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14. ABSTRACT During the first year of this grant, we have determined that intracranial injections of the Japanese macaque rhadinovirus (JMRV) can induce demyelinating disease, as assessed by histopathology, MRI, and symptoms. We have characterized the demyelinating lesions in these animals and show that they are very similar to demyelinating multiple sclerosis lesions. We have also developed novel tissue culture protocols to grow neurons and glial cells from fetal Japanese macaques, and we have begun testing how the virus influences these cells in vitro. Animals from specific maternal lineages are affected by virus injection while unrelated animals do not develop disease. We have determined that animals with specific haplogroups of the major histocompatibility complex (MHC) are more likely to develop spontaneous disease than others. In the next year, we will continue to analyze the mechanisms by which JMRV triggers disease onset, which genes are linked to disease susceptibility, and how the virus influences cells in lesion microenvironments.				
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Introduction

Particular viruses, especially gamma-herpesviruses, may act as a trigger of multiple sclerosis (MS; Ascherio and Munger, 2010). Furthermore, there is growing evidence that susceptibility to MS may be linked to polymorphisms at certain genetic loci, including major histocompatibility complex (MHC) genes (Ramagopalan et al., 2009) and the interleukin-7 α gene (Harley, 2007). This study is a collaborative effort between several investigators at the Oregon National Primate Research Center (ONPRC) who are interested in understanding the pathophysiological mechanisms that trigger MS and related inflammatory demyelinating disease. We have characterized a novel encephalomyelitis that occurs spontaneously in a small percentage of animals in a colony of Japanese macaques (JMs) at the ONPRC. The disease, called Japanese macaque encephalomyelitis (JME), occurs in both progressive and relapsing-remitting forms and is characterized by brain and spinal cord demyelination that is accompanied by extensive astrogliosis. Affected animals develop debilitating motor and ocular disturbances. Approximately 10% of the animals in this colony appear to have chronic, subclinical lesions as evaluated by magnetic resonance imaging (MRI). Pedigree analysis indicates that particular lineages of animals are substantially more susceptible to this disease than others, suggesting a genetic pre-disposition to JME. Furthermore, we have cloned a gamma-herpesvirus (called Japanese macaque rhadovirus; JMRV) from animals in this colony that is found within demyelinated JME lesions.

This highly integrated, multidisciplinary application is focused on developing JME as a pathophysiological and genetic model of MS whose etiology and progression more closely resemble MS in humans than other EAE and viral models in non-human primates and rodents. We aim to use this model to better understand how gamma-herpesviruses trigger MS; whether polymorphisms in gene loci that have been linked to MS in humans also predispose JMs to JME; to evaluate the lesions in both symptomatic and subclinical animals to determine if they model numerous aspects of human MS lesions; and to test if gamma-herpesvirus infection directly influences astrogliosis and the accumulation of factors, such as hyaluronan (Back et al., 2005), that can inhibit remyelination in demyelinated lesions. Our long-term goal is to develop JME as a model for testing immunological and neurobiological processes underlying MS, and to use this model in pre-clinical screens of novel agents with the potential to inhibit MS attacks and to promote remyelination and regeneration.

The first year of the project has been focused on the following areas:

1. Testing the effects of intracranial JMRV infections on Japanese macaques from previously affected and unaffected lineages
2. Using MRI screens to identify animals with sub-clinical disease and to expand the pedigrees of affected animals
3. Performing an analysis of MHC haplotype association with disease susceptibility
4. Developing an in vitro culture system to assess the effects of JMRV infection on neuronal and glial cell behaviors.

As outlined below, we have achieved a significant portion of our initial goals and we are now poised to conduct each of the studies outlined for the second year of the project.

Body

Aim 1: To test the hypothesis that intracranial infection with Japanese macaque rhadovirus (JMRV) can experimentally induce Japanese macaque encephalomyelitis (JME)

Four Japanese macaques (JM) that had been confirmed to be sero-negative for infection with JMRV were experimentally inoculated intracranially (right hemisphere) with JMRV at a dose of 5×10^6 plaque forming units (PFU). These animals were negative for pre-existing lesions by magnetic resonance imaging (MRI). Following inoculation the animals were scanned by MRI to detect any anomaly that could be associated with virus infection. Of the four animals, two (21572 and 26114) developed significant inflammation resulting from the injection, while the two other animals (21713 and 21754) developed no inflammation or minor inflammation, respectively (**Figure 1**). Interestingly, the two animals that developed significant inflammatory responses after the inoculations are directly related (mother and offspring), supporting, but not proving, host genetic factor(s) may play a role.

