURE 9: Testing the effectiveness of the positive control (anti-Vpr mouse anti sera) by ELISA.

Method used is as described in Fig. 1, with the exception of the use of 1ug/ml FENO antigen for wells that were tested for binding to anti-FENO mouse antisera. Anti-FENO anti mouse sera and hybridoma supernatant from DF.VPR.A5.C12 were introduced in order to make comparison with the amount of binding of the anti-Vpr mouse anti sera. Data represents the mean +/- SD for two replicas. The two graphs are identical, except that the Y axis in Figure 9A was expanded to allow better visualization of the error bars.

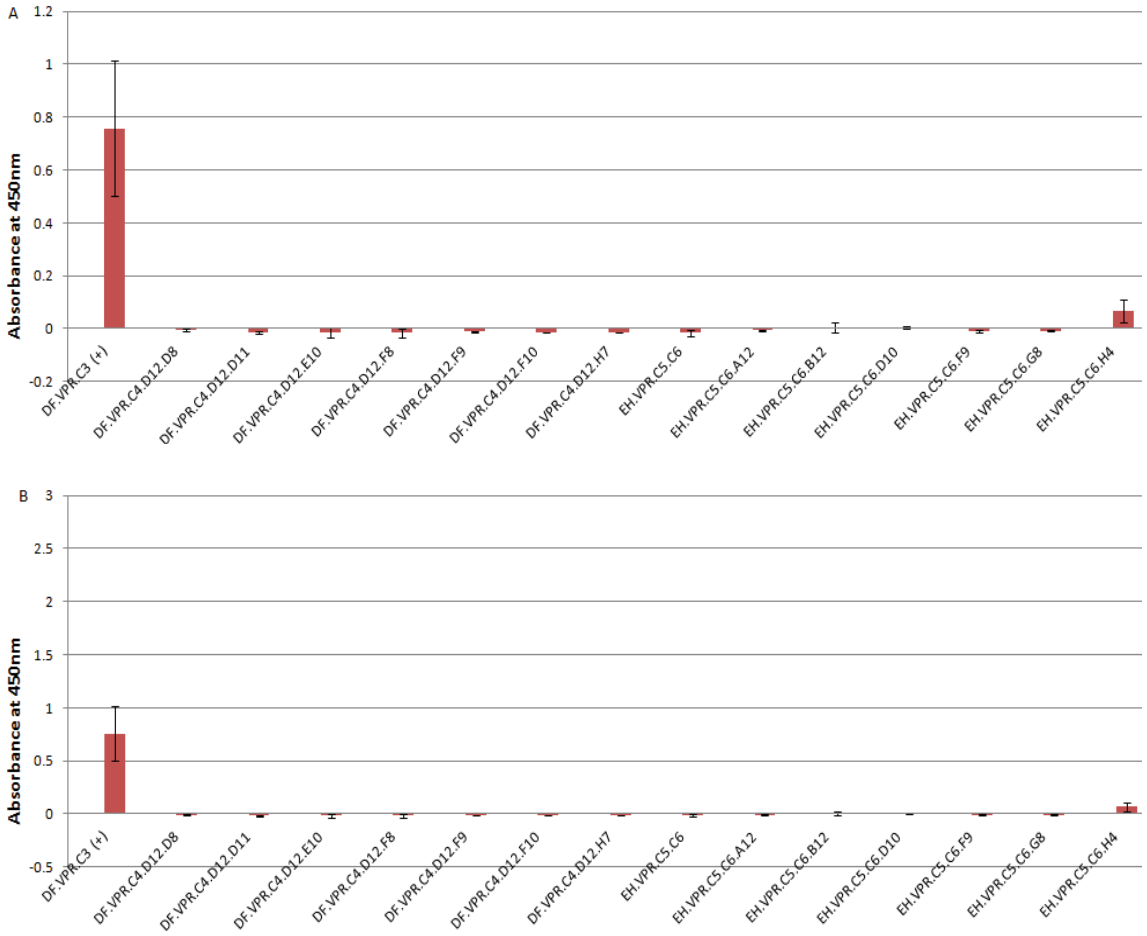


FIGURE 10: ELISA demonstrating the amount of binding of the subclones of EH.VPR.C5.C6 and DF.VPR.C4.D12 subclones to Vpr antigen.

