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Evaluation of the Efficacy of a Recombinant Simian Varicella Virus Vaccine Expressing Simian Immunodeficiency Virus Envelope and Capsid Proteins

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EVALUATION OF THE EFFICACY OF A RECOMBINANT SIMIAN VARICELLA VIRUS VACCINE EXPRESSING SIMIAN IMMUNODEFICIENCY VIRUS ENVELOPE AND CAPSID PROTEINS

By
Thomas Grant Wichman

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford
May 2019

Approved by:

________________________________
Advisor: Dr. Wayne Gray

________________________________
Reader: Dr. Brian Doctor

________________________________
Reader: Dr. Patrick Curtis
DEDICATION

I would like to dedicate this Capstone Project to my parents, John and Sharmon Wichman. I am more than lucky to have parents who constantly love and support me in anything that I do. It is because of them that I had the privilege to come to The University of Mississippi and can now culminate that time into my senior thesis for the Sally McDonnell Barksdale Honors College.
ACKNOWLEDGEMENTS

I first would like to thank Dr. Wayne L. Gray and The University of Mississippi Biology Department for the opportunity to participate in this research project, and to use this virology lab to expand my own knowledge. Dr. Gray’s innovation and willingness to teach at all scales has been both beneficial and inspiring to me during my time at Ole Miss.

I also would like to extend my thanks to Michael Hohl for always being willing to take time to answer my questions and guide me throughout new or complex lab protocols required for my thesis research.

I finally would like to thank the Honors College for the numerous opportunities throughout college to expand my academic career, and my thesis committee for taking time to read, edit, and further improve this manuscript.

This study was funded by NIH grant R01-AI123029 and NIH Contract HHSN272201300041.
ABSTRACT
THOMAS GRANT WICHMAN: Evaluation of the Efficacy of a Recombinant Simian Varicella Virus Expressing Simian Immunodeficiency Virus Envelope and Capsid Proteins
(Under the direction of Dr. Wayne L. Gray)

Human Immunodeficiency Virus (HIV) is a retrovirus that infects CD4+ T-lymphocytes, which when left untreated, later develops into Acquired Immunodeficiency Syndrome (AIDS). Up to 25,000 people die every week from AIDS infection, making HIV and AIDS research a very high priority for virologists. While highly researched by scholars around the world, no person has been able to develop a successful vaccine, as the retroviral nature of the virus and its high mutation rate make vaccine development incredibly difficult. However, recombinant genetic technology will hopefully allow the revolutionization of vaccines which have already proven effective in immunization. The already developed varicella-zoster virus (VZV) vaccine’s safety, effectiveness, and infection range limited to humans make it a great vector for the creation of a recombinant HIV Vaccine. In this study, simian varicella virus (SVV) and simian immunodeficiency virus (SIV) act as a model to evaluate a recombinant vaccine’s effectiveness on non-human primates’ humoral responses.

The wide-scale goal of HIV and concurrent AIDS and SIV research is to both cure and prevent these diseases; in the research this thesis follows, the aim is to create a recombinant SVV vaccine which can manifest immunity in non-human primates, with
hopes to parallel that research for a similar vaccine for human use. The specific aims of this thesis include: the development of an optimal protocol for antibody response analysis, and the evaluation of the efficacy of a recombinant SVV vaccine expressing SIV \textit{gag} and \textit{env} proteins, either with a protein boost or a DNA boost. After the protocol was modified and optimized, sera from rhesus macaques at different points throughout their immunization schedules were analyzed via western blot. Results showed that the prime-boost schedule is effective in inducing an antibody-mediated response, specifically after boosting, and that a protein vaccine boost rather than DNA vaccine boost is the most effective. This model will continue to be researched further in hopes of developing a successful SIV, and subsequently, HIV vaccine.
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<th>Full Form</th>
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<tr>
<td>1°</td>
<td>Primary</td>
</tr>
<tr>
<td>2°</td>
<td>Secondary</td>
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<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>BB</td>
<td>Blocking Buffer</td>
</tr>
<tr>
<td>D₂H₂O</td>
<td>Deionized Water</td>
</tr>
<tr>
<td>Env</td>
<td>Envelope</td>
</tr>
<tr>
<td>Gag</td>
<td>Group Antigen; Capsid protein</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>µg</td>
<td>Microgram</td>
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<td>µl</td>
<td>Microliter</td>
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<tr>
<td>mA</td>
<td>Milliamp</td>
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<tr>
<td>mL</td>
<td>Milliliter</td>
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<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
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<tr>
<td>NHP</td>
<td>Non-human primate</td>
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<tr>
<td>PB</td>
<td>Post-boost</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PI</td>
<td>Pre-immune</td>
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<tr>
<td>PP</td>
<td>Post-prime</td>
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<tr>
<td>PVDF</td>
<td>Polyvinylidene Fluoride</td>
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<td>rSVV-SIV</td>
<td>Recombinant SVV Vaccine Expressing SIV Proteins</td>
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<tr>
<td>SIV</td>
<td>Simian Immunodeficiency Virus</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>SIVmac251</td>
<td>Macaque Strain of SIV</td>
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<tr>
<td>STI</td>
<td>Sexually Transmitted Infection</td>
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<tr>
<td>SVEU</td>
<td>Simian Vaccine Evaluation Unit</td>
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<td>SVV</td>
<td>Simian Varicella Virus</td>
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<tr>
<td>TNPRC</td>
<td>Tulane National Primate Research Center</td>
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INTRODUCTION

Since as early as the 18th century, when Edward Jenner developed the world’s first vaccine, one that would later eradicate smallpox, vaccine research has remained among the top priorities of virology research across the globe. As new diseases are discovered, we are learning more about mechanisms of illness at both the global and molecular scales, and are today more equipped to fight illness than ever before. However, this does not mean that new viruses are of no challenge for scholars in this field. While only discovered in the early 1980’s, human immunodeficiency virus (HIV) has grown to affect tens of millions of people globally, and development of an HIV vaccine has become one of the most sought-out goals of the scientific community. While treatments have since become available to fight HIV (which left untreated develops into acquired immunodeficiency syndrome, or AIDS), none is sufficient in completely ridding a patient of viral particles. The research that is to be presented by this thesis is only a small portion of that which has been, and is being conducted, around the world on HIV/AIDS.

