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> Induction and Characterization of Immune Responses in small animals using a Venezuelan Equine Encephalitis Virus (VEE) Replicon System, Expressing Human Immunodeficiency Virus Type-1 (HIV-1) Envelope Genes

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14. ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) is the lentivirus responsible for the Acquired Immunodeficiency Syndrome (AIDS) pandemic. Currently, no approved vaccine exists to combat this global human health threat. One approach is to develop a vaccine that induces neutralizing antibodies. To date, the most promising platforms used for induction of neutralizing antibodies against HIV-1 is the Venezuelan equine encephalitis virus (VEE) replicon system. The studies detailed herein have characterized certain aspects of the immune responses induced by the in vivo VEE expression system. Following HIV-1 Env-encoding replicon preparation and immunization of mice, the analysis of those immune responses were carried out using enzyme immunoassays (EIAs) and virus neutralization assays. The data revealed significant anti-HIV-1 Env antibody induction, with intra-clade and to a lesser extent inter-clade neutralizing antibody reactivity. The data obtained from these studies will contribute to the goal of inducing broadly cross-reactive neutralizing antibodies and aid in the development of an effective vaccine for HIV-1.

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ABSTRACT

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Induction and Characterization of Immune Responses in Small Animals Using a Venezuelan Equine Encephalitis Virus (VEE) Replicon System, Expressing Human Immunodeficiency Virus Type 1 (HIV-1) Envelope Genes

Jennifer M. DeVol; Candidate, Masters of Science, 2003

Thesis Directed by: Gerald V. Quinnan, Jr., M.D., Professor and Chair, Department of Preventive Medicine

Human immunodeficiency virus type 1 (HIV-1) is the lentivirus responsible for the Acquired Immunodeficiency Syndrome (AIDS) pandemic. Currently, no approved vaccine exists to combat this global human health threat. One approach is to develop a vaccine that induces neutralizing antibodies. To date, the most promising platforms used for induction of neutralizing antibodies against HIV-1 is the Venezuelan equine encephalitis virus (VEE) replicon system. The studies detailed herein have characterized certain aspects of the immune responses induced by the *in vivo* VEE expression system. Following HIV-1 Env-encoding replicon preparation and immunization of mice, the analysis of those immune responses were carried out using enzyme immunoassays (EIAs) and virus neutralization assays. The data revealed significant anti-HIV-1 Env antibody induction, with intra-clade and to a lesser extent inter-clade neutralizing antibody reactivity. The data obtained from these studies will contribute to the goal of inducing broadly cross-reactive neutralizing antibodies and aid in the development of an effective vaccine for HIV-1.

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By

Jennifer M. DeVol

Thesis submitted to the Faculty of the
Emerging Infectious Diseases Graduate Program of
the Uniformed Services University of the Health
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Master of Science 2003

DEDICATION

To my Father, my hero, who believed in me
when I stopped believing in myself.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Etiology and the Epidemiology of HIV-1

Human immunodeficiency virus type 1 (HIV-1) falls within the Retroviridae family of viruses and is most closely related to the subfamily of lentiviruses, based on genetic and morphological criteria. Like all retroviruses, HIV is a single positive-stranded RNA virus, and viral particles are approximately 100nm in diameter (49, 69).

The HIV-1 genome consists of four major genes, which encode the structural proteins, *gag-pro-pol-env*. The *gag* gene, which is the 5′-most gene, encodes proteins that are necessary for transport, assembly and packaging of virus. It also encodes three proteins, which include the matrix (MA) protein, capsid (CA) protein, and nucleic acid-binding protein (NC). The *pro* gene encodes a protease (PR), which is responsible for the cleavage of the *gag* and *pol* polyproteins and sometimes part of *env* as well. The *pol* gene encodes the two proteins containing the two activities required by the virus early in infection, reverse transcriptase (RT) and the integrase (IN) protein, which is required for integration of the virus into host cell DNA. The *env* gene encodes the two envelope glycoproteins (44, 49, 69).

These envelope structures are derived from a 160-kDa precursor, gp160, which is cleaved, by cellular enzymes in the Golgi apparatus, into the gp120 external surface envelope protein and into gp41, the transmembrane protein (69, 76). These proteins are transported to the cell surface via the secretory pathway, where part of the amino-terminal portion of gp41 is also expressed on the outside of the virion. The

transmembrane protein binds to the surface gp120 in a noncovalent manner, primarily at two hydrophobic regions in the amino and carboxyl termini of gp120 (49, 56, 69). The virion gp120 contains the binding sites for the host cellular receptors and major neutralizing domains (20, 31, 69). The antigenicity of these components provides a means for detection of antibody, the basis for much HIV diagnostic testing.

The HIV-1 envelope glycoproteins, gp120 and gp41, are assembled on the surface of the virus particle as a trimeric complex that mediates virus entry into target cells (66, 67, 96). HIV-1 entry depends on the sequential interaction of gp120 surface Env glycoprotein with CD4, the cell surface receptor on the target cell. This interaction causes a conformational change, resulting in the binding of gp41 to one of the co-receptors, CCR5 and CXCR4, which are members of the chemokine receptor family (69). Macrophage-tropic (M-tropic) primary strains of HIV-1 use CCR5 as a co-receptor while T cell-tropic (T-tropic) primary strains use a CXCR4 co-receptor (69). The gp120 glycoprotein bound to gp41 by non-covalent interactions elicits both neutralizing and non-neutralizing antibodies during natural infection (4). HIV-1 strains that have been passaged in immortalized T cell lines are typically more sensitive to antibody neutralization than primary (PI) viruses (10, 79). Moreover, neutralization-sensitive epitopes may become resistant to neutralization during the course of infection (80, 81). Our laboratory has also described a global neutralization resistant phenotype resulting from the *in vitro* passage of a neutralization sensitive HIV-1 isolate variant in the presence of neutralizing serum (89, 90).

The pathogenesis of HIV infection involves infection of host cells that express CD4 molecules. The probability of infection is a function of both the number of infective

HIV virions within the host as well as the number of cells available at the site of HIV entry that express the appropriate CD4 molecules (44, 49).

HIV has the ability to mutate easily, in large part due to the error rate of the reverse transcriptase (RT) enzyme, which introduces a mutation approximately once per 2000 incorporated nucleotides. This high mutation rate leads to the emergence of HIV variants within the host that can resist immune attack, are more cytotoxic, can generate syncytia more readily, or can resist drug therapy (44, 49, 69). The phenotypic evolution of lentiviruses is thought to be significant in disease pathogenesis. These types of mutations in the HIV-1 envelope gene alter cellular host range and neutralization epitopes of the virus (12, 23, 55).

Phylogenetic studies can identify clusters of HIV-1 *env* genes which are known as subtypes, or clades, that have arisen with progression of AIDS epidemic worldwide (44, 69, 77). Since their initial expansion in humans roughly seven decades ago in Central Africa, the HIV-1 pandemic strains have 24 circulating genetic forms of the main (M) HIV-1 group, as shown in Figure 1. The M group consists of 11 subtypes and 13 recombinant forms, which are the result of extensive diversification through mutation and recombination. This high genetic diversity is a result of the extraordinary capacity of HIV-1 to evade selective pressures through genetic variation (114). Efficient targeting of this extensive genetic diversity of HIV-1 constitutes one of the major challenges in present efforts to control the AIDS pandemic (77, 78).

The AIDS epidemic claimed more than 3 million lives in 2002, and an estimated 5 million people acquired HIV in 2002, bringing the number of people living with the

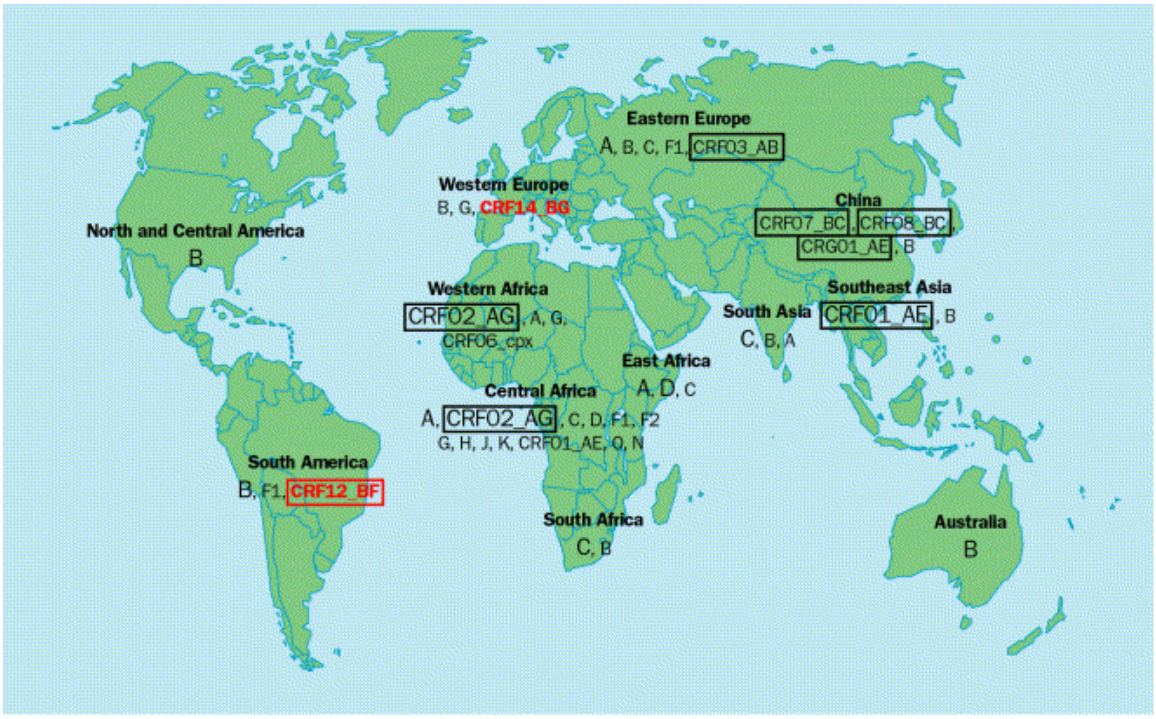

Figure 1. Geographical distribution of HIV-1 genetic forms.
These are the various circulating forms of HIV-1 in different areas of the world. The genetic forms that are dominant in an area are shown in letters of larger size. Epidemiologically relevant circulating recombinant forms (CRF) are boxed (114). The three CRF pertinent to this work are, CRF-1 AE, CRF-7 and CRF-8 BC.

virus globally to 42 million. In Asia and the Pacific, 7.2 million people are now living with HIV, a 10% increase since 2001. The growth of the epidemic in this geographic region is largely due to the increase in the number of cases in China, where a million people are now living with HIV (1, 27, 48, 70). Among Asian countries, the AIDS epidemic has been rampant for a number of years, but has recently begun to spread rapidly in China. Considering the large population of China, it is evident that a large number of new infections will occur in the next several years in China (48). The clades that are likely to be most prevalent during this time are clades C and D in Africa and clades B, C and E (CRF-1) in Asia. Clade E (CRF-1) strains have retained the cytoplasmic domain of gp41 from clade A. In the HIV-1 lineage, the great majority of isolates that have been genetically characterized belong to clades A through J in the main (M) group (77, 78).

The subtypes of HIV-1 found in China include B, Thai B, A, C, D, E, F, G, and BC and BB recombinants (111, 125). Among mainland China's 22 provinces, serious HIV epidemics are already raging in 7, and threatening to break out in another 9. Several HIV epidemics are being observed among certain population groups of drug users in nine provinces, as well as in Beijing Municipality (83). The most recent reported outbreaks of HIV among Chinese intravenous (IV) drug users have been in Hunan and Guishou provinces. Signs of heterosexually transmitted HIV epidemics are spreading in at least three provinces, Yunnan, Guangxi and Guangdong, as seen in Figure 2, where HIV

Figure 2. **Map of Chinese provinces.**
Among mainland China's 22 provinces, serious HIV epidemics are already raging in 7 and threatening to break out in another 9. The Guangxi province, circled in red, is where the HIV-1 envelope genes used in this study were obtained. The other provinces in close proximity to Guangxi, Yunnan and Guangdong are other areas where HIV-1 spread is rampant. (www.chinatour.com/maps/maps.htm)

prevalence in 2000 was as high as 11% among sentinel sex worker populations (1, 124, 125). UNAIDS warns that the disease, if left unchecked, could afflict 20 million people in China by 2010 (48). In certain parts of China the problem is already particularly acute. Along China's southern borders with the opium-growing regions of Burma, Thailand, and Laos, widespread IV drug use was an early source of HIV infection. Drug use, with the spread of HIV, has also extended along drug trafficking routes into China's northwestern province of Xinjiang (48). Although IV drug users constitute the largest proportion of HIV cases in China today, the fastest growing cause of the disease spread in the country is unprotected sex within the heterosexual population (48, 125).