EH.VPR.C5.C6 and DF.VPR.C4.D12 clones were subjected to limiting dilution as described in Fig. 2. Hybridoma supernatant from clones of DF.VPR.C3 was adopted as the new positive control due to its amount of binding, as shown in Fig. 9. The amount of binding of the subclones of EH.VPR.C5.C6 and DF.VPR.C4.D12 to Vpr antigen was determined by the indirect ELISA method, as described in Fig. 1. Data represents the mean +/- SD for two replicas. The two graphs are identical, except that the Y axis in Figure 10A was expanded to allow better visualization of the error bars.

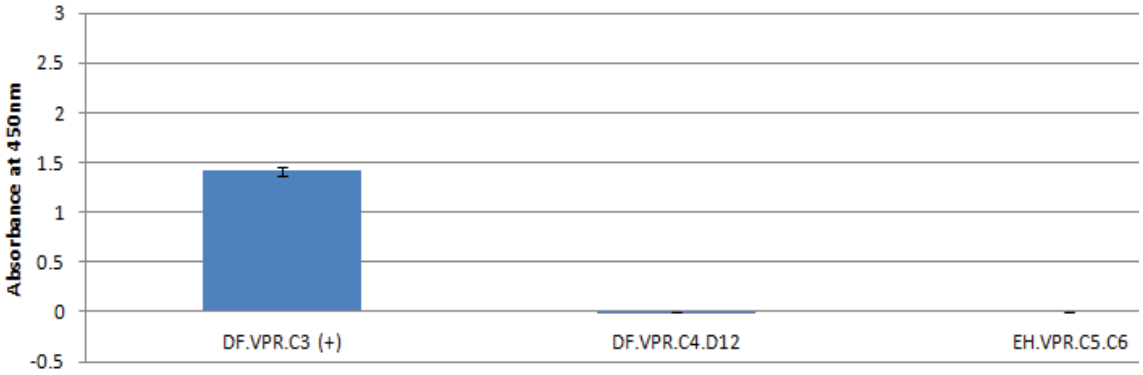


FIGURE 11: ELISA demonstrating the amount of binding of EH.VPR.C5.C6 and DF.VPR.C4.D12 monoclonal antibodies to the Vpr antigen.

Method used is as described in Fig. 1. Hybridoma supernatants from earlier versions of the frozen clones DF.VPR.C4.D12 and EH.VPR.C5.C6 were tested for their amount of binding to the Vpr antigen. Data represents the mean +/- SD for two replicas.