This project is being conducted to further research in the field of HIV and prevention of the virus, by use of a non-human primate model and recombinant genetic technology. It is hypothesized that by creating a recombinant simian varicella virus (SVV) vaccine expressing the simian immunodeficiency virus (SIV) capsid ($gag$) and envelope ($env$) protein, and vaccinating the non-human primates on a prime-boost method, the animals will be vaccinated against SVV, and in the process will also create
antibodies to SIV \textit{gag} and \textit{env} protein, effectively immunizing the animal against both viruses. The three primary goals of this study are listed as follows:

- To optimize conditions for running western blots of vaccinated rhesus sera and determine a preferred method of detection of antibodies to SIV capsid (\textit{gag}) and envelope (\textit{env}) proteins.

- To compare the efficacy of two different vaccination strategies utilizing a prime-boost technique employing the rSVV-SIV vaccine and either an SIV protein or SIV DNA vaccine boost.

- To evaluate the humoral immune response induced by recombinant SVV vaccines expressing the SIV \textit{gag} or \textit{env} protein in immune naive \textit{Macaca mulatta}.

By assessing the efficacy of the existing vaccines, future efforts can be further conducted, and even targeted or specified, and ineffective efforts can essentially be omitted from future studies.
CHAPTER I: BACKGROUND ON HIV/AIDS

Acquired Immune Deficiency Syndrome was first described in 1981 when there was an epidemic among young, homosexual men that was causing opportunistic infections and rare types of cancer (Sharp & Hahn, 2011). AIDS was previously known as GRID, Gay-Related Immunodeficiency, due to the demographic that was most affected, or A.I.D. for Acquired Immunodeficiency Disease. When AIDS was first discovered, the CDC and National Cancer Institute said that they were probably only seeing “the tip of the iceberg”, due to the idea that the disease was being spread long before symptoms were beginning to show (Altman, 1982).

As of 2017, almost 37 million people worldwide were living with HIV/AIDS, with 1.8 million of them being new infections that year. Only an estimated 75% of these citizens knew of their status, pointing to the need of more accessible testing. While the proportion of AIDS patients receiving antiretroviral therapy is increasing (up to 59% in 2017), accessibility and cost of treatment is still a hinderance. Mortality rates have also improved, with 940,00 having died from AIDS complications in 2017, compared to the almost 2 million people dead in 2004. Though previously mentioned that access to effective treatment is an issue, this mortality statistic at least shows that the scientific community is indeed moving in the right direction (“Global HIV/AIDS Overview”, 2019). While the 2010’s have shown great progress with making treatment accessible
across the globe, particularly in under-developed countries which are most affected by the disease, researchers are not done yet.

**Emergence of HIV**

AIDS is caused by either HIV-1 or HIV-2, with the former being the most common. Both viruses are classified as lentiviruses, which can cause chronic and persistent infections in several different species of mammals like horses, cats, and primates. These viruses integrate into the hosts’ germ cells, allowing it to pass through to offspring. Both come from cross-species infection of varying SIV strains, with HIV-1 specifically resulting from a chimpanzee strain of SIV (SIVcpz). Most primate species contain their own, specialized strains of SIV, but cross-species transmission has created a plethora of combination strains. When HIV-1 was discovered, one of the scientific community’s main goals was to find how the virus suddenly came about. The first clue was in 1986 in patients in western Africa, who were diagnosed with HIV-2. Structurally, HIV-1 and HIV-2 are very similar. Due to the two strains’ genetic differences, different diagnostic methods may be implemented. HIV-2 isn’t often tested for unless the patients’ behaviors call for it. HIV-2 is also typically less pathogenic than HIV-1 and less easily transmitted (German Advisory Committee Blood, 2016). Though HIV-2 and HIV-1 are distantly related, HIV-2 has shown to be very similar to a macaque strain of SIV. Later studies showed that this strain, SIVmac, couldn’t have been natural, but was likely generated within primate centers in the U.S. while cross-inoculating macaque species with sooty mangabey blood and/or tissue (Sharp & Hahn, 2011).
Meanwhile, SIV strains from chimpanzees and gorillas have nearly identical genomic structures to HIV-1, of which there are multiple subtypes, each having originated in different regions due to various transmission events between individuals of different species. The groups include M, N, O, and P, with M being the pandemic group, O accounting for less than 1% of total HIV-1 infections, and N and P only causing single- or double-digit numbers of infections. There are hypotheses around what species of primates these 4 different types of HIV-1 might have originated from, but it is known that transmission to humans must have occurred through some exposure to infected primate blood either at a mucous membrane or percutaneously (Sharp & Hahn, 2011).

