Understanding the Role of Predictive, Diagnostic and Pathogenic Autoantibodies in Systemic Lupus Erythematosus and its Central Nervous System (CNS) Involvement

by

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A Dissertation Presented in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Approved May 2011 by the
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ARIZONA STATE UNIVERSITY

August 2011

ABSTRACT

Systemic lupus erytematosus (SLE) is an autoimmune disease where the immune system is reactive to self antigens resulting in manifestations like glomerulonephritis and arthritis. The immune system also affects the central nervous system (known as CNS-SLE) leading to neuropsychiatric manifestations such as depression, cognitive impairment, psychosis and seizures. A subset of pathogenic brain-reactive autoantibodies (BRAA) is hypothesized to bind to integral membrane brain proteins, affecting their function, leading to CNS-SLE. I have tested this BRAA hypothesis, using our lupus-mouse model the MRL/lpr mice, and have found it to be a reasonable explanation for some of the manifestations of CNS-SLE. Even when the MRL/lpr had a reduced autoimmune phenotype, their low BRAA sera levels correlated with CNS involvement. The correlation existed between BRAA levels to integral membrane protein and depressive-like behavior. These results were the first to show a correlation between behavioral changes and BRAA levels from brain membrane antigen as oppose to cultured neuronal cells.

More accurate means of predicting and diagnosing lupus and CNS-SLE is necessary. Using microarray technology I was able to determine peptide sets that could be predictive and diagnostic of lupus and each specific CNS manifestation. To knowledge no test currently exists that can effectively diagnose lupus and distinguish between each CNS manifestations. Using the peptide sets, I was able to determine possible natural protein biomarkers for each set as well as for five

monoclonal BRAA from one MRL/lpr. These biomarkers can provide specific targets for therapy depending on the manifestation.

It was necessary to investigate how these BRAA enter the brain. I hypothesized that substance P plays a role in altering the blood-brain barrier (BBB) allowing these BRAA to enter and affect brain function, when bound to its neurokinin-1 receptor (NK-1R). Western blotting results revealed an increase in the levels of NK-1R in the brain of the MRL/lpr compared to the MRL/mp. These MRL/lpr with increased levels of both NK-1R and BRAA displayed CNS dysfunction. Together, these results demonstrate that NK-1R may play a role in CNS manifestations.

Overall, the research conducted here, add to the role that BRAA are playing in CNS-lupus.

DEDICATION

I would like to dedicate this dissertation to my family and friends who have been very supportive throughtout my graduate education. I would especially like to thank my parents Douglas and Martha for both their emotional and financial support. They have always emphasized the importance of obtaining an education and to always do the best at whatever you want to achieve in life. My parents have been there every step of the way throughout my graduate experience and have been very supportive of the choices that I have made.

I would also like to dedicate this dissertation to my sisters Tanya, Tiffany and Stacy. We have always been an encouragement to each other since we have all been in college around the same time.

And lastly I would like to dedicate this dissertation to my grandparents Elbert and Marcella and my grandmother Elaine who have always shown how proud they are of me.

ACKNOWLEDGMENTS

I would like to thank Dr. Steven Hoffman for all the help and support that he has provided to me throughtout my graduate education. Dr. Hoffman has dedicated a great deal of time to help me complete my lab experiments as well as the written portion of my graduate work. I would like to thank Dr. Hoffman for his time and patience over the past years.

Next, I would also like to thank the rest of my committee members Dr. Miles Orchinik, Dr. Cheryl Conrad and Dr. Julian Chen for all the time and help that they have given me to complete my graduate studies.

I would like to thank my collaborators Dr. Boris Sakic and Dr. Stephen Johnson for the opportunity to work with them. I would also like to thank all the members of the Hoffman lab and Johnston lab for all the help that they provided.

The research in chapter one has been supported by the Arizona Biomedical Research Commission (grant 9-073), NIH (grant 1R21 AR49163-01), and the Canadian Institutes of Health Research.

The research in the remaining chapters has been supported by the Arizona Biomedical Research Commission (grant 9-073), and the School of Life Sciences, Arizona State University, Initiatives Award (2008).

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INTRODUCTION

THE IMMUNE SYSTEM

The immune system is important in helping us fight foreign antigens such as viruses and bacteria. Therefore it is important for our immune system to be functioning properly in our battle with different illnesses. There are two types of immunity that we have; these include innate immunity and adaptive immunity (Abbas and Lichtman, 2003). The innate immune system responds to microbes. Components of this system include the complement proteins, the skin, cytokines, phagocytic and natural killer cells (Abbas and Lichtman, 2003). This system does not recognize specific details of their target antigen, but are important for the initial immune response.

The second type of immunity is the adaptive immune system. This system is quite specific in the antigen that it recognizes and can produce a much stronger response, but it takes a little longer to get started. Components of this system include the lymphocytes and the antibodies that are produced. There are two types of adaptive immunity; humoral and cell-mediated immunity (Abbas and Lichtman, 2003). In humoral immunity, antibodies are produced by B cells and these antibodies circulate throughout the body and target viruses and bacteria for elimination. Cell-mediated immunity on the other hand, involves the use of T cells to help eliminate cells that have become infected.

The adaptive immune system once activated has another important feature, viz., it produces memory cells. These memory cells are important because they

allow an individual to respond to an infection much faster the second time if the antigen that is presented is the same as the antigen that it recognized previously. Therefore, the proper functioning of both the innate and adaptive immune systems is important in fighting infections throughout the body.

I have barely touched on the complexity of the immune system by the brief description above, since that is not my focus here. It is, however, important to understand that any disruption to function of the immune system can be detrimental to the health of an individual. This is what occurs in the case of autoimmune disease, where the body is attacking itself. We have chosen to study the autoimmune disease systemic lupus erythematosus (SLE; or "lupus") in our lab and this disease will be the focus of all the research being described. Since the immune system is so complicated in its function, it can be noted that trying to understand the mechanisms that are occurring during disruption as in the case of an autoimmune disease is very difficult and complex.

SYSTEMIC LUPUS ERYTHEMATOSUS

There can be instances where the immune system does not function properly, leading to disorders in the body. Such cases can result in the development of autoimmune diseases, such as systemic lupus erythematosus, multiple sclerosis and arthritis (Blatt and Glick, 1999; Ballok et al., 2004a; Arabo et al., 2005; Williams et al., 2010). The immune system no longer functions solely to protect the body from harmful and foreign antigens, but begins to attack and affect the body as if it itself is foreign.

In SLE, the immune system affects and damages many organs of the body including the skin, liver, kidneys, spleen and central nervous system (the system of interest to us) (Sakic et al., 2005). The body produces autoantibodies to many different tissues that either cause damage to the tissue or affects the functions of the organs. Sometimes these autoantibodies are present as immune complexes (a complex between an antibody and antigen) and they get lodged in the kidneys as the blood is being filtered (Blatt and Glick, 1999; Bagavant et al., 2011). This will therefore lead to an immune reaction in the kidneys involving the complement cascade leading to damage in that area and affecting the function of the kidneys (Abbas and Lichtman, 2003). One way to tell that damage has occurred to the kidneys is the presence of proteins in the urine, since normally proteins are not found in the urine except for when there is damage to the kidneys (Blatt and Glick, 1999). This damage is known as glomerulonephritis (Blatt and Glick, 1999). The joints of some of these individuals are also affected leading to arthritis (Gao et al., 2009).

The presence of autoantibodies is not solely responsible for the damage being done as lupus progresses. There can be other factors that contribute to the damage such as cytokines since they may affect many areas of the body resulting in changes in normal functioning of those regions (Mondal et al., 2008; Calvani et al., 2005; Ballok et al., 2003). It has been suggested that when the neuropeptide substance P is stimulated by cytokines, it increases the permeability of the blood-brain barrier (BBB), which normally allows the brain to be an immune privileged site (Annunziata et al., 2002). This opening up of the BBB can cause the autoantibodies and other factors to enter the brain resulting in neuropsychiatric manifestations through alteration of normal brain function (Annunziata et al., 2002; Kowal et al., 2004). Neuropsychiatric manifestations of lupus is thought to be the result of the immune system affecting the brain and will be discussed below since we are mainly interested in the neuropsychiatric manifestations of lupus (CNS-SLE) (Williams et al., 2010). The complement system is also thought to play a role in the progression of lupus since mice lacking complement factor B have a lower level of immunoglobulin in the brain compared to mice that have complement factor B (Alexander et al., 2007). These mice that lack the complement factor B also displayed normal behavior in the open field test that looks at exploratory behavior. Therefore, the presence of complement seems to have an effect on behavioral performance of the lupus mice, indicating its role in CNS dysfunction. However, even though cytokines and other factors may contribute to disease progression in autoimmune disease, we are more interested in understanding the role of autoantibodies in causing SLE and CNS-SLE.

The etiology of lupus is currently unknown (Ma et al., 2006). There are some factors that are believed to help contribute to the development of lupus. These include environmental factors, such as microorganisms and the sun, genetics and drugs (Gordon et al., 2010). The sun is believed to play a role since UV light can cause damage to the DNA of an individual's cell and this damaged DNA may lead to the production of autoantibodies to body tissue (Gordon et al., 2010; Blatt and Glick, 1999). Secondly, the genetic makeup of an individual can help contribute to the development of lupus since mutations in certain genes like the major histocompatibility complex (MHC), which is important in the presentation of antigens to T cells, if not functioning properly, will affect antigen presentation (Blatt and Glick, 1999). However since the etiology of lupus is unknown, more studies are crucial to discovering information to help in the prevention and treatment of lupus.

In the general population of individuals affected by lupus, the vast majority of these individuals tend to be women (Kiss et al., 2002). The exact reason is unknown but it has been hypothesized that hormones may be involved. Some researchers have found that in one of the mouse models of lupus, there was reduced levels of testosterone along with signs of autoimmunity and so the presence of high levels of testosterone may actually help with ameliorating the effects of lupus (Sakic et al., 1998). It has also been found that specific subtypes of estrogen receptor may be responsible for the continual development of autoimmune diseases (Li and McMurray, 2007). More research is necessary to

further solidify the importance of hormones in the development and progression of lupus.

Overall, even though there are many researchers trying to understand what is occurring in lupus patients, a lot of information is still missing to understand what causes the development of lupus, what proteins/ molecules are affected in the body and why out of the affected general population the vast majority are women. Our laboratory has undertaken the goal of trying to understand the proteins and molecules being affected in the brain by attempting to determine the target proteins of the autoantibodies detected in lupus patients. That is, we believe that if we can discover some of the molecules that are affected, we may be able to comprehend why certain neuropsychiatric manifestations are present in lupus. More importantly, in the future, these molecules may provide useful targets (biomarkers) for the prevention and treatment of lupus.

ANIMAL MODEL OF SYSTEMIC LUPUS ERYTHEMATOSUS

In order to study lupus, there are several different mouse strains that are available including the NZB/W, BXSB and the MRL/lpr mice (Ballok, 2007; Burnett et al., 2004; Han et al., 2002; Sakic et al., 2005; Williams et al., 2010). We have chosen to use the MRL/lpr mice as our model of lupus because they start to develop lupus at about 2 months of age and about 4 months of age they have fully developed the disease (Williams et al., 2010). At about 6 months of age these MRL/lpr mice have 50% mortality, so progression of the disease is quite rapid (Williams et al., 2010). Also, the MRL/lpr mice have manifestations that are similar to humans including renal damage (glumerulonephritis), skin rashes and damage to the central nervous system, therefore making them a better model for comparison to human SLE (Sakic et al., 1992, 2005; Williams et al., 2010). The MRL/lpr mice develop this autoimmune disease partly due to the presence of the lpr gene. The lpr gene is a mutation of the fas gene which is very important in apoptosis. Because the fas gene is mutated, this means that the B cells that are producing the autoantibodies cannot undergo apoptosis and therefore are kept alive and keep producing these autoantibodies (Gao et al., 2009). This is also the reason that these MRL/lpr mice have such an enlarged spleen.

In previous studies conducted in our lab, we have detected high levels of anti-DNA autoantibodies in these MRL/lpr mice at 4 months versus 1.5 months of age, which is an indicator of disease progression in these mice (Williams et al., 2010). High levels of anti-DNA autoantibodies have also been detected in patients with lupus (Yung and Chan, 2008). We have also observed enlarged

spleens in our 4 month MRL/lpr, another indicator of disease progression (Williams et al., 2010). When we conducted our battery of behavioral tests to look at neurological deficits in these mice, altered behavior was observed in the forced swim test and the sucrose preference test, displaying a dysfunction in the central nervous system. The forced swim test has been used for testing antidepressants and so may be an animal model of depressive-like behavior, while the sucrose preference test may be an animal model of anhedonia (Ballok et al., 2003; Gao et al., 2009; Porsolt et al., 1977; Sakic et al., 1992, 2005; Williams et al., 2010). These dysfunctions are similar to the experiences of lupus patients with central nervous system involvement, allowing us to use these mice as a model to understand behavioral dysfunctions in humans. All of the examples listed above demonstrate why using the MRL/lpr mice as a model of human SLE in our studies is appropriate.

We also include two control strains of mice in our studies. The first strain is the MRL/mp mice. The MRL/mp are almost genetically identical to the MRL/lpr (Sidor et al., 2005; Stanojcic et al., 2009; Williams et al., 2010). This becomes very important when we are trying to understand what is causing the progression of lupus in these mice, since we want to ensure that it is not the normal genetics of the mice that is causing the observations being made but rather the disease itself that is responsible for the changes. The MRL/mp mice will eventually develop lupus because they have the same background as the MRL/lpr mice, but will develop lupus at a much later stage in life. Therefore, since we are interested in testing the MRL/lpr mice at 4 months of age (when they have lupus)

and the MRL/mp will not have any of the manifestations of lupus at that time, but are almost genetically identical to the MRL/lpr, then they are a good control for genetics (Sidor et al., 2005; Stanojcic et al., 2009; Williams et al., 2010). The MRL/mp mice have 50% mortality at about 12 months of age.

It is interesting to note that other researchers have found that these MRL mice may have regenerative capacities since when performing ear punches of the MRL mice full closure of the holes were observed (Clark et al., 1998). It is not believed that this ability is related to the fas gene mutation since the MRL/mp have this regenerative capacity, but they do not have the fas gene mutation (Clark et al., 1998). It may therefore be important to explore the relationship between this capacity and autoimmune diseases and determine if this capacity plays any role in the development of these diseases.

The second control strain of mice that we use in our studies is C3H/HeJ mice. The reason that we chose to use these mice is that we want to show that the normal aging process is not the reason for the different manifestations that are being observed. Since the C3H/HeJ mice will not develop lupus in its lifetime, it should not display any of the manifestations associated with lupus (Trune et al., 2007). Therefore by including this strain throughout our studies, we are able to show that as time progresses and our lupus-prone mice becomes more and more sick and we start seeing different manifestations, but not in C3H/HeJ whatsoever at each time point, we then know that the manifestations that we are observing are not the result of normal aging.

Overall, the use of the MRL/lpr mice in our studies should prove to be a good model of lupus. Using the MRL/mp and C3H/HeJ mice as our control strains, should also strengthen the findings in our studies since the absence of these manifestations in our control groups should help to show that the findings are as a result of the progression of lupus in our MRL/lpr mice.

CENTRAL NERVOUS SYSTEM – SYSTEMIC LUPUS ERYTHEMATOSUS (NEUROPSYCHIATRIC LUPUS)

One of the organ systems affected during lupus is the central nervous system. The immune system affecting the central nervous system is believed to be the reason for the development of the many different neuropsychiatric manifestations observed, including psychoses, seizures, depression and cognitive impairment (Gao et al., 2009; Ma et al., 2006; Sidor et al., 2005; Stanojcic et al., 2009; Sakic et al., 1992, 2005; Williams et al., 2010). This can occur in about 31% to 70% of lupus patients (Tin et al., 2005). However, exactly what occurs in the brain to cause these manifestations is currently unknown. Since there are many autoantibodies present during lupus, our lab hypothesizes that there are a subset of brain-reactive autoantibodies (BRAA) that react to the integral membrane proteins on the surface of the cells in the brain, thereby affecting the function of these structures leading to neuropsychiatric manifestations (Hoffman and Madsen, 1990; Hoffman et al., 1978, 1987; Narendran and Hoffman, 1989; Khin and Hoffman, 1993; Zameer and Hoffman, 2001, 2004; Williams et al., 2010). We titled these BRAA as pathogenic since not all BRAA are pathogenic due to the presence of autoantibodies in the sera of normal mice in low concentrations (Hoffman et al., 1987; unpublished data). There are other mechanisms that may be responsible for the development of these neuropsychiatric manifestations, but we are interested in the role these BRAA are playing. We have detected these BRAA in our animal model of lupus and have correlated the presence of these BRAA to different neuropsychiatric

manifestations (Williams et al., 2010). Exactly which receptors are affected in the brain and the pathway being undertaken to cause the different neuropsychiatric manifestations is currently unknown, however our data suggests that these BRAA are involved. Therefore, one of the main goals throughout my research was to look at what role these BRAA are playing by understanding what proteins they are affecting in the brain.

As mentioned, anywhere from 31% to 70% of lupus patients can have neuropsychiatric manifestations (Tin et al., 2005). Affective disorders such as depression have been found to affect about 40% of lupus patients with neuropsychiatric manifestations (Gao et al., 2009). In a number of studies, a large number of lupus patients with NPSLE had cognitive impairments (Stojanovich et al., 2007). Cognitive impairments can affect areas such as verbal learning and memory and attention/mental flexibility. It was also found that lupus patients with CNS-SLE displayed impairments in verbal and memory tests. High levels of anti-cardiolipin antibodies (cardiolipin is present in the inner mitochondrial membrane) have been hypothesized to play a role in causing these cognitive impairments.

Since we have hypothesized that BRAA play a role in causing these neuropsychiatric manifestations, in order to correlate the presence of these BRAA to the different neuropsychiatric manifestations, we used a battery of behavioral tests to observe these neuropsychiatric manifestations in the mice. There are many different tests available that can be used to look at how behavior is affected during the presence of lupus and below I will describe two of the tests that we

chose to use. The two tests include the forced swim test and the sucrose preference test, both of which are tests of affect (Ballok et al., 2003; Maric et al., 2001; Sakic et al., 2005; Williams et al., 2010).

The sucrose preference test looks at emotional dysfunction and may be a proposed model of anhedonia (the lack of the desire to seek out something that is pleasurable) (Sakic et al., 1996a; Sakic et al., 1997; Ballok et al., 2003). Mice normally like sucrose solution and therefore if given a syringe containing sucrose solution in their cages, they will more than likely seek out that solution and consume it. If the mice display anhedonic behavior, they will refuse to consume large amounts of the sucrose solution, not because they do not like sucrose, but because they do not have the desire to seek out the sucrose. In our control mice, we have observed more sucrose consumption on average compared to our MRL/lpr mice (Williams et al., 2010). This demonstrated that there is altered behavior in this test for the MRL/lpr. The reason that we also believe this behavior is indeed being observed and not due to joint pathology is that in the beam walk test the MRL/lpr are able to move across the beam to the larger platform (unpublished data). Also, since during the test there is a training and a testing phase, the lupus-prone mice had sufficient time to know that the solution is present.

The second test of affect is the forced swim test (Williams et al., 2010; Sakic et al., 1994a). This test has been used in other studies to test the effects of anti-depressants and may be a model of depressive-like behavior (Porsolt et al., 1977). Depression is one of the neuropsychiatric manifestations observed in

lupus patients and therefore using a behavior test that helps to explore this emotional dysfunction in our mice may help us to understand more of what is taking place to cause depression in humans (Williams et al., 2010). Again, knowing this information can help to provide targets for treatment and possibly prevention of this manifestation. The forced swim test has been used by many researchers including us, and we have found consistent results with this test in our lupus mouse model (Sakic et al., 2005; Williams et al., 2010). Our MRL/lpr mice have consistently displayed altered behavior compared to our controls in this test (Williams et al., 2010; unpublished data). The way that we measure this altered behavior is by recording float time in a swimming pool.

So, by using the tests above we can try to understand some of the CNS manifestations of lupus, however, we need to understand what proteins/molecules are affected in the brain and the areas of the brain that these proteins are located. For the two behavioral tests mentioned above, we expect once more research is done, it will be discovered that limbic structures are affected in lupus patients with depression or other emotional dysfunction since these structures are known to be involved with emotion as well as structures important in learning and memory since this deficits is observed in MRL/lpr mice (Ballok et al., 2004b). One of our future goals to confirm the autoantibody hypothesis is to use the BRAA that we isolate (and have correlated with specific behaviors) and inject them into control mice (e.g., MRL/mp) and see if we can replicate the behavior. If the behavior is replicated, this is the strongest test showing that specific BRAA was responsible for the CNS manifestation observed.

We hypothesized that these autoantibodies are partly responsible for the neuropsychiatric manifestations of lupus, by interacting with different cell surface proteins and altering their function. Therefore the BRAA will need to interact with brain proteins in order to cause some of the neuropsychiatric manifestations. But, how does the BRAA interact with the brain proteins with the presence of a blood-brain barrier (BBB)? A recent study was able to detect an anti-DNA autoantibody that cross-reacts with the NMDA receptor (Kowal et al., 2004). NMDA receptors are important for learning and memory and brain function across synapses, therefore if we have an antibody that can bind to these receptor, it may definitely alter normal functioning in the brain. These researchers found that if the blood-brain barrier is altered pharmacologically, allowing the anti-NMDA receptor antibody to enter, the mice displayed cognitive impairment (Kowal et al., 2004). Therefore this suggests that if in lupus patients, the barrier is allowed to be compromised and BRAA do enter, this may cause neuropsychiatric manifestations. Our lab has previously detected IgG in the brains of lupus-prone mice using immunohistochemistry as well as B and T cells (Zameer and Hoffman, 2001, 2004). This means that the IgG was able to pass through the BBB when it was somehow altered by an unknown process. Since we hypothesized that this barrier is altered through the help of the vasodilator substance P, we also looked at the expression level of the neurokinin-1 receptor, which is the receptor that substance P binds to, in the brain.

Other researchers have shown data to support the hypothesis that substance P is altering the BBB permeability (Annunziata et al., 2002).

Researchers found that two cytokines, tumor necrosis factor – alpha (TNF-α) and interferon-gamma (INF-γ), stimulated the release of substance P leading to the further alterations in the levels of different proteins (such ICAM-1 and MHC class I) and also causing an increase in the permeability of the BBB. This research suggests that cytokines along with substance P may be playing a role in causing an alteration of the BBB allowing the BRAA to enter and bind to their target antigens. We therefore hypothesize that substance P plays a role in CNS dysfunction and predict that there will be an increase in the levels of neurokinin-1 receptor since we infer that you will need an increase in this receptor in order for substance P to mediate its effects. Our initial findings so far support our hypothesis and prediction.

There are of course other possible ways that the barrier is altered during such a heightened immune state. However, the open barrier allows for the permeability of proteins that would normally not be allowed through, such as antibodies. Therefore our BRAA can enter through the open barrier, bind to the antigens that it recognized and cause various neuropsychiatric manifestations (Zameer and Hoffman, 2001). An alternative is that leukocytes are able to enter the barrier through the increased permeability and produce BRAA inside the brain (Zameer and Hoffman, 2004).

Overall, current research seems to suggest that BRAA are playing a role in causing these neuropsychiatric manifestations of lupus, but more work is needed to identify the targets of these BRAA. In this research project we tested multiple hypotheses. First we attempted to learn more about the targets of these BRAA

(pathogenic BRAA hypothesis) using different technologies. We tested the BRAA hypothesis in our first study, demonstrating further evidence for the correlation between the presence of BRAA and CNS dysfunction (Williams et al., 2010). Secondly, we hypothesize that since these autoantibodies are playing a role in lupus and CNS lupus, by detecting their presence early on before any disease manifestations, we should be able to predict if someone will get lupus or a specific CNS manifestation. Our data so far has shown this to be possible. We were also able to diagnose lupus and specific CNS manifestations. One of the key features of this second study was being able to predict and diagnose a specific CNS manifestation. To our knowledge no one has done this before. Our microarray chip will allow for a more accurate prediction and diagnosis of lupus and CNS lupus. Therefore in the second and third studies we tested what we called the predictive and diagnostic hypotheses (each of which will be discussed below). Lastly, we looked at the expression levels of NK-1R since we hypothesize that they play a role in causing CNS dysfunction through alteration of the BBB. Our initial results show that NK-1R levels are altered adding further support for its role in CNS dysfunction. All of this research should add more information to understand the role of autoantibodies in lupus and CNS lupus.

OVERVIEW OF RESEARCH PROJECT

Our main goal throughout this research project was to better understand how CNS manifestations in SLE occur, including an attempt to get closer to identifying the targets of the BRAA that are detected in lupus. The reason for the importance of the characterization of these BRAA is because our lab hypothesizes that there is a subset of BRAA that are responsible for some of the neuropsychiatric manifestations seen in lupus through binding to the integral membrane proteins on the surface of brain cells (Hoffman and Madsen, 1990; Narendran and Hoffman, 1989; Khin and Hoffman, 1993; Zameer and Hoffman, 2001, 2004; Williams et al., 2010). Few researchers have been able to identify the targets of these BRAA and therefore being able to characterize these BRAA would be essential to understanding what is causing these neuropsychiatric manifestations. One set of researchers identified one of the BRAA targets as dynamin-1, which may be important for synaptic vesicle endocytosis (Lawrence et al., 2007). A second researcher identified another brain target as the NMDA receptor (Kowal et al., 2004). However, we expect that there will be many more targets in the brain and therefore more work is needed to identify these targets.

To begin, below will be a summary of what will be discussed in the next few chapters. We used the MRL/lpr mice as our model of lupus in many studies. We use this model because previous research has shown them to have similar manifestations to humans and their disease state is accelerated compared to other autoimmune mice (Sakic et al., 1994b, 1996b; Ballok et al., 2003; Williams et al., 2010). However, a few years ago, we observed the MRL/lpr mice were not

performing similar to previous cohorts so we did a comparison of two groups of MRL/lpr from two different time points. We found that the MRL/lpr from the more recent cohort (second cohort) had a decrease in disease activity suggesting that the MRL/lpr mice were losing their autoimmune phenotype (Williams et al., 2010). Next, when correlating behavioral deficits to immunological measures, we found more correlations for the first cohort of MRL/lpr (Williams et al., 2010). These results kept demonstrating that the MRL/lpr mice were not as sick as in previous years. However, even with a reduced autoimmune phenotype we were able to detect a correlation between BRAA and altered behavior, for which possible molecular weights of brain targets as well as binding locations in the brain were identified. It is important to note that our BRAA were reactive to integral membrane proteins from brain tissue which is a novelty of this study since other researchers used Neuro-2A cells (Williams et al., 2010). This first study showed more support for the BRAA hypothesis and the results have been published in the Journal of Neuroimmunology.

In the second segment of our research we decided to use microarray technology to identify some of the targets of these BRAA as well as to provide a better diagnostic tool for lupus and CNS lupus. Current tests to accurately determine if someone has lupus is not available since lupus can look like so many other diseases and that is why it is called "the great imitator" (Liu and Ahearn, 2009). Measurements are taken to determine the levels of autoantibodies such as anti-nuclear antibodies for diagnosis, however our lab used a microarray chip containing random peptides of known sequence and based on the binding pattern

we were able to predict and diagnose lupus and of even more importance, specific CNS manifestations caused by lupus in our mouse model (Liu and Ahearn, 2009). In our first microarray study, we were able to identify peptides that could distinguish between deficits on the forced swim test and sucrose preference test as well as diagnose lupus. This is where we tested the diagnostic antibody hypothesis which states that there are different autoantibodies present during lupus and we can diagnose if someone has lupus or any of its CNS manifestations by the presence of these autoantibodies. The results in the first study were obtained from one set of mice, so we ran another study to determine which of the peptides may indeed be diagnostic peptides of lupus and each specific CNS manifestations. The predictive antibody hypothesis, which states that there are certain autoantibodies present early, even before any of the signs of lupus and its CNS manifestations and if we can detect these autoantibodies we can predict if someone is going to get lupus or a specific CNS manifestation, was tested here as well. Based upon commonality across both studies, we discovered 18 peptides that may be predictive of lupus, 58 peptides that are diagnostic peptides of lupus, 39 peptides that are diagnostic of altered behavior in the forced swim test and possible predictive peptides of altered behavior in the forced swim test.

This type of diagnostic technology is currently not available and the success of our tests would be very useful in providing better care to lupus patients since we will not only be able to tell that a patient has neuropsychiatric lupus, but specifically which neuropsychiatric manifestation is due to the autoimmune processes. Once we identified predictive and diagnostic peptides of lupus and

specific CNS manifestations, we took the peptide sequences of these peptides of interest and using an alignment program, we identified possible targets of these BRAA that corresponded to each data set. Also, using one mouse from the second study, MRL/lpr #2, we created five monoclonal. We then used microarray technique and Western blotting to best determine the possible identity of these BRAA targets. The identification of these proteins will provide much needed biomarkers for lupus and specific CNS manifestations and also targets for therapy.

One important feature of using our random microarray chip to predict and diagnose lupus and its CNS manifestations is the cost. Since our chip can be used and have been used in other studies to try and diagnose other diseases, this makes the use of our chip inexpensive since no specialized chip is needed specifically for lupus (Boltz et al., 2009; Morales Betanzos et al., 2009; unpublished data).

Lastly, we hypothesized that substance P plays a role in causing some the CNS dysfunction in lupus through alteration of the BBB when bound to its NK-1R (Annunziata et al., 2002). So in the final study of this dissertation we looked at the levels of NK-1R in the brains of our 4 month MRL/lpr in comparison to the 4 month MRL/mp. Our 4 month MRL/lpr with high disease activity, increased BRAA levels and increased NK-1R levels all displayed behavioral dysfunction as compared to the controls. The reason for this may be that if the NK-1R is indeed responsible for assisting in opening up the BBB and all of the mice had increased levels of NK-1R, then the high levels of BRAA that is present in their sera would be able to enter the brain and bind to its target antigen. These initial results help

to support the BRAA hypothesis, but also suggest a possible brain protein that may be affecting the BBB.

My research has added more information on the role that BRAA are playing in causing some of the neuropsychiatric manifestations of lupus. We tested the BRAA hypothesis and found this hypothesis to be a reasonable explanation for some of the CNS manifestations of lupus. We also identified the molecular weights of some of the potential BRAA targets that may be responsible for the deficits on the forced swim test as well as possible binding sites in the brain (Williams et al., 2010). In the future, as we continue to identify the names of the BRAA targets responsible for this altered behavior, we now have data on what the molecular weights of those targets could be. Also, based on the different proteins that have been found by other researchers to be affected during CNS lupus, we can relate this information back to the molecular weights we discovered. We have also used microarray technology to more accurately predict and diagnose lupus and specific neuropsychiatric manifestations, which is currently not possible for individuals with lupus. The accurate diagnosis of each specific CNS manifestation in our mouse model of lupus is a novelty of this study. This technology has also allowed us to provide the names of brain targets, which is highly needed since currently more biomarkers for lupus and CNS lupus are necessary (Liu and Ahearn, 2009). Lastly, we were able to put forth, for the first time, support that NK-1R levels are altered in mice displaying CNS manifestations which may play a role in allowing the pathogenic BRAA from our BRAA hypothesis to enter the brain and cause CNS dysfunction. Overall, we

have provided data on pathogenic, predictive and diagnostic autoantibodies in lupus and CNS-lupus and the use of these autoantibodies as future biomarkers and target for therapy.

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Chapter 2

CIRCULATING BRAIN-REACTIVE AUTOANTIBODIES AND BEHAVIORAL DEFICITS IN THE MRL MODEL OF CNS LUPUS

ABSTRACT

Brain reactive autoantibodies (BRAA) are hypothesized to play a role in the neuropsychiatric manifestations that accompany systemic lupus erythematosus (SLE). The present study tests the proposed relationship between circulating BRAA and behavioral deficits in lupus-prone MRL/lpr mice. Two age-matched cohorts born at different times were used to test the relationship in the context of altered disease severity. Significant correlations between autoimmunity and behavior were detected in both cohorts. These results are the first to report correlations between behavior and autoantibodies to integral membrane proteins of brain, supporting the hypothesis that BRAA contribute to the behavioral dysfunction seen in lupus.

Keywords: Brain-reactive autoantibodies, Lupus, CNS lupus, Behavior, MRL mice

INTRODUCTION

Almost seventy years ago, the German physician Lechman-Facius proposed the link between autoimmunity and mental illness. He observed that immunoglobulin/protein in sera and cerebrospinal fluid from psychiatric patients may react with neuronal antigens (Lehmann-Facius, 1939). The above notion did not attract significant attention until the 60's, when Fessel and Solomon published a series of reports on "macroglobulins" or "anti-brain factors" in psychotic patients (Fessel, 1962a, 1962b, 1962c; Fessel and Hirata-Hibi, 1963; Solomon et. al., 1966, 1969). Although recent work supports the hypothesis that brain-reactive autoantibodies (BRAA) play a role in the pathogenesis of some forms of mental illness, further evidence is required to establish the cause-effect relationship (Ganguli et al., 1993; Tanaka et al., 2003; Schott, Schaefer et al., 2003; Margutti et al., 2006).

One of the conditions with well-documented BRAA involvement is neuropsychiatric lupus (NP-SLE or CNS-SLE), an autoimmune disorder which affects both the central and peripheral nervous systems (Carr et al., 1978; Hoffman and Sakic, 2009). It was in this context that the autoantibody hypothesis received strong impetus from the findings that sera from lupus patients and autoimmune mice contain autoantibodies reactive with brain tissue (Martin and Martin, 1975; Bluestein and Zvaifler, 1976) and isolated neurons (Hoffman et al., 1978a,b; Harbeck et al., 1978). This hypothesis was refined by distinguishing non-pathogenic from pathogenic BRAA, or a sub-set that could induce neuropsychiatric manifestations (Narendran and Hoffman, 1989; Hoffman and

Madsen, 1990). Subsequently, NP-SLE has been found to be frequently accompanied by increased levels of serum autoantibodies [reviewed in (Hanly, 2005)], which cross-react with diverse brain-specific and systemic antigens (Zandman-Goddard et al., 2007).

Given that the blood-brain barrier (BBB) is compromised in SLE, it is still unclear whether autoantibodies passively diffuse from peripheral blood and/or become synthesized intrathecally [reviewed in (Hoffman and Harbeck, 1989; Abbott et al., 2003)]. The importance of a breached BBB in BRAA pathogenicity has been recently confirmed in animal models. In particular, active immunization with the NR2 antigens of the NMDA receptor (Kowal et al., 2004), or passive infusion of serum with reactivity to the NMDA receptor and DNA (Kowal et al., 2006) led to learning deficits when barrier permeability was increased by systemic administration of lipopolysaccharide. Compared to this antigen-induced, acute model of CNS-SLE, the inbred strain of MRL/MpJ-Fas^{lpr}/J (MRL-lpr) mice develops systemic autoimmune disease spontaneously. Although they show ~50% mortality between 5 and 6 months of age [reviewed in (Theofilopoulos, 1992)], serological changes, such as increased levels of IL-6, can be detected even at 3 weeks (Tang et al., 1991). Similar to SLE, the murine form of SLE has a progressive and chronic time-course, which is accompanied by a constellation of behavioral deficits, operationally labeled "autoimmunity-associated behavioral syndrome", or AABS (Sakic et al., 1997a,b). At the onset of autoimmunity the deficits are most consistently noted in tasks reflective of emotional reactivity and affective behavior (Szechtman et al., 1997), while at advanced stages of lupus-like

disease learning/memory deficits may emerge (Sakic et al., 1992; Hess et al., 1993). Autoimmunity-induced compromise of the BBB is evidenced in autoimmune mice by immunoglobulin binding in brain (Zameer and Hoffman, 2001), expression of cell adhesion molecules (Zameer and Hoffman, 2003), and infiltration of lymphoid cells into the choroid plexus (Zameer and Hoffman, 2004; Ma et al., 2006; James et al., 2006). Moreover, behavioral deficits and infiltration of immunocytes into the brain tissue coincide with markers of neuronal degeneration and brain atrophy (Sakic et al., 1998, 2000; Ballok et al., 2003a,b, 2004).

An earlier study, in which serum antibodies to Neuro-2A cells were measured, revealed that MRL-lpr mice with serum BRAA differ in behavioral performance from BRAA-negative cagemates (Sakic et al., 1993a). In particular, they moved slowly in a novel environment, groomed less, and showed increased thigmotaxis in comparison to mice that had no detectable levels of BRAA in their serum. The relevance for CNS involvement was tentative, however, because the Neuro-2A cell line is derived from peripheral nerves and surface antigens do not fully match the antigen profile on CNS neurons (Hoffman et al., 1988). In this study, we use a preparation of transmembrane proteins from normal mouse brain. We correlate indices of systemic autoimmunity, brain atrophy, and behavioral deficits in two cohorts of MRL-lpr mice that differ in disease severity. The selection of the transmembrane fraction is based on the evidence that a large set of serum BRAA from several autoimmune strains (including the MRL-lpr strain) are directed against integral membrane proteins extracted from brain homogenates

(Narendran and Hoffman, 1989; Hoffman and Madsen, 1990). In addition, using Western blotting to brain homogenates and immunohistochemistry to brain sections we further test the reactivity of BRAA to CNS tissue. Behavioral measures known to reliably detect aberrant performance in diseased MRL-lpr mice were selected (Sakic et al., 1994a, 1996).

Over the past several years, however, the immunological phenotype of the MRL-lpr substrain changed to the point that autoimmune manifestations became mild and their life span significantly extended. This unexpected phenomenon (http://jaxmice.jax.org/strain/006825.html) led to the re-coding of stock #485 to stock #6825, and re-development of the original MRL-lpr population (available from The Jackson Laboratory from fall 2007). This loss of phenotype was also noted at the behavioral level, prompting a comprehensive re-analysis of the MRL-lpr model using a large cohort of diseased animals (Sakic et al., 2005a,b). Multivariate analysis of immunological, neuropathological, and behavioral data revealed that approximately 30% of MRL-lpr mice (born between 1998 and 2000) show severe brain damage and behavioral dysfunction, however serum BRAA levels were not measured in this study and the current experiment is addressing the correlation between BRAA and brain atrophy/behavior. Therefore, as a secondary focus we also examined the relationship between BRAA and aberrant behavior in the context of declining immunological phenotype. We presently employ MRL/lpr cohorts that were produced over two subsequent years, and tested at these two points in time. The overall expectation was that regardless of diminishing autoimmune profile, increased production of serum BRAA would be

associated with impaired behavioral performance in measures of overall activity, motivated behavior, and/or emotional reactivity. There have been no previous reports of a decline in behavioral dysfunction in an autoimmune substrain in parallel with a decline in autoimmune phenotype. The comparisons between these two cohorts provided us with a rare opportunity to test the causal relationship between peripheral autoimmunity and CNS involvement.

