SELECTION AND QUANTIFICATION OF COMMERCIAL ANTIBODIES FOR SPECIFIC IDENTIFICATION OF VENEZUELAN, WESTERN, AND EASTERN (NORTH AMERICAN AND SOUTH AMERICAN) EQUINE ENCEPHALITIS VIRUSES

by

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(Under the Direction of David Hurley)

ABSTRACT

Screening of monoclonal antibodies for binding specificity and selectivity has been attempted using multiple methods. Flow cytometric analysis is rapid and non-subjective. These studies used flow cytometry to screen antibodies for their antigen specificity and intensity of fluorescent signal to cells expressing antigens from Venezuelan, Western, and Eastern (North American and South American) equine encephalitis viruses. Avian cells were infected with each of the strains, fixed and stained with primary antibodies and fluorescent conjugate. Four antibodies were chosen as most suitable to advance to quantitative testing on the basis of differential specificity, one for each viral strain. Three trials of seven replicates were used to establish that these antibodies generated reproducible and consistent signal that discriminate cells infected with each specific virus relative to uninfected cells. These flow cytometry measurements have demonstrated the essential specificity, selectivity and reproducibility of four antibodies for further development of assays to support vaccine production.

INDEX WORDS: Equine encephalitis virus, Monoclonal antibodies, Flow cytometry, Immunofluorescence assays

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DEDICATION

I dedicate this work to my very patient husband, Nathaniel and to my Mother and Father. Without their support and love this endeavor would have been impossible.

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I would like to thank my committee members for their patience and help. Their support has been wonderful. I want to also thank the management at Merial Limited in Athens, GA for their support and for providing me the means to pursue this degree. Lastly, I must thank my co-workers for their support and help. They have helped me in many ways and I will forever be grateful.

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CHAPTER 1

INTRODUCTION AND LITERATUE REVIEW

Introduction to the Thesis Problem

A standing problem in the development of multi-antigen vaccines for control of equine alphaviruses is the lack of methods to allow for the individual discrimination of viral antigens in a vaccine mixture. The basic problem addressed in this thesis is to establish the most feasible method to routinely measure the individual expression of exogenously expressed proteins from a group of alphaviruses for their use in combined antigen biological manufacturing. One method to attempt to resolve this problem is to exploit the unique epitopes in alphavirus structures by binding commercial monoclonal antibodies that recognize individual epitopes. To do this, one must demonstrate that each antibody binds to the viral antigen claimed by the manufacturer giving a signal that is clearly stronger than its binding of other alphavirus antigens, and in later studies to show that the staining can be used to measure the quantity of antigen present. This thesis addresses the issue of selective and specific binding, and evaluates potential methods to facilitate the quantization of antigen using the antibodies that have been characterized here.

To address the issues involved in this process, one must understand the biology of alphaviruses and the specific biology of the expression of viral proteins, understand the in vitro systems in which the viral proteins will be expressed, and the biological properties of the antibodies that have been proposed for use in measuring the expression of these viral proteins. Further, one must acknowledge the known limitations of the proposed methods.

Traditionally, monoclonal antibodies (Mab) are screened using immunofluorescence assays that are analyzed using a fluorescent microscope. This method allows for screening multiple antibodies at once. However, the level of fluorescence produced using a labeled secondary conjugate is subjectively determined. A sample demonstrating very bright fluorescence is determined to have stronger antibody binding to the specific target proteins than a sample with weaker fluorescence. Problems arise when the human eye is utilized to try to determine slight differences in brightness. Direct comparison by an observer allows for no quantitative value to be established for a sample, except that the observer believes that one sample is less bright or brighter than another. This problem is compounded by differences in the perception of brightness among different individuals when analyzing the same samples. To overcome the subjectiveness of using an immunofluorescence microscope, Flow Cytometric analysis can be used to assign more specific, quantitative values to antibody analysis. Slight differences in brightness seen with similar proteins bound by similar antibodies can be discriminated with Flow Cytometry.

When producing an assay for use in manufacturing scale testing, one must determine the brightest fluorescence producing antibodies for detecting the presence of virus among multiple antigens. The mean level of fluorescent intensity (MFI) resulting from an antibody binding to an expressed viral antigen is a measure of that antibody's affinity for the one antigen (Shapiro, 2003). The most common application of quantitative fluorescence cytometry (QFCM) is to utilize MFI to determine antibody-binding capacity (ABC) (Shapiro, 2003). ABC is defined as the number of antibody molecules bound by a particle, such as a cell, when all the specific binding sites are saturated (Shapiro, 2003). When staining viral antigens with antibodies, we are attempting to saturate the available epitope molecules to produce a reproducible MFI signal that

can be repeatedly measured. Therefore, MFI can be used to make qualitative measurements of an antibody's affinity for a specific viral antigen as long as a saturating concentration of antibody is used in the assays.

To ensure that the antibodies chosen produce the brightest signal, a screening process is completed to rule out cross reactions and poor binding. My research includes screening multiple antibodies that are reported to be specific for individual strains of Equine Encephalitis Virus (EEV). The proposed screening will allow for the selection of antibodies that not only produce the strongest binding, but also are able to differentiate among multiple viral strains. After choosing a group of antibodies, we will test them in repeated assays to determine reliability and robustness of the antibodies for discrimination among the antigens that are candidates for vaccine production. The results of these assays will provide a basis for future studies to determine the ability of the antibodies to measure the level of virus in constructs to be utilized as vaccines. By non-subjectively screening the antibodies for the strongest binding and greatest selectivity, less time will be needed for future studies to establish assays for use in vaccine validation and quality control.

Biology of the Alphavirus

The alphavirus, a genus of the Togaviridae family is an enveloped, plus-stranded RNA group of viruses with worldwide distribution. The alphavirus genus includes more than 20 species that are classified into 7 different complexes (Powers, 2001). Important alphaviruses include Semliki Forest Virus, Sindbis Virus, and the Equine Encephalitis (Encephalomyelitis) viruses. These viruses feature a complex transmission cycle and broad species tropism. Because they can be transmitted from insect vectors to avian and/or to mammalian species, they are of major epidemiological importance. Further, members of the alphavirus family have been

identified as the causative agent of a number of diseases of significant importance in horses and humans. It is therefore critical that good methods of biological control of these viral pathogens be developed.

Structure: Genome

The alphaviruses are enveloped positive sensed RNA viruses (Griffin, 2007). The nucleocapsid and envelope together are organized in T=4 symmetry (Frolov, 1996). The viral particle contains a single genomic RNA of 11-12kb complexed with 240 molecules of basic capsid protein surrounded by a lipid bilayer (Frolov, 1996). The genome is composed of domains encoding non-structural (NS1-NS4) and structural proteins (C, E1, E2, E3 and 6K). The 5'-terminal domain encodes the nonstructural replicase proteins, while the 3'-terminal domain produces the structural proteins of the virus (Strauss, 1994). The nonstructural proteins are translated from genomic RNA and the structural proteins are translated from subgenomic RNA (Strauss, 1994). The genome is nonsegmented, possesses a 5'methyl guanosine cap and a 3' polyadenylated tail (Griffin, 2007), making it truly mRNA-like. Presumably to strike an appropriate stoichiometry of viral non-structural to structural proteins, two species of mRNA-like genomic material are present in alphavirus infected cells; genomic (42S) and subgenomic (26S), respectively (Chang, 1987)

Viral Proteins

The proteins produced by the virus are used as structural elements and to mediate in cell assisted viral replication. There are two different polycistronic mRNAs that encode the viral proteins. 42S mRNA is packaged into mature virions and acts a message for nonstructural proteins while the 26S mRNA encodes the structural proteins (Chang, 1987). The lipid bilayer found within the virion envelope is embedded with copies of viral encoded glycoproteins

(Strauss, 1994). The lipid bilayer of the virus is produced using the host cell membrane. The glycoproteins found in the lipid bilayer undergo lateral interactions that are important for virus assembly and budding of the nucleocapsid for virion production (Strauss, 1994).

Viral Protein: Structural Proteins

The structural proteins of the alphavirus include C, E1, E2, E3, and 6K which are all encoded in a polyprotein. Structural proteins give the virus its shape. The shape of the virus is essential for transport within the cell and is critical for viral binding to its cellular receptor. The capsid protein has 2 domains, the N-terminal and the C-terminal. The N-terminal contains 96 residues rich in lysine and arginine giving it a positive charge (Strauss, 1994). The C-terminal begins at residue 97 and functions as a proteinase making it important for interactions during budding of the nucleocapsid (Strauss, 1994). The structural proteins have many functions. According to Strauss et al. (1994), the C protein binds viral genomic RNA and interacts with the cytoplasmic tail of E2 and copies of the C protein to form the nucleocapsid. E1 contains glycosylation sites and serves as a fusion peptide for virion entry into the cell (Griffin, 2007). The E2 glycoprotein is a transmembrane protein with 2 or 3 N-linked glycosylation sites. The primary function of E2 is for virus-specific attachment to the cell and provides epitopes for neutralizing antibodies (Griffin, 2007). E3 serves as a signal sequence for E2 function while 6K is a signal peptide for E1 and important for budding (Griffin, 2007).

Viral Proteins: Non-Structural Proteins

Nonstructural proteins are not included as a part of the virus particle but play an important role in viral replication and infection. Gorchakov et al. (2005), states that genomic RNA is translated by cellular translational machinery to produce viral nonstructural proteins 1 to 4. Nonstructural proteins form replicase/transcriptase and are required for viral genome

replication and transcription of subgenomic RNA (Gorchakov, 2005). The 4 different nonstructural proteins function as polyproteins during infection (Strauss, 1994). Strauss et al. (1994), comments that nsP1 appears to be required for initiation of, or continuation of, synthesis of negative stranded RNA and that nsP1 is the enzyme or a component of the enzyme needed to cap the genomic and subgenomic RNAs during transcription. The N-terminal of nsP2 is believed to be a RNA helicase while the C-terminal functions as a nonstructural proteinase (Strauss, 1994). Nonstructural protein 3 is required for RNA synthesis (Shirako and Strauss, 1994). Strauss et al. (1994), states that nsP4 is the RNA polymerase of the virus and is tightly regulated in infected cells.

Structure: Replication Cycle

Viruses replicate by copying their own genetic information and by producing the mRNA to encode viral proteins. Alphaviruses replicate in both arthropod and vertebrate host cytoplasm, producing a persistent lifelong infection in arthropods while leading to an acute, usually short-duration infection in vertebrates (Strauss, 1994). The viral particle matures by budding through the plasma membrane (Chang, 1987). The efficiency of entry is affected by host cell membrane cholesterol content. Cholesterol is important because alphaviruses enter cells via membrane fusion (Chatterjee, 2002). Membrane fusion begins with the virus binding to host cell membrane receptors. According to Frolov et al. (1996), upon entry into the cell, the genomic RNA serves as mRNA for translation of viral nonstructural proteins required for initiation of viral RNA amplification. Alphaviruses are able to interact with host cell proteins. The ability to adapt to host cell proteins may give alphaviruses the ability to shutdown host protein and mRNA synthesis (Gorchakov, 2005) consume NAD energy stores (Ubol, 1996) and induce the

malfunction of ATPase (Ulug, 1989). By stopping these processes and weakening the host cell, alphaviruses become efficient at replication and infection.

E1 and E2 glycoproteins, C protein, and small membrane-embedded proteins are the final products of replication (Frolov, 1996). Completed nucleocapsids are transported to the plasma membrane for interaction with viral glycoproteins allowing the enveloped virus to bud (Frolov, 1996). Once replication is complete, the virus will bud out of the infected cell. Because alphaviruses require a viral envelope for entry into a cell, they also require one to exit the cell. This envelope protects the viral proteins and contains the receptors needed to infect subsequent cells. The virus must use the host cell membrane to bud out and carry its genetic information.

Vectors

Vectors are any living organism that can carry and transmit an infectious antigen. The vector will carry the infectious agent from one host to another therefore maintaining the cycle of transmission. The alphavirus uses different species of insects as vectors and different hosts including birds, small rodents, horses, and humans. For most alphaviruses, mosquitoes are the common invertebrate hosts while birds and mammals are the common vertebrate host. Yet, other hematophagous arthropods and fish appear as amplifying and reservoir hosts (Griffin, 2007). Dead-end hosts tend to be large mammals due to their development of severe or fatal disease infections (Griffin, 2007). Studies have shown that avian hosts may be responsible for the global spread of alphaviruses (Strauss, 1994).

Transmission

The cycle typically begins with an insect biting an infected animal; also known as a blood meal. Griffin et al. (2007), states that the primary mode of alphavirus transmission to vertebrates is via the bite of an infected mosquito. The infectious antigen then replicates within the insect

making its way to the salivary glands. It is essential for virus to spread within an insect and reach the salivary glands to ensure the maintenance of the cycle of transmission (Woodward, 1991). The glycoprotein E2 spreads to the salivary glands by binding to and infecting midgut epithelial cells in the mosquito (Woodward, 1991). Dissemination to the salivary gland is dependent on ambient temperatures; higher temperatures can accelerate the transmission cycle (Griffin, 2007). Once it has reached the salivary glands, the insect is then able to infect birds and mammals by obtaining another blood meal.