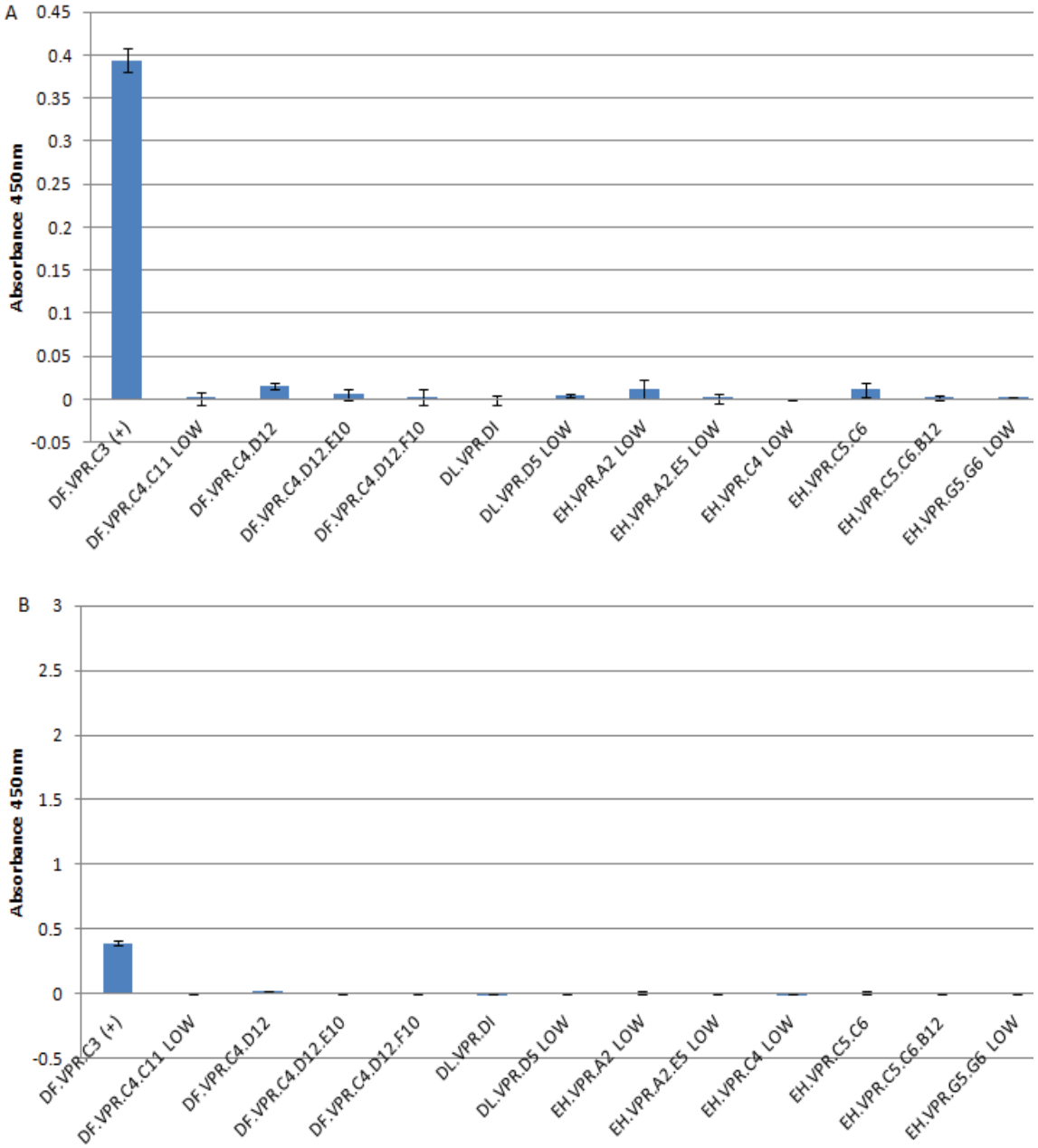
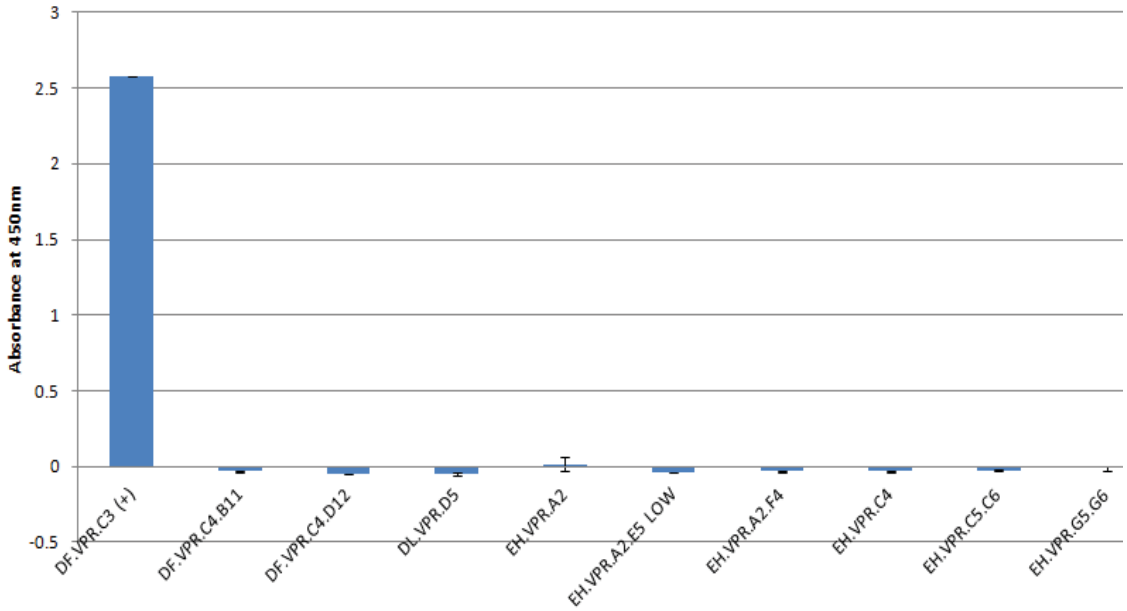