MATERIALS AND METHODS

ANIMALS

Compared to the congenic MRL/MpJ sub-strain, MRL-lpr mice (both male and female) develop an accelerated form of lupus-like disease, with ~50% mortality occurring between 5 and 6 months of age [reviewed in (Theofilopoulos, 1992)]. Serological changes (e.g. increased production of IL-6) can be detected as early as 3 weeks of age (Tang et al., 1991), followed shortly by an excessive production of autoantibodies and infiltration of leukocytes into the choroid plexus (Vogelweid et al., 1991; Ma et al., 2006).

To examine whether the autoimmune phenotype indeed diminished over time, the immune markers were compared at equivalent ages, between fourteen 3-5 month-old, male MRL/lpr mice (first cohort, N = 14, born in 2004) and forty 3-5 month-old, male MRL/lpr mice (second cohort, N = 40, born in 2005). For correlations between variables (primarily behavior and immunologic) all animals, from 5 to 34 weeks, were used in the first cohort (N = 30). Differences in numbers (N) between the figures was due to not being able to use some animals in the correlations or comparisons, e.g., because not all the data were available for specific animals in a group. The cohorts were maintained under comparable housing conditions and tested with the same apparatus one year apart. The 3 – 5 month time frame was chosen because CNS involvement and behavioral deficits can be readily detected, but potentially confounding clinical manifestations (e.g. dermatitis, lymphadenopathy, arthritis) are not present yet.

Mice were housed 4-5 per cage, and kept under standard laboratory conditions (light period from 8 A.M. - 8 P.M., room temperature ~22^{o}C, humidity ~62%, regular rodent chaw, and tap water *ad libitum*, bedding changed every 3 - 4 days). After acclimation and habituation, they underwent behavioral testing over 3 weeks. During the behavioral testing the animals were housed individually. The sequence of behavioral tests matched the order they are described below. One test was given daily, with no overlapping.

Brains from a 2 and a 4 month-old female C3H/HeJ mice were used for immunohistochemistry and Western Blotting, respectively. Sera from three 4 month-old female C3H/HeJ mice were also used as negative controls in the immunohistochemistry. The C3H strain has no autoimmunity and is a 12% background strain for the MRL mice. We could not use MRL brain, since we have previously reported that both MRL strains have in situ bound Ig (Zameer and Hoffman, 2001), while C3H had none, and this could interfere with testing for serum BRAA binding to brain. The CNS antigens from C3H were expected to be comparable to the antigens in the MRL strains, but free of pre-bound BRAA.

BEHAVIORAL TESTING

The specific behavioral tests selected were based on our previous research (Sakic et al., 1993a,b, 1996, 1997a,b, 2005a,b), which has reliably shown differences between MRL/lpr mice and controls. Most of the behavioral tests are related to affective disorders, which have been reported in CNS-SLE patients.

SPONTANEOUS NOCTURNAL ACTIVITY

Spontaneous ambulation was assessed by computerized activity monitors (AccuScan Instruments, Columbus, Ohio) from 6 P.M. – 8 A.M. During the testing period mice were taken out of home cages (4-5 mice housed per cage) and returned 14 h later. The testing room was equipped with ten activity chambers (40×40×35cm) with ventilated lids. The chambers were interfaced with a PC computer running VersaMax software from the same manufacturer. Total distance traveled, moving time, and ambulatory speed were assessed in 30-min intervals. These assessments were designed as a basic test of motor activity, which can be used to assess the effects of disease on motor functioning, as well as to supplement information on the tests of affective behavior. "Lights on" reflected cumulative measures from 6 P.M. – 8 P.M., while "lights off" reflected cumulative score from 8 P.M. – 8 A.M. During the 14-h testing mice did not have access to food and water.

SUCROSE PREFERENCE TEST

Reduced sucrose intake in a preference paradigm is proposed to measure sensitivity to reward and model anhedonia (Willner et al., 1992). More extensive analysis of the dose-dependent performance, post-ingestive factors, and taste responsiveness has been reported in our previous studies (Sakic et al., 1996, 1997a,b). Based on an established methodology (Ballok et al., 2003a,b), mice were trained to drink 3 ml of a 4% sucrose solution from a graduated syringe fastened to the cage lid with a 2.5" paper clip. They had 24-h access to sucrose

over three days (the training period), and free access to food and water. Training and testing was performed after the activity monitoring, but before the forced swim test. The criterion for training was that the mouse empties the syringe at least once before testing. All of the mice met this criterion. The solution was then removed for 24-h to allow sugars to clear from their circulation. A 1-h sucrose preference test (between the times 20:30-21:30h) was given over four subsequent nights. Each night syringes were filled with one of four sucrose solutions, presented in ascending order (i.e., 1, 2, 4, or 8%). The volume ingested over three trials (a trial being the 1 hour period) was used as an index of responsiveness to palatable stimulation.

FORCED SWIM TEST

Increased immobility of rodents in a no-escape situation was proposed to reflect a state of lower "mood", which can be reduced by antidepressants and electroconvulsive shock (Porsolt et al., 1977). It is a good complement, in testing affective behavior, to the sucrose preference test. Presently, a mouse was gently lowered into a circular pool filled with 25°C water and allowed to swim for 10min (Sakic et al., 1994a). The time spent in floating was measured by EthoVision XT video tracking software (Noldus, NL). Floating was defined when swimming speed was less than 5 cm/s.

TISSUE COLLECTION AND INDICES OF AUTOIMMUNE DISEASE

Mice were anesthetized with an intraperitoneal injection of Somnotol (65mg/kg) and body weight was measured on a digital scale (Sartorius 2024 MP, VWR, Scientific Canada Ltd.). Blood samples were collected by cutting the vena cava and exsanguinating (approximately 1 ml) within 10-15 seconds using a needle-free syringe, then left to coagulate for 1 h at 4^{o}C, and centrifuged for 3min at 7000rpm. Serum was separated from blood clots, aliquoted and plastic vials (containing 100 µl of serum/mouse) shipped on dry ice by over-night courier service for further analysis. Blood vessels were flushed with intracardial phosphate buffered saline (~40ml), brain was extracted within 2min, and weighed on an analytical scale (AB54-S, Mettler Toledo, Switzerland). Reliable signs of systemic autoimmune disease in MRL-lpr mice are splenomegaly and high serum levels of autoantibodies. Therefore, wet spleen weight was measured on an analytical scale immediately upon extraction. Serum levels of anti-DNA antibodies were measured by ELISA, as described below.

INTEGRAL MEMBRANE PROTEIN PREPARATION

The protocol used for the extraction of the integral membrane proteins used in the ELISA and Western blotting techniques was previously described by (Narendran and Hoffman, 1988). Briefly, the integral membrane proteins were suspended in phosphate buffer saline (PBS), if being used for ELISA, or 1.0M Tris (pH 6.8) if being used for Western blotting. The concentration of the integral membrane proteins used for the ELISA was determined using the BCA Assay Kit

(Pierce, USA) and the Bradford Test (Sigma-Aldrich, USA) when doing Western Blots.

ELISA FOR ANTI-DNA AND BRAA

The procedure for determining anti-dsDNA and BRAA levels were performed using previously described protocols (Aotsuka et al., 1979; Crimando and Hoffman, 1995; Zameer and Hoffman, 2003) and only brief descriptions, plus differences from the norm, are given here. To test anti-dsDNA antibody levels poly-L-lysine (Sigma, USA) was used to coat the 96-well plates. The odd wells of the plates were incubated with 10 µg/ml of calf-thymus DNA (Sigma, USA; purified for dsDNA), while the even wells received only PBS (paired control wells). One hundred µg/ml of poly-L-glutamate (Sigma, USA) dissolved in PBS was added to the plates and thereafter PBS containing 5% bovine serum albumin (BSA) (Sigma, USA). The plates were incubated with serial dilutions of mouse sera and then incubated with the secondary antibody goat anti-mouse IgG antibody conjugated with peroxidase (Caltag, USA) diluted at 1/1000 in PBS. Lastly citrate buffer containing 2,2'-azino-bis(3ethylbenzthiazoline) sulfonic acid and hydrogen peroxide was added for 30 minutes and incubated at 37^{o}C. The plates were then read at 405nm on a microplate reader. The data was corrected for background binding by subtracting the optical density (OD) values for the paired control wells from the OD values for the wells containing the calf-thymus DNA, which is known as the S-value. The S-value was used for the statistical analysis. Due to correcting for background binding, S-values were allowed to be

negative because occasionally the binding in the control well was greater, likely due to high levels of immunoglobulins in the mouse serum. This helps deal with a lot of the variability that occurs in autoimmune mice. Since this calculation was performed on all samples, the binding intensity in individual samples was relative to each other.

S-values were also calculated in the same way for the serum BRAA. We used 10 μg/ml of integral membrane proteins from a brain homogenate of healthy C3H/HeJ mice. The plates were treated with 5% BSA to reduce background reactivity and serial dilutions of the mouse sera were added to the wells. The secondary antibody, goat anti-mouse IgG antibody conjugated with peroxidase (Caltag, USA) was diluted at 1/1000 in PBS. The plates were read at a wavelength of 405 nm on a microplate reader.

IMMUNOBLOT ANALYSIS

We further tested the serum samples from the mice using immunoblotting techniques we have previously described (Narendran and Hoffman, 1989; Hoffman and Madsen, 1990) to determine the apparent molecular weight of antigens to which BRAA are binding. Briefly, a polyacrylamide gel was prepared using a 12% resolving gel layer and a 4% stacking gel layer. The stacking gel was then loaded with 0.668 μg/μl of integral membrane proteins from a 4 month C3H/HeJ mouse. The gel was then transferred to nitrocellulose paper (BioRad Laboratories, CA) and then cut into strips and incubated with blocking solution containing TBS, 0.1% Tween-20, 5% albumen, and 1% casein (Sigma). Serum

samples from the mice were diluted and added to the strip of nitrocellulose paper overnight. The secondary antibody, goat anti-mouse IgG antibody conjugated with peroxidase (Caltag, USA), was diluted at 1/5000 and the blots were then incubated with a detection solution from a Chemiluminescence Kit (Roche, USA). The blots were exposed to the X-ray film (Kodak, New York) for 1, 5, and 10 minutes and then processed. The molecular weight (MW) of the bands was determined from the graph with known MW markers and the distance that a given band traveled. Some strips were incubated with anti-mouse NK-1R antibody (Zymed, USA) as a positive control.

IMMUNOHISTOCHEMISTRY

Based on previous techniques of ours (Hoffman et al., 1978a,b; Zameer and Hoffman, 2001), brains from 2 month-old C3H/HeJ mice were blocked by cutting the entire brain into 3 blocks at an angle of 68° from the table top and freezing in Tissue Tek OCT Compound (Sakura, USA) using dry ice and 2-methyl butane. These were then cut on a cryostat into 8 µm sections and heat-fixed onto a microscope slide and acetone-fixed. Subsequently, the slides were immersed in 50^{o}C citrate buffer containing 1.92g/l of citric acid at a pH=6.0 to better expose the epitopes for antibody binding on the brain sections. A 1.5% blocking solution containing PBS and BSA was added to the sections and mouse sera at a 1/10 dilution was then added overnight at 4^{o}C. After washing, the secondary antibody, FITC-conjugated goat anti-mouse IgG (Caltag, USA**)**, which had been treated with rabbit liver powder (in order to prevent non-specific binding

of the secondary antibody to mouse tissue), was added to the brain section at a 1/10 dilution. A 1/10 dilution of propidium iodide (to stain the DNA in the cells) to Fluoroguard (to prevent fading of the fluorescence being detected) was added to the sections. Negative controls included slides without primary (i.e., sera) or secondary antibody (controlling for auto-fluorescence), as well as slides without primary antibody but having the secondary antibody (as a control for non-specific, secondary antibody binding). Pictures of the cortex and the hippocampus were taken at 100x objective magnification. The brain sections were analyzed using confocal microscopy, using the Keck Lab facilities at Arizona State University.

STATISTICAL ANALYSIS

The Student's *T*-test was used for all simple comparisons between cohorts (Fig. 1). Correlations (Tables 1 and 2, and Fig. 2A, B and C) between the immunological (BRAA, anti-DNA and spleen weight) and neurobehavioral variables (forced swim test, 4% sucrose consumption, activity measures with lights on and off, for distance travelled, movement time and speed, plus the brain weight) were also done for both cohorts. More specifically, linear relationships between scale variables were analyzed by partial correlation, with age and/or body weight as controlling factors, for the first cohort and bivariate correlations for the second cohort. Given that the direction of relationships was known *a priori*, one-tailed significance was used in the overall analysis. Fig. 1 shows mean values ± SEM. Significant differences of $p \leq 0.05$, $p < 0.01$ and $p < 0.001$

are indicated by *, **, and ***, respectively, in the figures. All computations were performed using the SPSS 15 statistical package.

RESULTS

LOSS OF AUTOIMMUNE PHENOTYPE

As expected, males from the first cohort had bigger spleens (t_{52}=4.238, $p<0.01$, Fig. 1A) and higher levels of (a 1:400 dilution for both cohorts) anti-DNA antibodies (t_{49}=10.012, $p<0.001$, Fig. 1B) than animals from the second cohort. Similarly, serum BRAA levels (done at closely comparable dilutions of 1:25 in the first cohort and 1:20 in the second cohort) were higher in mice from the first cohort (t_{50}=8.881, $p<0.001$, Fig. 1C). These results are consistent with the expectation that the autoimmune phenotype of the MRL/lpr substrain diminished over two years.

THE RELATIONSHIP BETWEEN IMMUNE MEASURES, BRAIN SIZE, AND BEHAVIOR

FIRST COHORT

The same tests and correlations were performed on both cohorts, only those correlations which were significant are shown in the Tables 1 and 2. It should be noted that there are 8 significant correlations to behavior in the first cohort and only 5 in the second cohort, consistent with the reduced autoimmune phenotype. Significant relationships obtained by partial correlations for the first cohort are shown in Table 1. Consistent with the hypothesis that BRAA play a role in the etiology of behavioral dysfunction, increased serum BRAA levels (1:20 dilution) were associated with impaired 4% sucrose consumption, (r_{29}=-0.348, p=0.041; Figure 2A) and reduced speed during spontaneous nocturnal

ambulation (r_{29}=-0.399, p=0.022). The latter result is consistent with our earlier report, in which serum levels of antibodies to the Neuro-2A cell line correlated with low ambulatory speed and increased anxiety-related behaviors (Sakic et al., 1993a).

As one may expect, increasing levels of anti-DNA autoantibodies (a serological marker of disease activity) were associated with increasing BRAA levels (r_{29}=0.550, p=0.002). More importantly, increased anti-DNA autoantibody levels correlated with shorter distances traveled at night (r_{24}=-0.388, p=0.025), as well as with shorter movement times (r_{30}=-0.366, p=0.033). As observed in our previous studies (Sakic et al., 2005a,b; Ma et al., 2006; Ballok et al., 2006), mice with bigger spleens had lower brain weights (r_{30}=-0.699, p<0.001). Conversely, increased brain weight positively correlated with distance traveled (r_{30}=0.469, p=0.008), movement time at night (r_{30}=0.461, p=0.009), and volume of 4% sucrose solution consumed (r_{30}=0.430, p=0.014). Splenomegaly was associated with reduced consumption of 4% sucrose (r_{30}=-0.352, p=0.039).

SECOND COHORT

Since the direction of relationships has been established previously, a one-tailed test of significance was accepted in the bivariate correlation analysis (selected data summarized in Table 2). Increased floating time in the forced swim test was associated with higher BRAA levels, tested at a dilution of 1:80 (r_{38}=0.463, p=0.002; Table 2 and Fig. 2B). Similar to the first data set, BRAA (1:80) levels correlated with splenomegaly (r_{38}=0.504, p=0.001) and anti-DNA

autoantibody levels (r_{37}=0.358, p=0.015), indicating a correlation to disease activity.

Anti-DNA autoantibody levels and splenomegaly correlated positively (r_{37}=0.633, p<0.001), further supporting the relationship between serological markers and organ pathology in lupus-like disease. Increased consumption of 4% sucrose (r_{37}=-0.324, p=0.025; Fig. 2C) correlated negatively with increased levels of anti-DNA autoantibodies, suggesting an impaired motivated response in mice with severe systemic autoimmunity. Further support for this notion came from the negative correlations between spleen weight and 4% sucrose consumption (r_{38}=-0.330, p=0.022), and the positive correlation between splenomegaly and floating time (r_{38}=0.308, p= 0.03). Lastly, brain weight correlated positively with increased 4% sucrose consumption (r_{38}=0.274 p=0.048), suggesting that mice with brain atrophy due to autoimmunity were also poor responders to a palatable stimulation.

WESTERN BLOT ANALYSIS

To characterize BRAA on the basis of molecular weight of the antigen bound, we used the remaining sera for the Western blot analysis. Those mice showing the greatest immobility in the forced swim test and the highest BRAA levels was used in the initial analysis. Fig. 3 illustrates BRAA banding patterns (and their approximate molecular weights) in these mice (samples 1, 17 and 25). For the mice that showed low BRAA levels and low float times (far left, Fig. 2B), three out of the five samples (i.e. #12, #18, and #33) had no bands. Bands were,

however, detected in sample #3 (Fig. 3D) and sample #14, suggesting that sensitivity and specificity of Western blotting and ELISA were different.

Bands were detected as listed in Table 3. No bands were detected in sample numbers 2, 6, 8-10, 12, 18-24, 26, 29, 30, 32-35, 38, and 39. Samples 11, 15, 27, 28, 31, 36, and 40 were not tested due to an insufficient amount of sera. As previously reported (Narendran and Hoffman, 1989), there was a diversity of antigens bound by the BRAA.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry was also performed using sera from eight MRL/lpr (the three samples with high float times and five samples with low float times in Fig. 2B) and three non-autoimmune C3H/HeJ mice, our normal age-matched control strain. Red propidium iodide (PI) staining (of DNA) was used to show cells, while the antibodies were detected using a green fluorescein isothiocyanate (FITC) stain, as described in the Materials and methods. In Fig. 4, all the figures on the right correspond to dual staining using both PI and FITC and all the figures on the left are showing staining with FITC only. No binding was seen in any of the controls where no primary (sera) or secondary (anti-Ig) was added, or where secondary antibody was added only, or in the C3H serum samples tested (Fig. 4C and 4D). Binding to brain sections was observed in all of the eight MRL/lpr samples, not distinguishing between mice with high or low BRAA levels (as determined by ELISA). Moderate binding to the hippocampus and the cortex (Fig. 4A,B) was seen in serum from mouse #1, who has shown excessive floating,

high ELISA BRAA levels, and BRAA positivity on Western blots. Fig. 4E and F shows moderate binding to the cortex for sample #33, however binding was also seen in the hippocampus for this animal (data not shown). Fig. 4G and H shows moderate binding in the hippocampus for sample #18. These mice (sample #33 and #18) showed low floating time, low BRAA levels and were negative for brain antigens on Western blotting. These results clearly show that there are autoantibody to brain in the sera of the MRL/lpr mice, but also suggest that the levels of BRAA detected in the sera by ELISA, or immunoblotting and the intensity of their binding to the brain (as determined by immunofluorescence) do not necessarily correspond with one another. The sample from mouse #12 (data not shown) further illustrated this lack of correspondence because in comparison to sample #1 (Fig. 4A and B), a stronger binding to the hippocampal region and throughout the brain was observed, even though this mouse had low BRAA levels, low float time and was negative by Western blotting.

What is interesting, however, is that mice #1, #12 and #33, even though they vary in float times, show low sucrose consumption, often considered to reflect a dysfunctional reward circuitry. This suggests that the immunohistochemistry is more sensitive in the detection of functionally important BRAA, likely because targeted brain antigens are not denatured as much as they are in ELISA and Western blotting. It is important to keep in mind that any of these methods for detecting BRAA can be valid and detect different autoantibodies, but will not necessarily correspond to one another.

Table 4 shows a comparison of data from the 8 MRL/lpr mice (from the second cohort) used for immunohistochemistry. The first 3 animals have high BRAA levels as determined by ELISA and also showed banding by the Western blot. These mice all showed abnormally high float times, indicative of depressive-like behavior. The other 5 mice had low ELISA BRAA levels, but were positive by immunohistochemistry. Some showed Western blot bands, while others did not. All had anhedonia as determined by the relatively low sucrose consumption.

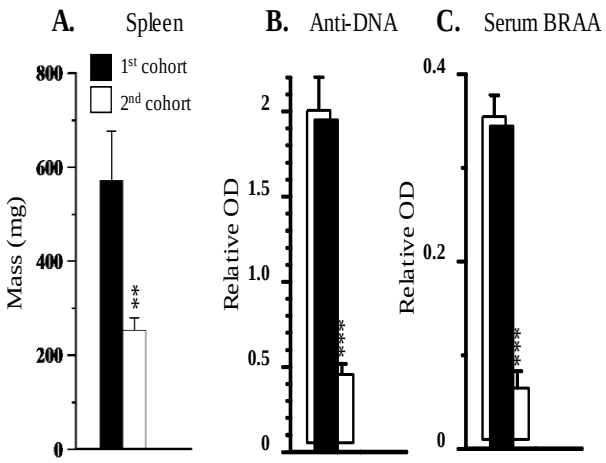

Fig. 1. Differences in spleen weight, anti-DNA autoantibody and BRAA levels between the first (N = 14) and second cohorts (N = 40) confirmed a production year-dependent decline in autoimmune phenotype. The mean +/- 2 standard errors of the mean are shown. The serum dilutions shown were at 1:400 for the anti-DNA autoantibodies, and 1:25 for the first cohort and 1:20 for the second cohort BRAA. There were statistically significant differences between the groups (** p = 0.01, *** p = 0.001).

Table 1. Significant partial correlations (controlling for age and/or body weight) for behavioral and immunological variables measured in the first cohort.

Behavioral / Immune Variables (N = 30)	Immune/Organ Weight Variables			
	Serum BRAA	Serum anti-DNA	Spleen Weight	Brain Weight
4% Sucrose consumption	-0.348*	-0.209	-0.352*	0.430*
Average speed (lights off)	-0.399*	-0.203	0.108	0.083
Distance traversed (lights off)	-0.140	-0.366*	-0.299	0.469**
Movement time (lights off)	-0.081	-0.352*	-0.318	0.461**
Brain weight	-0.123	-0.148	-0.699***	
Serum anti-DNA	0.550**			
Spleen Weight	0.231	0.015		

One tailed test; 5-34 weeks.
Significance: * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$.

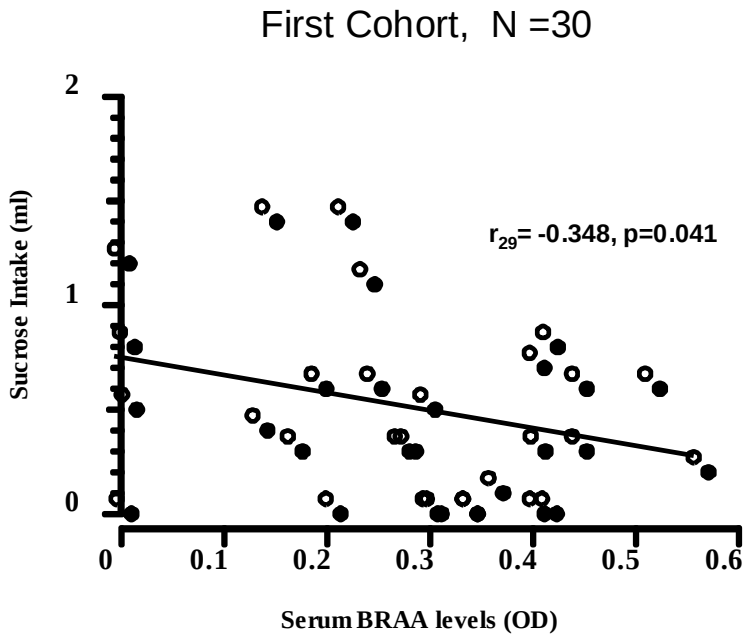

Fig. 2A. Negative correlation between performance in the sucrose test and serum BRAA levels (1:25 dilution), suggesting that decreased 4% sucrose consumption is associated with high levels of serum BRAA (r_{29}=-0.348, p=0.041). This figure shows data from the mice in the first cohort.

Table 2. Significant bivariate correlations for behavioral and immunological variables measured in the second cohort.

Behavioral / Immune Variables (N = 39)	Immune/Organ Weight Variables			
	Serum BRAA	Serum anti-DNA	Spleen Weight	Brain Weight
Floating Time	0.463**	0.240	0.308*	0.015
4% Sucrose consumption	0.031	-0.324*	-0.330*	0.274*
Serum anti-DNA	0.358*			0.061
Spleen Weight	0.504**	0.633***		0.006

Significance: * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$.

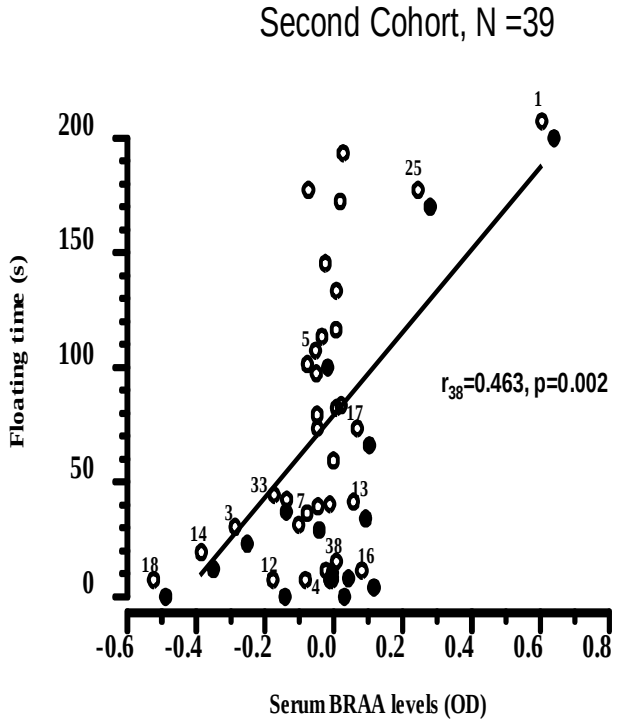

Fig. 2B. Positive correlation between performance in the forced swim test and serum BRAA levels (1:80 dilution), suggesting that increased immobility is associated with high levels of serum BRAA (r_{38}=0.463, p=0.002). This figure shows data from the mice in the second cohort. Solid symbols denote mice whose sera were used for Western blotting and immunohistochemistry.

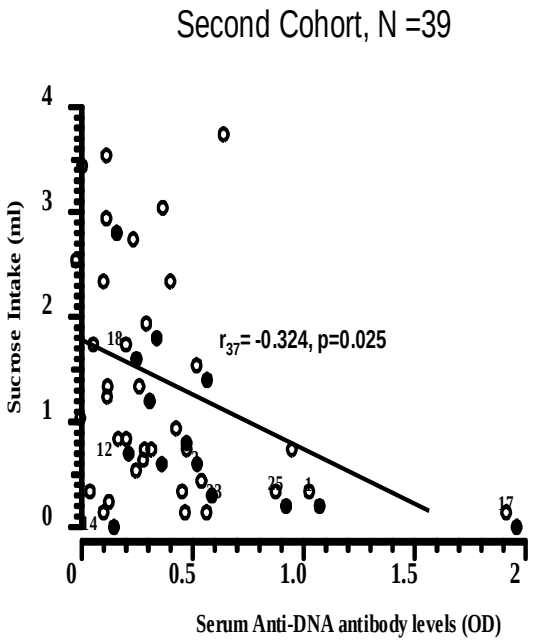

Fig. 2C. Negative correlation between performance in the sucrose test and serum anti-DNA antibody levels (1:400 dilution), suggesting that decreased 4% sucrose consumption is associated with high levels of serum anti-DNA antibodies (r_{37}= -0.324, p=0.025). This figure shows data from the mice in the second cohort. Solid symbols denote mice whose sera were used for Western blotting and immunohistochemistry.

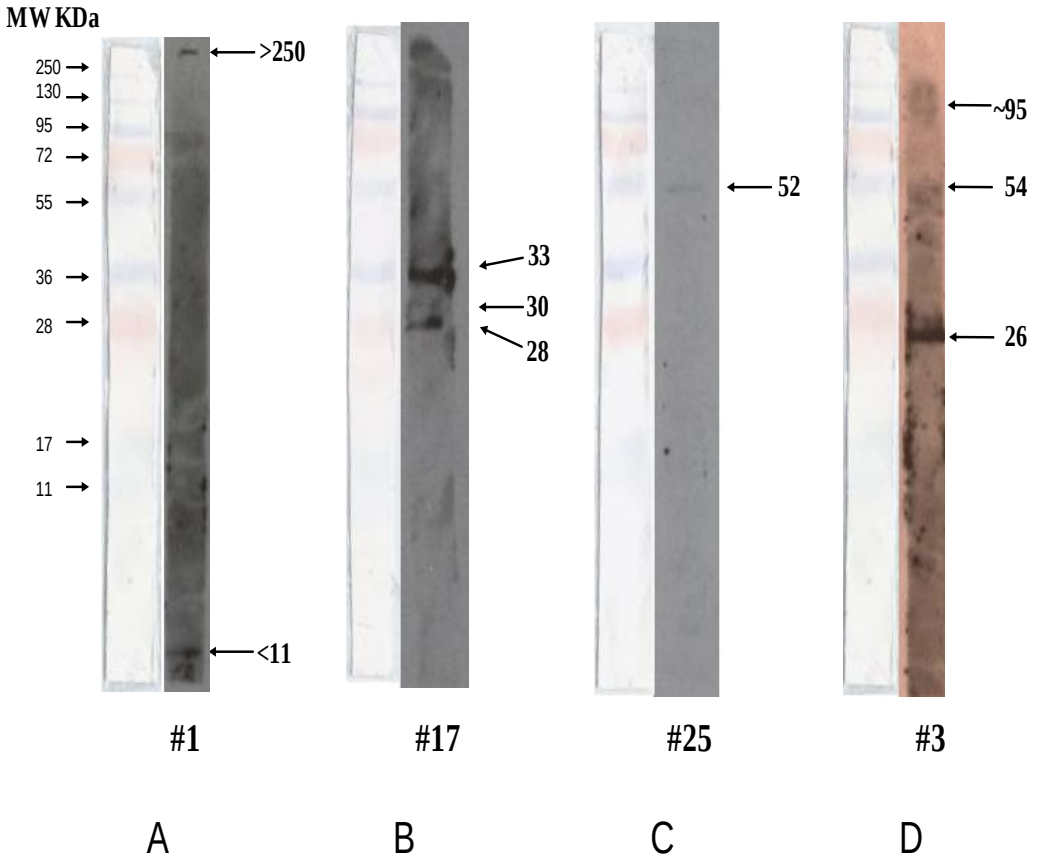

Fig. 3. Representative Western blots of MRL-lpr sera (A-D), corresponding to high or moderate float time animals 1, 17, 25 and low float time animal 3 (see Fig. 2B, from the second cohort), reactive with integral membrane proteins from brain of a non-autoimmune mouse. Different brain antigens were detected and their number or specificity did not match serum BRAA levels measured by ELISA. (Also see Table 4.)

Table 3. Molecular weights of Western blot bands (second cohort)

Mouse ID	Band MW (kDa)
1	>250, 12
3	54, 26
4	48
5	65
7	68, 59, 43
13	47
14	42
16	48
17	33.3, 30, 28
25	52
37	38

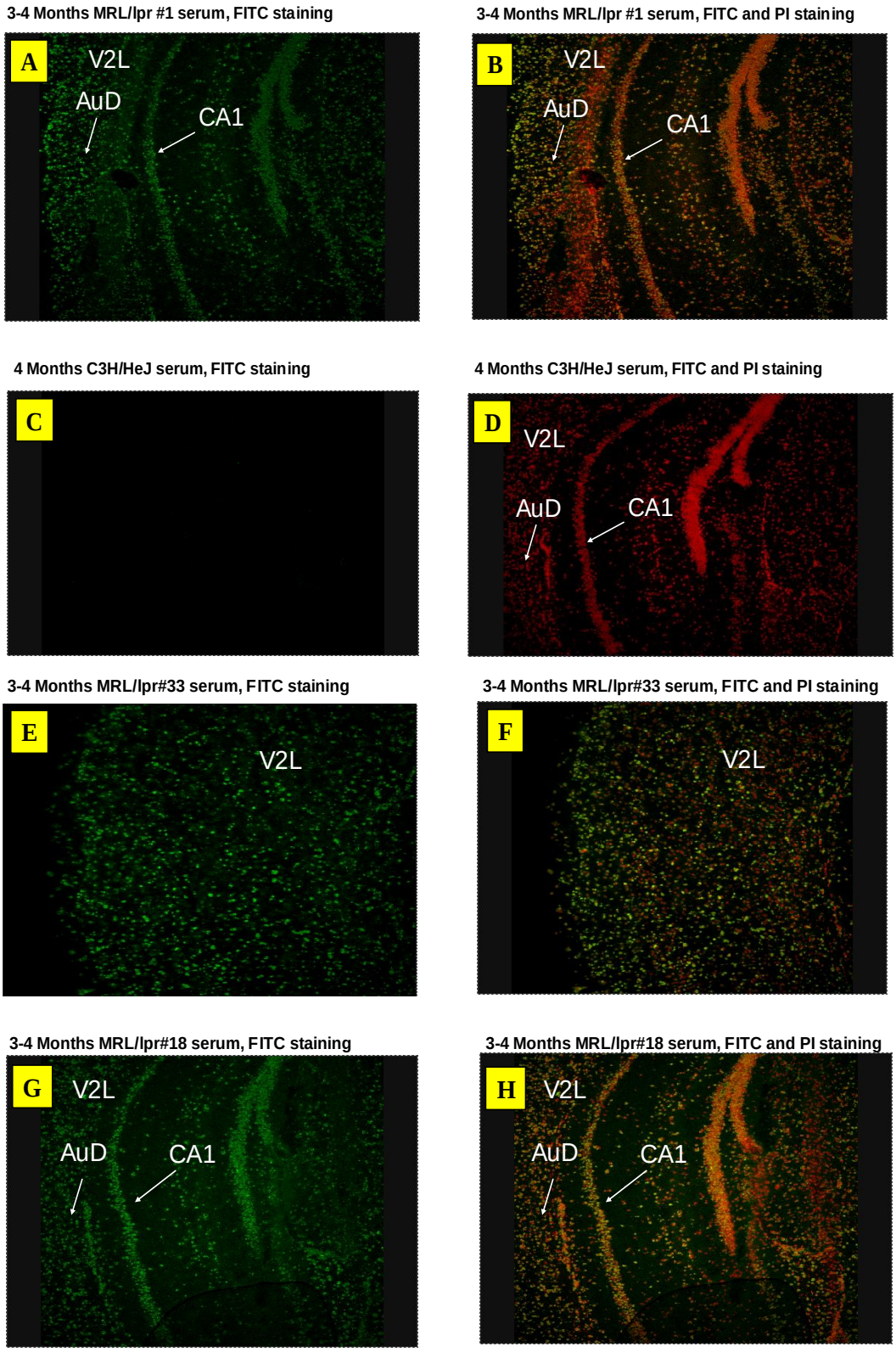
3-4 Months MRL/lpr #1 serum, FITC staining
A
V2L
AuD
CA1
3-4 Months MRL/lpr #1 serum, FITC and PI staining
B
V2L
AuD
CA1
4 Months C3H/HeJ serum, FITC staining
C
4 Months C3H/HeJ serum, FITC and PI staining
D
V2L
AuD
CA1
3-4 Months MRL/lpr#33 serum, FITC staining
E
V2L
3-4 Months MRL/lpr#33 serum, FITC and PI staining
F
V2L
3-4 Months MRL/lpr#18 serum, FITC staining
G
V2L
AuD
CA1
3-4 Months MRL/lpr#18 serum, FITC and PI staining
H
V2L
AuD
CA1

Fig. 4.

Fig. 4. Representative images showing binding between serum BRAA and brain sections from healthy C3H/HeJ mice by immunohistochemistry. The green fluorescence (FITC) shows antibody - antigen binding, while the red color shows propidium iodide (PI) staining of the cell nucleus which allows for easier identification of brain structures. Dual staining with PI and FITC is shown in figures B, D, F and H, i.e., all the figures on the right. A, C, E and G show the BRAA green fluorescence only, using FITC, i.e., all the figures on the left. **(A)** Hippocampus and cortex showing only green fluorescence after exposure to serum from MRL-lpr mouse #1. **(B)** Hippocampus and cortex showing both green and red fluorescence after exposure to serum from MRL-lpr mouse #1. The combination staining appears as yellowish/green. **(C)** Lack of binding in the hippocampus and cortex using a serum from a non-autoimmune C3H/HeJ mouse with green fluorencence only. No staining indicates no BRAA binding. **(D)** Red and green fluorescence showing binding in the hippocampus and cortex using a serum from a non-autoimmune C3H/HeJ mouse. Only the red PI staining appears, indicating lack of BRAA binding. **(E)** Green fluorescence in the cortex only, obtained with serum from MRL-lpr mouse #33. **(F)** Green and red fluorescence in the cortex only, obtained with serum from MRL-lpr mouse #33. **(G)** Green fluorescence in the hippocampus only, obtained with the serum sample from MRL-lpr mouse #18. (**H)** Green and red fluorescence in the hippocampus only, obtained with the serum sample from MRL-lpr mouse #18. Abbreviations: CA, hippocampal regions; AuD, secondary auditory cortex, dorsal area; V2L, secondary visual cortex, lateral part.

Table 4. Comparisons from Second Cohort

Mouse Number	Forced Swim Test	Sucrose Test	Western Blot Number of Bands (Molecular Weight)	Immunohistochemistry	BRAA Levels
1	High Float Time	Low Consumption	2 (>250, 12)	+	High
17	High Float Time	Low Consumption	3 (33.3, 30, 28)	+	High
25	High Float Time	Low Consumption	1 (52)	+	High
3	Low Float Time	Low Consumption	2 (54, 26)	+	Low
14	Low Float Time	Low Consumption	1 (42)	+	Low
12	Low Float Time	Low Consumption	0	+	Low
33	Low Float Time	Low Consumption	0	+	Low
18	Low Float Time	Moderate Consumption	0	+	Low

DISCUSSION

Our previous studies have shown that behavioral changes and disease-dependent overproduction of BRAA occur in parallel in the MRL/lpr substrain (Hoffman and Madsen, 1990; Zameer et al., 2001; Sakic et al., 1994b, 2005a,b). The levels of circulating BRAA peak around 3 months of age (Hoffman et al., 1987) with parallel deficits in spatial learning and memory (Sakic et al., 1993b). In addition, circulating autoantibodies to neuroblastoma cells were associated with changes in locomotor activity in a novel environment, short grooming episodes, and enhanced thigmotaxis (Sakic et al., 1993a). A neuroblastoma cell line does not, however, reflect the antigenic spectrum of a normal mouse brain. In addition, paradigms reflective of motivated behavior were not employed in these early behavioral studies. For the first time, the present study shows a consistent set of correlations between circulating BRAA to native brain antigens (as confirmed by Western blot and immunofluorescence) in the context of autoimmune phenotype (both declining and natural). As such, it provides further evidence of the importance of naturally occurring, peripheral BRAA in the etiology of structural and functional CNS damage during lupus-like disease.