The infectious agent can also be transmitted in other ways. Equines infected with Venezuelan Equine Encephalitis virus (VEEV) have been shown to shed virus in nasal, eye, and mouth secretions as well as in urine and milk (Griffin, 2007). Eastern Equine Encephalitis Virus (EEEV) has been found to reside in feather follicles in infected pheasants (Griffin, 2007). While the most common form of viral spread occurs via bloodmeal, viral shedding is an important factor in the spread of infection from one horse to another. Equines are considered as dead-end hosts for EEEV since the virus does not replicate to a sufficiently high titer to allow subsequent acquisition by the mosquito vector. However, VEEV does replicate to a high titer in equines. Therefore, equines may serve as a reservoir for the mosquito vector. In the case of VEEV, but not EEEV, viral shedding in secretions is suggested to be a possible route of spread for the infection (Griffin, 2007).

Virulence

Dubuisson et al. (1997), states that age of the animal, site of inoculation, dose, and viral strain are determining factors in susceptibility to virus-induced encephalitis. The virulence of alphavirus is easily maintained by inducing substantial plasma viremia in their amplifying hosts and those susceptible to disease (Griffin, 2007). This susceptibility is what allows mosquitoes to

be such sufficient vectors for alphaviruses. Alphaviruses can sustain the required viremia due their efficient production of virus at a primary site of replication, delivery of virus into the vascular system, and the slow clearance from the blood (Jahrling and Scherer, 1973). The ability to enter the central nervous system also allows alphaviruses to sustain a high virulence (Griffin, 2007). Neuroinvasiveness and neurotropism both contribute to virulence and are influenced by different molecular characteristics of the virus (Griffin, 2007).

Groups of Disease Causing Alphaviruses

EEEV: Classical Epidemiology

Equine encephalitis viruses have evolved over many years and have been studied extensively. Eastern Equine Encephalitis virus (EEEV) is the most virulent of all encephalitic alphaviruses (Griffin, 2007). It was first isolated in 1933 from the brains of infected horses in New Jersey, Virginia, and the costal areas of Delaware and Maryland (Tenbroeck, 1935). In 1938, 30 children living near infected horses developed fatal encephalitis; this was the first year where human cases were tied to an equine outbreak (Tenbroeck, 1935). Birds are the primary reservoir host even though they remain asymptomatic with a prolonged viremia (Komar, 1999). EEEV is enzootic in North America from New Hampshire southward along the Atlantic coast, Gulf coast to Texas, and in the Caribbean and Central America (Griffin, 2007). Forest-dwelling rodents, bats, and marsupials may provide an additional reservoir in Central and South America (Komar, 1999). Outbreaks tend to be every 5 to 10 years and are associated with seasons of heavy rainfall and higher temperatures (Griffin, 2007). Outbreaks among horses and humans tend to appear from July through October in North America yet can occur year round in South America and the Caribbean (Griffin, 2007). Males and females are equally susceptible to disease while children under 10 years old are most susceptible (Deresiewicz, 1997). The case-fatality

rate was determined to be 30 to 40 percent in the most recent studies of humans (Deresiewicz, 1997).

EEEV: Molecular Epidemiology

The antigenic differences between North and South American strains are distinguished by their reactivity with monoclonal antibodies to the E1 glycoprotein (Roehrig, 1990). Comparisons of their gene sequences show that the North and South American strains evolved independently over the past 1,000 years (Griffin, 2007). On the nucleotide level, North American and South American subtypes differ by 25 to 38 percent (Aguilar, 2008). The North American isolates are highly conserved and have a low yearly substitution rate of $1.6x10^{-4}$ bases per genome (Brault, 1999). However, the South American isolates comprise several genotypes and evolve at a higher yearly rate of $4.3x10^{-4}$ bases per genome (Weaver, 1994). Oligonucleotide fingerprinting and nucleic acid sequencing analyses confirm this finding by demonstrating remarkable genetic conservation among North American variety strains and genetic heterogeneity in strains of the South American variety (Strizki, 1996).

WEEV: Classical Epidemiology

In the summer of 1930, Western Equine Encephalitis virus (WEEV) was isolated from the brains of two horses and later used to infect other experimental animals (Meyer, 1931). It is widely distributed in the western plains and valleys of the United States, Canada, and South America (Griffin, 2007). WEEV epidemics in horses, humans, and emus have occurred in the western United States (Griffin, 2007). WEE virus is transmitted enzootically in western North America among passerine birds primarily by the mosquito *Culex tarsalis* (Weaver, 1997). The epizootic vector in South America is *Ae. albifasciatus* with rabbits serving as a possible amplifying host (Weaver, 1999). The risk for developing WEEV increases in rural areas and

during the months of July and August in North America (Griffin, 2007). Older children and adult males are 2 to 3 times more likely than females to develop disease (Griffin, 2007). Clinical disease is most common in the very young and those over 50 years old (Griffin, 2007). The overall case-fatality rate in humans is 3 percent but increases to 8 percent for those over 50 (Griffin, 2007).

WEEV: Molecular Epidemiology

The WEEV complex includes 5 strains in addition to WEE found in the New World: Highlands J, Fort Morgan, Buggy Creek, and Aura with the sixth virus, Sindbis, found in the Old World (Weaver, 1997). All 6 have different ecologic niches and vary in virulence (Griffin, 2007). It has been shown using phylogenetic data that most of the New World viruses in the WEE antigenic complex descended from an ancestral alphavirus due to a recombination event. This event created a recombination of the E2 and E1 envelope protein genes from a Sindbis-like virus with genes from an EEEV-like ancestor (Powers, 2001). WEEV isolates from South America are shown to have a monophyletic lineage with nucleotide identity greater than 90 percent within the E2/6K/E1 coding region when compared to isolates from California, Texas and Montana (Zacks, 2009). Within California alone, separately evolving lineages have been indentified among the central valley and southern part of the state (Kramer, 1999).

VEEV: Classical Epidemiology

The last of the equine encephalitis causing alphaviruses is VEEV. Venezuelan Equine Encephalitis has been a significant human and equine pathogen for much of the past century with recent epidemics indicating that VEEV is still a serious public health threat with a potential to become a biological warfare threat (Paessler, 2003). The complex includes 6 subtypes: VEE, Everglades, Mucambo, Pixuna, CAB, and Rio Negro (Calisher, 1985). The first isolation of

VEEV occurred in 1936. The brains of horses in Colombian and Guajra regions of Venezuela were used to isolate the virus (Griffin, 2007). The first human case was reported in a lab worker (Casals, 1943). Weaver et al. (2004), states that wild birds are susceptible but believes that mammals are the most likely reservoir hosts. Horses are important amplifying hosts during epizootics and potential for an epizootic to occur is related to mutations that increase the infectivity of *Oc. taeniorhynchus* (Sudia and Newhouse, 1975). Heavy rainfall, which can increase mosquito vectors in cattle ranching areas of Venezuela, causes outbreaks every 10 to 20 years (Rivas, 1997). Equine mortality rates are estimated to be between 19 and 83 percent, with infrequent human deaths and neurological disease occurring between 4 and 14 percent of cases (Weaver, 1999).

VEEV: Molecular Epidemiology

The Venezuelan Equine Encephalitis virus complex is comprised of 13 subtypes and varieties. Many methods have been used over the years to determine genetic differences among the strains. T1 oligonucleotide fingerprinting was initially used in many VEEV strain studies while serological assays followed. A distinct advantage appeared for molecular approaches over serological methods when determining epidemiological relatedness and patterns of alphaviral evolution (Weaver, 1999). Monoclonal antibody work and nucleotide sequencing were later used to determine which strains lead to outbreaks. Isolates from major outbreaks belong to specific serotypes that are designated as antigenic subtype I with varieties A, B, and C (Weaver, 1999). It was later discovered that IA and IB were not different and therefore, were grouped together. Epizootic viral subtypes include IAB, IC, and IE while enzootic subtypes include ID, IE, and IF (Griffin, 2007). The epidemic/epizootic VEE emerged from ID progenitors on more

than one occasion and this was shown to be true when IAB, IC, and ID viruses were found to share a recent, common ancestor (Weaver, 1999).

Genomic sequencing of prototype strains of subtypes IAB, IC, and ID were used to verify their genetic similarity (Weaver, 1999). Furthermore, the epizootic phenotype has evolved independently indicating that, even if extinction events occur, the potential for new outbreaks remains (Weaver, 1999). Subtype II was used to show the distinctness of the Everglades virus strain, which is more closely associated with the variety IAB/C/D viruses than the IE and IF varieties (Weaver, 1999). A monophyletic group describes the subtype III viruses, which are more closely related to most subtype I viruses than the IF virus isolates (Weaver, 1999). While the divergence among the VEEV strains is highly developed, analysis shows that the E2 glycoprotein has the greatest divergence while the C-terminal region of nsP3 is the most conserved (Meissner, 1999).

Viral Antigens of Alphaviruses

Hemagglutination, virus attachment and penetration are the biological activities possessed by the glycoproteins E1 and E2 (Pence, 1989). As the viral antigens of alphaviruses, they are responsible for many of the immune responses seen in an infected host. These glycoproteins carry antigenic determinants recognized by neutralizing and nonneutralizing antibodies (Flynn, 1988). When the virion enters the host cell, viral antibodies are stimulated for production that are of varying specificities and will act together to eliminate the infection (Roehrig, 1988). However, an antibodies' ability to inhibit infectivity can be hindered by the epitopes conformation (Roehrig, 1982a) and the accessibility of that epitope on the virion surface (Braciale, 2007). Hunt et al. (1985) confirms this in finding that antibodies reactive with E1 are very sensitive to conformation, while E2 is less sensitive. The antibody footprint, area of the

antigen that makes contact with the antibody, is between 400 to 1,000 Angstroms (Braciale, 2007).

Immunodominant Antigens (Neutralizing and Nonneutralizing)

Immunodominant antigens are subunits of the antigenic determinant that are most easily recognized by the immune system. They induce a more pronounced immune response than other epitopes (Kuby, 1996). Hunt et al. (1985) concluded that host protection via nonneutralizing antibody is not as efficient as that of neutralizing antibody. A majority of E1 epitopes are located on the surface of infected cells or in acid exposed virions (Griffin, 1995). E1-reactive monoclonal antibodies (Mab) have been studied for several alphaviruses including SINV, VEE, and WEE (Strauss, 1994). Antibodies to E2 have biological activities that include neutralization of virus infectivity and fusion inhibition (Roehrig, 1982). While the E1 protein tends to only produce nonneutralizing effects, studies of alphaviruses have repeatedly shown that nonneutralizing antibodies can be protective (Hunt, 1985). In vivo protection by nonneutralizing Mabs is done by lysis of virus-infected cells via complement-dependent, antibody-dependent, and cell-mediated lysis (LeFrancois, 1984). Griffin et al. (1995) showed that E1 Mabs were able to protect in vivo against other alphaviruses. This lack of neutralization by E1-specific antibodies is likely due to the fact that without a conformational change, E1 antibodies are unable to reach their epitopes such as when hemagglutination occurs causing a decline in pH (Griffin, 1995). Roehrig et al. (1982) states that another reason for weak neutralization by E1 antibodies are that they can only bind to mature forms of E1. Yet, E2 antibodies can react with mature E2 and its' cellular precursor, PE2.

Protective Antigens (Neutralizing)

Antibodies produced during a viral infection to envelope proteins, core proteins, and proteins associated with the viral genome can play a protective role (Kuby, 1996). Glycoprotein E2 antibody is more often neutralizing than antibodies reactive with E1 (Strauss, 1994). E2 antibody activities include hemagglutination inhibition (HI), neutralization of virus infectivity, and blocking of virus fusion (Roehrig, 1982a). The capsid protein has been determined to be a target for class I-restricted alphavirus-specific cytotoxic T lymphocytes that are important in viral neutralization (Linn, 1998). Neutralizing antibodies are defined as those that bind to surface structures on a virion causing interference with the uptake and uncoating of a virus (Wang, 1991). More specifically, the antibody neutralizes the antigen by binding to the structure on the virus that interacts with a receptor on the cell surface, thus directly blocking the virus from interacting with its receptor (Wang, 1991). Griffin et al. (1995) believes that neutralization also works by inhibiting viral budding by restoring cellular Na+ K+ ATPase function and K+ flux. Roehrig et al. (1988) determined that E2c and E2h epitopes elicit neutralization via blocking cell attachment and receptor binding. Neutralizing epitopes elicit antibodies with Fc regions which Griffin et al. (1995) stated are required by many Mabs for efficient neutralization. When neutralized by an antibody with an Fc region, the virus is cleared by a Fc receptor-bearing cell (Roehrig, 1988).

Strain Specific Marker Antigens

WEEV

The WEEV E1 glycoprotein has antigenic specificities and biological activities that are similar to Sindbis virus (Trent and Grant, 1979). Hunt et al. (1985) identified 8 epitopes on WEEV concerning glycoprotein E1. E1a1 and E1b1 are both involved in passive protection.

These specific epitopes are cryptic on the native virion with another, E1d1, being only partially exposed (Hunt, 1985). Mendoza et al. (1988) determined that epitopes on the E1 glycoprotein of WEEV possessed a mixture of neutralizing and hemagglutination-inhibiting Mab activities. As is the case with Sindbis, anti-E1 antibodies were used in mice that were later protected from challenge with a lethal dose of WEEV (Hunt, 1985).

