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THE KINETICS OF NEUTRALIZING ANTI-VIRAL ANTIBODIES AND THE PRODUCTION OF A T CELL-DEPENDENT ANTIBODY RESPONSE DURING VESICULAR STOMATITIS VIRUS INFECTION

Emily Claire Pyle

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ABSTRACT

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Antiviral T cell-independent responses provide early protection during infection that are essential for control of disease. However, the characteristics of the antigens which activate these T cell-independent pathways and the mechanisms by which the immune response proceeds are not yet completely understood. The research presented here focuses on infection with vesicular stomatitis virus (VSV), which induces a T cell-independent response. Using mice infected with VSV or immunized with ultraviolet light (UV)-inactivated VSV, the kinetics of neutralizing anti-viral antibodies were examined. Vesicular stomatitis virus and UV-inactivated VSV induce a rapid protective antibody response, with high titers of antibodies detected only three days after infection or immunization. Infection with VSV also induced a long-lasting antibody response, in contrast to mice immunized with inactivated VSV. This research also investigated the antibody response to an unrelated, T cell-dependent antigen (OVA) during VSV infection or after immunization with UV-inactivated VSV. Mice infected with VSV produced significantly more anti-OVA antibodies than non-infected mice, while no increase in the anti-OVA response was detected with inactivated VSV. These results suggest that infection with VSV increases the antibody response against unrelated antigens and thus, opens the possibility to use recombinant VSV as vectors in vaccines.

PREFACE

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LIST OF SYMBOLS AND ABBREVIATIONS

ABTS……..2,2`-Azinobis diammonium salt

ANOVA……..analysis of variance

BHK 21……...Baby hamster kidney 21 cells

cm……..……centimeter

CO_2……....…….……Carbon Dioxide

cc……..…cubic centimeter

˚C……...degrees Celsius

CD……...cluster of differentiation

EDTA……..ethylenediaminetetraacetic acid

ELISA……Enzyme linked immunosorbent assay

FCS…….Fetal calf serum

FMDV……...…Foot and mouth disease virus

g……..relative centrifugal force

Ig…….Immunoglobulin

Il……Interleukin

MHC…….….Major Histocompatibility Complex

ml…….……milliliter

mm……milimeter

µg……..microgram

µl……microliter

nm……..…nanometer

OVA……...…egg albumin

PBS……..Phosphate Buffered Solution

PBST……...Phosphate Buffered Solution with 0.05% Tween-20

RPMI ……...…...Roswell Park Memorial Institute 1640 (culture media)

$TCID_{50}$ ……...Tissue Culture Infective Dose at 50%

U…….....unit

UV……ultra-violet

UV-VSV……...…ultra-violet light inactivated vesicular stomatitis virus

VSV……..….Vesicular stomatitis virus

W…….....watt

X……times

INTRODUCTION

Overview of a typical immune response to an antigen

Through many complementary and sometimes overlapping mechanisms, the immune system provides defense against potential threats. Nonspecific (innate) immune protection arises from inherited structures such as evolutionarily conserved molecules anchored on membranes, acidic internal environments, phagocytic cells such as neutrophils and macrophages, and soluble molecules such as interferon and complement proteins. The ability to defend against specific pathogens is provided by lymphocytes in the adaptive immune response. The lymphocytes include cells derived in the thymus (T cells) and cells produced in the bone marrow (B cells). Because B cells can differentiate and proliferate into plasma cells, which secrete pathogen-specific antibodies, they are said to provide antibody-mediated (humoral) immunity. Cell-mediated immunity is provided by the various subpopulations of T cells. The subpopulations of T cells, as well as those of other immune cells, are differentiated based on cluster of differentiation (CD) molecules found on each cell's surface. Biologically, these function as ligands or receptors, and are used by researchers to characterize various cell populations, as each will have a different set of CD markers. T cells expressing CD8 are called cytotoxic T cells, and can kill their target cells by coming into physical contact with them. Helper T cells (CD4 positive) increase the activity of B cells and cytotoxic T cells.

T cells cannot bind free antigens; instead T cell response to antigens depends on antigen presenting cells. These include dendritic cells and macrophages, which engulf and

partially digest protein antigens. The resulting polypeptides are displayed on the presenting cell surface in association with major histocompatibility complex (MHC) molecules, for which T cells have receptors. Once stimulated by the MHC molecule and antigen, T cells are activated. Type 2 helper T cells then activate follicular B cells to become plasma cells. Two signals are required for this stimulation, one from the antigen itself and one from the T cell. B cells express antibodies on their surface that act as receptors for antigens. They also express CD40, which interacts with the CD40 ligand on a helper T cell recognizing the same antigen as the B cell. Because only activated T cells display CD40 ligand, the activity of antigen presenting cells indirectly controls the production of antibodies in this pathway. A typical adaptive immune response against a protein antigen would be its capture and processing by a dendritic cell, subsequent presentation to a T cell, and then the activation of a specific B cell (see Figure 1). In this T cell-dependent pathway, antibodies released by plasma cells are detectable in the blood after seven days. Antibodies bind with the antigen, leading to its neutralization and eventual destruction through complement system activation and by directing the immunological attack by phagocytes. Long-term immunity against a pathogen results after an infection through the induction of memory B and T cells.

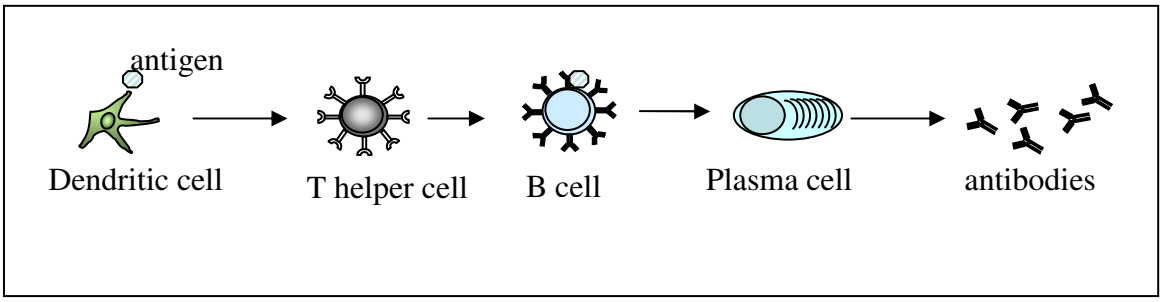

Figure 1. Illustration of cells interacting to provide humoral immunity.

The first antibodies released by plasma cells at the onset of an infection have lower affinity to the antigen than those appearing later in the infection. Increased affinity results from somatic hypermutations of the genes encoding the antibody variable regions. This greatly increases the diversity of antibodies produced. Mutations that increase the specificity of the antibody for the antigen increase its binding affinity for that antigen. This leads to selection for B cells producing these antibodies, since they receive more proliferation signals than the B cells without antigen. Research has found that considerable increases in binding affinity begin seven days after exposure to the antigen (1). Antibody isotype also changes throughout the course of infection. Before and immediately after antigen exposure, antibodies are mainly of the IgM isotype. After seven days, DNA recombination of regions in the antibody heavy-chain constant genes leads to the secretion of other antibody isotypes, such as IgG. Because of their structure, IgG antibodies have greater access to antigens in the interstitial space than IgM antibodies, and can also implement more effector functions (such as phagocytic cell recruitment). Isotype switching most often takes place within areas of secondary lymphoid tissue known as germinal centers through the activity of helper T cells and their release of molecules (cytokines) such as interleukin-4, interferon-γ (IFN γ), and transforming growth factor- β (TGF-β).

Early protection in the adaptive immune response

In the typical T cell-dependent immunity for model antigens discussed above, there is a delay between the onset of infection and efficient neutralizing antibody production. Affinity maturation of an IgG antibody response that takes more than a week may not

provide effective protection against those pathogens that require neutralizing antibodies, as too few antibodies may be secreted too late (2). However, protection against certain cytopathic viruses seems to arise through a faster, alternative pathway. Examples of viruses that induce this response include polyomavirus (3), foot-and-mouth disease virus (FMDV) (4), influenza virus (5, 6) and vesicular stomatitis virus (VSV) (7). Neutralizing antibodies produced in this pathway can be detected in the blood as early as three days after infection, as with mice experimentally infected with FMDV (4). In VSV-infected mice, high neutralizing titers of IgG with great affinity for the virus are detected on day six post infection, with no improvement in affinity for more than six months (2).

It is now becoming clear that this faster response occurs through the T cell-independent pathway, where the need of T cell involvement in antibody production is circumvented. Both euthymic and athymic mice infected with FMDV show the same neutralizing antibody kinetics, indicating that the primary antibody response is T cell-independent (4, 8). Polyomavirus infection of T cell-deficient mice induces both IgM and IgG responses (3), suggesting that T cells are not always required for isotype switching. Vesicular stomatitis virus initiates a T cell-independent neutralizing IgM response (7) and a dosage-dependent IgG response (9, 10), as seen in studies with nude mice. The importance of T cells later in infection is shown by the requirement of activated, pathogen-specific T cells for generating immunologic memory against FMDV (11).

The usual requirement for two signals to activate naïve B cells means that if a B cell receives only one signal, it will either become apoptotic or anergized. However, B cells

activated independently of T cells by VSV lack a CD40 ligand-derived second signal, and yet are not silenced (12). One possible explanation is that the repetitive, highly organized structure typical of many of the T cell-independent antigens is involved in the induction of antibody responses, perhaps through cross-linking of the B cell receptors (13-15). Other research suggests that it is the viral surface antigen itself that determines neutralizing antibody kinetics, regardless of its surface arrangement (16). However, this fails to explain the lack of T cell-independent responses to noncytopathic viruses that do have a highly organized surface, such as lymphocytic choriomeningitis virus (LCMV, 17-19). Also, experiments with polyomavirus have found that the structure of the virus does not account for T cell-independent IgG production, but instead seems to depend on signals generated during a live-virus infection (3, 20). Similarly, the immune response to noninfectious ultra-violet-inactivated VSV differs from that initiated by infectious VSV (12, 21). Treatment with ultra-violet (UV) light renders some viruses noninfectious by inhibiting their ability to replicate while leaving their surface structure unaltered. The importance of the virus being infectious has also been seen with FMDV. In contrast to the rapidly produced neutralizing antibodies with infectious FMDV, mice treated with an inactivated virus yielded a medium titer of neutralizing antibodies only after seven days. Different subclasses of the IgG antibodies were also produced. Infection with FMDV led to IgM, IgG3 and IgG2b secretion, whereas immunization with UV-inactivated virus induced IgG1 and IgG2a antibodies (22). This indicates that the two forms of the virus lead to different pathways of activation, despite having the same physical structure.

The most efficient initiators of the primary immune response are the dendritic cells (23). These antigen presenting cells undergo maturation upon contact with particular pathogens and migrate to secondary lymphoid organs where they present pathogen-specific antigens to T cells (24). The importance of dendritic cells as antigen presenting cells in T cell activation is highlighted by viruses, such as human immunodeficiency virus (25), herpes simplex virus type 1 (26), measles virus (27-29), and influenza virus (30), which target dendritic cells and induce an immunosuppressive state by impairing T cell responses. Dendritic cells can also enhance B cell responses to T cell-dependent antigens by non-specifically stimulating activated B cell proliferation and production of IgM and IgG (31). A role for dendritic cells in the process leading to plasma cell differentiation in T cell-independent responses has also been established. Early after infection with VSV, dendritic cells transport and release the virus in secondary lymphoid organs where B cells are directly stimulated and neutralizing IgM is produced independently of T cells (21).

One pathway of direct dendritic cell-B cell stimulation has been described. Blood-born $CD11c^{lo}$ dendritic cells capture and carry antigen to the spleen, where they provide crucial survival signals to marginal zone B cells and promote their differentiation into IgM-secreting plasma cells in the absence of T cells (32). The co-signal is provided by the interaction of $CD11c^{lo}$ dendritic cell derived ligands APRIL (*A PR*oliferation-*I*nducing *L*igand) and BAFF (B cell Activating Factor of the TNF Family) with their receptors TACI (Trans-membrane Activator and Calcium modulator cyclophilin Ligand Interactor), BCMA (B Cell Maturation Antigen), and BAFF-R. Signals through TACI

and BAFF-R can also mediate antibody isotype switching (32-34). It is interesting to note that FMDV-infected dendritic cells express low amounts of CD11c (35).

Splenic marginal zone B cells play an important role in T cell-independent antibody responses. These are a subset of B cells located in the marginal zone of the spleen that are much faster at proliferating and differentiating into antibody-secreting cells upon stimulation by a T cell-independent antigen than are follicular B cells (36, 37). Marginal zone B cells also differ morphologically from follicular B cells, with the former expressing $IgM^{high}IgD^{low}CD21^{high}CD23^{low}$ and the latter characterized by $IgM^{low}IgD^{high}CD21^{int}CD23^{high}$ (38). Marginal zone B cells have a low threshold of activation, and because of the anatomy of the spleen, have immediate access to blood-born antigens. This allows them to release an early wave of T cell-independent antibodies, as is the case with VSV. This virus is trapped in the marginal zone by cells expressing CD11b and CD11, and marginal zone B cells are then stimulated to produce neutralizing IgM and IgG3 antibodies (38, 39). The importance of marginal zone B cells in VSV infection is suggested by the correlation between a lack of protective IgM response to VSV and structural defects in the spleen (40). Marginal zone B cells may also be responsible for early anti-FMDV neutralizing antibody responses as well. These B cells mainly release isotypes IgM and IgG3, which are the isotypes of the first neutralizing antibodies produced during FMDV infection (22). Our laboratory has recently found that FMDV-infected dendritic cells can directly stimulate marginal zone B cells to secrete anti-FMDV antibodies (41).

Cytokines are small proteins that mediate many of the functions of the immune system. Most are not constitutively expressed, but are produced in response to signals. These potent chemicals have a high binding affinity for their receptor, and may act in either a paracrine or autocrine manner. Cytokines may also act in either an inhibitory or stimulatory fashion, sometimes activating one branch of the immune system while simultaneously inhibiting another. Interleukin-4 (IL-4) is mainly produced by activated CD4+ T cells (41, 42). This cytokine acts as a T cell growth factor, leads to the differentiation of CD4+ helper T cells, induces MHCII expression on B cells, promotes the proliferation and differentiation of B cells, and is necessary for antibody isotype switching (42). Interleukin-4 also antagonizes the response of some cells to the inflammatory cytokine IL-2, and so in this way can inhibit part of the immune response. Interleukin-6 is a major mediator of the inflammatory response during infection and is involved in the induction of the acute phase reaction (43). Interleukin-6 stimulates B cells to become antibody-producing plasma cells (44), and also can lead to T-cell growth and differentiation. Interleukin-10 is an inhibitory cytokine that limits and ends inflammatory responses (45). This molecule reduces activated monocyte/macrophage functions, including monokine synthesis, nitric oxide production, MHC class II and CD80/CD86 costimulatory molecule expression (45). Interleukin-12 (IL-12) is involved in the early defense against infections and is secreted by phagocytic cells, T cells and B cells (46). This cytokine leads to the production of interferon-γ (IFN- γ), which is itself an inflammatory cytokine.

Early immune response during FMDV and VSV infection

The T cell-dependent immune response is the best characterized pathway of the immune system. Research in our laboratory has focused on the mechanisms leading to an early protective immune response, especially that of a T cell-independent response. To this end, we are characterizing the immune responses to different cytopathic viruses, including FMDV and VSV.

Our laboratory was recently able to describe the basis for the T cell-independent response during FMDV infection (35, 41). We found that infection of mice with FMDV leads to a generalized immunosuppression of T cells. This immunosuppression is mediated by FMDV-infected dendritic cells, and occurs through at least two mechanisms: a decreased expression of molecules involved in the activation of T cells, and the secretion of inhibitory cytokines. Infected dendritic cells exhibited decreased expression of the T cell stimulatory molecules CD40 and MHC class II. Accordingly, these cells did not stimulate T cell proliferation *in vitro* or a T cell-dependent antibody response *in vivo* (35). Other surface marker changes on FMDV-infected dendritic cells include the increased expression of CD11b, and decreased expression of CD11c (35). It is hypothesized that dendritic cells of this phenotype provide the signals needed for T cell-independent marginal zone B cell differentiation into plasmablasts. The surface marker CD11b (also known as integrin αMβ2, complement receptor 3, and Mac 1) plays an important role in many polymorphonuclear neutrophil adhesion-dependent functions, such as phagocytosis (47). CD11c (integrin αXβ2, complement receptor 4, p150) is also involved in phagocytosis. In mice, this marker is found on most dendritic cells, but not

on most tissue macrophages, indicating a phenotypic change towards that of macrophages. Macrophages are not as efficient at activating T cells as are dendritic cells, again showing that FMDV-infected dendritic cells have decreased T cell stimulatory capabilities.

Our laboratory has found that FMDV-infected dendritic cells induce splenocytes to significantly increase their secretion of IL-10 and increase their secretion of IFN- γ. The production of these cytokines correlates with the suppression of T cell proliferation and of the response to the T cell-dependent antigen (35). As IL-10 and IFN- γ promote the isotype switch to IgG3 (48, 49), they may also explain the presence of this antibody isotype early in infection.

Vesicular stomatitis virus is similar to FMDV in that it too is a highly cytopathic virus that initiates an early T cell-independent IgM antibody response in experimentally infected animals (7). In mice, VSV is mainly neurotropic and causes paralysis and death if it reaches the neuronal tissue (50). This is seen in animals lacking a functional type I IFN system (51) or appropriate B cell responses (52, 53, 54). These responses include the production of neutralizing antibodies during the early phase of infection; neutralizing IgM antibodies are generally seen by day three or four (7), with isotype switch to IgG around day six (2). While the IgG response to VSV is generally considered to be strictly T helper cell dependent (55), some researchers have found T cell-independent production of IgG at high doses of VSV (9). T cells are important for long-term survival, with CD4 T cells being more effective than CD8 T cells in controlling infection (53).

Vesicular stomatitis virus is the type species of the genus *Vesiculoviridae,* a member of the family *Rhabdoviridae*. It is a bullet-shaped, enveloped, negative-stranded RNA virus, and is a relative of rabies virus (56). The sole protein found in the viral envelope is its glycoprotein. This is present in a highly organized form, and is able to induce B cells directly in the complete absence of T cell help (15). The glycoprotein is also the target for neutralizing antibodies (54, 57, 58). There are two distinct serotypes found in the Western Hemisphere, *New Jersey and Indiana*. These infect insects and mammals, such as horses, cattle, and swine. While infection is associated with significant disease, it is rarely fatal (56, 59). Outbreaks of VSV infection among livestock are economically important, and occur periodically in the United States, with widespread epizootics occurring roughly every 10 to 15 years (60, 61). The virus is likely spread through arthropod vectors, such as sandflies (62, 63) and *Aedes* mosquitoes (64). Human infection by VSV has been seen with people exposed to infected animals. Infection can results in influenza-like symptoms, but often is asymptomatic (56). High antibody titers to VSV are present in a large percentage of humans living in areas where VSV infection of animals is common (65).