It was found, over a ten year period, that there was a declining autoimmune phenotype in MRL/lpr mice (http://jaxmice.jax.org/strain/006825.html) produced in The Jackson Laboratories. This phenomenon of unknown etiology allowed us to test the hypothesis that behavioral abnormalities could occur even in weak autoimmune disease and correlate with BRAA. The present study confirms the declining phenotype and

extends this by showing a cohort-dependent decrease in splenomegaly, serum autoantibody levels, and their declining association with behavioral deficits. Nevertheless, associations were found in both cohorts (with mild and more severe autoimmune disease), supporting the hypothesis that circulating BRAA alter behavior, even with fading autoimmunity. The fact that there were fewer correlations between behavior and immunological parameters in the second cohort is consistent with our expectation, due to declining autoimmune phenotype in this cohort. We would also expect differences in correlations between the two cohorts, as were seen, due to the differences in autoimmune phenotype. Although these correlations suggest a link between BRAA and behavioral alterations, they do not prove a causal connection. Other researchers have supplemented this by using passive or active BRAA transfer to specific antigens (Kowal et al., 2004; Lawrence et al., 2007; Mondal et al., 2008). Given the diversity of behavioral manifestations in NP-SLE and AABS, as well as the results of our current study, it is expected that a variety of BRAA account for deficits in different domains of behavior.

In the first cohort high BRAA levels were associated with low sucrose consumption and low ambulatory speed. In addition, high anti-DNA autoantibody levels were a predictor of attenuated locomotor activity. Finally, splenomegaly was a predictor of reduced sucrose consumption and increased immobility. It should also be noted that speed, distanced traveled, and movement time reflect the same aspect of behavior, viz., reduced locomotor activity. Correlations between BRAA and ambulatory speed, and anti-DNA and

ambulation time and distance, may reflect an overlapping of autoantibody action. Taken together, these correlations suggest that more severe lupus-like disease is associated with the emergence of behavioral dysfunction, largely in the domain of locomotion and "affect". They also suggest that autoimmune factors different from BRAA (e.g., cytokines, immune complexes, complement components, vascular pathology) affect behavior. The relationship between brain weight and sucrose consumption/movement suggests that the reduction in brain mass affects behavioral performance of autoimmune animals. The negative correlation between brain and spleen mass further supports the possibility that brain growth is retarded during progression of lupus-like disease (Sakic et al., 2005a,b; Ma et al., 2006; Ballok et al., 2006). The second cohort confirmed the relationship between BRAA and behavior, but likely due to the declining autoimmune phenotype, fewer correlations were observed. Although the relationships were not identical to the pattern seen in the first cohort, they were consistent with the notion that circulating BRAA alter emotional reactivity by inducing functional deficits in the limbic system (Sakic et al., 1994a). Taken together, the above results suggest that the emergence of lupus-like manifestations is associated with brain atrophy and impaired exploration/motivated behavior in MRL/lpr mice. Importantly, although circulating BRAA may account for specific changes in behavior, they seem not to be related to the structural brain damage.

To confirm the presence of BRAA in these mice, we used Western blotting and immunohistochemistry, in addition to ELISA. When Western blot data are considered, antigen-specific bands were expected in three mice with high

serum BRAA levels (as determined by ELISA) and high float times. Although bands were detected in these animals, no bands were common, precluding inference on a unique, antigen-specific pathogenic BRAA. As would be expected, in three mice showing an absence of BRAA by ELISA and low float times, no bands were seen. There were, however, bands seen in two other such mice, suggesting, as we have previously predicted, that non-pathogenic BRAA can exist. With respect to immunohistochemistry of brain, all MRL/lpr sera showed different levels of fluorescence in the hippocampus and the cortex. The binding to the hippocampus is consistent with the notion that areas involved in emotional reactivity are affected, as evidenced by deficits in the forced swim and the sucrose preference tests.

In order for serum BRAA to mediate their effects, they must gain access to the brain. Increased BBB permeability was first shown in experimental models of immune complex disease (Harbeck et al., 1979; Hoffman et al., 1983), a pathogenic mechanism of lupus. Several lines of evidence suggest a breached blood-brain barrier in diseased MRL-/lpr mice. Perivascular leakage of IgG (Vogelweid et al., 1991), immunoglobulins bound to the MRL/lpr brain (Zameer and Hoffman, 2001) and increased levels of IgG in the CSF (Sidor et al., 2005) point to IgG diffusion via a more permeable blood-brain barrier. These observations are consistent with recent studies showing increased local expression of cell adhesion molecules and entry of immunocytes (Zameer and Hoffman, 2003, 2004; Ma et al., 2006; James et al., 2006). Therefore it is reasonable to assume that the principal pathogenic mechanisms include entrapment of

circulating leukocytes by cell adhesion molecules on endothelial cells, increased permeability of the BBB, and BRAA diffusion and/or intrathecal synthesis before they bind to brain tissue.

Except for a few monoclonal antibodies we have produced (Khin and Hoffman, 1993; Crimando et al., 1995) there have been limited attempts to systematically characterize the BRAA in autoimmune mice. The specificity of these antibodies is for the most part not known, nor where they bind in brain (e.g., what cell types bear the reactive antigens). Nonetheless, there are new attempts to characterize BRAA in SLE patients (Zandman-Goddard et al., 2007). One study (Gitlits et al., 2001) has gone in this direction, identifying synapsin I as a reasonable candidate autoantigen for mediating CNS manifestations. In addition, Kowal et al. (2004) have focused on the cross-reactivity between anti-DNA autoantibodies and the NMDA receptor, providing evidence for their role in neurobehavioral changes. Similarly, using the autoimmune NZM strain, Lawrence and colleagues produced a monoclonal autoantibody that was directed against mouse dynamin-1 (Lawrence et al., 2007; Mondal et al., 2008). More importantly, when the antibody was injected intravenously into non-autoimmune Balb/C mice, they developed behavioral manifestations similar to those seen in the NZM mice. Nonetheless, there are likely many more BRAA involved in neuropsychiatric manifestations, which have not been identified.

One of the weaknesses of our present study is in not knowing the identity of the bands in the Western blots. We focused on the relationship between overall BRAA and behavior. If we measured levels of specific BRAA (such as anti-NR2,

anti-ribosomal P, anti-cardiolipin, or others not identified), then we would be in a better position to find more significant correlations. In the future, we plan to develop studies that will do this, by identifying the molecules to which the BRAA bind and correlating them to behavior.

Our study also points to the possibility that BRAA binding is not the sole mechanism by which CNS dysfunction is induced. For example, much of the neurologic involvement in SLE patients has been attributed to vascular lesions (Bluestein et al., 1981; West, 1996). It is also well known that cytokines can affect brain function (Dunn, 1988; Bindoni et al., 1988; Bartholomew and Hoffman, 1993; Zalcman et al., 1994; Kim et al., 1998), as can immune complex disease (Hoffman et al., 1978a,b, 1998), which may be mediated by other soluble components, such as complement (Hoffman et al., 1982; Schupf and Williams, 1987). Along this line, it has been shown that complement can mediate apoptosis in the brains of MRL/lpr mice (Alexander et al., 2007). Therefore, it is likely that multiple mechanisms, including BRAA, mediate behavioral deficits in SLE-like disease.

In conclusion, dissimilar immune status between two cohorts produced in different years confirms fading autoimmune phenotype (which has since been corrected) in the MRL/lpr substrain. Despite this phenomenon of unknown origin, the relationship between serum BRAA to surface neuronal antigens and behavioral dysfunction is detectable. This study is the first to identify BRAA to naturally occurring integral membrane proteins of murine brain and correlate them to behaviors in autoimmune mice. Although the presence of BRAA did not

account for the entire constellation of behavioral deficits, the obtained results are consistent with the notion that a subset (rather than the entire class) of circulating BRAA is pathogenic. As such, they further support the hypothesis that peripheral BRAA enter the brain, bind to integral membrane proteins, and contribute to emergence of behavioral dysfunction in lupus-like disease.

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Chapter 3

EARLY DETECTION AND STRATIFICATION OF SLE USING PEPTIDE MICROARRAYS

ABSTRACT

We describe and test a microarray technique, using random sequence peptides (based on the binding pattern of serum antibodies), for diagnosing the onset of lupus and its neuropsychiatric manifestations, since there are no current methods to accurately do this. Significantly greater binding intensity and unique patterns were observed for the 4 month MRL/lpr in comparison to the 4 month MRL/mp and 4 month C3H/HeJ suggesting that this technique can be used in the diagnosis of lupus. Within the MRL/lpr group there was variation in behavioral performance and binding pattern of the peptides. There are compelling signs that the neurobehavioral phenotypes observed can also be diagnosed by this unique microarray technique.

Key Words: Lupus; Microarray; Diagnostic; Neuropsychiatric; Brain-Reactive Autoantibodies; MRL/lpr Mice**.**

SLE, Systemic Lupus Erythematosus; CNS, Central Nervous System; BRAA, Brain-Reactive Autoantibodies; IP, Intraperitoneal; PBS, Phosphate Buffered Saline; ELISA, Enzyme-Linked Immunosorbant Assay; BSA, Bovine Serum Albumin.

INTRODUCTION

It is well known that there is a large diversity of autoantibodies in systemic lupus erythematosus (SLE) along with numerous manifestations like glomerulonephritis, skin lesions, neurologic problems, etc (Sakic et al., 2005). Some of these autoantibodies are characteristic of specific forms of the rheumatic diseases, but there are no clear cut patterns for diagnosing specific diseases (Colasanti et al., 2009; Wandstrat et al., 2006). We are proposing the use of a random-peptide microarray technology for exactly this purpose. Researchers have used microarray technology to determine both possible predictive and diagnostic antigens of type 1 diabetes (Quintana et al., 2004). In this study we are testing two ideas, both using this unique, random-peptide, microarray. In the first part of this paper we will be using this technique to diagnose SLE in an animal model by determining possible diagnostic peptides of lupus. The second part is a bit more difficult, and interesting, since we also believe this microarray technology can be used for diagnosing specific neuropsychiatric manifestations of lupus in our mouse model. We are currently interested in determining both predictive and diagnostic peptides of lupus and CNS-lupus, as well as using them for identifying pathogenic autoantibodies, but in this paper we are only focusing on the diagnostic utility of this technique for lupus and CNS-lupus.

We are testing these ideas in a murine model (MRL/lpr), prior to moving on to human testing. The mouse model is a good one and has been used many times in the study of SLE (Sakic et al., 1994, 1992, 1993b, 1993c, 1995; Theofilopoulos, 1992). The lpr gene, which is related to a defect in fas,

accelerates and intensifies disease manifestations in the MRL mice. Although a fas gene defect is rare in humans, the autoimmune disease manifestations are very similar to what occurs in human SLE (Ballok et al., 2003a; Theofilopoulos, 1992). The congenic MRL/mp mice are a perfect control, since they are virtually, genetically identical, except for the lpr gene. These mice also develop an autoimmune state, but much more slowly and of less intensity. The C3H/HeJ strain was also analyzed as a normal mouse with a different genetic background. Behavioral testing was done and antibodies to random peptides were tested by microarray on these three groups of mice (MRL/lpr, MRL/mp and C3H/HeJ) at 4 months of age.

It would be very important to have a good, simple, yet rapid diagnostic technique for SLE and also be able to predict overt clinical manifestations months to years (in the case of humans) in advance. Not only would this provide for a clear-cut diagnosis of the disease, but it would allow for early treatment to begin, perhaps preventing more serious manifestations. The type of early treatment that could be administered for the different CNS manifestations may be also determined using the microarray technology since we intend to identify some of the brain targets using this technology. Central nervous system (CNS) involvement in SLE is a form of lupus (CNS-SLE) where the immune system affects central nervous system function, causing neuropsychiatric manifestations including psychoses, cognitive impairment and emotional dysfunction (Hoffman and Sakic, 2009). Psychiatric disorders can be observed in up to 70% of patients that have lupus (Lawrence et al., 2007). We believe that the presence of a subset

of brain-reactive autoantibodies (BRAA) is responsible for causing some of these neuropsychiatric manifestations, through interaction with the integral membrane proteins on the surface of cells in the brain (Narendran and Hoffman, 1989; Sakic et al., 1993a; Zameer and Hoffman, 2001). These BRAA can enter the brain through increased permeability of the blood-brain barrier as lupus progresses (Hoffman and Harbeck, 1989). The BRAA could enter directly, or antibody producing cells can enter the brain and produce the BRAA. One of our main goals is to characterize these BRAA by identifying the proteins they bind in brain, which will indicate which brain structures are being affected, possibly explaining these neuropsychiatric manifestations. The battery of behavioral tests included the forced swim test, which looks at behavioral despair seen through increased float times, and secondly the sucrose preference test, which looks at anhedonia observed through decreased consumption of a 4% sucrose solution (Sakic et al., 2005). The major goal of this study is to test the diagnostic autoantibody hypothesis, which will allow us to identify a pattern of autoantibody reactivity on an immune-display microarray. We posit that the microarray 'immunosignature' is diagnostic not only of lupus, but of different neurobehavioral manifestations. This study develops a microarray probe using a pattern of autoantibody binding as a diagnostic indicator of lupus and its CNS manifestations.

In order to do this we used behavioral tests to identify the mice with CNS manifestations and then performed a series of immunological assessments on their sera. BRAA and anti-DNA ELISA were performed using sera to measure disease activity. We also used a new immune-diagnostic microarray that uses 10,000

random-sequence 20-mer peptides to bind specific antibodies from disease-affected and healthy individuals. These arrays contain covalently-coupled peptides that contain random sequences of amino acids. This differs from epitope arrays in that there are no overlapping life-space sequences on this array; any reactivity of antibody to peptide is driven by amino acid sequences with no similarity to the actual antibody-eliciting antigen. These arrays bind and display differences in the level of antibodies raised to a broad variety of disease-specific immunogens. These arrays allow broad application across diseases - the same arrays can be used for any disease without the need to create new epitopes. Also, patients experiencing the same disease tend to produce antibodies that show common patterns of antibody binding, allowing us to cleanly distinguish naïve from affected samples (Legutki et al., 2010). In the current study, we looked at the binding pattern of sera from healthy vs. lupus mice. Affected mice perform differently on behavioral tests; some do better on one test and worse on another. If our hypotheses are correct, we would expect a link between autoantibodies and behavioral performance; this difference could be detected on the peptide microarray without having to know the identity of the antigen. These differences in patterns would allow us to diagnose the onset of lupus-like disease, as well as the different neurobehavioral manifestations in our mouse model. The data presented here is used to develop and test the diagnostic power of autoantibodies, both for the onset of disease and specific neurobehavioral manifestations.

MATERIALS AND METHODS

ANIMALS

The MRL/lpr strain develops lupus after 2 months of age and has 50% mortality at about 5 to 6 months of age. The MRL/lpr mice used in this study are a new lot (stock #000485) from Jackson laboratory with heightened autoimmune state. The congenic control, the MRL/mpJ substrain (stock #000486) develops at about 12 months of age. The C3H/HeJ is the normal control that does not develop lupus but serves as a control for normal aging. Results in the various tests were compared when these animals were 4 months of age. There were 3-6 animals in each group and all were females. The mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and individually housed under standard conditions. The light cycle was from 6:00 A.M. to 6:00 P.M. They were given food and water ad libitum. All behavioral tests commenced at 8:00 P.M. Integral membrane proteins from 2 month old C3H/HeJ mouse brain were used in the BRAA ELISA.

The mice were sacrificed with an intraperitoneal (IP) injection of Nembutal sodium solution. The blood was collected via cardiac exsanguination and allowed to coagulate in microcentrifuge tubes. The tubes were then centrifuged for 10 minutes at 8500 rpm (5200 x g). The serum was removed, aliquoted into 100 µl aliquots and frozen at -50^{o}C. The spleen and body weights of the mice were also measured. The spleen weight was divided by the body weight of the mouse since each mouse had a different body weight.

Animals are maintained in University facilities fully accredited by AAALAC and are registered with the USDA APHIS (Registration # 86-R-0002). An assurance is on file with the Office for Laboratory Animal Welfare (#3217-01). Animal husbandry programs and protocol review are in compliance with NIH and USDA standards.

BEHAVIORAL TESTING

SUCROSE TEST

The sucrose test looks at anhedonia. The mice were given 3ml of 4% sucrose solution for 24 hours over three days. They were then allowed to rest, i.e., no sucrose solution, for 24 hours. Following this a 7ml solution of 4% sucrose was given to the mice for one hour for three consecutive days. The total amount of sucrose consumed by these animals was recorded. The body weights of the mice were taken into account and therefore the amount of sucrose consumed was divided by their individual body weights.

FORCED SWIM TEST

The forced swim test looks at depressive-like behavior in the MRL/lpr mice compared to the controls. A circular pool (~6 ft in diameter and 2.5 ft high) was filled with water at about 25°C. All the mice were placed into the water at the same location and allowed to swim around for 10 minutes. The total float time for each animal was recorded. Floating is defined as the absence of movement in the tail and hind limb.

IMMUNOLOGICAL ASSESSMENT

INTEGRAL MEMBRANE PROTEIN PREPARATION

The technique used to extract the integral membrane proteins from the brain of the mice to be used in the BRAA ELISA was previously described (Narendran and Hoffman, 1988). Briefly, the whole brain was chopped and teased through a porous cloth. The cells were suspended in TBS and centrifuged for 10 minutes at 1200 rpm. The pellet was then suspended in lysis buffer (buffer containing 4.18g of Tris, 1.71g of NH_4Cl, 35mg of phenylmethanesulfonylfluoride (Sigma-Aldrich, USA) in 200ml of water at pH 7.2) for 15 minutes. The sample was then centrifuged again for 10 minutes at 15000 rpm and followed by a 10 minute incubation at 37^{o}C of the supernatant. The sample was then centrifuged for 10 minutes at 1000 rpm and the oily pellet collected. Once the integral membrane proteins were extracted we suspended the proteins in phosphate buffer saline (PBS) and the concentration determined using the BCA Assay Kit (Pierce, USA).

ELISA FOR ANTI-DNA AND BRAA

Anti-DNA and brain-reactive autoantibody levels were determined using previously described protocols (Aotsuka et al., 1979; Crimando and Hoffman, 1995; Zameer and Hoffman, 2003).

ANTI-DNA ELISA

To test for anti-DNA autoantibodies, 96 well plates were incubated with 50µg/ml of poly-L-lysine (Sigma, USA) dissolved in phosphate buffer saline (PBS) at room temperature for 1 hour. The plates were washed three times using 0.1% PBS-Tween in between the incubation steps. Ten µg/ml of calf-thymus DNA (Sigma, USA; purified for dsDNA) dissolved in PBS was added to the odd wells of the plate and PBS only to the even wells and the plates incubated for 1 hour. Next, 100ug/ml of poly-L-glutamate (Sigma, USA) dissolved in PBS was added to the wells. A 5% bovine serum albumin (BSA) (Sigma, USA) solution in PBS was then added followed by serial dilutions of the mouse sera. A 1/1000 dilution of the secondary antibody, goat anti-mouse IgG antibody conjugated with peroxidase (Caltag, USA) in PBS, was then added. Citrate buffer (1.04g/100ml of sodium citrate and 1.46g/100ml of citric acid (pH =4.4)) containing 2,2'-azino-bis(3ethylbenzthiazoline) sulfonic acid and hydrogen peroxide was added to plates for 30 minutes at 37^{o}C. The plates were then read at 405nm using a microplate reader. The optical density of the control wells (the even wells) were subtracted from the optical density of the odd wells to give an S-value showing the levels of anti-DNA antibodies. Some S-values may be negative, since the S-values are all relative to each other.

BRAA ELISA

Odd wells of 96 well plates were incubated with 10µg/ml of integral membrane proteins, isolated from a brain homogenate of healthy 2 month C3H/HeJ mice, in PBS, while PBS only was added to the even wells. The plates were incubated for 1 hour at room temperature. The wells were then washed with 0.1% PBS-Tween three times. A 5% BSA solution in PBS was added to all the wells and the plates incubated for 1 hour. Next, serial dilutions of the mouse sera were added to the wells, followed by a 1/1000 dilution of the secondary antibody goat anti-mouse IgG antibody conjugated with peroxidase (Caltag, USA). Finally, 2,2'-azino-bis(3-ethylbenzthiazoline) sulfonic acid (17mg/100ml) and hydrogen peroxide (100µl/100ml) in citrate buffer was added to the wells and incubated for 30 minutes at 37^{o}C. The plates were then read at a wavelength of 405nm and as with the anti-DNA ELISA, the S-Values were determined.

MICROARRAY ANALYSIS

Microarrays containing 10,000 randomly generated 20-mer peptides were obtained from the Center for Innovations in Medicine, Biodesign Institute, Arizona State University (Legutki et al., 2010). The arrays were pre-blocked with buffer containing mercaptohexanol for 1 hour in a humidity chamber to quench any remaining reactivity on the surface of the arrays. The plates were then washed once with Tris-buffered saline with Tween (TBST), twice with water and then spun dry for 5 minutes at 1500 rpm (2800 x g). A hybridization chamber enclosed a 1/500 dilution of the sera samples in 300 µl of hybridization buffer.

The slides were covered and incubated 1 hour at 37°C. The slides were washed three times with TBST, three times with water followed by secondary detection. Goat anti-mouse IgG biotinylated secondary antibody (Bethyl, USA), 6.67μM (1 nM final concentration) was added to the slides and the slides incubated for 1 hour at 37°C. A tertiary reagent, 5nM of streptavidin conjugated with AlexaFluor 555, was added to the slides and incubated for 1 hour at 37°C. The slide was removed from the hybridization chamber, washed three times with TBST, three times with water and spun dry at 1500 rpm for 5 minutes. Once dried, the slides were scanned with a Perkin Elmer Scan Array laser Scanner (543nm emission, 565 nm absorption, 75% PMT, 100% laser power).

STATISTICS

Analytical methods for conventional expression microarrays were used for the immunosignature microarrays and no unusual biases were noted. The performance of the microarrays has been tested using sera from influenza and healthy mice. The arrays yield 14% average slide-to-slide Coefficient of Variance across all peptides, and a 1.3-fold minimum detectable fold-change at the 95th percentile. All experiments were done with triplicate technical and triplicate biological samples (9 samples per condition). Tests were done on pooled sera from replicate mice but each mouse was also run independently to determine mouse-to-mouse variance and the robustness and representative nature of each immunosignature. Each slide is imaged using the Agilent 'C' scanner, producing a 16-bit TIFF image that was analyzed in GenePix Pro 6.0 (Molecular Devices,

Palo Alto, CA). Resulting gpr (GenePix Results) files were exported to GeneSpring 7.3.1 (Agilent, Palo Alto, CA) or R (CRAN GNU open-source) for analysis. Slides were median normalized and log-10 transformed. Peptides <2 standard deviations above the average background were discarded as non-informative. Statistical analyses were done on the remaining peptides, using the Welch's T-test using the Benjamini and Hochberg False Discovery Correction Rate set at 0.05. Statistical analysis for the results shown in figures 1, 2, 5, 6A, 6B, 7A, 7B and 8 used Model I 1-way ANOVA and LSD post-hoc analysis (SPSS 16.0).

RESULTS

DIAGNOSTIC MICROARRAYS FOR LUPUS

IMMUNOLOGICAL ASSESSMENT AND DISEASE ACTIVITY

Before we tested the murine sera using the microarray technology, we wanted to determine that lupus was progressing in the MRL/lpr mice, so we tested the level of anti-DNA autoantibodies in the sera and measured spleen weights. We used anti-DNA autoantibody levels as a confirmation of disease activity since we (Crimando and Hoffman, 1992; Zameer and Hoffman, 2001) have previously shown that these measures correspond to disease activity. Anti-DNA ELISA were performed on 3 randomly selected mice from each of the 3 groups (Fig. 1), and ANOVA analysis showed that the levels of anti-DNA autoantibodies was significantly different between the groups ($F=112.953$, $p < 0.001$). LSD post-hoc analyses at $p < 0.001$ revealed the following differences. MRL/lpr mice had significantly greater anti-DNA autoantibodies in comparison to both of the control groups. Spleen weights per body weight were used as another measure of disease activity (Fig. 2), since they become enlarged. ANOVA analysis showed that there was a significant difference between the groups ($F=18.365$, $p < 0.003$) and post-hoc analysis at $p < 0.007$ revealed that the MRL/lpr mice had greater spleen weights in comparison to the MRL/mp and C3H/HeJ. Since both increased spleen weights and higher levels of anti-DNA autoantibodies were detected in the MRL/lpr mice this indicates that lupus was progressing as expected.

MICROARRAY ANALYSIS

The sera samples from all nine mice were then analyzed using microarray technology. We ran each of the sera samples in triplicates to ensure consistency in results. Fig. 3 shows a representative slide from one animal from each of the groups. Fig. 3A is the control slide to which no serum was added. The secondary is titrated to the point where the primary is detected but essentially no 2^{o}-only binding is detected. Fig. 3B shows a C3H/HeJ mouse serum and, as in other experiments (Legutki et al., 2010 and unpublished observations) little reactivity is seen from naïve mice. Fig. 3C shows the MRL/lpr mouse serum. We have noted that there is consistently more overall fluorescence for infected/disease vs. naïve mice (personal observations), although the total amount of IgG incubated on the peptide microarray was identical as measured by Nanodrop 2000c (Thermo Scientific, Wilmington, DE). We empirically determined that a 1:500 dilution of serum or plasma in incubation buffer provides a uniform signal, low background, and a concentration of ~20ug/ml total IgG for samples tested. The coefficient of variance for this measure is 0.035 across 33 different BALB/cJ mice, 16 were day 21 post-influenza infection, 17 were naïve (personal observations from previous data). Fig. 3D shows MRL/mp serum; again the binding intensity was much lower than the MRL/lpr. According to the anti-DNA and BRAA ELISA results, higher levels of anti-DNA autoantibodies and BRAA were detected in the sera of the MRL/lpr mice in comparison to both control groups, so these high levels of autoantibodies are consistent with expectations in the microarray.

Each peptide on the microarray was synthesized and quality tested by Alta Biosciences (Birmingham, UK). The sequence, mass spectrometry results, and other chemical characteristics for each peptide is known and although each peptide's intrinsic properties vary widely (pI, hydropathicity, hydrophobicity, mass, etc.) these properties are fixed. Thus, statistical evaluation of the interaction between antibody and peptide can exclude these properties and only evaluate the differential binding of the antibodies as measured by fluorescence. Fundamentally, there are peptides that bind consistently to antibodies from autoimmune (MRL/lpr) mice vs. control mice. We were able to pick out 200 peptides where the binding intensity of the MRl/lpr was greater than controls. These were plotted on a line graph (Fig. 4A). This demonstrates a pattern which can distinguish the groups as well as lupus-like disease activity. Since these 200 peptides had greater binding intensities for the MRL/lpr mice in comparison to the controls, they could be specific for autoantibodies that are diagnostic indicators of lupus. These are the autoantibodies we are referring to as possible diagnostic autoantibodies for lupus. Fig. 4B is another graph showing the intensities of these peptides across the groups.

DIAGNOSTIC MICROARRAYS FOR CNS MANIFESTATIONS

BRAIN-REACTIVE AUTOANTIBODIES

BRAA levels were used as a measure of CNS involvement, which has been discussed previously in the literature (Sakic et al., 1993a;Williams et al., 2010). The ANOVA analysis of the BRAA ELISA results (Fig. 5) revealed that

there was a significant difference between the groups ($F=14$, $p < 0.005$). Post-hoc analysis at $p < 0.004$ showed that there was significantly greater levels of BRAA in the MRL/lpr mice in comparison to the MRL/mp and the C3H/HeJ mice at 4 months of age.

BEHAVIORAL DYSFUNCTION IN MRL/LPR MICE

We used two behavioral tests, sucrose preference and the forced swim test, as measures of CNS dysfunction in these animals. Even though there was not a significant difference ($F=4.150$, $p < 0.074$) in sucrose consumption between the groups (Fig. 6A), a decrease in consumption of the 4% sucrose solution for the MRL/lpr mice was seen in comparison to the congenic controls, the MRL/mp. A significant difference in floating time was detected between the groups for the forced swim test (Fig. 6B) ($F=12.068$, $p < 0.008$). Post-hoc analysis at $p < 0.007$ revealed increased floating time for the MRL/lpr mice in comparison to the MRL/mp and the C3H/HeJ. The decrease in sucrose consumption and increased floating times are indicative of the CNS involvement (possibly emotional dysfunction) that is expected with the MRL/lpr mice (Sakic et al., 2005; Ballok et al., 2003b).

GROUP SEPARATION WITHIN MRL/LPR BY NEUROBEHAVIORAL MANIFESTATIONS

Variations in behavior were observed within the 4 month MRL/lpr group of mice. Some of the mice perform differently depending on the behavior test. In

addition, these mice have different levels of anti-DNA and BRAA and have different binding patterns in the microarray analysis. Because of these differences, we believe that we will be able to use the microarray analysis to identify peptides that are bound by autoantibodies that are diagnostic of certain neuropsychiatric manifestations. In order to test this, we first grouped the mice according to their behavior, and then we looked for differences in peptide binding patterns using the microarray analyses.

We found in this study that there was a difference in behaviors for individuals within the MRL/lpr group, as has been previously reported by Sakic and colleagues (2005). In the sucrose preference test MRL/lpr #2, #3 and #5 were grouped as low consumers and MRL/lpr #1 and #4 were grouped as high consumers (Fig. 7a). Statistical analysis reveals that there was a significant difference between the groups ($F=8.785$, $p < 0.009$). Post-hoc analysis at $p < 0.03$ showed that there were statistical differences between the low consumers and high consumers, the low consumers and the MRL/mp (group3) and the low consumers and the C3H/HeJ (group4). There was no difference between the MRL/lpr high consumers and the MRL/mp and C3H/HeJ.

In the forced swim test MRL/lpr #2, #3 and #4 were grouped as having higher float times and MRL/lpr #1 and #5 had lower float times (Fig. 7b). There was an overall significant difference between the groups ($F=9.2$, $p < 0.008$). Post-hoc analysis at $p < 0.010$ revealed statistically significant differences between the MRL/lpr with high float times and those with low float times, as well as compared to the MRL/mp and the C3H/HeJ. There was, however, no significant

difference between the MRL/lpr with a lower floating time and the MRL/mp and C3H/HeJ.

The fact that there was no difference between the mice with high sucrose consumption and lower floating time when compared to the controls indicates that these mice performed similar to the controls, which becomes interesting when looking at the microarray results, since according to the grouping for each of the behavior tests, each group did not include the same MRL/lpr mice so some mice did better on one test and worse on another. These behavioral differences in the mice allowed us to pick out peptides that are specific to certain neuropsychiatric manifestations (below).

We also split the MRL/lpr mice based on their anti-DNA autoantibody levels (Fig. 8), as a measure of disease activity. MRL/lpr #2, #3 and #4 were grouped as having higher levels of anti-DNA autoantibodies and MRL/lpr #1 and #5 were grouped as having lower levels of anti-DNA autoantibodies. There was a significant difference between the groups ($F=91.176$, $p < 0.001$). Post-hoc analysis at $p < 0.004$ revealed that there was a significant difference between the 4 month MRL/lpr with greater levels of anti-DNA antibody and the 4 month MRL/lpr with lower levels of anti-DNA autoantibodies, as well as the MRL/mp and the C3H/HeJ. There was also a significant difference between the MRL/lpr that had lower levels of anti-DNA autoantibodies and the MRL/mp and the C3H/HeJ. It should be noted that this breakdown was the same as for the forced swim test groupings, which is suggestive of disease activity (other than BRAA) causing these behavioral manifestations in the forced swim test.

SELECTED MICROARRAY PEPTIDES BASED ON NEUROBEHAVIORAL TESTS

We next ran the microarray analyses based on the results of the behavior tests and the immunologic assessment described above. This allowed us to pick out diagnostic autoantibodies for the neurobehavioral manifestations. Using the groupings for the sucrose preference test we identified those peptides (Fig. 9a) where there was greater binding for the animals with lower sucrose consumption (group 2) in comparison to the animals with higher sucrose consumption (group 1). Some of these peptides are possibly detecting autoantibodies that are diagnostic of altered behavior in the sucrose preference test.

Grouping the animals based on the forced swim test, which also corresponded to the anti-DNA antibody grouping, identified peptides (Fig. 9b) associated with high float times and high disease activity as measured by anti-DNA autoantibody levels (group 2) in comparison to those with low anti-DNA levels and lower floating time (group 1). Again, some of these detected peptides are possibly diagnostic autoantibodies for deficits in the forced swim test, or increased disease activity, in the form of elevated anti-DNA levels. All of these differences were statistically significant, as shown in the figure.

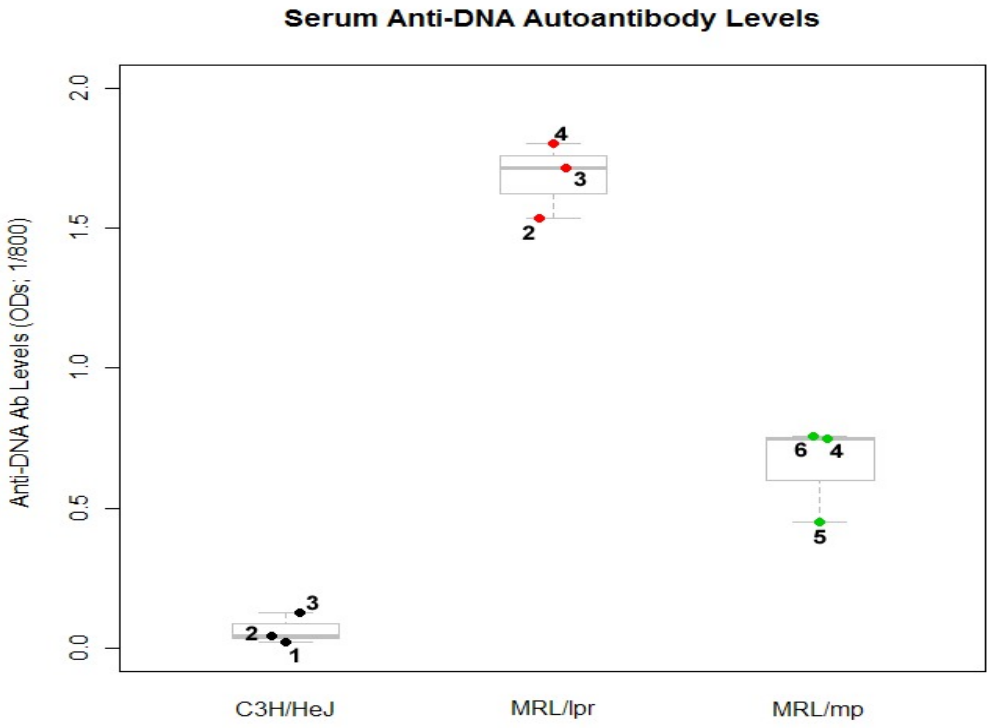

Fig. 1. Immunological assessment – serum anti-DNA autoantibody levels. Three randomly selected mice from each of the 3 groups were used for immunologic assessments (the numbers next to each dot represents the mouse number). ANOVA analysis showed that the levels of anti-DNA autoantibodies was significantly different between the groups ($F=112.953$, $p < 0.001$). Post-hoc analysis at $p < 0.001$ revealed that the 4 month MRL/lpr had significantly greater levels of anti-DNA antibodies (measured at a 1:800 serum dilution) in comparison to the controls. S-values (ODs; see Methods) were used to correct for background reactivity and assess antibody levels. In the box plot the middle line is the 50^{th} percentile, the top of the box is the 75^{th} percentile and the bottom of the box is the 25^{th} percentile.

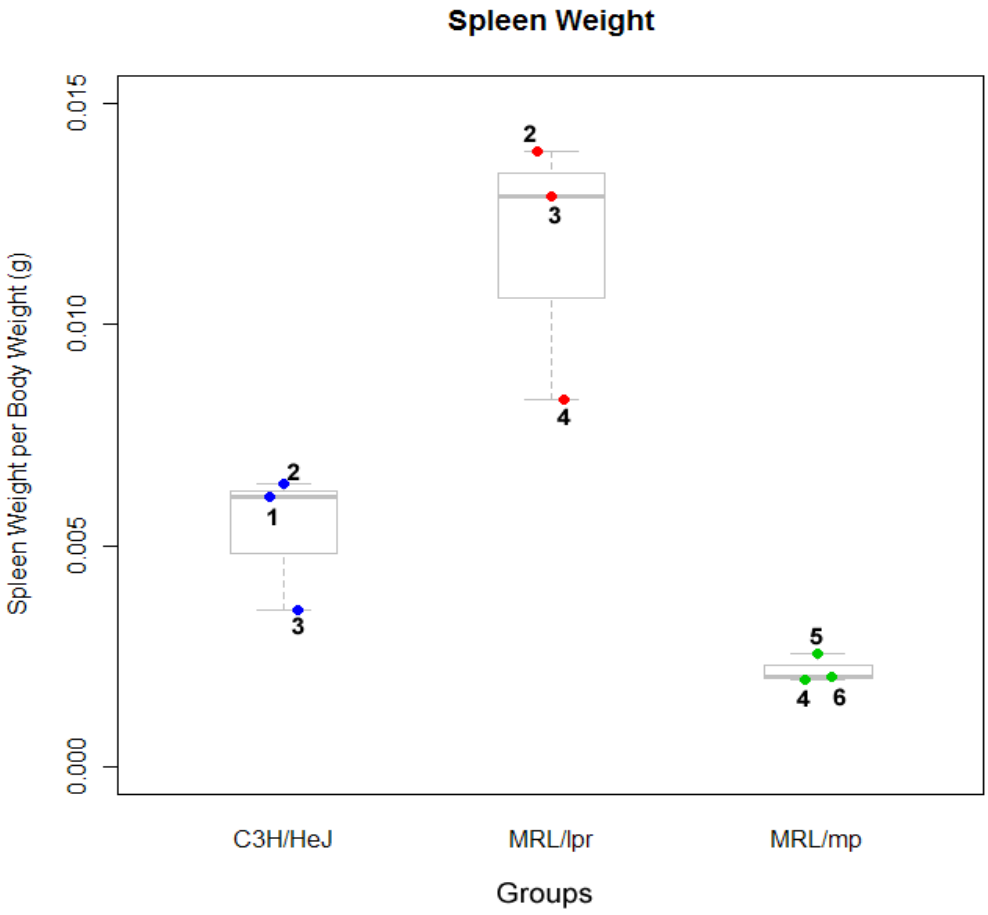

Fig. 2. Spleen weights of MRL/lpr, MRL/mp and C3H/HeJ. ANOVA analysis showed that there was a significant difference between the groups ($F=18.365$, $p < 0.003$). Post-hoc analysis at $p < 0.007$ revealed that the 4 month MRL/lpr had significantly greater spleen weights (per body weight) in comparison to the MRL/mp and C3H/HeJ. In the box plot the middle line is the 50th percentile, the top of the box is the 75th percentile and the bottom of the box is the 25th percentile.