VEEV

The major antigenic determinants for VEEV subtypes and varieties are found on the E2 glycoprotein (Wang, 2005). Twelve epitopes have been identified for the surface glycoproteins of VEE viruses. Some of these epitopes include critical neutralization domains for E2 (Roehrig, 1988). This domain was located between the sequence E2-182 and E2-209 (Johnson, 1990). The conserved epitopes of VEEV include E2c and E2h. Anti-E2c antibodies were determined to bind very well to viruses of IAB and IC but only IC showed the most efficient neutralization (Roehrig, 1985). Monoclonal antibodies for E1 and E2 are nonneutralizing and neutralizing but E2 is the most important for control of VEEV (Mendoza, 1988). Roehrig et al. (1985) found that the epitopes of E2 are conformationally stable even after denaturation whereas the same cannot be said for E1. The only epitope that lost any reactivity was E2d (Roehrig, 1985). A final statement about the importance of E2 antibody reactivity was shown by a Griffin et al. (1995) study where anti-E2 limited virus replication in vivo.

Immune Protection against Alphaviruses

Immune Response

Initiation of an immune response requires many systems within the body to work together. Innate immune cells must recognize the pathogen so the adaptive immune system can pinpoint the specificity of that pathogen to mark it for death. Recovering from encephalitis

CHAPTER 2

SCREENING OF COMMERCIAL ANTIBODIES FOR DIFFERENTIAL BINDING ACTIVITY, SPECIFICITY, AND CROSS-REACTIVITIY AMONG VENEZUELAN, WESTERN, AND EASTERN (NORTH AMERICAN AND SOUTH AMERICAN) EQUINE ENCEPHALITIS VIRUSES [1]

[1]Aitcheson, T.A.W. and D. J. Hurley. To be submitted to *Veterinary Microbiology*.

Abstract

The screening of monoclonal antibodies for binding specificity and selectivity has been attempted using multiple methods. Flow cytometric analysis is a non-subjective and rapid screening method. This study used flow cytometry to screen 15 antibodies for their specificity and binding strength to cells expressing antigens from Venezuelan, Western, and Eastern (both North American and South American strains) equine encephalitis viruses. An adherent avian cell line was infected with each of the strains. Cells were fixed and stained with primary antibodies and a fluorescent conjugate. Four antibodies were chosen to advance to quantitative testing that yielded the highest mean fluorescence intensity for one virus each. These antibodies yielded minimal cross-reactivity to other viruses. The use of flow cytometry allowed for the definitive determination of different levels of fluorescence each antibody generated between infected and non-infected cells in a rapid and repeatable fashion.

Introduction

Antibodies are powerful tools for the identification of viral antigens in either infected samples or in infected cells from culture. There are quite a variety of antibodies that are commercially available for equine encephalitis viruses (EEV). In general, these antibodies (as well as several that have not been released for commercial distribution) have been used to attempt to detect differences among wild-type EEV strains for the purpose of outbreak identification and disease control (Roehrig and Bolin, 1997) Antibodies have also been shown to react differently among strains found in the same family of viruses (Midgley, 2008). Some EEV antibodies have also been used for attempting to assess the immune responses induced by EEV infections (Calisher, 1988). Because outbreaks of EEV are quite common and very unpredictable, and some strains of EEV can cause severe disease in humans, the development of vaccines against EEV for use in horses is very important. To aid in the development of a new, more effective vaccine, better methods for quality control of antigen production and more precise methods of vaccine formulation are desirable. To contribute to the production of safe and efficacious vaccines, the antibodies and other reagents used to test vaccine components and mixtures must yield specific and robust results. Current methods for using antibodies in quality control include ELISA assays and the use of immune-fluorescence microscopy measurements. These studies conducted here will assess the use of commercial monoclonal antibodies and flow cytometry to determine if they will add a greater level of specificity and the capacity to make quantitative measurements of the target proteins for the development of a new generation of assays.

Classical fluorescent microscopy methods for screening antibodies to differentially identify related viral strains are subjective (Givan, 2001). Flow cytometry provides a rapid,

reproducible and quantitative measure of binding level and differential binding of antibodies to cellular targets. Flow cytometric analysis also provides a method for the rapid screening of antibodies without the subjectiveness of immunofluorscence microscope assays (Givan, 2001). Further, quantitative flow cytometry offers a simple and rapid method for objective quantization of antigens (Shapiro, 2003). This is extremely important when screening multiple antibodies that are to be used to discern between similar virus proteins based on binding efficacy and differential binding. By using flow cytometry, we were able to screen 15 different antibodies and choose those with the highest level of binding and greatest binding specificity for use in further development of assay to assess vaccines on the manufacturing level. The need for this assay is critical to the quality control in the process of vaccine manufacture.

An understanding of epitope binding and antibody specificity is based on good immunological and molecular level information about the antibodies and antigens involved. We used the available information on equine encephalitis viruses to explain our findings and to apply the results generated to choose which antibodies were best suited for use in potential assays to discriminate among VEEV, WEEV and EEEV. Our understanding of this area also allowed us to determine why certain errors occurred relative to the experimental methods. Therefore, through the use of flow cytometric analysis, antibodies were chosen that not only produced strong and distinct binding against VEE, EEE and WEE but that also have a reproducible binding pattern.

Materials and Methods

Cultured Cells

The initial cell line used for testing was an adherent avian cell line (ACL-1) that could be grown as a suspension culture or as an adherent monolayer (proprietary cell line). Cells were

grown in a commercially available serum free medium (SAFC) that was supplemented with 2.5mM L-Glutamine (Gibco catalog # 25030) and 10 micrograms per ml of gentamicin sulfate (SAFC catalog # G1264) in T-150 flasks (Corning catalog # 430823). The cells were incubated at 37°C (Celsius) with 5% CO_2. The cells were infected when the monolayer cultures were 96 hours old. The infection cycle was completed without changing the culture medium. At time of infection, the multiplicity of infection that would produce the most viral antigen was not known for these strains. Therefore, a calculated multiplicity of infection, 10.0 $TCID_{50}$ per cell, was used to ensure a high level of viral proteins would be produced. For this cell line, the average cell count for 96 hour old monolayers in T150 flasks is $3x10^7$ cells per milliliter. This is the cell count used at the time of infection. To ensure that the cells used for the uninfected control were in the same metabolic state, the medium was not changed. The infected cells were harvested 24 hours post infection showing greater than 85% cytopathic effect.

A second cell line was also used to assess the antibodies against EEV. This was the mammalian cell line, Vero (African Green Monkey Kidney origin line). The Vero cells were seeded in T-150 flasks at a density of $2.5x10^5$ cells per milliliter and incubated at 37°C with 5% CO_2 in Minimal Essential Medium containing 5% fetal bovine serum and 30 micrograms per milliliter of gentamicin. Cells were 48 hours old at infection. Fresh media was added at the time of infection. The flasks were harvested at 48 hours post infection. The uninfected cells were harvested when their monolayers reached 96 hours old, representing the same average culture duration as infected cells.

Virus Strains

Four different viral type strains were used for testing in this study. The strains used were VEEV, WEEV, North American EEEV, and South American EEEV type strains. These strains

are proprietary. The titers, in 50% infectivity units ($TCID_{50}$), used for our infection trials with these cells were: VEEV – $10^{8.9}$, WEEV – $10^{8.2}$, NA EEEV – $10^{7.7}$, and SA EEEV – $10^{7.4}$.

Monoclonal Antibodies and Secondary Conjugates

Fifteen different commercially available monoclonal antibodies were tested in these studies on the ACL-1 cells. Each had a specificity that was assigned by their manufacturer. (Table 2.1) Some were identified as recognizing multiple equine encephalitis virus strains, and others were claimed to recognize only individual, specific strains. Two secondary conjugates were tested in these studies. They were Sigma Anti-Mouse IgG (whole molecule)-FITC, antibody produced in rabbit (catalog # F9137, lot # 059K4871) at a 1:200 dilution, and the second was a Jackson ImmunoResearch Fluorescein (FITC)-conjugated AffiniPure Goat Anti-Mouse IgG (subclasses 1+2a+2b+3), Fc, Fragment Specific (minimal cross-reaction to Human, Bovine, and Rabbit Serum Proteins) (catalog # 115-095-164, lot # 85770) at a 1:24 dilution. The Sigma conjugate was marketed at a protein concentration of 12.6mg/ml, while the Jackson conjugate was marketed at a protein concentration of 1.5mg/ml. To make the assay easier to process, both conjugates were diluted to 62ug/ml so that 100µL could be used for each conjugates during staining with equivalent antibody concentration. All primary and secondary antibodies were diluted in phosphate buffered saline (PBS) (produced by Merial Limited) that contained 0.5% bovine serum albumin (Fraction V) (Sigma catalog # A7906, lot #120M1340V). All primary antibodies were tested at 1:100, 1:400, and 1:1600 dilutions. The quantity (mass) of antibody in each assay was not specifically normalized between monoclonal products. Antibodies 3, 4, 11 and 14 were tested using Vero cells at the same dilutions and with the same conjugated second antibody (Sigma) as described for the ACL-1 cells.

Fixative Agents

Preliminary testing included 2 fixative agents. 85% acetone was prepared by the addition of de-ionized water to absolute acetone. A stock of 2% para-formaldhyde (PFA) was prepared in PBS by bringing the PBS to a boil and dissolving the paraformaldhyde crystals. This solution was allowed to cool before use. The acetone fixative was stored at -20°C. The PFA fixative was prepared fresh immediately before use. Cells fixed with acetone were treated for 20 minutes at -20°C. Those treated with PFA were placed on ice for 30 minutes. It was determined that the more practical fixative agent for these experiments was 85% acetone. The decision was based on ease of use, since PFA must be prepared fresh, and that there was little difference seen in background between the fixatives. This data is not shown.

Screening of Antibodies

Ninety-six well U-Bottom polypropylene plates (USA Scientific catalog # 1830-9610) were used as vessels for the staining of cells. Cells were trypsinized with 0.05% porcine Trypsin EDTA (SAFC catalog # 63055, lot # 7C0278) to remove them from the T-150 flasks, prepared as single cells, and then centrifuged to remove the trypsin solution. The cell pellet was suspended in PBS. A cell count was not performed before the cells were placed in their respective wells, but the cells at confluence in T-150 flasks yield a consistent number of single cells from this line. All 15 antibodies were screened for their binding to cells infected with each of the 4 EEV strains. Each of the secondary conjugates was tested in combination with the 15 primary antibodies and cells infected with each of the 4 EEV strains. To achieve staining, cells were placed on ice for 1 hour with the primary antibody. The cells were washed 3 times with PBS containing 0.5% BSA between primary antibody staining and secondary conjugate staining. Once the secondary conjugate was applied to each sample, the cells were placed on ice in the dark for 30 minutes.

After incubation with the secondary antibody conjugate, the cells were washed 3 times. 200µL of PBS containing 0.5% BSA was added to each sample well and the contents thoroughly mixed before transfer to flow cytometer tubes containing 1.5ml of PBS containing 0.5% BSA. During testing, tubes were kept at 4°C in the dark.

Flow Cytometric Analysis

The flow cytometer used was a **Partec CyFlow Space**®. The software used for analysis was **FloMax**® version 2.70. The software was used to assign cell population gating based on the forward angle scatter (FSC) versus orthogonal side scatter (SSC) plots representing cell size (diameter) and internal granularity, respectively. Once a gate containing singlets of whole cells was applied, an analysis of FSC versus Green Fluorescence (detector 1) was made and the Mean Fluorescence Intensity (MFI) of each sample was measured. MFI is proportional to the binding affinity of antibody under saturation staining conditions (Shapiro, 2003). To compensate for the background present, the MFI of uninfected cells stained with the same antibody and secondary conjugate was subtracted from the infected cells stained with that combination. The antibody specific MFI value for each antibody (and conjugate) was determined using this method.

Results

Determination of Lowest Background Producing Secondary Conjugate for Flow Cytometric Analysis

Overall, each of the conjugated secondary antibodies was useful in demonstrating differential binding of the primary antibodies. Further, our testing indicated that the Sigma conjugated secondary antibody produced a higher specific MFI value for many of the monoclonal antibodies tested when reacted with cells infected with the appropriate EEV. The conjugate from Jackson Laboratories is commercially distributed at a lower antibody

concentration than the conjugate prepared by Sigma. Therefore, the Sigma conjugate was used for the remaining studies to reduce costs and in an attempt to optimize signal.

Antibody Binding Activity and Specificity for VEE, WEE, NA EEE, and SA EEE

Of the fifteen monoclonal antibodies tested, one antibody was selected for each strain that yielded the highest and most stable MFI for EEV. Four antibodies emerged as preferred primary antibodies for further study. The 1:100 dilution of each of these antibodies yielded the highest overall MFI signal with infected cells. It is important to remember that the following results are based on only a single replicate of specific MFI values observed for each primary, secondary, and dilution combination tested. A series of three dilutions were used for this study. This provided a measure of the level of binding saturation for each antibody on uninfected cells and cells infected with each viral construct. From this, we were able to estimate the level of antibody saturation at each specific dilution of each of the antibodies relative to the experimental conditions. This allowed us to work under conditions that yielded a good estimate of differential affinity of each antibody for the viral antigens.