Project objectives

The overall goal of my research was to characterize the early, T cell-independent immune response against VSV. Specifically, this research examined the kinetics of neutralizing anti-viral antibodies after VSV infection and after immunization with UV-inactivated

VSV. It also investigated the production of a T cell-dependent antibody response against an unrelated T cell-dependent antigen during VSV infection.

Project Significance

Many diseases that historically have been of great medical or agricultural importance can be prevented through vaccination, which uses an infectious agent in a form that cannot cause disease. Through this prior exposure, the immune system is able to gain the experience needed to generate a protective response in case of future exposure to the agent. The 20th century saw the development of vaccines against many infectious pathogens, leading to dramatically increased life expectancy. For example, the cowpox virus vaccine led to the eradication of smallpox. Attenuated vaccines also provide means for the successful control of measles, mumps, yellow fever, and polio. In contrast, attempts to create a protective antibody response against other viruses such as HIV, tuberculosis, leprosy, or most parasitic infections have thus far failed. Also, several antiviral vaccines using inactivated virus provide only weak, short-lasting immunity. Foot and mouth disease virus vaccine only protects for six months to one year. The yearly influenza vaccine will not provide protection against infection for the first fifteen days. Even effective vaccines carry risks of adverse immune reactions, and some are of limited availability due to their cost. Transportation of certain vaccines to third world countries is also difficult for a number of reasons. Only a few of the agents that cause disease have safe and effective vaccines. The lack of knowledge of the basic mechanisms involved in the response against these pathogens has hampered the development of effective vaccines. Increasing our knowledge of the immune system will increase our ability to

manipulate its responses, providing us with the ability to generate long-lasting, protective memory or alleviate unwanted immune responses. The research performed here will help expand our knowledge of the protective immune response against viruses that could be used in the future to formulate more effective vaccines.

Antiviral T cell-independent responses provide early protection during infection that are essential for control of disease, and can potentially impair viral spread before the inception of T cell-dependent responses. The early response may also help the later T cell-dependent response. The expansion of specific B cells without prior T helper cell induction increases the chances of cognate T-B cell interactions once T cell help has been induced. The T cell-independent pathway(s) also supply protection in organisms with impaired T cell functions. The number of cytopathic viruses that elicit a T cell-independent response indicate that virus-induced T cell-independent antibody responses are a general phenomenon. Despite their obvious importance and frequency however, the exact mechanisms of the immune system and the characteristics of the antigens which activate these T cell-independent pathways of B cell differentiation, antibody production, and isotype switching are not yet known. Through the study of VSV-infected mice, this research contributes to our knowledge of the means of the protective immune system response against viruses inducing tissue injury.

CHAPTER ONE: OPTIMIZATIONS

1.1 Materials/Methods

1.1.1 BHK 21 Cell Culture

Standard cell culture methods were used, as detailed below. All cells were handled under sterility and grown at 37˚C with 5% CO_2. Baby hamster kidney (BHK) 21 cells, originally obtained from the laboratory of Dr. M Osorio/University of Nebraska, were stored at -80˚C. When needed, a vial containing 1 ml of cells was quickly thawed and added to 4 ml of sterile RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 5% fetal calf serum (FCS). After centrifugation at 145g for five minutes, the supernatant was removed and the cells resuspended with 5 ml of 5% FCS-RPMI and 100 U-µg penicillin-streptomycin (Gibco). The cells were grown in 25 cc tissue culture flasks (Midwest Scientific) at 37˚C with 5% CO_2.

Cells were passed 1:3 after a monolayer was formed, usually once every three or four days. For this, the supernatant was removed and the cells rinsed with 1 ml 0.25% (1X) trypsin (Gibco) in RPMI. One milliliter of 1X trypsin was then added to the cells, until they appeared shrunken when viewed under the microscope. All but approximately 0.2 ml of the 1X trypsin was then removed, and the sides of the tissue culture flask gently tapped until the cells detached. Enough 5% FCS-RPMI to split the cells 1:3 was then added, and the cells were transferred to a new tissue culture flask of the appropriate size.

Various concentrations of FCS, ranging from 2.5% to 20%, were used in the growth medium. The cells responded best with a medium containing 5% to 10% FCS. Medium with 10% FCS required fewer days between passages, and so 5% FCS was used. Cells were first thawed and grown without penicillin-streptomycin, but a slow-growing bacterial contamination eventually required the use of antibiotics. The recommended concentration of antibiotic by the provider (Gibco) was deemed sufficient to contain the bacterial contamination.

To detach the cells for passage from one tissue flask to another, both 0.25% trypsin and 0.5% trypsin solutions were tried, with the addition of 1% EDTA (Sigma-Aldrich), in one attempt with the 0.25% trypsin. All three solutions were able to detach the cells from the tissue culture plastic. Subsequent experiments used the lowest concentration of trypsin (0.25%), without EDTA as this was sufficient and avoided potentially lysing the cells by using too harsh a treatment.

Preparation of a 96 well tissue culture plate with healthy, confluent BHK 21 cells was optimized by adding various concentrations of cells to each well of the plate. Cells from a 75 cc tissue culture flask were detached using 1X trypsin and diluted 1/30, 1/60 or 1/80 with 5% FCS-RPMI and 100 U-µg penicillin-streptomycin. Two hundred microliters of the cell containing solution were placed in each well of the plate and incubated at 37° C with 5% CO_2. Cells diluted 1/30 were too concentrated for the 200 µl growing environment of each well and metabolized too quickly. These cells were unable to survive in the 96 well tissue culture plate for four days. Cells diluted 1/80 were too dilute,

with too few cells seeded into each well and unable to form a monolayer (Figure 2). The 1/60 dilution allowed cells to grow to a monolayer after two days, and were able to survive until at least day five in the 96 well plate.

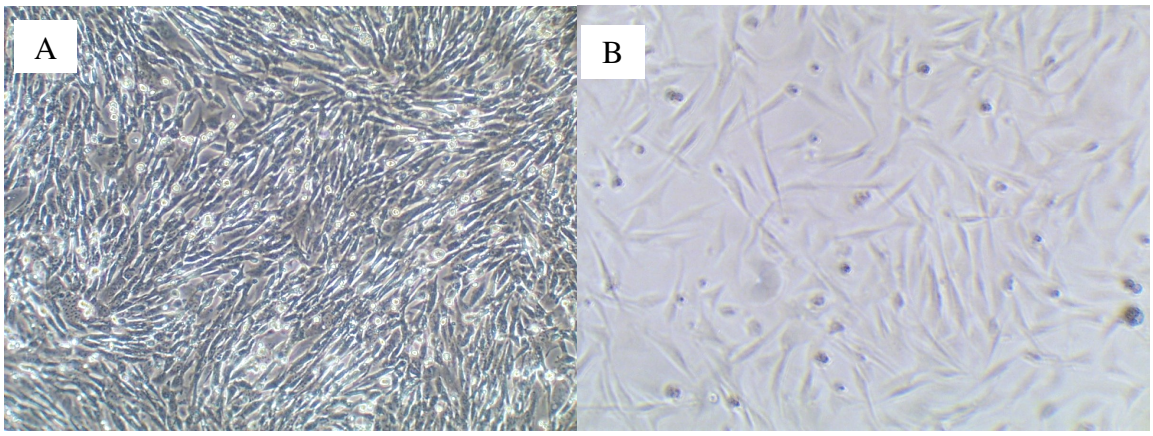

Figure 2. BHK 21 cells cultured at 37˚C with 5% CO_2. a) Healthy BHK 21 cells grown with 5% FCS-RPMI and 100 U-µg penicillin-streptomycin. b) Cells too diluted for passage are unable to form a monolayer after several days of culture. Cells were viewed with 100X magnification.

1.1.2 Vesicular stomatitis virus

1.1.2a Propagation

Vesicular stomatitis virus (VSV) serotype Indiana was originally obtained from the laboratory of Dr. Osorio (University of Nebraska, Lincoln). The virus was stored at -80˚C. BHK 21 cells were cultured in a 75 cc flask and incubated until a monolayer was formed. Then, one vial containing 1 ml of VSV was quickly thawed and mixed with 10 ml sterile RPMI. The supernatant from the BHK 21 cells was removed and replaced by the VSV-containing media. After 45 minutes at 37˚C with 5% $CO_{2.,}$ the virus-containing supernatant was replaced with RPMI supplemented with 5% FCS and 100 U-µg penicillin-streptomycin. After one day, almost all of the cells had been infected and killed, as assessed by observing cytopathic effect with 100X magnification (Figure 3). This indicated that the virus was infectious and had replicated.

The virus was collected from the infected cells by placing the tissue culture flask at -70˚C for 30 minutes, and then warming to room temperature. This was repeated three times to lyse the BHK 21 cells, causing the virus to be released into the supernatant. This supernatant was then collected, and centrifuged for 20 minutes at 580g. The cell pellet was then discarded, and the virus-containing supernatant was stored in 1 ml aliquots at -80˚C.

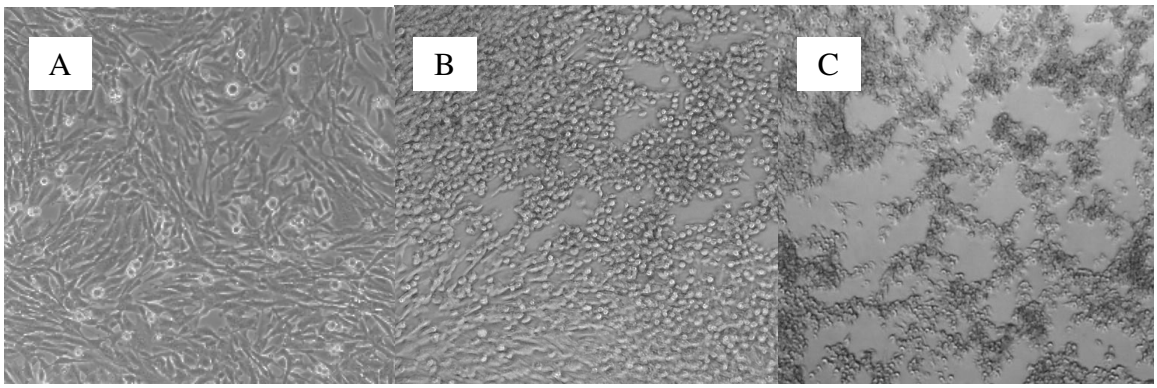

Figure 3. Infection of BHK 21 cells with vesicular stomatitis virus (VSV). a) BHK 21 cells before VSV was added to growth media. b) BHK 21 cells 18 hours after infection with VSV and c) 48 hours after infection with VSV. Cells were viewed with 100X magnification.

1.1.2b Titration

The virus was titrated using a semi-quantitative infectivity assay. BHK 21 cells were grown in a 96 well tissue culture plate for two days. One vial of VSV was quickly thawed, and 100 µl added to 900 µl RPMI. From this, 100 µl were taken and added to another 900 µl RPMI. This process was repeated 11 times, for a series of VSV dilutions ranging from 10^{-1} to 10^{-11}. The supernatant from the 96 well tissue culture plate containing the confluent BHK 21 cells was replaced with 100 µl of virus and 100 µl of RPMI supplemented with 5% FCS and 100 U-µg penicillin-streptomycin. The plate was

organized so that each column contained a different dilution of VSV. The last column did not receive virus (see Figure 4). The plate was stored at 37˚C with 5% CO_2, and examined after 24 and 48 hours. Cytopathic effect was used to find the tissue culture infective doses at 50% ($TCID_{50}$). Exactly half of the wells (four of eight) in column nine showed cell death, meaning that a dilution of 10^{-9} of the virus yielded 50% cell death per 100µl. Therefore, the titer of VSV was found to be 10^{10}/ml. The column that did not receive virus (column 12) did not have any cell death. Dilutions of virus below 10^{-7} resulted in 100% cell death. Virus titration was repeated whenever a vial was thawed, as repeated cycles of freeze/thaw may result in a lowered titer. In general, each aliquot of VSV could be thawed at least five times before it lost infectivity.

	1	2	3	4	5	6	7	8	9	10	11	12
A	10^{-1} VSV	10^{-2} VSV	10^{-3} VSV	10^{-4} VSV	10^{-5} VSV	10^{-6} VSV	10^{-7} VSV	10^{-8} VSV	10^{-9} VSV	10^{-10} VSV	10^{-11} VSV	No VSV
B												
C												
D												
E												
F												
G												
H												

Figure 4. Diagram illustrating organization of semi-quantitative viral titration. The diagram above represents a 96 well tissue culture plate with confluent BHK cells. Each well of column 1 received 100 µl of VSV diluted 1:10 with RPMI, each well of column 2 received 100 µl of VSV diluted 1:100 with RPMI, etc, up to column 11, which received 100 µl of VSV diluted $1:10^{11}$. Column 12 did not receive any VSV. All wells also received 100 µl 5% FCS-RPMI with 100 U-µg penicillin-streptomycin.

1.1.2c Inactivation

Vesicular stomatitis virus was inactivated using ultraviolet (UV) light. To optimize this inactivation, 1 ml aliquots of VSV (titer: 10^{10}/ml) were thawed and exposed to various sources of UV light for different lengths of time. After treatment, a portion of the sample was tested in a semi-quantitative titration assay using confluent BHK 21 cells, as described in detail in the section above. The virus was considered inactivated once it no longer infected BHK 21 cells at a 1:10 dilution. A handheld UV light (Mineralight) of 18W with wavelengths of 254 nm and 365 nm lowered the titer of the virus from 10^{10}/ml to 10^{6}/ml. The same result was obtained with a UV transilluminator from Fotodyne, also of 18W with a wavelength of 300 nm. The 30W UV bulb with a wavelength of 254 nm located in the Labconco Class II Biosafety cabinet was found to lower the virus titer to 10^{4}/ml after a 90 second exposure at a distance of approximately 15 cm. Increasing the exposure time to a total of six minutes using this UV light source inactivated the virus, so that it no longer killed BHK 21 cells at a 1:10 dilution. Because the virus was merely to be inactivated and not killed, the six minute treatment was broken into three segments of two minutes with thirty seconds of cooling away from the light. This prevented overheating the virus. Viral inactivation was confirmed by titration with BHK 21 cells as described above. One milliliter aliquots of inactivated virus were stored at -80°C.

1.1.3 Infection of mice

The animal experiments described here meet the Institutional Animal Care and Use Committee guidelines and have been approved (March 28, 2005). The mice used in these

experiments are of the C57BL/6 strain, except for one experiment which also used BALB/c. All experiments used male mice between eight to twelve weeks of age housed in the animal facility at Northern Michigan University. An aliquot of VSV was quickly thawed and diluted with sterile RPMI to a titer of 2 x 10^6 $TCID_{50}$ /ml. The titer was confirmed by a semi-quantitative assay (see *section 1.1.2b*). Mice were restrained, and injected with 0.5 ml of the diluted VSV through the intraperitoneal route, using a 3 ml syringe and a 27.5 gauge needle.

1.1.4 Optimization of serum neutralization assay

1.1.4a Serum sample collection

A mouse was securely held and a 5 mm GoldenRod Lancet (MEDIpoint, NY) used to puncture the sub-mandibular vein. This allowed the collection of approximately 200 µl of blood in a microcentrifuge tube before the blood flow stopped naturally. The blood samples were then incubated at 37˚C before centrifugation to separate the serum component from the blood. Several incubation times and centrifuge speeds were tested to find the optimal method of obtaining serum from the blood samples. Incubations of 20, 25, and 30 minutes were tested. In two experiments, this was followed by a 10 minute incubation at 4˚C. Different centrifugation speeds, ranging from 300 to 800 g (Eppendorf centrifuge 5415D), were used; in one test the centrifugation was repeated after removing the serum separated during the first spin. Some of these methods caused lysed red blood cells to leech into the serum component. The method that yielded the clearest serum separation was an incubation of the blood samples at 37˚C for 20 minutes followed by

4˚C for 10 minutes. These samples were then centrifuged for five minutes at 400g and the serum component removed with a pipette. The serum was stored at -20˚C.

1.1.4b *Neutralization assay*

Baby Hamster Kidney 21 cells were grown in a 96 well tissue culture plate for two days. Serum collected from a VSV-infected mouse (see *sections 1.1.3* and *1.1.4a*, above) was pre-diluted 1:20, 1:30, or 1:40 with 5% FCS-RPMI and de-complemented by placing in a 56° C water bath for 30 minutes. The inactivated serum was then further diluted with RPMI supplemented with 5% FCS and 100 U-µg penicillin-streptomycin in serial 1:2 dilutions. The supernatant in the 96 well plate of BHK 21 cells was aspirated, and each well received 100 µl of diluted serum. This was incubated for 60 minutes (in one experiment) or 90 minutes (in a second experiment) at 37° C with 5% CO_2. The serum was then removed, and replaced with 5% FCS and 100 U-µg penicillin-streptomycin. Health of the cells was assessed using 100X magnification after 24 and 48 hours. Serum diluted 1:20 killed the cells, while 1:30 and 1:40 dilutions of sera were non-toxic.

In the next set of optimization experiments, serum from a VSV-infected mouse (collected 21 days after infection) was diluted 1:30 and 1:40 with 5% FCS and 100 U-µg penicillin-streptomycin, and de-complemented for 30 minutes in a 56° C water bath. An aliquot of VSV was thawed and diluted with RPMI to 10^7 $TCID_{50}$ /ml, and 50 µl was pippetted into each well of the first ten columns of a sterile 96 well plate. The de-complemented serum was then further diluted in serial 1:2 dilutions (250 µl in 250 µl), and added in 50 µl increments to nine of the ten columns in the plate containing VSV, plus one additional

column without VSV. The plate was organized so that each column received a different dilution of serum, except for the last column, which remained empty. The serum and VSV were co-incubated for 90 minutes at 37° C with 5% CO_2. After 90 minutes, the media from a 96 well plate of BHK 21 cells was replaced with the serum-VSV mixture and left at 37° C with 5% CO_2 for 60 minutes. The serum-VSV mixture was again aspirated from the BHK 21 cells and replaced with 5% FCS in RPMI with 100 U-µg penicillin-streptomycin. Cells were viewed after 24 and 48 hours (Figure 5), and protection from cytopathic effect was used to titrate the neutralizing antibodies in the serum. Final serum dilutions less than 1:320 were not found to provide protection against infection by VSV.

