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14. ABSTRACT The major goal of this project was to characterize an encephalomyelitis that occurs spontaneously in a colony of Japanese macaques housed at the Oregon National Primate Research Center. We determined that this disease, Japanese macaque encephalomyelitis (JME), can be induced by an apparently unique virus found in this colony (Japanese macaque rhadinovirus; JMRV) but only in animals belonging to families of previously affected animals. We also determined that animals with certain major histocompatibility complex (MHC) polymorphisms are more likely to be affected than others, consistent with findings in humans with multiple sclerosis. Finally, we characterized both spontaneous and induced JME lesions using a combination of magnetic imaging and histopathological approaches, and found that infection with JMRV leads to demyelination, axonopathy, reactive astrogliosis and hyaluronan accumulation in affected white matter. All together, our findings indicate that JME is a unique and powerful non-human primate model of multiple sclerosis.					
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Table of Contents

	page
Introduction.....	2
Body.....	3
Key Research Accomplishments.....	7
Reportable Outcomes.....	8
Conclusions.....	8
Abbreviations.....	8
References.....	9

Introduction

Multiple Sclerosis (MS) is characterized by autoimmune destruction of myelin sheaths and axons, leading to conduction deficits that influence motor, sensory and cognitive function. Although the etiology of MS is still poorly understood, particular viruses, especially gamma-herpesviruses, may act as triggers of MS (Levin et al., 2010; 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 and the interleukin-7a gene (Ramagopalan et al., 2009; Harley, 2007).

This project involves a collaborative effort between several investigators at the Oregon National Primate Research Center (ONPRC) and the Oregon Health & Science University (OHSU) 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 (Wong et al., 2011). The disease, called Japanese macaque encephalomyelitis (JME), occurs in both progressive and relapsing-remitting forms and is characterized by brain and spinal cord demyelination and axonopathy 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 (Estep et al., 2013).

This highly integrated, multidisciplinary application was 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. Our major aim was to use this model to better understand how gamma-herpesviruses trigger demyelination and axon damage; 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, that can inhibit remyelination in demyelinated lesions. Our progress towards each of these goals is outlined below.

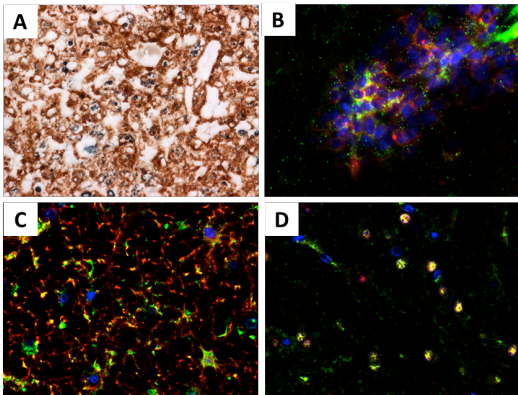
Based on our findings, we believe that JME is a unique and powerful model for testing immunological and neurobiological processes underlying MS, and can be used for pre-clinical screens of novel agents with the potential to inhibit MS attacks and to promote remyelination and regeneration.

Body

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

During the course of this study, we used a combination of diagnostic procedures, MRI-based analyses, and histopathology to fully characterize the course of spontaneous JME pathogenesis (reported in Axthelm et al., 2011); fully characterized the virus (JMRV) that is associated with JME lesions (reported in Estep et al., 2013); and determined that JME can be induced in animals with a genetic predisposition to the disease (see below and previous progress reports). All together, our findings are consistent with the notion that JME is an autoimmune demyelinating disease triggered by JMRV with a pathophysiology that is similar to MS.