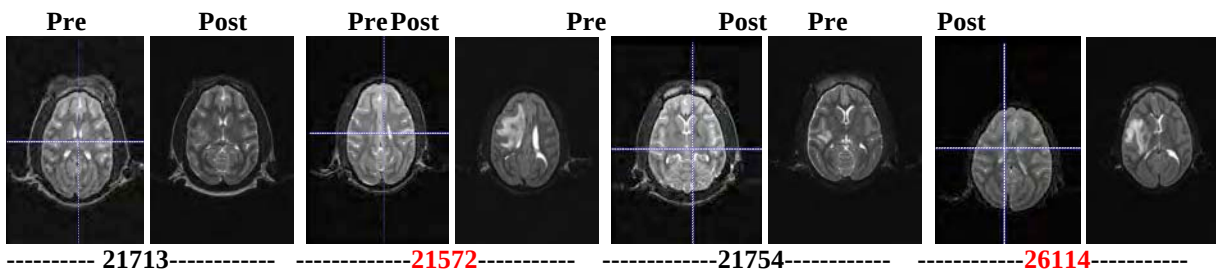


Figure 1: Magnetic resonance imaging (MRI) of animals 21713, 21572, 21754 and 26114 following JMRV inoculation. MRI scans of animals before (Pre) and after (Post) JMRV intracranial inoculation. The Post scan of animal 21713 was acquired at day 28 post-injection, whereas the scans of animals 21572, 21754 and 26114 were acquired at day 8 post-injection.

Of the two animals that exhibited significant inflammation by MRI, one (21572) was euthanized on day 8 post-infection with clinical signs manifesting as paralysis. The second animal (26114) developed partial loss of use of one forelimb (left), but has since recovered. Pathological examination of animal 21572 revealed the animal had pronounced inflammation in the right hemisphere co-incident with the site of inoculation (**Figure 2B**). Histopathological examination detected inflammatory cell infiltrates near the site of inoculation (**Figure 2C**), which were determined to be comprised primarily of microglia cells/macrophages as these cells were positive for ionized calcium binding adaptor molecule 1 (IBA-1), a marker for microglia and macrophages (**Figure 2D**). The affected area was subsequently stained for myelin basic protein (MBP) to assess the potential negative impact the inflammation had on surrounding neurons. The immunohistochemical staining indicates that myelin is destroyed in the affected area compared to an area distant from the inflammation (**Figure 2E and F**, respectively). The affected area was also analyzed for the presence of JMRV. As shown in **Figure 2G**, IBA-1 positive macrophages/microglia also stained positive for JMRV vIL-6 expression. This demonstrates that JMRV infection was associated with the inflammation. Additionally, we were able to recover JMRV from the brain when the affected area was homogenized and plated on primary fibroblasts for virus isolation.

The lineages of these four animals were evaluated to determine whether any were related to an animal diagnosed with JME. Pedigree analysis revealed animal 21572 is the offspring of animal 14839, who is a half sibling to animals 16387 (confirmed JME) and 17807 (positive for subclinical disease as assessed by MRI; see below). This suggests that animal 21572 may have harbored a specific genetic factor(s) that predisposed the animal to elicit an inflammatory response to JMRV, which may or may not coincide with development of JME. Nonetheless, the inflammatory response observed in 21572 suggests some genetic factor(s) may in fact be associated and that these were passed on to animal 26114. Interestingly, animal 21713, who is not related to an animal with history of JME did not exhibit detectable inflammation, whereas animal 21754 did by MRI analysis. Animal 21754 is related to animals 19085, 18270 and 22720, which all died of JME, implying this animal may have inherited a genetic factor(s) that made it susceptible to JMRV-induced disease.