FIGURE 12: ELISA demonstrating the amount of binding of hybridoma supernatant to the Vpr antigen.

Method used is as described in Fig. 1. Data represents the mean +/- SD for two replicas. The two graphs are identical, except that the Y axis in Figure 12A was expanded to allow better visualization of the error bars.



FFIGURE 13: ELISA demonstrating the amount of binding of hybridoma supernatant to the Vpr antigen.

Method used is as described in Fig. 1. Data represents the mean +/- SD for two replicas.

DL.VPR.D5 and the Vpr antigen (Figure 13). DL.VPR.DI and DL.VPR.D5 were hybridoma cell lines known to have some amount of binding with the Vpr antigen (Figure 1). This is an indication of the possibility of antibody degradation in the cell lines.

CHAPTER VI: DISCUSSION

The use of hybridoma technology to develop monoclonal antibodies has helped to solve some pitfalls in medical research that involve the use of polyclonal antibodies (Lipman et al., 2005). The lack of specificity, inconsistent production and short life span involved in the use of polyclonal antibodies has been a thing of the past since the advent of hybridoma technology. In order to study a viral protein in Human Immunodeficiency Virus 1 (HIV-1) called viral protein r (Vpr), this study was aimed at using hybridoma technology to help develop monoclonal antibodies specific to viral protein r (Vpr). In addition, the study was also aimed at purifying the developed monoclonal antibodies in order to get rid of impurities. It was hypothesized that the developed anti-Vpr monoclonal antibodies (MAbs) should be able to bind and identify Vpr antigen both as cell bound Vpr and free Vpr.

Diagnosis of HIV has been plagued with the problem of hypervariabltity of HIV strains. Studies have shown that Vpr is one of the most conserved HIV proteins (Stark and Hay, 1998). Our hypothesis was that development of MAbs capable of binding to the Vpr protein could help detect various subtypes of HIV. In addition, Vpr has been documented to be found as a circulating protein in the serum of 40 % HIV-seropositive patients and in the cerebrospinal fluid of AIDS individuals (Levy et al., 1994), thus, making it a good candidate as a viral protein that could be measured during the diagnosis of HIV infection. Furthermore, due to the importance of Vpr to HIV-1 replication, it would be useful to develop a MAb that can be used to help study where Vpr is expressed, when it is being expressed and under what condition it is made.

In my study, a mouse was immunized with Vpr antigen and the spleen from the immunized mouse was isolated and teased in order to release antibody-secreting spleen

cells. Isolated spleen cells were fused with myeloma cells using polyethylene glycol and then cultured in HAT medium in order to select for hybridomas. Hybridomas secreting anti-HIV-1 Vpr were screened by indirect ELISA (Figure 1). Positive wells were further subcloned by limiting dilution in order to develop monoclonal cells. Monoclonal cells still having the ability to produce antibodies that bind to Vpr antigen were identified by indirect ELISA (Figure 2).

In this study, hybridomas were developed and some of the developed hybridomas were able to identify cell-free Vpr antigen *in-vitro.* Worthy of mention, are DL.VPR.A3, DL.VPR.A6, DL.VPR.C1, DL.VPR.C2, DL.VPR.C4, DL.VPR.D1, DL.VPR.D4, and DL.VPR.D5. In addition, DL.VPR.DI.E12, DL.VPR.DI.D10 and DL.VPR.DI.G6 were developed MAbs that was able to identify cell-free Vpr antigen *in-vitro.* As the number of monoclonal antibodies produced by the fusion was limited, I expanded the work to developed monoclonal antibodies from this lab with known high amount of binding to Vpr (EH.VPR.C5.C6, and DF.VPR.C4.D12).