To further characterize the autoimmune mechanisms of JME, we evaluated JME cases for interleukin 17 (IL-17), a cytokine that plays an important role in host defense and inflammation, and is widely considered



to be associated with autoimmune diseases such as collagen-induced arthritis, colitis, psoriasis, experimental autoimmune encephalomyelitis (EAE) and MS Gold et al., 2008; Pierson et al., 2012), but not acute demyelinating encephalomyelitis (ADEM) (Ishizu et al., 2006). Utilizing immunohistopathological analysis, we stained JME lesions acquired from our most recent animals for IL-17, utilizing a murine monoclonal antibody directed against human IL-17. From these studies we observed IL-17 positive staining in eight out of nine lesion samples (89%). IL-17 staining was consistent with that reported by Tzartos et al. (2008), who reported IL-17 production in the microglia, oligodendrocytes, and infiltrating T cells within active areas of MS lesions (Fig. 1).

Figure 1. JME lesion of animal 26174. (A) Immunohistochemical staining of lesion revealing CD163+ cells (brown) engulfing myelin components (blue). Immunofluorescent analysis of lesion exhibiting IL-17 staining (green) localized in (B) CD3+ T cells (red), (C) Glial fibrillary acidic protein+ astrocytes (red) and (D) olig-2+ oligodendrocytes (red). Yellow indicates co-localization.

To investigate whether the animals suffering from JME have T cell responses to myelin components, we collected the bronchoalveolar lavage from these animals when they initially presented with JME and assayed the mononuclear cells for the presence of CD4+ and CD8+ T cells to myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP) and proteolipid protein (PLP) by intracellular cytokine staining flow cytometry. We choose bronchoalveolar lavage versus peripheral blood as the lung is a rich source of effector T cells and immune cells have been shown to traffic from the lung to the CNS. Overlapping peptides to macaque MOG, MBP and PLP were synthesized and used to stimulate cells and stained for CD4+ and CD8+ T cells expressing γ -interferon. Analyses were also performed in age and gender matched control animals (Fig. 2).

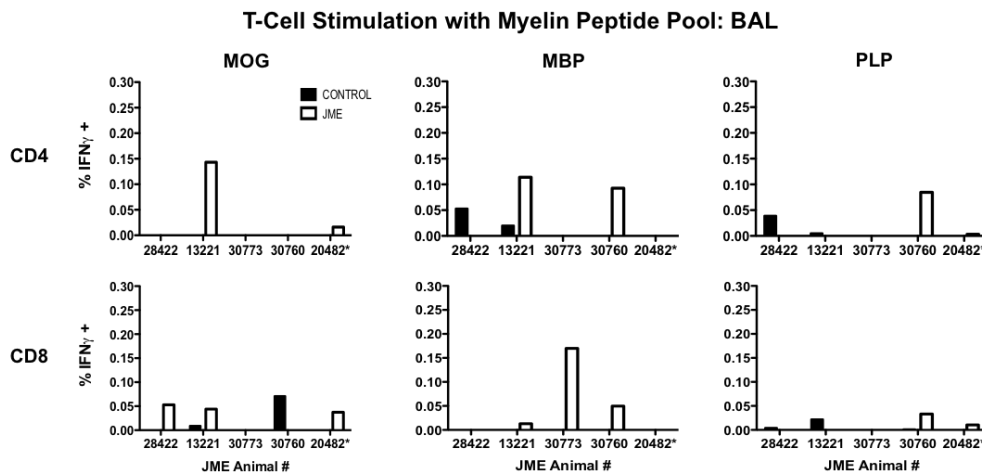
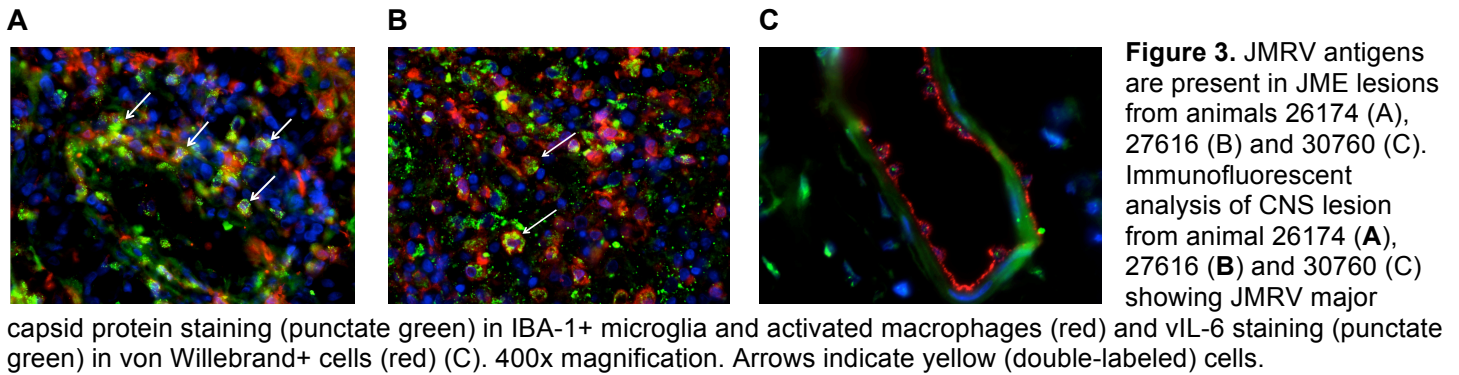