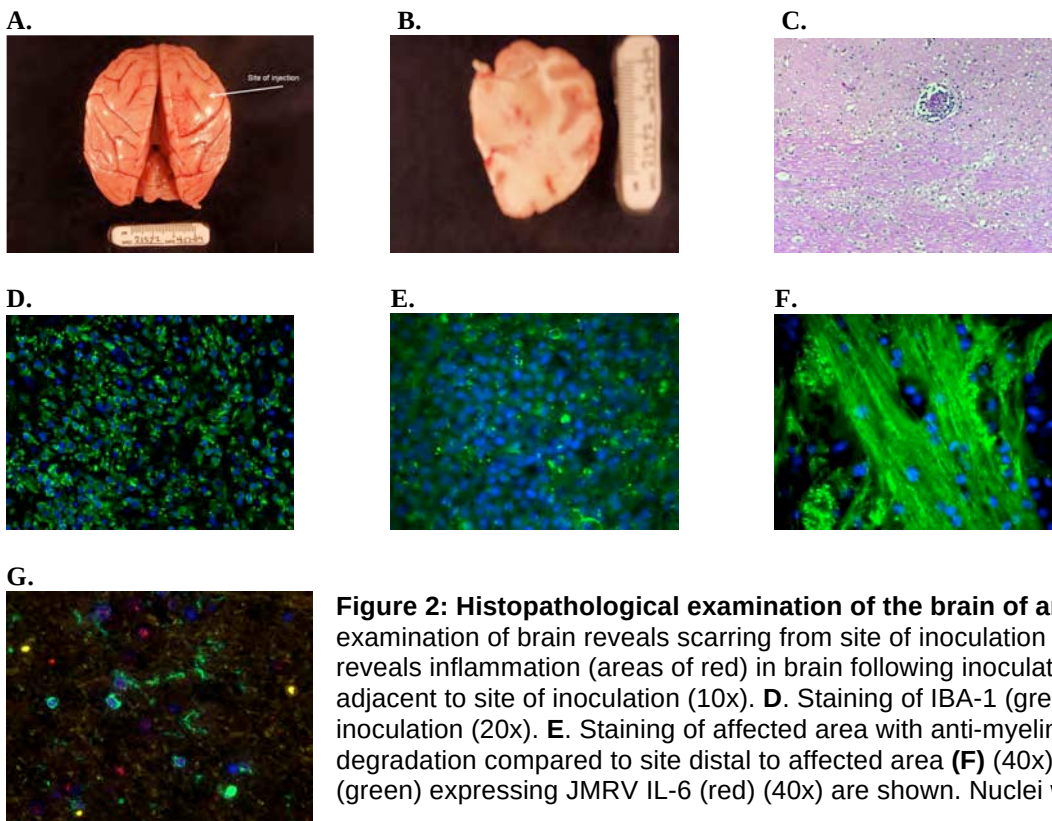


Figure 2: Histopathological examination of the brain of animal 21752. **A.** Gross examination of brain reveals scarring from site of inoculation (arrow). **B.** Section of brain reveals inflammation (areas of red) in brain following inoculation. **C.** H&E staining of area adjacent to site of inoculation (10x). **D.** Staining of IBA-1 (green) in area adjacent to site of inoculation (20x). **E.** Staining of affected area with anti-myelin basic protein (MBP) reveals degradation compared to site distal to affected area (**F**) (40x). **G.** Macrophages/microglia (green) expressing JMRV IL-6 (red) (40x) are shown. Nuclei were stained with DAPI (blue).

We expanded our experimental intracranial inoculations into a larger cohort of animals, all of whom were sero-positive for JMRV infection. A total of seventeen additional animals were identified and all were scanned by MRI prior to inoculation to ensure absence of lesions. In addition to the virus inoculation the animals also received a second inoculation containing PBS alone on the opposite hemisphere to confirm whether any inflammatory response observed post-infection is directly associated with the virus and not due to the procedure.