Mab 11 yielded the highest specificity and signal strength for cells infected with VEEV (49.40, 21.41, and 7.23 at 1:100, 1:400. and 1:1600, respectively). (Figure 2.1) The mean MFI for the other fourteen antibodies tested against VEEV infected cells were as follows: -1.28, 0.50, and -0.06 at dilutions of 1:100, 1:400, and 1:600, respectively. Antibody 11 had a considerably higher MFI at all 3 dilutions for VEEV than for the other antibodies evaluated in this study. Considering the decreasing specific MFI values of antibody 11 from 1:100 to 1:1600, it appears that antibody saturation occurs at 1:100. The mean MFI for antibody 11 on uninfected cells was as follows: 1.58, 0.61, and 0.27 (1:100, 1:400, and 1:1600, respectively). The standard

deviations (SD) for the mean MFI of both, the other fourteen antibodies tested and Ab 11 on uninfected cells were minimal

Mab 14 yielded the highest MFI for cells infected with the WEEV strain. (Figure 2.2) These MFI values measured were 8.97, 3.09, and 0.55 at dilutions of 1:100, 1:400, and 1:1600, respectively. The highest MFI value was produced at a dilution of 1:100, and MFI values above that of uninfected cells was still observed at a dilution 1:400 of the primary antibody when bound to cells infected with WEEV. Considering the decreasing specific MFI values of antibody 14 from 1:100 to 1:1600, it appears that antibody saturation occurs at 1:100. The mean MFI for the other thirteen antibodies tested against WEEV infected cells were -0.16, 0.58, and 0.07 at dilutions of 1:100, 1:400, and 1:1600, respectively. The SD for the mean MFI of the other thirteen antibodies infected were 2.17, 0.81, and 0.81 at dilutions of 1:100, 1:400, and 1:1600, respectively. These values represent about one-quarter of the mean MFI for antibody 14 on WEEV infected cells at the same dilution.

The nucleotide differences in the genome of North American and South American EEEV have been shown to be between 25 to 38 percent (Aguilar, 2008). Both antibodies 3 and 4 yield highly specific binding for these targeted EEEV strains. The binding of these two antibodies to infected cells resulted in evidence for strain specific differences between the SA and NA members of the EEEV family. Antibody 3 yields a slightly stronger affinity for the NA type strain of EEEV, and antibody 4 yields a slightly stronger affinity for the SA type strain for EEEV. However, both have a strong selective binding to EEEV over VEEV or WEEV. The specificity observed for these two antibodies are detailed below.

Mab 3 had slightly stronger MFI signal for the NA type strain of EEEV strain than the other primary antibodies tested at 1:400 and 1:1600 (Figure 2.3), but was similar to antibody 4 at

a dilution of 1:100. Considering the specific MFI values of antibody 3 from 1:100 to 1:1600, it appears that antibody saturation occurs at 1:400. The mean MFI values for antibody 3 on NA EEV infected cells were 3.66, 3.32, and 2.16 at 1:100, 1:400, and 1:1600, respectively. The mean MFI values observed for the other thirteen antibodies tested on NA EEV infected cells were -0.76, 0.24, and -0.07 at 1:100, 1:400, and 1:1600, respectively. The SD for mean MFI of the other thirteen antibodies tested were 1.88, 0.48, and 0.54 for each dilution. These values represent about one-third of the mean MFI for antibody 3 on NA EEEV infected cells at the same dilution. The mean MFI values yielded for antibody 3 on uninfected cells were 1.21, 0.23, and 0.21 at 1:100, 1:1400, and 1:1600, respectively.

Mab 4 yielded a slightly higher MFI for cells infected with the SA type strain of EEEV strain than the other monoclonals tested in this study. (Figure 2.4) The mean MFI for antibody 4 on SA EEEV infected cells was 1.58, 1.31, and 0.78 at dilutions of 1:100, 1:400, and 1:1600, respectively. These MFI values are the lowest MFI values we considered as virus strain specific for this study. Considering the specific MFI values of antibody 4 from 1:100 to 1:1600, it appears that antibody saturation occurs at 1:400. The mean MFI values yielded for the other thirteen antibodies tested on SA EEEV infected cells were -0.70, 0.11, and -0.09 at dilutions of 1:100, 1:400 and 1:1600, respectively. The SD for the mean MFI of the other thirteen antibodies tested was 1.46, 0.24, and 0.34 at dilutions of 1:100, 1:400 and 1:1600, respectively. These SD values represent one-third or slightly more of the MFI value of antibody 4 on SA EEEV infected cells. The mean MFI observed for antibody 4 on uninfected cells were 0.95, 0.37, and 0.26 at dilutions of 1:100, 1:400 and 1:1600, respectively. The data indicates that there is good selectivity for EEEV, but only a small difference in binding to the SA or NA type strains of EEEV by the antibody 11. Thus, we advanced both antibody 3 and antibody 4 for further testing.

Antibody 11 was excluded from the graphs for antibodies 3, 4, and 14 (Figures 2.2, 2.3, and 2.4, respectively). Antibody 11 yielded specific MFI values that either equaled or exceeded the specific MFI values for the chosen antibodies on their target strains (Table 2.2).

Cross-Reactivity of Antibodies for VEE, WEE, North American EEE, and South American EEE

Focusing on the four antibodies identified as yielding the highest MFI for each strain, 11, 14, 3, and 4, cross-reactivity with the other strains was analyzed. Monoclonal antibody 11 yielded the highest specific MFI values for cells infected with VEEV, higher than for the other EEV viruses tested. (Figure 2.5) Antibody 11 should be used at a dilution of 1:100 or 1:400 to ensure it yields high specific MFI for VEEV infected cells. Based on the cross-reactivity observations, the results for specific MFI on VEEV infected cells, and the differential binding yielded for the mean MFI for the other antibodies tested and for the uninfected cells stained with 11, we believe that antibody 11 should move forward for quantitative testing.

Monoclonal antibody 14 yielded higher MFI values on cells infected with WEEV than with the other EEV viruses tested. (Figure 2.6) This antibody should be used to stain WEEV infected cells at 1:100 to ensure it maintains a high level of specific MFI for WEEV infected cells. Because limited cross-reactivity was observed with the other antibodies selected for WEEV, and the strong differential binding to WEEV observed relative to uninfected cells stained with antibody 14, we believe that antibody 14 should move forward for quantitative testing.

The cross-reactivity of the antibodies tested showed that several are capable of recognizing EEEV. Mab 3 yielded the highest MFI values with cells infected with North American EEEV. (Figure 2.7) This antibody should be used at 1:100 to ensure it maintains its specific MFI value on NA EEEV infected cells. We feel that due to the limited cross-reactivity

and the differential binding intensity and pattern observed between specific MFI for NA EEEV infected cells relative to uninfected cells demonstrates that further testing for its quantifiable characteristics would be valuable.

Monoclonal antibody 4 yielded similar MFI values for cells infected with the North American EEEV strain as did antibody 3 stained against NA EEEV infected cells. (Figure 2.8) This data demonstrates that there is cross-reactivity for antibody 4 on NA EEEV infected cells. This cross-reactivity behavior decreased at 1:1600, but unfortunately so did the differential signal above uninfected cells. This antibody produces little specific MFI values against VEEV and WEEV strains. We believe that while this antibody yields similar specific MFI values for NA EEEV infected cells as antibody 3, the differential binding observed between specific MFI for SA EEEV infected and uninfected cells deems that further testing for its quantifiable characteristics would be valuable. Further testing will allow us to determine that while antibodies 4 and 3 both specifically bind NA EEEV infected cells, more replicates may yield a clearer specificity for the NA and SA EEEV type strains.

Quantification of the Chosen Antibodies Using Vero Cells

The antibodies that appeared to bind each of the EEV strains tested most brightly were tested on Vero cells under the same conditions as were used for ACL-1. The antibodies did not yield as high a level of MFI or as consistent a staining pattern as was observed with the ACL-1 cells. This data is not shown.

Discussion

This study covered the screening of 15 commercially available monoclonal antibodies that were claimed by the manufacturer to be specific for individual Equine Encephalitis Virus (EEV) strains. The strains tested included Venezuelan Equine Encephalitis Virus (VEEV),

Western Equine Encephalitis Virus (WEEV), and Eastern Equine Encephalitis Virus (EEEV). Further, both a North American and a South American strain of EEEV were tested in this study to determine if any of the antibodies could differentiate between these strain variants. Samples were scored on the basis of the mean fluorescence intensity (MFI) of the staining of infected cells relative to uninfected cells. Both staining of specific viral antigens (as assigned by the manufacturer) and cross-reactivity with proteins from other viral strains were scored using the same criterion. The results were collected using duplicate samples for each treatment, and placed in rank order based on their specificity and intensity. The results of the screening assay were used to pick 1 monoclonal antibody (Mab) that best and most uniquely identified each viral type strain used in these studies. These antibodies produced strong binding to cells infected with an individual strain that was measured by the MFI level.

Antibodies 11 and 14 yielded highly specific MFI values for VEEV and WEEV, respectively. Antibodies 3 and 4 yielded specificity for NA EEEV and SA EEEV type strains, respectively. Interestingly, the manufacturer of antibody 3 claims that it is specific for all EEV strains (VEEV, WEEV, and EEEV), but antibody 3 only had specific differential binding to cells infected with EEEV in our trials. These observations demonstrate that these antibodies are effective tools for specifically identifying an EEV type strain, but should be further developed for use in individual assays for determining the level of antigen present in a sample to avoid problems with viral cross-reactivity.

To further strengthen the findings regarding antibody saturation and the correlation between specific MFI values for an individual antibody and it's affinity for one strain over another, testing of a longer dilution series would be required. A more complete dilution profile that included testing 1:10, 30, 100, 300, 1000, 3000, 10,000 and 30,000 with a probit or logit

curve fitting would allow us to calculate the actual saturation concentration for each of the 4 antibodies chosen. Time and resources for this study would not allow for this more detailed testing.

This study has shown that flow cytometry can be used in determining the binding activity and specificity of monoclonal antibodies to discriminate among similar viral strains. A limitation of this method is apparent in attempting to resolve the small differences in MFI seen when comparing the antibodies for North American and South American EEEV in these assays. The specificity of the antibodies utilized and the presence of common epitopes in the surface proteins of the EEEV strains both probably contributed to the poor discrimination between North American and South American strains of EEEV. This too could have lead to the cross-reactivity observed for antibody 4 on NA EEEV infected cells.

Trying to detect the differences in the level of fluorescence produced by the antibodies 3 and 4 would have been beyond the sensitivity of the eye and would have required the use of sensitive image analysis systems and photon counting microscopy equipment with associated software. This would have been more labor intensive and time consuming than flow cytometry. By determining a definite value for the binding activity and specificity of these antibodies, we were able to produce more exact data to ensure repeatability. Producing more replicates will strengthen the findings seen here.

The rapid results yielded from flow cytometric analysis became even more important when ACL-1 was no longer available for the continuation of the work. Therefore, the chosen antibodies were tested for repeatability using similarly infected Vero cells (data not shown). Unfortunately, the results with infected Vero cells were not very similar to those of ACL-1. This may have been due to viral protein production differences between avian and monkey

(mammalian) cells. As vaccine production of the viral antigen is likely to be done in avian cells, the avian cell assessments were more critical. The poorer discrimination of viral antigen by the antibodies measured with infected Vero cells was more easily determined with the use of flow cytometry. Differences in antibody activity and specificity were easier and quicker to see. This alone allowed us to determine that the antibodies would not work in a similar manner. Therefore, no more time was spent on the Vero line as a tool for the assessment of the reproducibility of staining. With regards to the secondary conjugates tested, it is important to note that commercial reagents are often manufactured with levels of quality control that may be lacking. Therefore, screening of subsequent lots of conjugates relative to the current working antibody dilutions will be required to allow adjustments needed to maintain consistency of the assay system.