This experiment was repeated as detailed above, except that VSV was diluted with RPMI to 10^4 $TCID_{50}$ /ml, and sera was initially diluted 1:40 before inactivation. The organization for this assay is illustrated in Figure 6. Each 96 well plate of BHK21 cells was large enough for two serum neutralization assays. Generally, sera taken from two experimental animals on the same day were compared on the same plate; each sample was placed in four wells of each column. Neutralizing titers were calculated as the highest dilution of serum that protected 50% of the cells from death, as evaluated with 40X magnification.

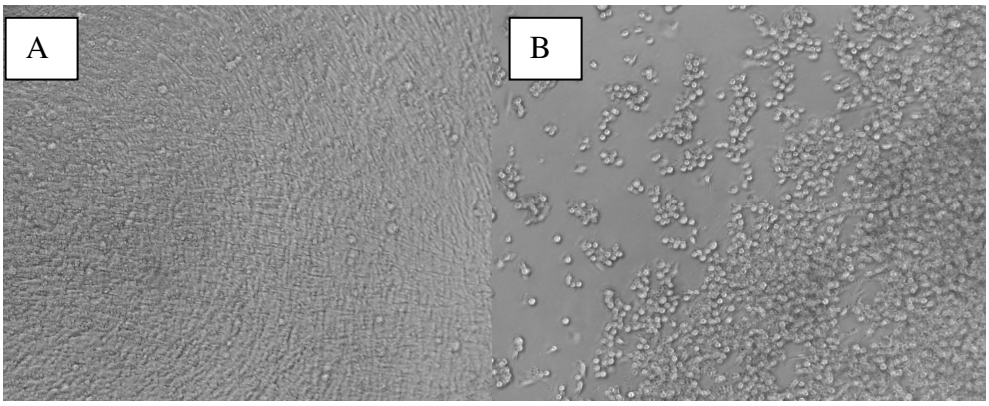

Figure 5. Protection of BHK 21 cells from VSV infection by neutralizing antibodies in a serum neutralization assay. a) BHK 21 cells are protected from VSV infection by neutralizing antibodies found in the sera of infected mice. b) BHK 21 cells infected by VSV; the concentration of antibodies added to the well was not enough to prevent infection. Cells were viewed 48 hours after assay with 40 X magnification.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1:40 sera 1 with 10^4 VSV	1:80 sera 1 with 10^4 VSV	1:160 sera 1 with 10^4 VSV	1:320 sera 1 with 10^4 VSV	1:640 sera 1 with 10^4 VSV	1:1280 sera 1 with 10^4 VSV	1:2560 sera 1 with 10^4 VSV	1:5120 sera 1 with 10^4 VSV	1:10240 sera 1 with 10^4 VSV	10^4 VSV	1:40 Sera 1	No sera or VSV
B												
C												
D												
E	1:40 sera 2 with 10^4 VSV	1:80 sera 2 with 10^4 VSV	1:160 sera 2 with 10^4 VSV	1:320 sera 2 with 10^4 VSV	1:640 sera 2 with 10^4 VSV	1:1280 sera 2 with 10^4 VSV	1:2560 sera 2 with 10^4 VSV	1:5120 sera 2 with 10^4 VSV	1:10240 sera 2 with 10^4 VSV		1:40 Sera 2	
F												
G												
H												

Figure 6. Diagram illustrating organization of sera-neutralization assay. The diagram above represents a 96 well tissue culture plate with confluent BHK cells. Each well of columns 1-10 received 50 µl of VSV diluted to 10^4 with RPMI. Additionally, each well of column 1 received 50 µl of sera diluted 1:40 with 5%FCS-RPMI; each well of column 2 received 50 µl of sera diluted 1:80 with 5%FCS-RPMI, etc, up to column 9, which received 50 µl of sera diluted 1:10240 with 5%FCS-RPMI. Column 10 received VSV only, and column 11 received sera diluted 1:40 only. Column 12 did not receive any VSV or sera. All wells also received 100 µl 5%FCS-RPMI with 100 U-µg penicillin-streptomycin.

1.1.5 Natural anti-VSV antibodies

To test for the possibility that the mice used in these experiments produce antibodies that neutralize VSV prior to exposure ('natural antibodies'), serum from a control mouse was

tested using a serum neutralization assay. A monolayer of BHK 21 cells was grown in a 96 well tissue culture plate. Serum drawn from a non-infected animal was diluted 1:40 with 5% FCS and 100 U-µg penicillin-streptomycin, and de-complemented for 30 minutes in a 56° C water bath. Vesicular stomatitis virus diluted with RPMI to 10^4 $TCID_{50}$ /ml was pippetted in 50 µl increments into each well of ten columns in a sterile 96 well plate. The de-complemented serum was then further diluted in serial 1:2 dilutions (250 µl in 250 µl), and 50 µl was added to nine of the ten columns in the plate containing VSV, plus one additional column without VSV. The plate was organized so that each column received a different dilution of serum, except for the last column, which remained empty. The serum and VSV were co-incubated for 90 minutes at 37° C with 5% CO_2, and then added to the 96 well plate of BHK 21 cells. After 60 minutes, the serum-VSV mixture was replaced with 5% FCS in RPMI with 100 U-µg penicillin-streptomycin. Cells were viewed after 24 and 48 hours, and protection from cytopathic effect was used to determine if the mice produced natural protective anti-VSV antibodies. All wells that received virus were killed, indicating that these mice do not produce anti-VSV antibodies prior to infection.

1.1.6 Optimization of ELISA to detect anti-OVA IgG

1.1.6a Vaccination of mice

Vaccines of 0.2 µg /ml, 10 µg /ml, and 40 µg /ml egg albumin (OVA; Acros Organics) in sterile RPMI were prepared. Six mice were restrained and injected with 0.5 ml of one of the three vaccines by the intraperitoneal route; two animals for each different concentration. A 3 ml syringe and a 21.5 gauge needle were used for the injections.

1.1.6b Blood sample collection

Once each week for three weeks after immunization, the mice were held securely and a heparanized capillary tube used to obtain approximately 400 µl blood retro-orbitally. Alternate eyes were used to access the retro-orbital vein each week of collection. The blood was transferred to microcentrifuge tubes, and then kept at 37˚C for 20 minutes, followed by 4˚C for 10 minutes. The samples were then centrifuged for five minutes at 400g and the serum component saved and stored at -20˚C.

1.1.6c Optimization of amount and type of reagents for anti-OVA ELISA

Immulon 2HB plates (Thermo Scientific/Fisher) were used for all enzyme-linked immunosorbent assays (ELISA) described in these experiments. Concentrations of 1, 5, 10, 20, and 50 µg/ml OVA in 0.1M Carbonate buffer were made, and 50 µl added to different columns of an ELISA plate (see Figure 7). The plate was covered and stored overnight at 4˚C. The plate was next washed three times using 100 µl 1X phosphate buffered solution with 0.05% Tween-20 (PBST) in each well, and then blocked for 70 minutes at 30˚C with a non-specific binding protein source. One experiment used 200 µl of 0.5% nutrient gelatin (DIFCO) in 1X PBST to block, and another used 1% milk in 1X PBST. The plate was washed again three times with 200 µl 1X PBST. Serum taken from a mouse immunized two weeks earlier with 20 µg OVA, and serum from a non-immunized mouse were diluted 1:25 and 1:50 with 1X PBST. Fifty microliters of these samples were then added to each well in different rows on the ELISA plate. The plate was incubated for 90 minutes at room temperature, and then washed three times with

200 µl 1X PBST. Lyophilized peroxidase-labeled anti-mouse IgG antibody (KPL) was rehydrated in sterility with a 50% water 50% glycerol solution. An aliquot of this was further diluted 1:1000 with 1X PBST, and 50 µl added to all wells of the ELISA plate. After 45 minutes at room temperature, the plate was washed three times with 100 µl 1X PBST and twice with 100 µl 1X PBS. The substrate was made by dissolving 2,2'-azino-bis (3-ethyl benzthiazoline-6-sulfonic acid) (ABTS; Pierce) in 100 mM phosphate citrate and 10 µl 30% hydrogen peroxide. Fifty microliters were added to each well and incubated at 30˚C for about five minutes. Absorbance was measured at 405 nm with Spectramax Plus spectrophotometer (Molecular Devices), and analyzed using Softmax PRO (version 3.1.1, 1999).

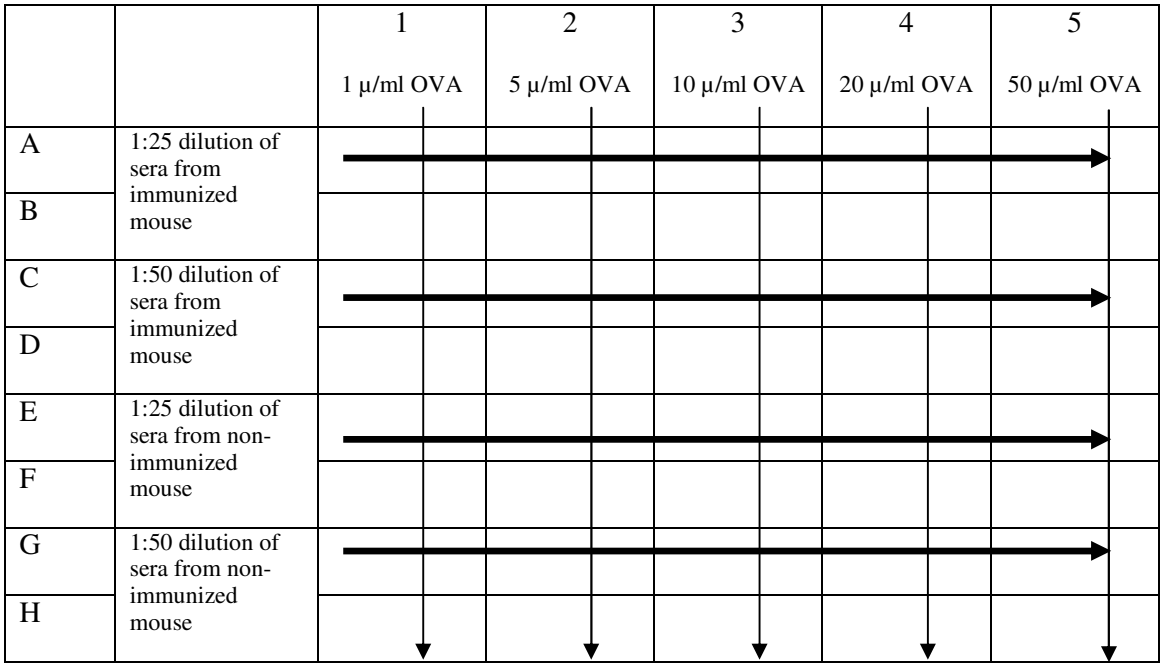

Figure 7. Diagram illustrating organization of ELISA optimization. Each column had 50 µl of the indicated concentration of OVA bound to each well. Rows A and B of all five columns were incubated with 50 µl sera from a mouse immunized with 20 µg OVA diluted 1:25 with 1X PBST. Sera was diluted 1:50 and added to rows C and D. Serum taken from a non-immunized mouse was diluted 1:25 and 1:50 and added to rows E and F, and G and H. The ELISA plate was blocked with 0.5% nutrient gelatin in 1X PBST, the secondary antibody was peroxidase-labeled anti-mouse IgG antibody, and ABTS was used as the substrate.

1.1.6d Optimization of amount of OVA to be given to mice

Mice were immunized with various concentrations of OVA as described above in section *1.1.6a Vaccination of mice*. Serum samples were obtained from these animals after two weeks, as described above in section *1.1.6b Blood sample collection.* Serum was also collected from a non-immunized mouse. An ELISA was then performed to find the minimum amount of OVA needed to reliably induce detectable antibody production against OVA in these mice. Immulon 2HB plates were coated overnight at 4˚C with 50 µl of OVA in 0.1M Carbonate buffer at a concentration of 20 µg/ml. After washing three times, the plate was blocked with 200 µl 0.5% nutrient gelatin in 1X PBST for 70 minutes at 30°C. The plate was washed three times with 200 µl/well 1XPBST. Serum samples were diluted 1:25 with 1X PBST, applied in duplicates to the plate (see Figure 8), and incubated for 90 minutes at room temperature. After washing, peroxidase-labeled anti-mouse IgG antibody diluted 1:1000 was added for 45 minutes, followed by washing and a 10 minute incubation with ABTS solution. Absorbance was measured at 405 nm with the Spectramax Plus spectrophotometer.

	1	2	3	4	5	6	7	8
A	Sera from non-immunized mouse		Sera from 0.1 µg immunized mouse		Sera from 5 µg immunized mouse		Sera from 20 µg immunized mouse	

Figure 8. Diagram illustrating organization of ELISA for optimization of OVA immunization. The diagram above represents the portion of an Immulon 2HB plate used. All wells received 50 µl of 20 µg/ml OVA that was bound overnight. Columns 1 and 2 were incubated with 50 µl sera from a non-immunized mouse diluted 1:25 with 1X PBST. Columns 3 and 4 were given sera from a mouse immunized with 0.1 µg OVA; columns 5 and 6 from a mouse immunized with 5 µg OVA; and columns 7 and 8 from a mouse immunized with 20 µg OVA. All sera was diluted 1:25 with 1X PBST. The plate was blocked with 0.5% nutrient gelatin in 1X PBST, the secondary antibody was peroxidase-labeled anti-mouse IgG antibody, and ABTS was used as the substrate.

1.1.7 Comparison of infection and immunization between two strains of mice

The response to infection and immunization with OVA between different strains of mice was compared. Four male, eight week old BALB/c mice and four male, eight week old C57BL/6 mice were infected and vaccinated as described above (*section 1.1.3* and *1.1.6a*; mice were given 10 µg egg albumin), and serum obtained after 7, 14, and 21 days (see *section 1.1.6b*). The sera were analyzed for neutralizing antibodies using a serum-neutralization assay (*section 1.1.4b*) and anti-OVA IgG by ELISA (*section 1.1.6c*).

1.2 Results

The protocols for BHK 21 cell culture, vesicular stomatitis virus titration and inactivation, serum neutralization assays, ELISA, the optimal dose to generate anti-OVA antibodies in mice, and serum collection were optimized. The optimized techniques described below are those used for the remainder of the research.

1.2.1 Optimization of BHK 21 cell culture

Different conditions were tested for the optimization of BHK 21 culture, as detailed in materials and methods (*section 1.1.1*). The optimal conditions for BHK 21 cell growth are described below; these techniques were used for the remainder of the research. The cells were grown at 37˚C with 5% CO_2 with RPMI supplemented with 5% FCS and 100 U-µg penicilin-streptomycin. Every three days, the cells were passed 1:3, using 0.25% trypsin to detach the cells from the tissue culture flasks. When needed, 96 well tissue culture plates were prepared by adding 200 µl of BHK 21 cells diluted 1:60 with RPMI supplemented with 5% FCS and 100 U-µg penicilin-streptomycin.

1.2.2 Production and titration of vesicular stomatitis virus

Vesicular stomatitis virus (VSV) serotype Indiana was propagated with BHK21 cells and clarified by centrifugation, as described in materials and methods (*section 1.1.2*). The virus was stored in 1 ml aliquots at -80°C; 20 ml were produced in total. The virus was titrated using a semi-quantitative infectivity assay. For this, serial 1:10 dilutions of virus were added to confluent BHK 21 cells in a 96 well plate and stored at 37˚C with 5% CO_2. Cytopathic effect was used to find the tissue culture infective doses at 50% ($TCID_{50}$). Fifty-percent of the wells in the column that received VSV at a 10^{-9} dilution were dead (Figure 9); therefore, the virus was titrated as 10^{10} $TCID_{50}$/ml.

	1	2	3	4	5	6	7	8	9	10	11	12
A	X	X	X	X	X	X	X	X	X	O	O	O
B	X	X	X	X	X	X	X	X	X	O	O	O
C	X	X	X	X	X	X	X	O	O	O	O	O
D	X	X	X	X	X	X	X	X	X	X	O	O
E	X	X	X	X	X	X	X	O	O	O	O	O
F	X	X	X	X	X	X	X	X	X	O	O	O
G	X	X	X	X	X	X	X	X	O	O	O	O
H	X	X	X	X	X	X	X	O	O	O	O	O

Figure 9. Diagram illustrating results of semi-quantitative viral titration. The diagram above represents a 96 well tissue culture plate with confluent BHK cells. Each well of column 1 received 100 µl of VSV diluted 1:10 with RPMI, each well of column 2 received 100 µl of VSV diluted 1:100 with RPMI, etc, up to column 11, which received 100 µl of VSV diluted $1:10^{11}$. Column 12 did not receive any VSV. All wells also received 100 µl 5%FCS-RPMI with 100 U-µg penicillin-streptomycin. An 'X' indicates the cells in that well had died 48 hours after VSV treatment; and 'O' indicates the cells were alive after 48 hours.

1.2.3 Inactivation of VSV

Several methods to inactivate VSV were tried. The most successful protocol of inactivating the virus was to transfer aliquots of infectious VSV frozen at 10^{10} $TCID_{50}$/ml to a small round tissue culture dish (15mm x 60 mm). This was held without a covering under a 30W UV lamp for two minutes, then removed for one minute and gently swirled. This was repeated three times. Inactivation of the virus was confirmed by titration with BHK 21 cells. The virus was inactivated so that it was no longer infected BHK 21 cells at a 1:10 dilution.

1.2.4 Infection of mice

Male, 8 to 12 week old C57BL/6 mice were infected through the intraperitoneal route with $10^{6}TCID_{50}$ VSV. One experiment used BALB/c mice in addition to C57BL/6 mice. The dose of VSV administered did not cause paralysis or death in these mice. Infection was confirmed by the presence of neutralizing anti-VSV antibodies in infected animals, as detected in serum-neutralization assays.

1.2.5 Optimization of serum neutralization assay

The best method for separating the serum from the blood was to keep each blood sample at 37˚C for 20 minutes, then at 4˚C for 10 minutes. The samples were then centrifuged for five minutes at 400g, and the serum component saved and stored at -20˚C.