All seventeen animals received the identical preparation and dose of virus as our initial group of four JM. Of these animals, nine developed noticeable inflammation detected by MRI by day 3 post-inoculation in the hemisphere receiving virus, but not in the side receiving phosphate buffered saline (PBS; data not shown). Analysis of the pedigrees for these animals revealed that eight were related to animals diagnosed with JME, while the ninth animal had no known relationship. No overt clinical manifestations or neurological deficits, other than the MRI analyses, were noted in these nine animals. These data indicate that intracranial JMRV inoculation can precipitate inflammation in animals that have been previously infected with the virus, and suggest that re-infection of sero-positive animals provides a potential model for JME. This is encouraging as most, if not all JM housed at the ONPRC are sero-positive for JMRV. The utilization of sero-positive animals could eliminate the strict requirement for sero-negative macaques. However, it is important to note that the sero-negative animals (at least two) exhibited more pronounced inflammation than sero-positive animals and exhibited neurological deficits, implying that the availability of sero-negative animals for these studies is still needed and warranted.

One hallmark associated with MS in humans is the development of autoimmune responses to components comprising myelin. In particular, MS patients have detectable CD8⁺ T cell responses to myelin oligodendrocyte glycoprotein (MOG). To determine if the JMRV-infected animals developed similar immune responses, we generated a recombinant vaccinia virus directing the expression of human myelin-oligodendrocyte glycoprotein (MOG; rVV-hMOG; Human and macaque MOG are >97% identical, so there is good likelihood that the animals can recognize human MOG if they develop an anti-MOG response). Utilizing the rVV-hMOG and wild type vaccinia virus (WT-VV), and established protocols for intracellular cytokine staining, we found that 4 of 5 animals tested so far

developed CD8+ T cell responses to MOG and 3 of these 4 animals were MRI-positive for inflammation. The remaining animals are being evaluated immunologically for anti-MOG activity.

Overall, these results suggest experimental intracranial inoculation of JM with JMRV can precipitate inflammatory responses which can lead to autoimmune responses to MOG and, at least in previously sero-negative animals, demyelinating disease. Whether or not these responses in the previously sero-positive animals can lead to multiple lesions in the white matter and clinical disease manifestations is not known at this time.

We have also been further characterizing spontaneous JME cases both clinically and by MRI. We performed MRI scans on 107 animals. Nine of these animals were positive for one or more white matter signal hyperintensities (WMSH) consistent with subclinical JME (animals 15548, 17252, 17807, 24027, 16754, 17261, 17787, 22001, 22723). All 9 animals were asymptomatic at time of study and there was no indication in their medical records of any history of neurological impairment. The age range of the WMSH positive animals was 2-20 y and 7 of the 9 were female. The locations of the WMSH were distributed throughout the brain and included periventricular, posterior fossa, and spinal cord white matter - similar to the distribution observed at necropsy of the JME confirmed animals. There was no association between WMSH and age in this cohort.

Over the last 28 months 8 animals developed acute behavioral disturbances suggestive of neurological involvement. These animals all underwent a comprehensive MRI examination of the brain and upper spinal cord. Five (4 male, 1 female; 218d-14.7y; animals 26174, 27616, 18276, 27571, 20482) of the animals had MRI findings consistent with acute JME; findings that included extensive proton-density and T₂-weighted hyperintensities pre-contrast and T₁-weighted hyperintensities post-contrast. Post-contrast T₁-weighted hyperintensities were most commonly observed in the upper spinal cord, cerebellum, pons, and peduncle and are indicative of focal breakdown of the blood-brain barrier. Representative post-contrast T₁-weighted images of an 11 y male with acute JME are displayed in **Figure 3**. Interestingly, MRI of this same animal acquired 30 months earlier was normal (data not shown). Quantitative estimates of blood-brain barrier permeability to low-molecular weight contrast agents are in very good agreement with similar measures obtained in human MS.

