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Being a mouse in a man's world: what TMEV has taught us about human disease

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1. ABSTRACT

Choosing an appropriate animal model to study a disease is guided by a variety of factors including but not limited to the questions being asked, availability of reagents, knowledge of the animal species, personal biases of the researcher, and in some cases, cost and availability of facilities to effectively investigate the model. The validity of an animal model can be further complicated when the etiology of the disease is incompletely defined. Examples of these diseases include multiple sclerosis (MS) and type 1 diabetes (T1D). In addition to host genetics, epidemiological studies have implicated infectious agents, in particular viruses as triggers of these diseases. Thus many studies of these diseases have focused on modeling the interactions of viruses and the host immune response *in vivo* in small animals. Theiler's murine encephalomyelitis virus (TMEV) infection of mice has been used for over 30 years as a model of virus-induced demyelination. TMEV induces a MS-like disease in susceptible strains of mice but does not cause pathology in humans. While some researchers may question the rationale for using a non-human pathogen to model human disease, the TMEV model of central nervous system (CNS) demyelination has permitted study of some aspects of human MS which would have been difficult to address in other models of the disease. Despite being 'merely a disease of mice,' many of the findings in the Theiler's virus model are directly applicable to the human condition, and studies from the model are responsible for our current understanding of mechanisms of pathology and clinical disability in human MS. In this review we will present some of the key findings from the TMEV model in the context of human disease.

2. AN OVERVIEW OF THE THEILER'S MODEL OF DEMYELINATING DISEASE

Theiler's murine encephalomyelitis virus (TMEV) is a positive-stranded RNA virus belonging to the family Picornavirade (genus *Cardiovirus*). There are two subgroups of TMEV – GDVII and TO. The GDVII subgroup viruses (FA and GDVII) are highly neurovirulent and cause necrotizing encephalitis and death in the murine host within 7 days of intracerebral (i.c.) infection even at very low doses (1). Intracerebral infection of mice with TO subgroup viruses (DA and BeAn) induces acute encephalitis between days 5 and 10 post-injection (p.i.) in all strains of mice. During this phase of infection virus replicates primarily in neurons and viral titers escalate rapidly (2). Within two weeks of infection, virus is cleared from the gray matter of the brain. In mouse strains resistant to persistent infection ($H\text{-}2^{d,\ b}$) (3-5)TMEV is effectively cleared from the central nervous system (CNS) by a strong cytolytic T lymphocyte (CTL) response directed against the VP2 viral capsid protein (6,7). These animals recover and experience no obvious functional deficits as a result of the infection. Some strains of mice are unable to efficiently clear virus from the CNS and a persistent infection is established in the white matter of the brain and spinal cord, where demyelination and chronic inflammation develops. The infiltrate is comprised of CD4+ T cells, CD8+ T cells, B cells, and activated microglia/macrophages (8). Low-levels of virus can be detected in oligodendrocytes (9,10), microglia/macrophages (2,11,12), and astrocytes (10). In the BeAn strain the macrophages are the main reservoir of virus in chronically infected mice (11). Despite the high degree of sequence homology between DA and BeAn (13), the disease observed in DA-infected mice is distinct from

BeAn-induced disease. DA-infected mice have higher levels of spinal cord demyelination, increased functional deficits, higher levels of virus-specific RNA and protein, and lower levels of TMEV-specific antibody compared to BeAn-infected animals (14).

The initial demyelination in the TMEV model is due to direct viral damage to the myelin-producing oligodendrocyte. Ongoing chronic demyelination is attributed to the development of autoimmune responses to previously sequestered self-epitopes, a phenomenon known as epitope spreading (15-17). Clinical signs of chronic TMEV infection are similar to those observed in patients with chronic progressive MS. These include spasticity, incontinence, weakness of the extremities, and eventually paralysis (2,18). Akin to the oligoclonal bands found in the cerebrospinal fluid (CSF) of MS patients, i.c. infection of susceptible strains of mice with TMEV results in intrathecal antibody production (19).