Table 2.1
Monoclonal Antibody Specificity

Mab Name	Claimed specificity	Isotype
1	WEEV	IgG2a
2	VEEV	$IgG_1$2a
3	EEEV, WEEV, and VEEV	IgG2b
4	NA EEEV and SA EEEV	IgG2b
5	VEEV	IgG2a
6	VEEV	IgG1, 2a
7	WEEV	IgG2a
8	WEEV	IgG2a
9	WEEV	IgG2a
10	EEEV	Unknown
11	VEEV	Unknown
12	VEEV	Unknown
13	VEEV	Unknown
14	WEEV	Unknown
15	WEEV	Unknown

Table 2.2
Antibody 11 Average Specific MFI for All EEV Strains Tested

EEV Strain	1:100	1:400	1:1600
VEEV	49.39	21.40	7.23
WEEV	9.17	2.33	0.58
NA EEEV	9.21	2.61	0.76
SA EEEV	7.54	2.34	0.61

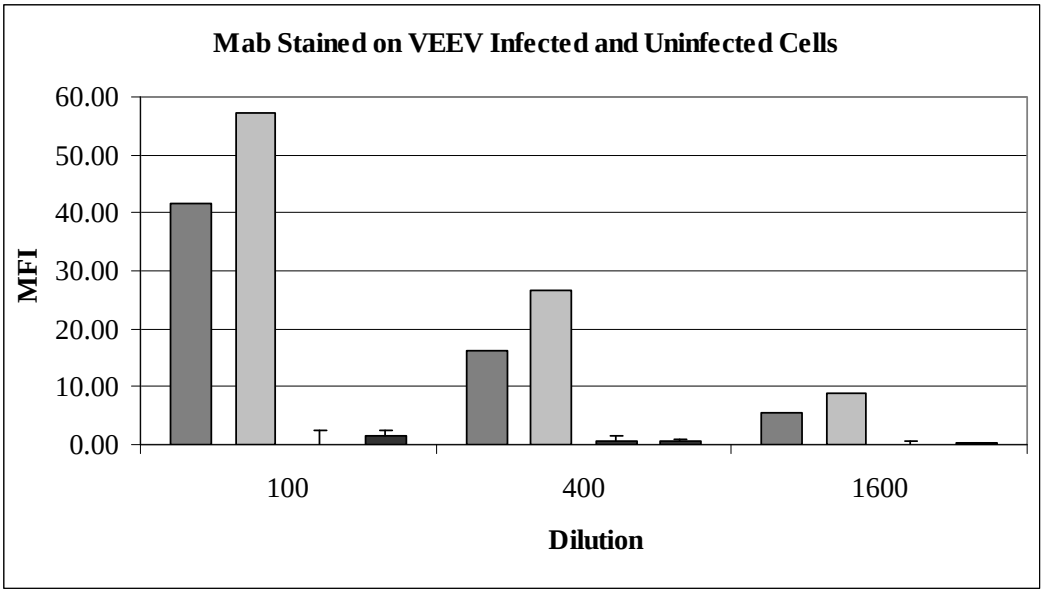

Figure 2.1
Monoclonal Abs stained against VEEV infected and uninfected cells.
For each dilution shown, the 1st and 2nd bars denote Ab 11 with either Jackson or Sigma conjugate. The 3rd bar is the average (+/- SD) MFI for the other fourteen antibodies tested and the 4th bar is the average MFI for uninfected cells.

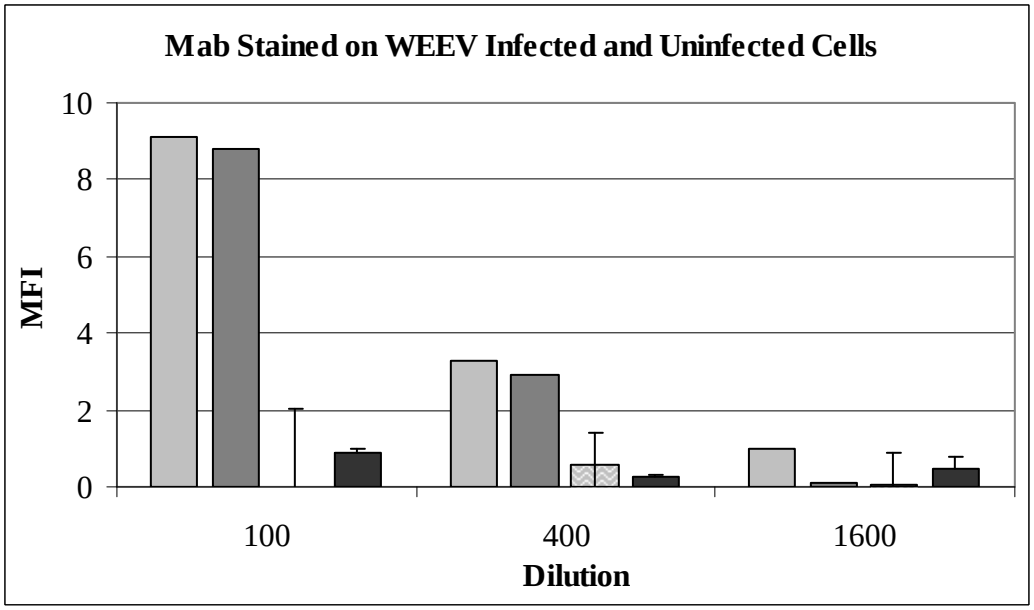

Figure 2.2
Monoclonal Abs stained against WEEV infected and uninfected cells.
For each dilution shown, the 1st and 2nd bars denote Ab 14 with either Jackson or Sigma conjugate. The 3rd bar is the average (+/- SD) MFI for the other thirteen antibodies tested (Ab 11 excluded) and the 4th bar is the average MFI for uninfected cells.

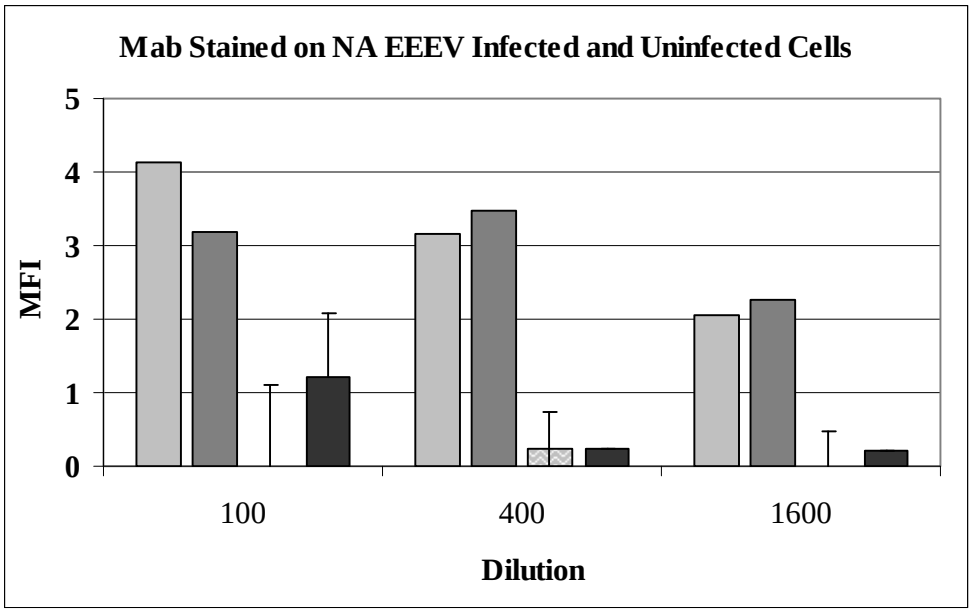

Figure 2.3
Monoclonal Abs stained against NA EEEV infected and uninfected cells.
For each dilution shown, the 1st and 2nd bars denote Ab 3 with either Jackson or Sigma conjugate. The 3rd bar is the average (+/- SD) MFI for the other thirteen antibodies tested (Ab 11 excluded) and the 4th bar the average MFI for uninfected cells.

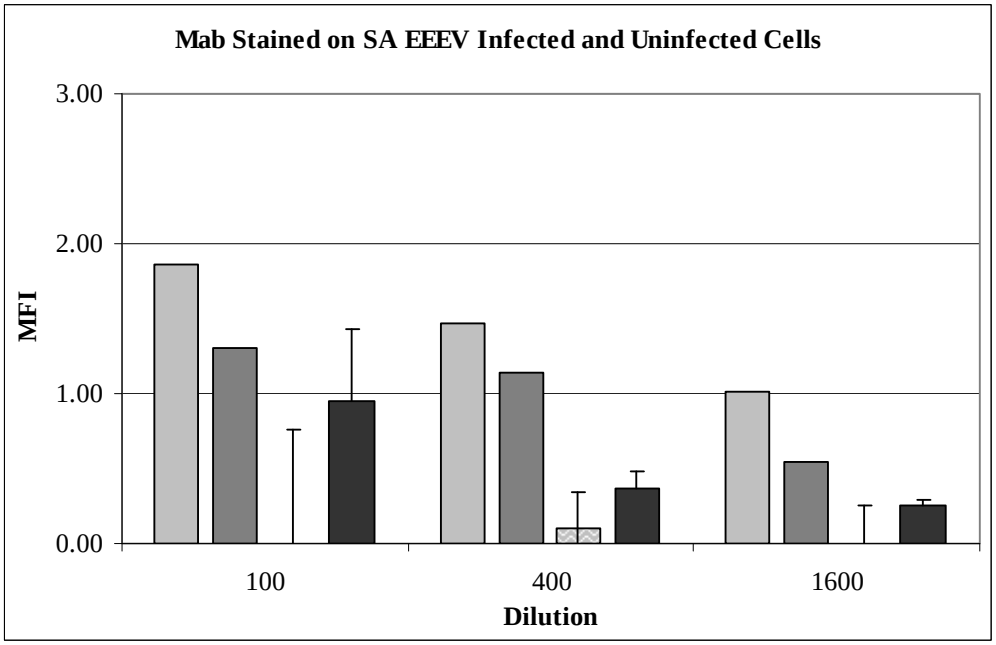

Figure 2.4
Monoclonal Abs stained against SA EEEV infected and uninfected cells.
For each dilution shown, the 1st and 2nd bars denote Ab 4 with either Jackson or Sigma conjugate. The 3rd bar is the average (+/- SD) MFI for the other thirteen antibodies tested (Ab 11 excluded) and the 4th bar is the average MFI for uninfected cells.

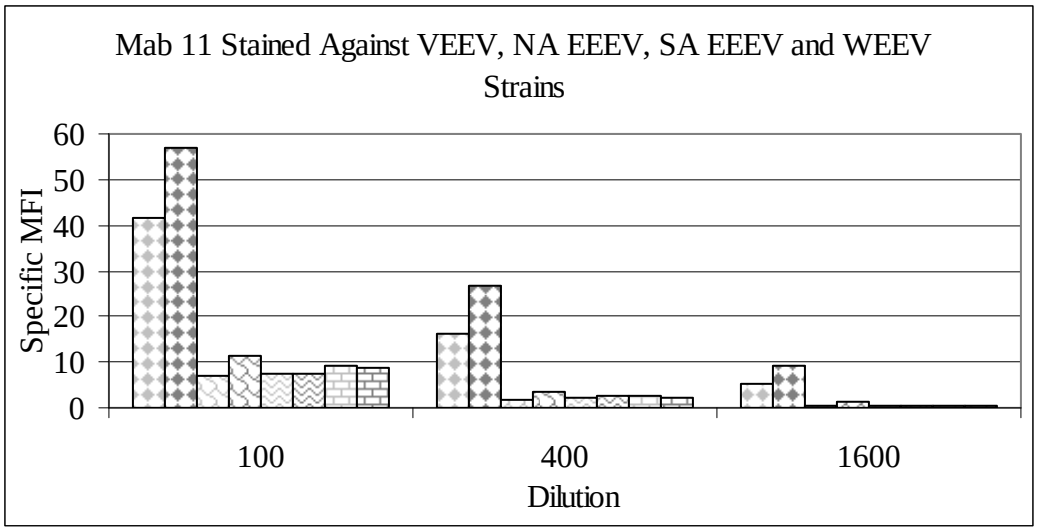

Figure 2.5
Specific MFI yielded on EEV infected cells for Ab 11.
The 1st bar denotes VEEV (J), the 2nd: VEEV (S), the 3rd: NA EEEV (J), the 4th: NA EEEV (S), the 5th: SA EEEV (J), the 6th: SA EEEV (S), the 7th: WEEV (J), and the 8th: WEEV (S) for each dilution represented. (J) = Jackson secondary conjugate. (S) = Sigma secondary conjugate.

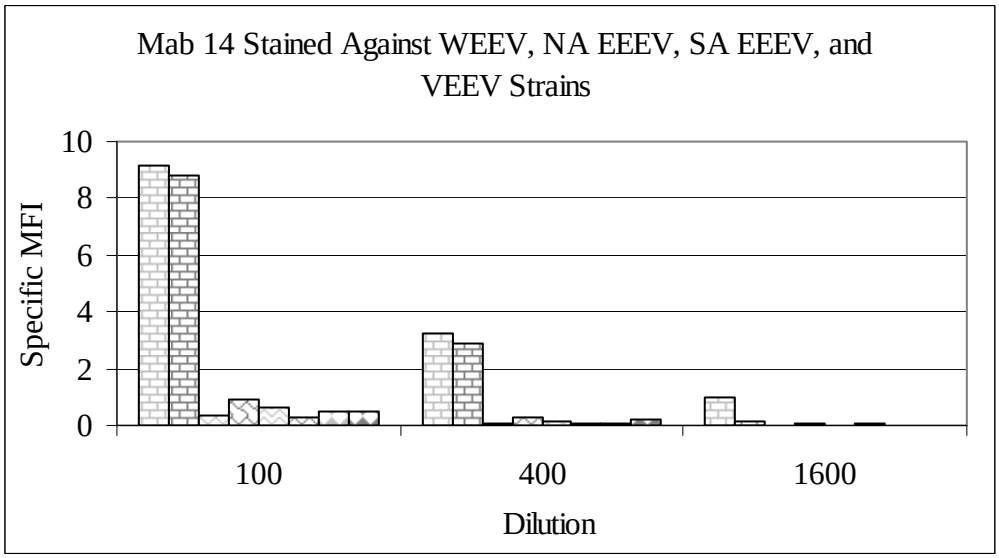

Figure 2.6
Specific MFI yielded on EEV infected cells for Ab 14.
The 1st bar denotes WEEV (J), the 2nd: WEEV (S), the 3rd: NA EEEV (J), the 4th: NA EEEV (S), the 5th: SA EEEV (J), the 6th: SA EEEV (S), the 7th: VEEV (J), and the 8th: VEEV (S) for each dilution represented. (J) = Jackson secondary conjugate. (S) = Sigma secondary conjugate.