Figure 2. JME animals possess CD4+ and CD8+ T cell responses to myelin components.

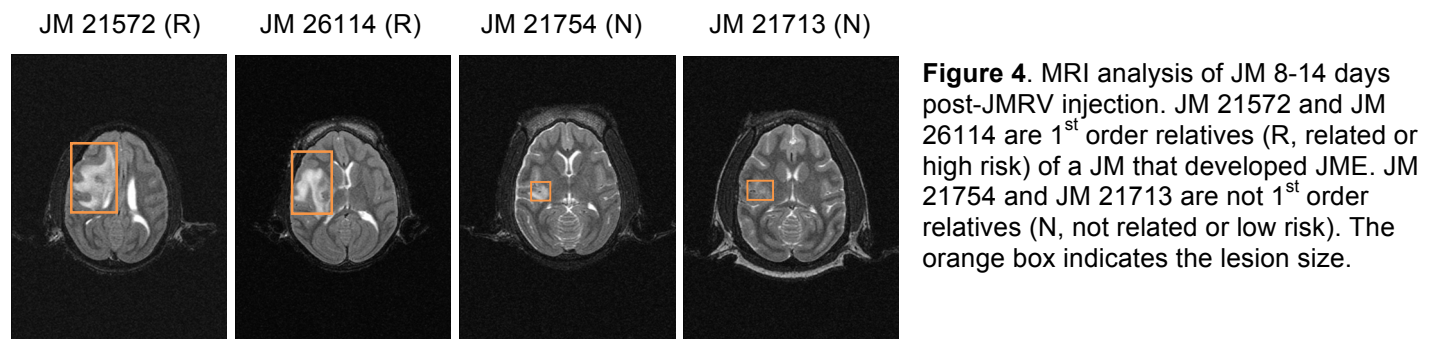
Bronchoalveolar lavage samples were collected from JME animals and gender and age-matched controls and stimulated with overlapping peptides to MOG, MBP and PLP, and subsequently stained for CD4 and CD8b, followed by intracellular staining for interferon- γ and tumor necrosis factor- α . Each JME animal is identified with its ID

number on the X axis in all the graphs and the gender and age match control response is included as a black bar. MOG, MBP and PLP-specific T cell responses are represented as the percentage of CD4+ or CD8+ T cells expressing interferon- γ after *ex vivo* peptide stimulation.