Over time, anti-Vpr mouse antisera (positive clone) lost its reactivity to the Vpr antigen. This might be due to improper storage or due to precipitation of antibodies following repeated freezing and thawing of the sample. Loss of reactivity to Vpr antigen was also noticeable in some of the subclones from cell lines (EH.VPR.C5.C6, DF.VPR.C4.D12) that were initially reactive to the Vpr. A study has shown that one of the possible reasons for the loss of specificity of the hybridoma is due to mutation in the genes that code for both the light and heavy chains of the immunoglobulins (Köhler, 1980). These mutations could lead to the production of immunoglobulins with truncated or excess light and heavy chains. These render the MAbs inactive and non-specific to

their target antigen. Another study correlated the frequency of hybridoma passage to the degree of chromosomal loss and ultimately to the degree of loss of specificity by the MAbs (Xin and Cutler, 2006). That is, the more times the cells were subcloned or passaged, the higher the chance of the occurrence of some important chromosomes being lost during hybridoma cell multiplication. Hybridomas are heterokaryons, having twice as many chromosomes as a normal cell. This makes accurate separation of chromosomes during mitosis problematic.

Other laboratories have identified hybridoma technology as a great tool in HIV research and there are quite a number of studies that have been done. One of the earliest works done on MAbs and HIV was aimed at developing techniques that would help in the rapid identification of the different isolates of HIV (Minnasian et al., 1988). This study was conducted when HIV-2 isolates were emerging. In this study, a mouse was intraperitoneally immunized with purified solubilized HIV-2 isolates emulsified in complete Freund's adjuvant on day 0 and the same amount of antigen in PBS on days 1, 2, 16, 17, and 18. Splenocytes were collected, fused with myeloma cells and subsequently grown in HAT medium. Hybridoma cells producing HIV-2 specific antibody were screened by an indirect immunofluoresence assay. In the immunofluoresence assay, HIV-2 infected T cells lines were spotted on slides, air-dried, and fixed in acetone/methanol. Hybridoma supernatants were spotted on the slide and incubated at room temperature for 30 mins. Fluorescein-conjugated goat antimouse immunoglobulin (IgA, IgM, and IgG) was used as the secondary antibody in order to aid the detection of positive clones. The Minnasian study also employed the use of the Ouchterlony double immunodiffusion technique for the isotyping of the MAbs produced by the hybridoma cells rather the

ELISA method of isotyping. The MAbs were found to belong to IgG1 class of antibody. Furthermore, the study employed the use of western blotting technique to assay the specificity of the developed MAbs to different viral structural proteins from HIV-1, HIV-2 and SIV isolates. In this immunoblotting method, the viral structural proteins of HIV-1, HIV-2 and SIV were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and were blotted onto a nitrocellulose sheet. The developed MAbs were then applied to the blotted sheet for binding. Interestingly, some of the developed MAbs were specific in their binding to structural proteins from HIV-2 isolates, while the majorities were able to cross-react with structural proteins form HIV-2 and SIV isolates. This helps to further validate that MAbs could help solve one of the major problem in HIV diagnosis (i.e. hyper-variability) due to its ability to identify conserved regions on a structural protein irrespective of the viral isolate. Most importantly, the specificity of some of the MAbs to a viral isolate indicated that MAbs could be used as a diagnostic tool in distinguishing HIV-2 isolates from other isolates.

A study by Gao and coworkers (Gao et al., 2009) aimed to develop a tool that would help overcome the hurdle of genetic variability in one of the most prevalent HIV-1 subclasses (group M). Interestingly, not only does HIV-1 group M have nine subtypes, but these subtypes have recombined and generated circulating recombinant forms (CRF are unique strains of HIV derived from the recombination of two or more HIV subtypes). In this study, the focus was to develop MAbs that would bind to all HIV-1 group M subtypes by detecting a conserved region in the envelope protein (one of the most divergent genes of HIV-1). The method used by this study and the screening for the specificity of the MAbs was similar to the study in our laboratory. The MAbs produced