The best method for determining neutralizing anti-VSV titers in the serum of infected mice was found by trying several serum neutralization assays; the optimal procedure is described below. Serum samples were pre-diluted 1:40 and de-complemented by placing

in a 56°C water bath for 30 minutes. Sera were then further diluted with RPMI containing 5% FCS and 100 U-µg penicilin-streptomycin. Serial two-fold dilutions of serum provided a good range to detect the presence of antibodies. This was determined based on having living cells in the column that received de-complemented serum only (no virus), having dead cells in the column that received virus only, having dead cells in all the wells in columns with virus and the highest dilutions of sera (around 1:10240) and having living cells in all the wells in columns with virus and the lowest dilutions of sera (around 1:40 to 1:80). Fifty microliter serial two-fold dilutions were co-incubated with 50 µl VSV diluted to 10^4 $TCID_{50}$/ml for 90 minutes. This dilution of VSV allowed some dilutions of the serum to protect cells, but not all dilutions. Supernatant media from a 96 well plate of confluent BHK 21 cells was replaced with the sera-VSV mixture and left at 37° C with 5% CO_2. After 60 minutes, the sera-VSV mixture was aspirated from the BHK 21 cells and replaced with 5% FCS in RPMI with 100 U-µg penicillin-streptomycin. Cells were viewed after 24 and 48 hours, and protection from cytopathic effect was used to titrate the neutralizing antibodies in the animal serum. Neutralizing titers were calculated as the highest dilution of serum that protected 50% of the cells from death, as evaluated with 100X magnification. These titers were then converted to the format conventionally used, by finding the $-\log_2$ of the dilution of serum that provided 50% protection (see *section 2.2.1*).

1.2.6 Natural neutralizing antibodies against VSV

A serum neutralization assay was used to see if the mice used in these experiments produce antibodies that neutralize VSV prior to exposure. Sera from non-infected mice

were tested. All wells that received virus showed cytopathic effect. The column that received de-complemented sera alone (no virus), and the control column (no treatment) did not have cytopathic effect. The sera did not protect the cells at any dilution after and including 1:40, indicating that the mice used in this thesis do not produce detectable neutralizing antibodies against VSV prior to exposure.

1.2.7 Optimization of ELISA to detect anti-OVA IgG

Several enzyme-linked immunosorbant assays were performed to optimize the detection of anti-OVA antibodies produced in mice. In these assays, the absorbance at 405 nm corresponds with the relative amount of IgG antibodies present. Various amounts of OVA bound to the plate and dilutions of mouse sera were tested. The results show absorbance increases with an increase in the amount of OVA bound to the plate up to 20 µg, with no difference after 20 µg (Figure 10). For this reason, 20 µg of OVA was used in future experiments. Between the two dilutions of OVA-inoculated mouse serum tested (1:25 and 1:50), the 1:25 yielded the greatest difference when compared to serum from a control mouse (Figure 10). This dilution was used in future experiments.

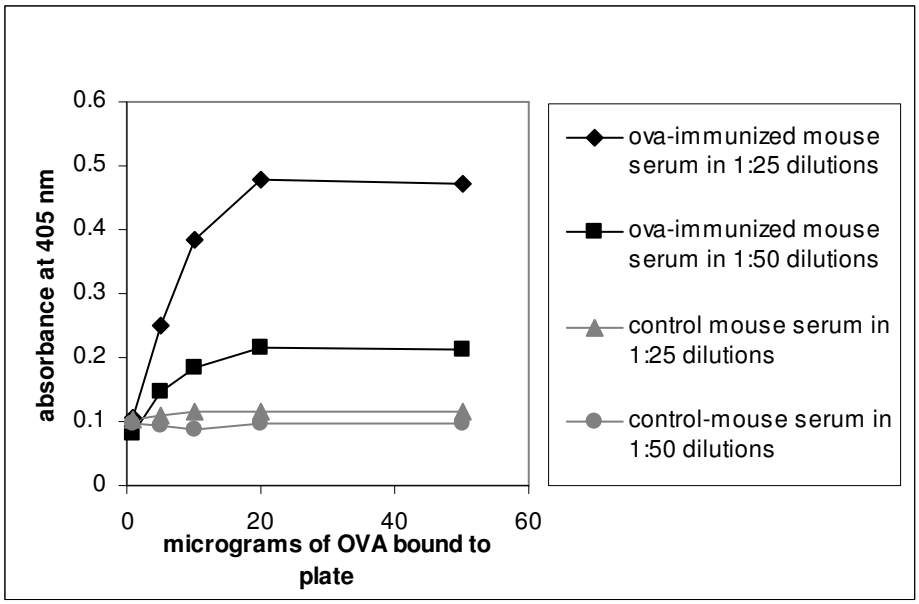

Figure 10. Optimization to detect anti-OVA antibodies in mice by ELISA. C57BL/6 mice were immunized with 10 µg OVA, and various dilutions of sera from this mouse in 0.1 M Carbonate buffer were tested with various amounts of OVA bound to a 96-well plate in an ELISA. Serum levels of anti-OVA IgG are expressed as the optical density at 405 nm. Results were compared to serum from a mouse not immunized (control-mouse, gray lines).

1.2.8 Optimization of the dose of OVA to be administered

To determine the optimal amount of OVA that should be administered for detectable antibody production, mice were injected with various amounts of OVA and bled after three weeks. An ELISA was performed to measure anti-OVA antibody production. No significant difference in absorbance was found between control mice and mice given 0.1 µg and 5 µg (Figure 11). Sera from mice given 20 µg yielded high absorbance values, indicating the presence of IgG antibodies in these sera. Ten micrograms were given to mice in future experiments.

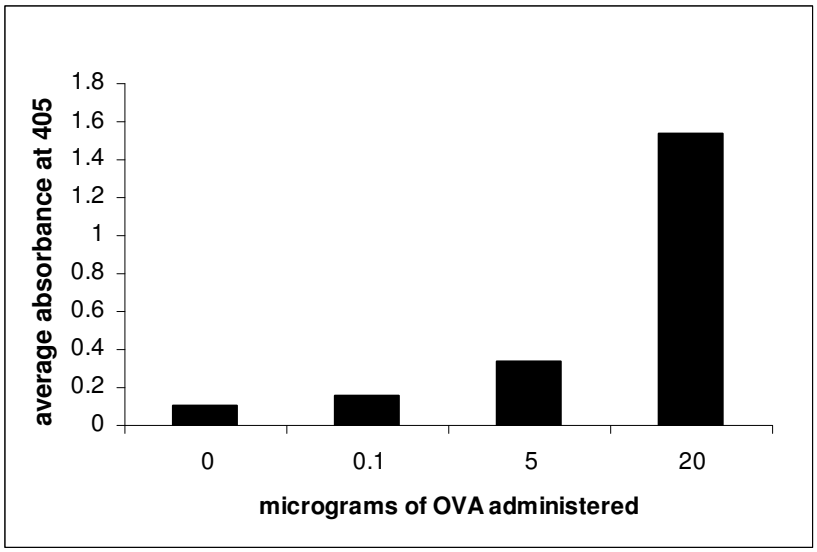

Figure 11. Optimization of anti-OVA antibody production in mice. C57BL/6 mice were immunized with the amounts of OVA indicated, and the serum levels of anti-OVA IgG after fourteen days were determined by ELISA.

1.2.9 Comparison of the immune response to infection with VSV between two strains of mice

Vesicular stomatitis virus-infected BALB/c and C57BL/6 produced similar neutralizing titers, although the standard deviation among the C57BL/6 strain was greater than that among the BALB/c strain. Four infected BALB/c mice produced an average of 12.176 (+/- .293) while four infected C57BL/6 mice produced an average of 12.314 (+/- .490) on day 21 (Figure 12). By convention, neutralizing titers are expressed as $-\log_2$ (dilution of serum that protects 50% of cells).

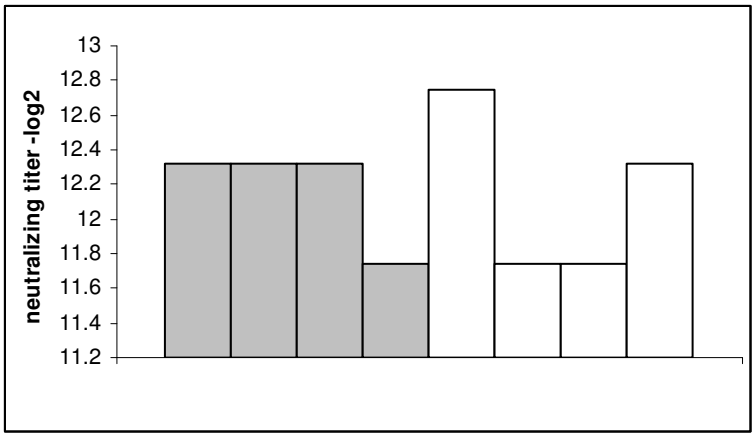

Figure 12. Neutralizing titers of sera from BALB/c and C57BL/6 mice infected with VSV 21 days earlier. Each column represents one mouse; the first four (gray) are BALB/c mice and the second four (white) are C57BL/6 mice.

1.2.10 Comparison of the immune response to immunization with OVA between two strains of mice

For three weeks following immunization, BALB/c mice produced similar anti-OVA IgG titers to the titers seen with C57BL/6 mice (Figure 13). Again the C57BL/6 mice had greater standard deviation in antibody titers (+/-0.128 on day 7 to +/- 0.131 on day 21) compared to BALB/c mice (+/-0.059 on day 7 to +/-0.063 on day 21).

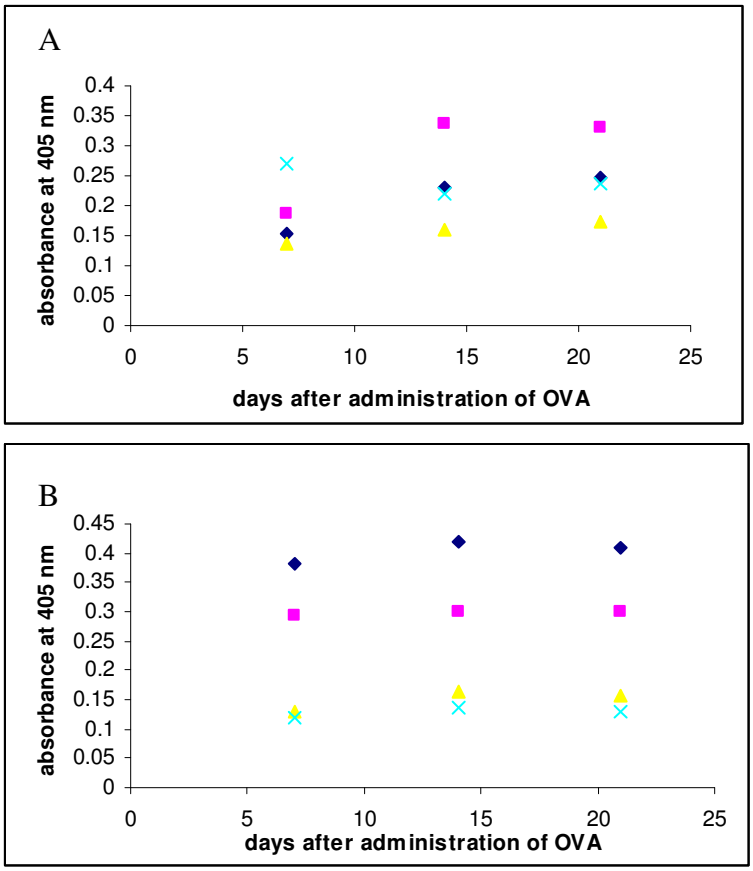

Figure 13. Anti-OVA IgGs produced in immunized BALB/c and C57BL/6 mice. Four BALB/c (a) and four C57BL/6 (b) mice were injected with 10 µg OVA, and serum samples collected after seven, 14, and 21 days. An ELISA was used to detect anti-OVA IgG in the sera, and is represented by absorbance at 405 nm. Each symbol represents one animal.

The difference in anti-OVA response between the two strains of mice was also similar after VSV-infection. Four BALB/c and four C57BL/6 animals were infected with VSV and immunized with OVA one day later. The anti-OVA IgGs produced in these mice after 7, 14, and 21 days was measured by ELISA. Although there is a greater standard deviation in the C57BL/6 mice (+/- 0.507 on day 21, compared to +/- 0.288 in the BALB/c mice on day 21), this strain of mice gave results similar to those obtained with BALB/c (Figure 14). The C57BL/6 strain was used for the experiments described in this work as there were more of these mice available at the time of this research.

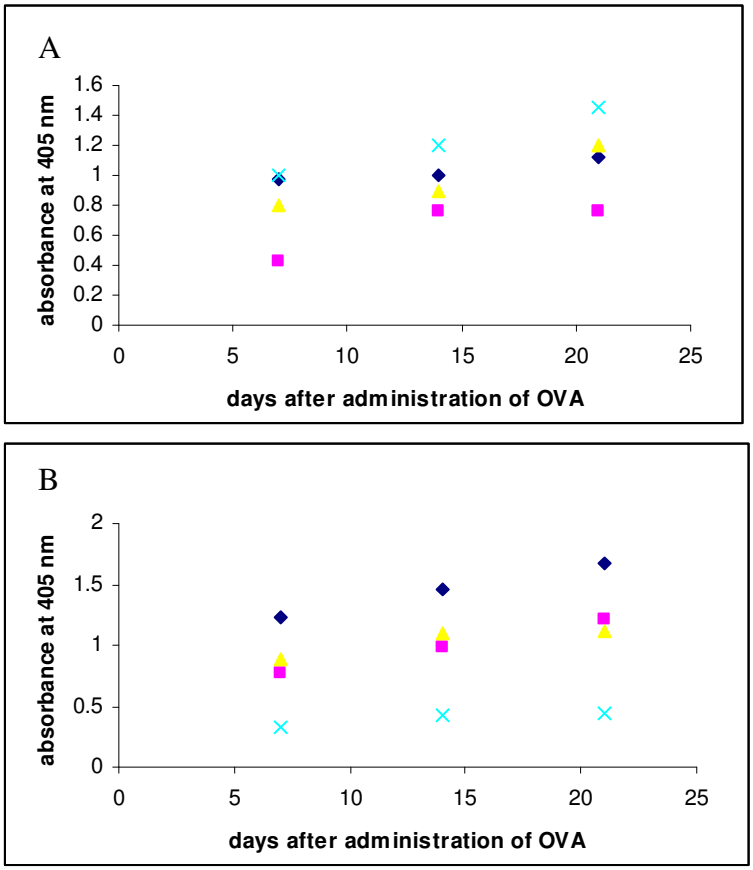

Figure 14. Anti-OVA IgG response in VSV infected BALB/c and C57BL/6 mice immunized with OVA. Four BALB/c (a) and four C57BL/6 (b) mice were infected with 10^6 $TCID_{50}$ doses VSV and injected with 10 µg OVA the next day. Anti-OVA IgG were detected in serum from these mice by ELISA, and is represented by absorbance at 405 nm. Each symbol represents one animal.

Based on the results of these optimizations, the remaining experiments used C57BL/6 mice injected with 10 µg OVA by the intraperitoneal route, 1:25 diluted sera and 20 µg OVA in the ELISA, and 10^4 $TCID_{50}$ VSV in the serum neutralization assays.

CHAPTER TWO: KINETICS OF ANTI-VSV AND ANTI-OVA PRODUCTION IN MICE

2.1 Materials/Methods

The methods used here were first optimized, as described in chapter one.

2.1.1 Kinetics of neutralizing anti-VSV antibodies

2.1.1a Infection of mice with VSV

Four male C57BL/6 mice were infected with10^6 50% tissue culture infectious doses ($TCID_{50}$) of VSV by intraperitoneal injection of 0.5 ml of $2x10^6$ $TCID_{50}$/ml VSV in sterile RPMI. The virus was back-titrated on a plate of confluent BHK 21 cells to confirm titer. A 3 ml syringe and a 21.5 gauge needle were used for the injections. This experiment was conducted twice. Each animal was housed in a separate cage.

2.1.1b Immunization of mice with inactivated VSV

Four male C57BL/6 mice were immunized with 2.5 x 10^9 $TCID_{50}$ of UV-inactivated VSV by intraperitoneal injection of 0.5 ml of 5 x 10^9 $TCID_{50}$/ml UV-VSV in sterile RPMI. The virus was back-titrated on a plate of confluent BHK 21 cells to confirm inactivation. A 3 ml syringe and a 21.5 gauge needle were used for the injections. This experiment was conducted twice. Each animal was housed in a separate cage.

2.1.1c Collection of serum samples

Serum samples were collected after 3, 5, 7, 14, 21, 28, 35, and 60 days. A mouse was restrained and a 5 mm GoldenRod lancet (MEDIpoint, NY) used to obtain approximately 200 µl blood from the sub-mandibular vein. Blood samples were kept at 37˚C for

20 minutes, then at 4˚C for 10 minutes. The samples were then centrifuged for five minutes at 400g (Eppendorf centrifuge 5415D), and the serum component saved and stored at -20˚C.

2.1.1d Serum neutralization assay

Serum-neutralization assays were used to calculate neutralizing antibody titers. Sera were prediluted 40-fold in 5% FCS-RPMI 1640 medium, and incubated for 30 minutes at 56˚C to decomplement the sera. Serial two-fold dilutions of this solution were then incubated in a 96-well plate for 90 minutes at 37˚C with 10^4 $TCID_{50}$/ml VSV. The serum-VSV mixture was then transferred onto confluent BHK 21 cells in a 96 well plate, and incubated for one hour at 37˚C with 5% CO_2. The mixture was then aspirated and replaced with fresh 5% FCS-RPMI 1640 medium. After 48 hours of incubation at 37˚C with 5% CO_2, cytopathic effect was evaluated visually using 40X magnification and serum neutralization titers were calculated.

2.1.2 Kinetics of anti-OVA antibodies

2.1.2a Immunization of mice with OVA

Four C57BL/6 mice were injected by the intraperitoneal route with 0.5 ml of a vaccine of 20 µg /ml egg albumin (OVA) in sterile RPMI (final concentration of 10 µg OVA). A 3 ml syringe and a 21.5 gauge needle were used for the injections. Each animal was housed in a separate cage. This experiment was conducted four times, using four animals in each except for one experiment, which used three mice.

2.1.2b Collection of serum samples

Blood samples were collected after 7, 14, and 21 days through the retro-orbital venous plexus using a heparanized capillary tube. The blood samples were kept at 37˚C for 20 minutes, then at 4˚C for 10 minutes. The samples were then centrifuged for five minutes at 400g (Eppendorf centrifuge 5415D), and the serum component saved and stored at -20˚C.

