Published in final edited form as:

J Neurovirol. 2015 February ; 21(1): 43–55. doi:10.1007/s13365-014-0297-8.

Manipulation of host factors optimizes the pathogenesis of western equine encephalitis virus infections in mice for antiviral drug development

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Abstract

While alphaviruses spread naturally via mosquito vectors, some can also be transmitted as aerosols making them potential bioterrorism agents. One such pathogen, western equine encephalitis virus (WEEV), causes fatal human encephalitis via multiple routes of infection and thus presumably via multiple mechanisms. Although WEEV also produces acute encephalitis in non-human primates, a small animal model that recapitulates features of human disease would be useful for both pathogenesis studies and to evaluate candidate antiviral therapies. We have optimized conditions to infect mice with a low passage isolate of WEEV, thereby allowing detailed investigation of virus tropism, replication, neuroinvasion, and neurovirulence. We find that host factors strongly influence disease outcome, and in particular that age, gender and genetic background all have significant effects on disease susceptibility independent of virus tropism or replication within the central nervous system. Our data show that experimental variables can be adjusted in mice to recapitulate disease features known to occur in both non-human primates and humans, thus aiding further study of WEEV pathogenesis and providing a realistic therapeutic window for antiviral drug delivery.

Keywords

Encephalitis; Alphavirus; Host factors; Pathogenesis; Western equine encephalitis virus

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Conflict of Interest

The authors declare that they have no conflict of interest.

Introduction

Unlike the old-world alphaviruses (Chikungunya virus, O'nyong-nyong virus, and Ross River virus) that cause acute febrile illnesses and debilitating polyarthritides, infections caused by the new-world alphaviruses (Venezuelan equine encephalitis virus (VEEV), eastern equine encephalitis virus (EEEV), and western equine encephalitis virus (WEEV)) cause overt encephalitis in a proportion of infected equine and human hosts. While VEEV usually precipitates widespread epizootics in horses that lead to spillover outbreaks of encephalitis in humans, EEEV and WEEV typically cause single cases or small clusters of human infections. The epidemiology of these diseases has been well described, including factors controlling the mosquito vectors that normally transmit them (Calisher 1994; Johnston and Peters 1996). Encephalitis caused by the unintentional aerosol exposure of humans to these pathogens has been documented in the laboratory setting (Hanson et al. 1967), and this secondary route of infection, coupled with their ease of production and their capacity to cause debilitating disease, make the encephalitic alphaviruses potential bioterrorism agents. Large alphavirus outbreaks have also recently been documented in previously unaffected geographical regions, making them important emerging pathogens from a public health perspective (Weaver 2014). The lack of any licensed vaccines or other disease countermeasures further underscores the risk posed by these viruses and highlights the need for a more detailed understanding of disease pathogenesis.

WEEV has been isolated from humans, horses, birds, and small mammals across a region spanning from western Canada to Argentina since 1930 (Calisher 1994; Johnston and Peters 1996; Meyer et al. 1931). Human cases of western equine encephalitis (WEE), once fairly common, have declined steadily since the mid-20th century (Calisher 1994; Forrester et al. 2008). As a result, detailed clinical and pathological findings associated with natural human infections are lacking. Limited epidemiological data suggest a case fatality rate ranging from 3-15%, with variable outcomes attributed to age of the host, viral strain, and the amount and route of virus exposure (Steele and Twenhafel 2010; Zacks and Paessler 2010). Up to 30% of human survivors incur permanent neurological deficits (Zacks and Paessler 2010). Mortality from the few accidental cases of WEE described amongst laboratory workers is 40% (Hanson et al. 1967), raising some question as to whether aerosol transmission facilitates neuroinvasion. Inhaled WEEV causes acute encephalitis in non-human primates, with overt neurological signs and death occurring over a period of 8-12 days in 75% and 25% of infected animals, respectively (Reed et al. 2005). This primate model will be useful for final preclinical testing of vaccines and other therapeutics once they become available, but it remains cumbersome for drug screening and disease pathogenesis studies. Rodent infections are much better suited for these experimental purposes, provided they adequately recapitulate disease features known to occur in higher mammals.