To determine if JMRV is more than just a passenger in cells that trafficked to the CNS lesions in JME cases, we utilized monoclonal antibodies that we generated against rhesus macaque rhadinovirus major capsid protein and viral interleukin-6 (vIL-6) that are cross-reactive with JMRV to interrogate CNS lesions obtained from animals that developed JME. Of the ten lesions analyzed, seven were positive for JMRV major capsid protein and four were also positive for vIL-6 (**Fig. 3**). Interestingly, we found JMRV vIL-6 staining in von Willebrand factor positive cells, demonstrating that JMRV can infect endothelial cells composing the blood-brain barrier, suggesting direct infection of the brain microvascular endothelial cells is the natural route of CNS infection.

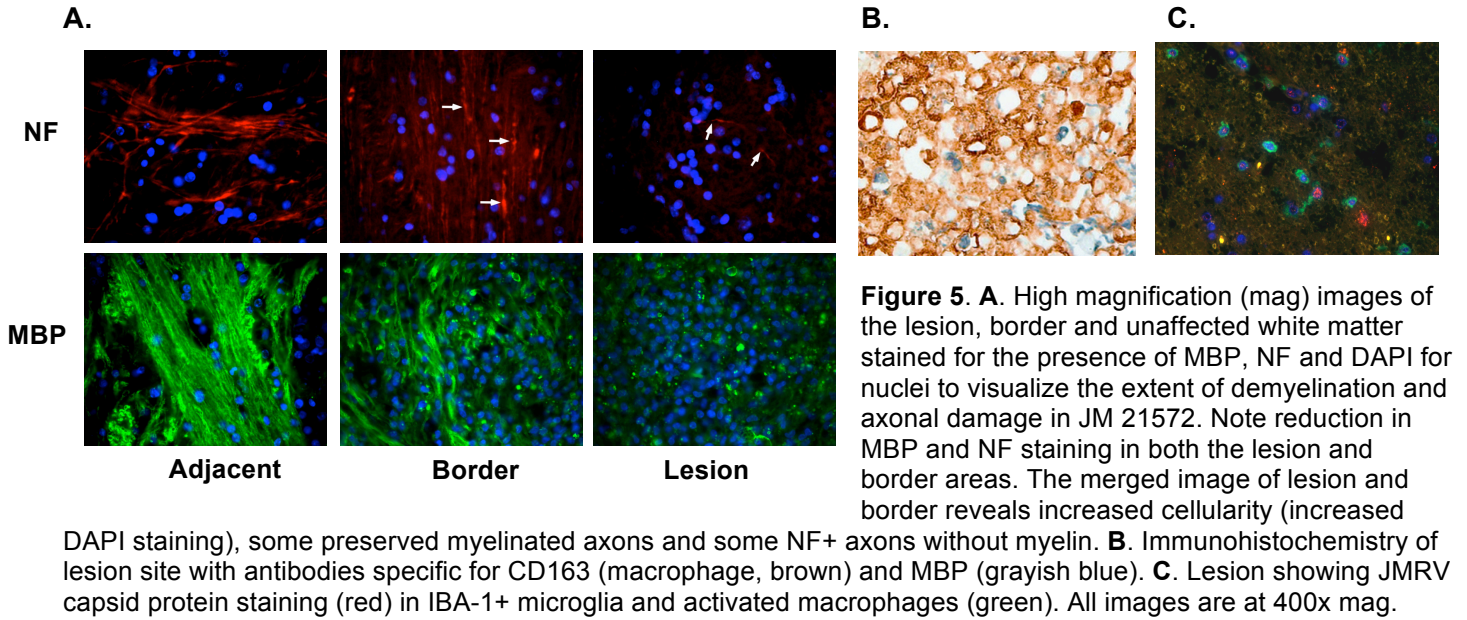


The isolation of JMRV from diseased tissue does not prove the virus is the etiological agent. To establish a causal relationship between JMRV and JME requires experimental animal infection studies to test whether JMRV can induce JME in healthy animals. For this, we identified four JMs and experimentally infected these animals by intracranial injection with 5×10^6 plaque forming units of JMRV (see progress report from year 2 of this project). We chose intracranial injection rather than other routes as this eliminates the virus having to cross the blood-brain barrier, which may or may not normally occur. Two of the JMs were 1st degree relatives of JM (offspring, sibling or parent) who developed JME, and thus may be genetically high risk for JME. The other two JMs were not 1st degree relatives and are thought to be at low risk to develop JME. Post infection analysis of the 4 JM revealed that JMRV inoculation is associated with larger T₂-weighted MRI lesions in the white matter of high risk JM, but much less in low risk JM (**Fig. 4**).