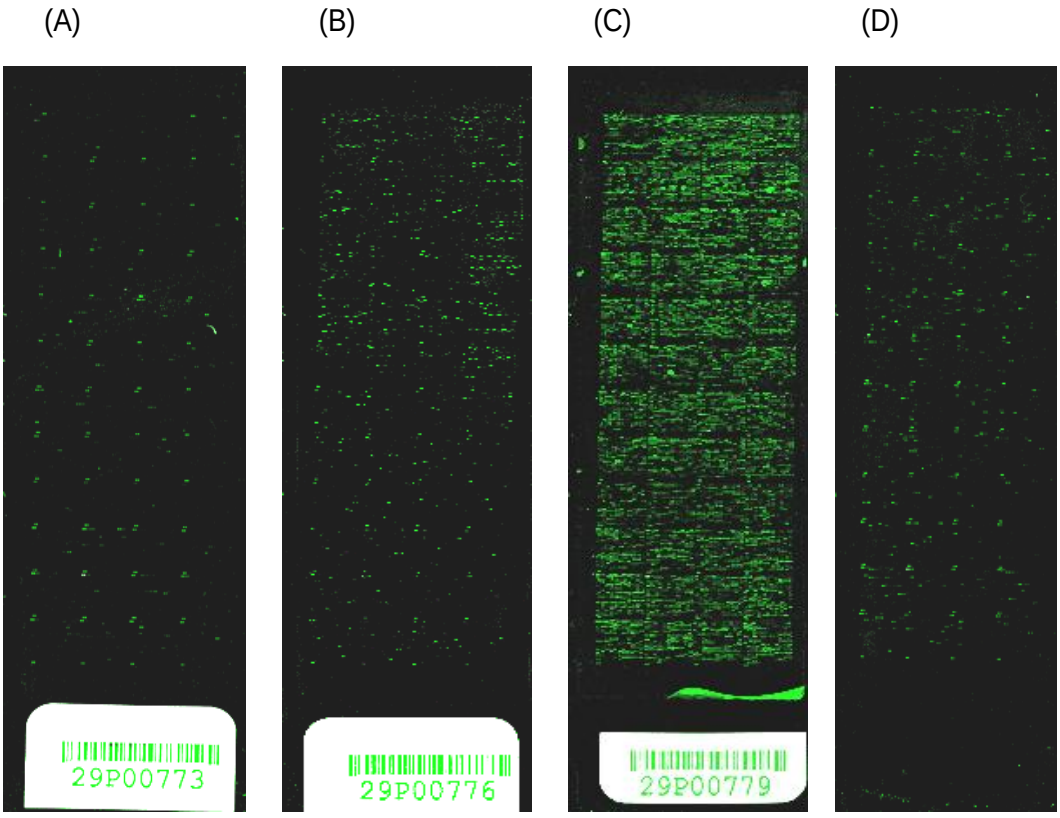

Fig. 3. Sample peptide binding intensities across pooled samples. This figure demonstrates the intensity pattern across individual mice of different strains. Each green dot is the binding of the serum to an individual peptide. **(A)** Secondary Only Control (only secondary and tertiary antibodies added). **(B)** C3H/HeJ **(C)** MRL/lpr strain. **(D)** MRL/mp. Identical amounts of IgG were added to each microarray.

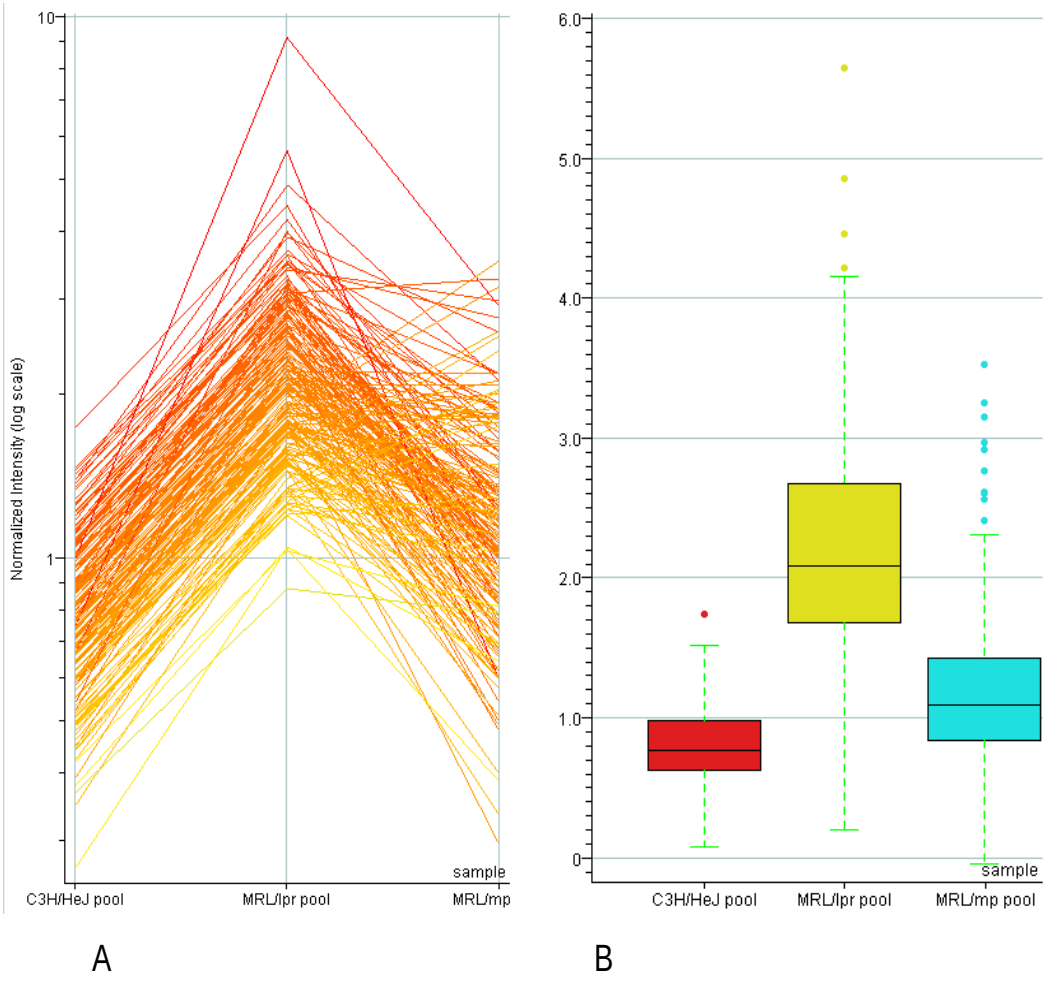

Fig. 4. Graphs showing results with only 200 chosen peptides. **4A.** Line graph of the intensity of two hundred peptides where MRL/lpr reactivity was greater than C3H/HeJ. The samples are the pooled C3H/HeJ sera, the pooled MRL/lpr sera, and the pooled MRL/mp sera. **(B)** Bar graph of the data in **A.** There were significant differences between MRL/lpr and both control groups ($p << 0.001$), but not between the two control groups. When these were run as individual samples, instead of pooled, there were still significant differences ($p << 0.001$).

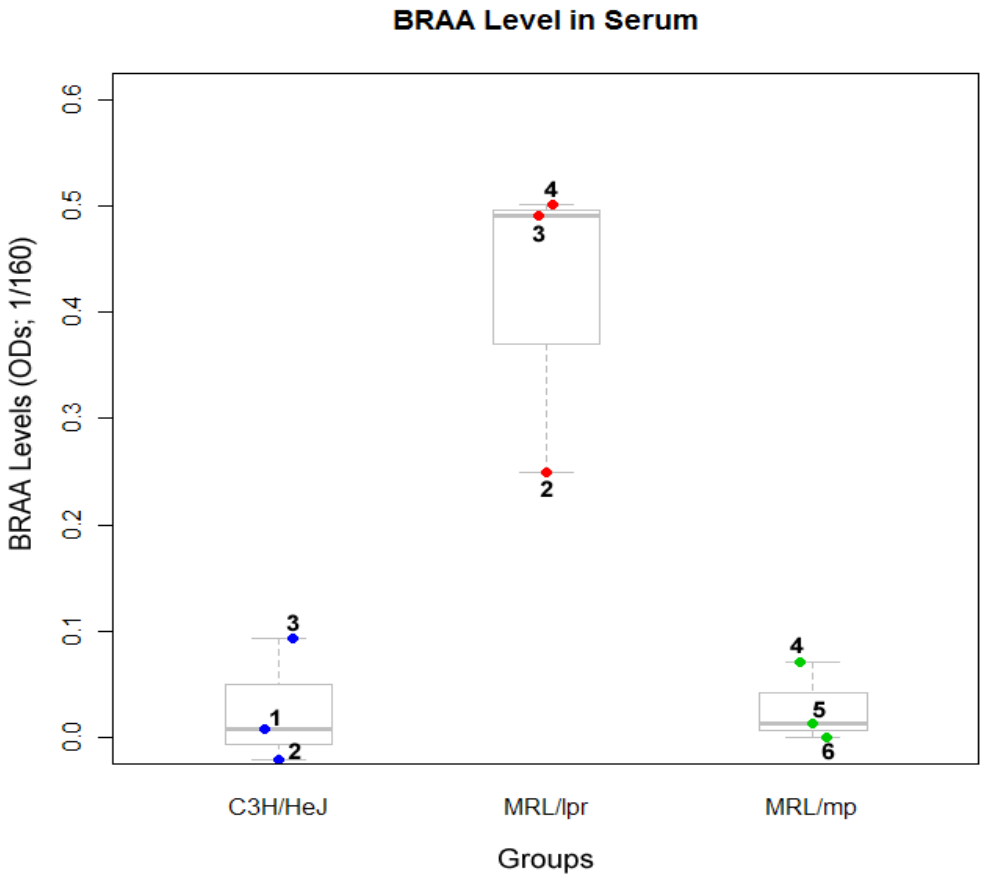

Fig. 5. Immunological assessment – Brain-Reactive Autoantibodies levels. The ANOVA analysis of the BRAA ELISA results revealed that there was a significant difference between the groups ($F=14$, $p < 0.005$). Post-hoc analysis at $p < 0.004$ revealed that the 4 month MRL/lpr had significantly greater levels of BRAA in comparison to the MRL/mp and C3H/HeJ. In the box plot the middle line is the 50th percentile, the top of the box is the 75th percentile and the bottom of the box is the 25th percentile.

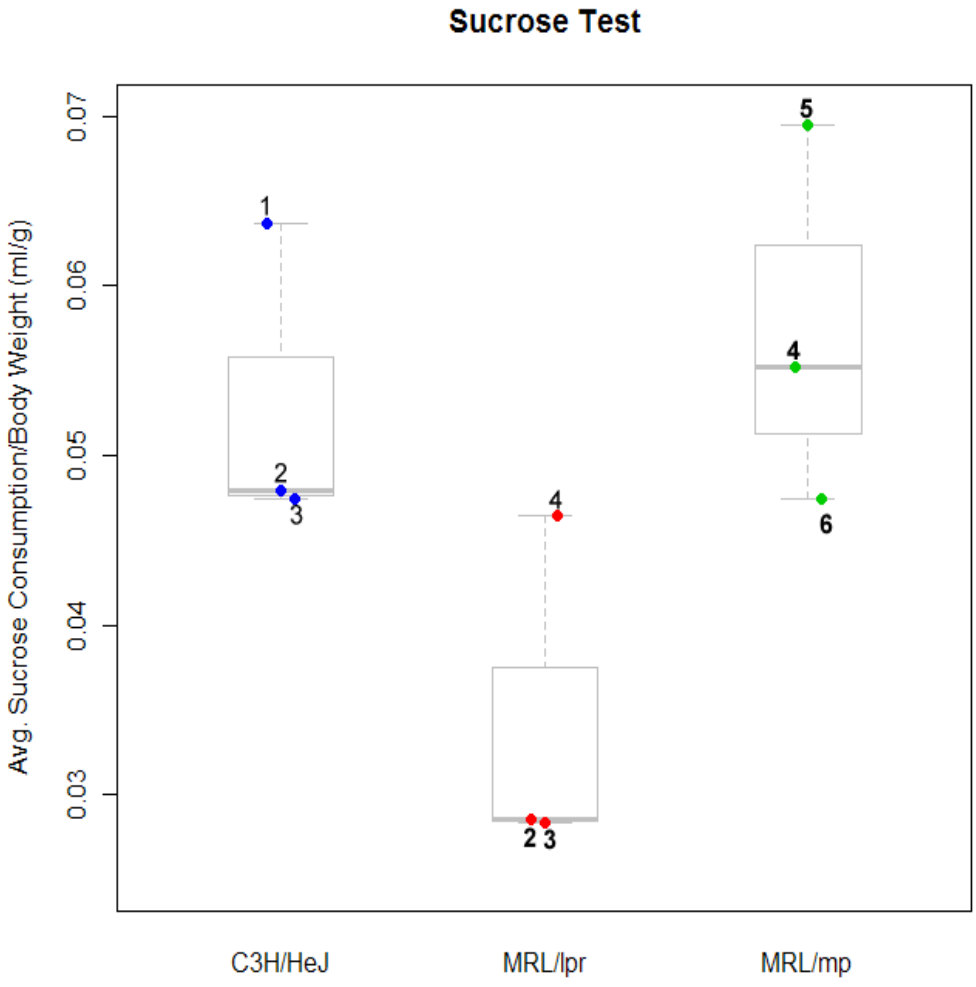

Fig. 6A. Measuring behavioral dysfunction. For the sucrose preference test there was not a significant difference ($F=4.150$, $p < 0.074$) in sucrose consumption between the groups. However, a decreased in consumption of the 4% sucrose solution for the MRL/lpr mice was seen in comparison to the congenic controls, the MRL/mp. In the box plot the middle line is the 50th percentile, the top of the box is the 75th percentile and the bottom of the box is the 25th percentile.

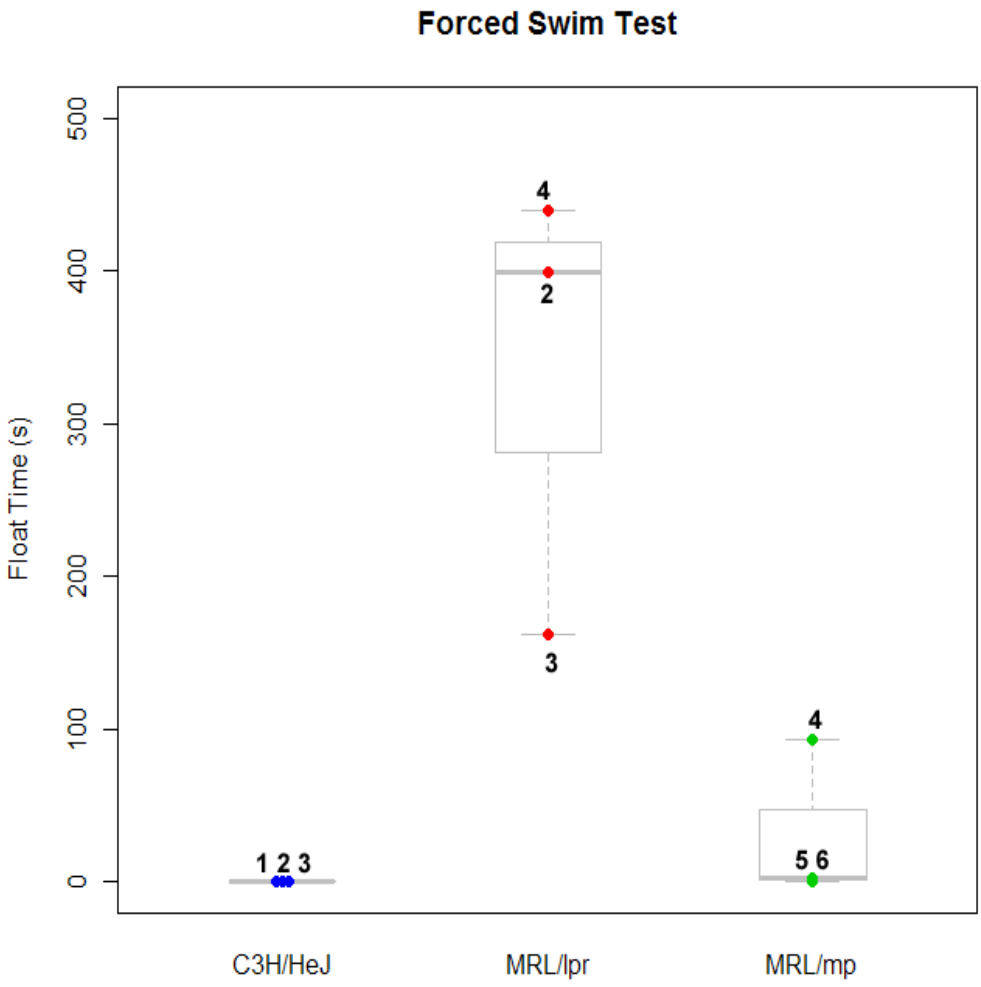

Fig. 6B. Measuring behavioral dysfunction – Forced Swim Test. A significant difference in float time was detected between the groups for the forced swim test ($F=12.068$, $p < 0.008$). Post-hoc analysis at $p < 0.007$ revealed that the 4 month MRL/lpr had significantly greater float time in comparison to the MRL/mp and C3H/HeJ. In the box plot the middle line is the 50^{th} percentile, the top of the box is the 75^{th} percentile and the bottom of the box is the 25^{th} percentile.

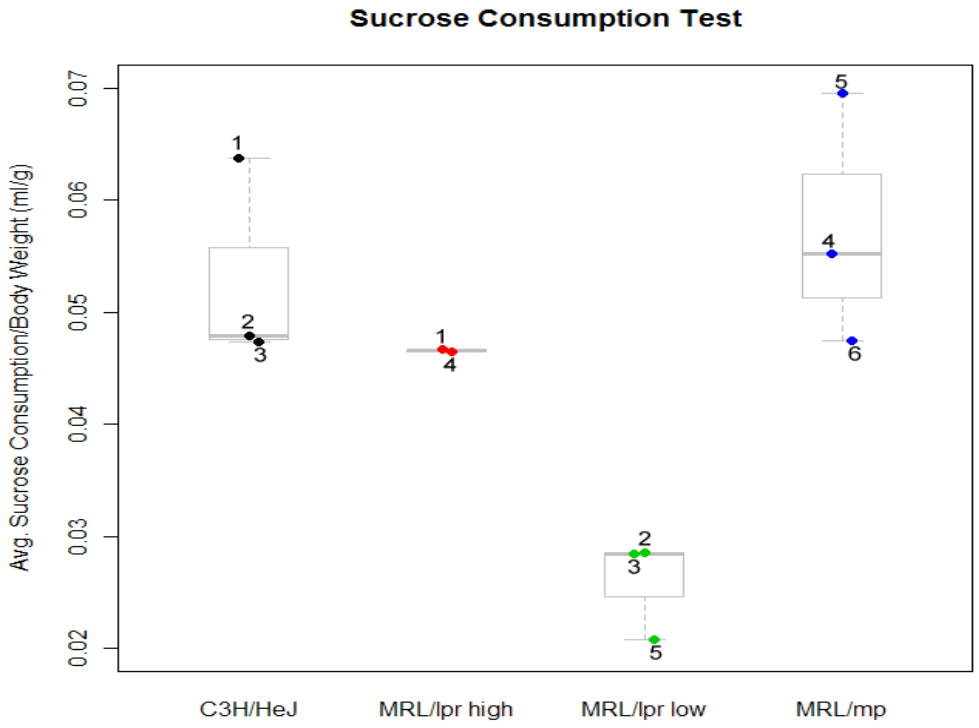

Fig. 7A. Behavioral dysfunction within the MRL/lpr mice group. There were five mice in the MRL/lpr group that were tested and so we included all 5 in this second set of analyses. These 5 mice were split into two groups based on their performance and the results analyzed for significance using one-way ANOVA plus post-hoc analysis. Statistical analysis reveals that there was a significant difference between the groups ($F=8.785$, $p < 0.009$). Post-hoc analysis at $p < 0.03$ revealed that the 4 month MRL/lpr low consumer (group 1) consumed significantly less sucrose in comparison to the MRL/lpr high consumer (group2), the MRL/mp (group3) and the C3H/HeJ (group 4). There was, however, no difference between the MRL/lpr high consumer, the MRL/mp and the C3H/HeJ. In the box plot the middle line is the 50th percentile, the top of the box is the 75th percentile and the bottom of the box is the 25th percentile.

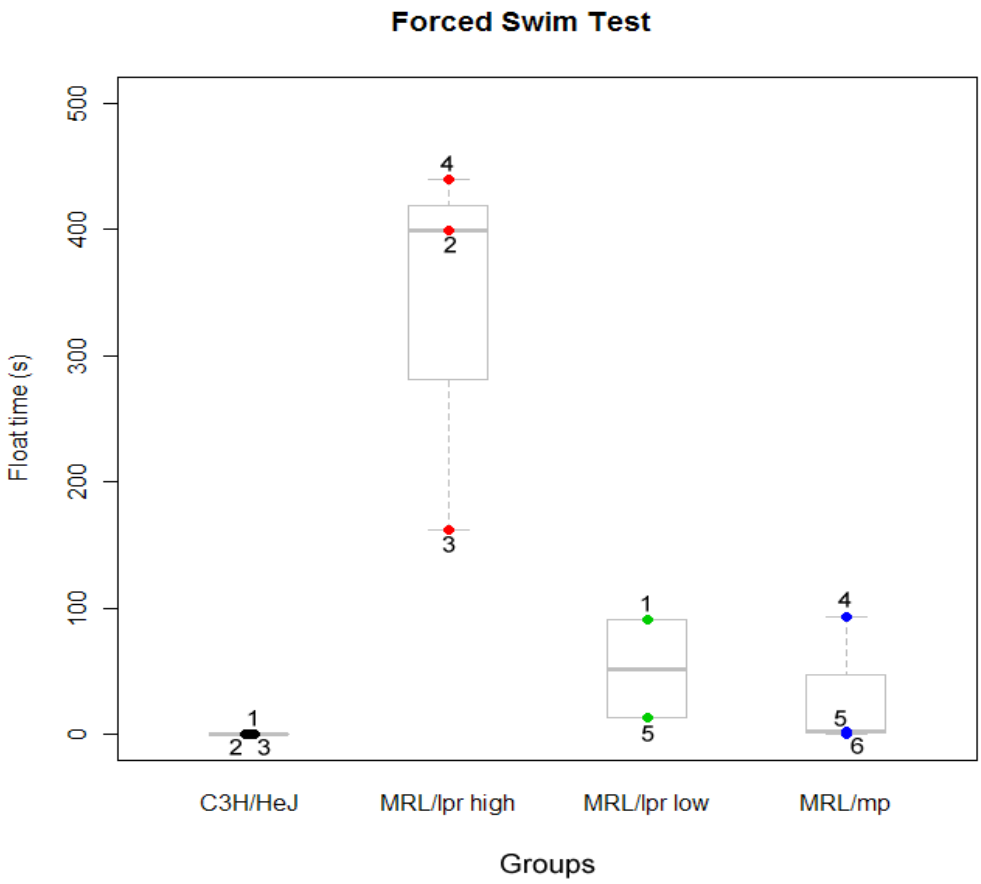

Fig. 7B. Behavioral dysfunction within the MRL/lpr mice group. There was an overall significant difference between the groups ($F=9.2$, $p < 0.008$). Post-hoc analysis at $p < 0.010$ revealed that the MRL/lpr mice with the high floating time (group1) had significantly greater floating time in comparison to the MRL/lpr mice with low floating time, the MRL/mp and the C3H/HeJ. There was, however, no significant difference between the MRL/lpr with lower floating time and the MRL/mp and the C3H/HeJ. In the box plot the middle line is the 50th percentile, the top of the box is the 75th percentile and the bottom of the box is the 25th percentile.

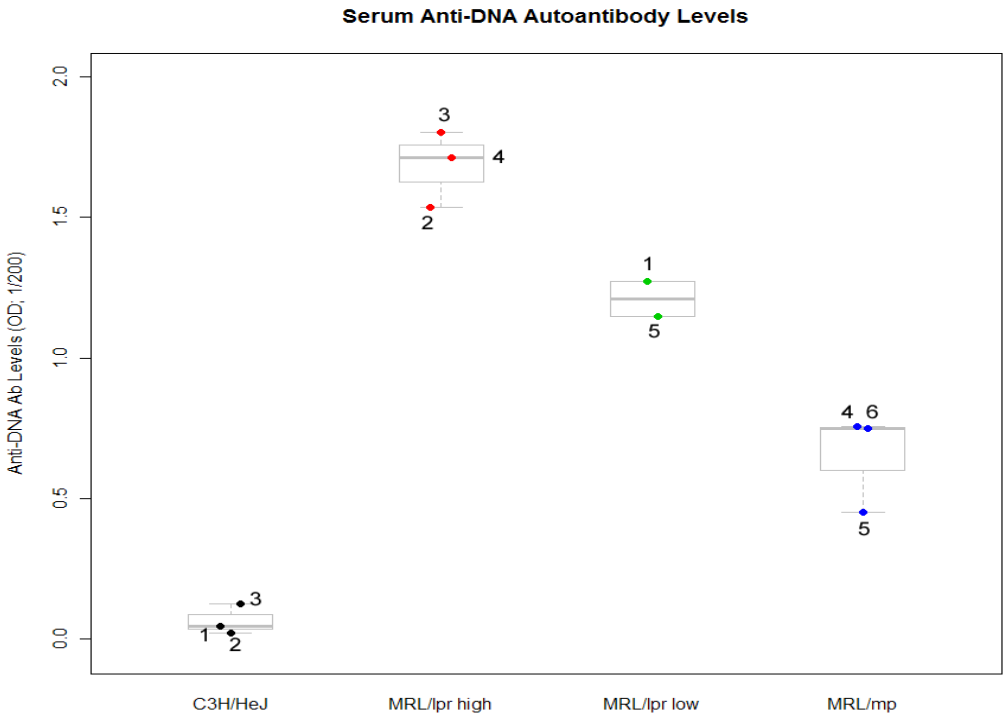

Fig. 8. Immunological assessment within the MRL/lpr group. The MRL/lpr mice were also split based on their anti-DNA antibody levels. There was a significant difference between the groups ($F=91.176$, $p < 0.001$). Post-hoc analysis at $p < 0.004$ revealed that there was a significant difference between the 4 month MRL/lpr with greater levels of anti-DNA levels and the 4 month MRL/lpr with lower levels of anti-DNA autoantibodies, the MRL/mp and the C3H/HeJ. There was, however, also a significant difference between the MRL/lpr that had lower levels of anti-DNA autoantibodies and the MRL/mp and the C3H/HeJ. Results were analyzed using one-way ANOVA plus post-hoc analysis. In the box plot the middle line is the 50th percentile, the top of the box is the 75th percentile and the bottom of the box is the 25th percentile.

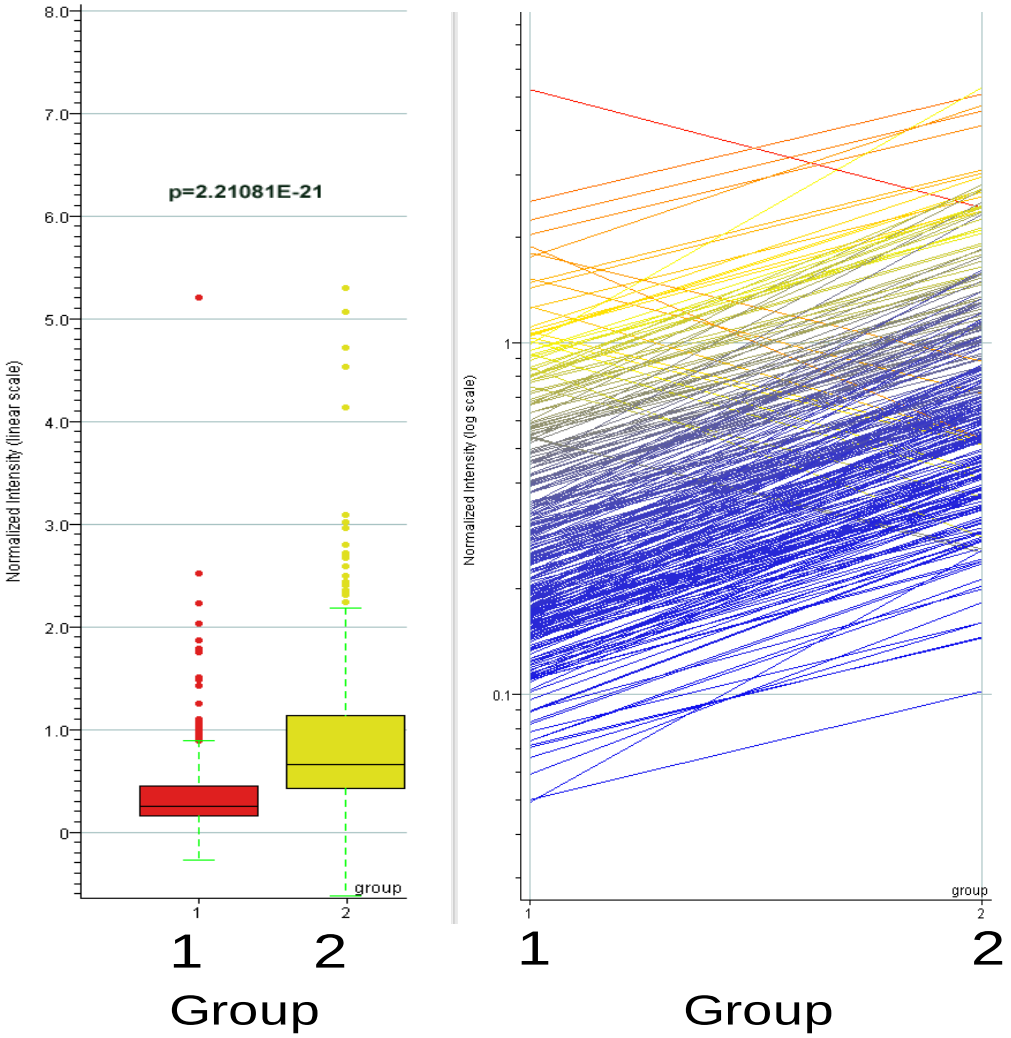

Fig. 9A. Line graph showing results after re-grouping five MRL/lpr mice. Group one consists of MRL/lpr #1 and #4 (higher sucrose consumers), group two consists of MRL/lpr #2, #3 and #5 (lower sucrose consumers). The two no sera controls (not shown) were not significantly different from group 1. This grouping of the MRL/lpr is similar to that seen in the sucrose test (fig. 7A). As can be seen there are a large quantity of peptides where there was greater binding in group 2 as compared to group one ($p < 0.001$).

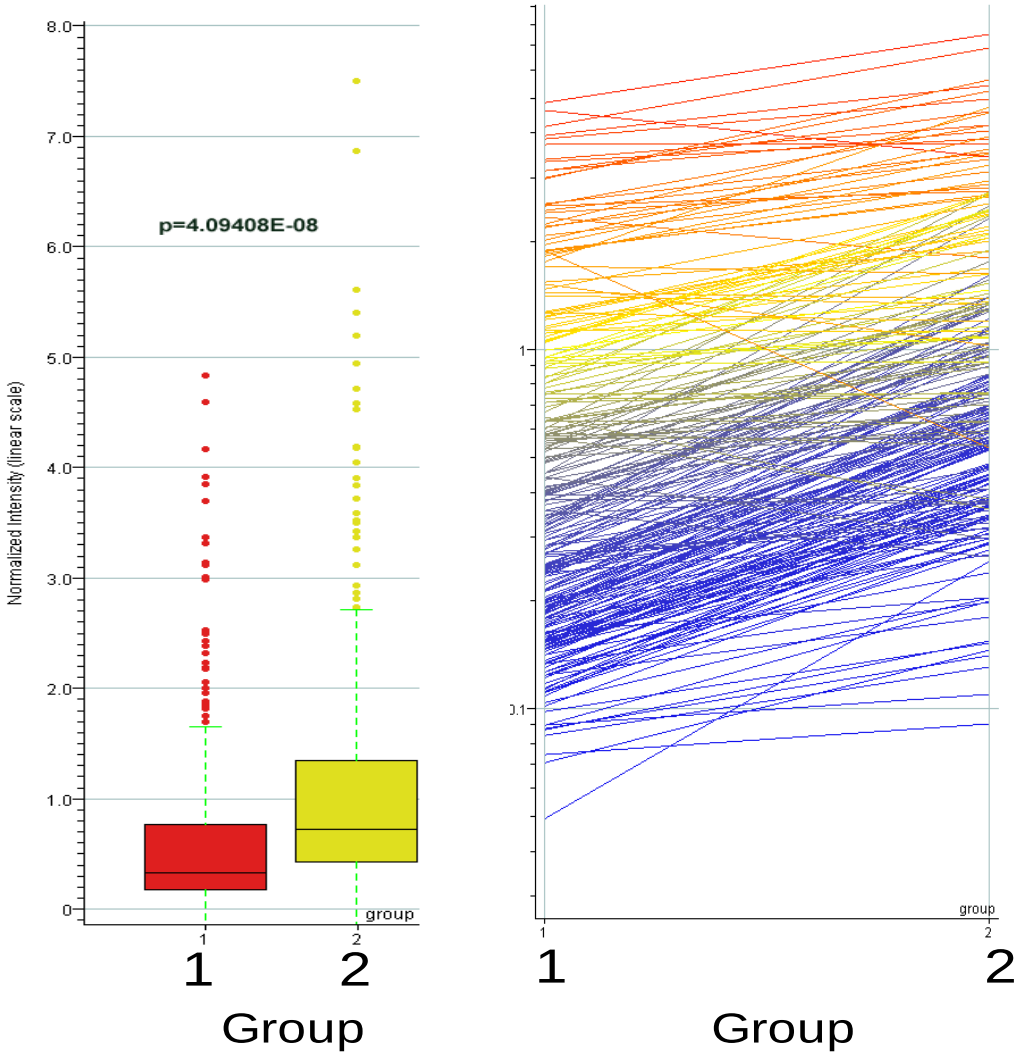

Fig. 9B. Line graph showing results after re-grouping five MRL/lpr mice. Group one consists of MRL/lpr #1 and #5 (low floating time and low anti-DNA levels), group two consists of MRL/lpr #2, #3 and #4 (high floating time and high anti-DNA levels). Once again the two no sera controls (not depicted) did not differ from group 1. This grouping is similar to that of the forced swim test (Fig. 7B) and the anti-DNA ELISA (Fig. 8). As can be seen there are a large quantity of peptides where there was greater binding in group 2 as compared to group one ($p < 0.001$).

DISCUSSION

The above studies describe a unique peptide microarray whose array patterns are characteristic of disease activity and neurobehavioral manifestations in MRL/lpr mice. We were able to detect peptides that had greater binding intensities for the MRL/lpr group in comparison to the MRL/mp and C3H/HeJ controls (Fig. 4). These microarray patterns are possibly detecting autoantibodies that are diagnostic for lupus. Indication of disease activity were the high levels of BRAA and anti-DNA autoantibodies detected in the MRL/lpr mouse sera similar to our previous studies (Zameer and Hoffman, 2001, 2004; Williams et al., 2010). The MRL/lpr mice also had greater spleen weights per body weight in comparison to the controls which along with higher levels of anti-DNA autoantibodies indicate that lupus was progressing in these mice (Figs. 1 and 2). Thus, our results so far suggest that there may be autoantibodies that could be diagnostic of lupus (Fig. 4). Even more interesting, the second part of our study further suggests that there are autoantibodies that may be diagnostic of specific CNS manifestations. This was detected when looking within the MRL/lpr group. We were able to observe that there was a difference in the performance of these animals in the behavioral tests and the autoantibody ELISA. These differences corresponded to the statistically significant differences in the peptide binding patterns in the microarray within the MRL/lpr mice (Fig. 9). We hypothesize that some of the autoantibodies that are binding to these peptides are involved in causing the behavioral changes and disease activity (viz., pathogenic autoantibodies). The behavioral changes that are observed are likely due to the

neuropsychiatric manifestations of lupus because when these MRL/lpr mice undergo other behavior tests such as the beam walk or open field test (previous studies) these animals are able to physically perform these tests as well as controls. Other researchers have also carried out these tests supporting that the performance in the Sucrose Test and Forced Swim Test are related to CNS manifestations (Ballok et al., 2003b; Sakic et al., 1994, 1996). Finally, if the behavioral manifestations were due solely to disease activity, the peptides that are thought to indicate disease activity would be the same as those that are thought to be responsible for the behavioral changes, but they are not, as detected by the microarray data.

Early diagnosis of lupus will be very useful in helping to treat individuals who are prone to this disease. We are trying to use microarray technology in order to create a test that would permit the diagnosis of lupus and are currently doing studies that will allow us to predict lupus. Furthermore, the lupus prone MRL/lpr mice are known to display neurobehavioral deficits in comparison to controls (Figs. 6A and B), based on previous studies (Sakic et al., 1993a, 1996; Williams et al., 2010). They had lower sucrose consumption and higher floating times, indicative of altered behavior. BRAA and anti-DNA ELISA testing revealed that our MRL/lpr mice on average also had greater levels of autoantibodies (Figs. 1 and 5), as expected. We have previously hypothesized (Hoffman et al., 1987; Narendran and Hoffman, 1989; Zameer and Hoffman, 2001) that there is a subset of (pathogenic) brain reactive autoantibodies that are responsible for some of the neuropsychiatric manifestations seen in SLE, which

has been supported by others (Schott et al., 2003; Tanaka et al., 2003). We also hypothesize that there are predictive autoantibodies for these neurobehavioral manifestations.