2.1.2c Analysis of sera by ELISA

Immulon 2HB plates were coated with 50 µl of OVA in 0.1M Carbonate buffer at a concentration of 20 µg/ml, and stored overnight at 4˚C. Sera samples were diluted 1:25 with 1X PBST, applied in duplicates, and incubated for 90 minutes at room temperature. Peroxidase-labeled anti-mouse IgG antibodies (KPL) in a 1:1000 dilution with 1X PBST were added for 45 minutes at room temperature. After each step, the wells in the plate were washed three times with 200µl 1X PBST, and twice with 1X PBS before the addition of the ABTS substrate. Absorbance was measured at 405 nm. Each ELISA included internal controls. Serum from a non-immunized mouse was used as a negative control, and serum from a mouse immunized with 20 µg OVA was used as a positive control. Analysis by ELISA was performed at the end of each repetition of this experiment, and also once using all of the sera collected from each experiment.

2.1.3 Kinetics of anti-OVA antibodies during VSV infection

2.1.3a Infection and vaccination of mice

Four male eight to twelve week old C57BL/6 mice were infected with10^6 50% tissue culture infectious doses ($TCID_{50}$) of VSV by intraperitoneal injection of 0.5 ml of $2x10^6$ $TCID_{50}$/ml VSV in RPMI. One day later, these four mice were injected intraperitoneally with 0.5 ml of 20 µg/ml OVA in RPMI (final concentration of 10 µg OVA). This experiment was repeated three times, once using three mice. Each animal was housed in a separate cage.

2*.1.3b Collection of serum samples*

Serum was collected 7, 14, and 21 days after OVA immunization using the method described in section *2.1.2 b 'collection of serum samples'*.

2.1.3c Confirmation of infection

To confirm that the mice were infected with VSV, serum drawn on day 21 from one VSV-infected mouse was tested for the presence of protective anti-VSV antibodies in a serum neutralization assay, as described in section *2.1.1d 'serum neutralization assay'*.

2.1.3d Analysis of sera by ELISA

Each serum sample taken over the three week period from the VSV-infected mice was tested for anti-OVA IgG by an ELISA, as described in section *2.1.2c 'Analysis of sera by ELISA'.* Analysis by ELISA was performed at the end of each repetition of this experiment, and also once using all of the sera collected from each experiment.

2.1.4 Kinetics of anti-OVA IgG after immunization with UV-VSV

2.1.4a Vaccination of mice

Four male C57BL/6 mice were immunized with 2.5 X 10^9 $TCID_{50}$ of UV-inactivated VSV by intraperitoneal injection of 0.5 ml of 5x10^9 $TCID_{50}$/ml UV-VSV in sterile RPMI. The virus was back-titrated on a plate of confluent BHK 21 cells to confirm inactivation. One day later, these four mice were injected intraperitoneally with 0.5 ml of 20 µg/ml OVA in RPMI (final concentration of 10 µg OVA). This experiment was repeated three times, once with three mice. Each animal was housed in a separate cage.

2*.1.4b Collection of serum samples*

Serum was collected 7, 14, and 21 days after OVA immunization using the method described in section *2.1.2 b 'collection of serum samples'*.

2.1.4c Confirmation of immunization with UV-VSV

To confirm that the mice were immunized with inactivated VSV, serum drawn on day 21 from one UV-VSV-immunized mouse was tested for the presence of protective anti-VSV antibodies in a serum neutralization assay, as described in section *2.1.1d 'serum neutralization assay'*.

2.1.4d Analysis of sera by ELISA

Each serum sample taken over the three week period from the mice given OVA and the mice given UV-VSV and OVA was tested for anti-OVA IgG by an ELISA, as described

in section *2.1.2c 'Analysis of sera by ELISA'*. Analysis by ELISA was performed at the end of each repetition of this experiment, and also once using all of the sera collected from each experiment.

2.1.5 Statistical analysis

To compare the effects of VSV infection or immunization on anti-OVA production in mice, an analysis of variance (ANOVA) test was used. Data were analyzed by ANOVA using SPSS version 15.0 (Statistical Software Package for Social Sciences 2006, Chicago). The absorbance at 405 nm, indicating the concentration of antibodies, was the dependent variable. The independent variable was one of three treatment groups for the mice, either injection of UV-VSV and OVA, OVA alone, or VSV and OVA. This variable was treated as a fixed factor. In addition, each of the three repetitions of the experiment were considered random, blocking effects. This controlled for any extraneous variation between the different experimental repetitions, as care was taken to keep all conditions the same. The results of the ANOVA indicate that variations present between different trials did not significantly contribute to overall variations between means. The blocks were random as each type of treatment occurred randomly within each repetition. The assumption of normality was not tested as the sample size was small, but was assumed to be met. Levene's test of equality of variance found that the data did vary with significance on days 7 ($F=4.255$, $p=0.003$), 14 ($F=2.665$, $p=0.030$), and 21 ($F=4.879$, $p=0.001$). Data from each of the three days post treatment were entered into SPSS, and then the GLM Univariate analysis module was selected. The 'Sum of squares' was set at 'Type III', as the model contained both random and fixed factors. An alpha

value of 0.05 was used, indicating that the conclusion reached will be accurate 95 times out of 100. Tukey's HSD test was chosen for the post-hoc comparison. Calculations of power and descriptive statistics were given by SPSS as well.

2.2 Results

2.2.1 Kinetics of neutralizing antibodies

The kinetics of the production of protective anti-VSV antibodies in mice infected with 10^6 $TCID_{50}$ VSV or immunized with 2.5 x 10^9 $TCID_{50}$ UV-inactivated VSV was measured in this thesis. Neutralizing titers were measured in serum neutralization assays. Both groups of mice produced antibodies able to protect BHK21 cells from infection with the virus (Figures 15, 16). The neutralizing titer is expressed as the $-\log_2$ of the sera dilution that protected 50% of the cells. The VSV-infected animals produced an average neutralizing titer that increased from 9.0719 three days post infection to 12.1343 on day 21 post infection. Neutralizing antibodies then reached a plateau and were still found in the sera on day 60 (titer of 11.3219). Animals immunized with UV-VSV did not produce as high a titer of neutralizing antibodies, except on day three, which had a higher titer (9.655 compared to 9.072 in VSV-infected animals). The peak was also reached on day 21 (8.1329) in the UV-VSV mice. No neutralizing titers were found in the blood of these animals by day 35 post-immunization. These results indicate that infection with VSV in these animals generates a long-lasting, protective antibody response. These results also show that immunization with UV-VSV also produces a protective antibody response in these mice, but that differences exist in titer and duration compared with infectious VSV.

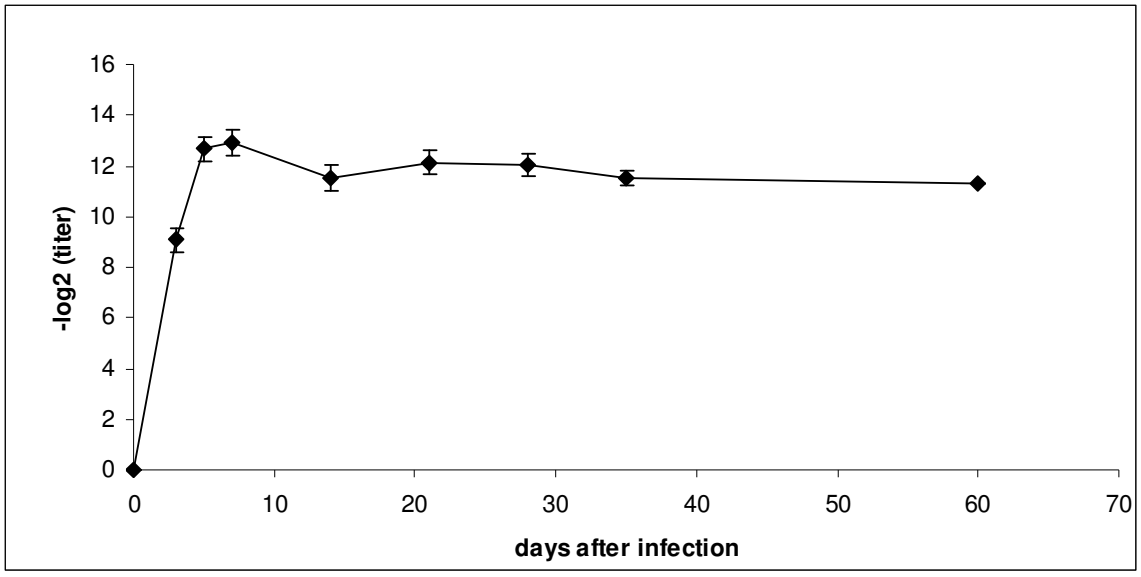

Figure 15. Kinetics of neutralizing anti-VSV production in mice infected with VSV. Four mice were infected with 10^6 $TCID_{50}$ VSV, and their serum analyzed by serum neutralization assays for the presence of neutralizing antibodies after days 3, 5, 7, 14, 21, 28, 35, and 60. Serum was also assessed for neutralizing titers before infection (day 0). Titers are expressed as the $-\log_2$ (dilution). Each point represents the average of four mice; standard deviation is indicated.

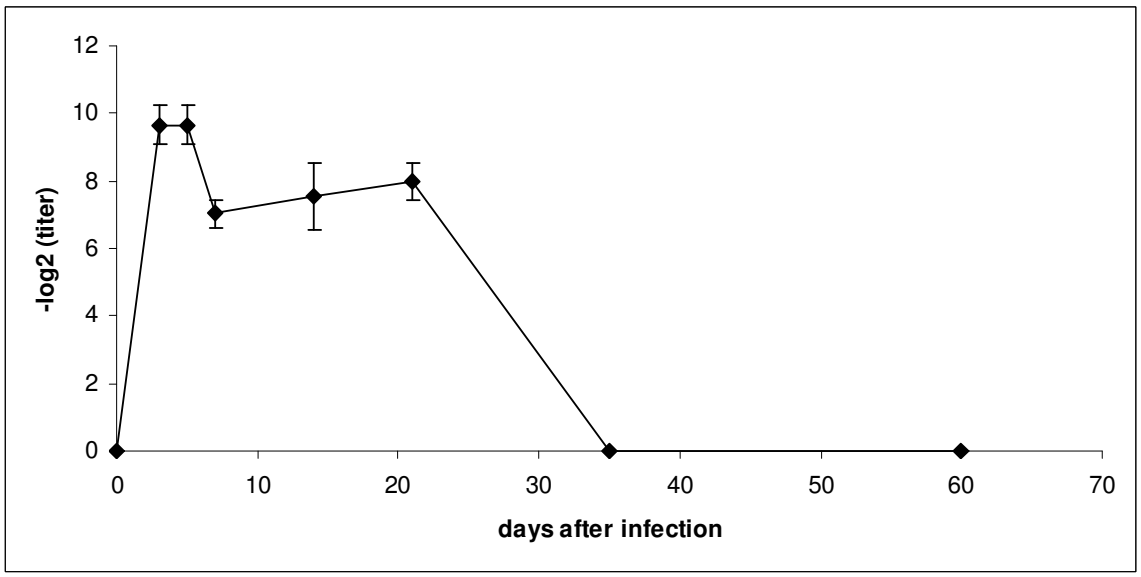

Figure 16. Kinetics of neutralizing anti-VSV production in mice immunized with UV-VSV. Four mice were immunized with $2.5x10^9$ $TCID_{50}$ UV-VSV, and their serum analyzed by serum neutralization assays for the presence of neutralizing antibodies after days 3, 5, 7, 14, 21, 35, and 60. Serum was also assessed for neutralizing titers before infection (day 0). Titers are expressed as the $-\log_2$ of dilution. Each point represents the average of four mice; standard deviation is indicated.

These results indicate that there are differences in the production of protective antibodies between infection and immunization with VSV. While both infectious and non-infectious VSV elicited rapid titers of neutralizing antibodies, only infection induced long-lasting antibodies. Higher titers were also found following infection compared to immunization.

2.2.2 Kinetics of anti-OVA antibodies

In order to compare the impact of infection on the immune response to an unrelated antigen, the kinetics of antibody production against the unrelated antigen alone were first studied. An ELISA was used to measure anti-OVA IgG production over three weeks in mice immunized with 10 μg OVA. An ELISA was performed at the end of each of three independent experiments to detect anti-OVA IgG antibodies in the sera taken from these mice. Each of the three experiments yielded similar results (Figure 17 a-c). Additionally, one ELISA was performed using all of the data collected in each experiment, so direct comparisons could be made (Figure 18 a). The results of each serum sample relative to one another remained the same when repeated. The average absorbance of each sera tested in this combined ELISA corresponded to the average amount of anti-OVA IgG produced in these mice. The antibody titer peaked on day 14 at 0.721. This was an increase from day seven (0.369), and there was a slight decline by day 21 (0.709) (Figure 18 b).

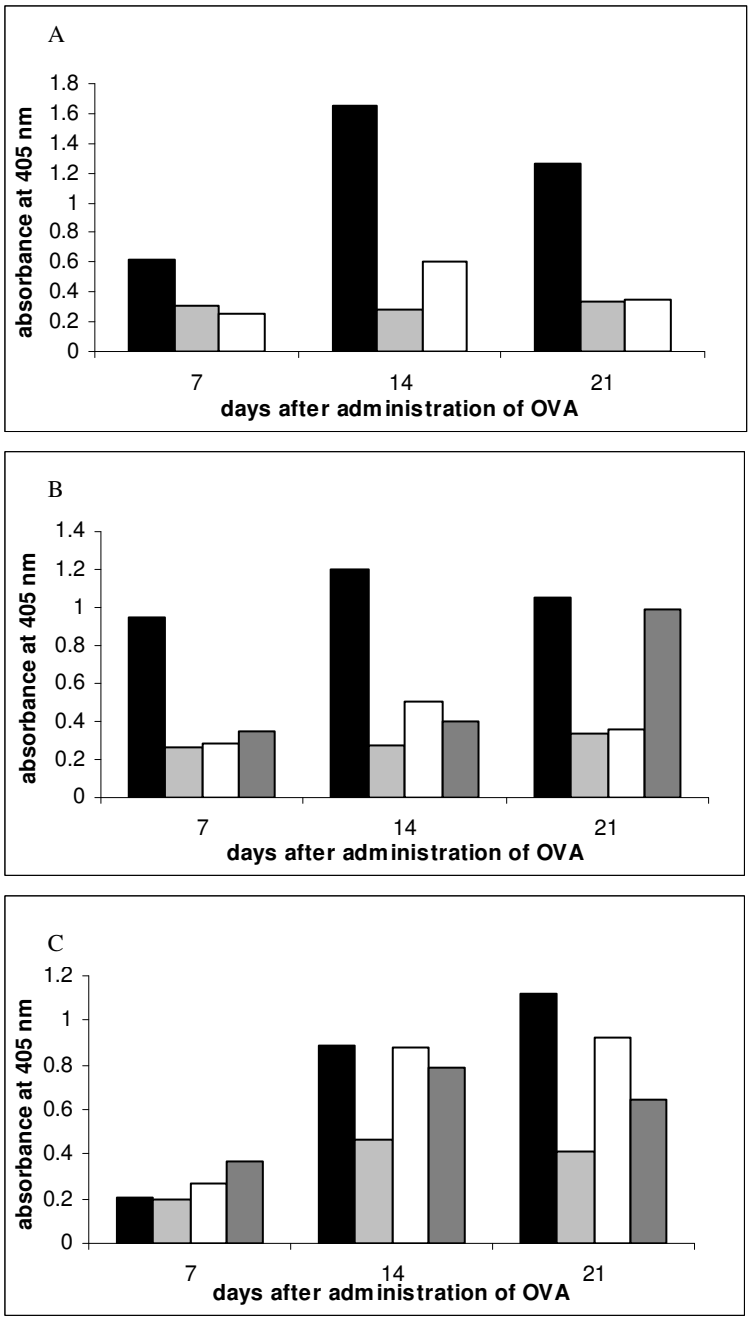

Figure 17 Anti-OVA IgGs produced in mice immunized with 10 µg OVA. Each box (a-c) shown represents one independent replication of this experiment. Serum was collected after 7, 14, and 21 days, and anti-OVA IgGs were detected by ELISA, as measured by absorbance at 405 nm. Each experiment included four male C57 mice, except experiment one (a), which had three. Each column represents one animal. In addition, each experiment included a negative control, or serum from a mouse not given OVA (data not shown). This control had an absorbance lower than any of the immunized animals.

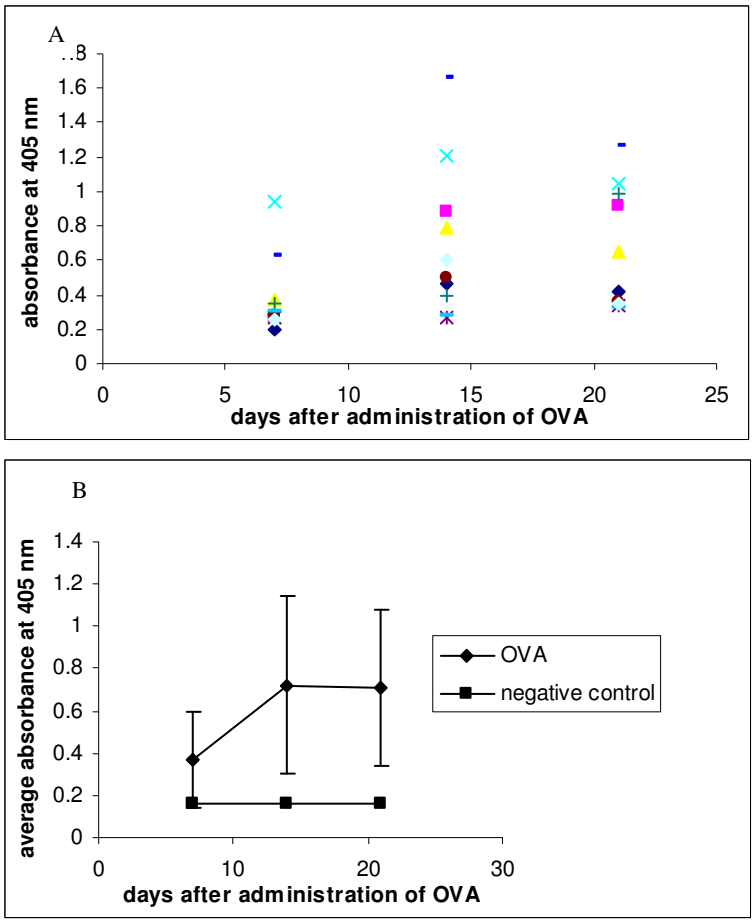

Figure 18. Summary of anti-OVA IgG production in mice immunized with 10 µg OVA. One ELISA was performed using all data collected; antibody titer is indicated by the relative absorbance at 405nm. a) Each symbol represents the measured absorbance from one serum sample from an OVA-immunized mouse. b) Absorbance at 405 nm averaged from all eleven serum samples on each day after administration of OVA, compared to serum from a non-treated mouse. Standard deviation is shown.