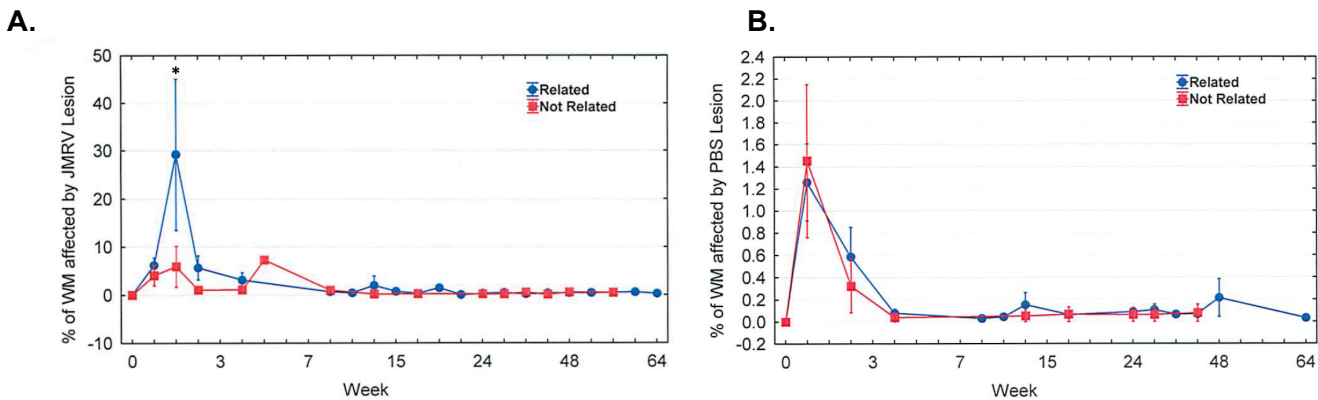


JM 21752 developed paralysis on day 8 post-infection and was euthanized. JM 26114 developed limited and temporary neurological deficits, and JMs 21574 and 21713 did not exhibit any deficits.

Histopathological examination of the injection site of JM 21572 revealed an inflammatory demyelinating lesion that was subsequently sectioned and stained for neural filament (NF) and myelin basic protein (MBP) (**Fig. 4A**). The staining of the area adjacent to the lesion site with NF specific antibodies reveals intact neurons as seen as long NF strands. The border area between the adjacent site and lesion site exhibits some blebbing of NF and less staining, whereas the lesion site shows almost no NF staining. Staining with MBP-specific antibodies revealed ordered MBP strands in the adjacent area, some loss of order in the border region and complete loss of order in the lesion site with numerous infiltrating cells observed by DAPI staining. These data indicate that JMRV inoculation can lead to an inflammatory demyelinating syndrome that resembles JME. Adjacent sections were also stained for CD163, a marker for macrophages and MBP (myelin) to visualize macrophages engulfing myelin, which is often observed in acute active MS lesions (**Fig. 4B**), and for the presence of JMRV and activated macrophages/microglia (**Fig. 4C**).