**Viral Transmission**

HIV-1 spreads between individuals through sexual intercourse, percutaneous routes such as using infected needles, or during childbirth. Most of the viral transmissions take place at mucosal surfaces, such as through sex, classifying HIV-1 as a sexually transmitted infection (STI) (Sharp & Hahn, 2011). Transmission by sex involves HIV being taken up by macrophages in the mucosal layer of tissue, whether it be rectally, vaginally, or orally. If the transmission occurs directly at the site of a wound or to the blood stream directly, a much smaller dose is required for infection to occur, and T helper cells can be directly infected rather than taking a pathway through M cells in a mucosal membrane. Transmission during pregnancy typically occurs in the last trimester, but can occur sooner; it can also occur via breast feeding (German Advisory Committee Blood, 2016).
Pathophysiology

Before discussing how HIV enters cells, it’s important to understand the virus’s structure. A mature HIV particle is round, containing an envelope which itself hosts 72 “knobs” that are composed of trimeric env proteins; the monomers are commonly known as the gp120 envelope protein. Those trimers are held onto the membrane by the transmembrane gp41 protein. Inside is a cone-shaped capsid made of capsid protein p24, a protein which is uncommonly, but at times, used in particular diagnostic tools for HIV infection. An HIV virus particle contains 2 identical ssRNA molecules within a core. The HIV genome also codes for different regulatory proteins, such as Tat, Rev, Nef, Vif, Vpr, and Vpu, which all play a role in replication and pathogenicity (German Advisory Committee Blood, 2016).

Initial infection and viral entry requires several steps of protein binding and conformational change. The gp120 glycoprotein of an HIV particle can bind to any host cell containing a CD4 receptor. After a conformation change in those proteins, there is additional space for the gp120 to bind to CD4’s co-receptor. Following that step of binding, yet another conformation change occurs in gp120 and gp41. The gp41 then has the ability to form a channel between virus and host, which fuses the two and allows the HIV capsid to enter the host. The virus’s genome is synthesized in the host’s cytoplasm by using reverse transcriptase, the viral RNA is degraded, and the viral DNA can integrate into the host genome to complete viral infection and establish latency. It can take as little as 24 hours for the entire process to occur, and for new viruses to be released from infected cells. Mode of transmission can also affect the infection’s timeline (German Advisory Committee Blood, 2016).
**Illness and Symptoms**

Symptoms are usually observed 3-6 weeks post-infection and can vary greatly. Most often, however, HIV infection presents as cold or flu, rendering it difficult to recognize for someone unaware of their status or risk behavior. Symptoms can last from 2 weeks to almost 2 months. After initial symptoms, a patient can go into an asymptomatic phase for years, and viral load can decrease by millions or even billions. One factor that makes HIV so dangerous is this asymptomatic period. Vaginal or seminal fluid is still infectious, even when the person shows no symptoms or the viral load is virtually undetectable in the blood (German Advisory Committee Blood, 2016). If we travel back to the 1980’s, we can get a glimpse into what early researchers or discoverers of this disease could have been thinking. People coming into a clinic with flu-like symptoms very well could have had HIV, but there were no tests as it was not a known disease at the time. Years go by and people’s HIV infections were developing into full-blown AIDS, while at the same time the disease was being spread at an exponential rate.

If HIV goes undetected and untreated, it will eventually result in AIDS. Because HIV infects CD4+ cells, it leaves the immune system very weak, hence the term “immunodeficiency”; the depleted CD4 supply is the main contender in leaving someone prone to further illness (Richman, 2000). When a person reaches this point, it is not necessarily the virus that kills them, but rather what the medical community knows as “opportunistic infections.” People come into contact with pathogens on a daily basis, but a healthy person with a competent immune system has the ability to fight most of them off. An AIDS patient, however, does not, and will experience these infections in much more lethal forms. There are also several rare cancers that may be observed in AIDS
patients, the most common being Kaposi’s Sarcoma. It is quite possible that a person who previously engaged in risky sexual behavior also be infected with other STI’s such as forms of herpes or human papillomavirus (HPV), both of which can also result in cancers throughout the body of an AIDS patient (German Advisory Committee Blood, 2016).

**Diagnosis**

It is extremely important to be tested for HIV, and other STIs regularly if a person is engaging in risky sexual behavior. HIV is diagnosed using 2 main forms of detection. These include both antibody and viral detection (HIV GAC). When someone donates blood, HIV RNA levels in the blood can be measured using a nucleic acid test (NAT), and this can be accurately measured around a week and a half after infection.

Antibody detection is the primary screening method for HIV diagnosis, and if the primary screening results are positive it is followed up by confirmatory tests such as western blot, or immunoblot. ELISA (enzyme linked immunosorbent assay) tests containing common HIV-1 subtypes and HIV-2, and sometimes additional antigens, or particle agglutination tests are the foremost ways of antibody detection for regular patient testing. ELISA is known for giving false-positives, as it is a highly sensitive test, but immunoblots have a much lower sensitivity, which can offer a more accurate result in the case of a false-positive ELISA (German Advisory Committee Blood, 2016).

Viral detection is much more costly and not very quick, so therefore is not used in the US anymore for diagnosis. What is observed in virus detection is the p24 antigen. Because this is simply shed from infected cells, with no association to the viral nucleic acid, no correlation be made with progression of infection and p24 antigen levels.
Treatment and Prevention

While there is not an absolute method of immunizing against HIV, there are still ways to treat the virus post-infection and minimize risk of contracting the virus.

Early Methods

There was about 15 years before any effective antiretroviral treatment developed for the fight against HIV, and the most that could be done in that time is try to ward off the opportunistic infections and manage the illnesses that come with AIDS infection (Arts & Hazuda, 2012). Hospital bills were around $64,000 a patient when HIV was first discovered, likely because medical professionals were quite in the dark on HIV/AIDS (Altman, 1982). The first antiretroviral treatments targeted reverse transcriptase and protease by inhibiting them, and were given independently, rather than a cocktail like today’s regimens (Arts & Hazuda, 2012).