In this study, microarray analyses of the sera show peptides where there was greater binding intensity for the MRL/lpr mice in comparison to the controls. Because there was greater binding with these peptides as seen in fig. 4, some of these peptides are likely diagnostic for lupus. Peptides can be synthesized either chemically or *in vivo* to perform biochemical pull-downs of the antibodies of interest. It would be technically challenging but these antibodies could then be used to isolate the original autoantigen. We are working on more sensitive mass spectrometry methods that would require much lower sample sizes. At a minimum, however, the 200 diagnostic peptides could be biomarkers for the disease, also making it likely that predictive autoantibodies also exist. This would have to be tested in future studies. Reducing the 200 peptides to a smaller set would require analysis of the variability of detectable antibodies in the population, but in our small test it is encouraging that our classification error is 0%. In addition to identifying diagnostic autoantibodies, we should be able to use the 200 peptides to identify the antibodies to the proteins in the brains of the MRL/lpr mice to which the BRAA are binding, possibly causing the neurobehavioral deficits. Identifying these BRAA will be critical in order to backtrack from an antibody response to specific proteins – the proteins will lead to identification of the regions within the brain that are affected, and what behavior is likely altered due to the binding of these autoantibodies. This would lead to the identification

of pathogenic autoantibodies. In an article published in *Nature Medicine*, researchers used autoantigen microarrays to look at the autoantibody binding of patients with SLE and other disorders(Robinson et al., 2002). Their work demonstrated how sensitive microarrays are, since only a small sample is needed, and provide better results than conventional methods. They also discussed the importance of this technology in identifying autoantigens (which is one of our goals).

We've seen remarkable results from the random sequence peptide arrays using other diseases as a case study (Boltz et al., 2009;Morales Betanzos et al., 2009; unpublished data). We have been very successful at detecting and classifying influenza pre-symptomatically and before ELISA is able to make a distinction. We've used the power of the arrays to classify disease states with very low classification error, and we've found diseases that are not typically associated with immune response or autoimmunity that can be detected and classified using these arrays. The significance of partial binding between mimotope (peptide) and antibody cannot be understated. Peptide arrays in the past relied on perfect interactions between antibody and peptide, but we've created a system where 1-2 kCal/mol (millimolar binding constants) are sufficient to create a stable interaction on the arrays that can be recapitulated in solution. Thus, the patterns of binding that we see on the arrays correspond to actual reagents that can be used to biochemically pull down antigens that remained heretofore undiscovered and undetectable. We believe these results are a good

indication of the power of immunosignaturing, and the resolution of 10,000 random-space peptides, which can be applied to SLE.

In addition to identifying diagnostic autoantibodies for lupus, we also believe that we can use these microarray techniques to diagnose diverse neuropsychiatric manifestations. Evidence of this came when we looked within the MRL/lpr group and found differences in behaviors and microarray patterns. We found that in the sucrose consumption test there were MRL/lpr mice that can be grouped as low sucrose consumers vs. high sucrose consumers, while in the forced swim test, there were mice with higher float times and mice with lower float times. Looking at the anti-DNA autoantibody levels, the MRL/lpr group can be split into mice having higher and lower anti-DNA autoantibody levels. When retesting only the MRL/lpr mice, using the microarray analysis, and grouping them based on these differences, we found that there were significant array differences between the groups (Fig. 9). This suggests that based on the binding patterns of the arrays we may be able to diagnose the specific type of neuropsychiatric manifestation which an individual has when applying this technology to human sera. The next step will be to see if we can use some of the above identified peptide arrays to detect the occurrence of disease activity, or neurobehavioral manifestations, long before manifestations. It should be noted that based on the similarity of the groupings for the forced swim tests and anti-DNA autoantibody levels, it may be that these anti-DNA autoantibodies may be responsible for some of the neuropsychiatric manifestations. This has been shown to be a reasonable hypothesis according to the literature (DeGiorgio et al., 2001).

An alternative is that the anti-DNA autoantibodies are indicative of an inflammatory response and that cytokines are mediating the behavioral changes.

Based on the above results, our microarray analysis appears to be a useful tool for aiding in the diagnosis and prediction of lupus and the associated different neuropsychiatric manifestations in our mouse model. Beyond this, the microarray technique will be a powerful tool in allowing us to identify the specific proteins that the BRAA bind allowing us to better understand neuropsychiatric involvement in SLE, as well as develop unique therapeutic techniques. As mentioned, our future studies are aimed at determining not only the diagnostic peptides of lupus and CNS-lupus, but also predictive peptides and their autoantibodies.

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Chapter 4

VALIDATING DIAGNOSTIC PEPTIDES OF LUPUS AND ALTERED BEHAVIOR AND DETERMING POSSIBLE PREDICTIVE PEPTIDES OF LUPUS AND ALTERED BEHAVIOR FROM LUPUS-PRONE MRL/LPR MICE USING MICROARRAY TECHNOLOGY

ABSTRACT

We believe antibodies are present early on which can be useful in predicting future occurrences of lupus and specific CNS manifestations. Some of these will be able to be used to diagnose disease and may even be pathogenic autoantibodies. We are using a random peptide microarray to identify these antibodies. In a previous study, possible diagnostic peptides of lupus and of specific CNS manifestations were detected. In the current study we are testing the possible validity of these peptides being diagnostic and identifying predictive antibodies. Furthermore, these techniques can be used to identify pathogenic antibodies. We validated 58 peptides as being diagnostic of lupus and identified 18 possible predictive peptides of lupus. Of more interest, validated diagnostic and possible predictive peptides of altered behavior in the forced swim test were identified. There were overlaps between the possible predictive and validated diagnostic peptides of lupus and altered behavior, but there were some that were unique to each. This provides us with a tool to not only diagnose and predict CNS-SLE, but also to better investigate pathogenic mechanisms. Also, using five created monoclonal brain-reactive autoantibodies and the different peptide sets, we were able to suggest possible targets of these autoantibodies.

INTRODUCTION

Systemic Lupus Erythematosus (SLE) is an autoimmune disease that affects many organs of the body including the joints, kidneys and brain (Sakic et al., 2005). Some of the symptoms include arthritis, seizures, rashes and psychoses (Sakic et al., 2005). One of the characteristics of lupus is the detection of autoantibodies to many different antigens in the body and it is the presence of these different autoantibodies that are thought to be partly responsible for some of the manifestations of lupus (Williams et al., 2010). As mentioned, the brain is one of the organs that is affected, causing neuropsychiatric manifestations, such as cognitive impairment and psychoses (Hoffman and Sakic, 2009). In about 31% to 70% of lupus patients there was some kind of neuropsychiatric manifestation observed (Tin et al., 2005). We have hypothesized that there are brain reactive autoantibodies (BRAA) that bind to integral membrane proteins of brain and this interaction is partly responsible for some of the neuropychiatric manifestations seen in lupus (Narendran and Hoffman, 1989; Zameer and Hoffman, 2001; Sakic et al., 1993).

Our model of lupus was the MRL/lpr mice. These mice start to develop lupus after 2 months of age and they have 50% mortality at about 5-6 months of age. The MRL/lpr have manifestations similar to humans such as rashes, swollen joints and neuropsychiatric manifestations (Ballok et al., 2003; Theofilopoulos, 1992). A mutant of the *fas* gene, the *lpr* gene, is thought to help accelerate lupus in these mice. Because of the similarity in manifestations to humans, the MRL/lpr mouse is a good model of SLE and has been used by many other

researchers as their model of lupus (Ballok et al., 2003;Theofilopoulos, 1992). As a control for the genetics of the MRL/lpr, we used the MRL/mp mice since they are almost genetically identical to the MRL/lpr (without the lpr gene) but have late-onset autoimmunity. We also included a C3H/HeJ group as a control for non-autoimmune murine aging.

We have multiple goals in this study. The first goal is to validate peptides previously identified as possibly being diagnostic peptides of lupus and specific CNS manifestations, using a high-throughput, random peptide, microarray. We had already performed a previous study (Study 1 – Chapter 3) that allowed us to suggest diagnostic peptides, but we ran the current study (Study 2 – Chapter 4), in a similar manner to study one, in order to validate which peptides are indeed diagnostic of lupus and specific CNS manifestations. Validated peptides are those peptides that would show up in both studies. Secondly, unique to this study, we wanted to be able to predict the onset of lupus, or a specific CNS manifestation, long before any observed symptoms and therefore looked at the mice at two ages (before and during the onset of the disease). A third goal is to get preliminary information on the utility of this technique for future studies in identifying pathogenic peptides. Pathogenic peptides are those peptides bound by autoantibodies, which may be responsible for a specific manifestation (may be CNS related or not) possibly through binding to protein targets in the body and altering their function. If we can identify the targets of these autoantibodies that correspond with a specific manifestation using the sequences of the pathogenic peptides, then we can target those proteins for therapy.

There are low concentrations of autoantibodies present in the sera even before any clinical signs of lupus are observed. If we can detect these autoantibodies, it is our hypothesis that we can predict if someone will get lupus or even specific neuropsychiatric manifestations. In order to detect these predictive autoantibodies we used microarray technology (Legutki et al., 2010) and the MRL/lpr mice at 1.5 months (before any symptoms are observed), as well as 4 months. We looked for peptides where there was greater binding in our 1.5 month MRL/lpr compared to our control C3H/HeJ (assuming these would likely be the antibodies of importance) and compared them to peptides obtained using the same type of analysis and our 4 month MRL/mp (low levels of predictive autoantibodies should be detected in these mice since this is before any manifestations are occurring). The peptides found to be in common between these groups are said to be identified are being predictive of lupus and is a unique attribute of the current study. In order to detect predictive peptides of CNS manifestations, we regrouped the 1.5 month MRL/lpr into two groups based on how they perform on a specific behavioral test at 4 months of age (the age when they have lupus). By doing this, we were able to determine which peptides may predict if a mouse will get a specific neurobehavioral manifestation (again unique to this study).

Since we wanted to validate peptides that would diagnose lupus and a specific CNS manifestation, we did the same type of analysis as above, but instead used the microarray and behavior data for the MRL/lpr at 4 months of age (when they have lupus). By repeating the study twice, it allowed us to be more

confident in our results and therefore we could say that we have validated the different groups of diagnostic peptides. If this is successful, then this same technology can be applied to human SLE. However, in order for this technique to be applicable to human lupus, we would need to run multiple trials using human sera samples. These studies would be conducted over many years and then based on the results this would allow us to see if our technique was accurate in predicting the onset of lupus and specific CNS manifestation and diagnosing patients that already had lupus and different CNS manifestations. The multiple trials would allow us to determine "false positives" and "false negatives" when using our microarray analysis results. Microarray technology has been shown to be able to determine both predictive and diagnostic peptides of diseases such as diabetes and therefore should be applicable to lupus (Quintana et al., 2004).

The manifestations of lupus resemble the manifestations of other diseases. This therefore makes the accurate diagnosis of lupus very difficult. In order for a patient to be diagnosed as having lupus, physicians use a set of 11 criteria and patients must satisfy 4 out of the 11 criteria (Liu and Ahearn, 2009). Antinuclear antibodies and anti-DNA autoantibodies have been used as some of the markers for the diagnosis of lupus (Colasanti et al., 2009). However, these markers are not able to accurately diagnose a specific manifestation. Therefore, being able to correctly diagnose lupus and its CNS manifestations is of high importance due to the lack of a means of accurately diagnosing lupus and its CNS manifestations (Colasanti et al., 2009). Since our microarray technology provides specific patterns (specific peptides) for specific manifestations, this technology may prove

to be more accurate in diagnosing and predicting lupus and its CNS manifestations. It is important to note that being able to predict the onset of lupus or specific CNS manifestations is of great significance since this will be very useful in providing early and accurate treatments.

The third goal of this study is to test preliminary ideas about developing a reliable method for characterizing these autoantibodies, especially the BRAA. If we can identify the specific targets of these BRAA, then we can better understand if they have any functional effects, whether they are pathogenic and if they may be mediating neuropsychiatric manifestations. Eventually, this may lead to new methods of treating CNS-SLE. Since we knew the sequence of the peptides on the microarray chip, once we determined which peptides could be predictive and diagnostic of lupus and specific CNS manifestations, we used those sequences and a computer program (Guitope) to determine possible proteins that these antibodies are targeting. Even though we did not directly determine the targets of these BRAA, this information helps to suggest some possible molecular targets.

As examples of the above, some researchers have found an autoantibody that reacts with double-stranded DNA and the NMDA receptor (Kowal et al., 2004). This autoantibody resulted in cognitive deficits in their murine model, which suggests that this NMDA receptor autoantibody is partly responsible for this CNS manifestation. Another researcher found an autoantibody that is cross-reactive with the dynamin-1 protein that also altered the behavioral performance of their autoimmune murine model in comparison to control samples (Lawrence et al., 2007). Research data is, however, limited on the identification of the

targets of these autoantibodies and correlations between these specific targets and specific CNS manifestations and therefore more research is needed on the identification of these targets and their relation to function. Using the microarray technology, we have a technique that will allow us to identify more targets of these BRAA (and not rely on chance findings to identify these), we can better understand what areas of the brain may be affected. And if we can identify the areas of the brain possibly being affected, it may help us to understand why certain CNS manifestations are observed.

One interesting way to do the above is to combine the microarray and hybridoma technologies. Using one of the MRL/lpr with behavioral dysfunction from our current study, we created five monoclonal BRAA. We tested these monoclonal BRAA using different immunological techniques to better determine the possible identity of their brain targets.

The current study allowed us to validate diagnostic peptides of lupus and altered behavior in the forced swim test, from the first study, as well as identify predictive peptides of lupus. We were also able to suggest possible predictive peptides of altered behavior in the forced swim test. Using computer analysis we were able to suggest natural proteins possibly being affected by autoantibodies from the sera. The latter shows how identifying the targets of the autoantibodies could be done, but it's only part of the story.

MATERIALS AND METHODS

ANIMALS

The MRL/lpr mice is our model of lupus which start developing overt signs of the disease after 2 months of age and have 50% mortality at about 5-6 months of age. The MRL/mp is the congenic controls which have 50% mortality at about 12 months of age and develop disease manifestations much more slowly. The C3H/HeJ strain serves as our age-matched control and is non-autoimmune. In the current study we used 9-10 MRL/lpr and MRL/mp mice, which were tail bled every two weeks starting at 1.5 months until 4 months of age. All the mice were obtained from Jackson Laboratory (Bar Harbor, ME). These mice underwent behavioral testing at 8:00 P.M after being in the ASU Animal facilities for one week. The mice were housed under standard laboratory conditions with a light cycle of 6:00 A.M. to 6:00 P.M. Water and food were available *ad libitum*.

We used data from animals in a previous study (see chapter 3) for comparison to the current study. These animals included 3-5 MRL/lpr, C3H/HeJ and MRL/mp mice at 4 months of age. These mice were housed under similar conditions as the current study and also underwent similar behavioral testing.

Mice were anesthetized with an intraperitoneal (IP) injection of Nembutal sodium solution and blood samples collected. The blood was allowed to clot and then the tubes were centrifuged at 8500 rpm for about 10 minutes. 100 ul aliquots of the removed sera were frozen for future analysis. The body weights of all the mice were measured. The brains of 2 month old C3H/HeJ mice were used for preparing integral membrane proteins for the BRAA ELISA.

Animals were maintained in University facilities fully accredited by AAALAC and are registered with the USDA APHIS (Registration # 86-R-0002). An assurance is on file with the Office for Laboratory Animal Welfare (#3217-01). Animal husbandry programs and protocol review are in compliance with NIH and USDA standards.

To detect peptides that were specific to the different CNS manifestations, we had to look at the behavioral tests since we needed to split the MRL/lpr group into two. The battery of behavioral tests that we used included the forced swim test and the sucrose preference test. The forced swim test is used to test anti-depressants and may be a model of depressive-like behavior and the sucrose preference test looks at affect and may be a model of anhedonia (i.e., the desire to seek out pleasurable stimuli). Both of these tests have often been used by other researchers with the MRL/lpr mice and behavioral abnormalities were observed, so therefore we expect these tests to be useful in looking at emotional dysfunction in these MRL/lpr mice (Sakic et al., 2005). The MRL/lpr mice are expected to have high float time and low sucrose consumption. That is, however, not always the case since some of the MRL/lpr mice will actually do better on one test compared to the others in the group. By splitting the MRL/lpr into two groups we are able to identify peptides specific to certain behavioral abnormality.

BEHAVIORAL TESTING

SUCROSE TEST

As previously described, for 24 hours a day and over three days the mice were given 3ml of 4% sucrose solution as a training phase (Williams et al., 2010). For 24 hours after the training phase the mice were given no sucrose solution. The following three days the mice were given 7ml of the 4% sucrose solution for 1 hour on each day, known as the testing phase. The amount of sucrose consumed on each of the days was recorded and divided by the body weight of each animal. Consumption was used to measure altered behavior in this test.

FORCED SWIM TEST

As previously described, using a circular pool filled (5 foot diameter, 2 feet high) with 25^{o}C water, each mouse was allowed to swim around for 10 minutes (Williams et al., 2010). All mice were tested in the same surroundings and also lowered into the pool at the same location. The total amount of time that each mouse floated was recorded. Floating was measured as the absence of movement in the tail and hind limbs. Float time was used as a measure of altered behavior in this test.

IMMUNOLOGICAL ASSESSMENT

INTEGRAL MEMBRANE PROTEIN PREPARATION

The protocol that was used to extract the integral membrane proteins from the brains of the mice has been previously described (Narendran and Hoffman, 1988). We used integral membrane proteins from a 4 month C3H/HeJ control mouse in the BRAA ELISA and Western blotting technique and their concentrations were determined using the Bradford Assay (Sigma-Aldrich, USA).

ANTI-DNA AND BRAA ELISA

We used previously described protocols to determine the levels of BRAA and anti-DNA autoantibodies in the sera of the mice (Aotsuka et al., 1979;Crimando and Hoffman, 1995;Zameer and Hoffman, 2003).

ANTI-DNA ELISA

Fifty ug/ml of poly-L-lysine (Sigma, USA) dissolved in phosphate buffer saline (PBS) was added to the wells of 96-well plates and incubated for 1 hour at room temperature. Using a 0.1% PBS-Tween solution the plates were washed three times. This washing step was performed in between each of the incubation steps that will be described below. Each of the incubations steps were done for one hour at room temperature unless noted otherwise. The plates were then incubated with 10ug/ml of calf-thymus DNA (Sigma-Aldrich, USA; purified for dsDNA) dissolved in PBS in the odd wells only and PBS was added to the even

wells. In the next incubation step, 100ug/ml of poly-L-glutamate (Sigma-Aldrich, USA) dissolved in PBS was added to the plates followed by incubation with a 5% bovine serum albumin (BSA) (Sigma-Aldrich, USA) solution dissolved in PBS. Mouse sera diluted in PBS was added to the wells and followed by one final 1 hour incubation with the secondary antibody, goat anti-mouse IgG antibody conjugated with peroxidase (Caltag, USA) at a 1/1000 dilution in PBS. In the final step, a solution containing citrate buffer (1.04g/100ml of sodium citrate and 1.46g/100ml of citric acid (pH =4.4)), 2,2'-azino-bis(3ethylbenzthiazoline), sulfonic acid and hydrogen peroxide was added to each well and incubated for 30 minutes at 37^{o}C. The absorbance was read at 405nm using a microplate reader. In order to obtain the S-Value, which demonstrates the levels of anti-DNA autoantibodies for each sample, the optical density of the control wells was subtracted from the optical density of the wells that contain the calf-thymus DNA. Sometime the S-Values may be negative, but this can occur because the values are all relative to each other.

BRAA ELISA

Ten ug/ml of the integral membrane protein extracted from the C3H/HeJ mouse brain diluted in PBS was added to the odd wells of 96-well plates and PBS was added to the even wells. All incubation steps were done for 1 hour at room temperature. After each incubation step, the plates were washed three times with a 0.1% PBS-Tween solution. For the second incubation step, a 5% BSA solution dissolved in PBS was added to the wells. Mouse serum diluted in PBS was the

third incubation step followed by incubation with a 1/1000 dilution of the secondary antibody, goat anti-mouse IgG antibody conjugated with peroxidase (Caltag, USA). In the final incubation step a solution containing citrate buffer (1.04g/100ml of sodium citrate and 1.46g/100ml of citric acid (pH =4.4)), 2,2'-azino-bis(3ethylbenzthiazoline), sulfonic acid and hydrogen peroxide was added to each well and incubated for 30 minutes at 37°C. The absorbance was read at 405 nm using a microplate reader and the S-Values calculated in the same manner as that performed in the anti-DNA ELISA protocol described above.

MICROARRAY ANALYSIS

Microarray slides containing 10,000 randomly generated peptides about 20-mer in size were blocked with buffer containing mercaptohexanol for 1 hour in a humidity chamber (Legutki et al., 2010). The slides were then washed with Tris-buffered saline solution containing Tween (TBST) once and then twice with water. The slides were then centrifuged at 1500 rpm for 5 minutes and a gene frame was applied. A 1/500 dilution of the sera samples in blocking buffer without mercaptohexanol was added to the slides in triplicates. No primary antibody was added to some slides as controls. The slides were then incubated for 1 hour at 37°C in the humidity chamber. The slides were then washed three times with TBST and three times with water after each of the remaining incubation steps. A 6.67μM (1 nM final concentration) of the secondary antibody, goat anti-mouse IgG biotinylated secondary antibody (Bethyl, USA), was added to the slides and incubated for 1 hour at 37°C. 5nM of streptavidin conjugated with

AlexaFluor 555, the tertiary reagent, was added to the slides for 1 hour at 37^{o}C. The slides were washed for a final time and spun at 1500 rpm for 5 minutes. The slides were then loaded into the Perkin Elmer Scan Array laser Scanner (543nm emission, 565 nm absorption, 75% PMT, 100% laser power) and scanned.

When the microarray analysis was performed on the monoclonal BRAA, we used a similar procedure to above except the process was done using the Tecan HS 4800 Pro Hybridization Station made by Tecan Group Ltd, Mannedorf, Switzerland. Therefore the entire process was automated. The tertiary antibody that was added was 5nM of streptavidin conjugated with AlexaFluor 647.

MONOCLONAL ANTIBODY PRODUCTION

Monoclonal BRAA were produced using a ClonaCell®-HY Hybridoma Kit purchased from STEMCELL Technologies (Vancouver, Canada). Briefly, mouse myeloma cells (Sp2/0-Ag14) were purchased from ATCC (USA) and grown to the desired concentration using ClonaCell®-HY Pre-Fusion Medium. A volume containing 2 X 10^{7} cells was removed for fusion. The spleen of MRL/lpr #2 was then removed and the spleen cells teased out. A volume containing 1 X 10^{8} cells was removed for fusion. Cell viability was determined using trypan blue. The spleen cells and myeloma cells were combined, centrifuged for 10 minutes at 1500 rpm and then all the supernatant was removed. The cells were then fused using ClonaCell®-HY PEG Solution (PEG). The PEG was then removed, followed by the addition of ClonaCell®-HY Medium B and ClonaCell®-HY Medium C. The cells were then placed in the incubator

containing 5% CO_2 at 37°C. The following day, the cells were removed, centrifuged for 10 minutes at 1500 rpm, re-suspended in ClonaCell®-HY Medium C and then added to ClonaCell®-HY Medium D. The cells were then plated out onto petri dishes and placed in the incubator for 14 days. Visible colonies were removed from the petri dishes and suspended in 150 µl of ClonaCell®-HY Medium E in 96-well plates. The wells were tested for the presence of BRAA using ELISA technique. The cells that were positive were transferred to 24-well plates. If the cells were still positive for production of BRAA, the cells were transferred to petri dishes. The cells were allowed to expand and aliquots of the different cells were frozen at -80°C. Using this kit, 5 monoclonals were produced and tested using different techniques such as Western blotting and microarray in order to discover as much information about the identity of the proteins that the monoclonal BRAA are binding to.

WESTERN BLOTTING

The Western blotting protocol used has been previously described (Williams et al., 2010). Briefly, brain membrane antigen from a 4 month C3H/HeJ was loaded in the wells of a 12% resolving and 4% stacking gel. The gel ran for 2 hours at 102 volts. The gel was then transferred to nitrocellulose membrane (BioRad Laboratories, CA) for 25 minutes at 98 volts. The membrane was then blocked overnight at 4°C in blocking solution containing 0.1% Tris-buffered saline with Tween-20 (TBST), 5% bovine serum albumin (BSA) (Sigma-Aldrich, USA) and 1% Casein. The membrane was then cut into strips

and incubated with the primary antibodies (the 5 monoclonal antibodies, sera from MRL/lpr #2 and mouse anti-NK-1R (Zymed, USA) overnight at 4^{o}C. The membranes were then washed 3 times for 10 minutes using 0.5% TBST. Then they were incubated with the secondary antibody, goat anti-mouse IgG HRP conjugated (Caltag, USA) at 1/1000 dilution overnight at 4^{o}C. The membranes were washed 3 times for 10 minutes with the 0.5% TBST. The blots were visualized using a Chemiluminescence kit (Roche, USA) and detected on X-ray film (Kodak, New York). For some of the strips it was necessary to wash more times in order to better see the bands. The size of the bands were detected from a linear graph plotted using the molecular weight and distance traveled by the bands of the protein marker (Fermentas, USA).

STATISTICS

Standard microarray statistical methods were utilized to identify peptides that correspond to the disease conditions of interest (Stafford, 2009; Stafford and Brum, 2007). Each array of 10,000 peptides was tested for significant peptides by using either a Welch-corrected t-test or 1-way ANOVA with a FWER (family-wise error rate) adjustment of 5% ensuring that all peptides selected are significant given multiple testing correction. T-tests were typically done using multiple control animals against multiple animals at a particular disease state. Each sample was run on three technical replicates, the number of biological replicates varied per experiment.

Classification and clustering: each set of peptides that corresponded to those that reacted to antibodies differentially between control animals and those at a certain disease state were used to create heatmaps –hierarchical clusters of peptides against hierarchical clusters of samples using Euclidean distance as the similarity measure. Each heatmap shows how well the animals group based on the reactivity of the selected peptides. Note that we selected *all* significant peptides, rather than only those that were higher in the animals with disease, so some peptides may be *lower* in the disease state rather than only higher. Classification was done using linear discriminant analysis as the classifier and t-test or ANOVA as the feature selection method. Cross-validation was done using Leave One Out as per (Stafford and Brun, 2007).

In order to determine matches to natural proteins, the peptides were aligned individually to all proteins in the mouse proteome, using a gapless local alignment and scoring based on physiochemical similarity. Alignment scores were summed along each protein and proteins were ranked by their maximum score. The same numbers of peptides were randomly selected from the entire 10K to estimate the null distribution of these scores. An empirical one-sided p-value was reported based on the precentage of proteins having higher scores with the randomly selected peptide sets. This analysis was performed using an application called guitope (Halperin, et al., manuscript in preparation). Halperin and colleagues have previously shown that a similar method of aligning random-sequence peptides selected from array experiments has shown some value in predicting epitopes (Halperin et al., 2011).

Statistical significance for figures 1, 2, 3, 4 and 5 were determined using PASW Statistics 18. We used Model I 1-way ANOVA and LSD post-hoc analysis.

RESULTS

LUPUS

DISEASE ACTIVITY

The level of anti-DNA autoantibody levels was measured for the 9-10 MRL/lpr and MRL/mp in the current study to confirm disease activity. ANOVA revealed that there was a significant difference between the groups ($F=44.067$, $p < 0.001$). LSD post-hoc analysis at $p < 0.001$ revealed, as expected, that the 4 month MRL/lpr had significantly greater levels of anti-DNA autoantibody levels compared to the 4 month MRL/mp, 1.5 month MRL/lpr and 1.5 month MRL/mp (Fig. 1). This increased level of anti-DNA autoantibodies in the 4 month MRL/lpr is an indicator of disease activity in these mice (Crimando and Hoffman, 1992). The 4 month C3H/HeJ from Study 1 were used as a control group in both studies since these are non-autoimmune mice. The 4 month MRL/mp from Study 1, was useful in determining possible predictive peptides of lupus since they are lupus-prone mice but develop the disease at a much later time compared to the MRL/lpr. Therefore by comparing Study 1 and Study 2 (the current study) we were able to begin a validation of which peptides were diagnostic, as well as begin an identification of probable predictive peptides.

MICROARRAY ANALYSIS

VALIDATING DIAGNOSITC PEPTIDES OF LUPUS

In order to validate the diagnostic peptides of lupus identified in study 1 (chapter 3), we needed to see if the same peptides showed up as being diagnostic

in study 2. In order to do this we look at binding intensities on the microarrays when the animals have disease manifestations; therefore, we looked at the average binding intensities of the 4 month MRL/lpr. We divided the average binding intensities of these 4 month MRL/lpr by that of the average binding intensities of the 4 month C3H/HeJ. This picked out antibodies favoring autoimmune mice, but in order to be more conservative in our selection of diagnostic peptides, we selected only those peptides where the ratios were greater than the mean plus 1.5 SD of the binding intensity of the 4 month C3H/HeJ. There were 172 possible diagnostic peptides that were detected after this analysis for our current study. In study 1, we had also identified possible diagnostic peptides of lupus using the 4 month MRL/lpr in that study. In order to validate that these were diagnostic peptides, and to pare down the number of diagnostic peptides, we first took the average binding intensities of the 4 month MRL/lpr from study 1 and divided them by the average binding intensities of the 4 month C3H/HeJ from study 1. Then we chose those peptides with ratios greater than the mean plus 1.5 SD of the binding intensity of the C3H/HeJ. There were 193 possible diagnostic peptides of lupus detected. When comparing the 193 possible diagnostic peptides of lupus from Study 1 and the 172 possible diagnostic peptides of lupus from the current study, 58 peptides were found to be in common. Since these 58 peptides are reoccurring in both studies, they are validated as being diagnostic (Table 1). Of these 58, 3 were found to be in common with the predictive peptides of lupus, 23 were found to be in common with the diagnostic peptides of altered behavior in the forced swim test (discussed below) and 20 were found to be in common with

the possible predictive peptides of altered behavior in the forced swim test (Table 1). Possible matches to natural mouse proteins using our computer analysis program include 40S ribosomal protein S10 (sp|P63325), 60S ribosomal protein L22-like 1 (sp|Q9D7S7), Histone H3-like centromeric protein A (sp|O35216), Follistatin-related protein 4 (sp|Q5STE3) and H-2 class II histocompatibility antigen, A-D alpha chain (sp|P04228).

IDENTIFYING PREDICTIVE PEPTIDES OF LUPUS

Microarray analysis was performed on 10 of the 1.5 month MRL/lpr and 9 of the 4 month MRL/lpr. Overall, greater binding intensities were detected on the microarray slides for the 4 month MRL/lpr compared to the 1.5 month MRL/lpr. However there were still slightly greater binding intensities for some of the peptides for the 1.5 month MRL/lpr compared to the control C3H/HeJ group from Study 1. In order to identify possible predictive peptides of lupus, long before any manifestations would be observed, we compared the binding intensities of the 1.5 month MRL/lpr to that of the non-autoimmune 4 month C3H/HeJ from Study 1. The idea is that predictive peptides (actually antibodies binding to these peptides) will be those found in autoimmune mice, but not normal mice. We divided the average binding intensities of 1.5 month MRL/lpr by that of the C3H/HeJ and selected peptides where this ratio was greater than the mean plus 1.5 SD of the binding intensities of the C3H/HeJ. After this analysis 518 possible predictive peptides of lupus were detected in the current study. Since the 4 month MRL/mp are also lupus-prone but much later on in their life, at 4 months of age

they can also be used to detect predictive peptides due to no manifestations being present at that time. We performed the same analysis where we took the binding intensities of the 4 month MRL/mp (from Study 1) and divided them by the binding intensities from the 4 month C3H/HeJ and then chose any peptides where the ratio was greater than the mean plus 0.25 SD of the binding intensities of the C3H/HeJ. From this analysis 143 predictive peptides of lupus were detected from Study 1. When comparing the 143 possible predictive peptides of lupus from Study 1 and the 518 possible predictive peptides from the current study, 18 peptides were found to be in common between these two groups. These 18 peptides are the ones identified as being predictive peptides of lupus (Table 2). Possible matches to natural mouse proteins include complement C1q tumor necrosis factor-related protein 6 (sp|Q6IR41), Histone H3-like centromeric protein A (sp|O35216), Alpha-actinin-2 (sp|Q9JI91) and 60S ribosomal protein L22 (sp|P67984).

CNS-LUPUS

BRAA

We also measured the levels of BRAA in the sera of the mice since BRAA levels are expected to be higher in the 4 month MRL/lpr if they are one of the causes of the neuropsychiatric manifestations of lupus. ANOVA revealed that there was a significant difference between the groups ($F=9.746$, $p < 0.001$). LSD post-hoc analysis at $p < 0.001$ revealed that the 4 month MRL/lpr had greater BRAA levels compared to the 4 month MRL/mp, 1.5 month MRL/lpr and 1.5

month MRL/mp in the current study (Fig. 2). In study 1, the 4 month MRL/lpr also had significantly greater levels of BRAA compared to the 4 month MRL/mp and 4 month C3H/HeJ (Chapter 3).

BEHAVIOR TESTING

In order to look at some of the neurobehavioral manifestations of the lupus mice, we performed the sucrose preference test and the forced swim test, which are both considered to be measures of affective dysfunction in murine models of lupus. In the sucrose preference test a significant difference was found between the groups ($F=12.950$, $p < 0.001$). Post-hoc analysis at $p < 0.05$ showed that the 4 month MRL/lpr consumed significantly less sucrose compared to the 1.5 month MRL/mp and 4 month MRL/mp (Fig. 3). There was, however, no difference between the 4 month MRL/lpr compared to the 1.5 month MRL/lpr, which may mean that this manifestation is present early on even before any of the overt signs of lupus disease (Sakic et al., 1997). In the forced swim test, there was an overall significant difference between the groups ($F=11.057$, $p < 0.001$) and post-hoc analysis at $p < 0.05$ revealed that the 4 month MRL/lpr floated significantly more than the 4 month MRL/mp, 1.5 month MRL/mp and 1.5 month MRL/lpr (Fig. 4). This indicates that the 4 month MRL/lpr were displaying altered behavior in the forced swim test. In study 1, the 4 month MRL/lpr also had significantly greater float time compared to the 4 month MRL/mp and 4 month C3H/HeJ making them a good comparison for this behavior (shown in Chapter 3).

GROUP SEPARATION BASED ON THE FORCED SWIM TEST

Overall, the 4 month MRL/lpr had significantly greater float times compared to all the control groups; however, there was a large variation in this behavioral performance within this group with some mice doing worse in this test versus others, so we split the mice in this group into two. ANOVA revealed that there was a significant difference between the groups ($F=30.253$, $p < 0.001$). Of the nine mice in the 4 month MRL/lpr group, numbers 5, 6, and 7 were grouped as low floaters, due to being similar to the control groups. This group of low floaters was significantly different compared to the 4 month MRL/lpr that were high floaters ($p < 0.001$). Numbers 1, 2, 4, 8, 9 and 10 were grouped as high floaters. This group of mice, having very high float times, were significantly different from all the other groups (1.5 month MRL/lpr, 1.5 month MRL/mp, 4month MRL/mp and 4 month MRL/lpr low floaters) ($p < 0.001$) (Fig. 5).

In study 1, we had also split the 4 month MRL/lpr into 2 groups since some animals did worse in this test compared to others. Overall, the 4 month MRL/lpr high floaters had significantly higher float time compared to the 4 month MRL/lpr low floaters, 4 month MRL/mp and 4 month C3H/HeJ (data not shown).

MICROARRAY ANALYSIS

Since we were able to split the 4 month MRL/lpr into two groups for both study 1 and study 2 for the forced swim test, we were able to validate diagnostic peptides from Study 1 and identify possible predictive peptides of this behavior from our current study.

VALIDATING DIAGNOSTIC PEPTIDES FOR ALTERED BEHAVIOR IN THE FORCED SWIM TEST

The 4 month MRL/lpr were split into two groups as mentioned above for the current study. We then selected peptides where the 4 month MRL/lpr high floaters had greater binding intensities than the 4 month MRL/lpr low floaters, to get the highest of the antibodies in the autoimmune strains of mice binding to the peptides in the microarray. Next, the binding intensities of these peptides were divided by their respective 4 month C3H/HeJ binding intensities. We then selected only peptides that had ratios with greater binding intensities than the mean plus 1.5 SD of the binding intensities of the 4 month C3H/HeJ. This allowed us to conservatively identify those peptides with reactivity to autoimmune mouse sera that were greater than non-autoimmune mice, picking out potentially diagnostic peptides. From study 1, there were 261 possible diagnostic peptides of depressive-like behavior detected. In this current study 190 possible diagnostic peptides of depressive-like behavior were detected (Fig. 6). To "validate" the diagnostic peptides from study 1, we compared those to the ones seen in the current study, and found that there were 39 in common. These 39 peptides are therefore partially validated as being diagnostic peptides of depressive-like behavior in lupus (Table 3), more studies would have to be run to fully validate the selected diagnostic peptides. When using our computer analysis program to determine matches to natural proteins using all 39 diagnostic peptides of depressive-like behavior, some of the resulting proteins that were of interest to

use include metabotropic glutamate receptor 4 (sp|Q68EF4), 60s ribosomal protein L22-like 1 (sp|Q9D7S7), Calcium/calmodulin-dependent protein kinase kinase 1 (sp|Q8VBY2) and 40S ribosomal protein S9 (sp|Q6ZWN5).

IDENTIFYING PREDICTIVE PEPTIDES FOR DEPRESSIVE-LIKE BEHAVIOR

In order to determine possible predictive peptides of altered behavior in the forced swim test, we split the 1.5 month MRL/lpr into two groups identical to the grouping that we used for the 4 month MRL/lpr for the forced swim test. The logic here is that these (1.5 month) mice would later (at 4 months) display altered behavior in the forced swim test. We classified these as "1.5 month MRL/lpr high floaters" and "1.5 month MRL/lpr low floaters". In order to pick out predictive peptides, we averaged the binding intensities for the 1.5 month MRL/lpr in each of the 2 groups and chose peptides where the binding intensities for the 1.5 month MRL/lpr high floaters was greater than the 1.5 month MRL/lpr low floaters. Then the binding intensities of these peptides was divided by their respective binding in the 4 month C3H/HeJ and then we selected only those peptides where the ratios were greater than the mean plus 1.5 SD of the 4 month C3H/HeJ. From these analyses we selected 354 possible predictive peptides of altered behavior in CNS-lupus (results not shown due to large number of peptides). Of these 354 possible predictive peptides of altered behavior, 15 were found to be in common with the possible diagnostic peptides of altered behavior in the forced swim test, 20 were found to be in common with the possible diagnostic peptides of lupus and

8 were found to be in common with the possible predictive peptides of lupus. These are identified in the respective Tables.

CHARACTERIZING POTENTIALLY PATHOGENIC AUTOANTIBODIES

Another important objective is to identify "pathogenic" BRAA. One way this might be done is to produce monoclonal antibodies from mice displaying behavioral manifestations and use the microarray technique to help characterize these antibodies. We produced some monoclonal antibodies in order to get a preliminary idea of how this might work. The five monoclonal BRAA (labeled F9, G10, G4, D1 and D9) that were produced had S-Values above 0.1 OD on the BRAA ELISA, which we believe shows high reactivity of these antibodies (Fig. 7). The mouse, MRL/lpr #2 that was used to produce these monoclonals also had high levels of BRAA (Fig. 7). Next, the approximate molecular weight of the targets of these BRAA was determined using Western blotting (Fig. 8).