Since the original study in 4 JMs, we have subsequently infected 20 JMs by ic injection. These animals were identified as high risk or low risk based upon their relationship to JME animals. To ensure that the injection procedure itself was not responsible for the T₂-weighed MRI lesion, the animals were injected with JMRV in one hemisphere and vehicle (phosphate buffered saline) in the contralateral hemisphere, and followed by longitudinal MRI analysis to document changes to the WM. Blood draws were also obtained at multiple times post-injection to measure viral load in the periphery and changes in host responses to JMRV over time. Lesion size was calculated as percent of WM volume with hyperintense signal (voxels) detected by T₂-weighed MRI imaging in high risk (related) and low risk (not related) JM. Utilizing this method, we found that high risk JMs had larger T₂-weighed MRI lesions following JMRV infection than low risk JMs (**Fig. 6A**). Importantly, the ic injection was not responsible for the larger lesion size, as there was no difference between lesion size in the two groups when injected with vehicle alone (**Fig. 6B**).



All together, these data are consistent with the notion that JME is more similar to MS than to ADEM, and that JME can be induced in susceptible JM lineages by intracranial infection with JMRV. In future studies, it will be important to determine if systemic infection with JMRV is sufficient to induce JME and to better characterize the immune response to JMRV infection. These studies will provide additional clues about the viral etiology of JME and possibly MS.

Task 2: To test the hypothesis that common genetic variants in the JM genome increase the susceptibility of developing demyelinating disease

We analyzed 288 Japanese macaques including 27 spontaneously occurring JME affected animals, 4 experimentally induced JME affected animals, and 7 animals without clinical symptoms but with evidence for brain lesions by MRI analysis (MRI+) for associations between MHC haplotype and JME susceptibility. The study set also included 161 unaffected first-degree relatives (dams, sires, siblings or offspring) of JME affected animals, 48 unaffected first-degree relatives of MRI+ animals and 41 unaffected animals with no known first-

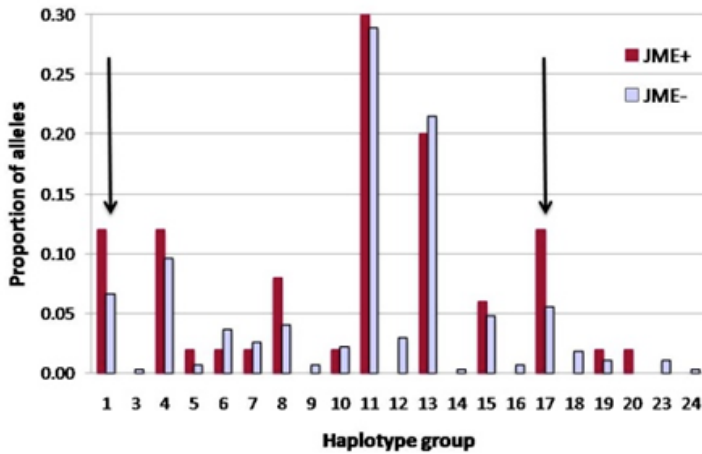


Figure 7. Distribution of 24 MHC haplogroups, by portion of chromosomes within JME cases and non-affected individuals.

degree JME relatives. As shown in our annual progress report (dated May 31, 2012; see Fig. 7), MHC haplogroups 1 and 17 had a statistically suggestive association with JME affected status. We subsequently used RNA sequencing (RNAseq) to identify expressed MHC alleles within the 24 haplogroups, focusing on the Class II DRB locus due to its connection to MS in humans. RNAseq from 26 individuals provided data on 15 of the 24 haplogroups. Suggestive associations include *DRB*014*, *DRB*015*, *DRB*016* and *DRB*017*, although additional studies, with additional individuals and full MHC expression analysis will be necessary to confirm potential association.

Several variants in the interleukin-7 receptor (IL7R) gene and interleukin 2 receptor-alpha (IL2RA) gene are reported to be associated with MS. To examine if these reported single base polymorphisms (SNPs) also occur in Japanese macaques, and whether they are associated with JME disease, we PCR amplified target regions of these genes to sequence in 38 animals of known phenotype (see Preogress Report dated May 31, 2012). The study set included 17 JME affected animals, 9 MRI+ animals, and 11 unaffected animals with no evidence for brain lesions by MRI analysis (MRI-). Although one of the SNPs reported as associated with human MS, rs2104286, was also polymorphic in the Japanese macaques we did not identify any significant associations between SNP alleles and either spontaneous JME or MRI+ status in this study set.