Current Treatment

Today, however, we have a quite effective HIV treatment known as highly active antiretroviral therapy (HAART), which involves combining multiple antiretroviral treatments; today it is the standard of care for HIV patients who are fortunate enough to access it. This treatment can lower viral load as much as to make it undetectable, in one way by making sure production of new CD4 cells isn’t being inhibited by HIV infection (Richman, 2000). Additionally, reverse transcriptase is very error prone, and as many as 10 mutations can arise in just one replication cycle, creating many HIV-1 variants. HAART’s success can also be attributed to its ability to decrease likelihood of selecting clones that are extremely mutated. The use of HAART to treat HIV must be maintained for the remainder of a patient’s lifetime, or viral rebound can occur. (Policicchio, et al.,
Past research on patient quality of life and neuropsychological function during HAART has been conducted alongside the medical signs of illness. Some studies have shown that HAART’s benefits go beyond the effects on the immune system, even in patients with advanced-AIDS. Among those benefits were weight gain, which is a good sign in AIDS patients, and decreased depression (Brecht, et al., 2001). Although HAART has proved to be a very effective method of treatment, it is still no means to cure HIV/AIDS.

Preventing Transmission

There are several ways to help halt the spread of HIV, and other STIs as well. The most important steps are to know one’s own HIV status, to practice safe sex, and in general to avoid high-risk behavior, such as having multiple partners or using drugs, specifically ones injected by needle. Pre-exposure prophylaxis (PrEP) is also an option for someone who knows that they are of high risk for contracting HIV. Truvada, an FDA-approved drug and pre-exposure prophylactic, is a combination of two compounds that help prevent HIV particles from taking hold of and infecting cells. If it’s used as prescribed on a daily regimen, the risk of getting HIV through intercourse is lowered by over 90%, and through infected needles is lowered by around 70%. An infected person undergoing treatment for HIV can also exhibit much lower titres of virus, which can help prevent the spread of the virus (“Pre-Exposure Prophylaxis”, n.d.).

Research Model and Experimental Counterparts

Now that effective treatment does exist, the next step is to find an effective mode of prevention, and to do this it is necessary to have the proper models for research. There
currently exists feline, mouse, and non-human primate models, the latter of which is used for conducting the research for this project (Policicchio, et al., 2016). The ultimate goal of this research, as mentioned previously, is to create a safe and effective recombinant varicella-zoster virus (VZV) vaccine that could also immunize against multiple HIV proteins. Studies cannot be conducted on humans prior to success with an animal model, due to ethical reasons and the risk of infecting more people than already are. The immediate goal, then, is to create a recombinant simian varicella virus (SVV) vaccine that also immunizes non-human primates from SIV (rSVV-SIV). This chapter will sum up the similarities and differences in HIV and SIV; and VZV and SIV, in order to demonstrate the potential efficacy of the simian model.

Because HIV originally emerged from primate species, primates are looked at as one of the most accurate models for studying HIV. Non-human primates (NHPs) relatively larger size also makes for the possibility of larger samples when collecting blood and sera (Policicchio, et al., 2016) The study undertaken by our lab in collaboration with the Tulane National Primate Research Center utilizes rhesus macaques, *Macaca mulatta*, as do many HIV researchers. Pathogenically, SIV and HIV infection in primates and humans, respectively, are very similar. For example, they are common in the way they both integrate into host genomes, and the way they distribute across the body in multiple tissues. However, with many research models there are still limitations. For example, during SIV or HIV-2 infection, NHPs exhibit elevated viral loads, both in acute and chronic stages, in comparison to HIV-1 infection in humans. This makes control with antiretroviral treatment much more difficult (Policicchio, et al., 2016).
The study presented in this thesis is a continuation of research that has been conducted for over a decade. The hypothesis that a rSVV-SIV vaccine would be effective against SIV pathogen has been tested previously and proven quite effective. Rhesus macaques were inoculated with the recombinant vaccine using a homologous prime-boost method, with boosters administered at 35 and 70 days following initial immunization. Six months later, the animals were challenged with SIVmac251 intravenously. The experimental group of animals showed much higher increases in SIV antibody after the challenge, demonstrating that the rSVV-SIV was at the very least effective and eliciting an antibody-mediated response in the animals. While all of the animals eventually became infected with SIV, an initial reduction in viral load shows promise for this experimental model (Traina-Dorge, et al., 2010).

**VZV and SVV**

Despite the possibility of limitations, the Simian model for this study has great ability to parallel potential vaccination for humans using VZV as a vector. The varicella-zoster virus, commonly known as chickenpox, vaccine was first approved for use in the United States in 1995. The current regimen has children receive a primary inoculation at 12-15 months and a booster between 4-6 years of age, both received intradermally. The vaccine, known as VZV VARIVAX, is a live, attenuated vaccine derived from a clinical isolate known as VZV Oka. It can establish latent infection and later cause shingles, but the Zostavax vaccine exists to immunize against that. Though past studies have shown that VZV inoculation in non-human models does not create the same type of disease as it does in humans, there is a similar disease which affects non-human primates, known as
simian varicella virus (SVV). SVV is very similar genetically to VZV, has similar pathophysiology, and also establishes latent infection in neural ganglia (Gray, 2013).
CHAPTER II: BACKGROUND ON RECOMBINANT VACCINES

The first question that could be asked is: why not use an attenuation method on HIV, like many of the other vaccines that have been developed over the past 2 centuries? Like with many decisions, there is a balance of cost and benefit, even in vaccine development. In the chickenpox vaccine, for example, there is some risk, although very minute, that the patient can have a full-blown infection post-inoculation. However, at the age of chickenpox vaccination, that disease it not particularly dangerous to the patient, so it is worth the small risk to most likely never contract the disease. Attenuating a virus like HIV and administering it as a vaccine would be far too dangerous. Fortunately, vaccinology has moved through several waves of new vaccine strategies. It began with administration of live virus, then attenuated or killed pathogens, but today’s scientific advances are now allowing strategies involving recombinant proteins and recombinant genomes. These advances continue to challenge scholars in the vaccinology field to develop vaccines that can indeed fend off intracellular pathogens, such as HIV, and it is possible that this could be done through use of recombinant vaccine methods (Nascimento & Leite, 2012).