The wide availability of mutant and knockout mice has permitted the study of the contribution of various immune system components on susceptibility or resistance to demyelination (20-31), neuronal damage (20,21,32-34), and in some cases, death of the host (24,32,35). Mice lacking adaptive immune systems die within two weeks of i.c. infection even when on a genetic background that is resistant to infection (32,35,36). In addition, some immune component deficiencies may result in altered patterns of brain pathology in the acute phases of disease (20,21,32). While Theiler's virus infection of mice has been used primarily as a model of demyelination, TMEV infection of mice has been used to explore many more scientific questions beyond modeling MS. TMEV infection of mice has also been successfully used to model T cell priming and antigen presentation in the CNS (17,37-41), mechanisms of virus transport from the PNS to the CNS (42-44), and understanding mechanisms of myelin damage (45) and repair (46-48).

3. MULTIPLE SCLEROSIS – A SHORT COURSE

Multiple sclerosis is the most common demyelinating disease of the CNS in humans, with more females affected than males (49-51). The disease is focal in nature and the lesions contain inflammatory infiltrates consisting of T cells, B cells, and macrophages (52). The disease course in individuals diagnosed with MS is unpredictable although most individuals experience increasing loss of function as time progresses. Autoreactive T cells have been identified against several myelin components including myelin basic protein (MBP) (53-55) and proteolipid protein (PLP) (53, 56, 57). As most studies have focused on individuals after diagnosis, the contribution of the autoreactive T cells to disease onset is murky.

The etiology of MS is unknown, but both host genetics and environmental factors are likely involved in disease development (58-64). A variety of genetic associations have been made between various HLA alleles and an increased relative risk rate of MS development (58, 59, 65-68). The first genes implicated in the development of MS were HLA class I alleles – key participants in the immune response to viruses (61), suggesting that the response to intracellular pathogens may be important in disease development. In addition to particular HLA alleles, polymorphisms in other genes that affect immune function have been made (69-76). Despite extensive study, no consensus has been reached with regard to which genes are most important in MS susceptibility. The results of the genetic studies do support a role for multiple genes in determining MS risk.

While the risk of disease development increases when a first degree relative is affected, genetics alone do not adequately explain disease development (77-83). Long-term studies in the Faroe Islands in the decades following World War II and migration studies have supported a role for infectious agents in disease onset (60, 62-64, 84, 85). Since these original epidemiological studies, much work has focused on identification of pathogens that may trigger disease. The most commonly implicated class of pathogens in the development of MS is viruses (86-94). Viruses have been implicated or eliminated as causes of the disease based on their presence or absence in a demyelinating lesion, or the level of virus-specific antibody in the patient at or near diagnosis. Some of the pathogens proposed as triggers of MS include rabies (86,87), human herpes virus 6 (95, 96), measles (96-100), adenovirus (88-89), parainfluenza type 1 (90-93, 101), rhinovirus (102), and Epstein Barr virus (94, 103, 104).

Based on studies in the TMEV model of MS, mechanisms of demyelination that are triggered by virus may or may not require the presence of viral antigen at the lesion site at the time of clinical disease onset. Studies from the laboratory of Stephen Miller have demonstrated that while virus is required to initiate demyelination, it is ultimately the ongoing immune response to newly exposed self-epitopes that is responsible for the chronic, increased levels of demyelination that are observed (15-17). The specificity of the autoreactive cells that develop changes over time in a predictable manner (15-17). Extending these findings to the human disease, one could postulate that while a virus (or viruses) may trigger the disease in humans, it is long-gone by the time of diagnosis.

Diagnosis of MS is difficult, as similar symptoms related to other disease conditions must be considered. Diseases that must be excluded include Lyme Disease, sarcoidosis, vascular disease, syphilis, genetic diseases, or structural conditions such as herniated disks or tumors that may impair nervous system function. Other diagnostic criteria include the presence of oligoclonal bands in the CSF and gadolinium-enhanced plaques in the spinal cord and brain upon magnetic resonance imaging (MRI) (105-107). Currently lesion activity, as defined by MRI visualization, is the key pathological feature of MS both in diagnosis and in monitoring disease progression.