were not all specific to the Env proteins of the individual subtypes, some of the MAbs were able to cross-react with envelope proteins from subtype A, B and C. In addition, these MAbs were able to cross-react with envelope protein from SIV. The results obtained from surface Plasmon resonance also help to show that not only do these cross reactive MAbs have a high amount of binding, they have a low dissociation and a high association binding kinetics to the Env proteins from subtype A, B and C. The result obtained by Gao and coworkers proved that hybridoma technology could once again help solve the problem of hyper-variability in HIV diagnosis. However, the disadvantage of the MAbs developed from the Gao was that binding did not necessarily mean inhibition of replication. A neutralizing assay showed that even though the MAbs were bound to the envelope proteins, the MAbs could not prevent HIV-1 viruses from infecting T cell lines. Irrespective of this short coming, these cross reacting antibodies can be used to better our understanding about the biology of HIV-1 envelope proteins.

Another study conducted using hybridoma technology on HIV research was aimed at developing and characterizing anti-HIV Vpr monoclonal antibodies (Sabbah et al., 2006). In terms of experimental design, one of the few differences between Sabbah's study and the current one were the immunization of mice in the rear footpad, and fusion of isolated cells from popliteal and inguinal lymph nodes with myeloma cells rather than the fusion of spleen cells and myeloma cells as it is been done in this study. Also, in Sabbah and coworkers the mice with either the N- (Vpr 1–51 amino acids) or the C-terminus domain of Vpr (Vpr 52–96 amino acids), rather than the whole Vpr protein. During the screening of anti-Vpr MAbs by the ELISA and immunoblotting method the MAbs displayed a high degree of specificity. The MAbs against the N-termini domain of

Vpr was specific only to the fragments of Vpr derived from 1-51 amino acids. This degree of specificity was also applicable to the MAbs developed against the C-termini domain of Vpr. Classification of the Vpr MAbs into five groups was determined by what type of amino acid Vpr fragments they bind to. Most importantly, the developed MAbs was able to immunoprecipitate Vpr protein from a lysate derived from the lysis of HIV-1 infected promonocytic cells and HIV-1 infected peripheral blood mononuclear cells. However, the introduction of a mutation at the C terminus region of the Vpr by substituting an alanine residue at 73, 77 and 80 for the existing arginines impaired the binding of the MAbs to the Vpr antigen. This is an indication of the importance of the integrity of the amino acid sequence. In addition, the binding of the MAbs was able to prevent interaction of the Vpr with adenine nucleotide translocator. Vpr interaction with adenine nucleotide translocator has been documented to be the key initiator of apoptosis in immunocompetent cells. Thus, this study produced antibodies that may be used to study Vpr nuclear localization. In addition to the anti-Vpr antibodies produced by the Sabbah lab, two biotech companies, Santa Cruz Biotechnology, Inc. and Cosmo Bio, have also recently commercialized the production of anti-Vpr MAbs. This is an indication that Vpr protein has been noted as a good candidate for the diagnosis of HIV research (COSMO, 2014; Santa Cruz, 2015).

In summary, from this study we have proven our hypothesis, that MAbs are able to identify Vpr and displayed some level of specificity by binding to cell free HIV-1 Vpr. The loss of antibody production by these clones was likely due to chromosome loss during cell replication in culture. It is possible that future cell lines may be maintained by producing a larger pool of frozen lines to work from. The use of phage display

technology can also be explored to produce antibody fragments that would still retain high specificity of binding to HIV-1 Vpr without having the inherent instability associated with monoclonal antibodies. A molecule (FENO) believed to be part of the Vpr antigen could also be worked on for future studies (see appendix). Amidst the obvious limitations encountered during this study, the advantages of using MAbs during anti-HIV research outweigh the use of polyclonal antibodies. The antibodies could be tested in drug trials with the anti-HIV-1 Vpr MAb coupled to drugs which would be targeted against HIV-1Vpr. This would allow localization of the drug of interest to the target site. In addition, the anti-HIV-1 Vpr MAb should be tested against other HIV isolates and subtypes in order to ascertain the ability of the anti-HIV-1 Vpr MAb to identify, bind and cross react with several Vpr from different isolates of HIV.