Role of Neutralizing Antibodies

The ability of an antiviral vaccine to elicit antibodies capable of neutralizing viral infectivity has been widely used as a benchmark for vaccine potency and efficacy (86). Some examples of such benchmark include smallpox, polio, and measles, mumps and rubella. Although the mechanisms of protection by many successful vaccines remain poorly defined, multiple arms of the immune response, cellular and humoral, may be involved in the antiviral activity of vaccines, with neutralizing antibodies (NA) being the most important immunological markers of response to vaccines (86, 87, 120). NA play a crucial role in a wide range of viral infections and are associated with recovery from disease, clearance of virus, and protection from reinfection. NA usually develops within weeks of the initial infection (119, 128). Neutralization of HIV-1 involves the binding of

antibodies to the native, fusion-competent envelope glycoprotein (Env) complex on the surface of the infectious virions (67, 91, 94, 108).

Neutralizing antibodies can protect against primary HIV-1 challenge *in vivo* (5, 47, 71, 75, 85, 92, 109), and, therefore any HIV vaccine preparation should ideally induce neutralizing antibodies. Although many HIV-1 immunogens elicit good antibody responses to HIV-1 envelope proteins, many such antibodies do not have neutralizing activity. Unfortunately, neutralizing antibody responses, especially against conserved epitopes on the virus are generally rather poor (15, 16, 95, 121).

A better understanding of the immune response that is more effective at preventing virus infectivity will facilitate the successful development of a vaccine against HIV-1. The induction of neutralizing antibodies is a major concern regarding the efficacy of a vaccine (7, 13, 38, 93). Neutralization of HIV-1 involves the binding of antibodies to the native, fusion-competent envelope glycoprotein complex on the surface of infectious viruses (67, 94). Different approaches attempt to express and immunize *in vivo* with the intact envelope protein complex where exposed immunogenic epitopes can induce specific cross-reactive neutralizing antibodies.

Virus neutralizing antibodies are commonly involved in protective immunity against viruses (100). Non-neutralizing antibodies and cellular immunity have been shown to contribute to protective immunity, but antibodies that prevent viruses from infecting cells appear to be of major importance (100). In the case of HIV-1, neutralizing antibodies generally develop within six months of initial HIV infection. There is also a response to new antigenic variants in patients, which may have developed in a similar time period (127, 128). HIV-1 neutralizing antibodies target the envelope glycoprotein

complex. This complex is a multimeric structure composed of three or four copies each of the gp120 surface and gp41 transmembrane glycoproteins (101). There are a number of neutralization domains on each of the three or four heterodimeric components of the complex (26). It is thought that potent neutralization of primary virus isolates may only be achievable by immunogens that explicitly incorporate the functioning uncleaved Env protein (86). Antibody-mediated neutralization of HIV-1 may take place either by blocking gp120 from binding its cellular receptor (CD4) or coreceptor (CCR5 or CXCR4) or by preventing gp41 from mediating fusion with the target cell membrane (14). Antibodies could also agregate viral particles or mediate antibody-dependent cellular cytotoxicity (ADCC).

Considerable geographic diversity in the genotype of HIV-1 exists, both in nucleotide and amino acid sequence. Linear neutralization epitopes in HIV-1 *env* gene products have now been defined within variable regions (V3 loop), in the transmembrane region, and also via CD4 binding site inhibition (22). It was once thought that the principal type-specific neutralizing determinant of HIV-1 lies within gp120 in the V3 loop (22, 50, 51, 107, 126), but not only are there several other important neutralizing epitopes on gp120 and gp41, recent data suggests that the V3 loop is not as type specific as once thought; e.g., the neutralization of anti-V3 monoclonal antibodies is not readily predicted by the linear V3 sequence (68).

It is thought that the incorporation of envelope proteins of primary neutralization-resistant viruses into vaccine constructs may be necessary in order to induce neutralizing antibody responses that are broadly cross-reactive among primary viruses (82, 127). A vaccine must be useful across the many clades because HIV-1 is so variable. Previously,

our laboratory has analyzed clade B virus envelope and its capacity to induce cross-clade neutralization (116). Clade B viruses are of interest and importance because this clade is the prevalent subtype found in the US and Europe. We are also interested in clade C and E (CRF-1) viruses, which are prominent in China (14, 125).

The range of neutralizing antibodies in sera from HIV-1-infected subjects is broad both in magnitude and in breadth (72). It has been shown that neutralization serotype does not correspond to virus clade (72, 82); e.g., sera from clade B and E HIV-1-infected subjects preferentially neutralized viruses from B and E clades respectively (72, 82, 97). Such preferential clade-specific neutralization elicited by natural infection, suggests that neutralizing antibodies are an important component of protection and thus, a multivalent immunogen will likely offer a broader range of protection against diverse strains of HIV-1 (72, 127). For any HIV-1 vaccine to be truly successful globally, it should be able to counter viruses from all of the clades, which may require multiple genotypes to be included in a complex vaccine formulation.

VEE Expression System

The use of live-virus vaccine vectors for the expression of HIV-1 proteins is an active area of investigation. A number of studies employing pox viruses, picornaviruses, adenoviruses, herpesviruses, or influenza virus, and alphaviruses, as expression vectors for HIV-1 genes all have shown the ability to elicit some degree of immunity to HIV (58).

Venezuelan equine encephalitis (VEE) is a member of the *Alphavirus* genus, a group of viruses that also includes Sindbis virus and Semliki forest virus. The single-stranded positive-sense RNA genomes are infectious, as are genomic replicas derived from full-length cDNA clones (34). These positive-stranded RNA viruses infect a broad range of host cells and initiate a rapid replication cycle within the cytoplasm. Expression of the structural proteins is controlled by a subgenomic mRNA promoter which transcribes message at levels 10-fold more than that of the genome. *Alphavirus* expression vectors have utilized this strong viral subgenomic mRNA promoter (18).

There are three key biological features of VEE suggest that this virus could be an unusually effective *in vivo* expression vector for vaccination with heterologous gene products. First, parenteral immunization of rodents and humans with live-attenuated VEE vaccines results in protection, not only against parenteral challenge, but also against intranasal and aerosol challenges (21, 33, 60, 63). Second, VEE replicates first in the lymph nodes draining the site of inoculation targeting dendritic cells (54, 59), where high-level synthesis of antigens might result in efficient immunization of the host. Finally, in contrast to several other viral vectors such as vaccinia or adenoviruses, most humans and animals are not immune to VEE. Therefore, prior immunity to the vector itself would not limit a VEE-based vector system (34).

Pushko and colleagues have reported on the development of a novel VEE based *in vivo* expression system (17, 18, 35, 99). This system has been remarkably effective for induction of immune responses to heterologous glycoproteins (17, 18, 34, 99). Alphavirus replicon systems are the most efficient systems that have been established to date for *in vivo* expression and immunization with conformationally intact envelope

protein complexes (18, 35, 99). VEE replicon particles have been used to induce very potent responses to a variety of heterologous gene products (17, 34, 36, 99). These vector expression systems have been developed from full-length cDNA clones of VEE (99). Alphavirus expression systems hold great promise as vaccine delivery vehicles, and the VEE-based replicon-helper system previously described (99) is especially well suited for this purpose (59). The system is illustrated in Figure 3.

Experiments have been reported by Caley *et al*. demonstrating that VEE vaccine vectors: 1) are efficient expression systems for *in vivo* delivery of HIV immunogens; 2) induce responses to the expressed gene product that can be enhanced by booster inoculation; and 3) stimulate cellular as well as humoral and mucosal arms of the immune system (18). Expression levels of the foreign protein generated by the VEE vector are comparable to levels of expression achieved in Sindbis virus, vaccinia virus, and adenovirus vector systems (17, 18, 122).

The VEE vaccine vector system is composed of a self-replicating RNA, or replicon, containing all of the VEE nonstructural genes. Cotransfection, by electroporation, of cells *in vitro* with a recombinant VEE replicon and two helper RNA molecules, the latter of which encodes all of the VEE structural proteins, results in the production of propagation-deficient VEE replicon particles (99). The 26S mRNA promoter drives transcription of the subgenomic RNA such that it is present at up to 10 times the amount of genomic RNA. VEE replicons were prepared from a plasmid carrying a complete cDNA copy of VEE genome modified to contain a second 26S promoter followed by a multiple cloning site. The VEE cDNA is downstream from a T7 RNA polymerase promoter such that linearization of the clone downstream of the VEE

sequences, and subsequent *in vitro* transcription with T7 polymerase, yields infectious VEE genomic replicas (33, 36, 99). The system is illustrated in Figure 3.

When administered to animals, the VEE replicons infect host cells and lead to the production of immunogens that stimulate an immune response (98). In our case, the replicons encode HIV-1 Env glycoproteins. Since the replicons lack any VEE structural genes, the infected cells will not produce progeny viral particles.

Potent immune responses have been induced against proteins of at least 12 different viruses using this VEE-based expression vector, including protection against experimental challenge with SIV and influenza. Monkeys immunized with VEE replicons that expressed SIV envelope genes and *gag* genes were protected from progressive infection and death (35). It has also been shown that VEE particles administered peripherally are rapidly taken up by lymphoid cells (53, 54). Therefore, the mechanism of efficacy of VEE as an immunogen may involve delivery of a highly efficient gene expression vector to professional antigen presenting cells in lymphoid tissues. As a result of the previously described studies, this vector system may provide a mechanism for induction of high potency systemic neutralizing antibody responses against HIV-1 antigens.

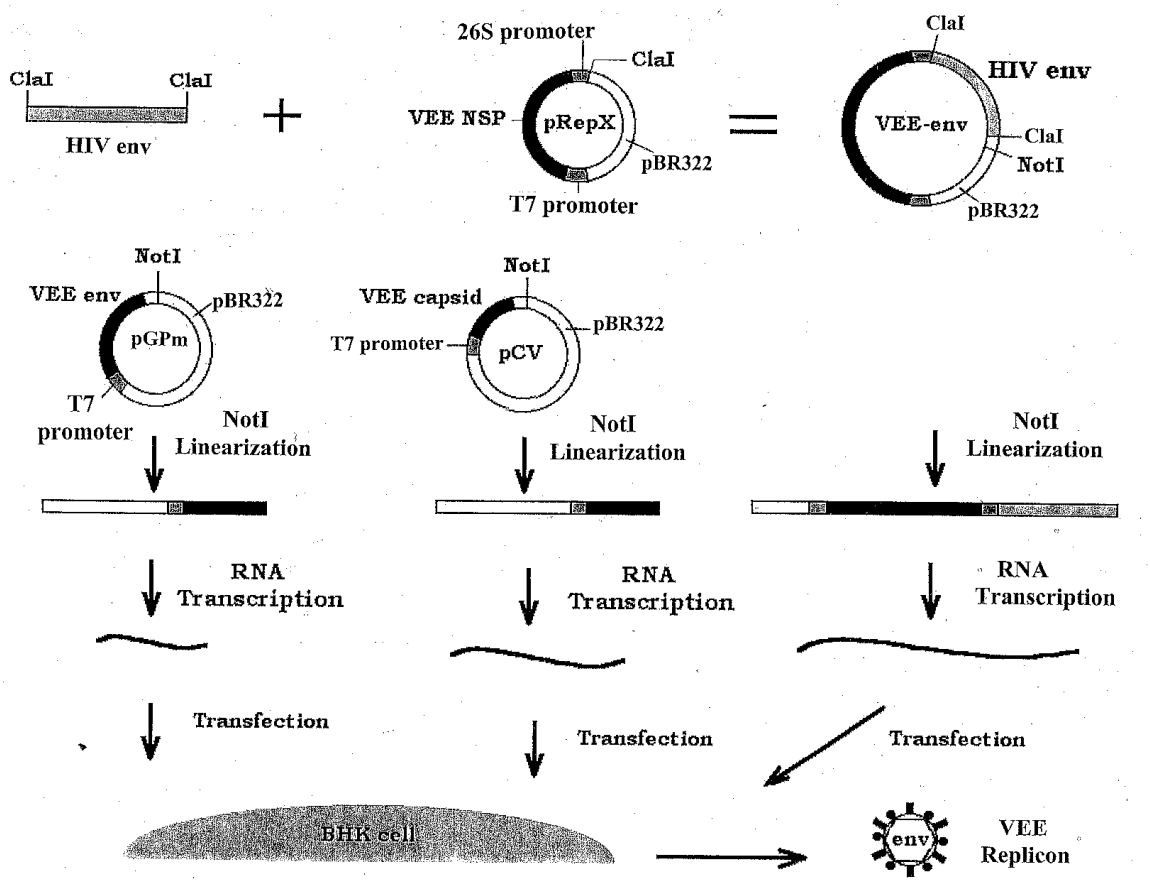

Figure 3. VEE expression system.
The VEE expression system used to prepare replicon particles used for immunization. The three plasmids used were, the pRepX VEE vector with a second 26S promoter, pGPm, the glycoprotein plasmid used with the back mutation of pGP to wild type and pCV, the capsid plasmid. All three were transcribed into RNA and transfected into BHK cells, from which replicon particles were produced. Modified and reproduced with the permission of Dr. Peng Fei Zhang.