4. AXONAL INJURY IN DEMYELINATING DISEASE. DOES DEMYELINATION REALLY MATTER?

4.1. Axonal injury in multiple sclerosis

Human multiple sclerosis has long been characterized as a primary demyelinating disease. The hallmark pathology of the disease is a loss of myelin, with relative sparing of the axon (108). Despite this categorization, the clinical course of the disease is perplexing because no firm association between the extent of demyelination lesions and patient disability has been described. This lack of correlation between lesion load and clinical disability is referred to as the clinico-radiological paradox (109,110). Until relatively recently, axonal damage has been thought to be a long-term sequelae that results from the assault on the denuded axon by the immune response (108). Increasing evidence however currently suggests that axonal damage occurs significantly earlier than previously thought, and demyelination alone is not the cause of patient disability (108,111-116). Axonal injury results in axonal transection, a condition for which there is no treatment. In the past decade numerous studies have demonstrated that at least in some instances, axonal damage occurs in areas of normal appearing white matter of MS patients (111-114).

To examine whether there were alterations in axonal density in normal appearing white matter in patients with MS, Bjartmer *et al.* examined autopsy tissue from a patient with acute MS. Using immunohistochemical staining with an antibody to neurofilament protein as a marker for axons, these studies demonstrated that there was a decrease in axonal density of approximately 22% in normal appearing white matter in the the MS patient as compared to the axonal density in the CNS tissue from individuals with no known neurological pathology (111). In the course of these studies, the authors also demonstrated that despite the significant level of axonal dropout, myelin sheaths devoid of axons were also apparent. These myelin sheaths were either intact or collapsed upon themselves. In addition, macrophages containing myelin debris were also detected, indicating that myelin was also disrupted (111). Similar immunohistochemical studies have confirmed the observation that axonal dropout occurred in normal appearing white matter in autopsy tissue from MS patients. Decreases in axonal density of up to 65% were observed (112-114). The axons at greatest risk were the small axons (113,114). Despite the sex bias in MS patients, no difference in the level of axonal dropout has been reported between the sexes (114).

While these findings were of interest, these studies did not address whether the axonal pathology was old or relatively recent. To address this question, immunohistochemical staining of biopsy tissue from MS patients was performed using amyloid precursor protein (APP) as a marker of acute axonal injury (117). Acute axonal injury was defined as damage that occurred within the last month. The amount of acute axonal injury varied with the subtype of MS in the patient. Patients with primary progressive MS had reduced levels of APP staining; patients with secondary progressive MS had much higher APP-positive staining than other forms of MS (117). APP was found in areas of demyelination, remyelination, and normal appearing white matter (117) indicating that axons in areas undergoing damage, invoking repair mechanisms, and normal appearing tissue were all vulnerable to axonal damage.

The main focus of treatments used in MS patients is targeted toward reducing lesion activity and relapses as a measure of success (118-122). Given that significant numbers of axons are damaged early on in some forms of MS, the view that clinical sequelae can be prevented if myelin sheaths can be repaired relatively quickly after the onset of demyelination may be naïve and outdated. This is not to say that reducing or repairing demyelination in the treatment of MS is not warranted. As myelination impacts conduction velocities along the axon, reduced myelination most certainly negatively impacts clinical symptoms in the patient. However, it is imperitive that alternative measures be used to assess treatment efficacy.

4.2. What has Theiler's virus taught us about demyelination and axonal injury?

The study of chronic infection of mice with Theiler's virus has been used to demonstrate the disparity between the extent of demyelination in the host and the level of clinical disability. Studies from this model first provided an experimental model demonstrating that clinical deficits and demyelination were independent of each other and ultimately providing a potential mechanism explaining the clinico-radiological paradox (123-126). Using beta2 microglobulin-deficient mice on a background resistant to demyelination (C57Bl/6J x 129), Rivera-Quinones *et al.* demonstrated that demyelinating lesions developed following intracerebral infection with Theiler's virus (123). These beta2 microglobulin-deficient mice, devoid of both MHC class I expression and CD8+ T cells, were unable to mount CTL responses (127). Despite the presence of these large areas of demyelination, spontaneous clinical activity and hindlimb evoked potentials in the virus-infected mice were similar to those observed in mice that were infected and efficiently cleared TMEV from the CNS (123). The retention of normal function in the TMEV-infected beta2 microglobulin-deficient mice parallels human cases of asymptomatic MS (128). TMEV-infected beta2 microglobulin-deficient mice also had increased sodium channel levels in the CNS, as well as relatively well-preserved axons, findings that would provide the basis for the normal evoked potentials measured in these animals (123). The development of this model permitted further studies on the role of CD8+ T cell/MHC class I interactions in the development of functional deficits in demyelinating diseases.