These results show that mice can be induced to produce antibodies against OVA following immunization with 10 µg, and that these antibodies can be detected by ELISA. Absorbance values from immunized-mouse sera are higher than background absorbance values obtained with serum from a non-immunized mouse (Figure 18b). Furthermore, the highest titer of anti-OVA IgG is not detected until two weeks following immunization. This titer decreases slightly by week three.

2.2.3 Kinetics of anti-OVA antibodies during VSV infection

These experiments investigated the ability of VSV-infected mice to mount an antibody response against an unrelated antigen during infection. To this end, mice were infected with VSV and immunized with OVA, and anti-OVA IgG detected in the mice after one, two, and three weeks. The presence of neutralizing antibodies against VSV 21 days after infection was used to confirm that the mice were infected with VSV. Earlier studies had shown that non-infected mice do not produce neutralizing antibodies as detected by serum-neutralization assays (see chapter one*, section 2.6*).

2.2.3a Confirmation of infection

To confirm that the mice were infected, serum collected on day 21 from a VSV-infected mouse from each experiment was tested for its ability to protect BHK21 cells from infection with VSV at 10^4 $TCID_{50}$/ml. All animals tested produced roughly the same titer of neutralizing antibodies against VSV, as determined by serum neutralization assays. At a dilution of sera of 1:2560 (two experiments) and 1:3840 (one experiment), half of the wells showed cell death, and half were protected by the serum. Therefore, the sera were found to contain a neutralizing antibody titer of 11.322 and 11.737, as expressed as $-\log_2$ of the sera dilution. Serum from a non-infected animal did not protect the cells at any dilution. Photographs of infected BHK 21 cells and protected BHK 21 cells are shown in Figure 5. These results indicate that the mice used in these experiments were infected with VSV.

2.2.3b Anti-OVA IgG production

An ELISA was used to measure anti-OVA antibody production over three weeks in VSV-infected mice. In three independent experiments, C57BL/6 mice were infected with 0.5 ml of 2 x 10^6 $TCID_{50}$/ml of VSV and the next day given 10 µg OVA. An ELISA was performed at the end of each of these three experiments to detect anti-OVA IgG antibodies in the sera taken from these mice. Each of the three experiments yielded similar results (Figure 19 a-c). Additionally, one ELISA was performed using all of the data collected so direct comparisons could be made across all experiments (Figure 20). The results of each serum sample relative to one another remained the same when repeated. The average absorbance of each sera tested in this combined ELISA corresponded to the average amount of anti-OVA IgG produced in these mice. The average antibody titer increased from day 7 (0.932) to day 21 (1.27) (Figure 20 b).

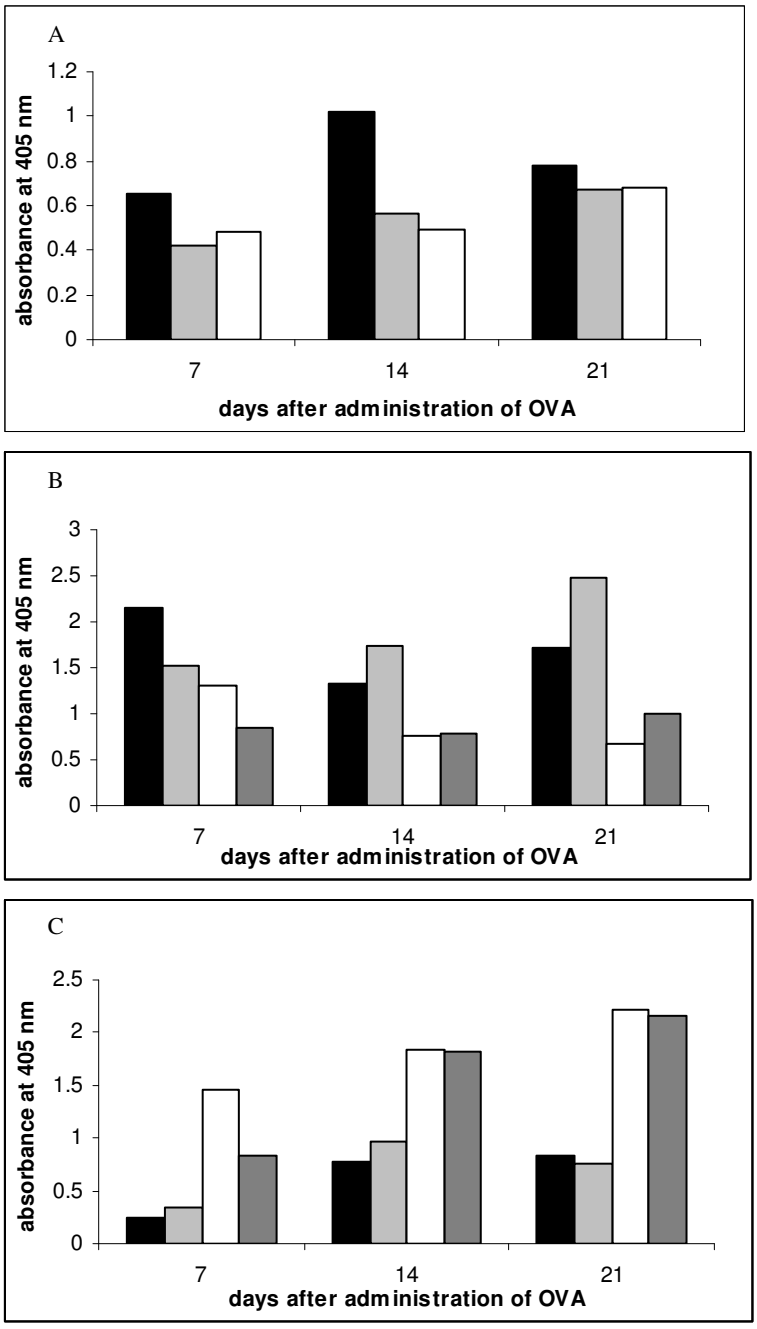

Figure 19. Anti-OVA IgGs produced in mice infected with VSV and immunized one day later with 10 µg OVA. Each box (a-c) shown represents one independent replication of this experiment. Serum was collected after 7, 14, and 21 days, and anti-OVA IgGs were detected by ELISA. Each experiment included four male C57 mice, except experiment one (a), which had three. In addition, each experiment included a negative control, or serum from a mouse not given OVA (data not shown). The absorbance of this serum was less than the absorbance of the serum from the immunized mice.

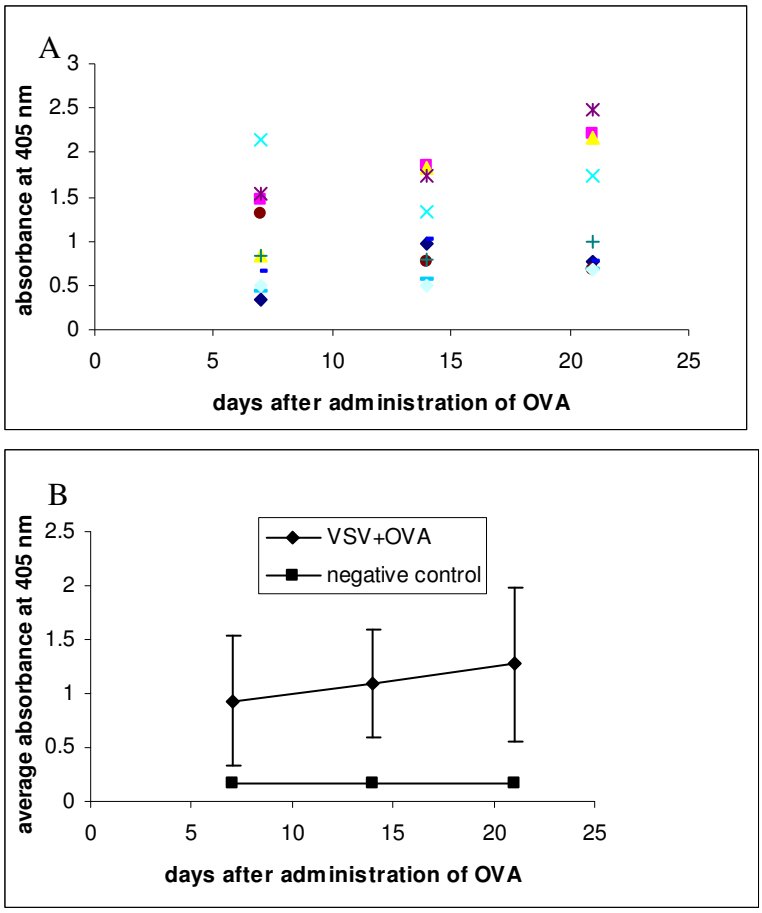

Figure 20. Summary of anti-OVA IgGs produced in VSV-infected mice immunized with 10 µg OVA. Antibodies were detected by ELISA and are indicated as absorbance at 405 nm. Samples collected from three independent experiments were analyzed by the same ELISA. a) Each symbol represents absorbance from one serum sample from one VSV-infected and OVA-immunized mouse. b) Absorbance at 405 nm averaged from all eleven serum samples on each day after administration of OVA, compared to serum from a non-treated mouse. Standard deviation is shown.

Serum neutralization assays confirmed that mice were infected with VSV, with neutralizing titers similar to those obtained in earlier results (Figure 15). These infected mice were able to mount an antibody response to OVA. The absorbance values from the immunized-mouse sera are higher than background absorbance values obtained with serum from a non-immunized mouse (Figure 20b). The IgG titer increased for three weeks following infection.

2.2.4 Kinetics of anti-OVA IgG after immunization with UV-VSV

These experiments investigated the ability of mice to mount an antibody response against an unrelated antigen in the presence of noninfectious VSV. For this purpose, mice were immunized with UV-inactivated VSV and also immunized with OVA, and anti-OVA IgG was detected in the mice after one, two, and three weeks. The presence of neutralizing antibodies against VSV 21 days after immunization was used to confirm that the mice had been exposed to VSV antigen, as previous results had shown that UV-VSV immunized mice do have neutralizing titers on this day (Figure 16).

2.2.4a Confirmation of exposure to VSV antigen

To confirm that the mice were immunized with UV-VSV, serum collected on day 21 from a UV-VSV-immunized mouse from each experiment was tested for its ability to protect BHK 21 cells from infection with VSV at 10^4 $TCID_{50}$/ml. All animals tested produced roughly the same titer of neutralizing antibodies against VSV, as determined by serum neutralization assays. At a dilutions of sera of 1:240 (two experiments) and 1:320 (one experiment), half of the wells showed cell death, and half were protected by the serum. Therefore, the sera were found to contain a neutralizing antibody titer of 7.737 and 8.322, as expressed as the $-\log_2$ (sera dilution). Serum from a non-immunized animal did not protect the cells at any dilution. Photographs of infected BHK 21 cells and protected BHK 21 cells are shown in Figure 5.

2.2.4b anti-OVA IgG production

An ELISA was used to measure anti-OVA antibody production over a series of three weeks in UV-VSV-immunized mice. In three independent experiments, C57BL/6 mice were injected intraperitonealy with 0.5 ml of 5 x 10^9 $TCID_{50}$/ml of UV-inactivated VSV, and the next day given 10 µg OVA. The inactivated virus used in each experiment was back-titrated on BHK21 cells to confirm its inability to replicate. The virus used was not cytopathic at a 1:10 dilution. An ELISA was performed at the end of each of these three experiments to detect anti-OVA IgG antibodies in the sera taken from these mice. Each of the three experiments yielded similar results (Figure 21 a-c). Additionally, one ELISA was performed using all of the data collected so direct comparisons could be made across all experiments (Figure 22). The results of each serum sample relative to one another remained the same when repeated. The average anti-OVA antibody production peaked on day 14 (0.288), rising from 0.173 on day 7, and falling to 0.238 on day 21 (Figure 22 b).

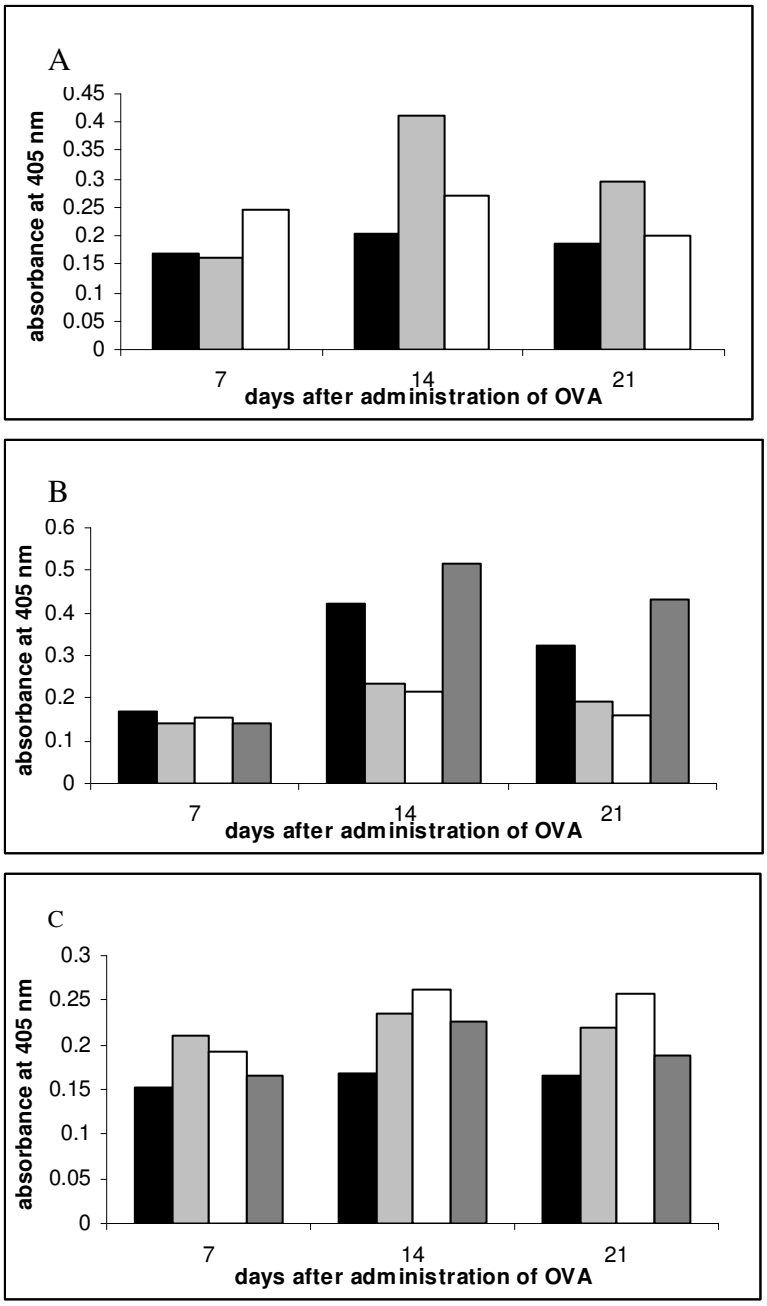

Figure 21. Anti-OVA IgGs produced in mice immunized with UV-VSV and immunized one day later with 10 µg OVA. Each box (a-c) shown represents one independent replication of this experiment. Serum was collected after 7, 14, and 21 days, and anti-OVA IgGs were detected by ELISA. Each experiment included four male C57 mice, except experiment one (a), which had three. In addition, each experiment included a negative control, or serum from a mouse not given OVA (data not shown). The absorbance of the control serum was lower than the absorbance of the immunized mice.

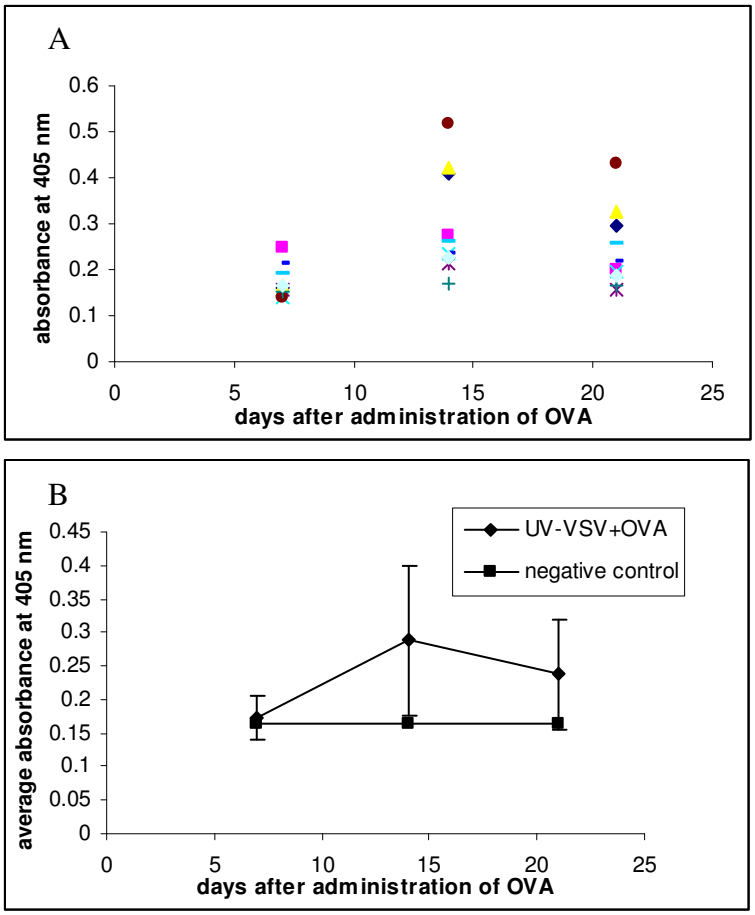

Figure 22. Summary of anti-OVA IgG produced in UV-VSV immunized mice also given 10 µg OVA. Antibodies were detected by ELISA and are measured by absorbance at 405 nm. Samples were collected in three independent experiments and analyzed by the same ELISA. a) Each symbol represents serum from one UV-VSV-immunized and OVA-immunized mouse. b) Absorbance at 405 nm averaged from all eleven serum samples on each day after administration of OVA, compared to serum from a non-treated mouse. Standard deviation is shown.

These results show that the mice were immunized with UV-VSV, with neutralizing titers similar to those obtained in earlier results (Figure 16). The inactivation of the virus was confirmed by back-titration. These mice were able to produce antibodies against OVA following immunization with 10 µg. The absorbance values from the immunized-mouse sera are higher than background absorbance values obtained with serum from a non-

immunized mouse (Figure 22b). The highest titer of anti-OVA IgG is not detected until two weeks following immunization, and decreases again by week three.