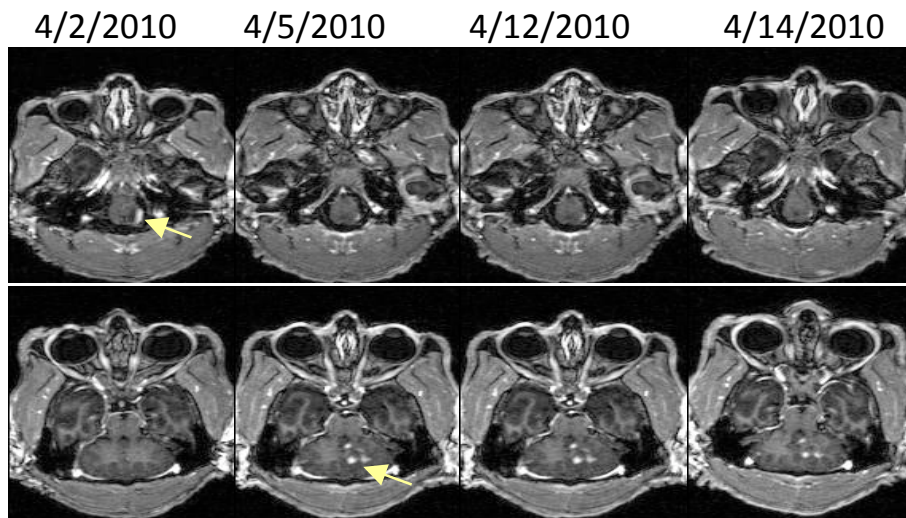


Figure 3. Time series showing post-contrast T₁-weighted images from an 11 y M presenting with left-head tilt, ataxia, and balance problems on 3/31/2010. Upper cord involvement is clearly visualized (see arrow) on the 4/2/2010 top image that partially resolves over a two week period perhaps the result of corticosteroid treatment (145 mg hydrocortison i.v. followed by oral prednisone). Cerebellar post-contrast T₁-weighted hyperintensities became apparent on 4/5/2010 (arrow) and persisted through 4/14/2010. MRI findings are consistent with acute JME.

Aim 2: Test the hypothesis that common genetic variants in the JM genome increase the susceptibility of developing demyelinating disease.

Based upon numerous reports of an association between certain HLA alleles and MS in humans, we aimed to investigate a similar association between MHC alleles and JME risk. Since no direct allele information is currently known about the MHC locus in JMs, we first characterized the heterogeneity of the MHC region in the JM colony using a set of 14 linked microsatellite/short tandem repeat (STR) markers that span the entire MHC locus [D6S291, D6S2741, 9P06, DRA, MICA, 246K06, 162B17A, 162B17B, 151L13, MOG, 268P23, 222I18, D6S276, D6S1691]. DNA from a total of 222 JMs were extracted and genotyped; the study set included 28 clinically affected animals, 7 animals with evidence of brain lesions by MRI analysis (MRI+) but who were otherwise asymptomatic, 171 1st degree relatives of those affected animals, and 23 control animals with no symptoms and no direct relationship to affected animals. The STR genotypes revealed 24 different haplogroups, each carrying the same STR alleles throughout the MHC region. Only 13 of the 24 MHC haplogroups were present in JME affected animals, with the most common haplogroups, 1, 4, 11, and 13 also highly represented in the affected animals (**Figure 4A**). Of greatest interest are haplogroups 1 and 17, which have a statistically suggestive association with JME affected status. In contrast, none of the clinically normal, MRI+ animals carried MHC haplogroup 17 and few had haplogroup 1; instead, these MRI+ animals had an over representation of MHC haplogroup 10 (**Figure 4B**). These results suggest that JME risk alleles may be contained within MHC haplogroup 1 or 17, and thus carriers of these alleles have an increased frequency of clinical JME. Haplogroup 10 may include alleles that result in an increased risk for a more moderate inflammatory response.