To advance the discovery of sequence variants for JME genetic analysis, we have established exon-sequencing data sets from twelve Japanese macaques and have identified gene variants in 90% of the exonic regions. Functional predictions for the discovered SNPs were generated using ANNOVAR tools. Such genomic approaches will not only expedite the discovery of variants that contribute risk for JME, but they also will provide an important tool for mapping genomic haplotypes that co-segregate with disease across multiple generations.

Similarly, we are developing the first Japanese macaque genome reference sequence, which will improve all sequence mapping and variant detection efforts in the future. In collaboration with Chris Mason, Ph.D., at Cornell Medical School we generated 60x short-read coverage using the Illumina HiSeq platform, and together with PacBio long-read sequence, are assembling a reference genome. The genome, as predicted, is similar in structure to the rhesus macaque, though sequence differences are clearly evident. This points to the fact that as the reference is completed, read mapping will be improved.

Genetic risk factors that underlie the macaque demyelinating disease share at least two key parallels with human MS. First, JME risk is correlated with degree of kinship. Incidence of spontaneous disease has been documented in only 6 of 11 matrilineal ONPRC macaques, and first-degree relatives of JME cases have shown a more robust inflammatory response following intracranial infection of JMRV than those without a family history of JME. Second, the HLA alleles of the MHC locus provide the most consistent and compelling genetic risk factors for MS, and similarly in Japanese macaques, specific MHC alleles suggest an association

with JME susceptibility. In contrast to humans, we found no variants in the IL7R or IL2RA genes that were significantly correlated with macaque demyelinating disease phenotype, although the study size was limiting. We conclude from these studies that JME disease risk mirrors MS disease risk in that it is likely the result of complex interactions of both genetic and environmental factors. We will continue to collect tissue from all new spontaneous JME cases for use in future genetic analyses, enabling improved refinement of the Japanese macaque MHC allele and increased power for testing candidate gene association with JME.

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

As outlined in the year 2 progress report, using primary cultures of dissociated fetal Japanese macaque cerebral cortex, we have been able to demonstrate that *in vitro* infection with JMRV infects microglia, oligodendrocyte lineage cells, and neurons. Infection leads to cell death by oligodendrocytes and to a lesser degree by microglia. These findings complement our studies in JME lesion sections.

Previous studies from the Sherman lab and others indicated that the glycosaminoglycan hyaluronan (HA) accumulates in demyelinating MS and EAE lesions coincident with elevated expression of the HA receptor, CD44 (Back et al., 2005). Digestion products of this HA inhibit oligodendrocyte progenitor cell maturation and prevent remyelination (Preston et al., 2013). We therefore analyzed lesions from both spontaneous (not shown) and induced (**Fig. 8**) cases of JME. CD44 was elevated throughout demyelinating JME lesions in conjunction with elevated HA (**Fig. 8**, arrows). Consistent with our observations in MS patient lesions, HA accumulation was also coincident with astrogliosis (not shown). Overall, these data indicate that JME is both an excellent etiological model of MS and is also appropriate for studies involving interventions for remyelination failure.