2.2.5 Comparison of anti-OVA production by infected, immunized, and control mice

In order to test the hypothesis that infection with VSV alters the immune response to an unrelated antigen, the anti-OVA production in the three groups of mice (VSV-infected and OVA immunized, UV-VSV and OVA immunized, or OVA only) were compared by ELISA. Serum was collected after 7, 14, and 21 days following OVA immunization. This experiment was repeated three times, and all sera collected were analyzed by the same ELISA. The average anti-OVA IgG production for each treatment group is shown in Figure 23.

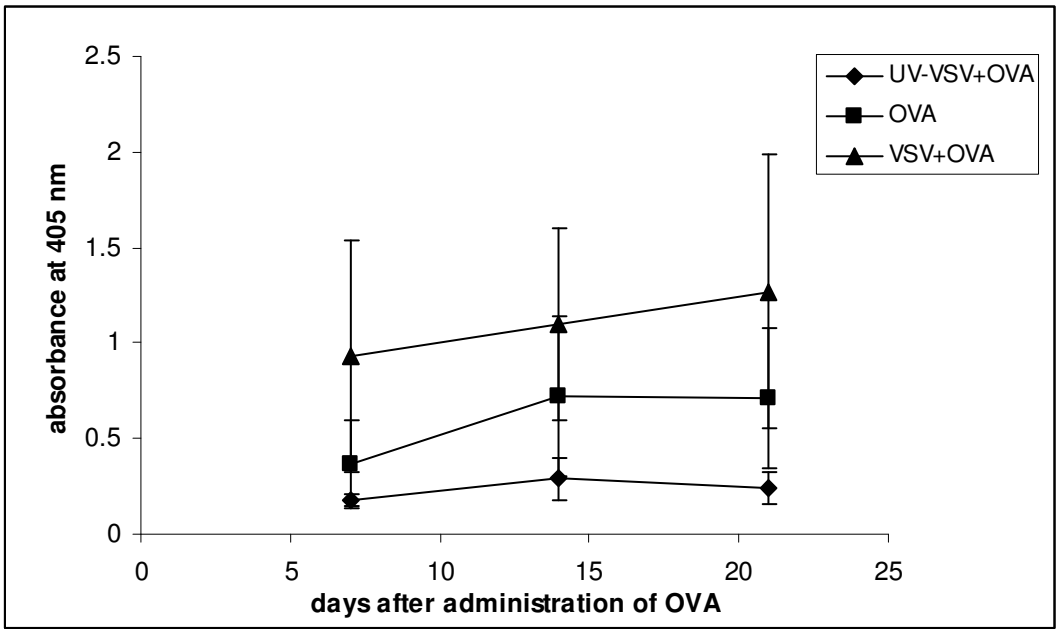

Figure 23. Summary of anti-OVA IgG produced in VSV-infected, UV-VSV immunized and non-treated mice also given 10 µg OVA. Antibodies were detected by ELISA and are measured by absorbance at 405 nm. Samples were collected in three independent experiments and analyzed by the same ELISA. Absorbance at 405 nm averaged from all 33 serum samples on each day after administration of OVA. Standard deviation is shown.

The differences in anti-OVA production in these three groups of mice were compared by one way ANOVA (α=0.05). Data collected after one, two, and three weeks were analyzed by three ANOVAs. The dependent variable was anti-OVA IgG concentration (given as absorbance of serum at 405 nm). The eleven mice that were immunized with UV-VSV and also with OVA were considered treatment group one. The eleven mice that were only immunized with OVA were treatment group two, and the eleven mice that were infected with VSV and then immunized with OVA were placed in treatment group three (Table 2). Although Levene's test of equality of variance found significant variation within the data, the F values were close to the typical cut-off of 2, and so the ANOVAs were run despite not meeting the assumption of equality of variance. The significance and power given in each ANOVA confirm that this was a reasonable decision. All three ANOVAs for each day of data collection showed that the means differed significantly ($p<0.0001$) with high power ($\geq$.987) (Table 1). The ANOVAs looked for variation in anti-OVA antibody concentration due to placement in each of the three treatment groups, and also due to variations between each of the three replications (trials) of this experiment. The three trials were included as a random blocking effect. The ANOVAs show that almost all of the differences in antibody concentration are due to treatment (Table 1).

Table 1. Summary of results from the three ANOVAs used to analyze between subjects effects; different ANOVAs were used on data collected after days 7, 14, and 21.

Day of data collection	Source of variation	ANOVA F	Significance	Observed Power
7	treatment	12.695	<0.0001	0.993
7	trial	1.275	0.295	0.253
14	treatment	11.426	<0.0001	0.987
14	trial	0.668	0.521	0.151
21	treatment	12.949	<0.0001	0.994
21	trial	1.351	0.275	0.267

To find where the means differ in each ANOVA, a post-hoc Tukey HSD test was used. Table 2 summarizes the results of the three Tukey tests. The ANOVA and Tukey HSD post-hoc comparison show that the UV-VSV mice differ significantly in anti-OVA production from the OVA-only mice on day 14 ($p<0.05$). The UV-VSV infected mice also differ significantly in anti-OVA production from the VSV-infected on days 7, 14, and 21 ($p<0.0001$). The VSV-infected mice differ significantly in anti-OVA production compared to mice given OVA alone on days 7 and 21 ($p<0.05$).

Table 2. Summary of Tukey HSD post-hoc comparisons of the effect of different treatments on anti-OVA antibody production in mice. Three different Tukey post-hocs were used on the data from three separate ANOVAs.

Day	P value	Treatments compared
7	0.453	UV-VSV (1) and OVA (2)
	<0.0001	UV-VSV (1) and VSV (3)
	0.004	OVA (2) and VSV (3)
14	0.037	UV-VSV (1) and OVA (2)
	<0.0001	UV-VSV (1) and VSV (3)
	0.074	OVA (2) and VSV (3)
21	0.060	UV-VSV (1) and OVA (2)
	<0.0001	UV-VSV (1) and VSV (3)
	0.021	OVA (2) and VSV (3)

These results show that there are significant differences in the ability of mice to produce anti-OVA IgG antibodies following infection with VSV (Table 2). Infected mice produced the highest titer of IgG on days 7, 14, and 21 post-immunization. Mice immunized with UV-inactivated VSV produced the lowest titer of IgG on days 7, 14, and 21 post-immunization (Figure 23). Also, UV-VSV immunized mice and mice given OVA alone had a peak antibody titer after 14 days, but VSV-infected mice had an even higher titer after 21 days. Statistical analysis shows that these differences are mainly due to infection status, and not due to any variation within each treatment group or between the three replications (Table 1).

DISCUSSION

Optimization of experimental procedures

The exact details of each technique to be used in this research needed to first be optimized for the Immunology/Virology laboratory at Northern Michigan University. One of the first protocols optimized was BHK 21 cell culture, as many of the experiments used these cells. The culture media consisted of RPMI supplemented with 5% FCS and 100 U-µg penicillin-streptomycin, and cells in this media formed a monolayer within four days. Growing the cells without antibiotics eventually resulted in bacteria in the supernatant, and so 100 U-µg penicillin-streptomycin was added to the culture media to prevent contamination. To transfer cells from a 75 cc flask to a 96 well plate for titration experiments, cells were detached from the flask using 0.25% trypsin and diluted 1:60 with culture media. Lower dilutions yielded too high of a concentration of cells for the size of each well, and higher dilutions did not provide enough cells in each well to create a monolayer within two days.

The vesicular stomatitis virus used in this work was propagated and titrated here. The original vial of virus provided by Dr. Osorio (University Nebraska, Lincoln) was added to BHK 21 cells and clarified by centrifugation. The titer of this virus was calculated by finding the dilution of virus that killed 50% of the cells, or the tissue culture infective doses at 50% ($TCID_{50}$). Vesicular stomatitis virus is highly cytopathic to BHK 21 cells, and cell death was easily discernable with 40X magnification. Half of the wells (four of eight) that received 100 µl of virus diluted 10^{-9} showed cell death after 48 hours. Taking

into account this dilution factor, the titer of VSV was found to be $10^{10}TCID_{50}$/ml. Another way of expressing the functional titer of a virus is to measure the number of viral particles able to infect cells and form plaques; these titers are given in plaque-forming units (pfu). These two measurements, tissue culture infective doses at 50% and plaque-forming units, are similar and will give comparable values in viral titrations.

One objective of this thesis was to compare the immune response against infectious and noninfectious VSV, and so a protocol to inactivate the VSV was optimized. Formalin treatment renders VSV noninfectious by altering the surface structure of the virus. Changing the structure of VSV would introduce another variable in the comparison, and so UV-light was used. This inactivation method affects the RNA of the virus by inducing pyrimidine dimers that cannot be repaired; it does not change the surface structure of the virion. The shortest treatment of UV-light that rendered VSV noninfectious at a 1:10 dilution on BHK 21 cells was investigated. Low wattage (18 W) UV bulbs were not strong enough to inactivate the virus. A 30 W bulb with 254 nm UV waves was able to inactivate the virus when directly exposed for a total of six minutes. Overheating, which could denature proteins in the viral capsid, was prevented by using three segments of two minute exposure.

In order to study the effect of infection with a T cell-independent virus on immune responses, mice were infected with $10^{6}TCID_{50}$ VSV by the intraperitoneal route. While lower doses of VSV have been shown to be dependent on T cells for IgG antibody production, $10^{6}TCID_{50}$ VSV is T cell-independent (9). This dose of VSV was not lethal

and did not cause paralysis, one symptom noted in studies conducted by other researchers using higher doses of VSV. Low doses of inactivated VSV (such as $2x10^4$ pfu) have been shown to be unable to stimulate neutralizing IgG responses, but high doses (10^8 pfu) create an early response similar to that seen after live-VSV infection (66). For this reason, a high dose ($2.5x10^9$ $TCID_{50}$) of UV-inactivated VSV was used in this research.

The route of administration of the virus causes the antigen to be brought to different lymphoid organs, and has been shown to determine the mode of the B cell activation (39). Antigens injected into the peritoneal cavity, as performed in this work, will be brought to the spleen. Other research studying the immune response to VSV has administered the virus by intravenous injection (7, 9, 66).

Neutralizing titers produced by infected or immunized mice were detected using serum-neutralization assays, where the presence of protective antibodies in serum prevented the virus from infecting cultured cells. In these experiments, the sera tested were first incubated for 30 minutes as 56° C to remove any complement that might be toxic to the BHK 21 cells. The complement-depleted sera were pre-diluted 1:40 to avoid background due to the presence of natural anti-VSV antibodies (54). Serum dilutions combined with 10^4 $TCID_{50}$ /ml of VSV yielded a good range to detect the presence of antibodies. At these concentrations, the dilution of sera that provided 50% of the wells with protection occurred towards the middle of the series of dilutions used. All experiments included internal controls, where one column of the 96 well plate received de-complemented serum only (no virus), one column received virus only, and one column received culture

media only. Only assays that had 100% living cells in the serum-only and culture-media only columns, and 100% cell death in the virus-only column were used. In some initial experiments, 10^7 $TCID_{50}$ /ml was used instead of 10^4 $TCID_{50}$ /ml. Diluted sera from VSV-infected mice were able to protect BHK 21 cells from this high dose of VSV, but only at very low dilutions. Lower doses of VSV were not tried, as the error would be too great. While just one infectious particle should be enough to cause infection, random variations in distribution of the viral particles might mean that one well of cells gets many particles, and another well none at all. Therefore, a dose of 10^4 $TCID_{50}$ /ml of VSV was used in the experiments described in this work.

To study the production of neutralizing antibodies from the initial exposure of the mice to VSV, it was important to use mice with no prior existing antibodies able to neutralize the virus. One initial experiment examined the mice housed in the animal facility at NMU for the presence of these antibodies, called 'natural' antibodies. This experiment used serum from a non-infected mouse in a serum-neutralization assay. The serum from this mouse was de-complemented and pre-diluted to 1:40. All sera used to detect anti-VSV antibodies in this thesis was diluted to at least 1:40, and so any protective effects at lower dilutions were of no concern. No protective natural antibodies against VSV were detected with sera diluted 1:40. Therefore, any neutralizing antibodies calculated in the serum of these animals resulted only from injection with VSV.

Enzyme-linked immunosorbant assays were used to detect anti-OVA antibodies. Immulon 2HB plates were sensitized with 20 µg of OVA overnight at 4°C. Lower

concentrations of OVA led to lower absorbance values in an ELISA that used serum from an OVA-immunized mouse, indicating that these concentrations were below the limit of detection for anti-OVA antibodies. Higher concentrations of OVA did not show an increase in absorbance values (Figure 10), and so were not used. Different concentrations of serum were also tested. Serum diluted 1:50 did not show absorbance values much higher than serum from non-immunized mice. Serum diluted 1:25 was concentrated enough so that antibodies present were detected in these assays, and yet also insured that the serum collected would last for multiple tests. Dilutions lower than 1:25 were not tested, as this might lead to a higher background.

The amount of OVA required to induce detectable levels of anti-OVA IgG in the C57BL/6 mice located in the animal facility at Northern Michigan University when given by intraperitoneal injection was also optimized. Mice were injected with different concentrations of OVA in sterile RPMI, and anti-OVA antibodies produced were detected by an ELISA (Figure 11). Mice given 0.1µg OVA did not have antibodies present in the sera that yielded an absorbance higher than that shown by the negative control (serum from non-immunized mouse). Sera from mice given 5 µg OVA gave slightly higher absorbance values; mice given 20 µg OVA gave much higher absorbance values. Based on these results, 10 µg OVA in RPMI were used to immunize the mice in the remaining experiments. This avoided potentially masking variations between different treatment groups by eliciting a very large anti-OVA production from immunizing with a high concentration of OVA.

This work compared antibody responses between mice that were non-infected, VSV-infected, or immunized with UV-inactivated VSV. It was assumed that the only difference between each group was the particular treatment administered. Variations that might occur between individual mice within a treatment group were minimized by using mice of the same gender (male), and of relatively the same age (eight to twelve weeks). This age group of male mice was chosen because it is the largest cohort seen in similar research from other laboratories.

Preliminary experiments for this work also considered which of two strains of mice located in the Northern Michigan University animal facility would be best for this research. These mice, C57BL/6 and BALB/c, were inbred and so each strain was homozygous. The two strains of mice differed in MHC haplotype. The C57BL/6 has an $H2^b$ MHC haplotype, and BALB/c mice have an $H2^d$ haplotype. These differences in MHC mean that the epitopes of antigen presented will differ between these strains, and so the antibody response to VSV and to OVA was compared (Figures 12-14). The neutralizing anti-VSV titer in VSV-infected mice was similar, as was the anti-OVA titer in immunized mice. Also, the differences in the anti-OVA IgG response between VSV-infected and non-infected mice was similar for C57BL/6 mice and BALB/c mice, with both strains of mice exhibiting increased anti-OVA antibodies during infection. However, the standard deviation of the calculated titers in all three experiments was much greater in the C57BL/6 strain than with the BALB/c mice (*sections 1.2.9 and 1.2.10*). Despite the greater standard deviation, the experiments for this work used the C57BL/6 strain. These mice had a much greater population than the BALB/c in the

animal facility at Northern Michigan University, and so enough male mice of the same age group could be obtained for each experiment.

The experiments to measure differences in anti-OVA antibody production between non-infected, VSV-infected, and UV-inactivated VSV-immunized mice were each repeated three times. Differences between each of these three independent replications were controlled for by the ANOVA model used for statistical analysis. The three experimental trials were placed in the ANOVA as random, blocking effects, which controlled for any extraneous variation that did exist between the different trials. The results of the ANOVA indicate that variation between trials did not significantly contribute to the overall variation between means (Table 1). Although Levene's test of equality of variance found that the variance in antibody response across each treatment group did vary with some slight significance (appendix B), it was relatively small and so it was ignored. This decision was supported by the very high power reached in each ANOVA tests for difference between means.

Kinetics of anti-VSV production

Anti-VSV titers in infected mice

Mice were infected with 10^6 $TCID_{50}$ VSV by the intraperitoneal route, and neutralizing antibodies were detected by serum-neutralization assays. Three days following infection, protective antibodies were found in the serum (Figure 15). This early response indicates that B cells were stimulated to produce antibodies without T cell involvement. The antibody response against infectious VSV given by the intraperitoneal route is long-

lasting in mice. In the experiments conducted here, high titers of neutralizing antibodies were still found 60 days post infection (Figure 15).

The immune response to VSV in mice has been studied for many years, most often by administering the virus by intravenous injection, which differs from the work here. Researchers have found that early after infection with VSV, dendritic cells transport and release the virus in secondary lymphoid organs where B cells are directly stimulated to produce neutralizing IgM independently of T cells (21). In particular, VSV antigen accumulates in the splenic marginal zone (38). The humoral response is crucial for recovery from infection and protection from paralysis caused by VSV. Antibody production occurs very quickly after infection by intravenous injection, with high titers detected within three days (7, 9, 66). Antibody secretion continues for at least 300 days (66). The long term maintenance of antibody titers requires persistent antibody production, because the half-life of antibodies in serum is less than 3 weeks (67). One hypothesis to explain the continual antibody production is the presence of persisting antigen on dendritic cells in germinal centers in secondary lymphoid organs, which stimulate memory B cells to differentiate to antibody secreting plasma cells. Memory B cells are long-lived in the absence of antigen, but their switch to plasma cells requires both the presence of viral antigen and T helper cells (66). Therefore, the protective neutralizing antibody titers are maintained by these cells only in the presence of antigen. In this way, dendritic cells infected with live-VSV may cause both the early, T cell-independent response and the long-lasting response.

Around six days after VSV infection, antibodies switch isotype from IgM to IgG (2, 9, and 55). Researchers have found that both the IgM and IgG response to infectious VSV at high doses are T cell-independent (9), since neutralizing titers of both isotypes can be induced in mice lacking T cells. The IgG production in these mice is dependent on the dose of VSV administered, with 10^6 pfu VSV inducing the highest titer of IgG. Although the research for this thesis used intraperitoneal injections instead of intravenous injections, the same dose was administered. This means there may have been T cell-independent IgG production early after infection.