Using our microarray technology along with Western blotting results we tried to determine the best possible matches to natural proteins. In Table 4, we have included six possible protein matches for each of the monoclonals using a computer analysis program and the approximate molecular weights of the targets based on the Western blot (proteins of interest to us have been highlighted and will be discussed). Starting with D9, the approximate molecular weight of its target was determined to be 53 kDa. Two interesting natural protein matches using our computer analysis program was the D (1B) dopamine receptor (DRD5)

and Galanin receptor type 3. The detected molecular weight of D (1B) dopamine receptor (DRD5) is ~54 kDa (The UniProt Consortium, P21918) (Fig. 8). We also detected a ~39 kDa band using the supernatant from D9, which has been detected at ~40 kDa by other researchers when using anti-serum to this receptor (Centonze et al., 2003).

Using D1 on the Western blot, we detected two bands, one band at 55 kDa and a second at 95 kDa (Fig. 8). After microarray analysis, possible matches of interest to us could be the gamma-aminobutyric acid receptor subunit rho-1 (GABRR1) and endothelin B receptor. GABRR1 has a molecular weight of ~56 kDa (The UniProt Consortium, P24046). The 95 kDa band that was detected, we believe is some kind of modification to the protein or a dimer between this protein and another Gamma-aminobutyric acid receptor subunit. On the antibody data sheet for GABRR1 antibody, two bands were detected at 55 kDa and ~95 kDa (ProSci Incorporated, USA).

For G10 on the blot, we detected one band at ~69 kDa (Fig. 8). This band could possibly be leucine-rich repeat-containing protein 4C or matrix metalloproteinase-14. With BRAA G4 a band at ~69 kDa was seen on the blot (Fig. 8). This protein could possibly be GRB2-associated-binding protein 2 or vesicular glutamate transporter 3. Lastly, for F9, on the blot, a band at ~158 kDa was detected (Fig. 8) and identified using the microarray as possibly being synaptojanin-2, epidermal growth factor receptor or glutamte [NMDA] receptor subunit epsilon-3.

Immunological Assessment

Anti-DNA Autoantibody Levels

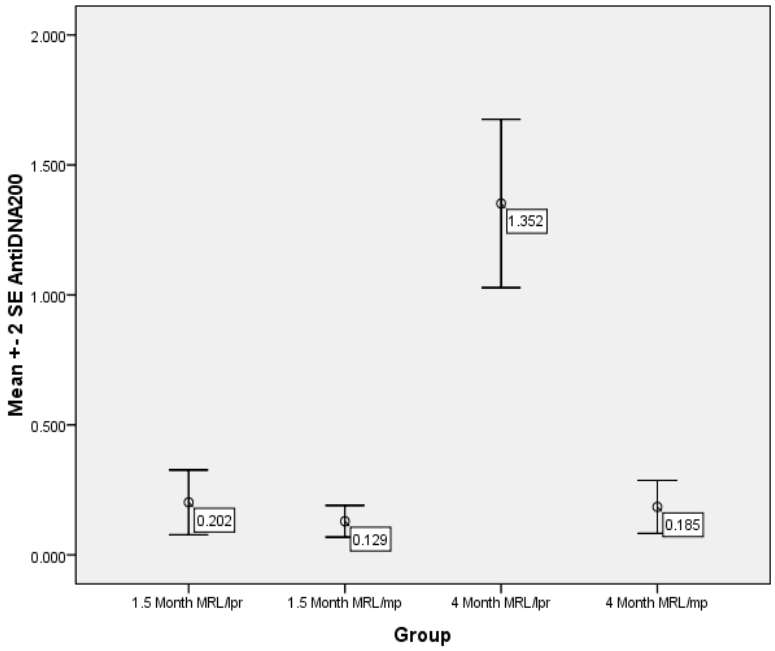

Figure 1. Williams et al. 2011

Fig. 1. The level of anti-DNA autoantibodies was measured for the MRL/lpr (N=9-10) and MRL/mp at 1.5 and 4 months of age. ANOVA analysis revealed that there was a significant difference between the groups ($F=44.067$, $p < 0.001$). LSD post-hoc analysis at $p < 0.001$ revealed that the 4M MRL/lpr had significantly greater levels of anti-DNA autoantibody levels compared to the 4M MRL/mp, 1.5M MRL/lpr and 1.5M MRL/mp.

Immunological Assessment

BRAA Levels

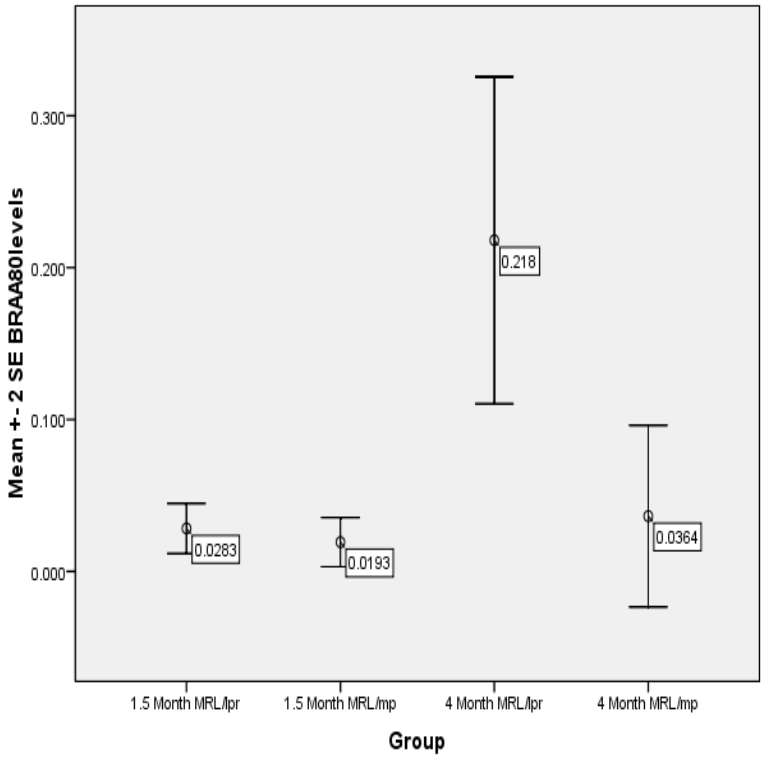

Figure 2. Williams et al. 2011

Fig. 2. The level of BRAA was measured in the MRL/lpr and MRL/mp at 1.5 and 4 months of age and ANOVA analysis revealed that there was a significant difference between the groups ($F=9.746$, $p < 0.001$). Post-hoc analysis at $p < 0.001$ revealed that the 4M MRL/lpr had significantly greater levels of BRAA compared to the 4M MRL/mp, 1.5M MRL/lpr and 1.5M MRL/lpr.

Behavioral Dysfunction

Sucrose Test

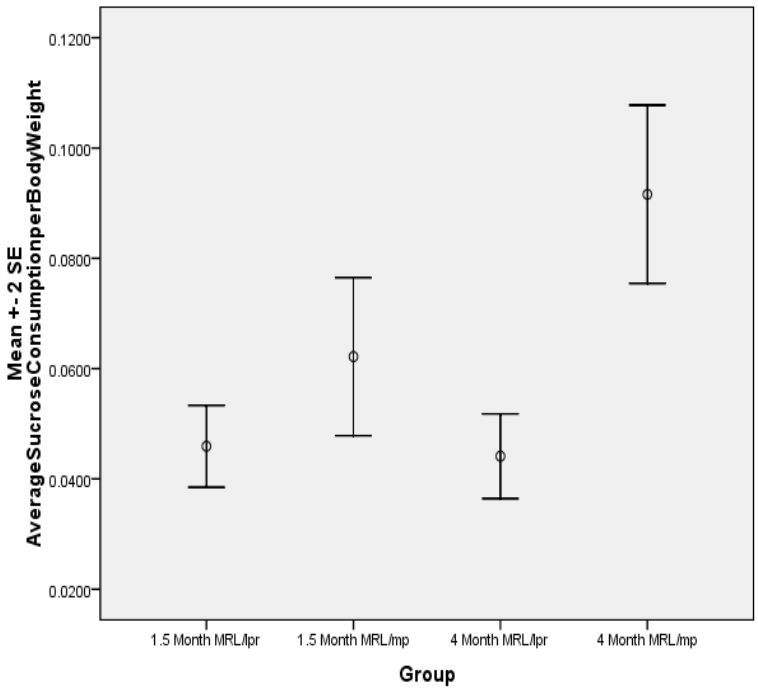

Figure 3. Williams et al. 2011

Fig. 3. The level of sucrose consumption was measured and a significance difference was determine between the groups ($F=12.950$, $p < 0.001$). Post-hoc analysis at $p < 0.05$ showed that the 4M MRL/lpr consumed significantly less sucrose than the 1.5M MRL/mp and 4M MRL/mp, but not significantly less than the 1.5M MRL/lpr. There was also not a significant difference between 1.5M MRL/lpr and 1.5M MRL/mp.

Behavioral Dysfunction

Forced Swim Test

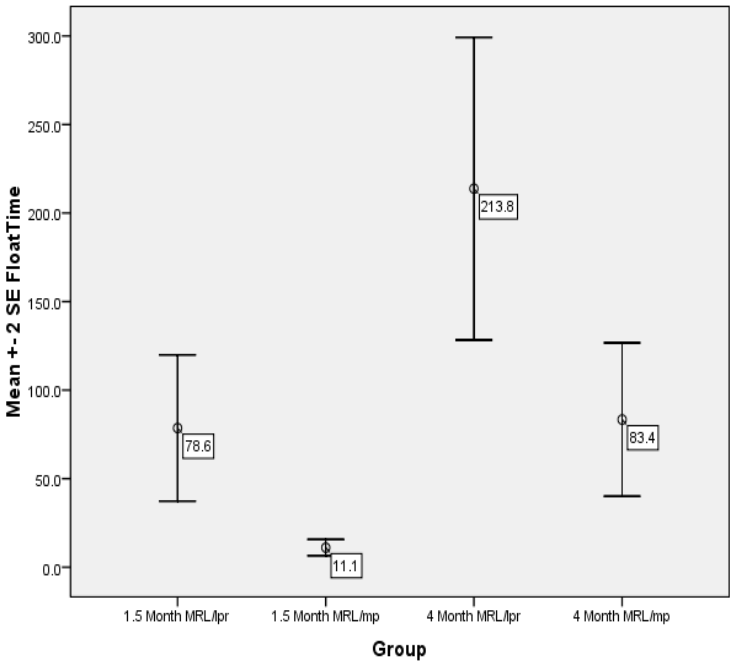

Figure 4. Williams et al. 2011

Fig. 4. For the forced swim test there was an overall significant difference between the groups ($F=11.057$, $p < 0.001$) and post-hoc analysis at $p < 0.05$ revealed that the 4M MRL/lpr floated significantly longer than the 1.5M MRL/lpr, 1.5M MRL/mp and 4M MRL/mp. The 1.5M MRL/mp was significantly different than the 4M MRL/mp.

Splitting Groups Forced Swim Test

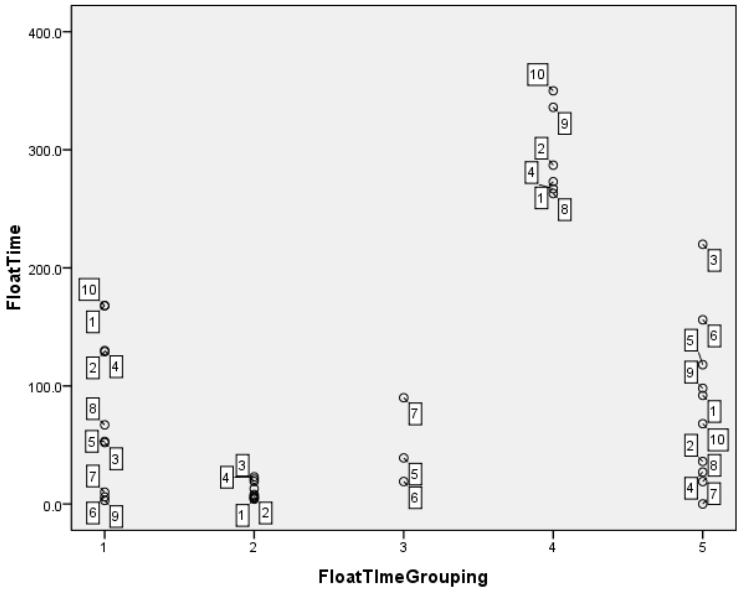

Figure 5. Williams et al. 2011

Fig. 5. The 4M MRL/lpr were separated into two groups, a group of high floaters (mouse numbers 1, 2, 4, 8, 9 and 10) and low floaters (mouse numbers 5, 6 and 7), based on their float times. In the figure above the numbers on the x-axis represents: 1) the 1.5M MRL/lpr, 2) the 1.5M MRL/mp, 3) the 4M MRL/lpr low floaters, 4) the 4M MRL/lpr high floaters and 5) the 4M MRL/mp. ANOVA analysis revealed that there was a significant difference between the groups ($F=30.253$, $p < 0.001$). Post-hoc analysis at $p < 0.001$ showed that the 4M MRL/lpr low floaters were only significantly different from the 4M MRL/lpr high floaters. The 4M MRL/lpr high floaters were also significantly different from the 1.5M MRL/lpr, 1.5M MRL/mp and 4M MRL/mp.

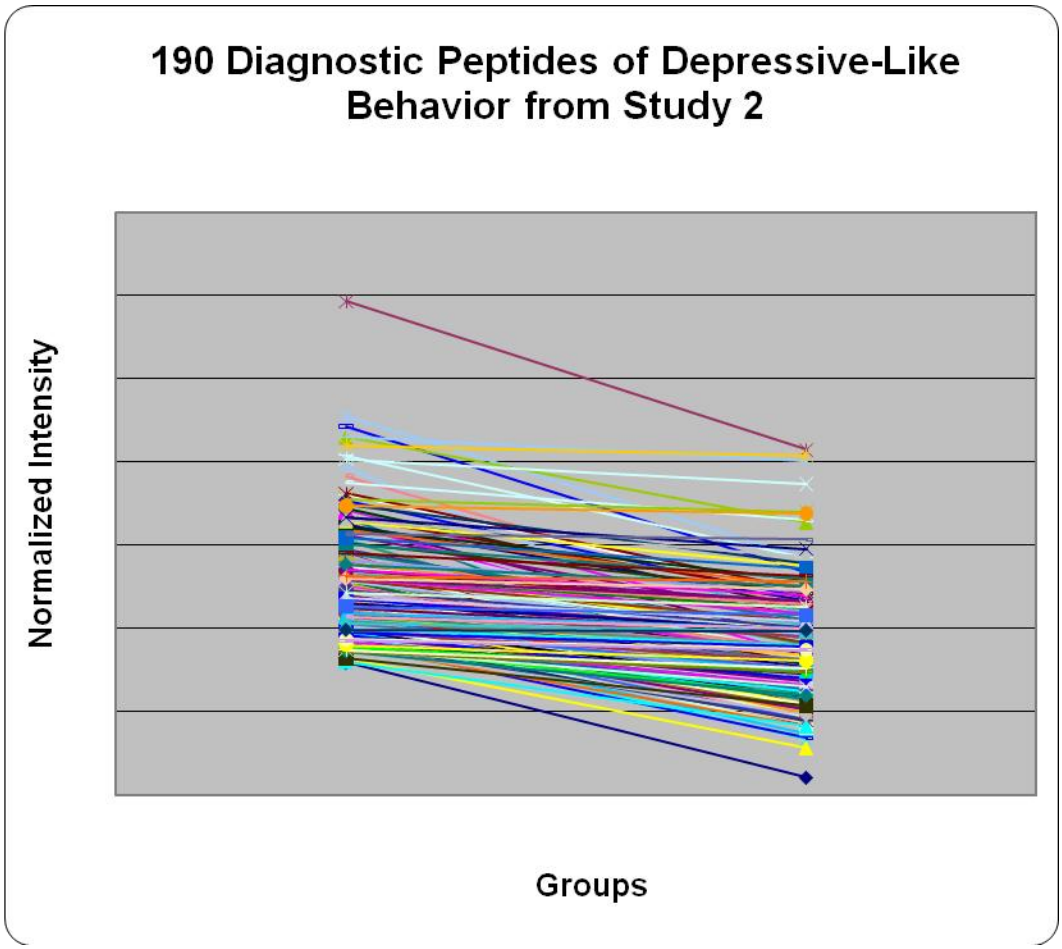

Fig. 6. Graph showing the 190 peptides that were determined to be possibly diagnostic of altered behavior in the forced swim test in study 2. The graph shows the peptides with greater binding intensities for the 4 month MRL/lpr high floaters (which had greater binding intenstities that the 4 month C3H/HeJ and 4 month MRL/lpr low floaters) (group on left) as compared to the 4 month MRL/lpr low floaters (group on right).

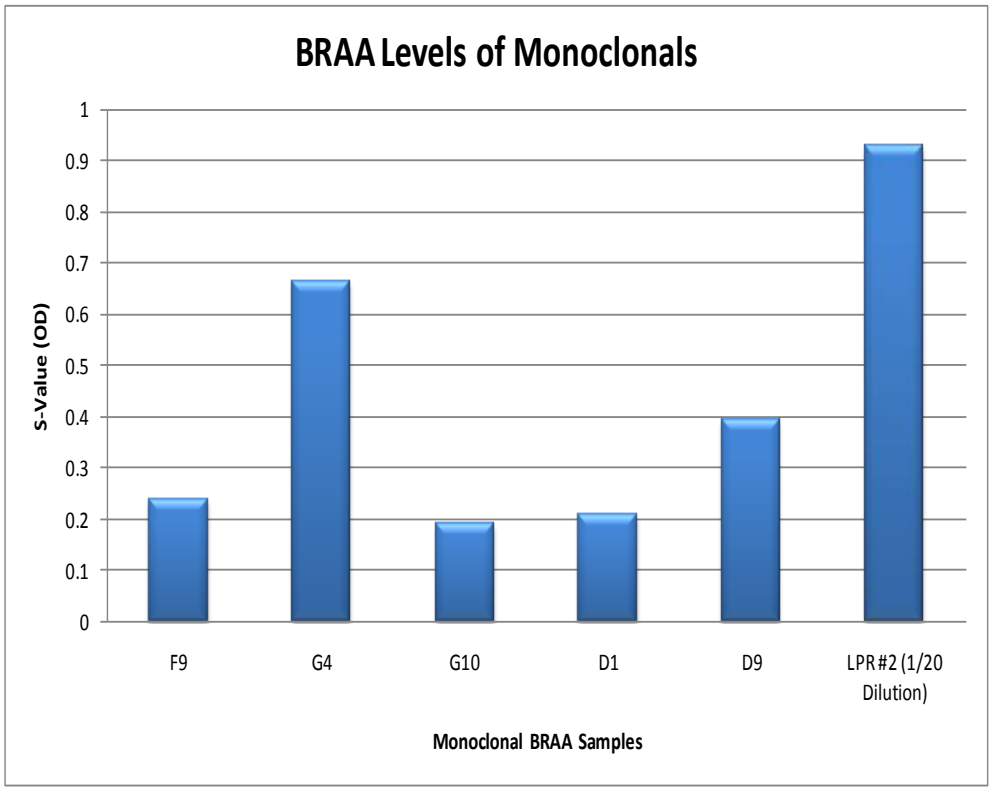

Figure 7. Williams et al. 2011

Fig. 7. BRAA ELISA results of our potential pathogenic monoclonal BRAA. The graph shows that all of these monoclonal had high levels of reactivity (above 0.1 OD). Non-reactive monoclonal antibodies show S-values at 0 or very close to 0.

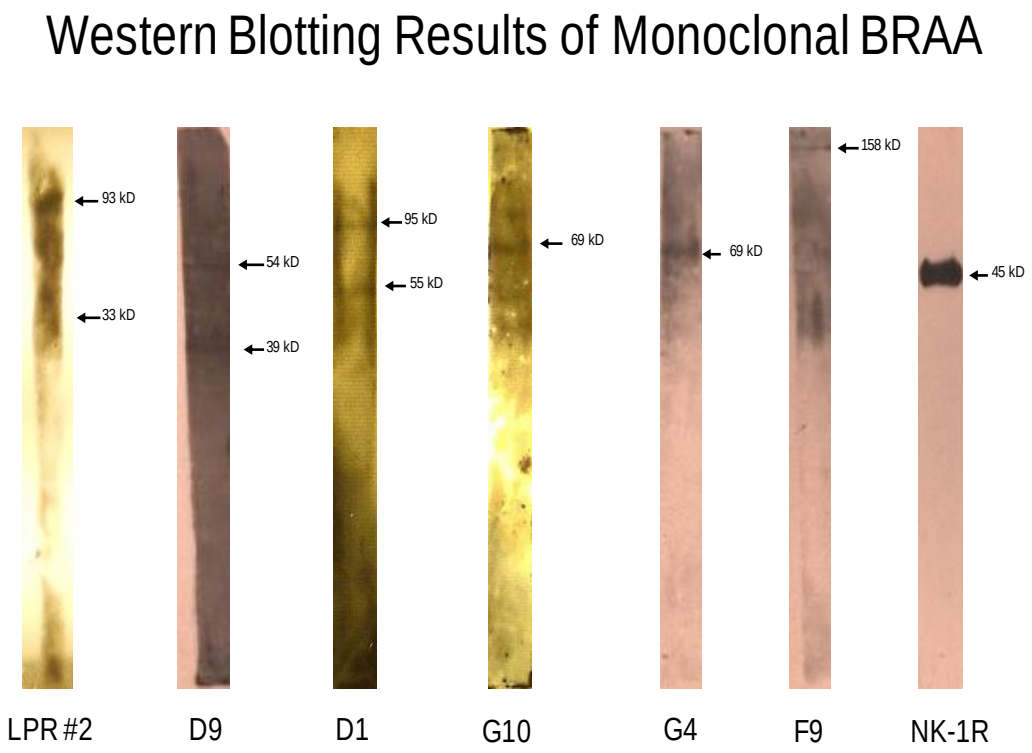

Figure 8. Williams et al. 2011

Fig. 8. Western blotting results showing the banding pattern of the five potential pathogenic monoclonal BRAA.

Table 1: Diagnostic Peptides of Lupus

Peptide Sequence	Binding Ratios		In Common with Predictive Peptides of Lupus	In Common with Diagnostic Peptides of Depressive-Like Behavior	In Common with Predictive Peptides of Depressive-Like Behavior
	Study 1 4MLPR/ 4MC3H	Study 2 4MLPR/ 4MC3H			
ADGSNWAARHWIPRMPRGSC	3.404555813	2.332923454			
AMSFHRGWDRKYRMSNIGSC	2.599608572	2.484332734			√
AQLGMYGVYRPVEIWPDGSC	2.872747926	2.519910261		√	√
ATDKTRFHFLYDYIRSNGSC	2.399571763	2.880058556			
DDTLYNAHKHLKWFGFIGSC	2.515491313	3.037141329		√	
EATGNDWVITRGGMRRYGSC	2.556966833	3.171502541			
EMNNGRFHRWAQQERHPGSC	2.880990297	2.507493739			
EMSWPRKPWRSKYYHEIGSC	2.353443025	2.435039315	√		√
ENILPTGRDRVAGWYRYGSC	2.825255829	2.675812354	√		
EPKLWFKPRRGGYRHRHGSC	2.679076813	2.434485702		√	
ERIYRDHFIHEHKANIIGSC	2.389911067	2.665777953			√
EWYYDPRGGTGSFYMRTGSC	2.826268307	3.144085944			√
FNRDHREFFEHFGFDEPGSC	4.422628739	2.314403551	√		
FPGDRRSGRAFPEVRWRGSC	2.66924554	2.635674506		√	
FTLMTGKKMIVWDWQRDGSC	2.564037604	2.381362551			√
FWEHHVFHSSRRDGWASGSC	2.951177541	2.464272834			
GLVSRIPSVPKHDEWTFGSC	2.38601487	2.329539073		√	
GRVPQDFNTPSFDRVFWGSC	2.513399666	2.788409089		√	
GWLKAMGPFPWGRLVQNGSC	2.681235945	3.008923213		√	√
IEAMGPSQRYRGRYELIGSC	2.377783579	2.371993113			
IGQRLKGKDENIRFENFGSC	2.347244686	3.594919118		√	√
ILDRRETAWNEHFSKFRGSC	2.781744911	2.979425643		√	
KAMSIHQLANPFDWHFWGSC	2.316559723	2.705671849			
KGYSIRHTEHAWPDIYVGSC	2.579657821	3.104342319			√
KLLMTDFMAKWPRNGWYGSC	3.325866727	2.467180958		√	√
KQHPIYIAHFLGTIVKRGSC	2.850248411	2.447758339			
KVDYVNQWARRRIFMAPGSC	2.933406801	2.826782754		√	
KWLQTQLNSAMYYIRLYGSC	3.442674101	2.816729922			
LAFAWKPDPWQSLVTKFGSC	3.276131888	2.698697705		√	
LFSFKEPQPFMWNKWQQGSC	3.390564552	2.759114763			
LRKISRGIWGMREAGEFGSC	2.476731381	3.603591875			
MFARAHNFDWVKWPLNRGSC	2.705759435	2.921507814			√
MWMSWGWAMLWLNGMMQGSC	4.177408974	2.432118292			
PLVHPWYPTYIPGRHNMGSC	3.630154471	2.495553997			√
PMLFWKWHRQLNQQGRRGSC	2.75721307	2.335852256		√	√
PNPEAWARSFKRWNRKFGSC	3.313709957	3.508821684			√
PSAWEWIPRNQHLNKFRGSC	3.112407959	2.391409291		√	√
PTWRLPPYTDPPKYWHPGSC	4.201093721	3.373945327			
PYRFDWAALPLKKPMWRGSC	2.40624731	2.565057719		√	
QKKPPDYRTWHHPFYNGGSC	2.604969331	2.754487673		√	
QKRWLQLPRNLMWRRETGSC	2.664022568	2.529899901		√	
QRKIFFNYKLHKIWFTAGSC	2.362843141	2.462441712			
QSHWFYDRTKDVYPGRHGSC	3.992759382	2.329563875		√	
RAAMHESLKNWRVYREWGSC	2.388166554	2.725739509		√	√
RPAFDKFADSYWYPPNLGSC	2.471919459	2.483799875			
RRLTKGIIRQYESQLWDGSC	2.38838007	3.049171397			
RTIYRWSQGALSWYMDAGSC	2.443171562	2.802208961			√
SDQVIRGFKDVWQYKWFGSC	3.018394899	2.579167723			
SRDAGLQYPYHRWLTGWGSC	2.452812911	2.732221588			
SRLEQQHFATIPQIWYTGSC	2.462810107	2.571973953			
SRQGLHYNLDGLKPIFPGSC	2.652106822	2.808739776		√	
TLQRTWRRPLLEDLPWWGSC	5.695014524	3.855075257			
VQERMHNRTWKRFGGSMGSC	2.754519694	2.496803929			√
WKPIWHSFHKRRPQILNGSC	2.50380818	2.372369774		√	√
WNGPEWKYSEKSKRILFGSC	2.445136421	2.433467124			√
WSYKYKKKQAWDWPWDPGSC	2.443311505	2.436968425		√	
WTWPSIRFVKGEEYGRFGSC	2.991053751	2.626530982			
YYNVQQVDRWVKLQWGLGSC	2.441114219	2.553038205		√	

Table 1. Williams et al. 2011

Table 2. Predictive Peptides of Lupus

Peptide Sequence	Binding Ratios		In Common with Predictive Peptides of	In Common with Diagnostic Peptides of	In Common With Diagnostic Peptides of
	Study 1 MP/C3H	Study 2 1.5M LPR/C3H			
DKFHYWMYMLYGINDKIGSC	2.122578617	2.341589487			
DKLWKQIWTERHFMSHKGSC	1.56585119	4.3041941	√		
DWDSRQINPHIIHHVGRGSC	1.412922851	2.776999584			
EEHAHNKLFWWHRSRALGSC	1.605761133	2.522392064	√		
EMSWPRKPWRSKYYHEIGSC	1.463541142	3.359490756	√		√
ENILPTGRDRVAGWYRYGSC	1.512851886	3.095766385			√
FNRDHREFFEHFGFDEPGSC	1.403104158	3.610817264			√
GYNYWIVEWDQDQWLMNGSC	1.39497628	2.759472191			
HWKRRHKHKWPKRHPHKGSC	1.915619411	2.63694364			
KIWAMRKPRYQYWNQPAGSC	1.407379051	2.859787278	√		
KWDHGQNGLFPPMHYIPGSC	1.544327953	3.07086271			
LEAHYKRSMHAQNWWEAGSC	1.406975222	2.595243258	√		
QYLWWQMLKIEWNSTYAGSC	6.223795435	2.613516832	√		
RHWYQDGSPLLAPVYKVGSC	1.480252182	3.07210387			
SYQRENESDEEEKNNEDGSC	1.638884707	2.375427636			
VEDNYGVTLRQPKYMGWGSC	1.406415063	2.363605066	√		
WNAMGKWKAMVDKTGDFGSC	2.101647607	2.402774023	√		
WNIHERHRFDQPYDYGHGSC	1.490400579	2.85033043			

Table 2. Williams et al. 2011

Table 3: Diagnostic Peptides of Altered Behavior in the Forced Swim Test

Peptides Sequence	Binding Ratios		In Common with Predictive Peptides of Lupus	In Common with Diagnostic Peptides of Lupus	In Common with Predictive Peptides of Depressive-Like Behavior
	Study 1 4M LPR High Floaters/ 4MC3H	Study 2 4M LPR High Floaters/ 4MC3H			
AGAFRERRYKPMMWLHVGSC	2.3585698	3.2438923			√
AGVRHKFHPYLMQFRRHGSC	2.4804174	2.4405015			
AQLGMYGVYRPVEIWPDGSC	3.2461162	2.5778561		√	√
DDTLYNAHKHLKWFGFIGSC	2.7148917	3.2115472		√	
EKFKRPRWPHLPFTHWDGSC	2.6595031	2.459389			
EPKLWFKPRRGGYRHRHGSC	2.8665683	3.0177305		√	
EPSLQVITEYNINFLTIGSC	2.3961576	3.031862			√
EQEDYDDDEEQEQDEDDGSC	2.3678429	2.3572539			√
ERNRRESDSKERKNYDHGSC	3.2522315	2.6460973			
FPGDRRSGRAFPEVRWRGSC	3.2688023	2.7553224		√	
GFHGPGMLGKTGRLSYGGSC	2.7249439	2.4930268			
GLVSRIPSVPKHDEWTFGSC	2.4475854	2.4896951		√	
GRVPQDFNTPSFDRVFWGSC	2.720075	2.8850922		√	
GWLKAMGPFPWGRLVQNGSC	2.9327554	3.1019225		√	√
IGQRLKGKDENIRFENFGSC	2.3991006	4.2322413		√	√
ILDRRETAWNEHFSKFRGSC	3.3735432	3.2197232		√	
IPDGWLKNVYRVRVPWPGSC	2.6438333	2.340745			
IRFVAILVFVIIILIARGSC	2.3412987	2.9533411			√
KLLMTDFMAKWPRNGWYGSC	3.7772183	2.7224506		√	√
KTHHSMWKGRITHELFAGSC	2.4523179	2.5201615			√
KVDYVNQWARRRIFMAPGSC	3.274227	3.3530846		√	
LAFAWKPDPWQSLVTKFGSC	3.4876927	2.7242745		√	
PMLFWKWHRQLNQQGRRGSC	3.1315924	2.4418162		√	√
PSAWEWIPRNQHLNKFRGSC	3.3805445	2.7599227		√	√
PYRFDWAALPLKKPMWRGSC	2.565745	3.1616486		√	
QKKPPDYRTWHHPFYNGGSC	3.0421354	3.0142199		√	
QKRWLQLPRNLMWRRETGSC	2.9189665	2.7744303		√	
QRVPIVKWLLWEPRALPGSC	2.9902886	2.4816832			
QSAYHNHRMKWRKIGIEGSC	2.4692871	3.2059303			√
QSHWFYDRTKDVYPGRHGSC	4.7885077	2.6374662		√	
RAAMHESLKNWRVYREWGSC	2.487184	2.9067771		√	√
SRQGLHYNLDGLKPIFPGSC	2.7656199	3.3371676		√	
SSELDFRKYSFYVHRPDGSC	2.7384221	2.5383546			
TLNKRRSWRDGFTADEYGSC	2.3085759	2.3886609			
VDARMETFYDMQYPYYLGSC	2.3683189	2.4565583			√
WKPIWHSFHKRRPQILNGSC	3.0463089	2.7121597		√	√
WRTKAAMKWQKYQREHRGSC	2.597044	2.6153741			
WSYKYKKKQAWDWPWDPGSC	2.9343494	2.7360999		√	
YYNVQQVDRWVKLQWGLGSC	2.6651713	2.6993872		√	

Table 3. Williams et al. 2011

Table 4: Possible Natural Protein Matches for Five Monoclonal BRAA

Monoclonal Antibody	Protein Name
D1	MLX-interacting protein
D1	Endothelin B receptor
D1	Gamma-aminobutyric acid receptor subunit rho-1
D1	CAS1 domain-containing protein 1
D1	Disintegrin and metalloproteinase domain-containing protein 1a
D1	Protein tweety homolog 3
G4	GRB2-associated-binding protein 2
G4	Diacylglycerol kinase epsilon
G4	Synaptotagmin-10
G4	Differentially expressed in FDCP 6
G4	Vesicular glutamate transporter 3
G4	Carbohydrate sulfotransferase 15
D9	Vacuolar protein sorting-associated protein 4B
D9	Transmembrane protein 164
D9	Cytohesin-1
D9	Galanin receptor type 3
D9	D(1B) dopamine receptor
D9	Fas apoptotic inhibitory molecule 2
F9	Serine/threonine-protein kinase TAO2
F9	Epidermal growth factor receptor
F9	Glutamate [NMDA] receptor subunit epsilon-3
F9	Nuclear pore complex protein Nup155
F9	Synaptojanin-2
F9	Astrotactin-1
G10	Autophagy-related protein 9A
G10	Leucine-rich repeat-containing protein 4C
G10	Matrix metalloproteinase-14
G10	Alpha-1,3-mannosyl-glycoprotein 4-beta-N-acetylglucosaminyltransferase A
G10	N-acetylgalactosaminyltransferase 7
G10	Tyrosine-protein kinase Fyn

Highlighted proteins are of interest.

Table 4. Williams et al. 2011

DISCUSSION

We are currently characterizing antibodies in three categories: 1) Diagnostic, 2) Predictive, and 3) Pathogenic. Diagnostic (auto)antibodies are simply those autoantibodies which can be reliably used for diagnosing a specific disease. Predictive (auto)antibodies are those which can predict the future onset of that disease long before it occurs. Finally, pathogenic (auto)antibodies are those which are responsible for pathogenic mechanisms in the disease causing symptomatology. There is probably a good overlap between these 3 categories, but they can also be different. Thus, diagnostic antibodies need not be predictive (although they can be), and predictive antibodies need not be diagnostic. Likewise, pathogenic antibodies need not be predictive, although they will almost certainly be diagnostic. The focus of this study was on the central nervous system manifestations of lupus.

Based on the above, our first goal in this (and our previous) study was to create a detection kit to diagnose and predict lupus and its CNS manifestations using a unique, high-throughput microarray technology. This technology has been used in other studies to determine binding patterns specific to that disease, and we expect that for each disease there will be a different binding pattern that will allow us to distinguish one illness from another (Boltz et al., 2009; Morales Betanzos et al., 2009; unpublished data). Therefore, based on the binding patterns of the autoantibodies present in lupus patients, we should be able to use this technology to distinguish lupus from other diseases. In our case, because of the different symptoms of lupus such as different CNS manifestations and the idea

that certain autoantibodies are partly responsible for each CNS manifestation, the binding pattern of these different autoantibodies, should also allow us to distinguish one CNS manifestation from another. Therefore, this technology may provide a more accurate means of diagnosing lupus. Also, as mentioned above, we are also interested in predicting lupus. Since we believe that autoantibodies are present in low amounts even before any manifestations are observed, by detecting these low levels of autoantibodies on the microarray chip, we may also be able to predict lupus and its CNS manifestations long before the onset of the disease. Being able to predict and diagnose lupus will not only help in providing proper treatments to patients, but also better care during their lifetime, as well as novel therapies in the future. In this study we identified predictive peptides. Our second goal for this study was to better identify (i.e., "validate") which peptides may indeed be diagnostic peptides from our first study. Our third goal was to develop a reliable means for detecting pathogenic antibodies.

We ran the same protocols in two different studies because the results of the first study identified possible diagnostic peptides of lupus and its CNS manifestations and we wanted to validate these peptides. Also, we used MRL/lpr at two different ages in the current study in order to identify predictive peptides of lupus and of altered behavior in the forced swim test. In this study we were able to validate diagnostic peptides and identified predictive peptides, and produced a way of identifying pathogenic antibodies. By comparing the results of our previous study to the current study we could validate which peptides were likely diagnostic and narrow this category even further. We measured anti-DNA

antibody levels in the mice in both studies, as a measure of disease activity. In both studies, our 4 month MRL/lpr mice had significantly greater anti-DNA autoantibodies compared to the control groups verifying that the MRL/lpr mice had disease activity. Disease activity in these mice have been detected in past research (Zameer and Hoffman, 2001; Zameer and Hoffman, 2004; Williams et al., 2010). After performing different analyses on the peptide data using set criteria described above, we were able to better narrow our list down to 58 diagnostic peptides of lupus. Better methods are needed to diagnose patients for lupus and this microarray technology should allow us to do this.

The 1.5 month MRL/lpr from our current study and the 4 month MRL/mp from study 1 did not display high levels of anti-DNA autoantibodies, indicating little or no disease activity; therefore we were able to use microarray data from these animals to determine predictive peptides of lupus since we know that in time both groups of animals will develop lupus. Comparison of the peptides in our data analyses revealed 18 peptides in common between both groups identifying these 18 peptides as being able to predict if an individual is lupus-prone.

Of more interest was trying to identify diagnostic and predictive peptides for specific neuropsychiatric manifestations. A certain percentage of lupus patients develop neuropsychiatric manifestations and therefore not only being able to predict and diagnose lupus is important, but also being able to predict and diagnose these specific neurologic and psychiatric manifestations (Lawrence et al., 2007). All the MRL/lpr mice eventually develop lupus, but even though they

will all develop lupus, some of the CNS manifestations are more severe in some versus others (Sakic et al., 2005). Some of the mice will display one CNS manifestation in the behavioral tests and not show any deficit in another (Sakic et al., 2005). Therefore, we took advantage of this diversity and use this performance to re-group our microarray results to determine predictive and diagnostic peptides specific to different CNS manifestations. We hypothesized that there are a subset of BRAA that are pathogenic and are at least partly responsible for some of the CNS manifestations that are observed (Hoffman et al., 1978; Narendran and Hoffman, 1989; Zameer and Hoffman, 2001; Sakic et al., 1993). Therefore we expect that our MRL/lpr mice at 1.5 months of age should have low levels of these BRAA and the 4 month MRL/lpr should have higher levels of BRAA. As expected, this is what was detected since our 4 month MRL/lpr in both studies had significantly greater levels of BRAA compared to the controls. By detecting antibodies that are specific to each of these CNS manifestations, we can predict and diagnose the different CNS manifestations.