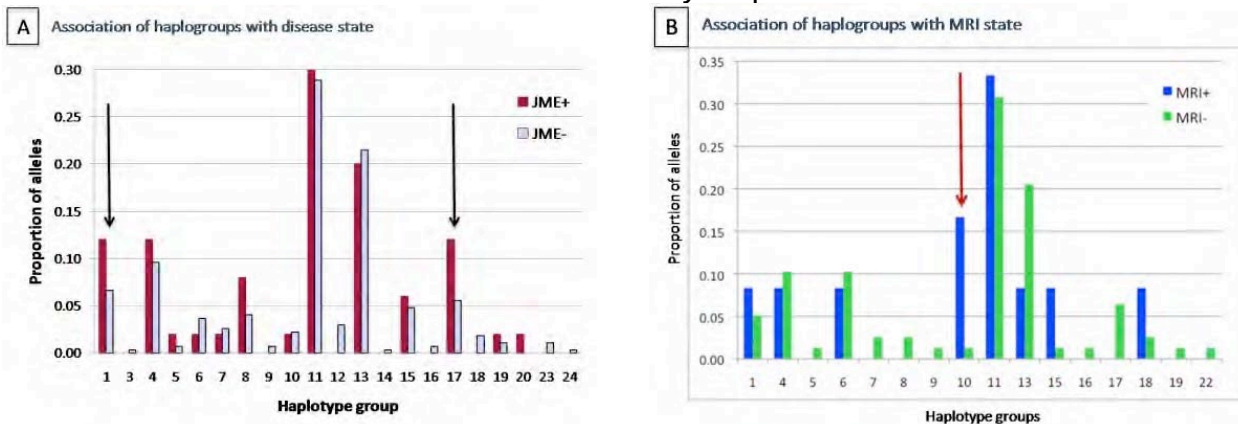


Figure 4. A (left) and B (right) show the proportion of chromosomes by category (JME and MRI) that occurred in each haplogroup, compared to the total number of chromosomes screened in either all affected or unaffected individuals.

To further investigate these findings, we have established a collaboration to sequence all Class I and Class II MHC expressed alleles in each of the 24 haplogroups identified in the JMs. These data will enable us to deduce the full allele representation in each individual, providing improved resolution for identifying alleles that alone, or in combination, increase or reduce risk for JME. To increase the power of this study, we will continue to MHC genotype any new animals that are diagnosed with JME or are identified with MRI+ brain lesions.

Aim 3: To test the hypothesis that JMRV infection of glial cells directly influences demyelination and remyelination failure.

One of the original goals of this aim was to grow neurons and glia derived from JM embryonic stem cells and test the effects of JMRV infection on cellular behaviors. However, we found that the neural stem cells derived from JM embryonic stem cells did not reliably give rise to each of the cell types we hoped to analyze (e.g. we observed neuronal differentiation but little or no oligodendrocyte progenitors or astrocytes). We therefore turned to acutely dissociated cultures of fetal JM cerebral cortex. We dissociated late term fetal temporal lobe cortex in trypsin-EDTA then grew cells in medium that we previously used to grow mouse neural progenitor cells (Matsuomoto et al., 2006).

Under these culture conditions, we observed a mixture of cell types (**Figure 5**) including GFAP⁺ astrocytes, map2⁺ neurons, and numerous olig2⁺ oligodendrocyte progenitors. We also observed small numbers (<5% of the total population) of microglia, detected with the anti-IBA1 antibody as above (data not shown). Small neurospheres growing in these cultures could be propagated and could differentiate into neurons, oligodendrocytes and astrocytes for >5 passages. We are now using these cultures to examine the effects of JMRV infection on the different cell types.

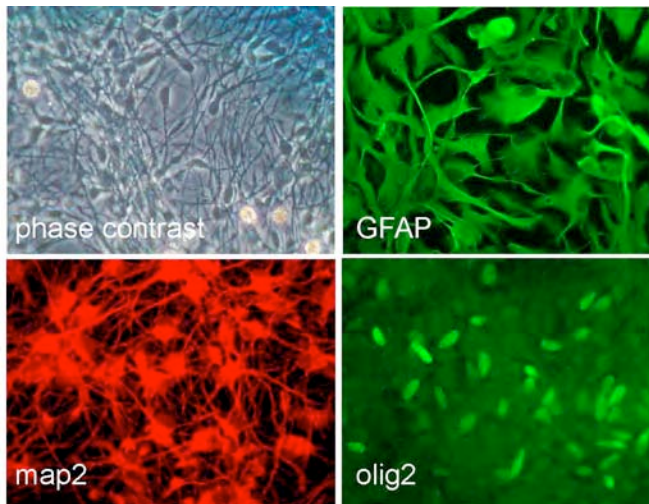


Figure 5. Microphotographs of acutely dissociated cultures of fetal Japanese macaque cortical cells. Cultures were grown on poly-l-lysine coated glass coverslips in a mixture of DMEM/F12 medium supplemented as previously described by us (Matsumoto et al., 2006) then processed for immunocytochemistry. GFAP = glial fibrillary acidic protein, a marker of astrocytes; map2 = microtubule associated protein, a marker of neurons; olig2 = a marker of oligodendrocyte progenitor cells.