The beta2 microglobulin knockout mice have been the focus of intense study since their initial characterization (23, 124-126). One possibility for the observed differences in clinical function between beta2 microglobulin-deficient mice and immunocompetent control mice susceptible to the development of large areas of demyelination and functional deficits was that the two

strains of mice developed lesions in different areas of the spinal cord. Simply put, the lesions in the beta2 microglobulin-deficient mice, while large, were in areas of the spinal cord that were less critical to motor function. To address this potential mechanism of clinical function preservation in the beta2 microglobulin-deficient mice, geographic distribution of the lesions and the extent of remyelination were examined. Beta2 microglobulin knockout mice and SJL/J mice, a strain of mice that experience significant loss of function following TMEV infection were used in these studies (125). The hypothesis being examined was that the location of the demyelinated lesions was the key determinant as to whether clinical deficits developed. Morphometric studies determined that both the lesion size, geographic distribution of the lesions, and the degree of remyelination in these two strains of mice were similar. Using retrograde neuronal labeling to measure the level of axonal injury, it was determined that compared to beta2 microglobulin-deficient mice, SJL/J mice had reduced retrograde labeling of neurons in the major motor tracts compared to the beta2 microglobulin-deficient mice (125). Together, these studies provide further evidence for the hypothesis that demyelination and axonal damage are independent of each other. These studies also implicate a role for CD8+ T cells in impacting axonal health.

4.3. Mechanism of axonal injury in the TMEV model

Key to induction of CTL response are that MHC class I molecules displaying a peptide in the binding cleft that corresponds to an appropriate T cell receptor on a CD8+ T cell. Under normal conditions, MHC molecules are not expressed in the brain and spinal cord. However, damage (infection, trauma, physiological) can result in the induction of MHC molecules in the affected area (129, 130). Thus, following damage or infection, CNS resident cells acquire the ability to present antigen to T cells. Increased expression of class I on the demyelinated axons in patients with MS has been reported, thereby demonstrating that one of the requirements for CTL-mediated damage to the axons is fulfilled (131). In studies exploring the interactions between CD8+ T cells and neurons in an *in vitro* setting, the hypothesis that cytotoxic T cells were directly responsible for damage to neuritis was tested (132). Murine neurons were pulsed with lymphocytic choriomenigitis virus (LCMV)-derived peptides and then co-cultured with LCMV-specific CD8+ T cells. The CD8+T cells attached to the neuritis and within 3 hours, changes to the neurite cytoskeleton consistent with transaction of the neurite were observed (132). No structural abnormalities were observed in neuritis when control peptides or neurons devoid of class I expression were used, indicating that the structural changes were the result of antigen-specific class-I mediated responses (132).

While studies in the beta2 microglobulin-deficient mice demonstrated that demyelination and axonal damage were not interdependent, they did not address the observation in human tissue that there are damaged axons in the normal appearing white matter (111-114). The concept that axonal injury is a sequela to demyelination has also been examined in the TMEV model. Using nonphosphorylated neurofilament protein as a marker of axonal damage, studies using SJL/J mice demonstrated that axonal injury was detected by one week p.i. with the DA strain of TMEV (133). At this time-point in infection the majority of virus was localized to the neurons. As the infection progressed the number of nonphosphorylated NPP immunoreactive axons increased in the spinal cord. Histologically there was an increase in the amount of axonal swelling in normal appearing white matter over time. These studies were significant as they provided evidence that axonal injury did not occur solely as a secondary event following demyelination (133). TMEV antigens rarely co-localized with axons indicating that direct virus-induced axonal damage was likely. Furthermore, similar to the pathology described in MS, empty myelin sheaths were observed indicative of axonal degeneration (111,133). Using the highly neurovirulent GDVII strain of TMEV, similar studies determined that this strain of TMEV also induced high levels of axonal swelling and degeneration in normal appearing white matter. As GDVII-infected animals do not demyelinate, these data demonstrate the independence of these distinct pathologies.