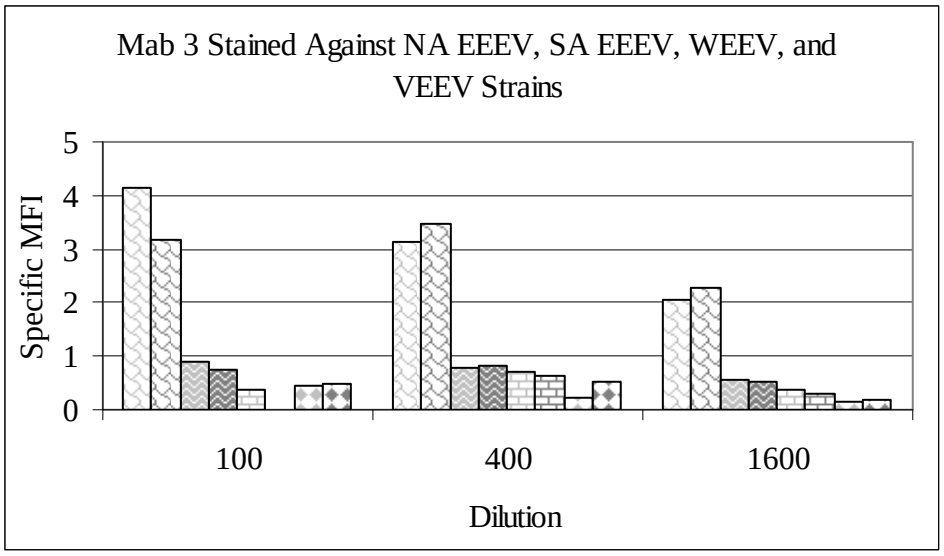

Figure 2.7
Specific MFI yielded on EEV infected cells for Ab 3.
The 1st bar denotes NA EEEV (J), the 2nd: NA EEEV (S), the 3rd: SA EEEV (J), the 4th: SA EEEV (S), the 5th: WEEV (J), the 6th: WEEV (S), the 7th: VEEV (J), and the 8th: VEEV (S) for each dilution represented. (J) = Jackson secondary conjugate. (S) = Sigma secondary conjugate.

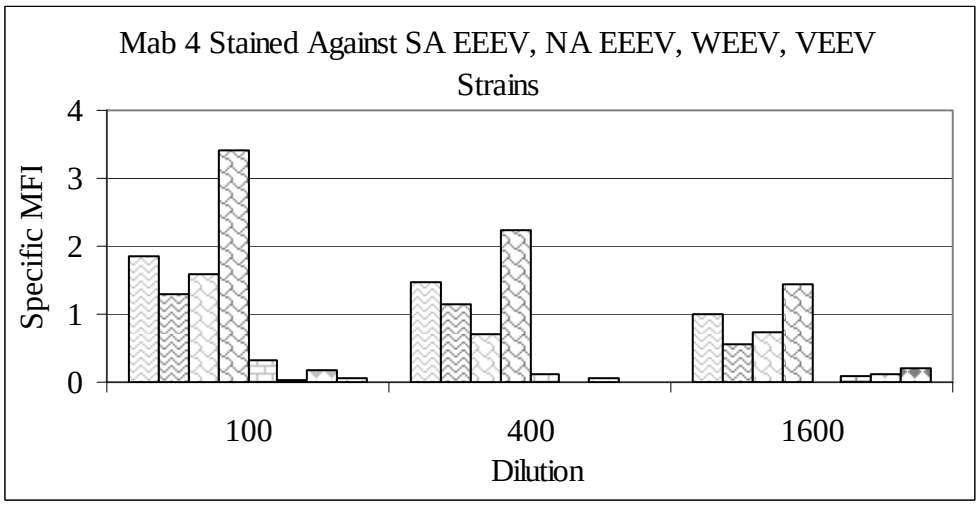

Figure 2.8
Specific MFI yielded on EEV infected cells for Ab 4.
The 1st bar denotes SA EEEV (J), the 2nd: SA EEEV (S), the 3rd: NA EEEV (J), the 4th: NA EEEV (S), the 5th: WEEV (J), the 6th: WEEV (S), the 7th: VEEV (J), and the 8th: VEEV (S) for each dilution represented. (J) = Jackson secondary conjugate. (S) = Sigma secondary conjugate.

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CHAPTER 3

QUANTIFICATION OF BINDING ACTIVITY OF HIGH-SPECIFICITY COMMERICAL ANTIBODIES FOR ASSESSMENT OF VENZUELAN, WESTERN, AND EASTERN (NORTH AMERICAN AND SOUTH AMERICAN) EQUINE ENCEPHALITIS VIRUSES [1]

[1] Aitcheson, T.A.W. and D.J. Hurley. To be submitted to *Veterinary Microbiology*.

Abstract

Four candidate monoclonal antibodies, each proven in screening studies to recognize avian cells expressing antigens from only one chimeric virus expressing specific antigen from Venezuelan, Western, and Eastern (North American or South American strains) of equine encephalitis viruses were tested to determine if the binding specificity was reproducible and robust to provide the basis for a method to assess viral antigen production for vaccine preparation. An adherent avian cell line was infected with each of the type strains. Cells were fixed and stained with primary antibodies and a fluorescent conjugate. The 4 antibodies chosen were tested in 3 trials to assess their ability to reproduce high mean fluorescence intensities for one specific strain of EEV. The results of this study indicate that flow cytometry, in conjunction with highly specific monoclonal antibodies, is useful for the definitive determination of antigen expression in infected avian cells in a rapid and repeatable fashion.

Introduction

Equine encephalitis viruses (EEV) have major infectious disease impact across the world. There are many strains of EEV and they are ubiquitously distributed (Powers, 2001). Because some strains of EEV are highly pathogenic in horses, and can cause disease in humans, methods of biological control of EEV are important. Further, methods for rapid identification of EEV in the diagnostic process are also important. Monoclonal antibodies (Mab) have already been used for the identification of outbreak strains (Roehrig and Bolin, 1997) and the detection of infection in horses (Passler and Pfeffer, 2003). The methods currently used for detection EEV in clinical samples and for differential identification of specific strains are often subjective and slow (Givan, 2001).

In the vaccine industry, there is a call for the ability to screen for the presence and quantity of specific EEV strain proteins as part of the production of viral antigen from viral constructs. It is important to use methods that provide objective quantitative information about staining patterns. Qualitative methods do not provide an objective method to determine a cutoff value that represents an acceptable antigen level in cell culture. Clear, objective quantitative assessment is even more important when testing the level of viral antigen in a vaccine. In our studies, we have utilized flow cytometry to assess quantitative fluorescence of avian cells infected with chimeric constructs that include proteins found in several EEV strains stained with antigen specific monoclonal antibodies.

Our earlier study involved the screening of 15 commercial monoclonal antibodies for their ability to specifically identify individual EEV strain infected avian cells. We identified 4 Mab that were able to selectively provide strong binding to Venezuelan Equine Encephalitis Virus (VEEV), Western Equine Encephalitis Virus (WEEV), and Eastern Equine Encephalitis

Virus (EEEV). These antibodies yielded minimal cross-reactivity above antibody staining with viral strains other than their intended target. Therefore, we moved forward with the 4 antibodies from an earlier study in order to test their ability to reproduce the specificity observed against infected cells as a method to identify the expression of viral antigen under simulated vaccine production conditions. Due to lack of availability, testing of the antibodies using ACL-1 was stopped. A new target cell line (ACL-2) was chosen that was also an avian cell line and compatible with the requirements for production of vaccine antigen.

Materials and Methods

Cultured Cells

The cell line used for testing was an adherent avian cell line (ACL-2) that was developed at Merial. Cells were grown in 60% Ham's F-10 with L-Glutamine (Mediatech, Inc. catalog # 10-070-CV, lot # 10070078) and 40% Medium 199 with Earle's Salts and L-Glutamine (Mediatech, Inc. catalog # 10-060-CV, lot # 10060168) that was supplemented with 5% fetal bovine serum (HyClone catalog # SH30071.03, lot # ARA25563) in T-75 flasks (BD Falcon catalog # 353136). The cells were incubated at 37°C (Celsius) with 5% CO_2. The cells were infected when the monolayer cultures were 48 hours old. A medium change was completed at infection using the same media formulation as above excluding the serum. The cultures were infected at a target of 0.001 viruses $TCID_{50}$ per cell. This multiplicity of infection (MOI) was chosen based on a previous study to determine the MOI that yielded the highest levels of viral antigen expression. This data is not shown. For this cell line in T-75 flasks, the average cell count is $1x10^7$ cells per flask. This cell count was used to adjust the volume of virus suspension added at time of infection. To ensure that the cells used for the uninfected control were in the same metabolic state, the medium was changed. The infected cells were harvested 24 hours post

infection showing greater than 85% cytopathic effect. The uninfected cells were harvested when their monolayers reached 72 hours old.

The flasks used for trials 1 and 3 were infected when their monolayers were no greater than 80% confluent. The flasks used for trial 2 were infected when their monolayers were greater than 90% confluent.

Virus Strains

Four different viruses were used for testing in this study. The strains used were VEEV, WEEV, North American EEEV, and South American EEEV. These strains are proprietary. The titers, in 50% infectivity units ($TCID_{50}$), used for infection of the cells were: VEEV – $10^{7.2}$, WEEV – $10^{6.5}$, NA EEV – $10^{6.8}$, and SA EEV – $10^{6.2}$.

Monoclonal Antibodies and Secondary Conjugate

Four different commercially available monoclonal antibodies were tested in this study. These Mab had been identified as the best candidates for quantitative assessment of viral antigen in our earlier study on ACL-1, and one was tested on a non-target strain as an alternative due to the removal from the market of one of our target antibodies to allow this study to have the greatest long term benefit. The 4 principal monoclonal antibodies (see Table 3.1) were chosen after screening in the first phase of this project (Aitcheson et al, companion paper). The secondary conjugate used was Sigma Anti-Mouse IgG (whole molecule)-FITC, antibody produced in rabbit (catalog # F9137, lot # 059K4871) at a 1:200 dilution, which was also validated in our screening assay. All primary and secondary antibodies were diluted in phosphate buffered saline (PBS) (produced by Merial Limited) that contained 0.5% bovine serum albumin (BSA) (Fraction V) (Sigma catalog # A7906, lot #120M1340V). All primary antibodies were tested at 1:100, 1:400, and 1:1600 dilutions.

Fixative Agent and Conditions

85% Acetone was prepared with the addition of de-ionized water. The acetone fixative was stored at -20°C. Cells fixed with Acetone were treated for 20 minutes at -20°C.

Screening of Antibodies

Ninety-six well U-Bottom polypropylene plates (USA Scientific catalog # 1830-9610) were as vessels for the staining of cells. Cells were trypsinized with **TrypLE**™ Express without Phenol Red (Gibco catalog # 12604, lot # 1029805) to remove them from the T-75 flasks, prepared as single cells, and then centrifuged to remove the trypsin solution. The supernatant pellet and trypsinized cell pellets were suspended in PBS and combined. A cell count was not performed before the cells were placed in their respective wells, but the cells at confluence in T-75 flasks yield a consistent number of single cells from this line. The primary antibodies were only tested against their respective EEV strain. The Sigma secondary conjugate was tested against all 4 antibodies and strains. To achieve staining, cells were placed on ice for 1 hour with the primary antibody. The cells were washed 3 times with PBS containing 0.5% BSA between primary antibody staining and secondary conjugate staining. Once the secondary conjugate was applied to each sample, the cells were placed on ice in the dark for 30 minutes. After incubation, with the secondary antibody conjugate, the cells were washed 3 times. 200µL of PBS containing 0.5% BSA was added to the each sample well and the contents thoroughly mixed before transfer to flow cytometer tubes containing 1.5ml of PBS containing 0.5% BSA. During testing, tubes were kept at 4°C in the dark.

Flow Cytometric Analysis

The flow cytometer used was a **Partec CyFlow Space**®. The software used for analysis was **FloMax**® version 2.70. The software was used to assign cell population gating based on the

forward angle scatter (FSC) versus orthogonal side scatter (SSC) plots representing cell size (diameter) and internal granularity respectively. Once a gate containing singlets of whole cells was applied, an analysis of FSC versus Green Fluorescence (detector 1) was made and the mean fluorescence intensity of each sample was measured. To compensate for the background present, the MFI of uninfected cells stained with the same antibody and secondary conjugate was subtracted from the infected cells stained with that combination. The antibody specific MFI value for each antibody (and conjugate) was determined using this method.