Key Research Accomplishments

- Determined that intracranial injection of JMRV is sufficient to cause an MS-like disease in genetically susceptible Japanese macaques that had not previously been exposed to this virus, thus demonstrating that a gamma-herpesvirus can trigger an autoimmune demyelinating disease in susceptible individuals. These data shed light on a potential pathophysiological mechanism for the onset of MS in humans.
- Determined that animals that had previously sero-converted to JMRV developed subclinical disease following intracranial injection of JMRV.
- Determined that there is a significant population of animals in the ONPRC JM colony with MRI findings that are consistent with subclinical disease
- Determined that animals with specific MHC haplotypes have an increased risk of developing JME
- Developed a highly novel culture system for JM neurons, glia and progenitor cells that will allow us to perform the *in vitro* studies outlined in the proposal focused on defining how JMRV influences cellular survival and behavior.

Reportable Outcomes

Julie A. Hollister-Smith, M. Cecilia T. Penedo, Larry S. Sherman, Scott W. Wong, Michael Axthelm, Bill Rooney, Steve Kohama, Beth Wilmot, Daniel Bottomly, Peter A. Ryabinin, Samone Khouangsathiene, Roger Wiseman, Julie Karl, David O'Connor and Betsy Ferguson. Genetic exploration of Japanese macaque encephalomyelitis (JME), a promising new animal model for multiple sclerosis. Presented at the 4th International Conference on Primate Genomics, Seattle, WA (April 13-16, 2010). See abstract in appendix.

Conclusion

During this first year of funding, we have established that intracranial infection with a gamma-herpesvirus (JMRV) can induce an MS-like disease in non-human primates, but only in subsets of animals related to animals that had previously had a spontaneous disease. These findings are consistent with a genetic predisposition for virus-induced disease onset. Consistent with this hypothesis, we found that animals with particular MHC haplotypes were most likely to develop this disease. All together, these findings support an etiological mechanism for MS that is consistent with the disease being triggered in genetically susceptible individuals by a gamma herpesvirus. We are now poised to further investigate the mechanisms underlying the pathophysiology of JME during the second and third years of this grant.

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Appendix

Genetic exploration of Japanese macaque encephalomyelitis (JME), a promising new animal model for multiple sclerosis

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Multiple sclerosis (MS) is a debilitating disease that involves demyelination and axonal damage in the central nervous system. Its causes are unknown, however, disease susceptibility has been tied to polymorphisms in Major Histocompatibility Complex (MHC) genes and disease onset is likely connected to viral trigger(s) of which gamma-herpesviruses may be most relevant. Since 1986, approximately 5% of individuals in the Japanese macaque (*Macaca fuscata*) colony at Oregon National Primate Research Center have developed a demyelinating disease, Japanese macaque encephalomyelitis (JME) that shares many of the hallmarks of MS. Key similarities include spontaneous onset of disease, neurological pathologies and resultant physical manifestations, comparable disease courses, which may be acute, relapse/remitting, or chronic, and unequal prevalence across population lineages and gender. Finally, analogous to MS, a novel gamma-herpesvirus, identified in the colony in 1995, provides a possible trigger for JME disease. Although the strongest genetic association for MS is found within the MHC, the association is multifaceted involving haplotypes rather than single alleles and likely involves epistatic interactions and modulators of gene expression. As a part of the larger study to develop the JME model we genotyped 225 colony animals at 14 microsatellite markers. These markers spanned the entire macaque MHC locus and included 3 flanking markers. Our study set comprised 25 confirmed JME cases, six animals with chronic subclinical brain lesions who were otherwise asymptomatic (MRI+), 171 first-order relatives of JME case and MRI+ animals, and 22 control animals. All relatives were asymptomatic for JME disease; a subset, 39 animals, showed no visible MRI brain lesions and the remainder were without brain scan data. Genotype data segregated into 24 distinct MHC haplotype groups, however just four haplotype groups encompassed 64% of individuals. JME cases occurred in 13 of the 24 haplotypes including both common and rare haplotypes. Initial analyses showed that the familial structure of the colony impacts the sensitivity and specificity of the test statistics used for association analysis. Current work is thus focused on evaluating strategies to clarify these effects and maximize our ability to identify genetic variants involved. Expressed MHC alleles from 21 haplotypes, including all those with JME cases, are currently being characterized with next generation sequencing technology.

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