Past Successes in Recombinant Vaccinology

The utilization of recombinant technology like that in this thesis is not new. There are multiple studies that have demonstrated success in, or at least progress towards
immunization using recombinant methods, and these have also discovered limitations in this type of technology. For example, the amount of protein that can be expressed from a recombinant vaccine, as well as how it may be translated and/or modified within a cell all matter when developing a recombinant vaccine (Nascimento & Leite, 2012).

Recombinant Viral Vectors

Rather than directly introducing proteins into an individual, using viral vectors allows the introduction of a genomic system that can induce an immune response upon entrance of the target antigen; this is the method employed in this study, as well. As mentioned previously, it is a priority to find vaccination methods that can induce intracellular, or even all forms of immune responses, and scientists hypothesize that this can be achieved by using recombinant viral vectors. For a virus to be a desirable vector for recombinant vaccination, it should have a large enough genome for incorporating new genetic material, be genetically stable, have capability of growing large numbers and be able to be isolated and purified, lack ability to integrate into host genome, and as always, be completely safe. Current viruses that meet these criteria and are currently used for vaccine development research include vaccinia, adenovirus, and herpes virus, to name a few. This method has been used widely in the development of an effective HIV vaccine. One example comes from adenovirus, a non-enveloped, icosahedral virus that invades multiple locations across the body. Adenovirus serotype 5 (Ad5) is the strain used, as it meets all criteria for a safe and effective viral vector. The HIV antigens expressed by the recombinant Ad5 include gag, pol, env, and nef. This system has shown to be quite competent in controlling viral infection of SIV in monkeys, even after an HIV-SIV challenge. However, after this study moved into human trial, it proved unsuccessful in
vaccination against HIV. Additionally, patients who were already immune to Ad5 were more likely to become infected by HIV (Nascimento & Leite, 2012). While this may have been unsuccessful, it helped progress the entire field closer to a possible cure or vaccine, and also demonstrated the limitations of certain models.

**Prime-Boost Method**

Throughout testing of many successful and unsuccessful vaccine developments, one fact that is now widely understood is that immunization against a disease as complex as HIV/AIDS will most likely require a combination of vaccination methods. One such method is prime-boosting, which simply means that multiple inoculations are administered over a designated time span. When the same vaccine is used in both priming and boosting, it is considered homologous prime-boosting, and if varying vaccines are used, it is called heterologous prime-boosting (Nascimento & Leite, 2012). An example of homologous prime-boosting is the vaccine for VZV. A study performed in Germany assessing the efficacy of one-dose vs. two-dose vaccination against varicella proved that the homologous prime-boost method was much more successful. Children who received the two-dose regimen were much less likely to get varicella than those who only received one dose, validating the prime-boost method’s potential (Wutzler, et al., 2008).

The prime-boost method has shown great potential for vaccine research for HIV and is the chosen immunization method used in the research presented by this thesis. One such success was demonstrated in an immunization schedule with a priming recombinant vaccinia virus expressing SIVmne gp160, followed up by a booster of a
recombinant gp160 protein. Success in the prime-boost method was also demonstrated in mice which were primed with a DNA vaccine that encoded genetic material from influenza, and were boosted with a recombinant poxvirus encoding the same material (Nascimento & Leite, 2012). While the mechanisms behind the prime-boost method are poorly understood, the scientific community has enough knowledge to continue on researching with it in hopes of curing varying infectious diseases. Hopefully with different combinations of DNA vaccines, protein vaccines, and recombinant vaccines, the right regimen for curing will be eventually be discovered. This thesis explores and compares two heterologous prime-boost studies, each employing either a DNA or protein vaccine as a booster.
CHAPTER III: METHODS AND PROTOCOL OPTIMIZATION

To target the first aim of this study, various parameters were altered and analyzed for quality and/or accuracy based on hypothesized results. Various amounts (0.3 μg, 0.5μg, and 1μg) of the gag and env proteins were separated on 10-20% tris-glycine gels to gauge the minimal protein dilution that can be transferred to a PVDF membrane, but that can still provide clear imaging for analysis. Additionally, dilution of primary antibody (NHPs LK40 and LK47 at the PB stage) to blocking solution (5% blotto) was tested at the 1:100 and 1:200 increments to determine if a higher dilution improved level of detection on the blots. These experiments were performed at the onset of this study in order to standardize the results, control and experimental, attained throughout the remainder of the project, and also assist in attaining the most accurate results in later studies.

Experimental Design

The study explored in this thesis employs a heterologous prime-boost method, rather than a homologous method like previous studies involved with this project. Research done under a contract with the National Institutes of Health (NIH) Simian Vaccine Evaluation Unit (SVEU) includes a protein vaccine boost, and our other study presented here provided by resources from an NIH R01 grant utilizes a DNA
vaccine boost. I will focus primarily on the SVEU study, but later compare the results between the protein and DNA vaccine boost studies.