PRELIMINARY DATA

Our laboratory has conducted studies regarding HIV-1 primary virus cross-reactive neutralization and mechanisms of primary virus neutralization resistance, which are relevant to our current work with VEE replicons. We have found that the HIV-1 Neutralizing Serum (2) (HNS2), and HIV-1 envelope gene obtained from the same donor, clone R2, both exhibit extensive neutralizing cross-reactivity (101, 120). The human serum, HNS2, neutralizes many primary isolates of different clades of HIV-1 and virus expressing envelope from the same donor, clone R2, is neutralized cross-reactively by HIV-immune human sera (126). Neutralizing cross reactivity of the R2 envelope extends to numerous clade B sera, the majority of clade A, C, and F sera tested, and some clade D and E sera (101). A similar spectrum of cross-reactivity has been observed with HNS2. The finding of neutralizing cross reactivity in the donor serum indicates that these donors had been infected with a strain of HIV-1 that expressed primary virus cross-reactive neutralization epitopes.

Previous studies from our laboratory have demonstrated that infection and immunization, using a VEE replicon expression system results in cross-reactive neutralizing antibodies effective against primary isolates of HIV-1. Cross-reactive neutralization epitopes on HIV-1 envelopes are generally classically conformation-dependent epitopes, or are sensitive to conformational changes. It has been found that conformation independent epitopes are not functional for neutralization of primary isolates (88, 89, 126), with certain exceptions like monoclonal antibodies, 2F5 and Z13 and α-gp41. While some of the genetic variation that characterizes HIV-1 envelopes

affects the sequence of linear epitopes, much of it affects conformation dependent infectivity functions of the envelope complex. These functions determine the neutralization resistance of primary isolates, and the nature of the neutralization epitopes that are functional on primary isolates (88, 89, 113).

The presence of antibodies that have broadly neutralizing activity against primary isolates of many subtypes of HIV-1 in sera from infected people is unusual, but the nature of envelope proteins in individuals with such antibodies may be of interest for defining the epitopes that may be broadly immunogenic in vaccines (101). Our laboratory has recently demonstrated the induction of neutralizing antibodies by *in vivo* expression of the HIV-1 Env protein by using a VEE replicon system (39). The results of this study suggest that efficiency of this immunization system is likely related to the manner in which the replicon targets expression to lymphoid tissues, a preferred site for induction of immunity and the production of high levels of foreign protein (35).

Our laboratory has studied the induction of neutralizing antibodies by *in vivo* expression of the HIV-1 envelope using a VEE replicon system (39). This replicon system is comprised of 2 helper plasmids, pCV and pGP. Our lab reversed two mutations in pGP, to yield pGPm, in which the VEE envelope genes comprised wild-type, rather than host adapted sequence. The results we obtained demonstrate that the pGPm replicons induce significantly faster and more potent neutralizing responses to HIV-1 envelope in mice than pGP replicon particles, even when used at substantially lower infectivity titers based on assays of BHK cells (39). Our laboratory has also looked at the induction of neutralizing antibodies by the previously described HIV-1 envelope clone, R2. R2, is an HIV-1 clade B clone. Clade B HIV-1 is the prominent form in the United

States and Europe. R2 pseudovirus was neutralized by sera from people known to be infected with clade A, B, C, D, E, and F strains of HIV-1 (101).

In previous work we have found that using the VEE replicon system, R2 was neutralized by sera from clades A, B and C the most and to a lesser extent D or CRF-1 (Chinese E). These results demonstrate that neutralizing antibody responses can be induced in mice within 2 to 3 months that are similar to those found in the chronically infected, long-term nonprogressive donor of HNS2 (HIV-1-neutralizing serum 2) (39). R2 has broad sensitivity to cross-reactive neutralization, and was obtained from a donor with broadly cross-reactive, primary virus neutralizing antibodies (39).

The overall objective of this thesis was to gain knowledge on the cross-reactivity of responses induced by VEE replicons, containing Chinese HIV-1 envelopes, in small animals. The aims of the present study were to; 1) induce clade C and E neutralizing antibodies in small animals with VEE replicons; 2) determine intra- and inter-clade cross-reactivity of responses induced in Aim #1; 3) determine comparability of neutralizing responses detected using pseudotyped and recombinant viruses.

MATERIALS AND METHODS

Preparation and Characterization of VEE Replicons

Preparation and characterization of env DNA

Chinese donors having virus-serum combination with neutralizing cross-reactivity were the source of HIV env genes. Donors were individuals who were diagnosed with HIV infection for longer than two years. The clade C and E viruses used in this project are from the Guangxi province in southeast China, as shown in Figure 2 (14, 125).

Functional clones of the Chinese clade C and E viruses were constructed in our lab by Dr. Peng Fei Zhang. Briefly, supernatant-containing HIV-1 virus (a gift from Dr. X.F. Yu, Johns Hopkins University School of Medicine) was used to amplify envelope genes by RT-PCR. These genes were cloned into the pSV7d expression vector and were screened for function in pseudotyped virus infectivity assays using HOS-CD4-CCR5 cells (39)

To prepare the full-length HIV-1 genes for cloning into the VEE expression vector, it was necessary to introduce specific sequences at the 5′ and 3′ ends of the gene (99). As previously described, the sequences were introduced at the ends of an HIV-1 envelope gene by PCR (99). At the 5′ end, a *Cla*I recognition sequence was introduced, followed by a 14-nucleotide VEE promoter sequence, followed immediately by the envelope gene initiation codon. This 14-nucleotide VEE sequence between the *Cla*I recognition sequence and the envelope gene initiation codon is necessary for protein

translation by the VEE 26S subgenomic promoter (99). At the 3′ end of the gene, a *Cla*I recognition sequence was introduced. Each primer contained an additional restriction enzyme recogntion sequence for use in cloning of the HIV-1 gene into the VEE expression vector. The HIV-1 envelope gene was amplified by using the primers and cloned into a shuttle vector (39).

Clones GX-C #44 and GX-E #14 were two of the functional clones constructed by Dr. Peng Fei Zhang. PCR, using rtTh DNA polymerase, was done to amplify the envelope genes. PCR products were treated with DpnI at 37ºC for one hour. Treatment with DpnI endonuclease is specific for methylated and hemimethylated DNA and was used to digest the parental DNA template, leaving the PCR products intact. DNA isolated from almost all *E. coli* strains is *dam* methylated, and, therefore, susceptible to DpnI digestion.

PCR products were gel purified and digested with *Cla*I to excise the envelope gene. At the same time, the VEE vector, was also digested with *Cla*I and treated with Calf Intestinal Alkaline Phosphatase (CIP). This procedure removed the 5′-phosphate group from the vector, preventing self-ligation, therefore decreasing the vector background and increasing the chances of a successful ligation.

Env obtained from GX-C #44 and GX-E #14, was then ligated into the double promoter VEE replicon and transformed into DH5α competent cells. Briefly, 1µl of ligation reaction DNA was added to 100µl of DH5α competent cells, kept on ice. This DNA-cell mixture was incubated on ice for 30 minutes. The mixture was heat-shocked at 42ºC for 45 seconds and then incubated on ice for 2 minutes. Next, 900µl of SOC medium (Invitrogen, Carls Bad, Calif.) was added to the mixture and incubated at 37ºC

for 1 hour in a shaking incubator. Following this incubation, mixtures were plated on LB-amp agar plates and incubated overnight at 37ºC. The following day, colonies were selected and screened for correct size.

Clones with correctly sized bands on agarose gel electrophoresis were digested with ClaI to verify that the insert was the *env* gene of interest. Clones were then sequenced to verify the correct orientation of *env*. The mRNAs of most RNA viruses, like VEE, are not complete copies of the viral RNA but are subgenomic. However, to assemble infectious viral particles, exact replicas of the RNA genome must be made. The 26S mRNA promoter drives transcription of the subgenomic RNA (44, 99). In Alphavirus-infected cells three different multisubunit RNA polymerases are produced from a single polyprotein precursor, P1234. Cleavage at different sites in the precursor produces enzymes that contain the same protein sequences in different polypeptides and recognize different initiation for site for RNA synthesis (44). Therefore, correct orientation of *env* is necessary, and an incorrect orientation will cause the reading frames to be incorrect and the 26S promoter will not be able to read for protein synthesis.

Protein characterization and preparation of RNA

Clones with the correct *env* orientation following sequencing were linearized by digestion with *Not*I, the recognition sequence for which is located upstream of the VEE primary promoter sequence. Linearized plasmid DNA that contain an RNA polymerase promoter site can be used as template. In general, any DNA with a promoter site that is digestable by restriction enzymes can be used for *in vitro* transcription studies. These

digestion products were visualized on a gel, column purified and DNA was quantified using optical density. We used 0.05μg DNA of column-purified clones for *in vitro* RNA transcription using T7 RNA polymerase (Ambion, Austin, TX). *In vitro* synthesis of capped RNA mimics most eukaryotic mRNAs found *in vivo*, because it has a 7-methyl guanosine cap structure at the 5′ end. Following transcription, RNA was purified and electrophoresed to quantify and stored at -80ºC.

Baby hamster kidney (BHK) cells were transfected by electroporation with recombinant replicon vector containing clade C clone C25 and clade E clone E18. After 27 hours, cells were harvested and lysed. Supernatant was collected and characterized for expression of protein using SDS-PAGE and Western blot. Briefly, the sample to be characterized was boiled with 3x buffer for 3 minutes and then directly loaded onto the SDS gel. The sample was electrophoresed at a constant 125V for one and a half hours. Next, the SDS gel was transferred to a nitrocellulose membrane, to later be used in a Western blot assay. For Western blot analysis, the membrane was blocked overnight at 4ºC with TBS-Blotto. The following day the membrane was washed three times with TBST. The primary antibody, LIC-1, human HIV-1 positive serum, was added at a 1:500 dilution and incubated at room temperature for one hour. The membrane was washed and the secondary antibody, goat α-human IgG conjugated to Alkine Phosphatase, was added and incubated at room temperature for one hour. Membrane was washed and color developed by adding AP developing buffer. The reaction was stopped by a 10 minute water wash.

Two clones showed good protein expression on Western blot, as seen in Figure 4, and were used throughout the rest of the study.

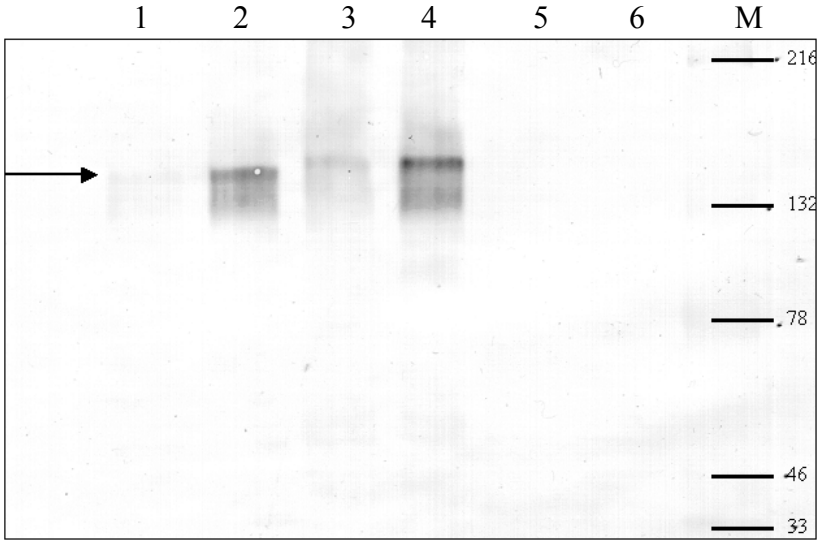

Figure 4. Western Blot of Envelope Proteins.
Western blot analysis showing protein expression of HIV-1 envelope genes used in VEE replicon immunization of small animals. Marker (M) is in Kilodaltons (KDa). Arrow is shown at 160 KDa band, gp160. Lane 1 is E18. Lane 2 is C25. Lane 3 is MACS#9. Lane 4 is R2. Lane 5 is pRepX, VEE vector. Lane 6 is the BHK cell control.