A handful of prior studies have shown that rodents can be infected with WEEV. In hamsters, either intranasal or intraperitoneal viral challenge leads to rapid central nervous system (CNS) spread and 100% mortality without causing overt neurologic signs over 4-5 days (Julander et al. 2007). This extreme host susceptibility differs from what occurs in primates and humans, and its rapid course requires that candidate therapies work in a very narrow therapeutic window. Murine infections typically evolve over a somewhat longer time frame

(Logue et al. 2009; Phillips et al. 2013; Phillips et al. 2014), and peripheral inoculations do not always cause encephalitis in some host strains (Nagata et al. 2006; Julander et al., 2009). Different WEEV isolates segregate into two general groups in mice based on mortality when the interval between virus inoculation and death is measured (Logue et al. 2009; Mossel et al. 2013; Nagata et al. 2006). On the other hand, a thorough characterization of host determinants that influence disease outcome has never been reported. Extensive data generated in the murine Sindbis virus (SV) encephalitis model, a related alphavirus that does not infect the human CNS but that does have significant sequence homology with WEEV (Hahn et al. 1988), show that age, genetic background, and virus-induced host responses all strongly influence survival (Byrnes et al. 2000; Irani and Prow 2007; Johnson et al. 1972; Kimura and Griffin 2000; Kimura and Griffin 2003; Liang et al. 1999; Rowell and Griffin 2002; Ryman et al. 2000; Stanley et al. 1986; Thach et al. 2000; Tucker et al. 1996). These findings suggest that similar host factors could also influence disease outcome in mice infected with WEEV.

In the present study, we sought to better characterize murine WEEV infections from the standpoint of the host. Our goals were to define a model that closely resembles human disease for further studies of WEEV pathogenesis, and to optimize an experimental system that would allow testing of novel antiviral compounds presently under development in our group (Delekta et al. 2014; Peng et al. 2009; Raveh et al. 2013; Sindac et al. 2012; Sindac et al. 2013). Here, we report that host factors exert substantial effects on WEE outcome in mice, and that experimental conditions can be adjusted so that the clinical, virological, and histopathological findings are made to resemble those occurring in both non-human primates and humans.

Materials and Methods

Virus

The Cba 87 strain of WEEV was generated from the cDNA clone pWE2000 as previously described (Castorena et al. 2008; Peltier et al. 2013). A low passage stock of infectious virus generated in Vero cells was expanded twice in C6/36 mosquito cells to produce viral stocks containing 1×10^7 plaque-forming units (pfu)/ml. All virus aliquots were stored in single use vials at -80°C and were not subjected to repeated freeze-thaw cycles. Experiments using infectious WEEV were conducted in an animal-compatible, federally certified Biosafety Level-3 (BSL3) containment facility in strict accordance with standard operating procedures and protocols approved by the University of Michigan Institutional Biosafety Committee.

Animal manipulations

Inbred mice used for all our experiments were obtained from Harlan Laboratories (South Easton, MA) or Jackson Laboratories (Bar Harbor, ME). All animal procedures were undertaken using BSL3 containment in strict accordance with protocols approved by the University of Michigan Committee on Use and Care of Animals. Virus inoculations and tissue collections were performed under isoflurane anesthesia (Abbott Laboratories, Chicago, IL). Mice were housed in our animal BSL3 facility on a 10-hour/14-hour light/dark cycle with food and water available *ad libitum*.

To induce WEEV infection, recipient mice were inoculated with $10^2 - 10^6$ pfu of Cba 87 suspended in a total volume of 50 μ l of PBS via intraperitoneal (i.p.), subcutaneous (s.c.), or intranasal (i.n.) routes. Both i.p. and s.c. doses were directly injected using a 26-gauge needle, while i.n. dosing was accomplished by gently pipetting 25 μ l of the inoculum into each nostril of supine anesthetized animals. Cohorts of infected mice were weighed and scored daily using a clinical neurobehavioral rating scale previously established for WEEV infection (Wu et al. 2007) as follows: 0, normal; 1, slightly ruffled fur, no visible signs of infection; 2, very ruffled fur, definite signs of infection with reduced cage activity; 3, very ruffled fur, hunched posture, reduced mobility due to paralysis; 4, very ruffled fur, hunched posture, little to no mobility, rapid breathing (moribund); 5, dead. Animals reaching a score of 4 with severe neurologic signs were euthanized immediately and were considered to have died the next day for all statistical comparisons.