Sera was taken from the NHPs housed at the Tulane National Primate Research Center at multiple points throughout the study for analysis of potential immune responses. Sera was taken before initiation of any immunizations to assure that the animals were not already infected with SIV (and SVV), and this was confirmed by ELISA at TNPRC, and by immunoblot in our lab. The experimental group received inoculations of the rSVV-SIV gag and env vaccines at months 0 and 2, and received the SIV gag/env protein vaccines at months 6, 9, and 12 (Figure A-5). The animals will have been challenged with the macaque strain of SIV (SIVmac251) in month 15, which will ideally be administered at a low dose either intrarectally or intravaginally, depending on the sex of the animal. Because HIV is primarily sexually transmitted rather than intravenously, this method of viral challenge could potentially provide more realistic parameters and results than introduction of a high-dose bolus directly into the blood stream.

**Handling of NHP Sera**

Working with animal sera can be very dangerous, so it is of the utmost important to be cautious when working with it. Any protocol that required direct interaction with the test subjects was dealt with at TNPRC. After drawing blood from the animals, sera was isolated, frozen, shipped to Oxford, MS, and analyzed in the laboratory at the University of Mississippi. Gloves were used at all times while handling any animal sera, and any disposable materials that came in contact with the animal sera were disposed of
properly. Sera was also heat-inactivated at 56°C for 30 minutes before being aliquoted into separate tubes. Repeated heating and freezing of animal sera could cause the quality of target proteins to decrease; aliquoting helps to preserve the quality of the sera. Protease and phosphatase inhibitors in the Lamelli sample buffer used for running the proteins during gel electrophoresis are also used to help preserve sera and antigen quality (Jensen, 2012).

**Initial Protocol**

A protein molecular weight ladder and one µg of purified gag and env protein, each diluted in Lamelli sample buffer, were loaded as according to Figure 1, and separated on a 10-well Invitrogen 10-20% tris-glycine gel via sodium dodecyl sulfate polyacrylamide gel electrophoresis, a common method of protein analysis also known as SDS-PAGE. Gel electrophoresis was carried out in running buffer at 100V and 60mA for 75 minutes. After electrophoresis, the proteins were transferred to a PVDF membrane using pre-prepared transfer buffer at 20V and 180 mA for 75 minutes. The PVDF membrane was allowed to air dry, and was cut into separate testing strips (containing the molecular weight ladder, controls, and experimental proteins) before storing in the 4°C refrigerator.

The immunoblot was blocked overnight in a 5% non-fat milk solution, or blocking buffer (BB). Following this step, the blot was soaked in 1:200 primary antibody (positive or negative control, or non-human primate sera: 5% BB) overnight on a gyratory shaker and then washed the next day using wash buffer. Secondary antibody (2º Ab) (HRP-conjugated mouse anti-monkey IgG, in 1:1000 dilution to 5% BB) was then
added to the blots to soak for 2 hours on the gyratory shaker and then washed again before adding 1:1 ECL Clarity luminol:peroxide solution. The enzyme conjugated to the 2\(^o\) Ab (horse radish peroxidase) reacts with the peroxide and luminol solution to emit light which is detectable by the BioRad Chemidoc system; blots soaked in this solution for approximately 5 minutes and were then dried to image using the BioRad Chemidoc system with the “high-resolution” program. The same parameters (program and IV exposure time) were used when imaging any western blots, with exception of taking additional images at various parameters, such as using “high-sensitivity” for an image with low antibody detection.

A protocol which did not require digital imaging was also performed once at the beginning of this study, but it was decided that imaging via chemiluminescence and the BioRad system was sufficient for the research being conducted, and was also easier to image, store, and organize.

![Sequence for loading wells prior to performing SDS-PAGE.](image)

**Figure 1. Sequence for loading wells prior to performing SDS-PAGE.** Throughout this study, gels were loaded in a consistent manner so that accurate comparisons could be made. The molecular weight ladder (MW) was always loaded in front of the purified env and gag proteins. Each PVDF membrane supplied 3 separate blots for later analysis.
Protocol Modifications

Initial protocol instructed that the blots be washed for 2-3 minutes, 5 times, in 2-3 mL of wash buffer after the 1º Ab and 2º Ab soaks. However, there was less background noise in the images when the blots were washed for longer periods, so the protocol was later changed to an 8 minute wash, 5 times, in 5mL wash buffer. With this, the membranes were also imaged while they were still wet rather than dry, and sometimes allowed to dry for reimaging. Dilutions of the protein ran on the gel and of the primary antibody were analyzed to see if further minimization of use of these solutions was possible. For the gel, 0.3μg, 0.5μg, and 1μg of each protein were ran to see if a difference was observed. Primary antibody dilutions were performed at a 1:200 dilution and a more concentrated 1:100 dilution.

Optimization of Immunoblot Conditions

Longer periods of washing as well as imaging while the blot was still wet after the luminol and peroxide soak did show better imaging in terms of background noise, so this protocol was adopted for the remainder of the blot imaging. There were no consistent differences observed between 1:200 vs. 1:100 dilution of primary antibody (Figure 2), so it was decided to continue on with the 1:200 dilution, for the purpose of conserving the serum stock. Results from testing various amounts of the gag and env proteins (Figure 3) included a noticeable reduction in antibody detection in the blot that was ran with .3μg of each protein. The 1μg dilution seemed to be slightly stronger than the .5μg one, so this dilution was used throughout the remainder of the project.
Figure 2. Western blot analysis using different concentrations of $1^\text{st}$ Ab. Blots A and B are analyses of NHP LK40 PB serum, at 1:100 and 1:200 $1^\text{st}$Ab:5%BB, respectively. Blots C and D are analyses of NHP LK47 PB serum, also at 1:100 and 1:200 $1^\text{st}$Ab:5%BB. Various $1^\text{st}$Ab concentrations were analyzed to find a concentration that allowed conservation of animal sera, while also producing quality images.