Transfection and preparation of replicons particles

BHK cells were transfected with recombinant replicon RNA and two helper RNAs synthesized, using the plasmids pCV and pGPm. These plasmids encode the VEE capsid proteins and glycoproteins, respectively. Use of a bipartate helper system greatly reduces the generation of replication-competent virus (99). pGPm contains mutations that were introduced to reverse mutations that were accumulated previously in VEE during attempts to adapt the virus to growth in BHK cells (99). These back mutations to wild-type affected lymphoid trafficking of the replicon particles and enhanced immunogenicity in mice (54). The plasmid with the back mutations was designated pGPm (39).

The day before the transfection, BHK cells were seeded at 10^7cell/ml for each T75 flask to be used. For the transfection, seeded cells were harvested by centrifugation for 10 minutes in culture medium to pellet cells, and for a second time in cold 1x PBS to wash the cells. Washed cells were resuspended and brought to a volume containing $2x10^7$ cells. We added 0.8ml of cell suspension to 60µl of a mixture containing VEE vector expressing the HIV-1 env of interest, pCV and pGPm RNAs, 20µl each. This mixture was then transferred into a 0.4-cm cuvette and electroporated at 0.85kV with 25uF capacitance, using a Bio-Rad Gene Pulser (Bio-Rad, Hercules, Calif.). The electroporation mix containing RNA and cells remained at room temperature for 10 minutes before being added to 24ml of culture medium. Post-transfection, before replicon particles began to assemble, flasks were moved to the Biosafety level 3 (BSL-3) suite.

27 hours post-transfection, the replicons were harvested in BSL-3 suite. A 30-minute 10,000 rpm centrifugation was used to separate the replicons from the cell debris. The culture medium containing the replicons were then underlayered with 5ml of 20% sucrose and centrifuged for 3 hours at 24,000 rpm. Following centrifugation, the culture medium was discarded, and the replicon pellet was covered with 0.5ml D-PBS and 0.1% FBS overnight. The following day, the replicon preparations were aliquoted and stored at -80°C.

A schematic showing plasmids used and steps involved in replicon preparation can be found in Figure 3.

Replicon infection and titer calculation

A 16-well chamberslide was seeded with $2x10^7$ BHK cells/ml at 200µl/well to prepare for infection. This procedure will permit the infectivity of the replicon particle preparations to be determined by immunofluorescence assay (IFA). Replicon suspensions were diluted from 10^{-2} to 10^{-7} in D-PBS and 0.1% FBS. The culture medium was decanted and 50µl/well of replicon suspension dilution was added to each well and incubated at 37°C for 1 hour. After 1 hour, 150µl of culture medium was added to each well and chamberslides were incubated at 37°C for 24 hours. After 24 hours, the slides were fixed in acetone at -20°C for 20 minutes and the IFA was then performed.

The slides were air-dried and 50µl of 7.5% BSA and 1x PBS was added for 15 minutes to block unbound sites. Human HIV-1 antibody positive serum (LIC-1) and negative serum were each diluted 1:200 in 1x PBS/0.1% BSA, 50µl/well was added, and

allowed to bind at room temperature for one hour. Slides were then washed twice with 0.1% BSA and 1x PBS for 15 minutes/wash. Following the washes, goat α-human IgG antibodies conjugated to fluorescein isothiocyanate (FITC), was diluted 1:25 in 1x PBS/0.1% BSA and 50µl/well was added and allowed to bind for 1.5 hours in the dark. Slides were washed twice and mounted with glass coverslips. Replicon titer was determined by fluorescence microscopy. For the calculation of titer the number of fluorescent cells was determined using an ocular grid. The average number for three fields was determined and converted into titer/ml.

Replication competency tests

Replicon preparations were checked for replication competence by passage three times in BHK cells. 1:10 dilutions of replicon preparations were incubated in six-well plates that contained confluent BHK cells for one hour at 37°C; then the plates were washed three times with 3ml of 1x PBS. 3ml of culture medium were added to each well and incubated at 37°C for 24 hours. After 24 hours, culture medium was collected and filtered through 45µm pore size filters and stored at -80°C. After these three passages, the stored medium was inoculated into chamberslides and tested by IFA, similar to that following infection of replicon particles. By the third passage, no fluorescence was detected which confirmed that the replicon preparations are not replication competent and able to be used in immunogenicity studies.

Immunogenicity Experiments

Immunization of small animals

The mice used for immunization were 6 month old, female C3H/He mice (Jackson Laboratories, Bar Harbor, ME). The mice were given $4x10^7$ focus-forming units (FFU) of HIV-1 gp160-expressing replicon particles. All mice were immunized by footpad inoculation of 50µl suspensions of replicon particles. This regimen yields $1x10^6$FFU/dose. Mice were bled from the tail vein, except at euthanasia when they were bled from the axillary artery. The immunization/boost/bleed schedule can be found in Table 1.

Determination of serum immunoglobulin responses

To study the humoral response to VEE replicons containing HIV-1 env genes in the context of oligomeric protein, an enzyme immunoassay (EIA) that utilized soluble, oligomeric Env protein was used. Use of oligomeric gp140 EIA allows for rapid and quantitative assessment of the levels of antibodies to linear and conformational determinants present in mouse sera to oligomeric Env as well as detection of a greater fraction of total anti-Env activity in sera (104). The EIA makes it possible to quickly and

quantitatively assess the capacity of different serum samples to react with oligomeric forms of Env protein (104). In the EIA, antigen was fixed to the interior

Week	Primary Immunogen (twice per week)	Secondary Immunogen (twice per week)	Bleed
1	C25 MACS#9 E18 R2		
2	C25 MACS#9 E18 R2		
3	C25 MACS#9 E18 R2		
4			BLEED
5		**(#1 and #2)** C25 MACS#9 E18 R2	
7			BLEED
11			BLEED
12		**(#3 and #4)** C25 MACS#9 E18 R2	
15			BLEED

Table 1. Immunization schedule showing weeks at which animals were immunized, boosted or bled. This Table also shows which HIV-1 envelopes the animals were given throughout the experiment. C3H/He mice were immunized for three weeks, twice a week, and bled 10 days after last immunization (4 week bleed). At week 5 mice were boosted twice and bled 10 days later (7 week bleed). Mice were also bled at week 11 prior to a final set of boosts at week 12 with the final bleed at week 15.

Surface of adsorption of serum proteins to the wells (30). The microtiter wells were washed to microtiter wells. First plates were blocked and then serum was added in blocking buffer to prevent nonspecific remove unbound serum proteins. Antibodies conjugated with Horseradish Peroxidase enzyme and directed against IgG were added for detection of any bound IgG present. The microtiter wells were washed to remove unbound conjugate and then chromogen/substrate was added. In the presence of peroxidase enzyme the colorless substrate is hydrolysed to a colored end-product. The color intensity is proportional to the amount to antibodies present in the serum (41, 52, 117).

Enzyme immunoassays were used to determine serum Immunoglobulin responses. This type of assay is extensively used for the detection of specific antibodies from serum samples. The specificity of the assay is directed by the antigen on the solid-phase, which may be either highly purified and characterized or relatively crude and noncharacterized. The antigen being used in our assays was highly purified and well characterized, oligomeric proteins, which was provided as a gift. (Dr. C. Broder, Microbiology, USUHS)

Two EIA formats were compared to determine the optimal conditions. One assay was based on use of Concanavalin A (ConA) for antigen capture, and the other assay involved direct binding of antigen to plates. For the first assay, ConA was absorbed to wells of microtiter plates. Antibodies binding to ConA-immobilized viral antigens were detected by peroxidase-conjugated antibodies and the appropriate enzyme substrate. Since ConA has high affinity for glycoproteins, it is possible that some epitopes of HIV-1

gp120 are rendered inaccessible to antibody when the gp120 is bound to ConA, or that non-specific binding of the glycoproteins may occur (106).

In this study, the relative merits of different substrate systems were investigated with regard to assay sensitivity. The substrates tested include a colormetric substrate, 2,2′ azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) in hydrogen peroxide solution, and one form of tetramethyl benzidine (TMB), were investigated with regard to assay sensitivity (112, 123). ConA and direct antigen binding EIAs were compared and optimized for detecting murine immune responses following replicon immunizations.

EIAs were performed by using 96-well Immulon II plates. Unless otherwise noted, all incubations were done at room temperature, using a volume of 100 µl per well for at least 1 hour followed by three washes (200 µl per well) with PBS containing 0.05% Tween 20 (wash buffer) (PBST).

For the ConA EIAs, Immulon II 96-well plates were coated with 100µl/well of ConA (100µg/ml in PBS containing 10mM HEPES [pH 8.5] and 0.1mM $CaCl_2$) for at least 2 hours at room temperature. Plates were washed six times with wash buffer (PBST), and cleaved R2 protien was added at 40ng/well in PBST. The plates were incubated at 4°C overnight, washed and blocked at room temperature with block buffer (5% Blotto in PBS) for 1 hour. For the rest of the experiment, plates were washed six times between each step. After blocking, mouse sera was diluted using BlottoT, starting at 1:200 and doing four-fold dilutions, then incubated at room temperature for 1 hour. Following mouse sera incubation, α-mouse IgG conjugated to horseradish peroxidase (1/10,000 dilution with BlottoT) was added and incubated at room temperature for 1 hour. Following conjugate incubation, TMB substrate and Peroxidase B, at 1:1 dilution,

were added and incubated for 15 minutes, absent light. Color development was stopped with 1M H_3PO_4. The plate was read at 450nm wavelength.

For the direct antigen binding EIAs, Immulon II 96-well plates were coated with 1M $NaHCO_3$ and 1M Na_2CO_3. The electric charge of the coat buffer helps the antigens adhere to the plates. Coat buffer was used to make a 120ng/well solution of cleaved gp140 which was used as an antigen. Plates were incubated overnight at 4ºC, washed and blocked at room temperature with blocking buffer (5% Blotto in PBS) for one hour. Mouse serum was diluted with Blotto T, starting at 1:200 and doing four-fold dilutions, then was incubated at room temperature for one hour. α-mouse antibody conjugated to horseradish peroxidase diluted, 1:1000 with Blotto T, was added and incubated at room temperature for one hour. ABTS substrate and Peroxidase B were diluted 1:1, added, and incubated for 30 minutes. The plates were read at 405nm.

Infectivity assays

The use of pseudoviruses as reagents for neutralization assays helps to facilitate the study of the genetic basis for the neutralization sensitivity phenotype variation during chronic infection and antigenic variation among different strains of HIV-1 (102). Pseudotyped HIV-1 virons have been, and continue to be, important in the study of HIV-1 replication. HIV-1 pseudotypes are virons that contain all the proteins and core structure of HIV-1 but are packaged inside the envelope protein of another HIV-1 viruses (8, 28, 89). Previously our laboratory developed a pseudovirus neutralization assay system permit the subsequent comparative study of *env* clones. For example, clones

obtained from a given individual at different time points have been compared (23, 37, 102).

The pseudotyped viruses used in our assays were prepared using two plasmids: pNL4-3.luc.E-R- and pSV7d-HIV-1env plasmids. Vector pNL4-3.luc.E-R- is a construct that expresses the HIV-1_{NL4-3} provirus but contains a frameshift at the 5′ end of the *env* gene (nucleotide 5950) that prevents the expression of the envelope glycoproteins. The vector also contains a reporter gene for firefly luciferase inserted into the *nef* gene of pNL4-3 and defective *env* and *vpr* genes (28, 90). This system is shown in Figure 5.

Pseudoviruses expressing envelope glycoproteins derived from various *env* plasmids were constructed as described previously (28, 89, 90, 127). Briefly, 293T cells were cotransfected with *env*-expressing plasmids and with the complementary viral genome-reporter gene vector, pNL4-3.luc.E-R-. Cell culture fluid harvests containing Pseudoviruses were used to infect either HOS-CD4-CCR5 or HOS-CD4-CXCR4 cells, depending on virus trophism. The luciferase activity of infected cells was measured after 48 hours using a MicroLumat *Plus* luminometer (Wallac, Gaithersbury, MD.).