To measure the amount of infectious virus present in tissues and body fluids, mice were euthanized via an overdose of isoflurane, cerebrospinal fluid (CSF) gently aspirated by cisternal puncture using a pulled glass capillary pipette, and blood collected by cardiac puncture. Serum was isolated from whole blood by centrifugation, and serum and CSF samples were stored at -80°C until further use. Once blood and CSF samples were collected, animals were then perfused extensively with ice-cold PBS, and individual tissues removed, weighed, snap-frozen on dry ice, and stored at -80°C until virus titrations assays could be performed. For histopathology studies, donor mice were sequentially perfused with PBS and then PBS containing 4% paraformaldehyde (PFA). Brains were carefully removed and post-fixed overnight in PBS with 4% PFA before processing.

Virus titration assays

Frozen samples were thawed, and 20% (w/v) homogenates were prepared in PBS from each solid tissue sample. Serial 10-fold dilutions of each homogenate or each liquid phase sample were then assayed for plaque formation on monolayers of Vero cells as described (Peltier et al. 2013; Raveh et al. 2013). Results presented are the mean or the mean $\pm$ SEM of the $\log_{10}$ of viral pfu/gm of tissue (all solid tissues) or the mean or mean $\pm$ SEM of the $\log_{10}$ of viral pfu/ml (serum, CSF) derived from 3 animals at each time point.

Measurement of tissue viral RNA levels

Tissue RNA extraction, primer sequences specific for WEEV envelope glycoprotein 1, and quantitative reverse transcription-PCR (qRT-PCR) was all performed as previously described (Peltier et al. 2013). In brief, cDNA was generated from total tissue RNA using the iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA) in strict accordance to the manufacturer's instructions. qRT-PCR was then performed in triplicate samples from each group using iQ SYBR Green Supermix (Bio-Rad) and a CFX96 Real Time thermal cycler (Bio-Rad), and fluorescence threshold cycles (Ct) was determined using the CFX96 Manager software. Viral RNA levels were normalized to 18S rRNA levels by calculating the Ct value of each sample, and reported as $1/ -Ct$ as shown.

Anti-WEEV capsid antisera

To generate polyclonal antisera against the WEEV capsid protein, the capsid region from pWE2000 (Castorena et al. 2008) was cloned into the pET-28a(+) expression vector (EMD Millipore, Billerica, CA) that included both C- and N-terminal 6His epitope tags to facilitate purification. Recombinant WEEV capsid protein was then expressed in *E. coli* KRX cells (Promega, Madison, WI), purified by Ni²⁺-affinity chromatography, and used to immunize rabbits for antisera production (Harlan). Rabbit antisera was tested for both WEEV reactivity and specificity by immunoblotting with lysates derived from mock- or WEEV-infected BHK-21 or BE(2)-C cells, and then used for immunohistochemical analyses as described below.

Histopathology

Post-fixed brains were cryopreserved in 30% sucrose overnight and flash frozen in optimal cutting temperature (OCT) compound for cryosectioning. Ten µm sections were first treated with 1% hydrogen peroxide in methanol to block all endogenous tissue peroxidase, and then blocked in 2% normal goat serum (Vector Laboratories, Burlingame, CA). Slides were washed, incubated with a 1:250 dilution of the astrocyte marker, glial fibrillary acidic protein (GFAP, Abcam, Cambridge, MA), for 1 hour at room temperature (RT), washed extensively again, and then treated with biotin-labeled goat anti-mouse IgG (Vector Laboratories) at a 1:200 dilution for another hour at RT. Other sections were treated with 1:500 dilution of anti-WEEV capsid rabbit antisera for 1 hour at RT, washed, and then treated with 1:200 goat anti-rabbit IgG (Vector Laboratories) for another hour at RT. These steps were followed by sequential incubations with avidin-DH-biotin complex (Vector Laboratories) and then 0.5 mg/ml 3,3'-diaminobenzidine (Sigma-Aldrich, St. Louis, MO) in PBS containing 0.01% hydrogen peroxide. All slides were counterstained with hematoxylin and mounted with coverslips for light microscopy. Selected slides were imaged using a Nikon Ti-U inverted microscope equipped with a DS-Fi-1 digital camera and supported by the NIS-Elements Basic Research acquisition and analysis software package (Nikon Instruments Inc., Melville, NY).