Anti-VSV titers in UV-inactivated VSV-immunized mice

Vesicular stomatitis virus was inactivated so that the virus was unable to replicate and cause infection. Ultra-violet light was chosen as the means of inactivation so as to leave the surface structure unaltered, meaning that any differences noted in antibody responses after vaccination compared to after infection would not be due to structural differences of the two treatments. The immune response to an inactivated viral vaccine is limited by the presence of viral antigens in the vaccine, because inactivated virus cannot undergo a productive replication cycle. Therefore, antigen that stimulates antibody production comes directly from the initial dose given. The research presented here used very high doses (2.5×10^9 $TCID_{50}$) of UV-inactivated VSV so that early after injection, the amount of VSV-antigen present was comparable in infected and immunized mice. This explains the similar titer of neutralizing antibodies seen on day three (Figures 15 and 16). Unlike infectious VSV, noninfectious UV-VSV does not elicit a long-lasting antibody response.

Antibodies were not found in the animals after four weeks post-immunization (Figure 16).

Other researchers have also studied the immune response in mice to inactivated VSV. Many have used formalin-inactivated VSV, and have used intravenous injections, instead of intraperitoneal as in this work. Formalin inactivates VSV by cross-linking surface proteins, and so this inactivation technique results in altered structures of VSV. Despite these differences, researchers have reported results similar to those obtained here, where high doses of inactivated virus induce high titers of neutralizing antibodies early after immunization (66, 68).

Kinetics of anti-OVA antibodies

This research investigated the impact of a viral infection on the production of antibodies against an unrelated T cell-dependent antigen, by comparing antibody production against OVA in VSV-infected and non-infected mice. Egg albumin (OVA) is a protein antigen that induces a T cell-dependent antibody response. Mice injected with 10 µg OVA by the intraperitoneal route produced antibodies that were detected by ELISA, where the absorbance value at 405 nm corresponded to the amount of antibodies present in the serum of the mice. OVA-immunized mice produced anti-OVA antibodies, with the highest titer of anti-OVA IgG detected two weeks following immunization. This titer decreased slightly by week three (Figure 18). Infection with VSV led to increased titers of anti-OVA IgG after one, two, and three weeks (Figure 23). A post-hoc test revealed that this increase was significant on weeks one and three ($p<0.05$, Table 2). The presence

of infection also increased the duration of the antibody response to OVA. After three weeks, mice that were given OVA alone had decreased antibody titers compared to two weeks post-immunization. In contrast, VSV-infected mice had the highest titer of anti-OVA antibodies after three weeks (Figures 20 and 23).

To isolate the role of infection in modulating the anti-OVA antibody response, this research also studied the effect of VSV antigens on anti-OVA antibody production. Ultra-violet-inactivated VSV is a source of VSV antigen that has the same structure as infectious VSV, but is unable to cause infection. Therefore, a third group of mice were immunized with inactivated virus and the anti-OVA production was measured by ELISA. Immunization with UV-VSV prior to OVA administration led to decreased anti-OVA production compared to results seen with mice given OVA only (Figure 23). This decrease was significant two weeks after immunization ($p<0.05$), as revealed by post-hoc analysis (Table 2). The UV-VSV immunized mice also had significantly decreased anti-OVA production compared to the results seen in VSV infected mice on all three weeks ($p<0.0001$, Figure 23). The only difference between these two groups of mice was the presence of infection after injection of VSV instead of UV-inactivated VSV. This indicates that it is infection, and not just the presence of VSV antigen, that led to the increased anti-OVA production in VSV mice compared to mice that received OVA alone.

Recent research has found that the antibody response to OVA is up-regulated after infection with influenza virus as well (69), although the OVA and influenza were both inhaled in those experiments, instead of intraperitoneal injection as in this work.

However, these results are the opposite of those obtained in similar experiments with FMDV infected mice. Researchers found significantly decreased anti-OVA production in FMDV infected mice compared to mice immunized with OVA only, or compared with UV-inactivated FMDV (35). Foot and mouth disease virus is a T cell-independent virus like VSV and influenza. However, unlike VSV, FMDV only undergoes abortive infection of dendritic cells (35). In this sense, inactivated VSV is similar to infectious FMDV. Inactivated VSV does not replicate, and it also leads to a decrease of T cell-dependent antibody responses.

The decreased anti-OVA response found during FMDV-infection is caused in part by a decreased ability of infected-dendritic cells to stimulate T cells (35). This contrasts with influenza virus-infected dendritic cells, which show an increase in maturation (69). Dendritic cells are important antigen presenting cells in the immune response to influenza and VSV. Murine dendritic cells exposed to VSV carry the replicating virus to secondary lymphoid organs where they induce B cell responses by releasing low levels of virus (21). Dendritic cells stimulated by infection with influenza or VSV may cause these cells to capture and present a mix of antigens to T cells, both infectious and innocuous, such as OVA. This bystander effect would explain the increased T cell-dependent response against OVA.

It would be interesting to see if the order of the treatments has an impact on the kinetics described here. If the mice were first immunized with OVA, and then vaccinated with UV-VSV, perhaps the immunsuppression would be instead noted with a suppressed anti-

VSV response. Another variable may be the route of infection. Both OVA and UV-VSV were administered by the intraperitoneal route, and so both vaccines were sent to the same lymphoid organ (the spleen). Perhaps if different injection sites were used, different lymphoid organs would be recruited to the response and no difference would be seen in anti-OVA production.

In conclusion, these results show that the immune response to infectious VSV and inactivated VSV is different. High titers of neutralizing antibodies were produced following infection and immunization, but only infectious VSV maintained antibody titer for longer than one month. The different response observed may be because antigen is no longer present in the mice, and so there is no stimulus for antibody production. Alternatively, a different pathway of activation occurs between infection and vaccination with inactivated virus. Further evidence for an alternative pathway is seen with the changes in the T cell-dependent antibody response to OVA during VSV infection. Infected mice produced the highest titer of anti-OVA IgG on days 7, 14, and 21 post-immunization, while mice immunized with UV-inactivated VSV produced the lowest titers over three weeks. Also, UV-VSV immunized mice and mice given OVA alone had a peak antibody titer after 14 days, but VSV-infected mice had an even higher titer after 21 days. This indicates that infection with VSV increases the generalized activation of T cells, which leads to an increase in the T cell-dependent antibody response against an unrelated antigen (OVA). Vesicular stomatitis virus can now be genetically engineered to produce recombinant vaccines with foreign antigens or epitopes. The results presented here suggest that infection of dendritic cells with VSV could act as a potent adjuvant for

T cell responses, and so increase the production of T cell-dependent antibodies to a weak immunogenic antigen.

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APPENDIX A

The Preparation of Solutions Used in this Work

0.1 M Carbonate Buffer (pH 9.6)
250 ml dH_2O with 5.3 g Sodium Carbonate
250 ml dH_2O to 4.2 g Sodium Bicarbonate
Adjust pH of each to 9.6
Combine 16 l Sodium Bicarbonate solution with 34 ml Sodium Carbonate solution
Add 50 ml dH_2O

0.1 M Citrate Phosphate Buffer
9.05 g Na_2HPO_4
4.71 g Citric acid
Adjust pH to 5
Add dH_2O to make 500 ml

10% FCS-RPMI
10 ml sterile fetal calf serum
90 ml sterile RPMI (Invitrogen)
Combine in sterility

5% FCS-RPMI with Penicillin-Streptomycin
1 ml sterile penicillin-streptomycin (Gibco)
5 ml sterile fetal calf serum
94 ml sterile RPMI (Invitrogen)
Combine in sterility

0.5% Gelatin
0.5 g gelatin
100 ml 1X PBS with 0.01% Tween-20
Combine, dissolve with heat and stir bar.

10X PBS pH 7.4
2.62 g NaH_2PO_4
11.5 g Na_2HPO_4
43.84 g NaCl

Combine and adjust pH to 7.4
Add dH_2O to 500 ml
Autoclave

1X PBS with 0.01% Tween-20 (PBS-t)
450 ml dH_2O
50 ml 10X PBS
0.05 ml Tween-20

1 X Trypsin
10 ml 2.5% Trypsin (Gibco)
90 ml RPMI (Invitrogen)
Combine in sterility

APPENDIX B

Statistical analysis: detailed ANOVA results

The output from SPSS version 15.0 (Statistical Software Package for Social Sciences 2006, Chicago) is shown below. Data entered were the absorbance values from an ELISA of serum samples collected on day seven from non-infected, VSV-infected, and UV-VSV immunized mice that had been immunized with OVA.

```
UNIANOVA
  antibody  BY treatment trial
  /RANDOM = trial
  /METHOD = SSTYPE(3)
  /INTERCEPT = EXCLUDE
  /POSTHOC = treatment ( TUKEY )
  /EMMEANS = TABLES(OVERALL)
  /PRINT = DESCRIPTIVE OPOWER HOMOGENEITY
  /CRITERIA = ALPHA(.05)
  /DESIGN = treatment trial.
```

Between-Subjects Factors

		N
treatment	1	11
	2	11
	3	11
trial	1	11
	2	12
	3	10

Descriptive Statistics

Dependent Variable: antibody

treatment	trial	Mean	Std. Deviation	N
1	1	.19304	.047114	3
	2	.14980	.013687	4
	3	.18047	.026492	4
	Total	.17275	.032690	11
2	1	.25875	.081164	4
	2	.46025	.324946	4
	3	.39533	.197419	3
	Total	.36927	.223268	11
3	1	.71975	.561529	4
	2	1.25025	.737826	4
	3	.79200	.459039	3
	Total	.93236	.603711	11
Total	1	.40846	.398325	11
	2	.62010	.641431	12
	3	.42839	.357034	10
	Total	.49146	.486499	33

Levene's Test of Equality of Error Variances(a)

Dependent Variable: antibody

F	df1	df2	Sig.
4.255	8	24	.003

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: treatment+trial

Tests of Between-Subjects Effects

Dependent Variable: antibody

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Noncent. Parameter	Observed Power(a)
treatment	Hypothesis	3.452	2	1.726	12.695	.000	25.391	.993
	Error	3.807	28	.136(b)				
trial	Hypothesis	.347	2	.173	1.275	.295	2.549	.253
	Error	3.807	28	.136(b)				

a Computed using alpha = .05
b MS(Error)

Expected Mean Squares(a,b)

Source	Variance Component		
	Var(trial)	Var(Error)	Quadratic Term
treatment	.000	1.000	treatment
trial	10.909	1.000	
Error	.000	1.000	

a For each source, the expected mean square equals the sum of the coefficients in the cells times the variance components, plus a quadratic term involving effects in the Quadratic Term cell.
b Expected Mean Squares are based on the Type III Sums of Squares.

Grand Mean

Dependent Variable: antibody

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
.487	.064	.355	.618

Multiple Comparisons

Dependent Variable: antibody
Tukey HSD

(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Upper Bound	Lower Bound
1	2	-.19653	.157233	.435	-.58558	.19252
	3	-.75962(*)	.157233	.000	-1.14867	-.37057
2	1	.19653	.157233	.435	-.19252	.58558
	3	-.56309(*)	.157233	.004	-.95214	-.17404
3	1	.75962(*)	.157233	.000	.37057	1.14867
	2	.56309(*)	.157233	.004	.17404	.95214

Based on observed means.
* The mean difference is significant at the .05 level.

Tukey HSD

treatment	N	Subset	
		2	1
1	11	.17275	
2	11	.36927	
3	11		.93236
Sig.		.435	1.000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .136.
a Uses Harmonic Mean Sample Size = 11.000.
b Alpha = .05.

The output from SPSS version 15.0 (Statistical Software Package for Social Sciences 2006, Chicago) is shown below. Data entered were the absorbance values from an ELISA of serum samples collected on day fourteen from non-infected, VSV-infected, and UV-VSV immunized mice that had been immunized with OVA.

```
DATASET ACTIVATE DataSet2.
UNIANOVA
  antibody  BY treatment trial
  /RANDOM = trial
  /METHOD = SSTYPE(3)
  /INTERCEPT = EXCLUDE
  /POSTHOC = treatment ( TUKEY )
  /EMMEANS = TABLES(OVERALL)
  /PRINT = DESCRIPTIVE OPOWER HOMOGENEITY
  /CRITERIA = ALPHA(.05)
  /DESIGN = treatment trial.
```

Between-Subjects Factors

		N
treatment	1	11
	2	11
	3	11
trial	1	11
	2	12
	3	10

Descriptive Statistics

Dependent Variable: antibody

treatment	trial	Mean	Std. Deviation	N
1	1	.29528	.105715	3
	2	.34719	.146611	4
	3	.22319	.039751	4
	Total	.28794	.110701	11
2	1	.75450	.197635	4
	2	.59425	.418090	4
	3	.84633	.718149	3
	Total	.72127	.422957	11
3	1	1.35050	.562648	4
	2	1.08575	.549342	4
	3	.78500	.231525	3
	Total	1.10000	.501173	11
Total	1	.84599	.552330	11
	2	.67573	.488503	12
	3	.57868	.470405	10
	Total	.70307	.501734	33

Levene's Test of Equality of Error Variances(a)

Dependent Variable: antibody

F	df1	df2	Sig.
2.665	8	24	.030

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: treatment+trial

Tests of Between-Subjects Effects

Dependent Variable: antibody

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Noncent. Parameter	Observed Power(a)
treatment	Hypothesis	3.445	2	1.723	11.426	.000	22.852	.987
	Error	4.222	28	.151(b)				
trial	Hypothesis	.202	2	.101	.668	.521	1.337	.151
	Error	4.222	28	.151(b)				

a Computed using alpha = .05
b MS(Error)

Expected Mean Squares(a,b)

Source	Variance Component		
	Var(trial)	Var(Error)	Quadratic Term
treatment	.000	1.000	treatment
trial	10.909	1.000	
Error	.000	1.000	

a For each source, the expected mean square equals the sum of the coefficients in the cells times the variance components, plus a quadratic term involving effects in the Quadratic Term cell.
b Expected Mean Squares are based on the Type III Sums of Squares.

Grand Mean

Dependent Variable: antibody

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
.701	.068	.563	.840

Multiple Comparisons

Dependent Variable: antibody
Tukey HSD

(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Upper Bound	Lower Bound
1	2	-.43333(*)	.165571	.037	-.84301	-.02365
	3	-.81206(*)	.165571	.000	-1.22174	-.40238
2	1	.43333(*)	.165571	.037	.02365	.84301
	3	-.37873	.165571	.074	-.78841	.03095
3	1	.81206(*)	.165571	.000	.40238	1.22174
	2	.37873	.165571	.074	-.03095	.78841

Based on observed means.
* The mean difference is significant at the .05 level.

Tukey HSD

treatment	N	Subset	
		2	1
1	11	.28794	
2	11		.72127
3	11		1.10000
Sig.		1.000	.074

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .151.
a Uses Harmonic Mean Sample Size = 11.000.
b Alpha = .05.

The output from SPSS version 15.0 (Statistical Software Package for Social Sciences 2006, Chicago) is shown below. Data entered were the absorbance values from an ELISA of serum samples collected on day 21 from non-infected, VSV-infected, and UV-VSV immunized mice that had been immunized with OVA.

```
DATASET ACTIVATE DataSet3.
UNIANOVA
  antibody  BY treatment trial
  /RANDOM = trial
  /METHOD = SSTYPE(3)
  /INTERCEPT = EXCLUDE
  /POSTHOC = treatment ( TUKEY )
  /EMMEANS = TABLES(OVERALL)
  /PRINT = DESCRIPTIVE OPOWER HOMOGENEITY
  /CRITERIA = ALPHA(.05)
  /DESIGN = treatment trial.
```

Between-Subjects Factors

		N
treatment	1	11
	2	11
	3	11
trial	1	11
	2	12
	3	10

Descriptive Statistics

Dependent Variable: antibody

treatment	trial	Mean	Std. Deviation	N
1	1	.22721	.058906	3
	2	.27612	.124549	4
	3	.20722	.039414	4
	Total	.23773	.082515	11
2	1	.77650	.309325	4
	2	.68425	.389138	4
	3	.65067	.535616	3
	Total	.70864	.366876	11
3	1	1.49425	.802572	4
	2	1.47050	.796449	4
	3	.70700	.059775	3
	Total	1.27091	.718008	11
Total	1	.88769	.711068	11
	2	.81029	.697542	12
	3	.49019	.353418	10
	Total	.73909	.623894	33

Levene's Test of Equality of Error Variances(a)

Dependent Variable: antibody

F	df1	df2	Sig.
4.879	8	24	.001

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a Design: treatment+trial

Tests of Between-Subjects Effects

Dependent Variable: antibody

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Noncent. Parameter	Observed Power(a)
treatment	Hypothesis	5.541	2	2.771	12.949	.000	25.898	.994
	Error	5.991	28	.214(b)				
trial	Hypothesis	.578	2	.289	1.351	.275	2.702	.267
	Error	5.991	28	.214(b)				

a Computed using alpha = .05
b MS(Error)

Expected Mean Squares(a,b)

Source	Variance Component		
	Var(trial)	Var(Error)	Quadratic Term
treatment	.000	1.000	treatment
trial	10.909	1.000	
Error	.000	1.000	

a For each source, the expected mean square equals the sum of the coefficients in the cells times the variance components, plus a quadratic term involving effects in the Quadratic Term cell.
b Expected Mean Squares are based on the Type III Sums of Squares.

Grand Mean

Dependent Variable: antibody

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
.731	.081	.565	.896

Multiple Comparisons

Dependent Variable: antibody
Tukey HSD

(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Upper Bound	Lower Bound
1	2	-.47091	.197240	.060	-.95895	.01713
	3	-1.03318(*)	.197240	.000	-1.52122	-.54514
2	1	.47091	.197240	.060	-.01713	.95895
	3	-.56227(*)	.197240	.021	-1.05031	-.07423
3	1	1.03318(*)	.197240	.000	.54514	1.52122
	2	.56227(*)	.197240	.021	.07423	1.05031

Based on observed means.
* The mean difference is significant at the .05 level.

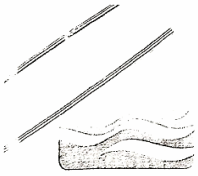
Tukey HSD

treatment	N	Subset	
		2	1
1	11	.23773	
2	11	.70864	
3	11		1.27091
Sig.		.060	1.000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .214.
a Uses Harmonic Mean Sample Size = 11.000.
b Alpha = .05.

APPENDIX C

IACUC approval letter



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May 10, 2005

TO: Osvaldo Lopez
Biology Department

FROM: Cynthia A. Prosen, Ph.D.
Dean of Graduate Studies & Research

RE: **Application to use Vertebrate Animals**
Application # IACUC 029
Approval Period: April 28, 2005 – May 1, 2008

The Institutional Animal Care and Use Committee has approved your project to use vertebrate animals in research entitled "Protective Immunity Elicit by Infection with Vesicular Stomatitis Virus."

If you have any questions, please contact me.

ljh

cc: Biology Department