Pseudotyped viruses were prepared by cotransfection of 50% to 70%-confluent 293T cells with pNL4-3.luc.E-R- and pSV7d-HIV-1env plasmid using the calcium phosphate/HEPES buffer technique, according to manufacturers' instruction (Promega, Madison, Wis.). Sixteen hours after the transfection, the medium was removed and replaced with medium supplemented with 0.1mM sodium butyrate and cells were allowed to grow for an additional 24 hours. The supernatant, containing pseudotyped particles, was harvested, centrifuged at 16,000 rpm for 5 minutes at 4ºC, filtered through a 0.45µm pore filter and used fresh for infectivity and neutralization assays. Clade A, B,

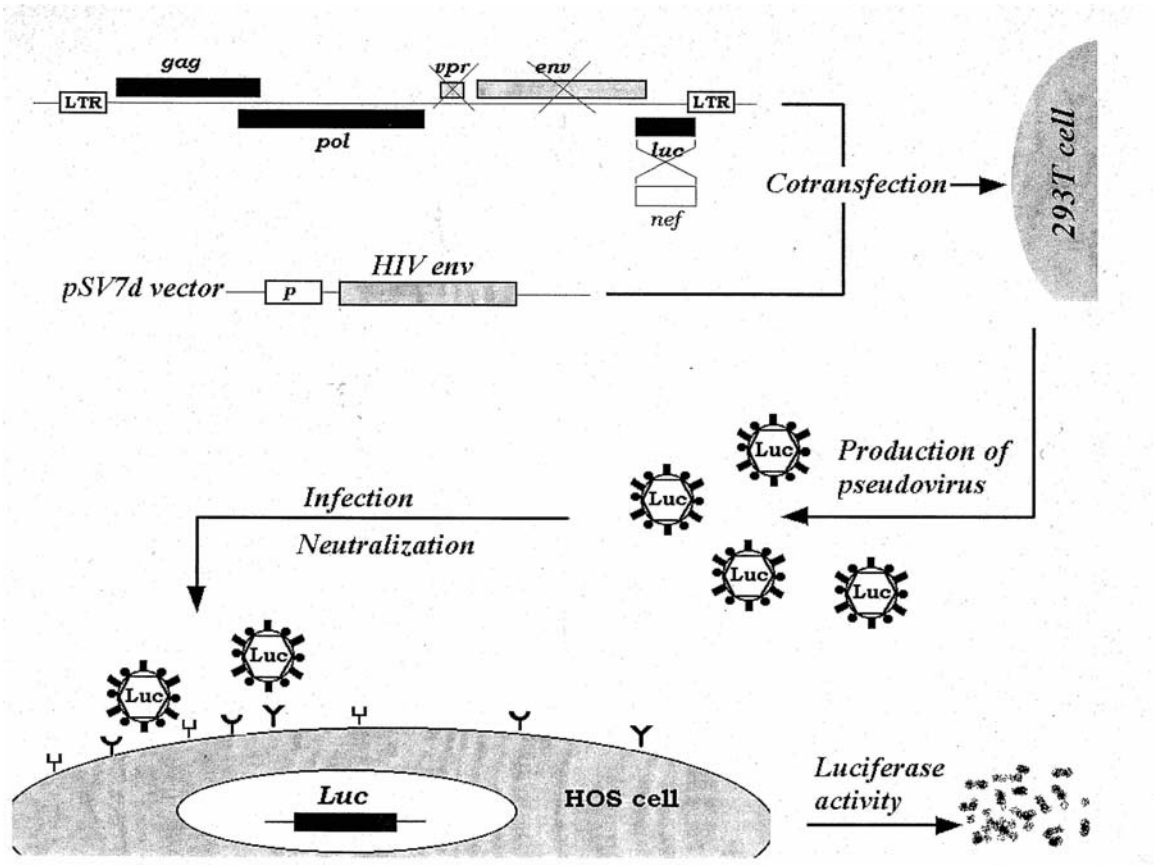

Figure 5. ***In vitro*** **pseudovirus construction.**
Pseudoviruses, most commonly used in neutralization assays, were constructed using two plasmids, pNL4-3.luc.E-R- and pSV7d-env. pNL4-3.luc.E-R- expresses the HIV-1_{NL4-3} provirus but contained a 5′-frameshit preventing the expression of the envelope glycoproteins. This plasmid also contains a reporter gene for firefly luciferase inserted into the *nef* gene and a defective *vpr* gene. The pSV7d-env plasmid contains the HIV-1 env of the virus to be used in pseudotyping. These plasmids were cotransfected into 293T cells, from which the Pseudoviruses were produced. These pseudoviruses were then used for neutralization assays with HOS-CD4-CCR5 cells. Non-neutralized Pseudoviruses will infect the cells and luciferase activity can be detected. Reproduced with permission of Dr. Peng Fei Zhang.

C, and E pseudotyped viruses were constructed to determine the intra- and inter-clade cross-reactivity of immune mouse sera obtained from VEE replicon immunization

Infectivity assays were carried out using HOS-CD4^{+}-CCR5^{+} cells because they have been shown in the past to be readily infected by pseudotyped vectors (90, 103). Infectivity titers were determined on the basis of luminescence measurements at 3 days post-infection of the HOS-CD4^{+}-CCR5^{+} cells by the pseudotyped viruses.

Neutralization assays

Most studies on neutralizing antibody sensitivities using serum samples from HIV-1-infected people and primary viral isolates have concluded that neutralization serotypes do not correlate with HIV-1 genetic subtypes, (65, 82). However, several studies (72, 74) have reported that subtypes B and E do correspond to different neutralization serotypes relative to each other. Few studies have examined the elicitation of cross-clade neutralization by HIV-1 vaccines, since antibody responses with neutralizing capacity against primary isolated or interclade activities, have not usually been obtained (114). It is thought that potent neutralization of primary isolate viruses may only be achievable by using immunogens that explicitly incorporate the functioning *env* (86).

It has been shown that immunization with envelope glycoproteins elicits NAs primarily against the strain of virus from which the immunogen was derived (62). The objective of inducing broadly cross-reactive neutralizing antibodies against HIV-1 is problematic because of the high sequence variability of the viral envelope proteins and

the general resistance of primary isolates to neutralization (126). Individual isolates of HIV-1 may differ by more than 20% in envelope glycoprotein amino acid sequence (46, 62). Thus, vaccines containing HIV-1 envelope proteins or glycoprotein antigens will elicit NA against only a fraction of the variants of HIV-1.

The basis behind the neutralization assays is that as the sera to be tested is diluted and equal amounts of pseudovirus is added, there is less neutralization, and, therefore more pseudovirus available to infect cells. Briefly, 48 hours after transfection, the pseudovirus-containing supernatants were harvested, filtered through 45µm-pore-size sterile filters (Millipore Corp., Bedford, Mass.), supplemented with additional fetal bovine serum to a final concentration of 20%, and stored at -80ºC (90).

Neutralization assays were carried out in 96-well plates with a clear flat bottom. Pseudovirus suspensions were diluted appropriately and 25µl of each pseudovirus suspension was incubated with 25µl of serially diluted mouse serum for one hour at 4ºC, in triplicate in 96-well plates. HOS-CD4-CCR5 cells were prepared and calculated to 10^4 cells/ml. 150µl/well was added to each well and incubated at 37ºC for 3 days. The pseudovirus dilutions were chosen to yield input inocula that gave luminescence generally between 10 to 100 times the background, in the absence of neutralization.

After 3 days incubation the plates were assayed for luciferase activity. The plates were centrifuged at 1,700 rpm for 10 minutes in a Sorvall RT6000B ultracentrifuge, the cells were washed with 150µl of cold PBS, and the plates were drained. Next, 15µl of 1x Luciferase Assay System cell lysis buffer (Promega, Madison, Wis.) was added per well. The plates were shaken for 30 minutes at room temperature. Luciferase Assay System

reporter substrate (Promega, Madison, Wis.) was added, and luciferase activity was measured using a MicroLumat *Plus* Luminometer (Wallac, Gaithersburg, MD.).

Neutralization assays were carried out in triplicate by preincubation of serial serum dilutions with pseudotyped viruses for 1 hour at 4ºC followed by infection of $2x10^4$ cells. The mean luminescence readings for triplicate wells were determined, and the endpoint was considered to be the last dilution of sera at which the mean results from the test samples were less than 50% of the non-neutralized control mean. Neutralization assays for each envelope clone against the sera were carried out at least 3 times.

The 50% neutralization endpoints were approzimately four- to eightfold higher dilutions than the 90% neutralizing endpoints. Since the 50% endpoints were at the middle of the titration curves the calculated values were less variable than the 90% endpoints. Also, because of the need to predilute sera substantially before testing, there were some sera that did not achieve 90% neutralization but mediated statistically significant neutralization of ≥50%.

Neutralization Assay Comparison Experiments

Virus neutralization assays are designed to measure a reduction in virus infectious titer mediated by exposure to antibody. In initial HIV-1 neutralization assays infection was monitored by counting virus-induced syncytia (57, 105, 119). Since each T-cell syncytium corresponds to infection of a single cell, the fraction of virus neutralized is related to a reduction in the number of syncytia (25, 84). This type of assay is quantitative and reproducible but is limited to syncytium-forming viruses that infect T cell lines. Since the minority of HIV-1 isolates infect T cell lines or form syncytia, this assay is not applicable to the majority of strains of HIV-1 (24, 43).

To assay infectivity of viruses that do not infect T cell lines, PBMC are often used as target cells. Most PBMC neutralization assays monitor virus growth by assaying for extracellular production of viral capsid protein, p24, or reverse transcriptase (3, 4, 118). These assays require several rounds of virus replication before expressed proteins can be quantified and thus only indirectly by measurement of the number of target cells infected. The accurate measurement of antibody-mediated neutralization of primary HIV-1 isolates is important for studies of virus neutralization and for the assessment of immune responses to candidate vaccines (73).

Folghera *et al*. have used flow cytometric detection of intracellular p24 to measure neutralization (45). Darden *et al*. have reported the use of a flow cytometric primary isolate HIV-1 neutralization assay that enumerates the number of infected PBMC after 4 to 7 days in culture (32). Mascola *et al*. have taken flow cytometric PBMC assays a step further by including a protease inhibitor in culture with target PBMCs (73). This assay measures single-round infection of individual PBMCs. This *in vitro* neutralization

assay directly quantifies the inactivation of infectious virus mediated by exposure to antibody.

Recombinant virus preparation

Our current pseudotype neutralization assay was initially compared to the PBMC p24 neutralization assay and was found to be comparable. Some advantages to using the pseudotype assay is that results can be obtained quicker, are more reproducible, and safer to laboratory personal then the conventional p24 assay (127). With the recent report of a new assay that measures single-round infection of individual PBMCs (73), we sought to compare our pseudotype assay to this new PBMC assay.

The strategy for the recombinant virus construction utilized pBluescript as a temporary vector for the mutagenesis reactions. The rationale behind this approach was that we didn't want any unnecessary manipulations to the R2 env gene, which may have affected its infectivity. pBluescript was also chosen because in its multiple cloning site (MCS) the EcoRI site was located 5′ and XhoI was 3′, which was necessary for correct cloning of the pNL4-3 env. For recombinant viruses, the full-length infectious clone, pNL4-3, was used (129). We used pNL4-3 because it is a commonly used molecular virus clone and readily available to our lab. pNL4-3 was obtained and re-transformed to get large quantities of DNA for cloning. pNL4-3 DNA was digested with EcoRI and XhoI to excise the envelope gene. Once the NL4-3 env was correctly cloned into pBluescript, it was excised and the envelope clone, R2, was used to replace it. Once R2

was in pBluescript it was excised and cloned into pNL4-3, resulting in a recombinant virus clone of pNL4-3 containing the R2 envelope gene.

The vector, pBluescript, was used as a temporary vector for envelope gene site-directed mutagenesis. Therefore, pBluescript and pNL4-3 were digested with EcoRI and XhoI. Vector, pBluescript, and insert, NL4-3 env, were ligated and transformed into XL1-Blue supercompetent cells and recombinant clones were identified by blue-white colony selection. Insert size and identity were verified using restriction enzymes. These results were confirmed by sequencing.

Next, site-directed mutagenesis (Stratagene, La Jolla, CA) was used to introduce a *Sal*I site at the 3′ end of the NL4-3 env gene, while in the pBluescript vector. The plasmid was denatured and annealed to the oligonucleotide primers containing the desired mutation. Using the nonstrand-displacing action of *Pfu Turbo* DNA polymerase, the primers were extended to incorporate the mutagenic primers into nicked circular strands of DNA. The methylated, nonmutated parental DNA template was then digested with *DpnI*, the circular, nicked dsDNA was transformed into XL1-Blue supercompetent cells. After transformation, the XL1-Blue supercompetent cells repair the nicks in the mutated plasmid. A second mutagenesis reaction, using site-directed mutagenesis, was done to remove a *Sal*I site located upstream of the NL4-3 env gene, in the non-coding region. This *Sal*I site was removed so there was no interference when we excised the NL4-3 env.