Neuronal damage was assessed in cryosections made in a coronal plane at -0.5 mm relative to the bregma from the brains of WEEV-infected mice. Before staining, each section was incubated in 0.1% Triton X-100 for 15 minutes to expose intracellular antigens. Slides were then incubated with NisslRed (NeuroTrace 530/615 fluorescent Nissl stain, Invitrogen, Grand Island, NY) diluted 1:100 for 20 minutes, washed, incubated in a 0.06% potassium permanganate solution, washed again, and stained in 0.0001% Fluoro-Jade C compound (EMD Millipore) in 1% acetic acid for 10 minutes. After further washing, slides were dried, dehydrated in xylene and coverslipped using VectaMount permanent mounting media (Vector Laboratories). Ten random microscope fields from each animal were imaged at 20x magnification using a Nikon Ti-U inverted microscope. The total number of Fluoro-Jade-positive neurons per 20x microscope field was counted in 10 random fields from 4 mice of each experimental group on day 4 post-infection and in 10 random fields from 3 mice of each experimental group on day 7 post-infection.

Statistical comparisons

The Prism 5.0 software package (GraphPad Software, La Jolla, California, USA) was used for all statistical analyses. Statistical comparisons between multiple groups at a single time point were performed by one-way analysis of variance (ANOVA) tests. Unpaired Student's *t* tests were used to assess differences between paired experimental groups at a single time point. Differences in survival among individual cohorts of infected mice were determined using a log-rank (Mantel-Cox) test. In all cases, differences at a $p < 0.05$ level were considered significant.

Results

Host determinants influence disease outcome following WEEV infection

Different strains of WEEV have been reported to infect BALB/c and CD1 mice, although spread to the CNS, severity of neurologic signs and disease mortality depend on the route and dose of virus inoculation (Julander et al. 2009; Logue et al. 2009; Nagata et al. 2006; Phillips et al. 2013; Phillips et al. 2014). To investigate whether host determinants influence WEEV pathogenesis, we undertook studies using the Cba 87 isolate of WEEV. Starting with 5-week-old female C57BL/6 mice, we found that intraperitoneal (i.p.), subcutaneous (s.c.), and intranasal (i.n.) virus challenges all caused lethal infections, with weight loss, severity of neurobehavioral signs and death being somewhat more accelerated in direct proportion to the amount of virus inoculated (Table 1, Experiments 1-3). To determine the extent that age of the host contributes to disease outcome, 5-, 10-, and 15-week-old female C57BL/6 mice were given identical s.c. viral challenges. We observed a significant drop in both clinical disease severity and mortality between hosts of 10 and 15 weeks of age, even though the magnitude of weight loss and the mean day of death were relatively unaffected across all three groups (Table 1, Experiment 4). Regarding differences in gender susceptibility, male mice proved to be significantly more resistant to lethal infection compared to matched female cohorts, both in terms of neurobehavioral deficits as well as survival (Table 1, Experiment 5).

Turning to the complex influences of host genetics on disease outcome, different inbred strains of mice were compared to outbred CD1 hosts in separate assays where age, gender, and s.c. viral dosing were all held constant. We found that CD1, BALB/c, A/J, and C57BL/6 mice were all highly susceptible to WEEV infection, but that DBA/2 mice as well as a BALB/c sub-strain, BALB/cBy, were notably more resistant to a lethal viral challenge (Fig. 1A, B and D). Animals that proved more resistant to infection also retained body weight to a much greater degree over time (Fig. 1C), and had lower mean neurobehavioral disease scores (Fig. 1E). Taken together, these data show that age, gender, and genetic background are all important host factors that influence WEEV susceptibility. Furthermore, the consistent disease induced in female C57BL/6 mice means that the wide variety of transgenic and gene knockout animals bred on this genetic background are appropriate for future use in studies of WEEV pathogenesis.