Looking at anhedonia (making the assumption that this is what we are testing in our sucrose preference test) in our current study, the 4 month MRL/lpr consumed significantly less sucrose compared to the control groups, except for the 1.5 month MRL/lpr. This may mean that this manifestation is present even before any of the signs of lupus are detected (Sakic et al., 1997). We also found that in both studies the 4 month MRL/lpr floated significantly more in the forced swim test compared to the control groups. This altered behavior is a manifestation that had been used by other researchers as a measure of CNS

dysfunction in the MRL/lpr mice (Sakic et al., 2005; Williams et al., 2010), specifically as a measure of depressive-like behavior. As mentioned, some of the 4 month MRL/lpr did worse in this test compared to others and because of this, we split the 4 month MRL/lpr into two groups, high floaters (animals showing deficit) and low floaters (animals behaving similar to control groups). We found that after statistical analysis, the 4 month MRL/lpr that were high floaters, floated significantly more than the 4 month MRL/lpr that were low floaters and all the other control groups. Therefore because we knew which 4 month MRL/lpr were displaying the altered behavior we re-grouped the microarray results according to this test and analyzed the results to better identify diagnostic peptides of altered behavior in the forced swim test. After this type of analysis was done in studies 1 and 2, there were found to be 39 peptides, validating these 39 peptides as diagnostic of altered behavior relevant to the forced swim test (possibly depressive-like behavior) in CNS-SLE.

Furthermore, we are also interested in predicting altered behavior in the forced swim test and since we had the 1.5 month MRL/lpr in our current study and we knew which of these animals would display altered behavior at 4 months of age, we regrouped the microarray results for the 1.5 month MRL/lpr into a group of high floaters and low floaters. We then determined possible predictive peptides of this altered behavior and found 354 peptides. In order to further narrow down the true number of predictive peptides we would need to run another study and compare these peptides to the peptides that would be detected in that future study. We cannot use the MRL/mp as a control here since we do not have

data on these animals at a much later time (viz., 10 months of age) when this altered behavior would likely be observed.

Another major goal was to use the peptide sequences to see if we can determine possible matches to natural proteins, particularly for characterizing the brain antigens which might be mediating CNS manifestations. Using Guitope computer analysis program, we were able to determine possible matches to natural protein (the corresponding synthetic peptide, is given in parentheses). When looking at the diagnostic peptides of lupus natural protein matches include 40S ribosomal protein S10 (sp|P63325), 60S ribosomal protein L22-like 1 (sp|Q9D7S7), histone H3-like centromeric protein A (sp|O35216), follistatin-related protein 4 (sp|Q5STE3) and H-2 class II histocompatibility antigen, A-D alpha chain (sp|P04228). These results are very interesting since autoantibodies to these proteins have been detected in lupus patients (Sherer et al., 2004). Autoantibodies to the 60S ribosomal protein L12, which is important in protein synthesis, has also been detected in 3-28% of lupus patients (Sherer et al., 2004). Even though this protein is not the same as the 60s ribosomal subunit we detected, it is possible that autoantibodies to our 60S ribosomal protein L22-like 1 protein can be affecting protein synthesis or by some other mechanism affecting areas of the body. Autoantibodies to 40S ribosomal protein S10, which is also important in protein synthesis, have been detected in 11-40% of lupus patients (Sherer et al., 2004). The histone H3-like centromeric protein A, is like the H3 nucleosome which is important for packaging the DNA in the cell, and autoantibodies to this protein have been detected in 50-90% of lupus patients (Sherer et al., 2004; Su et

al., 2007). These anti-histones antibodies are thought to play a role in lupus nephritis, which is one of the manifestations of lupus (Sherer et al., 2004). The exact mechanism that is occurring and how these autoantibodies are altering body function is not known, but these results help to confirm that these proteins are being affecting during disease activity.

When looking at the identified predictive peptides of lupus proteins of interest include C1q tumor necrosis factor-related protein 6 (sp|Q6IR41), histone H3-like centromeric protein A (sp|O35216), alpha-actinin-2 (sp|Q9JI91) and 60S ribosomal protein L22 (sp|P67984). The collagen-like region of C1q protein is believed to play a role in lupus nephritis and the autoantibodies to this protein occurs in about 30-50% of lupus patients (Sherer et al., 2004). Anti-alpha-actinin-2 antibodies have been detected in patients with lupus nephritis (Croquefer et al., 2005). It was interesting that these researchers detected anti-alpha-actinin-2 antibodies even before lupus nephritis was present and anti-alpha-actinin-2 autoantibodies is a possible match to the peptides that we identified as predictors of lupus (Croquefer et al., 2005). Autoantibodies to histone H3-like centromeric protein A and 60S ribosomal protein L22 that were determined using the predictive peptides of lupus were in common with the diagnostic peptides of lupus, so these autoantibodies may be present early on as biomarkers and remain throughout the disease process.

Natural protein matches for the diagnostic peptides of altered behavior in the forced swim test included metabotropic glutamate receptor 4 (sp|Q68EF4), 60s ribosomal protein L22-like 1 (sp|Q9D7S7), calcium/calmodulin-dependent

protein kinase kinase 1 (sp|Q8VBY2) and 40S ribosomal protein S9 (sp|Q6ZWN5). The 60s and 40s ribosomal protein shows up again as a possibility, demonstrating that the proteins involved in protein synthesis are affected during lupus and CNS lupus. Calcium/calmodulin-dependent protein kinase kinase 1 is thought to play a role in synaptic plasticity and learning and memory and our peptides suggesting that this protein is a target of autoantibodies may indicate that this protein is being affected during this altered behavior (Kaitsuka et al., 2011). It is very interesting that the metabotropic glutamate receptor 4 is one of the matches since researchers found that using an agonist to this receptor helps to decrease the float time in the forced swim test, therefore this receptor may play a role as an anti-depressant (Palucha and Pilc, 2007). One can hypothesize that autoantibodies binding to this metabotropic glutamate receptor 4 may be preventing its anti-depressant properties and therefore allowing this CNS manifestation to develop.

Other researchers have found autoantibodies that seem to be responsible for different CNS manifestations like the NMDA receptor being involved in causing cognitive deficits (Kowal et al., 2006). Therefore, being able to determine proteins that can match specific CNS manifestations will help to provide better targets for specific manifestations. Of course, being able to distinguish one manifestation from another is the key and this is where our diagnostic kit becomes very important.

Using one MRL/lpr #2 we created five monoclonal BRAA. We ran the supernatants collected from these BRAA on Western blotting (which gave us the

molecular weight of the unknown) and on a microarray chip (to determine the possible identity of the target). To begin, for D9, we identified one of the targets as possibly being D (1B) dopamine receptor. This receptor is expressed in the limbic system and plays a role in neurotransmission, which is important for cell to cell communication and it helps to regulate the release of glutamate (Centonze et al., 2003; Mair and Kauer, 2007). Because this receptor is important for cell to cell communication, a dysregulation of this receptor would likely result in some neurological deficit, such as affecting memory (Mair and Kauer, 2007). Since learning and memory deficits are observed in lupus, this could mean that this receptor could be involved in altering the learning and memory process (Arabo et al., 2005). BRAA D1 was identified as possibly being the gamma-aminobutyric acid receptor subunit rho-1. GABA binds to this receptor and has an inhibitory effect on the brain, which may be important in the synaptic plasticity in the amygdala (Rodrigues and Schafe, 2004). Emotional dysfunction occurs in our lupus-prone mice, so it would be interesting to see the role that the GABA receptors are playing in lupus progression (Williams et al., 2010).

For G10 the BRAA was identified as possibly targeting leucine-rich repeat-containing protein 4C. This protein is also known as Netrin-G1 ligand (NGL-1) (Woo et al. 2009). NGL-1 may be associated with schizophrenia and since schizophreniform disorder is observed in lupus, this protein possibly being targeted may help to explain some of these neuropychiatric manifestations. G4 may be targeting GRB2-associated-binding protein 2. This protein may play a role in susceptibility to Alzheimer disease (Zhong et al., 2011). Exactly what role

this protein is playing in neuropsychiatric lupus is unknown, but it would be interesting to further investigate how this protein is being affected. Lastly, one of the possible targets of F9 could be synaptojanin-2, which is important in the secretion of vesicles in the synapse. If there is a decrease in this protein then there is a decrease in the secretion of the vesicles (Jospin et al., 2007). If these vesicles are not released into the synapse, then this would disrupt normal brain functioning. As mentioned, in Table 4, we also listed other possible identities of the targets of these BRAA. Future experiments must be run to determine which of the six possibilities for each BRAA is the correct target. Knowing the identity of these BRAA will help us to understand what proteins are affected in the brain (since it is believed that many of the brain targets are not yet identified) and why these different CNS manifestations occur. Eventually it would be necessary to inject these monoclonal BRAA and see if we can replicate these behavioral dysfunctions in control mice. This would help to confirm the role of these BRAA in CNS-SLE.

As mentioned, we are testing this technology in our lupus mouse model. The results here are only preliminary and have not been applied to human sera. In order to truly validate the results of our study, we would need to run multiple trials using human sera and see how well our proposed set of diagnostic or predictive peptides work in being diagnostic or predictive. Likewise, the proposed pathogenic autoantibodies will also have to be tested in future studies to confirm their role in lupus.

One very important asset of using our chip in predicting and diagnosing lupus and its CNS manifestations is that it is inexpensive since this chip is created to be used for any disease. There will be no need to develop a specialized chip just to detect lupus. Overall, this random peptide microarray analysis is able to identify both predictive and diagnostic markers of lupus and depressive-like behavior. This technology therefore looks promising as a detection assay for lupus and its neuropsychiatric manifestations.

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Chapter 5

NEUROKININ-1 RECEPTOR CHANGES IN CNS-SLE

ABSTRACT

We hypothesized that the neuropeptide substance P is involved in altering permeability of the blood-brain barrier (BBB), during lupus progression, and this would be reflected in increased levels of its receptor, neurokinin-1 receptor (NK-1R). This would allow immune components, such as brain-reactive autoantibodies (BRAA), or lymphocytes, to have easier access to the brain, contributing to the diverse neuropsychiatric manifestations. We measured the levels of the NK-1R in the brains of our lupus mouse model, the MRL/lpr mice, and our control groups and found that there was a significant increase in the expression of this receptor in the MRL/lpr in comparison to the controls. These 4-6 month old MRL/lpr also had significantly greater BRAA and anti-DNA autoantibody levels in comparison to the controls. When looking at behavioral performance, we found that they consumed significantly less sucrose in comparison to the controls, a possible indicator of anhedonia. These results suggest that increased NK-1R receptor levels may play a role in contributing to CNS dysfunction in SLE, perhaps through alteration of BBB function due to substance P binding.

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disorder that affects many organs/areas of the body including the liver, kidneys, skin, joints, spleen, central nervous system, etc (Sherer et al., 2004; Blatt and Glick, 1999; Williams et al., 2010). Butterfly rashes are known to appear on the skin and when the joints are affected, this leads to arthritis (Blatt and Glick, 1999; Ghosh, 2007). Women are the vast majority of patients that are affected by SLE. The reason for this is unknown, but hormones are believed to play a role in this occurrence since lower levels of testosterone have been detected in a male murine model of lupus (Sakic et al., 1998).

The etiology of lupus is currently unknown. Many different autoantibodies have been detected in lupus and it is these autoantibodies interacting with their targets antigens that are known to play a role in the manifestations of lupus (Colasanti et al., 2009; Williams et al., 2010). For example, an antigen-antibody complex that gets lodged in a filtering organ like the kidney leads to an immune reaction in that region, causing kidney damage known as glomerulonephritis (Colasanti et al., 2009).

As mentioned one of the areas that is affected during lupus is the central nervous system. This CNS involvement during lupus leads to many different neuropsychiatric manifestations in about 15% to 80% of patients (Borchers et al., 2005). Such manifestations can include cognitive impairment, psychoses, anxiety and depression (Mondal et al., 2008). We have hypothesized that some of these neuropsychiatric manifestations are due to the presence of brain-reactive

autoantibodies (BRAA) binding to proteins in the brain and interfering with the function of those proteins (Hoffman and Madsen, 1990; Hoffman et al., 1978, 1987; Narendran and Hoffman, 1989; Khin and Hoffman, 1993; Zameer and Hoffman, 2001, 2004; Williams et al., 2010). Specific BRAA have been detected by other researchers and have been shown to be involved in causing behavioral dysfunction. BRAA to dynamin I protein has been shown to cause behavioral dysfunction in the elevated plus maze test and BRAA to the NMDA receptor has been found to correlate with cognitive impairment in murine models of lupus (Kowal et al., 2004; Lawrence et al., 2007).

So far, research is demonstrating that these BRAA are playing a role in neuropsychiatric manifestations. Our goal in this study is, however, to understand how these BRAA are able to enter the brain in the first place in order to cause these dysfunctions. The brain has always been thought to be an immune privileged site, protected by the BBB (Shatz, 2009). Therefore in order for these BRAA to enter the brain, there has to be some alterations in this barrier. We know that these BRAA do enter the brain because we have detected them in the brains of our murine model of lupus (Zameer and Hoffman, 2001). Our prior research has also shown that there is increased BBB permeability during immune complex disease (Harbeck et al., 1979; Hoffman et al., 1983). The underlying mechanisms are, however, unknown.

Substance P is a neuropeptide that has many functions in the central nervous system including being a vasodilator (Annunziata et al., 2002). Researchers have found that substance P was released when stimulated by two

pro-inflammatory cytokines, TNF-α and IFN-γ. They also found that the release of substance P leads to an increase in the permeability of cultured rat brain endothelium. Pro-inflammatory cytokines are believed to play a role in the pathogenesis of lupus and CNS lupus (Mondal et al., 2008; Williams et al., 2010). This could mean that the increased levels of these pro-inflammatory cytokines are affecting the levels of substance P in lupus patients and thereby leading to an opening of the BBB.

Substance P binds to the neurokinin-1 receptor (NK-1R) in the brain (Annunziata et al., 2002). Since we hypothesized that increased activity of substance P is involved in altering permeability of the BBB, in this study we decided to measure the levels of the NK-1R. We infer that if increased activity of substance P is needed to open the BBB, then there should be an increase in binding of substance P to its receptor and therefore a change in the receptor levels. Our hypothesis is that increased levels of NK-1R plays a role in causing the neuropsychiatric manifestations of lupus due to increased binding by substance P, subsequently bringing about the opening of the BBB, allowing lymphocytes, autoantibodies and other molecules to enter the brain and alter brain function.

We decided to look at the expression levels of NK-1R in the brains of our murine model of lupus, the MRL/lpr mice. We chose to use this mouse because it has been a good model of human lupus and CNS involvement (Sakic et al., 1994b,1996b, 2005; Ballok et al., 2003; Williams et al., 2010). It has demonstrated altered behavior in different behavioral tests such as the forced

swim test (which has been used as a measure of depressive-like behavior) and the sucrose preference test (which is often used as an indicator of anhedonia). Both of these behaviors are consistent with the emotional disorders seen in human lupus. In our case, however, we are simply using these tests as an indication of altered behavior as compared to the controls. Our control group is the MRL/mp, because they are virtually genetically identical to the MRL/lpr but do not develop the manifestations until much later on. We included MRL/lpr from 4-6 months of age (when the symptoms of lupus are present) and the MRL/mp at 4 month of age (when they have no manifestations of lupus). Levels of anti-DNA autoantibodies and BRAA were assessed to determine if the MRL/lpr have lupus and CNS lupus, respectively. The mice were also subjects in a battery of behavioral tests to determine if they were displaying neurobehavioral manifestations. The level of NK-1R was also measured to determine if there was a difference between our MRL/lpr and MRL/mp. We do expect that the MRL/lpr mice with greater levels of NK-1R and the presence of high levels of BRAA to display neurobehavioral deficits.

Understanding the role of the NK-1R in CNS lupus is important, not only for better understanding the mechanisms underlying CNS-SLE, but because if we know that this receptor is involved in opening up of the BBB, resulting in diverse neuropsychiatric manifestations, we can target this receptor for therapy. This can then help prevent CNS manifestations from occurring in the lupus patient. Some researchers have found that when cultured rat brain endothelium cells were given spantide, a substance P antagonist, the permeability of the cells decreased

(Annunziata et al., 2002). In the same manner, NK-1R in the brains of CNS lupus patients could also be targeted to prevent further increase in BBB permeability.

MATERIALS AND METHODS

ANIMALS

The mice used in this study were five 4-6 month MRL/lpr and six 4 month MRL/mp. The MRL/lpr mice start to develop lupus after 2 months of age and at about 5-6 months of age they have 50% mortality. The MRL/mp mice are a congenic control for the MRL/lpr because they are virtually genetically identical to the MRL/lpr, but they do not develop the disease until after 7 months of age, with 50% mortality at about 16 months of age. Therefore we compared the MRL/lpr at 4-6 month of age to the MRL/mp at 4 month of age since this is after the MRL/lpr have lupus, but just before their high 50% mortality rate. The mice were purchased from Jackson Laboratory (Bar Harbor, ME). They were housed individually under standard laboratory conditions. Light cycle was from 6:00 A.M. to 6:00 P.M. Behavioral testing commenced at 8:00 P.M. The mice had food and water ad libitum. The mice used in this study were from two different cohorts. The two cohorts were subjected to a battery of behavior tests less than one year apart. The conditions under which these behavior tests occurred were similar for each cohort. The behavioral tests were performed at 8:00 P.M.

An intraperitoneal (IP) injection containing Nembutal sodium solution was used to sacrifice the mice. The brain and blood samples were collected from each mouse. The blood was allowed to clot, the tubes centrifuged for 10 minutes at 8500 rpm and the serum was removed. The serum was aliquoted into 100 ul samples and tested on the BRAA and anti-DNA ELISA. Integral membrane proteins were extracted from the brains that was collected and used in Western

blotting to detect the levels of NK-1R and B-Actin. Integral membrane proteins from a 2 month normal control C3H/HeJ mouse were used in the BRAA ELISA test.

The body weight and spleen weight of each animal was measured prior to being sacrificed. The spleen weights for 3 of the 5 MRL/lpr and all 6 of the MRL/mp were included in this study.

BATTERY OF BEHAVIOR TESTS

FORCED SWIM TEST

As mentioned, the forced swim test is a possible indicator of depressive-like behavior and since depression is one of the CNS manifestations in lupus, this test is important to investigate. It has also been used to test novel anti-depressants. This test has been previously described (Williams et al., 2010). To begin, each mouse was lowered into a swimming pool in the same location facing the same direction. The mouse was then allowed 10 minutes to swim around and the amount of time that they spend floating was measured. Floating is when there is no movement in the tail and hind limbs. Increased floating is an indicator of this altered behavior and therefore increased float time is expected in our 4-6 month MRL/lpr. The pool was kept at a temperature of 25^{o}C.

SUCROSE PREFERENCE TEST

The sucrose preference test, a possible indicator of anhedonia, has been previously described (Williams et al., 2010). Anhedonia is the inability to experience pleasure, or participate in an activity that is normally pleasurable. There were two phases in this test. There was a training phase, which is where the mice were made aware of the sucrose solution and a testing phase, which is where we looked at the difference in consumption between our MRL/lpr and the MRL/mp. In the training phase, the mice were given 3 ml of 4% sucrose solution for 24 hours for three days. Then there was a 24 hour rest period. In the next three days the mice were given 7 ml of the 4% sucrose solution for 1 hour and the amount of sucrose consumed was measured. The sucrose consumption across the three testing days was measured for each mouse and divided by their individual body weights (to take into account differences in body weight affecting consumption).

IMMUNOLOGICAL ASSESSMENT

INTEGRAL MEMBRANE PROTEIN EXTRACTION

The integral membrane proteins from each mouse were extracted individually as previously described (Narendran and Hoffman, 1988; Williams et al., 2010). The proteins were suspended in phosphate buffered saline (PBS) when used in the BRAA ELISA and in Tris-buffered saline (TBS) when used in the Western blot. We tested the concentration of the proteins used in the BRAA

ELISA using a BCA Assay Kit (Pierce, USA) and used the Bradford test (Sigma-Aldrich, USA) to test the concentrations of the proteins used in the Western blot.

ANTI-DNA ELISA

The protocol used to measure the levels of anti-DNA autoantibody levels in the mice sera has been previously described (Aotsuka et al., 1979; Crimando and Hoffman, 1995; Zameer and Hoffman, 2003; Williams et al., 2010). Briefly, 96 well plates were incubated with 50ug/ml of poly-L-lysine in PBS (Sigma-Aldrich, USA) for 1 hour. The plates were then washed three times using 0.1% PBS-Tween solution. This washing step was performed after each incubation phase (each of which was 1 hour long). The even wells of the plates were incubated with PBS and the odd wells were incubated with 10 ug/ml of calf-thymus DNA (Sigma-Aldrich, USA; purified for dsDNA) in PBS. 100 ug/ml of poly-l-glutamate (Sigma-Aldrich, USA) in PBS was added to the wells, followed by incubation with a 5% bovine serum albumin (BSA) (Sigma-Aldrich, USA) in PBS. Serial dilution of the sera was added to the wells followed by a 1/1000 dilution of the secondary antibody, goat anti-mouse IgG conjugated with horse-radish peroxidase (Caltag, USA) dissolved in PBS. Citrate buffer containing hydrogen peroxide and 2,2'-azino-bis(3ethylbenzthiazoline) sulfonic acid (Sigm-Aldrich, USA) was added to the wells and the plates were then incubated at 37^{o}C for 30 minutes. The optical density was read at 405 nm on the microplate reader. The optical density of the control wells was subtracted from the optical density of

the wells containing DNA and these (S) values were used as a measure of the levels of anti-DNA autoantibodies in the sera.

BRAA ELISA

The protocol used to measure BRAA levels in the mice sera has also been previously described (Aotsuka et al., 1979; Crimando and Hoffman, 1995; Zameer and Hoffman, 2003; Williams et al., 2010). The odd wells of the 96 well plates were incubated with the brain integral membrane proteins and the even wells received only PBS. The plates were then washed with 0.1% PBS-Tween, followed by incubation with 5% BSA in PBS. Serial dilution of the mouse sera was added to the wells followed by a 1/1000 dilution of the secondary antibody (Caltag, USA), goat anti-mouse IgG HRP conjugated. Hydrogen peroxide (100µl/100ml) and 2,2'-azino-bis(3-ethylbenzthiazoline) sulfonic acid (17mg/100ml) dissolved in citrate buffer was added to the wells for 30 minutes at 37^{o}C. The optical densities of the wells were measured on a microplate reader at 405 nm and the optical densities of the control wells were subtracted from the wells containing brain membrane antigens. These S-values were then used as a measure of the levels of BRAA in the sera samples.

WESTERN BLOTTING

The Western blotting protocol used to measure the levels of NK-1R and β-actin in the brains of the mice has been previously described (Narendran and Hoffman, 1989; Hoffman and Madsen, 1990; Williams et al., 2010). The gel

concentration used in the Western blot was a 12% resolving gel and a 4% stacking gel. The brain membrane protein from the each mouse was loaded into the wells of the gel and run for 1.5 hours at 102 volts. The gel was then transferred to a nitrocellulose paper (BioRad Laboratories, CA) and the membrane was then incubated with blocking solution. Mouse anti-β-Actin antibody (the loading control) was added to the membrane for one hour. The membrane was washed for ten minutes with 0.5% TBS-Tween and then visualized using a Chemiluminescence kit (Roche, USA). The intensity of the β-actin bands detected on the X-ray film (Kodak, New York) was measured. The blots were then stripped and re-probed with mouse anti-NK-1R antibody (Zymed, USA) at a 1/2000 dilution overnight. In order to strip the blots, they were incubated with a solution containing 1M Tris solution, mercaptoethanol, 10% sodium dodecyl sulfate solution and water for 10 minutes at 50^{o}C. The blots were then washed 5-6 times for 10 minutes each with TBS-Tween, before adding the mouse anti-NK-1R antibody. After the primary antibody incubation, the blots were washed three times for ten minutes each and the secondary antibody, goat anti-mouse IgG HRP conjugated, at 1/1000 dilution was added to the blots. The blots were then washed again three times for 10 minutes each and then visualized using the methods described above. The level of the NK-1R detected on the X-ray film was divided by the level of the β-actin. The intensity of proteins detected on the X-ray film was measured using the Image J program (http://rsb.info.nih.gov/ij/).

STATISTICS

The data collected for the immunological assessments, behavioral tests and NK-1R levels were analyzed for statistical significance using the PASW 18 statistical program (SPSS 18.0). Statistical significance was measured using a one-way analysis of variance (ANOVA). Figure 6 was plotted using the Microsoft Excel 2007 program.

RESULTS

LUPUS IMMUNOLOGICAL ASSESSMENT

When we measured the level of anti-DNA autoantibody in the sera of the mice (Fig. 1), the 4-6 month MRL/lpr were found to have significantly greater levels in comparison to the 4 month MRL/mp ($F=17.697$, $p < 0.002$). The MRL/lpr also had significantly greater spleen weights in comparison to the MRL/mp (Fig. 2) ($F=28.628$, $p < 0.001$). Since increased levels of anti-DNA autoantibodies and enlarged spleens are markers of disease activity in these lupus-prone mice (Williams et al., 2010), this therefore indicates that lupus had progressed in our MRL/lpr.

CNS-LUPUS IMMUNOLOGICAL ASSESSMENT

We have hypothesized that increased levels of BRAA detected during lupus progression is responsible for some of the neuropsychiatric manifestations observed in CNS lupus through interaction with brain proteins (Narendran and Hoffman, 1989; Zameer and Hoffman, 2001; Sakic et al., 1993). Therefore, greater levels of BRAA are expected in our MRL/lpr displaying neuropsychiatric manifestations in comparison to control mice (Williams et al., 2010). As expected, our 4-6 month MRL/lpr had significantly greater levels of BRAA in comparison to the 4 month MRL/mp ($F=16.276$, $p < 0.003$) (Fig. 3).

BEHAVIORAL DYSFUNCTION

The forced swim test and the sucrose preference test were used in this study to look at behavioral dysfunction in the MRL/lpr. Decreased consumption in the sucrose preference test is indicative of altered behavior (possibly anhedonia since reduced consumption is associated with anhedonia). Our 4-6 month MRL/lpr consumed significantly less sucrose in comparison to the 4 month MRL/mp ($F=8.382$, $p < 0.018$), displaying this CNS manifestation (Fig. 4). There was not a significant difference between the two groups of mice in the forced swim test (data not shown), although in past studies, we and others have found significant differences in this test between our 4 month MRL/lpr and the control groups (Maric et al., 2001; Williams et al., 2010).

NK-1R RECEPTOR LEVELS

Western blotting was used to detect the levels of NK-1R and β-actin (our loading control) in the brains of the mice (Fig. 5). The level of NK-1R was divided by the level of β-actin for each mouse since we want to ensure that the overall brain protein concentrations were similar. It was expected that there would be an increase in the levels of this receptor in the brains of our 4 month MRL/lpr as compared to the controls since we hypothesize that this receptor is involved in the opening of the BBB allowing the BRAA, or lymphocytes to enter. As hypothesized, we did find an overall significant increase in the levels of NK-1R in the brains of our 4 month MRL/lpr as compared to the 4 month MRL/mp

($F=9.376$, $p < 0.014$), demonstrating that there is an alteration in the levels of this receptor in the brains of mice with CNS lupus (Fig. 6).

SUMMARY OF RESULTS

We included a summary of the NK-1R levels, behavioral dysfunction and immunological assessments of lupus and CNS lupus for each of our five MRL/lpr mice (Table 1). For each variable, except in the case of the sucrose preference test, "High" is defined as greater than the level of significance set for that specific variable, which differs for each (please see Table 1 for the levels of significance for each variable). "Medium" is defined as in between the average for the MRL/mp and the level set for "High". "Low" is defined as less than the average of the MRL/mp. For the sucrose preference test, it is the opposite since we are looking for a decrease as oppose to an increase. So, "Low" is defined as consuming less than the level set for significance, "Medium" is defined as consuming between less than the average of the MRL/mp and the value set for "Low" and "High" is defined as having greater consumption than the MRL/mp. The reason for this is that it is important to look at each individual animal because even though the MRL/lpr mice are genetically identical there is behavioral heterogeneity within this group of mice (Sakic et al., 2005).

All the MRL/lpr developed lupus, as can be seen by the medium to high levels of anti-DNA autoantibodies detected in their sera. For the 3 MRL/lpr that we had spleen weight measurements, high spleen weights was detected in all three, again demonstrating the development of lupus. Also, 4 out of the 5

MRL/lpr had high levels of BRAA and only one had medium levels, therefore CNS dysfunction is possible in all these mice if the BRAA detected were pathogenic. In regards to the levels of NK-1R receptor, 4 out of the 5 MRL/lpr had high levels of NK-1R and only one had medium levels of NK-1R, therefore BBB damage is possible in all these mice if our hypothesis is correct. Since increased levels of NK-1R was detected in all the MRL/lpr and they all had high BRAA levels that can enter the brain and bind to brain proteins, we did expect some kind of CNS dysfunction in these mice. The first MRL/lpr #1 did not display altered behavior in the forced swim test due to having low float time, but showed some dysfunction in the sucrose-preference test, by consuming less sucrose than the average MRL/mp. It is important to note that this mouse could have other CNS manifestations that were not measured in this study. MRL/lpr #2 and #3 displayed CNS manifestations in both the sucrose preference test and the forced swim test. MRL/lpr #4 did not display dysfunction in the forced swim test, but did show CNS manifestation in the sucrose preference test. MRL/lpr #5 showed some dysfunction in the forced swim test by having float times that was greater than the average MRL/mp, but not greater than the level set for significance, however there was definitely dysfunction detected in the sucrose preference test for this animal. Overall, behavioral dysfunction was detected in all the 4-6 month MRL/lpr in at least one of the behavioral tests which corresponded to the increased levels of NK-1R detected in all of the 4-6 month MRL/lpr.

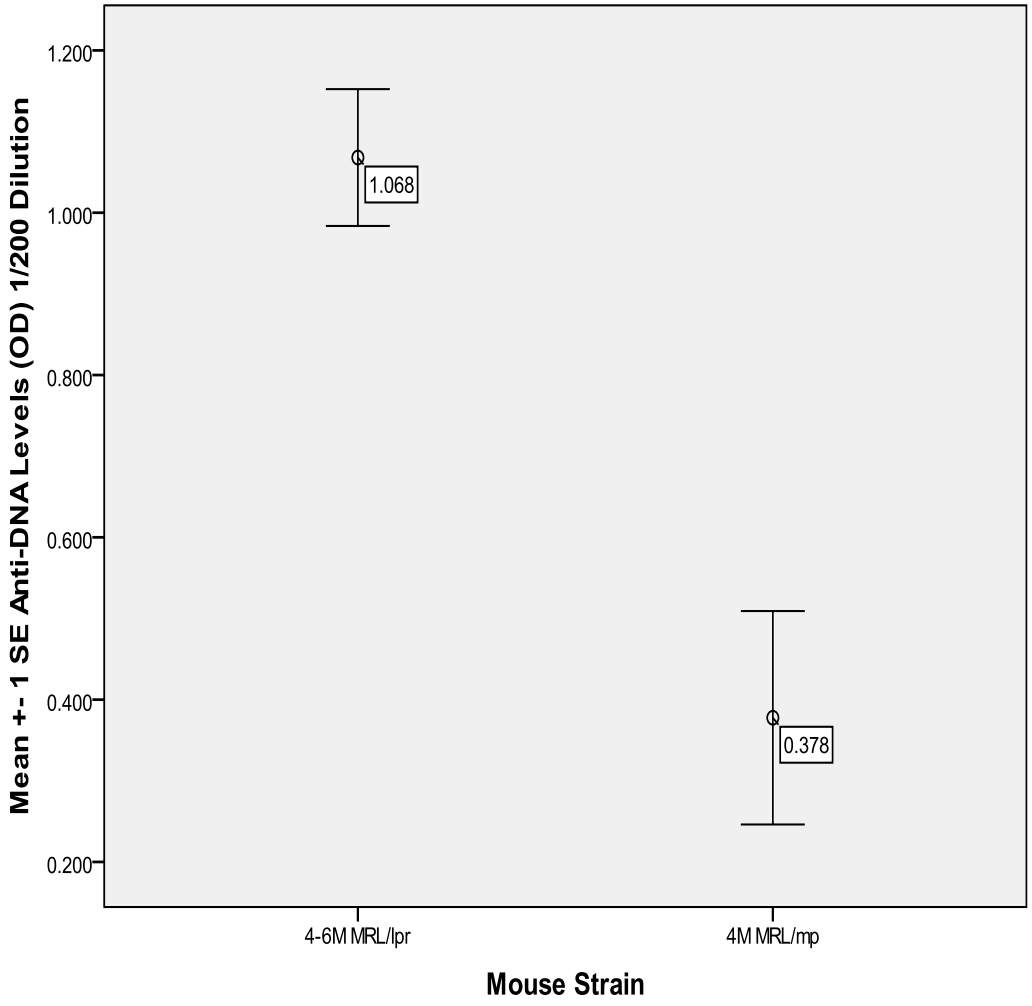

Fig. 1. Immunological Assessment – Anti-DNA Autoantibody Levels. Disease activity in the mice was determined by measuring the serum Anti-DNA autoantibody levels. The 4-6 month MRL/lpr had significantly greater levels of anti-DNA autoantibodies in comparison to the 4 month MRL/mp controls ($F=17.697$, $p < 0.002$). Anti-DNA autoantibody levels was measured at a 1/200 dilution.

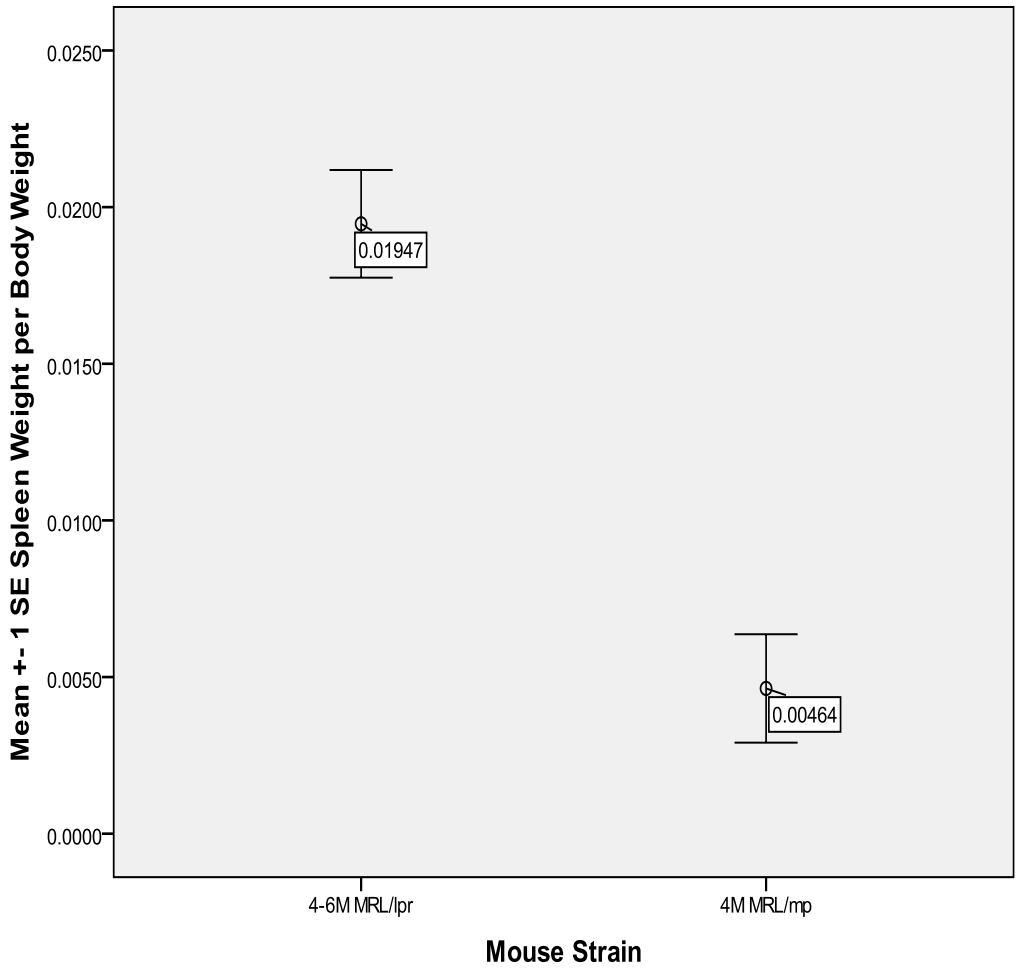

Fig. 2. Immunological Assessment – Spleen Weight. Disease activity in the mice was determined by also measuring the spleen weights. The 4-6 month MRL/lpr had significantly greater spleen weights per body weights in comparison to the 4 month MRL/mp controls ($F=28.628$, $p < 0.001$). We had spleen weights for only 3 of the 5 MRL/lpr, but for all 6 MRL/mp. However, there was still a significant difference between the two groups.