Once the *Sal*I site, downstream of the NL4-3 env gene was in place, most of the env was excised using *Kpn*I and *Sal*I. The corresponding fragment of our env of interest, R2, was inserted into the pBluescript vector. pBluescript vector containing the R2 env

gene was digested with EcoRI and XhoI, to remove the fragment of interest. The EcoRI-XhoI fragments containing these recombinant genes were then transferred back into pNL4-3. These recombinant pNL4-3 env plasmids will next be used to produce infectious viruses by transfection into HeLa cells.

Virus preparation for testing in PBMC assay

The full length infectious virus clones were transfected into HeLa cells by the calcium phosphate technique. After 2 days, the transfected HeLa cells will be cocultivated with H9 cells, and virus production were monitored by reverse transcriptase assay (2, 40, 90).

Infectivity of virus harvests were be monitored in GHOST cells using fluorescence microscopy. GHOST cells are human osteosarcoma cells (HOS) that express CD4 and one of several HIV coreceptors. These cells also contain a gene for green fluorescent protein (GFP) under the control of the HIV-2 promoter, which, in the presence of Tat, acts as an indicator of infection, generating a fluorescent cytoplasmic signal which can be detected and enumerated by flow cytometry or fluorescence microscopy (19, 115). Infectious virus pools will then be provided to Dr. John Mascola for testing in his neutralization assay, which uses flow cytometric quantitation of single-round infection of primary human T cells.

Virus neutralization assays are designed to measure a reduction in virus infectious titer mediated by exposure to antibody (73). J. Mascola *et al*. have developed an assay that directly enumerates the first-round infection of individual lymphocytes. This

enumeration of first-round infection of PBMC provides quantitative data on the number of infectious virus particles, as measured by the number of target cells infected (73). Thus, this *in vitro* PBMC neutralization assay can directly quantify the inactivation of infectious virus mediated by exposure to antibody. Results from assays performed in the Mascola laboratory were compared to results of pseudotyped virus assays obtained in our laboratory, as part of an effort to validate the relationship between the two tests.

RESULTS

CHARACTERIZATION OF IMMUNE RESPONSES FOLLOWING VEE REPLICON IMMUNIZATION

Enzyme immunoassay Optimization

Two types of enzyme immunoassays (EIAs) were compared for their ability to optimally detect immune responses in mouse sera following immunization with VEE replicons expressing HIV-1 envelope genes. These assays, a Concanavalin A (ConA) antigen-capture and a direct antigen binding EIA, were compared with varying amounts of antigen, either 40ng/well or 80ng/well. These experiments showed that the direct antigen binding EIA was more sensitive for the detection of serum immune responses. There was a 16-fold increase in the detectable titer and a 3-fold decrease in background with the direct antigen binding EIA. These results are shown in Figure 6. From these results it was determined that the direct antigen binding EIA was superior. Therefore, further experiments were conducted to optimize the direct antigen binding EIA in regards to suitable antigen for detection of the Chinese sera immune responses and conjugate used.

The results showed that R2 (clade B) was not a suitable antigen for detection of the Chinese clade C and E sera. Therefore, other antigens were tested and the two optimal preparations were VAT3 (clade C) and vGK4 (clade E) which were a gift from Dr. C. Broder, MIC, USUHS. These antigens were tested and optimized to determine the quantity of antigen necessary per well for detection of immune sera. The amounts of

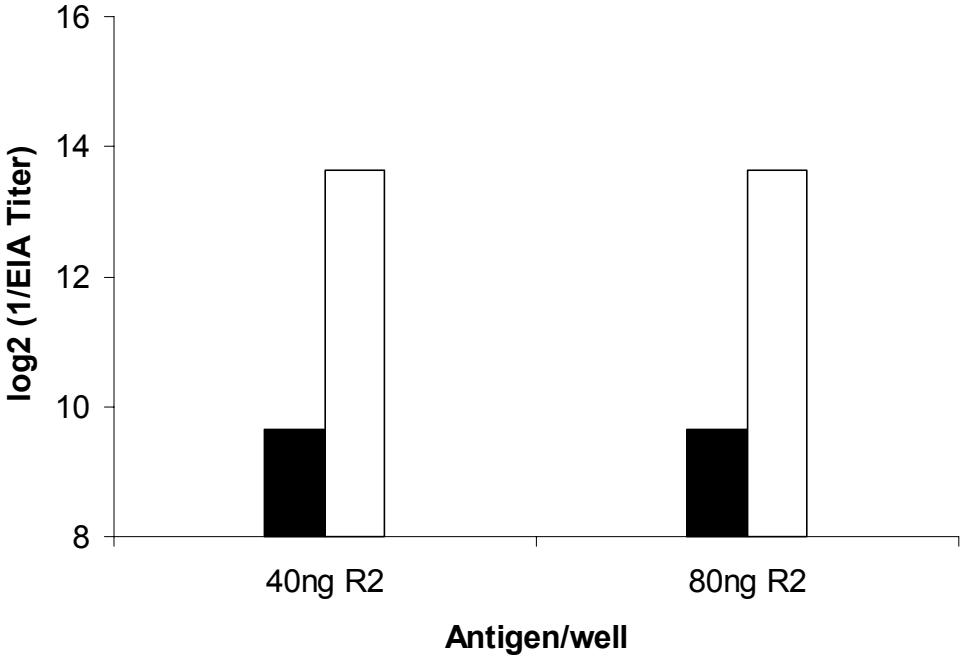

Figure 6. EIA optimization.
ConA (closed columns) and direct antigen binding (open columns) enzyme immunoassays (EIAs) were optimized for detection of serum immune responses following VEE replicon immunization. Two concentrations, 40ng or 80ng per well, of R2 antigen were used. Results showed that the ABTS substrate EIA was superior, regardless of the amount of R2 antigen used, with a 16-fold increase of titer detectable.

antigen that were tested were 40, 80 and 120ng/well. These experiments demonstrated that the best detection with least amount of background was achieved by using 120ng/well, compared to 40ng and 80ng/well, Figure 7.

Finally, conjugate dilution was optimized. When the direct antigen binding EIAs was initially tested we used a conjugate dilution of 1:10000 and 1:5000 but were unable to detect any immune responses at such high conjugate dilutions (results not shown). Therefore, we tested lower dilutions, 1:2500, 1:1000 and 1:500. These results showed that a 1:1000 dilution of α-mouse IgG conjugate was optimal, giving four-fold higher titer levels then a 1:2500 dilution and 16-fold higher titer levels then a 1:500 dilution, Figure 8.

Serum Immunoglobulin Responses

After optimizing the EIA, we next sought to determine the immune response levels in mice in response to immunization with the VEE replicon. Four groups of mice were immunized, boosted and bled according to the regimen shown in Table 1, along with one group of control mice that were immunized with PBS. Mice were bled via the tail vein at weeks 4, 7, 11, and 15. Following the collection of blood, it was allowed to clot overnight at 4ºC. The following day the serum was harvested by two, 10 minute, 2,000 rpm centrifugations. The serum was then heat inactivated at 56ºC for 30 minutes and stored at -20ºC.

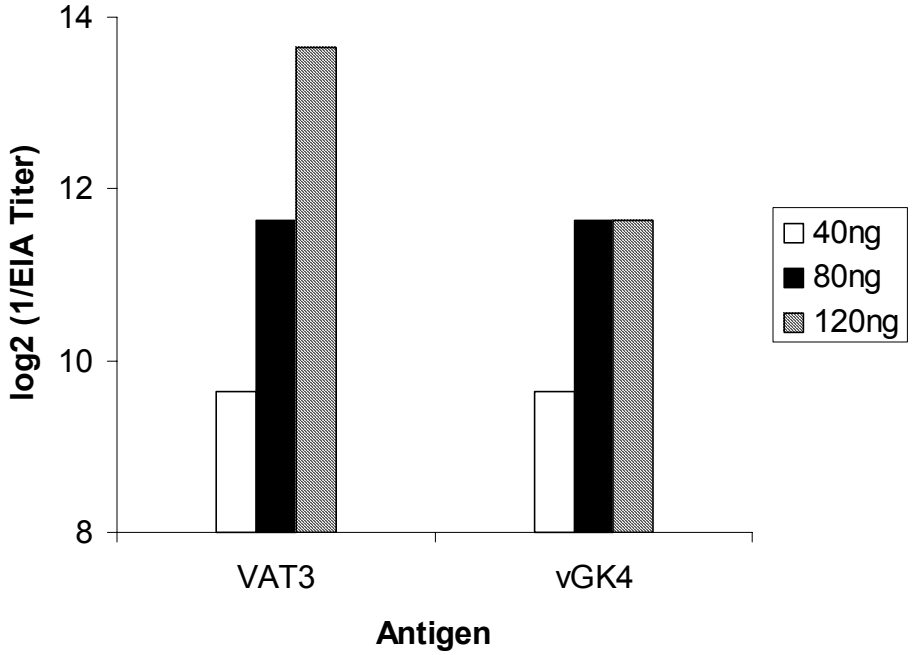

Figure 7. **Protein optimization.**
Our clade B antigen, R2, was not sufficient for detection of antibodies in the sera from Chinese clade C and clade E immunized mice. Two other antigens, VAT3 (clade C) and vGK4 (clade E), were obtained and tested at 40ng, 80ng, and 120ng per well, were tested. Results showed a consistent 4-fold increase with each additional 40ng of VAT3 antigen used. There was a 4-fold difference between 40ng and 80ng of vGK4, but no difference was observed when 80ng or 120ng of antigen was used.

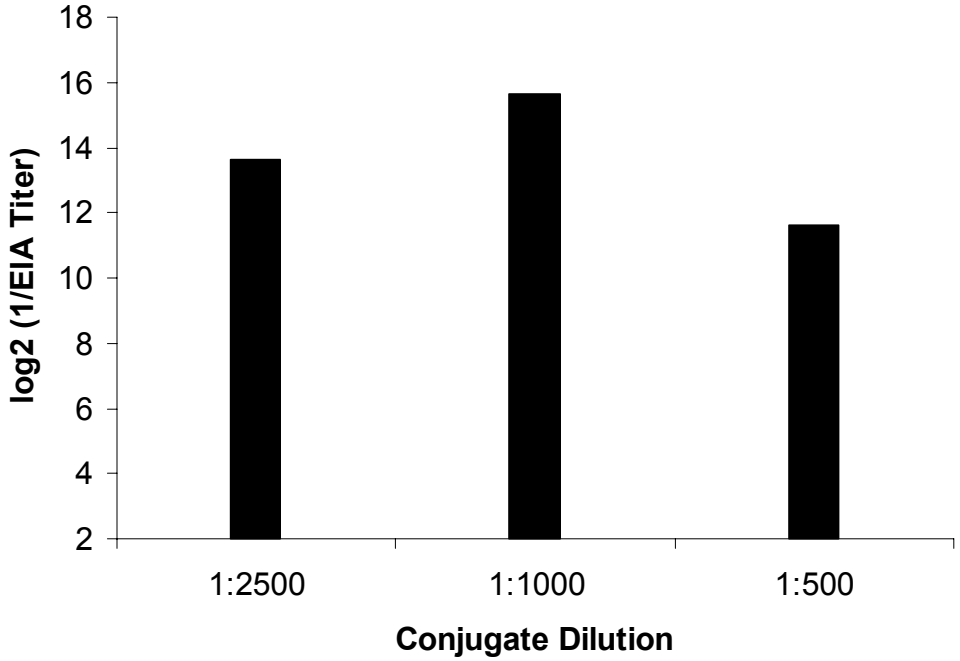

Figure 8. Conjugate optimization.
Varying concentrations, 1:2500, 1:1000 and 1:500, of α-mouse conjugate was tested. A four-fold increase from 1:2500 and 1:1000 was found. However, a 16-fold decrease was found from 1:1000 and 1:500. A 1:1000 dilution of α-mouse conjugate was used to test immune sera.

After each time-point, the sera were assayed using the optimized direct antigen binding, utilizing 120ng/well of the appropriate antigen and a 1:1000 dilution of conjugate. EIA titer results are shown in Figure 9. The resulted demonstrated that antibody titers had the greatest increase following one set of boosts. Groups C25, MACS#9 and E18 showed a large increase in EIA titers following the week 5 boost. A slight titer increase in the C25 and E18 groups following the week 12 boost was observed, but a minimal decrease in titer occurred in the MACS#9 group. The R2 group of mice consistently showed higher titers than the other 3 groups.