Data Manipulation and Statistical Analysis

To improve the resolution of the fluorescent peaks associated with the infected cells, particularly when the uninfected fluorescence overlapped the infected peak, peak subtraction was used. This was achieved using the Multi-Sample Compare option in **FlowJo**® version 9.4.5 software (Tree Star Inc, Ashland, OR). This method involves subtracting the area of the uninfected peak from the area of the infected peak with the same antibody and dilution. This yielded a new MFI value and fluorescent histogram. All infected samples from the quantitative trials were analyzed using this method. These new MFI distributions were tested for normality using **Statgraphics**® version 16.1.11 software (Statpoint Technologies Inc., Warrenton, VA). This normality testing was performed for each antibody at each dilution for each trial resulting in 12 normality plots for each trial. Therefore, Dixon's Q-test for data rejection of replicate measures was used to remove one proven outlier from some data sets. [Source http://www.chem.uoa.gr/applets/AppletQtest/Text_Qtest2.htm] With outliers removed, the data sets were tested again for normality. Once all infected peak subtraction MFI data sets were determined to be from normal populations, the data generated by trials 1 and 3 were combined (14 replicate samples in total) and a new mean for each antibody at each dilution was

determined. The uninfected MFI samples were also combined for trials 1 and 3 for each antibody at each dilution. The uninfected data sets were not tested for normality, as the staining was not different from irrelevant antibody. The means for the infected samples for each antibody at each dilution were then compared to the means of the uninfected samples for each antibody at each dilution. This comparison was performed using an unpaired T-test. (Statgraphics v16.1.11) For each antibody and the 3 dilution treatments, the unpaired T-tests confirmed with a 95% confidence interval and P-values less than 0.05 that the means were statistically different.

Results

Preliminary Testing of Antibodies on ACL-2 to Ensure Similar Results Seen on ACL-1

During the time between the original screening and the preliminary testing of the 4 chosen Mab on ACL-2, antibody 4 was removed from commercial distributions. However, enough 4 antibody remained in our stocks to complete the quantification assays. As a precaution, antibody 3 was also tested against the SA EEEV strain to determine if it would be a suitable replacement for antibody 4 in the production setting. Antibodies 11, 14, and 4 all yielded a slightly lower peak MFI against cells infected with the EEV type strain of their specificity when compared to ACL-1 screening results. Antibody 3 yielded a higher MFI against the NA EEEV strain on ACL-2 than ACL-1. While some of the ACL-1 MFI values were higher, the differences observed were minimal. Antibody 3 also yielded a higher MFI against SA EEEV on ACL-2 than antibody 4 infected with the same cells. These results indicated that ACL-2 is a good replacement for ACL-1.

Quantification of Antibody Binding Activity and Specificity for VEE, WEE, NA EEE, and SA EEE

To produce quantifiable results for these four antibodies, 3 assays yielding 7 replicates of each antibody and dilution treatment were tested. Unfortunately, the second trial did not yield data that passed normality testing, so the data was not included in the statistical analysis of binding specificity and reproducibility. Trials 1 and 3 both yielded similar results. For trials 1 and 3, some of the dilution data populations did not pass normality. These were Q-tested to remove the proven outliers within the populations. After removal, the data from trials 1 and 3 were tested for normality again and all passed.

Peak subtraction was used to clearly define the specific binding peaks of the infected samples. This generally yielded a right shift in MFI value, and removed overlap with the uninfected peak. All 4 antibodies yielded higher means in MFI, reflecting the distribution of specific binding of infected cells, when peak subtraction was performed. The average MFI yields were higher for these trials than the MFI values observed during preliminary testing. Antibody 11 yielded higher MFI values at all 3 dilutions. (Figure 3.1) This antibody repeatedly yielded bright fluorescence and highly specific binding when VEEV infected cells were stained. Antibody 14 yielded the highest MFI value at the 1:100 dilution. (Figure 3.2) Antibody 3 yielded the most distinguishable results for MFI values at 1:100 and 1:400 dilutions. (Figure 3.3) Antibody 4 gave the most specific MFI values at 1:100. (Figure 3.4) The MFI values yielded for both trials of 7 replicates we assessed statistically and demonstrated reproducible and quantitative results.

The dilutions used in this study and the previous study using ACL-1 were chosen to demonstrate if the antibody concentrations followed a downward trend as the antibody

concentration decreased. This was seen for all 4 antibodies when stained against their targeted EEV strain. From this data we have determined that the 1:100 dilution is a reliable dilution for staining for all 4 antibodies. Further, as we have indicated, some of the antibodies are also able to produce a bright fluorescence that is highly specific at 1:400 along with 1:100. This is important for future studies to ensure the proper dilution is used for staining and to lower study expenditures.

Discussion

The results of this study are very promising. It is clear that these 4 antibodies were able to not only specifically bind to their targeted viral antigens, but do so with reproducible staining patterns over multiple replicates. The data presented here indicates that these 4 antibodies are robust and will make definitive reagents for use in the validation of EEV vaccine testing. Furthermore, these antibodies will make testing of EEV vaccine titers more robust and validate the presence of viral antigen in the vaccine itself. More studies should be conducted using these antibodies in separate assays to determine levels of viral antigen expressed in EEV infected cells.

It is important to note that the trial 2 data was removed from the final analysis of this study. Overall, trial 2 yielded decreased specific peak subtraction MFI values for all 4 antibodies tested. This is not surprising considering that for this particular trial, the cells were infected when their monolayers were greater than 95% confluent. For ACL-2, it has been determined during the assessment of ACL-2 as a candidate for viral antigen production that the cell cultures should be split or infected before they reach a monolayer confluency greater than 85% (internal Merial data). Our previous experience with these cells has indicated that when these cells are split after the monolayers are greater than 85% confluent, cell viability and density is lower in subsequent passages. We believe that due to the high cell density and possibly the reduced cell

health from overcrowding, there was less viral antigen produced upon infection. The higher cell density at infection may have also caused sub-saturation of the antibodies at staining. We believe that trial 2 represents a technical failure to repeat rather than a failure of the assay to discriminate. This decision was not taken lightly as the results for peak subtraction MFI for antibody 14 appeared to more similar for trials 2 and 3. However, due to the high cell density observed at infection and the decreased peak subtraction MFI values determined for trial 2 for all antibodies, we still believe that trial 2 was not a reliable set of data for this study. We also believe that trial 2 holds a valuable lesson for the application of the methods presented here, that careful attention must be paid to all cellular and viral production parameters if one hopes to obtain maximal antigen yield, and that reduced yield can readily be detected using this method.

In conclusion, the statistical analysis results shown here and the similar specific MFI values for these antibodies across both trials and those observed during preliminary testing infer that these antibodies are highly specific for their targeted viral antigen and are able to repeatedly yield valuable fluorescence staining patterns against the tested EEV type constructs. To improve upon these results, we suggest that possibly trying antibodies 3 and 4 at higher concentrations. This may increase their specific MFI values. Increasing their concentration for staining against their respective viral antigens could increase the fluorescence observed. However, it is important to remember that non-specific binding could increase as the antibody concentration increases. Care must be taken to ensure this does not occur and that multiple washing steps remove as much excess antibody as possible. One could also increase the incubation time from 1 hour to see if increases in specific MFI values increase. Again, it is important to remember that increased incubation time could lead to increases in non-specific binding.

Table 3.1

Monoclonal Antibody Specificity

Mab Name	3	4	14	11
Claimed Specificity	EEEV, WEEV, and VEEV	NA EEEV and SA EEEV	WEEV	VEEV
Isotype	IgG2b	IgG2b	Unknown	Unknown

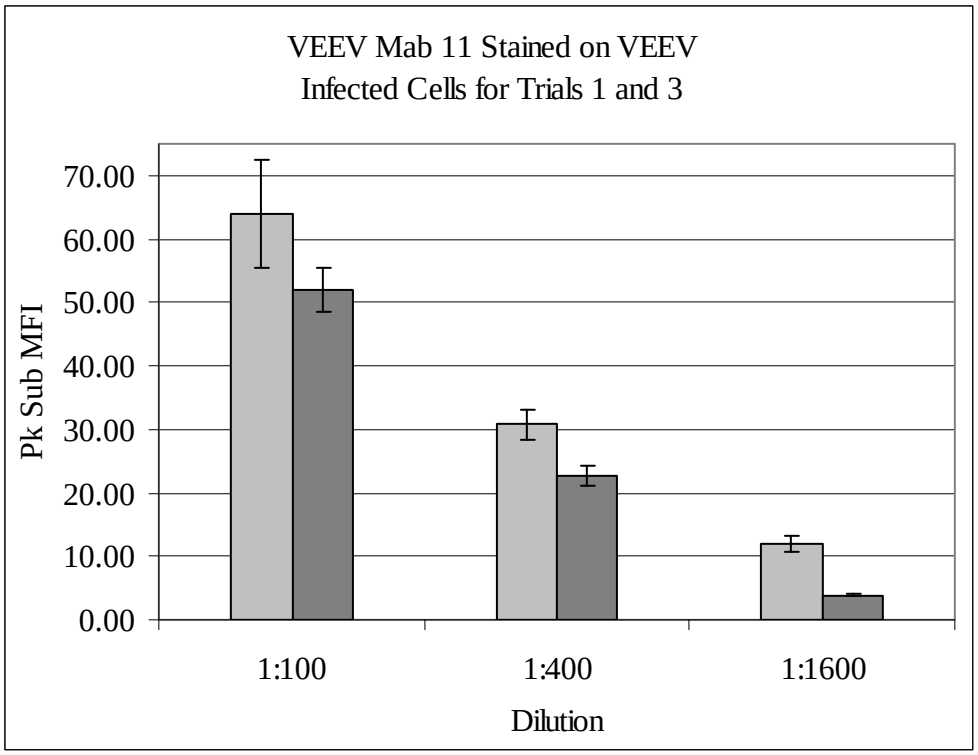

Figure 3.1
VEEV Mab 11 Peak Subtraction Results for Trials 1 and 3 on VEEV Infected Cells. The lighter colored bars are for trial 1 while the darker colored bars are for trial 3.

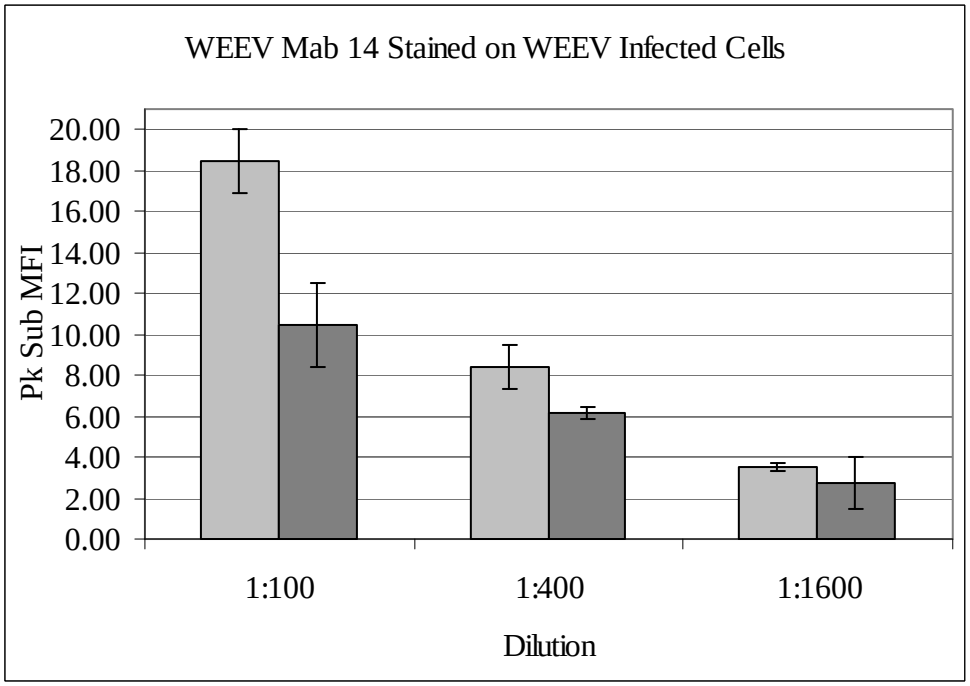

Figure 3.2
WEEV Mab 14 Peak Subtraction Results for Trials 1 and 3 on WEEV Infected Cells. The lighter colored bars are for trial 1 while the darker colored bars are for trial 3.

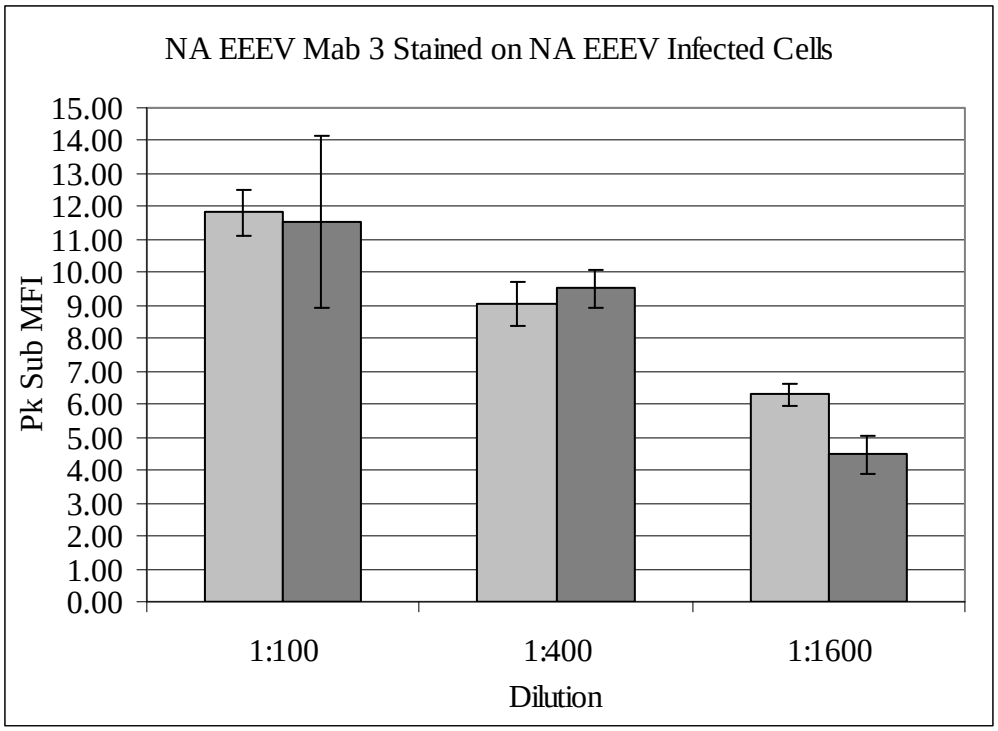

Figure 3.3
NA EEEV Mab 3 Peak Subtraction Results for Trials 1 and 3 on NA EEEV Infected Cells. The lighter colored bars are for trial 1 while the darker colored bars are for trial 3.