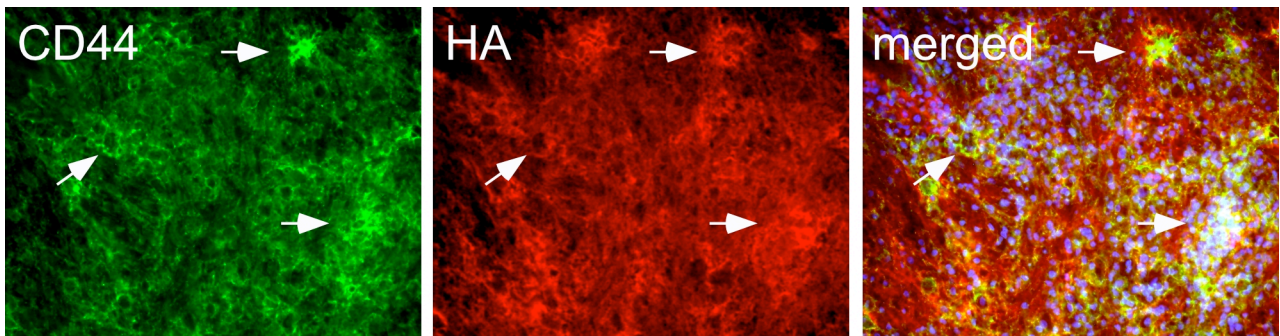


Figure 8: CD44 and HA accumulation in JMRV-induced demyelinating JME lesions from animal JM21572. Green = CD44; red = HA; blue = DAPI (to stain cell nuclei).

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 allowed us to demonstrate that JMRV infects numerous cell types including glia, and that infection induces cell death in oligodendrocyte lineage cells
- Determined that spontaneous and induced JME lesions are characterized by astrogliosis and the accumulation of HA, consistent with observations in human MS.

Reportable Outcomes

Rooney WD, Kohama SG, Wang P, Njus JM, Wong SW, Sherman LS, Axthelm MK, Marracci G, Bourdette D. 2009. MRI Estimation of Sub-Clinical Disease in Japanese Macaque Encephalomyelitis. Proc. Intl. Soc. Mag. Reson. Med. 17:836. Abstract.

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Axthelm MK, Bourdette DN, Marracci GH, Su W, Mullaney ET, Manoharan M, Kohama SG, Pollaro J, Witkowski E, Wang P, Rooney WD, Sherman LS, Wong SW. 2011. Japanese macaque encephalomyelitis: a spontaneous multiple sclerosis-like disease in a nonhuman primate. Ann Neurol. 70(3):362-73.

Estep RD, Hansen SG, Rogers KS, Axthelm MK, Wong SW. 2013. Genomic characterization of Japanese macaque rhadinovirus, a novel herpesvirus isolated from a nonhuman primate with a spontaneous inflammatory demyelinating disease. J Virol. 87(1):512-23.

Conclusions

We have demonstrated that JME is an MS-like disease, sharing many of the pathological characteristics of MS in both spontaneous and induced cases. We have established that intracranial infection with a gamma-herpesvirus (JMRV) can induce JME, 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. Furthermore, we have shown that JMRV can infect microglia, lymphocytes and macroglia, and is cytotoxic to cells in the CNS. HA accumulates in JMRV-induced lesions, suggesting a mechanism for remyelination failure in JME. 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. JME therefore represents a novel model of MS that can be used to explore ways to prevent disease onset, prevent attacks, and repair the damaged CNS.

Abbreviations

ADEM	-	acute demyelinating encephalomyelitis
EAE	-	experimental autoimmune encephalomyelitis
HA	-	hyaluronan
IL2RA	-	interleukin 2 receptor-alpha
IL7R	-	interleukin-7 receptor
IL-17	-	interleukin-17
JM	-	Japanese macaque
JME	-	Japanese macaque encephalomyelitis
JMRV	-	Japanese macaque rhadinovirus
MBP	-	myelin basic protein
MHC	-	major histocompatibility complex
MOG	-	myelin oligodendrocyte glycoprotein
MRI	-	magnetic resonance imaging
MS	-	multiple sclerosis
ONPRC	-	Oregon National Primate Research Center
PLP	-	proteolipid protein
SNPs	-	single base polymorphisms
vIL6	-	viral interleukin-6

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