Neutralization Responses

Neutralization assays were used to assay the ability of immune mouse sera, to inhibit pseudotyped virus infection of CCR5 cells. Sera from mice immunized with VEE replicons containing Chinese C and E, R2 and MACS#9 envelope genes, were compared to sera from non-immunized control mice maintained in parallel.

The first group of mice, immunized with C25 expressing replicons, was tested against two viruses: SF162, a clade B virus, and C44, a clade C virus. For these sera 5 out of 5 animals neutralized SF162 and 1 out of 5 neutralized C44. These results are interesting since SF162, the clade B virus, was neutralized better then C44 the clade C virus from which the C25 envelope was derived. One caveat is that SF162 virus is moderately sensitive to neutralization and therefore somewhat easily neutralized by homologous and heterologous sera. Theses results are shown in Figure 10.

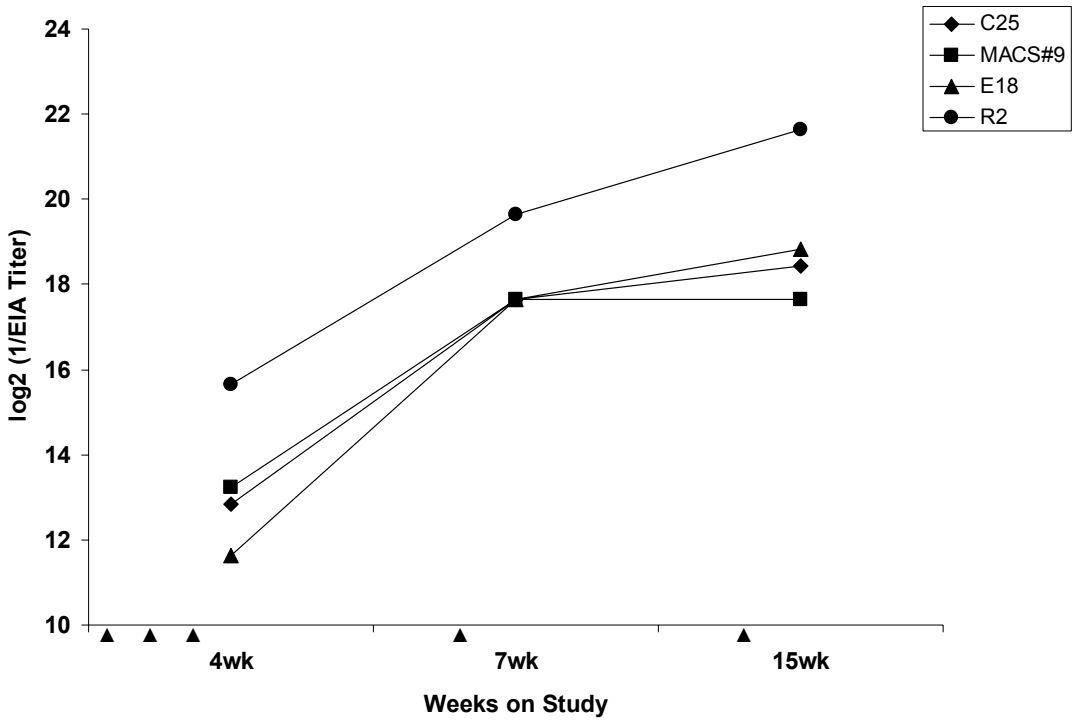

Figure 9. Immunogenicity of VEE replicons expressing HIV-1 envelope proteins. C3H/He mice were immunized by footpad inoculation at the times indicated by the arrowheads. EIA titers are presented for groups of 5 mice each immunized with $4x10^7$ FFU of the respective *env* gene per dose.

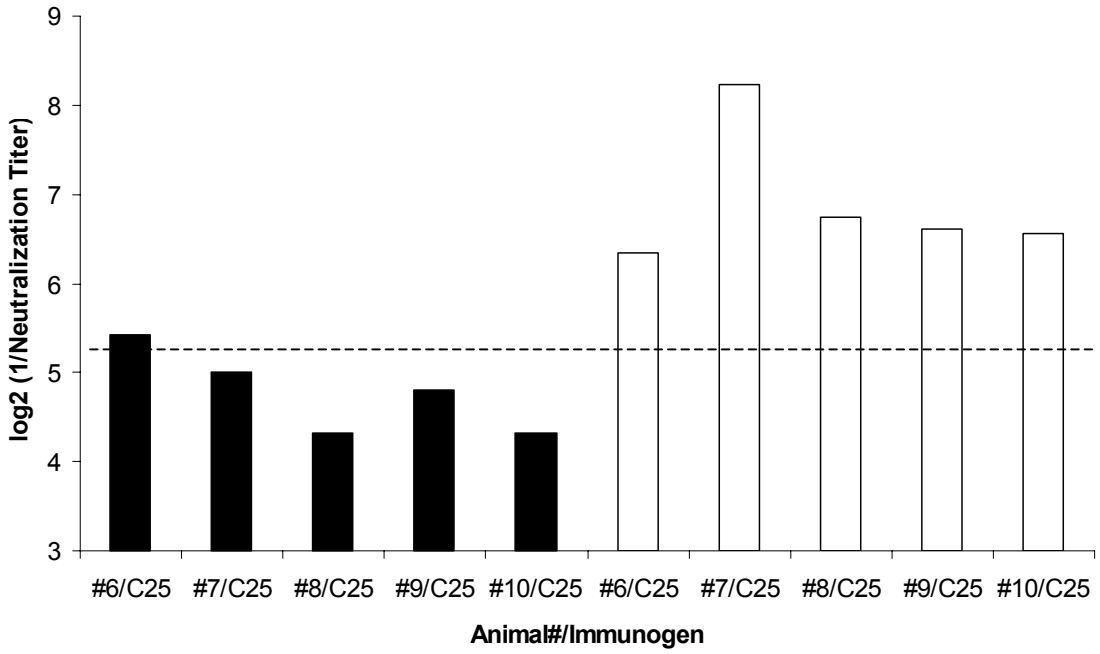

Figure 10. C25 sera neutralization.
Sera from mice immunized with VEE replicons expressing Chinese clade C env C25 was tested against the viruses: C44 (closed column) and SF162 (open columns) a clade C and clade B virus respectively. The dashed line represents a 1:40 dilution and any sera which falls below is considered to be negative. C44 was 50% neutralized by sera from 1 out of 5 animals, at a dilution of 1:43. SF162 was 50% neutralized by sera from 5 out of 5 animals, with dilutions of 1:107 and 1:300. There was some inter-clade neutralization between SF162 and the clade C sera. Neutralization titers were determined as the highest serum dilutions that caused reductions in mean luminescence results greater than 50 percent, compared to non-immune serum.

The second group of mice immunized with MACS#9 expressing replicons, was tested against SF162 and MACS#9 virus. The MACS#9 virus is a clade B virus from a donor enrolled in the Multicenter AIDS Cohort Study and is also the virus from which the MACS#9 envelope clone was derived. For this serum, 1 out of 5 animals neutralized MACS#9 and 1 out of 5 neutralized SF162. These results are shown in Figure 11.

The third group of mice, immunized with E18 expressing replicons were tested against two clade E viruses, TH966 a Thai clade E and E14 a Chinese clade E. E14 was also the virus from which the E18 envelope clone was derived. For the E18 sera, 3 out of 5 animals neutralized TH966 and 1 out of 5 neutralized E14. These results are shown in Figure 12.

The fourth and final group of mice immunized with VEE replicons, immunized with R2, and tested against SF162 and R2 viruses. The R2 immunized group was the group to which the three other groups were compared against, since our lab has extensive experience with this particular envelope clone (39, 101). The R2 sera from all 5 animals immunized, neutralized both viruses tested. Unfortunately, the neutralization titers were not high enough to test neutralization responses to other viruses from different clades. These results are shown in Figures 13.

Our previous studies used pGPm-gp160 replicons that were given at $5x10^5$FFU/dose, compared to the current dose of $1x10^6$FFU. Even though our overall antibody titers were high, antibody concentration does not necessarily correlate to the neutralizing antibodies in the sera. Animals #7 and #8, immunized with Chinese clade C, clone C25, neutralized SF162. SF162 virus was only neutralized at moderate levels, with

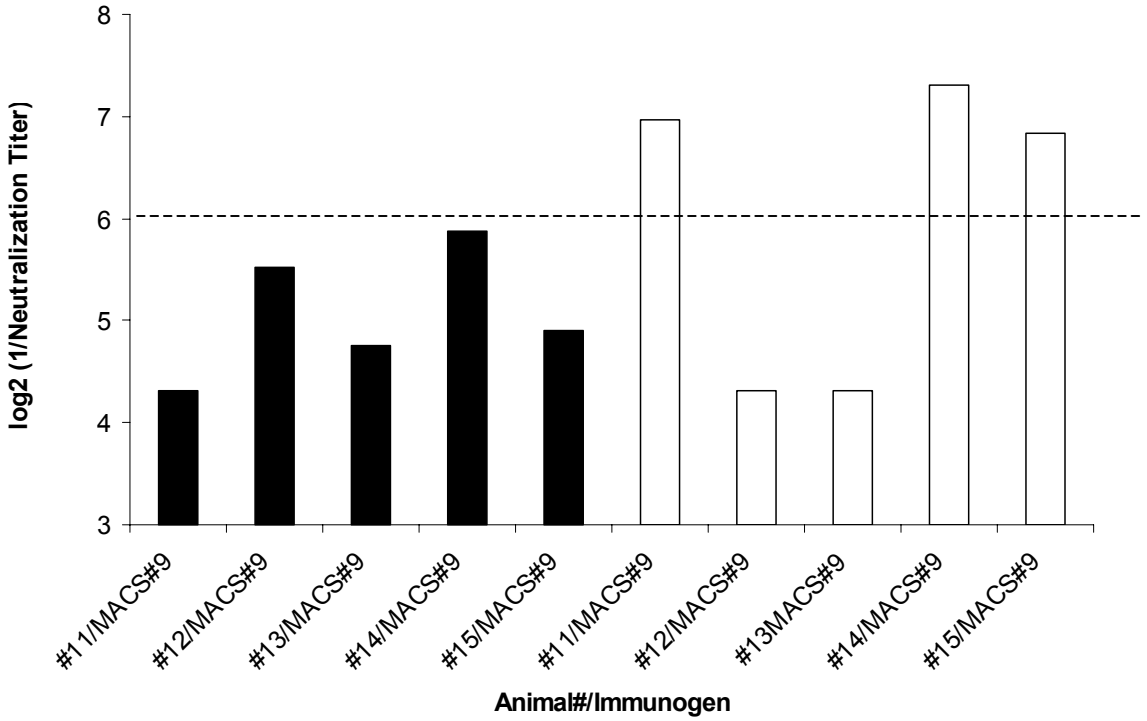

Figure 11. MACS#9 sera neutralization.
Sera from mice immunized with VEE replicons expressing North American clade B env MACS#9 was tested against varying viruses. MACS#9 (closed column) and SF162 (open column) both are clade B viruses. The dashed line represents a 1:40 dilution and any sera which falls below is considered to be negative. MACS#9 was neutralized 50% by 1 out of 5 animals, at a dilution of 1:59. SF162 was 50% neutralized by 1 out of 5 animals, with a dilution of 1:159. Neutralization titers were determined as the highest serum dilutions that caused reductions in mean luminescence results greater than 50 percent, compared to non-immune sera.