Two proteins are involved in CTL-mediated killing. Perforin is responsible for the generation of channels on the target cells, and granzymes enter the cell and cause damage to the target cell's DNA. To test the contribution of perforin to axonal degeneration and clinical deficits, perforin-deficient animals on a C57BL/6J background (that is, animals that can mount a vigorous CTL response and clear virus) were infected with TMEV and examined six months later. TMEV-infected perforin-deficient mice developed demyelinating lesions throughout the spinal cord but motor function and large diameter axons remained preserved (126). The levels of function and axonal preservation were similar to those observed in immunocompetent wild-type mice. In contrast, TMEV-infected mice with CD4+ T cell deficits experienced similar levels of demyelination as the perforin deficient mice but also experienced a loss of function. In addition, CD4-deficient mice infected with TMEV experienced a loss of large diameter axons (126). In these studies, demyelination alone was not a predictor of clinical function although the extent of loss of large diameter axons could be correlated to clinical disease. Similar to the *in vitro* studies demonstrating the antigen-specific nature of the CTL-mediated damage to the neuritis *in vivo* studies demonstrated that depletion of the VP2 121-130-specific T cells significantly reduces the damage in the TMEV model (124).

Because MS is typically categorized as an autoimmune disease the main focus of study in MS patients, as well as animal models, had been the host immune response. Despite the obvious interest in the immune response, the role of non-immune factors in the establishment of disease has also been of great interest. Recently, the role of myelin in the establishment of TMEV persistence has been explored. In these studies two mouse strains with myelin defects were studied and have presented us with new paradigms to understand the mechanism of viral persistence in this model. *Shiverer* mice have a large

deletion in the myelin basic protein gene resulting in extremely low levels of myelin production (134-137). Rumpshaker mice have an X-linked mutation in proteolipid protein gene which results in dysmyelination and increased numbers of oligodendrocytes (138). Even when infected with high doses of TMEV, it is not possible to induce a persistent infection in these mouse strains (139). In contrast, infection of wild type control mice with much lower doses of TMEV results in virus persistence and subsequent demyelination. These data cannot be explained solely by the immune response in the context of epitope spreading as the immune response to myelin basic protein is not one of the early identified self-reactivities (16).

To explore the basis of this protection from persistent TMEV infection, the optic nerve was used to model axon, myelin, and virus interactions (45). These studies demonstrated that the axons of infected neurons are a key component in permitting infection of the cytoplasmic channels of myelin. It was postulated that the virus attempts to gain a survival advantage in the host by establishing itself in an environment distal from the demyelinating lesion, which is the main target of the immune response (45). What is the relevance of these findings in the context of human multiple sclerosis? The data, while relatively new, provide a mechanism by which viruses could induce MS in humans, and provide an explanation as to the lack of viruses that have been identified at the lesion site.

5. LESSONS FROM THE ACUTE PHASE OF TMEV INFECTION

All strains of mice, regardless of their genetic background, experience the acute phase of TMEV infection characterized by high levels of virus replication in the neurons (2, 18). This virus-induced encephalitis has permitted the study of the role of immune system components in protection of discrete areas of the brain from TMEV-mediated disease (20, 21, 32-34). It has been observed in viral encephalitis in humans that certain viruses induce distinctive patterns of pathology in the brain. For example, rabies localizes primarily to the pons and medulla, while herpes simplex virus-1 induces disease that is localized to the frontal and temporal lobes (140). While one possibility is that specific patterns of brain disease are related to virus receptor distribution, the host immune response also appears to significantly impact where pathology will occur.