Figure 3. Western blot analysis of varying amounts of gag and env proteins using positive control sera. In western blot A, 0.3µg of purified of gag and env protein was loaded onto the gel; in blot B, 0.5µg was loaded; and in blot C 1µg of purified protein was loaded. All 3 gels were transferred to a PVDF membrane for analysis using positive control SIVmac251 serum to assess what amount of protein would allow for the best imaging technique.
Controls

Using the above protocol, a positive control blot (Figure 4A) using SIVmac251-positive sera as the 1º Ab, and a negative control (Figure 4B) using SIVmac251-negative sera as the 1º Ab were first attained for later comparison to blot analyses of pre-immune (PI), post-prime (PP), and post-boost (PB) sera. The only further modifications made to the protocol were for acquisition of a higher-quality negative control blot image (Figure 3C) for comparison of PI sera.

Figure 4. Optimization and assessment of negative control blots. Blot A is a positive control blot for comparison to blots B and C, which were both analyzed using SIV-negative control sera. Blots B and C are from separate analyses performed on different days. Blot C was obtained using a specialized sterile protocol to attain a higher quality and more accurate image.
Several images of both negative control blots, as well as analysis of assumedly seronegative PI sera showed light streaking on both the env and gag lanes of the blot. Due to these results, measures were taken to perform a highly sterile protocol. No dishes or tools that previously came in contact with control or experimental sera were used to handle the western blots, and fresh sterile petri dishes were used throughout any soaking processes to assure that unwanted antibodies were not capable of binding to the membrane.

Another limitation that was encountered while using chemiluminescence and digital imaging was that the computer auto-adjusted based on the amount of protein detected on the blots, and how bright that the blot was illuminating into the BioRad system’s camera, due to protein presence. This limitation was not truly recognized until the immune profiles for all of the subjects were completed (Appendix A). Essentially, a blot that may have had virtually undetectable amounts of protein, such as our negative controls or PI blots appeared very grainy, and even splotchy occasionally, while our positive control or PB blots, which were expected to have high antibody detection were of much higher quality. Quality of the digital images by the computer was and is considered in the results and discussion of further projects in hopes of keeping results standardized.
CHAPTER IV: HUMORAL RESPONSES INVOKED BY THE RSVV-SIV VACCINE

In the 2nd part of the study, sera from 4 non-human primates from the Simian Vaccine Evaluation Unit (SVEU) study at 3 different stages were analyzed by western blot to detect presence of antibodies to both the \textit{gag} and \textit{env} proteins. The three stages are pre-immune (PI), while the non-human primate (NHP) was still immune naïve, or prior to exposure to varicella or SIV; post-prime (PP), the period after 3 scheduled inoculations of the recombinant SVV-SIV vaccines; and post-boost (PB), or sera taken from the non-human primates after 2 additional inoculations of a protein vaccine developed by outside researchers. For the final project of this study, sera from the post-boost period of non-human primates which were given a DNA vaccine, rather than a protein vaccine, were analyzed by western blot to compare to the NIH SVEU contract animals.

\textbf{SVEU (Protein Boost)}

A protein vaccine is simply a purified or recombinant protein derived from a pathogen with the purpose of eliciting an immune response in the target subject. Recombinant protein methods of vaccination have proven quite successful in the fight against both Hepatitis B and human papillomaviruses, making recombinant technology a
popular choice. Serum from 4 rhesus macaques (LK40, LK43, LK44, and LK47) was taken at multiple points throughout this study (pre-immune, or PI; post-prime, or PP; and post-boost, or PB), and sent to the lab in Oxford for western blot analysis. The SVEU study employs a prime-boost method, using the rSVV-SIV as the priming agent, and the protein vaccine as the boosters, which here were purified gag and env proteins, supplied by another company and acquired by TNPRC. When analyzing the blots, we expect to see bands for the env protein at approximately 140 kDa, and bands for the gag protein at 51, 27, and 17 kDa.

Results and Discussion

Analysis of the PI blots following imaging showed some questionable results at first. There was a double-streaking pattern, seen in the negative control (Figure 5B) as well as in some of the NHPs’ sera analyses (Figure 5D-F). Considering these sera were taken before the NHPs were exposed to any virus or immunization methods, there should have been absolutely no antibody detection or bands on the immunoblot. However, it was determined that there was a certain amount of antibody detection present even on the negative control (Figure 4), so any streaking patterns or bands present on the negative control were also considered irrelevant in the immune profile presented on the western blots. This assumption was made also in conjunction with TNPRC’s negative ELISA test results for the animals prior to any immunizations. The grainy appearance of the blots could also indicate a low amount of antibody binding on the membrane.
Figure 5. Western blot analysis of SVEU NHP PI sera. A molecular ladder (A) is used in evaluating any blots for env and gag antibody detection, while a negative control (B) is used for specifically assessing PI blot analyses (C-F) from SVEU NHPs LK40, LK43, LK44, and LK47, respectively. Anything showing on the negative control blot is henceforth omitted from experimental analyses.

The main goal of analyzing the post-prime sera (Figure 6) was to assess the strength of the immune response before and after boosting. Based off of the quality of the images, there were likely low levels of antibody detected in PP analyses compared to the PB analyses (Figure 7), except for NHP LK43 (Figure 6C). The quality of the images of PB sera analyses (Figure 7) likely indicate that there was a sufficient amount of antibody bound to the membranes prior to imaging, demonstrating that the strongest immune response came after the protein boost.
Figure 6. Western blot analysis of SVEU NHP PP sera. A positive control blot (A) is included for comparison in any blots that are expected to have positive env and gag antibody detection, such as with PP and PB analyses. Experimental blots (B-E) were analyzed using SVEU NHP LK40, LK43, LK44, and LK47 PP sera, respectively.