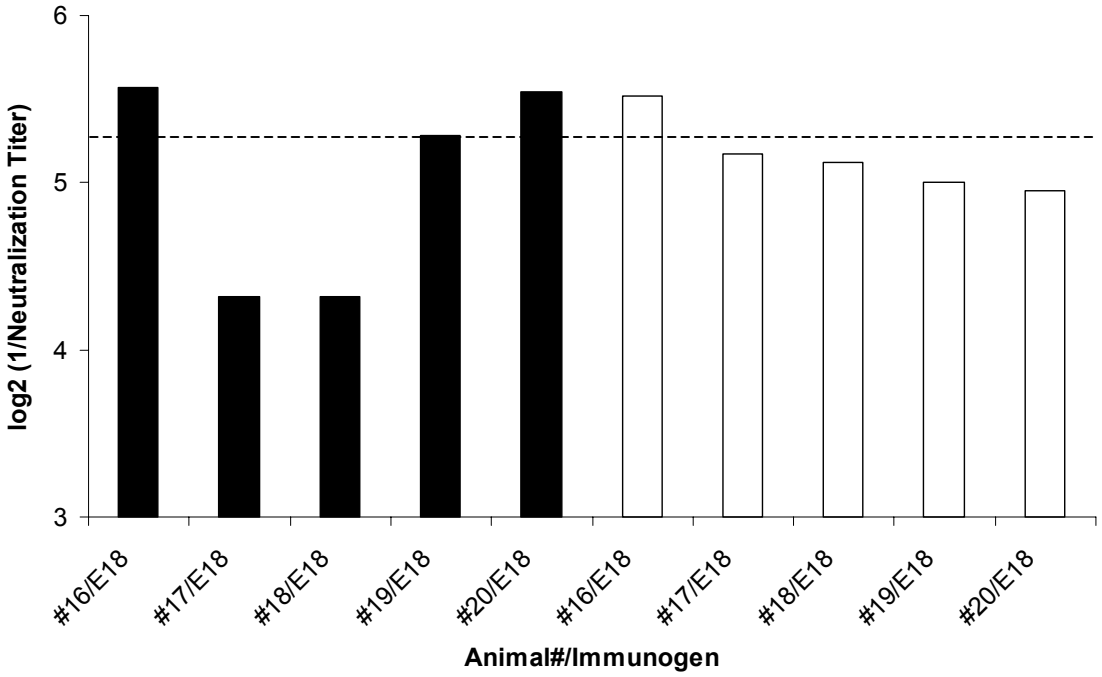

Figure 12. E18 sera neutralization.
Sera from mice immunized with VEE replicons expressing Chinese clade E env E18 were tested against varying viruses. TH966 (closed columns) and E14 (open column), both clade E viruses. The dashed line represents a 1:40 dilution and any sera which falls below is considered to be negative. TH966 was 50% neutralized by 3 out of 5 animals, with dilutions varying from 1:38 to 1:48. E14 was 50% neutralized by 1 out of 5 animals, at a dilution of 1:46. Some low intra-clade neutralization was seen. Neutralization titers were determined as the highest serum dilutions that caused reductions in mean luminescence results greater than 50 percent, compared to non-immune serum.

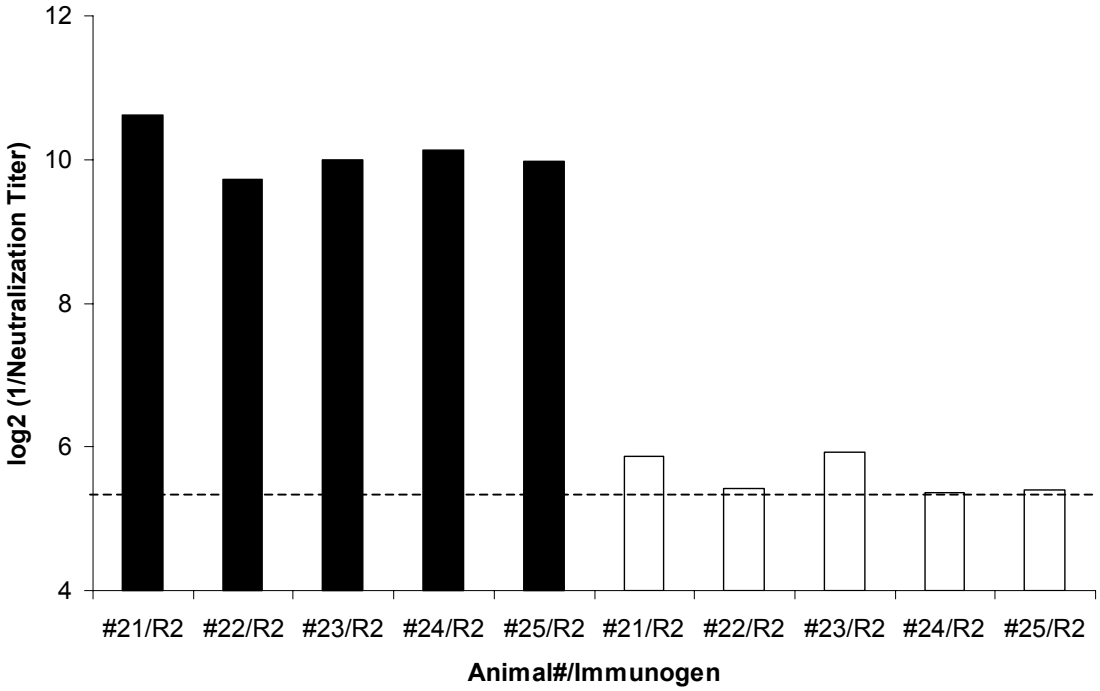

Figure 13. R2 sera neutralization.
Sera from mice immunized with VEE replicons expressing North American clade B env R2 was tested against the viruses: SF162 (closed columns) and R2 (open columns), both clade B viruses. The dashed line represents a 1:40 dilution and any sera which falls below is considered to be negative. All five animals were able to neutralize the SF162 virus at moderate titers. These titers ranged from dilutions of 1:850 (animal #22) to 1:1596 (animal #21). All five animals were able to neutralize the R2 virus, but at considerably low titers. These titers ranged from dilutions of 1:41 (animal#24) to 1:60 (animal #23). Neutralization titers were determined as the highest serum dilutions that caused reductions in mean luminescence results greater than 50 percent, compared to non-immune serum.

the highest neutralization titer being 1:300. This was lower then what we had expected, considering SF162 is a neutralization-sensitive virus.

The Chinese clade E sera derived, from immunizations with clone E18, as shown in Figure 12, was unable to neutralize any virus other then clade E. This finding was expected as reported in previous studies (65, 97). These studies found that clade E viruses grouped together and their neutralization serotypes correspond to genetic subtype for clade E viruses.

DISCUSSION

The principal mechanism by which effective viral vaccines confer protective immunity is by induction of antibodies capable of neutralizing prevalent strains of the particular virus (87, 100). This mechanism is especially relevant for HIV-1 virus which has many "strains" (clades) or subtypes, as well as variants within subtypes that are involved in the current pandemic. Serum that is able to neutralize across clades with significant potency has been characterized and is a necessary reagent for research on the feasibility of a broadly effective HIV-1 vaccine involving humoral immunity (116).

It has been argued that resistance to neutralizing antibodies is essential for the persistence of a lentiviruses such as HIV-1 *in vivo* (91, 94). This argument is supported by observations that primary isolates of several other lentiviruses are neutralization resistant (6, 29, 80, 81) and that neutralization-sensitive lentiviruses revert to a resistance phenotype *in vivo* (9, 11, 42, 61, 110). HIV-1 variants with a global neutralization-resistant phenotype have also arisen after the passage of neutralization-sensitive viruses in the presence of neutralizing serum *in vitro* (64, 88).

Based on the EIA comparison experiments, we came to the conclusion that the direst antigen binding EIA was superior. We initially used the antigen-capture ConA EIA in our laboratory because it required less antigen, but from the results shown in this thesis equivalent amounts of antigen could be used in the direct antigen binding EIA, with superior detection of immune responses. Following optimization of antigen and conjugate levels in the assay, we decided on the use of the direct antigen binding EIA for detection of immune responses following VEE replicon immunization.

Neutralizing antibodies are necessary for a protective immune response against many viral infections. Eliciting such antibodies is not an easy task, but the *in vivo* VEE expression system is one of the most promising delivery systems currently in use, as evidenced from the results in this thesis and previous results (39).

The preparation of VEE replicons containing Chinese and North American HIV-1 envelopes and their use in small animal immunization, along with their ability to induce immune responses has been described herein. These studies were undertaken to explore ability of mice to induce immune responses and to determine the extent of cross-reactivity of the responses. The studies conducted in this thesis are relevant to understanding vaccine parameters because the Chinese HIV-1 envelope proteins used are the prominent clades in the current HIV-1 epidemic in China. It is important to characterize the types of immune responses that can be induced in support of future vaccine efficacy trials. We were able to induce antibodies, and in some groups, cross-reactive neutralizing antibodies with our current VEE expression system. These findings are important and can be used in further studies for the development of a potential vaccine. The results shown in this thesis are significant because they are the first to show clade C and E neutralizing immune responses induced by the VEE replicon system. Therefore, these data are extremely important for the development of a vaccine against Chinese clade C and E viruses.

Antibodies were induced by VEE replicon immunization. We anticipated a greater cross-reactive response so a larger number of viruses from clades A, D and F could be tested. Unfortunately, the immune responses that were elicited did not have sufficient neutralizing activity to test additional clade B and clade A viruses. There was a

low level of neutralization of SF162, a moderately sensitive clade B virus, by clade C derived sera.

We had expected that clade E sera would neutralize clade E virus. In previous work done, we had observed that Chinese clade E virus was neutralized better by Thai clade E sera than Chinese E sera. This observation was considered most likely to be a result of the retention of neutralization epitopes and greater duration of infection in the Thai donors. The results in this thesis were consistent with the expectations in that the clade E sera neutralized only other clade E viruses. The viruses that were used were Thai clade E and a Chinese clade E, with little difference in neutralization titer levels. The study design used in this thesis compared the results of the two Chinese HIV-1 envelope proteins and the one North American clade B to R2, our previously described highly cross-neutralizing, North American clade B HIV-1 envelope protein (101, 120, 127). This comparison to R2 was done because the R2 envelope had been extensively studied in our laboratory and was used as a positive control for induction of immune responses, especially neutralizing antibodies.

Several difficulties arose that should be addressed when studying induction of virus-neutralizing antibodies in animal models. In studies of the induction of HIV-1-neutralizing antibodies, one particular difficulty often observed is the presence of nonspecific virus inhibiting activity in animal sera (39). It has been shown in control animals that nonspecific inhibitory activity in mouse sera appears to increase with mouse age (39). The mice that were used in this study were 6 months old at the beginning of the study.

One of the limitations with this study is that the immunofluorescence assay (IFA) used may not be equivalent in sensitivity for measuring envelope protein expression by replicons expressing *env* from different clades of HIV-1. The reagents used might not detect different envelope proteins to the same degree. Thus, preparations determined to have equivalent potency in terms of FFU may have different immunogenicities. This concern regarding protein detection and correlation to immunogenicity is also evident to the Western Blot assay as is illustrated in Figure 4. As seen in the Western blot, the C25 protein expression is better than the MACS#9 protein expression, yet the MACS#9 replicon induced better immune response then C25. Future studies to possibly eliminate these limitations is to use monoclonal antibodies for both the Western Blot and IFA assays. By using monoclonal antibodies we would be able to select for cross-reactivity. Hopefully with this type of selection we would be able to enhance our VEE expression and the detection of immune responses induced by it.

The immune responses that were induced by our immunization protocols were significant but not optimal. Variations that could be implemented to optimize the immune responses induced by our VEE replicons are: 1) increase the dose of replicons given; 2) lengthen the immunization regimen by addition of more boosts; and 3) the use of gp160 genes encoding protein with a deleted cytoplasmic tails. Each of these approaches has been shown to enhance immune responses induced by VEE-HIVenv replicons (39).

The current system to assay virus neutralization in our laboratory uses pseudotyped viruses. There are some concerns in the scientific community about this system and its artificial aspect. The cells used, HOS-CD4-CCR5, are engineered to

express more receptors and co-receptors than found on PBMCs. The pseudotyped viruses are useful tools, but the extent to which they produce results that can be directly used to predict protection against HIV-1 in exposed individuals has not been established yet. Because of this uncertainty we sought out to compare our current pseudotype neutralization assay to the Mascola PBMC, single-round infection assay. This PBMC assay has been shown to be a more sensitive and consistent assay as compared to p24 and RT assays (73). From the results of the neutralization assay comparison experiments we will be able to further validate which assay would be better utilized in our lab. Further preparation of recombinant virus is also necessary. Other envelopes of commonly used viruses in our pseudotype assay will be cloned and tested similar to the comparison of the R2 recombinant virus, described in this thesis. This library of recombinant viruses will be useful addition to the current available HIV-1 reagents used in vaccine development.

In conclusion, present evidence supports the conclusion that an alphavirus expression system can serve as a versatile platform for immunization with native HIV-1 envelope protein for induction of HIV-1-neutralizing antibodies. The HIV-1 envelope-specific antibody responses, measured by EIA and neutralization, were significantly enhanced, in both magnitude and rate of development, by the use of wild-type VEE envelope sequences for replicon packaging. The experiments presented here was a preliminary look at the capabilities of Chinese clade C and E envelopes to elicit immune responses using a VEE replicon system. With further testing we are confident that broader cross-neutralizing antibodies can be raised and applied towards vaccine development.

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