To examine the role of specific immune system components on brain pathology, a series of mice with various immune system participants knocked-out were i.c. infected with TMEV and sacrificed at day 16 p.i. This time-point was chosen as by this time virus has been cleared from the brains of immunocompetent mice that are capable of generating CTL responses sufficient to clear virus from the host. Using mice deficient in MHC class I or II, alpha/beta TCR or antibody, it was demonstrated that class I-mediated immune responses are critical in clearing virus from areas of the brain rich in white matter, while areas abundant in neurons (i.e., gray matter) are protected primarily by antibody (32). Given that white matter areas profoundly upregulate MHC class I levels following virus insult (141), it is logical that protective responses are induced that exploit this arm of the immune system. As neurons less efficiently upregulate MHC after virus infection, the dependence on antibody-mediated protective responses would be expected. Further studies using mice deficient in other immune systems components (ICAM-1, CD40, IL-6) have supported these initial observations (20, 21, 33, 34).

The continuous stream of knockout mice available to investigators will permit further dissection of immune system components to protection from virus-induced damage. Furthermore, utilization of this approach with different viruses will allow us to determine whether the patterns of brain pathology are unique for TMEV or reflect general patterns for particular classes of viruses.

6. INFECTION OF THE PERIPHERAL NERVOUS SYSTEM WITH TMEV

Peripheral nervous system infection with TMEV is an area of research that has been examined in a minimal number of studies. The natural route of CNS infection with TMEV in the wild is unknown. Because TMEV is transmitted via an oral-fecal route in the wild, it is likely that the CNS infection occurs via the peripheral nervous system or possibly, the blood. A small number of studies have examined the dynamics of virus spread from the peripheral to the central nervous system (42-44). To examine whether TMEV could enter the CNS from the PNS via axonal transport, mice were injected into the footpad with the highly neurovirulent GDVII strain of TMEV (44). Within one week, virus was detected in the spinal cord. Initially paralysis was observed in the injected limb, and subsequently in the contralateral hindlimb. Cholchicine, an inhibitor of fast axonal transport, was used and prevented transport of the virus into the CNS, demonstrating a microtubule-dependent mechanism of transport of TMEV from the periphery to the CNS (44).

More recently, studies were performed that propose a route for infection of the CNS with TMEV in the wild. Injection of either the tongue or the hypoglossal nerve with TMEV resulted in spread of the virus to the CNS as measured by the induction of paralysis (43). The results of the intratongue injections are significant, in that one could envision a scenario in the wild whereby a natural infection could travel to the CNS via a breach in the surface of the tongue, similar to one of the proposed mechanisms of transmission of prion diseases (142).

Our laboratory recently developed a model of direct injection of virus into the sciatic nerve with a goal of using this model to study myelin repair of the peripheral nervous system (42). While it has been well-described that the PNS is more efficient at repair than the CNS, few opportunities exist to directly examine the differences in the processes, as the lesioning methods used in the PNS and CNS vary. Further development of this sciatic nerve model, as well as our model of direct CNS lesioning (143),

will permit study of these processes without the complication of an additional variable (that is, the method by which the lesion was made).

7. SUMMARY AND PERSPECTIVES

The study of non-human pathogens that are not of agricultural interest is sometimes denigrated by those working with human pathogens (aka, 'my virus is better than your virus'). The concept that one cannot advance the understanding of human disease by studying a mouse virus is, in our view, short-sighted. Certainly, our understanding of axonal damage in multiple sclerosis would not be as advanced as it is without the TMEV model. The ability to utilize a small animal model in concert with human histopathological studies provides investigators with an excellent opportunity to test and understand mechanisms of pathology, and to gain confirmatory data from human samples. Furthermore other aspects of the model, such as the acute phase of disease or infection of the peripheral nervous system provide ample opportunity for further study of human diseases other than multiple sclerosis.

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Abbreviations: CNS: central nervous system; CSF: cerebrospinal fluid; CTL: cytotoxic T lymphocyte; i.c.: intracerebral; MBP: LCMV: lymphocytic choriomeningitis virus; MRI: magnetic resonance imaging; MS: multiple sclerosis; NFP: neurofilament protein; p.i.: post-infection; PLP: proteolipid protein; PNS: peripheral nervous system; TCR: T cell receptor; TMEV: Theiler's murine encephalomyelitis virus

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