Kinetics of CNS invasion and appearance of neurobehavioral deficits following peripheral WEEV inoculation

Prior studies have reported that peripheral WEEV challenge does not always cause encephalitis in certain mouse strains (Nagata et al. 2006), while more recent reports suggest that i.n. inoculation accelerates mortality by allowing virus particles direct access to the CNS via olfactory pathways (Logue et al. 2009; Phillips et al. 2013; Phillips et al. 2014). Another paper reported that a high proportion of C57BL/6 mice developed overt neurologic signs following i.p. challenge with the VR-70 strain of WEEV (Julander et al. 2009). In our hands, C57BL/6 mice given 10^5 pfu of Cba 87 i.n. did not die faster than animals challenged via i.p. or s.c. routes, and peripheral inoculation did not lower the risk of death compared to mice challenged via an i.n. route (Table 1). To further investigate the process of neuroinvasion in cohorts of C57BL/6 mice infected with Cba 87 via these three routes, the onset and progression of symptoms were rated using a neurobehavioral disease scoring scale previously established for WEEV infection (Wu et al. 2007). We found that symptoms typically began about a day earlier among i.n. compared to i.p. or s.c. inoculated animals, but progressed in a sequential manner in all groups (Fig. 2). By the time the first mice became moribund, disease severity was equivalent across all three cohorts (Fig. 2). We also observed that animals reaching a clinical score of 3 frequently exhibited transient and stereotyped episodes suggestive of seizures that also would indicate neuroinvasion. These findings imply that WEEV rapidly gains access to the CNS of C57BL/6 mice, and that it may replicate there for period of up to 72-96 hours before causing enough cellular damage to compromise vital host function(s) leading to death.

Natural WEEV infections are transmitted s.c. via mosquito vectors, and CNS involvement occurs in only a subset of equine and human hosts (Calisher 1994; Johnston and Peters 1996). We thus investigated the tropism and replication of Cba 87 in different tissues of C57BL/6 mice following s.c. challenge. Plaque titration assays revealed evidence of a viremia as well as low-level replication in abdominal viscera such as the liver and spleen during the first few days of infection (Fig. 3). Rapid spread to other vital tissues such as the heart and lungs was also observed, and replication at these sites was sustained at variable levels over time (Fig. 3). Kidneys were the extraneural site where virus replication peaked the latest prior to death (Fig. 3).

The first evidence that virus had reached the CNS was found on day 2 when infectious particles were detected in the brainstem, spinal cord and cerebrospinal fluid; virus then appeared in the cerebrum and cerebellum on day 3 (Fig. 3). Titers in various brain regions peaked at levels consistently 1,000- to 10,000-fold higher than extraneural sites, showing variable degrees of clearance thereafter (Fig. 3). When compared to the timing of symptom onset (Fig. 2), these data confirm that WEEV replicates in the CNS of C57BL/6 mice for up to 48 hours before the first overt neurobehavioral signs of disease become apparent. Peak CNS replication occurs well before animals succumb to infection.

Consequences of WEEV replication in the CNS versus the periphery

A recent study using a sensitive, non-invasive bioluminescence imaging technique showed that WEEV replication was not detected outside the CNS of CD1 mice following i.n. viral

challenge (Phillips et al., 2013). Likewise, we could not identify infectious virus in extraneural tissues of C57BL/6 mice challenged via an i.n route across multiple time points using more conventional but sensitive plaque titration assays (Fig. 4). Thus, widespread replication of virus in peripheral tissues does not appear to be required to cause fatal disease if virus particles can gain direct access to the CNS. Given the appearance and sequential progression of neurobehavioral deficits over the course of acute infection (Fig. 2), the high levels of virus replication in the CNS versus the periphery following s.c. challenge (Fig. 3), and the absence of measurable extraneural virus replication in animals challenged via an i.n. route (Fig. 4), CNS damage rather than direct failure of a critical visceral organ system appears to be the main cause of death in WEEV-infected mice.