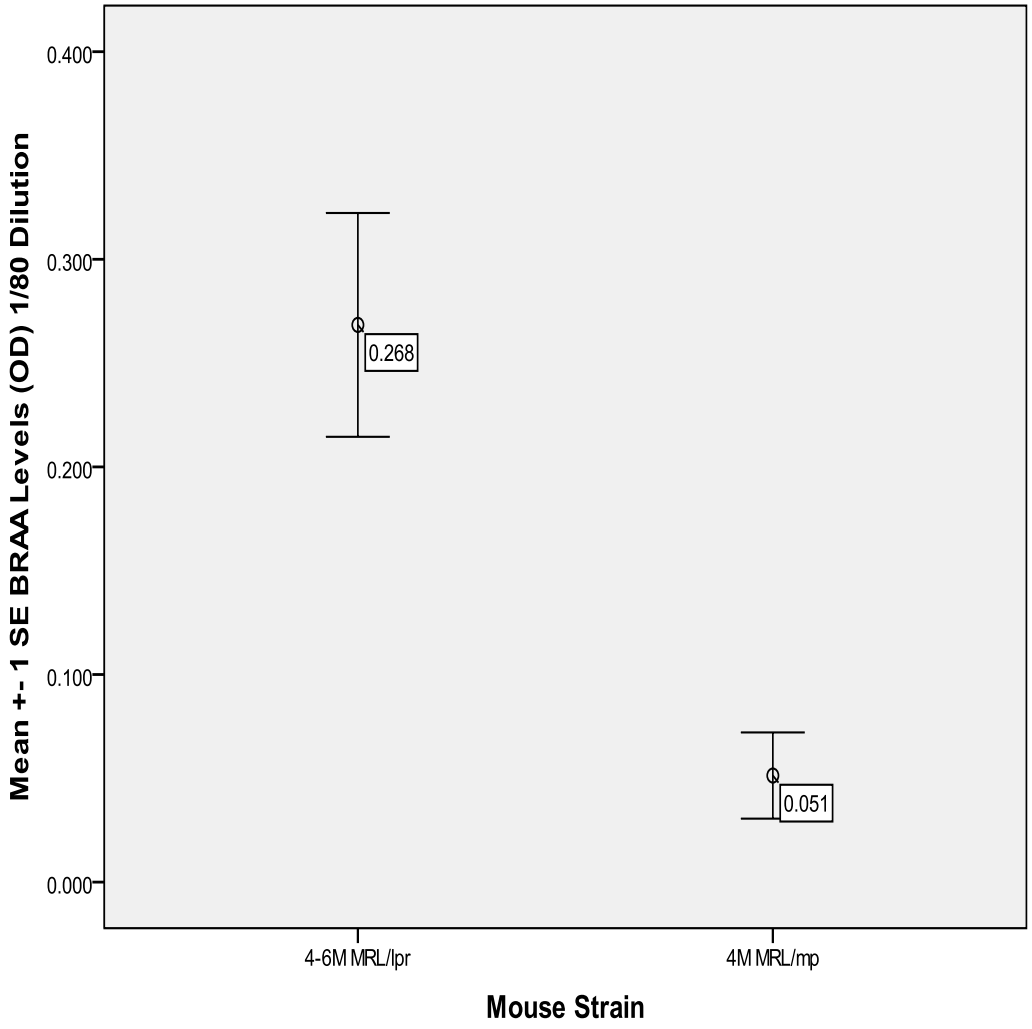

Fig. 3. Immunological Assessment – BRAA Levels. Significantly greater levels of BRAA levels were detected in the serum of the 4-6 month MRL/lpr in comparison to the 4 month MRL/mp ($F=16.276$, $p < 0.003$). Serum BRAA levels was measured at a 1/80 dilution.

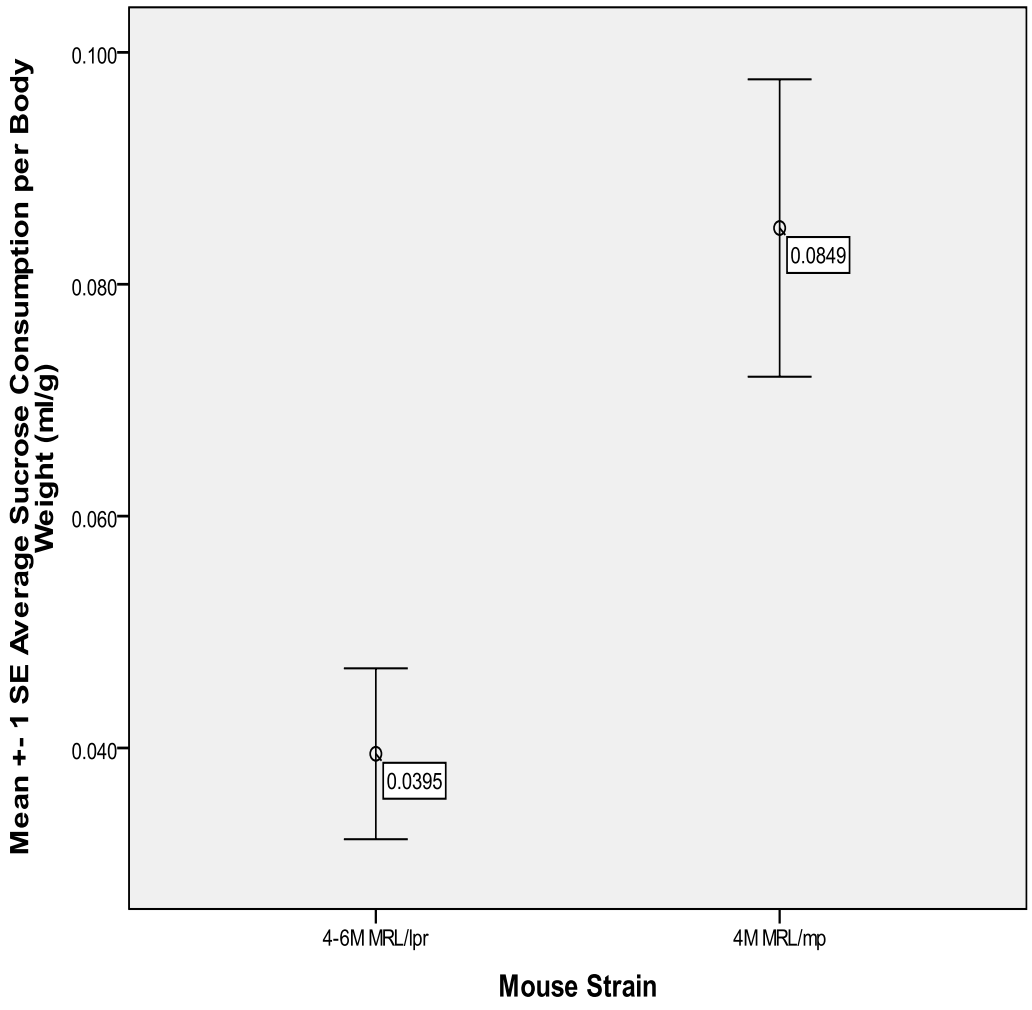

Fig. 4. Behavioral Dysfunction – Sucrose Preference Test. When looking at behavioral dysfunction using the sucrose preference test, the 4-6 month MRL/lpr were found to consume significantly less sucrose in comparison to 4 month MRL/mp, indicating anhedonia ($F=8.382$, $p < 0.018$).

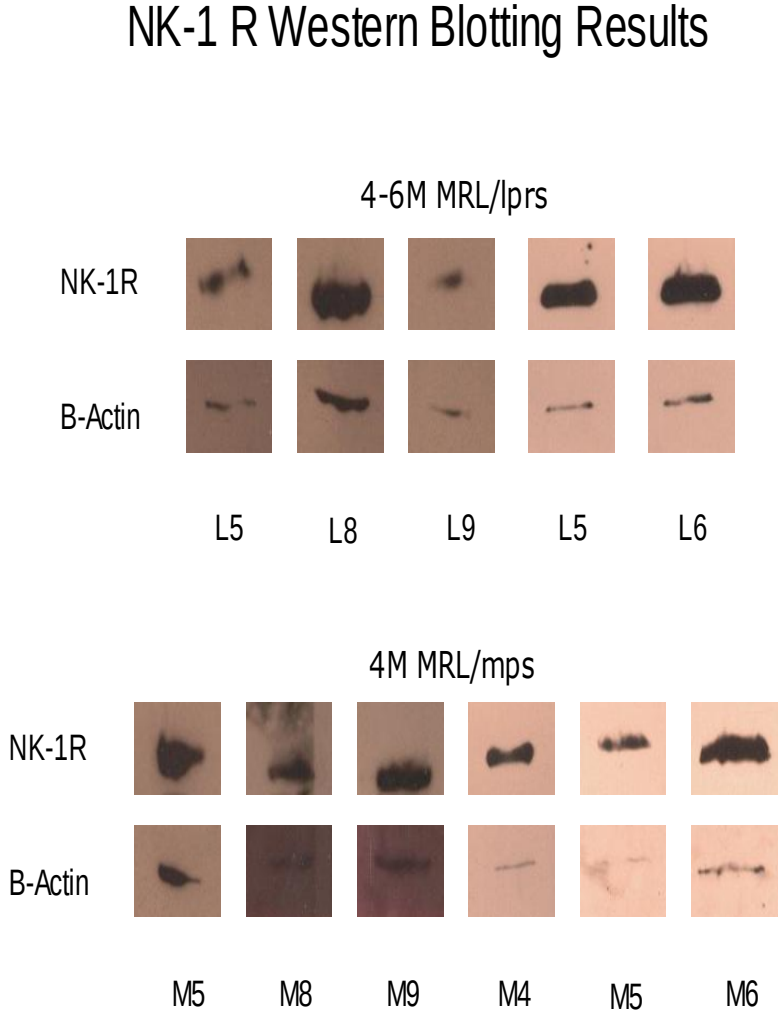

Fig. 5. NK-1R Western Blot. Western blotting results showing the level of neurokinin-1 receptor and β-actin in the brains of the five 4-6 month MRL/lpr and six 4 month MRL/mp.

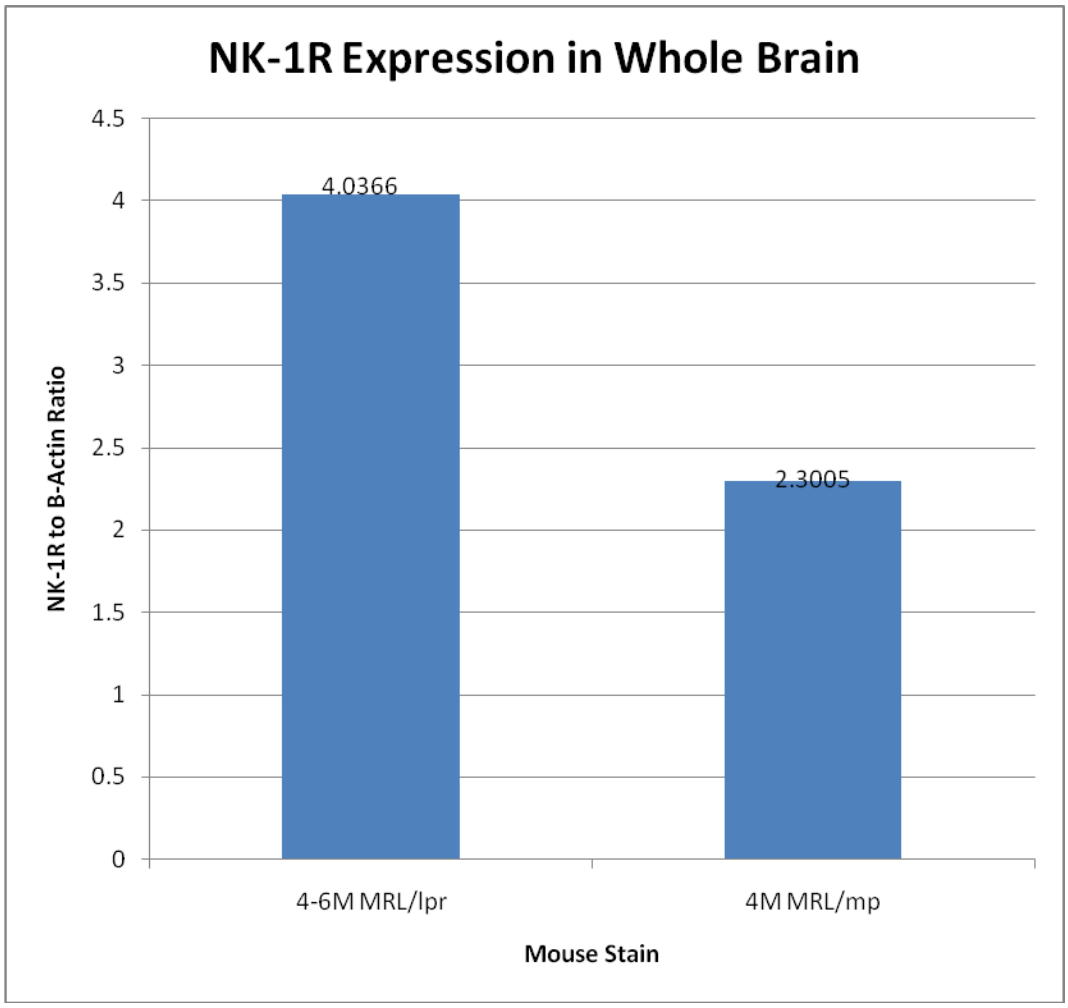

Fig. 6. NK-1R Levels Analysis. Graph showing that the 4-6 month MRL/lpr have significantly greater levels of NK-1R in comparison to the 4 month MRL/mp ($F=9.376$, $p < 0.014$). The level of NK-1R was divided by the level of β-actin detected in the brain of each mouse.

Table 1. Summary of Immunological Assessments, Behavioral Dysfunction, NK-1R Levels in the Five MRL/lpr Mice

Mouse Number	NK-1R to Beta-Actin Ratio Analysis	Float Time Analysis	Average Sucrose Consumption per Body Weight Analysis	BRAA Levels (1/80) Analysis	Anti-DNA Levels (1/200) Analysis	Spleen Weights per Body Weights Analysis
MRL/lpr #1	High	Low	Medium	High	Medium	High
MRL/lpr #2	High	High	Low	High	High	High
MRL/lpr #3	Medium	High	Low	High	Medium	High
MRL/lpr #4	High	Low	Low	Medium	High	
MRL/lpr #5	High	Medium	Low	High	High	
	High = Greater than Mean + 1.25SD of MRL/mp	High = Greater than Mean + 2 SD of MRL/mp	Low = Less than Mean - 1SD of MRL/mp	High = Greater than Mean + 2SD of MRL/mp	High = Greater than Mean + 2SD of MRL/mp	High = Greater than Mean + 2SD
	Medium = Between Mean of MRL/mp and Mean + 1.25SD of MRL/mp	Medium = Between Mean of MRL/mp and Mean + 2SD of MRL/mp	Medium = Between Mean of MRL/mp and Mean - 1SD of MRL/mp	Medium = Between Mean of MRL/mp and Mean + 2SD of MRL/mp	Medium = Between Mean of MRL/mp and Mean + 2SD of MRL/mp	Medium = Between Mean and Mean + 2SD
	Low = Less than Mean of MRL/mp	Low = Less than Mean of MRL/mp	High = Greater than Mean of MRL/mp	Low = Less than Mean of MRL/mp	Low = Less than Mean of MRL/mp	Low = Less than Mean

DISCUSSION

Substance P has been shown to play a role in increasing the permeability of rat brain endothelium cell cultures when stimulated by pro-inflammatory cytokines (Annunziata et al., 2002). In our lupus mouse model, the MRL/lpr mice, increasing levels of BRAA have been detected in their brain, indicating that somehow the BBB is being altered to allow these BRAA, or the corresponding B cells, to enter (Zameer and Hoffman, 2001). We hypothesized that substance P plays a role in altering this barrier and therefore we measured the levels of the NK-1R that substance P binds to in the brain.

To review our findings, when we measured the levels of anti-DNA autoantibodies in the sera of our mice we found that our 4-6 month MRL/lpr had significantly greater levels of anti-DNA autoantibodies in comparison to the MRL/mp. We also found increased spleen weights in all three of the MRL/lpr that we had measured in comparison to the controls. This therefore demonstrates that the MRL/lpr had lupus. Next, we measured the levels of BRAA autoantibodies in the sera, because this is an immunological assessment of CNS lupus since we believe that some of these BRAA play a role in causing the neuropsychiatric manifestations of lupus (Hoffman and Madsen, 1990; Narendran and Hoffman, 1989; Khin and Hoffman, 1993; Zameer and Hoffman, 2001; Zameer and Hoffman, 2004; Williams et al., 2010). The 4-6 month MRL/lpr had significantly greater levels of BRAA in comparison to the MRL/mp.

In order to observe CNS dysfunction, we looked at the forced swim test that looks at altered behavior (which is suggestive of depressive-like behavior

since floating in this test is used to test anti-depressants) and the sucrose preference test (for which decreased consumption is suggestive of anhedonia). The MRL/lpr consumed significantly less sucrose in comparison to the MRL/mp indicating that the MRL/lpr displaying altered emotions. For the forced swim test, 3 out of the 5 MRL/lpr display "medium" to "high" float times, but there was not a statistically significant difference between the two groups. This may be because the other two MRL/lpr, had very low float times (lower than the average for the MRL/mp) and the one MRL/lpr that had medium float time had a value that was more than three times less than the average of the two MRL/lpr with "high" float times. Therefore the overall level of significance was not present because 3 of the 5 MRL/lpr had float times similar to or lower than the controls. However as mentioned, this test in the past has revealed significant behavioral dysfunction in the MRL/lpr (Maric et al., 2001; Williams et al., 2010).

So far, all our MRL/lpr mice have high levels of BRAA and did display CNS dysfunction in one or both of the behavioral tests. So there seems to be an association between the presence of these BRAA and the observed CNS dysfunction. This association is, however, better discussed in one of our previous studies (Williams et al., 2010). Next, we are trying to understand how these BRAA would enter the brain to cause these neuropsychiatric manifestations and so the level of NK-1R was measured. It has been shown that opening the BBB was necessary for observing behavioral manifestations (Kowal et al., 2004). This seems to suggest that increased BBB permeability is needed for CNS manifestations. Our results showed that the 4-6 month MRL/lpr mice had

significantly greater levels of NK-1R in their brains as compared to the 4 month MRL/mp. These results show that NK-1R levels are altered in our lupus mouse model. Since we hypothesized that increased levels of NK-1R are allowing for more substance P to bind and bring about the opening of the BBB to allow the BRAA or lymphocytes to enter resulting in CNS dysfunction, this detected alteration in the levels of NK-1R is the first step to showing that the SP system is being affected in lupus. More research is necessary to determine exactly how this system is affected in CNS lupus and it is also essential to demonstrate that NK-1R is indeed involved in alteration of the BBB, however our initial finding does allow us to suggest NK-1R as one possible target affecting the BBB.

During immune complex disease (which occurs during lupus), it has been found that there is a change in the permeability of the blood-CSF barrier (Hoffman et al., 1983). Immune complexes have been detected in the choroid plexus of these animals with immune complex disease, suggesting a role for these deposits in causing some of the CNS manifestations of lupus (Harbeck et al., 1979). It has also been suggested that the deposition of these immune complexes in the choroid plexus is partly responsible for changing the permeability of the BBB, but are not the sole factor responsible (Hoffman and Harbeck, 1989). It may be that the immune complexes are mediating their effects on the BBB via substance P and its NK-1 receptor. It is also possible that the inflammatory response associated with immune complex deposition could be stimulating the release of substance P, through the release of pro-inflammatory cytokines.

As mentioned increased pro-inflammatory cytokine levels have been shown to increase the permeability of rat brain endothelium cells by stimulating an increase in substance P levels (Annunziata et al., 2002). These researchers also found that a substance P antagonist also significantly reduced the expression of ICAM-1, an adhesion molecule that helps with the trafficking of leukocytes to their location. These results show that substance P plays a role in up-regulating the expression of ICAM-1. We have also found an increase in the expression of ICAM-1 and VCAM-1 in the brains of our MRL/lpr (Zameer and Hoffman, 2003). Together, these suggest that substance P could be responsible for the increase in ICAM-1 expression found in the brains of MRL/lpr and along with opening up of the BBB, substance P may help to attract leukocytes to enter the brain, through increasing ICAM-1 expression. Our past research also revealed an increase in the B and T cells in the brains of the autoimmune mice indicating that these cells do enter the brain (Zameer and Hoffman, 2004). It should be mentioned that the BRAA are hypothesized to enter the brain directly or the lymphocytes are thought to enter the brain and produce the BRAA in situ and therefore finding increased levels of ICAM-1 supports this idea.

The substance P antagonist was also found to block the up-regulation of MHC class I, so this indicates that substance P also plays a role in the increased expression of MHC class I (Annunziata et al., 2002). We are currently testing the levels of MHC class I in the brains of our autoimmune mice and we have preliminary data showing an increase in this receptor level in comparison to our controls. Adding all our data and other publish research together we hypothesize

that pro-inflammatory cytokines are increasing the levels of substance P in lupus mice, which then binds to increased levels of the NK-1R, increasing the permeability of the BBB and at the same time helping to increase ICAM-1 levels attracting leukocytes to the barrier, thereby allowing the BRAA producing-cells to enter or even the BRAA themselves (Annunziata et al., 2002; Zameer and Hoffman, 2001; Zameer and Hoffman, 2003). All this then helps to result in some of the behavioral dysfunction that we have seen in past studies (Sakic et al., 1994a, 1996a, 1998, 2005; Williams et al., 2010).

Substance P's involvement in increasing MHC class I levels also adds an interesting piece to the puzzle. Datwani and colleagues (2009) found that mice lacking some MHC class I genes had increased synaptic plasticity (Datwani et al., 2009). Shatz proposed a hypothesis stating that MHC class I binding to its receptor PirB in the brain, is important in regulating plasticity (Shatz, 2009). This could suggest that if a decrease or lack of MHC class I increases synaptic plasticity, then too much MHC class I would decrease synaptic plasticity and learning and memory. Going back to our MRL/lpr, the increased NK-1R levels, suggesting increased substance P levels, could be causing an increase in MHC class I in our mice, which in turn could alter their ability to learn. This could help to explain the cognitive impairments that are observed in our mice, as well as in the human condition (Shucard et al., 2007). Shatz also suggested that MHC class I is located next to glutamate receptors and therefore can regulate the trafficking of these receptors (Shatz, 2009). We are also looking at the expression levels of

glutamate receptor in our 4 month MRL/lpr and have preliminary data that these levels are also altered.

Our current results show that the NK-1R which substance P binds to is altered in our lupus mouse model. Due to other research demonstrating the involvement of substance P in altering membrane permeability, we hypothesized that substance P is binding to the increased NK-1R and then altering BBB functioning, allowing BRAA to be present in the brain and change the function of their target proteins. As discussed, other data suggests that substance P may also be involved in causing CNS manifestations through alteration of expression of other molecules such as MHC class I. It is important to note that the opening up of the BBB and the presence of BRAA during lupus progression are two different processes that together may be responsible for some of the neuropsychiatric manifestations seen in lupus. Future research is necessary to establish NK-1R's role in alterating the BBB, but our current research demonstrates that this receptor's level is altered in our lupus prone mouse and therefore provides a possible target when investigating how changes occur at the BBB in CNS lupus.

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Chapter 6

CONCLUSION

Understanding the role of BRAA in CNS lupus has been the main goal of our research. We hypothesize that BRAA bind to integral membrane proteins in the brains of patients with CNS lupus and interfere with the function of those proteins, resulting in different CNS manifestations (Hoffman and Madsen, 1990; Narendran and Hoffman, 1989; Khin and Hoffman, 1993; Zameer and Hoffman, 2001, 2004; Williams et al., 2010). One of our objectives is to identify the brain proteins being bound by these BRAA. This will help us to understand why different CNS manifestations occur. If a specific protein is targeted, for example, in one of the limbic system structures, then this may account for emotional dysfunction in CNS-SLE. Furthermore, we could then target these proteins for therapy. For example, we could provide an inhibitor protein that would bind to these receptors preventing the receptors from being activated by the BRAA and thereby preventing the CNS manifestation. An alternative would be to create B cell vaccinations to eliminate the specific B cells producing the pathogenic BRAA. This is significant since current therapies are far from being specific in preventing disease or targeting specific CNS manifestations. They suppress the immune system in general, leading to complications like increased infections, or incidence of cancer (Yildirim-Toruner and Diamond, 2011). Therefore the identification of brain targets for the BRAA is an important goal.

Researchers have identified some of the BRAA targets, as being the NMDA receptor, synapsin I and dynamin-1 protein (Kowal et al., 2004; Lawrence

et al., 2007). There are, however, possibly thousands of autoantibodies in lupus. We have detected a large diversity of BRAA in the past and therefore our goal was to identify more of their targets using different techniques (Narendran and Hoffman, 1989; Williams et al., 2010). It seems that only a subset of BRAA can cause CNS dysfunction (called pathogenic BRAA) since BRAA have been detected in non-autoimmune mice and humans (called non-pathogenic BRAA) without CNS manifestations (Williams et al., 2010).

To summarize, our first study showed that there is good evidence for the BRAA hypothesis. The MRL/lpr mouse we used has been used by other researchers and has proven to be an excellent model to study lupus and its CNS manifestations (Sakic et al., 1994, 1996, 2005; Ballok et al., 2003; Williams et al., 2010). A few years ago, we started to notice the MRL/lpr were not displaying the same level of sickness as previously (which Jackson Laboratory confirmed). So in our first study, a comparative study over one year, we showed that there was a decrease in the autoimmune phenotype of the MRL/lpr and fewer correlations between immunological variables and behavioral deficits (Williams et al., 2010). This demonstrated reduced sickness in the MRL/lpr mice. Even with the reduced autoimmune phenotype as long as these MRL/lpr displayed elevated BRAA levels we expected behavioral deficits should still be observed. This was indeed the case, plus behavioral deficits in the forced swim test correlated with elevated BRAA levels. This helped to support the BRAA hypothesis since the detected BRAA may be responsible for this altered behavior. Although correlation does not imply causation, it is a necessary component of the causal link.

We also used the Western blotting technique to identify some of the molecular weights of potential BRAA targets. All the MRL/lpr that displayed high float times had binding on Western blotting, confirming autoantibody reactivity to brain and showing the potential molecular weights of the BRAA targets. Using immunohistochemistry, we also showed binding in the hippocampus and cortex, as well as other areas of the brain using the MRL/lpr sera. The binding helps to suggest what structures in the brain are affected. What is interesting is that behaviors associated with emotional dysfunction were observed in these animals and the hippocampus was bound, which may explain the emotional dysfunction. When detecting the BRAA we chose to use different techniques and each technique had varying sensitivity. By using all these techniques we were able to learn more about potential BRAA targets. One unique attribute of our study was that when we measured the levels of BRAA, we used integral membrane proteins from control mouse brain as our antigen, so the autoantibodies were reacting to natural brain proteins unlike cultured Neuro-2A cells used in another study (Sakic et al., 1993).

Many researchers have provided support for the autoantibody hypothesis including in human studies. For example, Tin and colleagues (2005) found BRAA that correlated with the presence of psychosis and/or seizures (some of the neuropsychiatric manifestations observed in lupus patients). Sakic and colleagues (1993) found that mice that had high levels of BRAA displayed behavioral abnormalities. Our first study further confirmed the BRAA hypothesis in a unique way, since the MRL/lpr displayed altered behavior which correlated with

BRAA levels (while the controls did not). There is much support for the BRAA hypothesis; however, data has yet to be shown which establishes a strong causal relationship between BRAA and CNS manifestations.

The current method of diagnosing lupus is not accurate since the manifestations of lupus are similar to other diseases. Physicians use a set of 11 criteria to diagnose lupus. In order for the patient to be diagnosed as having lupus they must have satisfied 4 out of the 11 criteria (Liu and Ahearn, 2009). Levels of anti-dsDNA antibodies and anti-nuclear antibodies are used as criteria in the diagnosis, but again these methods are not reliable. We were therefore interested to see if we can provide a more accurate means of diagnosing lupus.

Our second study, used peptide microarray technology to see if we can use the binding pattern on a microarray chip to accurately diagnose lupus and specific CNS manifestations. Results revealed that we were able to determine peptides that were likely diagnostic of lupus and two CNS manifestations (altered behavior in the forced swim test and sucrose preference test). To our knowledge, no one else has created a random peptide microarray chip that can be used to diagnose lupus and specific CNS manifestations in the MRL/lpr mice. In order to verify that some of these peptides were diagnostic and to identify predictive peptides of lupus and CNS-lupus, we ran another study using the same conditions as in the previous study, except with a larger number of animals and at different ages. A comparison of both studies allowed us to narrow down the diagnostic peptides of both lupus and its CNS manifestations (specifically, deficits in the forced swim test). Furthermore, we were able to identify predictive peptides of lupus, as well

as provide a preliminary list of predictive peptides of deficits in the forced swim test. It is important to note here that this test is so specific that we were able to diagnose each specific CNS manifestation. What is also of high interest to us was that this technology seemed to provide enough sensitivity that we could predict if the mice were going to get lupus and also display deficits in the forced swim test long before any signs. These diagnostic and predictive peptides will have to be further refined and validated. If we can make such predictions early on in humans, this can allow the individual to get treatments early on, which may prevent or lessen the severity of the disease. To our knowledge, no such accurate test exists to predict or diagnose lupus or its CNS manifestations and therefore this technology, will be very important in helping individuals that have lupus or are lupus-prone (Yildirim-Toruner and Diamond, 2011). This technology should be able to be extended to predict neuropsychiatric manifestations of many immunologically mediated disorders, not just lupus.

Other researchers have applied microarray technology to lupus, but not peptide microarray technology (Rus et al., 2002). These researchers used cDNA microarrays to look at the changes in gene expression in lupus patients versus controls and did find genes with greater expression levels in lupus patients versus the controls. In contrast, our microarray chips used peptides as opposed to DNA, because an increase in gene expression does not always indicate an increase in protein expression, it may be more accurate to look at the changes in peptide expression levels. This type of microarray technology has been used to identify predictive and diagnostic peptides of another autoimmune disease, diabetes

(Quintana et al., 2004). After analysis, they were able to find a set of antigens on the chip that was specific to pre-diabetes development and post-diabetes development. Therefore if such a test provides results that can predict and diagnose diabetes, this provides support to the idea that it can be applied to lupus. Other researchers have also used the same chip that we are currently using to study influenza and other diseases (Boltz et al., 2009; Morales Betanzos et al., 2009; unpublished data). Due to its applicability to multiple diseases, this means that the cost of using such a chip will be cheaper since we do not need a special chip for lupus.

Since we were interested in identifying the targets of the BRAA and we knew the sequence of the peptides on the chip, we used a computer program to see if there are potential matches to natural proteins for each set of the peptides (sets include diagnostic and predictive peptides of lupus and its CNS manifestations). We found interesting proteins matches for each set and these results are important since we may be identifying biomarkers and potential targets for therapy based on the disease manifestation. One of the mice from our last study was used to create five monoclonal BRAA. Even though we are not 100% sure as to the identity of the targets of these BRAA, we used the same computer analysis program. This again becomes important in the overall goal of understanding the mechanisms of CNS lupus and for developing better future therapies.

Using the computer analysis program and the microarray results, we were able to suggest the identities of the targets of the five created monoclonal BRAA.

However, it would be very helpful to actually identify the BRAA targets. One possible means of identifying these targets is using affinity chromatography. The monoclonal BRAA would be immobilized and used to isolate its corresponding antigen from a prepared mouse brain homogenate. We would then identify the antigen using mass spectrometry. It would be interesting if the identified target is one of the possible natural protein matches identified using the computer analysis program for the five monoclonal BRAA. This would be a confirmation of the utility of the microarray analysis and our computer program in identifying brain targets of any antibody of interest. Next, to determine if the BRAA is pathogenic, we would take each of our monoclonal BRAA and inject them into the low-autoimmune MRL/mp at an age prior to disease development, or the non-autoimmune C3H/HeJ mice. If behavioral deficits are produced similar to what was observed in the original MRL/lpr that was used to generate the monoclonal BRAA, then this would be strong evidence that this is a pathogenic BRAA. Since we would know the identity of the target of this BRAA, which corresponds with the CNS manifestations, then targeting this protein could be used to develop specific therapies. Our means of using the computer and microarray analysis to identify the targets of our monoclonal BRAA was just one step in this overall goal.

Of course, the BRAA hypothesis is not the only hypothesis, other than vascular damage, that is proposed as an explanation for why we observe different CNS manifestations in lupus. Other researchers have proposed that cytokines play a role since increased expression of three cytokines, TNF-α, IL-1β and IL-6,

have been associated with increased abnormalities in sucrose consumption for the MRL/lpr mice in comparison to control groups (Ballok et al., 2003). Another study also found increased expression of IL-1β in the MRL/lpr with lupus nephritis in comparison to the controls (Boswell et al., 1988). Increased expression of adhesion molecules have also been detected in brains of our murine models of lupus, which suggest that they play a role in causing these CNS manifestations (Zameer and Hoffman, 2003). ICAM-1 and VCAM-1 are two adhesion molecules that are important in allowing leukocytes to gain entry to tissues through binding with their receptors. Using immunhistochemistry, previous research in our lab found that in the brains of 4 month MRL/lpr, there was increased expression of both of these adhesion molecules in comparison to the controls (Zameer and Hoffman, 2003). This indicates that there is an immune response occurring in the brain. It could also mean that the adhesion molecules are allowing leukocytes entry into the brain, which in turn gives the autoantibodies access to the brain, resulting in different neuropsychiatric manifestations. Alteration of BBB permeability by itself could cause CNS manifestations. Lastly, researchers also believe that components of the complement system may play a role in lupus (Liu et al., 2005). However, exactly what role each of these factors is playing is not certain.

For the BRAA to enter the brain and cause change of function there needs to be an alteration of the BBB. It is hypothesized that the BBB is altered during CNS-SLE development, but this is still not completely understood (Zameer and Hoffman, 2001; Hoffman and Harbeck, 1989). We have in the past detected

increased BRAA levels in the brains of our autoimmune mice in comparison to the controls (Zameer and Hoffman, 2001) as well as the presence of B and T cells in the brains of these mice (Zameer and Hoffman, 2004). Because of the presence of the immunoglobulin in the brain during disease progression, this suggests that somehow these BRAA are able to cross the BBB and affect function or the leukocytes that produce these BRAA are somehow able to enter the brain and produce these BRAA in situ.

Also, there is evidence that there is functional damage to the barrier (Hoffman and Harbeck, 1989). During immune complex disease, changes in the permeability of the blood-CSF barrier have been detected. Immune complex deposition found in the choroid plexus has been thought to affect the permeability of the blood-brain barrier and may be partly responsible for some of the CNS manifestations of lupus (Hoffman and Harbeck, 1989; Harbeck et al., 1979). As further evidence of BBB involvement, other researchers have found that their antibodies to the NMDA receptor were able to enter the brain of control mice and affect behavioral performance, but only after compromising the BBB (Kowal et al., 2004). These mice displayed cognitive impairment showing that if the BRAA are able to enter the brain through a disrupted barrier, then behavioral dysfunction is observed.

We believe that multiple factors contribute to the opening of the BBB and generating CNS manifestations. We hypothesize that substance P is involved in opening the barrier through binding to its neurokinin-1 receptor (NK-1R). If increased substance P activity is necessary for opening the BBB, one way this

could occur is through increased levels of the NK-1R. Researchers have found that pro-inflammatory cytokines caused an increase in the levels of substance P, which seems to affect barrier permeability (Annunziata et al., 2002). They also found that this increase in substance P levels cause an increase in the expression of ICAM-1, since using a substance P antagonist causes a decrease in the expression of this adhesion molecule. As mentioned, we also found an increase in the expression of ICAM-1 and VCAM-1 in our lupus mice (Zameer and Hoffman, 2003). Therefore in the presence of a certain level of pro-inflammatory cytokines, substance P seems to help in opening the blood-brain barrier as well as causing an increase in the expression of the adhesion molecules that attract the leukocytes which produce the BRAA.

To test this hypothesis we looked at the levels of the NK-1R in the brains of MRL/lpr mice. These MRL/lpr displayed lupus disease progression when measuring different immunological variables as well as increased BRAA levels. Altered behavior was observed in the MRL/lpr in the sucrose preference test (used to measure anhedonia) in comparison to controls. When measuring the levels of NK-1R protein, our initial results showed a significant increase in the expression of this receptor. These results (see chapter 5) suggest a role for this receptor in contributing to altered BBB permeability and CNS dysfunction, but more research is needed to solidify these findings.

It is interesting to note that researchers have found that using an antagonist to substance P caused a decrease in the levels of MHC class I (Annunziata et al., 2002). This implies that an increase in substance P levels should cause an

increase in MHC class I levels. It has also been found that mice lacking MHC class I have increased synaptic plasticity (Datwani et al., 2009). Therefore, substance P causing an increase in MHC class I could be one reason for the learning and memory deficits seen in humans (Shucard et al., 2007) and MRL/lpr mice Shatz proposed a hypothesis that MHC class I interacts with a protein in the brain called PirB and this helps to regulate synaptic plasticity (Shatz, 2009). We have been looking at MHC class I levels in the brains of our MRL/lpr mice and our preliminary results suggest an increase in MHC class I in the brains of our MRL/lpr. Taken together, this could suggest that an increase in pro-inflammatory cytokine levels causes an increase in substance P levels and an increase in NK-1R binding (through an increase in NK-1R levels). This increase in substance P levels causes an increase in ICAM-1 levels which attracts leukocytes to the brain. Substance P then alters the blood-brain barrier, allowing leukocytes to enter, or the BRAA themselves. The BRAA are then able to bind to their target proteins and cause a variety of neuropsychiatric manifestations. Substance P also causes an increase in the levels of MHC class I which, through binding to its ligand PirB, affects brain functioning resulting in CNS manifestations like cognitive deficits through decreased synaptic plasticity. Of course, this is all hypothetical but the results that we and other researchers are finding seem to support these ideas.

Overall, our studies have added a better understanding of the involvement of BRAA in causing neuropsychiatric manifestations. We have developed a general procedure for determining the identities of pathogenic BRAA which likely will provide biomarkers and targets for therapy. More research is needed to

solidify these techniques and the role that each of these proteins is playing in CNS lupus. In order to confirm that these BRAA are indeed pathogenic, in future studies we would need to inject these BRAA into normal mice and see if we can replicate the altered behavior. This would provide strong causal support for the BRAA hypothesis. We have also discovered a better diagnostic and predictive technology for lupus and CNS lupus. Our detection system is very noteworthy because it has allowed us to predict and diagnose CNS manifestations in our lupus mouse model, which at the current time is difficult to do. The next step would be to apply this microarray technology to sera obtained from patients with lupus and CNS lupus. We expect that this technology should prove to be very useful in accurately predicting and diagnosing lupus and each specific CNS manifestation in humans and also in providing better treatments since accurate diagnosis is the first step in providing better care. Lastly, we have provided results to further suggest that the NK-1R may be playing a role in CNS lupus. Of course, more studies are necessary to validate all of our results as well as to discover the exact role that BRAA are playing in neuropsychiatric lupus. However, our findings are adding more information to what is necessary to better understand the mechanisms underlying CNS-SLE, and to help those individuals affected by this autoimmune disease.

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APPENDIX A

REFERENCES FOR JOURNAL ARTICLES AND MANUSCRIPTS INCLUDED AS CHAPTERS

Chapter Two:

Circulating Brain-Reactive Autoantibodies and Behavioral Deficits in the MRL Model of CNS Lupus

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Journal Refernce:
Williams,S., Sakic,B. and Hoffman,S.A., 2010. Circulating brain-reactive autoantibodies and behavioral deficits in the MRL model of CNS lupus. J. Neuroimmunol. 218, 73.

I have obtained permission from co-authors to include this chapter in my dissertation.

Chapter Three:

Early detection and stratification of SLE using peptide microarrays
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Chapter Four:

Validating Predictive and Diagnostic Peptides of Lupus and Depressive-Like Behavior from Lupus-Prone MRL/lpr Mice Using Microarray Technology
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Chapter Five:

Increased levels of substance P Receptor (Neurokinin-1) detected in the Brains of Lupus-Prone MRL/lpr Mice

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