Figure 7. Western blot analysis of SVEU NHP PB sera. Blots B-E, analyses of SVEU NHPs LK40, LK43, LK44, and LK47 PB sera, respectively, are compared against the positive control (A) for env and gag antibody detection.
DNA Boost Analysis

DNA vaccines have been used for HIV studies for the majority of the time HIV vaccine research has been conducted, most likely due to their ability to evoke cell-mediated immunity (Chen, et al., 2014). DNA immunization involves using genetic engineering to encode antigens on a plasmid, and then inoculating a person or subject with that genetic material. The desired genes are coupled with a promoter, and are either injected percutaneously or shot into the skin using a gene gun. The genes are expressed in cells that take up the plasmid, as well as any antigen presenting cells (APC’s) that recognize them as well. The plasmid will only express the desired protein, a target antigen, and will not incorporate into the host genome. After cells take in the plasmid, the encoded genes can be expressed and presented on major histocompatibility complexes (MHC). While this method has been successful in smaller animal models, DNA vaccination has not yet been promising with humans. However, immunogenicity of these vaccines have been improved by modifying the vector. It is recognized that DNA vaccination could be an important step for immunization against HIV, as it is safe even upon repeated inoculation (Khan 2013).

A new cohort of NHPs was used in the DNA vaccine study, which also employed a prime-boost method for rSVV-SIV vaccination, but with use of a DNA vaccine booster, rather than protein vaccination used in the SVEU study. The DNA vaccine was composed of a plasmid which encoded the genes for both gag and env proteins. The sera analyzed from this cohort of animals was only from the PB period. Comparison of the results from the SVEU study (Figure 7) and R01 study (Figure 8) demonstrates that a protein vaccine is overall more successful in eliciting an immune response, in terms of resulting in
immunogenicity to both target proteins. After boosting with the DNA vaccine, there was strong *gag* antibody detection but little to no *env* antibody detection. Meanwhile, the protein-boosted immunization schedule elicited a very strong response to *env* and, while less prominent than the *env* response, there was still a detectable amount of antibody to *gag* present in the sera.

**Figure 8. Western blot analysis of R01 NHP PB Sera.** Blots B-D, analyses of R01 NHPs LL83, LM28, and LM48, respectively, are compared against the positive control (A) for detection of *env* and *gag* antibodies.
CONCLUSION AND FUTURE EFFORTS

Through using different methods or making modifications throughout the research process, the protocol for detection of antibodies to \textit{gag} and \textit{env} protein created in response to the rSVV vaccine by western blot was ultimately optimized. The protocol modification testing period also showed us that there is flexibility in several aspects of the protocol, allowing it to be well suited to different time frames and parameters. Data collected throughout the semester shows that the prime-boost strategy using a protein vaccine seems to induce a stronger immune response than the DNA vaccine boost. For more accurate results, our strategy could be carried out in the future with a larger cohort. Repeat imaging including a longer wash period would also increase the quality of some of the blot images.

Up to the present, only sera from non-human primates that were immune-naive prior to their respective vaccination strategies have been analyzed via western blot. There is, however, a potential study which includes an additional group of non-human primates which have previously been exposed to SVV before vaccination that can be tested, which would show the effects of pre-existing SVV antibodies on the recombinant vaccine’s ability to illicit an immune response. This model will hopefully shed insight to the immune response to a theoretical recombinant VZV-HIV vaccine administered after a person has already been exposed to varicella.
Currently, the vaccine only includes the structural *gag* and *env* genes, but has potential for addition of new models as well. Our lab is currently working to develop a DNA vaccine and a rSVV vaccine, each of which includes the “Retanef” (*rev, tat, nef*) genes. *Tat* is the primary activator in transcription, *Rev* regulates RNA splicing and the export of viral RNA, and *nef* is an accessory gene which has affects on replication, budding, and pathogenicity (German Advisory Committee Blood, 2016). The hypothesis for this study is that adding the Retanef vaccine to the current cocktail will help increase the humoral response that the *gag* and *env* are already eliciting. This recombinant technology will continue to be modified, and has been, is being, and will likely be used for future research in vaccinology, and not only restricted to HIV cure research. The overarching goal of this project was to contribute to HIV research via an SIV model, and the findings show hope towards one day finding a vaccine for HIV.
Figure A-1. *Gag* and *env* antibody detection of NHP LK40 at the PI, PP, and PB stage of immunization. Blots C-E are compared with the negative control (A) and positive control (B) for the absence or presence of antibodies to the target antigens.

Figure A-2. *Gag* and *env* antibody detection of NHP LK43 at the PI, PP, and PB stage of immunization. Blots C-E are compared with the negative control (A) and positive control (B) for the absence or presence of antibodies to the target antigens.
Figure A-3. Gag and env antibody detection of NHP LK44 at the PI, PP, and PB stage of immunization. Blots C-E are compared with the negative control (A) and positive control (B) for the absence or presence of antibodies to the target antigens.

Figure A-4. Gag and env antibody detection of NHP LK47 at the PI, PP, and PB stage of immunization. Blots C-E are compared with the negative control (A) and positive control (B) for the absence or presence of antibodies to the target antigens.
Figure A-5. Immunization schedule of the R01 grant and SVEU contract rhesus macaques. The stars indicate the time periods from which the lab received sera samples from the animals, while the upward-pointing arrows indicate the time periods at which animals underwent vaccinations, SIV challenge, or euthanization.
BIBLIOGRAPHY