CHAPTER VII: REFERENCES

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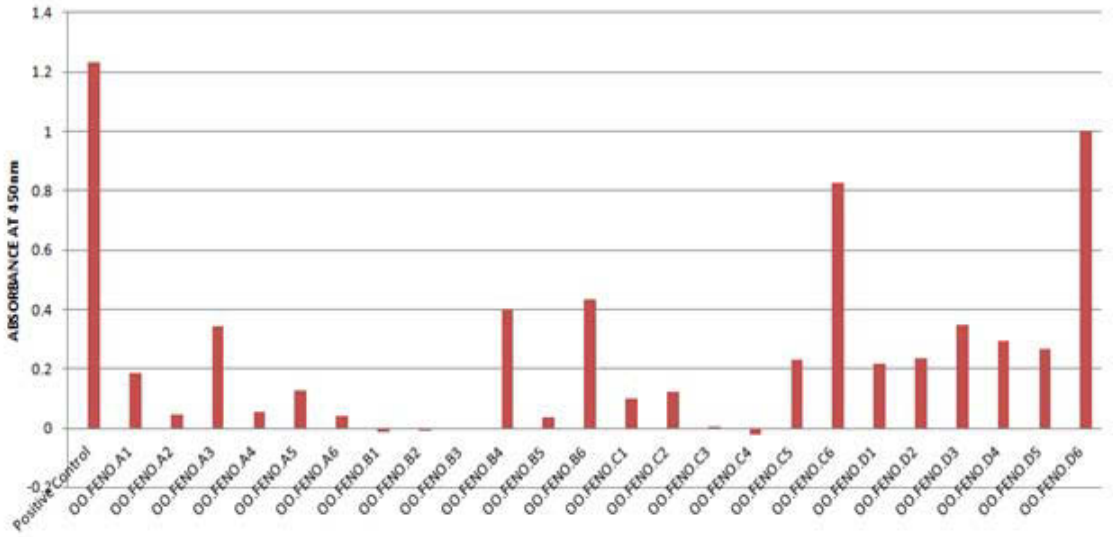
APPENDIX



ADDENDUM 1: ELISA demonstrating binding of anti-FENO polyclonal antibodies to Vpr antigen.

The hybridoma supernatants from the different pools of fused cells were used to determine the amount of binding of the soluble anti-FENO polycolonal antibodies to the FENO antigen. The mouse was immunized with three different boosts of the FENO antigen. The spleen cells from the immunized mouse were isolated and fused to myeloma cells using 50% polyethylene glycol and 10 % DMSO in RPMI. The cells were cultured in 1X HAT for 2 weeks. For each well that was two-thirds confluent, supernatant was collected and screened by indirect ELISA for antibody binding to FENO antigen. Absorbance at 450nm is indicated on the Y axis.

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Monday, February 18, 2013

Dr. Diana Fagan
Department of Biological Sciences
UNIVERSITY

Re: IACUC Protocol # 02-13
Title: Use of animals in research, immunization, collection of cells and cell culture.

Dear Dr. Fagan:

The Institutional Animal Care and Use Committee of Youngstown State University has reviewed the aforementioned protocol you submitted for consideration and determined it should be unconditionally approved for the period of February 18, 2013 through its expiration date of February 18, 2016.

This protocol is approved for a period of three years; however, it must be updated yearly via the submission of an Annual Review-Request to Use Animals form. These Annual Review forms must be submitted to the IACUC at least thirty days ***prior*** to the protocol's yearly anniversary dates of February 18, 2014 and February 18, 2015. If you do not submit the forms as requested, this protocol will be immediately suspended. You must adhere to the procedures described in your approved request; any modification of your project must first be authorized by the Institutional Animal Care and Use Committee.

Good luck with your research!

Sincerely,

[redacted]

Dr. Scott Martin
Interim Associate Dean for Research
Authorized Institutional Official

sm:dka

C: Dr. Walter Horne, Consulting Veterinarian, NEOUCOM
Dr. Robert Leipheimer, Chair IACUC, Chair Biological Sciences
Dawn Amolsch, Animal Tech., Biological Sciences