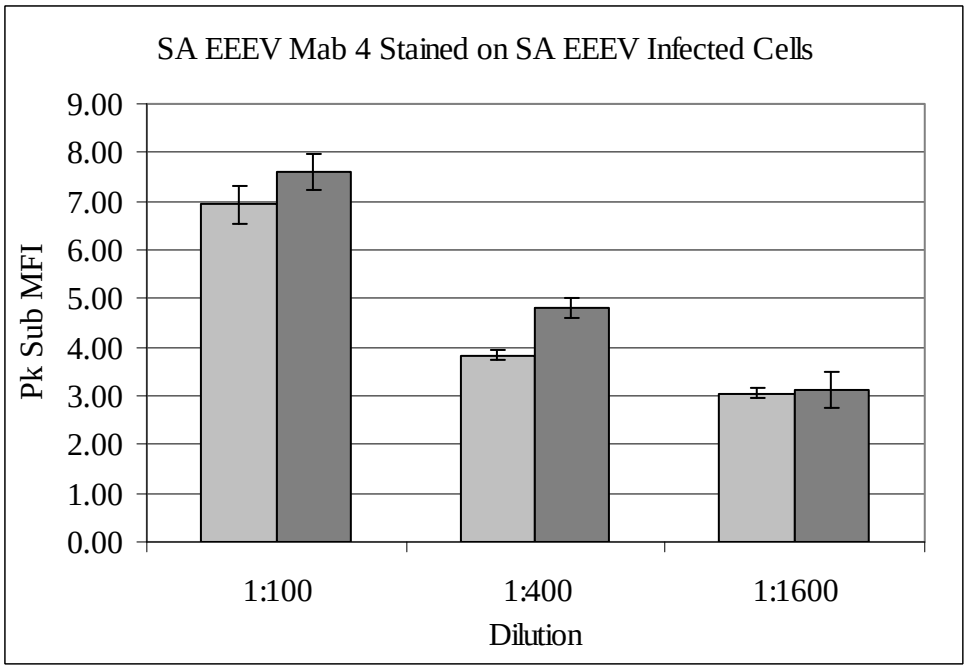

Figure 3.4
SA EEEV Mab 4 Peak Subtraction Results for Trials 1 and 3 on SA EEEV Infected Cells. The lighter colored bars are for trial 1 while the darker colored bars are for trial 3.

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CHAPTER 4

DISCUSSION

The principal objective of the research conducted for this thesis was to move closer to a rapid, real-time method for the quantitative assessment of multiple Equine Encephalitis Virus antigens produced in avian cells from chimeric constructs for use as a multi-component vaccine. Toward this end, the hypothesis tested was: "From among the commercially available monoclonal antibodies, antibodies that bind strongly and distinctly to VEE, EEE and WEE can be identified and shown to have a reproducible binding pattern that can be identified using conventional flow cytometry analysis of fluorescent brightness." This hypothesis was tested by carrying out two objectives:

1) A series of commercial monoclonal antibodies were assessed using two different second antibody preparations to determine the antibodies that most specifically and brightly bound to antigen specific for VEE, EEE and WEE. This study was done using an avian cell line.
2) The antibodies identified as most specific were tested repeatedly to demonstrate how reproducible the level and pattern of binding of each was for the appropriate virus using a second avian cell line.

The results of these studies have proven the hypothesis and provided characterized reagents and methods for the next step toward establishing a standard protocol for quantitative antigen assessment in multi-antigen vaccine production.

The studies conducted for this thesis demonstrate that flow cytometry is a useful tool for screening multiple antibodies that are specific for Venezuelan, Western, and Eastern (both North American and South American strains) Equine Encephalitis Viruses, and to detect high

specificity staining patterns repeatedly over multiple replicates. It was our goal to demonstrate these findings in a manner that would improve the current state of the art of flow cytometry and immunological assay development used in vaccine manufacture. Using antibodies for the detection of viral strains is not a novel idea (Passler and Pfeffer, 2003). Many assays exist today that utilize antibodies to detect the presence of virus in clinical samples. However, these existing assays were focused on the clinical aspect of viral research. We wanted to demonstrate that antibodies are useful for the quality control testing and development of vaccines. Using methods that are well established in the practice of flow cytometry, we have shown that binding of protein and detection by flow cytometry provide a feasible method of detecting highly specific antibodies to VEEV, WEEV, and North American and South American EEEV from among a large pool of candidates. Based on these findings, assays to improve quality control testing and manufacture during vaccine development can be improved. The new methods will provide an enhanced confidence that production results will be robust and reliable.

Our first study was designed to address the first objective. We screened fifteen commercially available monoclonal antibodies for which there was a claim of specificity for VEEV, WEEV, or EEEV by the manufacturer. To do this, we utilized an indirect fluorescence method where the primary monoclonal antibody binds to viral antigens and a secondary antibody is used to recognize the species and isotype of the monoclonal that has been bound. The secondary antibody is conjugated to a fluorochrome (Kuby, 1996). This method allowed us to analyze the specificity of the antibodies and determine their cross-reactivity among the different viral type strains. We designed this study to bind intracellular proteins that are produced during equine encephalitis cellular infection. To allow the antibodies to cross the cellular membrane and bind these internal proteins, we treated the cells with a permeabilizing fixative agent. The

85% acetone used for these studies disrupted the membrane allowing the antibodies to enter the internal cellular compartments and bind to their specific viral antigens (Givan, 2001). From this study, we determined that antibodies 11, 14, 3, and 4 all yielded specificity for individual targeted viral antigens (VEEV, WEEV, NA EEEV, and SA EEEV, respectively) in infected avian cells. This study also yielded results about the level of cross-reactivity among the four antibodies chosen for further study. This was important to discern so that for future studies these antibodies could be used on samples containing more than one viral strain. For the application intended, it would be acceptable to have only very minor cross-reaction of each antibody with the other strains of EEV. In our studies, one of the antibodies, antibody 11, had very strong MFI signal with VEEV and strong signal relative to the rest of the antibodies with the other viral antigens. Yet, the signal for antibody 11 was not strong enough so that it provided better differential binding to WEEV and EEEV (either NA or SA) than the three other antibodies forwarded. All other antibodies tested had little specific signal with viral antigens (except as noted for their selectivity in this thesis), so that there was generally very minor cross-reactivity. Unfortunately, because the antibody for VEEV (antibody 11) has considerable general cross talk, and the two EEEV antibodies identified (3 and 4) recognized both types of EEEV virus infected cells similarly, the set of four antibodies characterized here were not good candidates for combined antigen assessment assays. Therefore, it is recommended that these antibodies be used separately in further development of assays for the determination of levels of antigen present in virus infected cells.

Our next step was to address the second objective of our study. This was to demonstrate that the highly specific staining patterns observed in our screening study on the original avian cell line (ACL-1) were repeatable with other infected cells. This would prove that these

antibodies were screened properly using flow cytometry, and that good immunological fluorescence techniques were employed. Attempts at repeating the results observed for ACL-1 failed when staining with the same antibodies on EEV infected Vero cells. Vero cells were chosen because it had been previously demonstrated that the virus would replicate in these cells. As Vero cells are from mammals (African Green Monkey kidney), it is not unexpected that the level of viral antigen and its intracellular processing may have been different than avian cells. Because flow cytometry yields results in real time, decisions about the success or failure of antibody staining or lack of specificity can be made quickly (Carter and Ormerod, 2000). Those results demonstrated that flow cytometry could rapidly and specifically present definite values that were not similar to those seen on ACL-1 (Carter and Ormerod, 2000).

The next part of our study was designed to show that the specific staining patterns observed were repeatable over multiple trials. ACL-1 was no longer available for the assessment of the robustness of the method. Therefore, an alternative avian cell line was selected (ACL-2). This cell line was developed within Merial and according to its previous characterization, appeared suitable as a substitute. Preliminary testing with the four antibodies selected yielded similar results to ACL-1. The definite staining patterns and MFI values yielded were compared, and it was determined that ACL-2 was a good replacement for assessing the specificity and reproducibility of infected cell antibody staining. Three trials were attempted to assess specific staining by the four Mab selected, each against cells infected with its specific viral target. Each assessment included seven replicates of each staining combination. It was using this design that we were able to demonstrate that these antibodies were highly specific and were able to repeatedly yield their specific staining behavior. Trial two was removed from the study after the data failed a test for normal distribution. The data in trials one and three were normally

distributed and could be tested in the desired statistical analysis. Several statistical tests were utilized to demonstrate that the staining specificities observed for the infected samples were indeed significant when compared to uninfected samples treated with the same antibody dilutions.

The failure of trial two to produce data that passed normality testing is not a major setback, and provided us with useful information about the test system The failure here was basically related to not keeping adequate control of the cells and virus infection for staining, rather than the ability of the antibodies to reproduce their staining patterns. At the time of infection, the cell density was higher than what has been shown to be ideal for infection of ACL-2. One possible cause for the reduced specific MFI values observed could be the decreased amount of viral antigen expression due to the over crowding of the cell monolayer impacting the efficacy of viral replication. The definite values yielded by flow cytometry allowed us to quickly determine that a failure had occurred for all samples of trial two.

During our testing, we observed that the MFI values and staining patterns for antibodies 3 and 4 tested against North American (NA) and South American (SA) EEEV were very similar. This might be explained by the similarities that exist between these two strains. (Griffin, 2007) The nucleotide differences in the genome of North American and South American EEEV have been shown to be between 25 to 38 percent (Aguilar, 2008). Common antigen domains between these two families of EEEV have been demonstrated. Both NA EEEV and SA EEEV share the E1 epitope for antibody neutralization (Griffin, 2007). This could also explain why antibody 3 yielded a higher specific MFI value than did staining with the antibody 4 on ACL-2 infected with SA EEEV. Further, by comparing the quantitative MFI values, we were able to determine that antibody 4 was a proper choice for targeting SA EEEV infected cells. While the specific

fluorescence observed with both antibodies, 3 and 4 were very similar, a reproducible difference was determined.

Flow cytometry is not a new method for use in immunological assays. However, its use for screening antibodies to determine antibody specificity and cross-reactivity behavior for assessment of viral antigen in cells is new. The highly reproducible staining patterns and clear viral antigen specificity of the four antibodies evaluated provide a solid basis for further studies for use in vaccine production and quality control. By testing these antibodies against their targeted strain in separate assays, the level of antigen present in a vaccine could be determined. By using them separately, issues of cross-reactivity would be avoided but the value of their highly specific antigen binding can still be utilized. We believe that this would allow for increased confidence in titer assay development and results. First, would be to stain samples that contain cells infected with the targeted EEV strain. Next, testing to verify the level of mean fluorescence intensity produced by each antibody binding to its targeted viral strain and the results could then be correlated to differences in specific MFI values and the amount of viral antigen present in the samples. The specific MFI values for each antibody and EEV strain could be correlated to the titer values observed for the same samples. The antibodies could be used in serial assessments to measure the amount of each antigen within a single sample.

We feel that the ideas for future studies detailed here would improve process development of vaccines. By designing and testing an assay that uses these antibodies, a single-step assay would improve confidence in the production of the vaccine itself. This would provide another step toward ensuring our ability to safely and efficaciously protect horses from the family of EEV. Bringing a vaccine to market is a multiple year endeavor. By determining the correct reagents more quickly and with more confidence, one should be able to decrease the time

needed for vaccine testing development. We have shown that flow cytometry provides a rapid and non-subjective method to determine both antibody specificity and cross-reactivity among different strains of equine encephalitis viruses. The benefits of a rapid assay with flow cytometry was observed twice during our studies; when the antibodies yielded lower specific MFI values for EEV infected Vero cells and in assessment of the failure of trial two assay data to produce data within the design of the experiment. The testing of antibodies to determine their specificity towards viral antigens is an important tool in the development of viral antigens that will later be used in vaccines. Using flow cytometric analysis improves the screening of the antibodies by removing subjective assessment, and ensuring repeatability of the antibody measurements themselves. Had we tested antibodies 3 and 4 using an immunofluorscence microscope assay, the small differences in specific MFI would have been beyond the sensitivity of the human eye. Because an assay is only as good as its reagents, using immunological methods that increase confidence in the assay and its reagents only improves biological testing and the development of vaccines that can advance animal and human health.

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