Virus replication in the CNS of susceptible and resistant hosts

To further investigate the outcome differences observed between BALB/c and BALB/cBy mice (Fig. 1B), we measured CNS viral titers following s.c. viral challenge in these two closely related host strains. Plaque titration assays showed that virus replication was not different in any CNS region examined on either day 4 or day 7 post-infection (Fig. 5A). Likewise, we found no obvious differences in viral replication or spread in extraneural tissues collected from these two host strains (data not shown). To confirm and extend these observations, tissue viral RNA levels at these same time points were measured by quantitative reverse transcription-PCR. Once again, no differences between the two hosts were observed (Fig. 5B). Our findings show that the notable survival advantage observed in BALB/cBy mice cannot be explained by delayed neuroinvasion or suppressed CNS virus replication at peak stages of disease. As proposed in the SV encephalitis model (Griffin 2005; Thach et al. 2000; Tucker et al. 1996), it appears that the enhanced survival of WEEV-infected BALB/cBy mice is due to an intrinsic host factor, perhaps something that limits virus-induced neuronal cell death, rather than anything that curtails the capacity of the virus to infect or replicate in its main target tissue.

Histological and histochemical features of WEEV infection of the CNS

WEEV infects neurons in non-human primates with fatal encephalomyelitis (Reed et al., 2005), and its tropism for neurons in mice has only been confirmed following i.n. infection (Phillips et al. 2013). Using polyclonal rabbit antisera raised against recombinant WEEV capsid protein, we found abundant cytoplasmic staining in cells throughout multiple brain and spinal cord regions of s.c. challenged mice having the morphological appearance of neurons (Fig. 6A). Infected cells were first consistently found in the brain on day 4 post-infection; their identification in different brain regions at early time points implies that virus gains access to the CNS via hematogenous dissemination rather than by direct cell-to-cell spread (Fig. 6B). Using Fluoro-Jade staining, an established histochemical marker of neuronal injury (Schmued et al. 1997), we identified target cell damage in brain regions such as the hippocampus where infected cells were abundant (Fig. 6C).

Immunohistochemical staining for glial cells showed morphological changes consistent with astrocyte activation (Fig. 7), while hematoxylin counterstaining of these sections revealed the presence of both submeningeal and perivascular inflammation (Fig. 7, arrowheads). Taken together, these microscopic analyses show that murine WEEV infection induces

inflammation, glial activation, and neuronal damage similar to what has been reported in the CNS of infected non-human primates (Reed et al. 2005).

Fluoro-Jade staining was then used to determine whether variability in the amount of neuronal damage could explain outcome differences between BALB/c and BALB/cBy mice of the same age (Fig. 1B), and between C57BL/6 mice of varying ages (Table 1). Consistent with our survival data, we found significantly less neuronal damage in the CNS of 5-week-old BALB/cBy mice compared to 5-week-old BALB/c mice over the course of infection (Fig. 8A). Similarly, there was a measurable drop in the amount of neuronal damage found in the brains of 15-week-old C57BL/6 mice compared to mice of the same strain that were 5-weeks-old (Fig. 8B). Although correlative in nature, these data further support our hypothesis that the extent of neuronal damage, due either to some intrinsic difference in the susceptibility of target cells or variability in some host response that impacts neuronal survival, explains outcome differences among WEEV-infected mice.

Discussion

Mosquito-borne alphaviruses are important natural causes of human encephalitis throughout the Western hemisphere. Past experience has also demonstrated their pathogenicity as aerosols (Hanson et al. 1967); although not normally transmitted via this route, aerosol dissemination could be exploited as a means to disperse an encephalitic alphavirus during a bioterrorism attack. When combined with their ease of production, their capacity to induce severe disease, and the lack of any known countermeasures, New World alphaviruses are considered Category B Priority Pathogens by the National Institute for Allergy and Infectious Diseases and the Centers for Disease Control and Prevention. Yet the rarity of fatal human alphavirus encephalitis cases means that our basic understanding of disease mechanisms following either subcutaneous or inhaled routes of infection derives entirely from animal models. Non-human primates can develop fatal neurologic disease following aerosol exposure to WEEV (Reed et al. 2005), but this model is both expensive and cumbersome to use. Hamsters have been used for experimental purposes, but WEEV is highly virulent in these hosts and successful treatment has to be administered prior to virus challenge (Julander et al. 2007). For all these reasons, an optimized mouse model would be useful for both pathogenesis studies and to develop protective interventions that could be implemented at or even after clinical disease onset. In experiments reported here, we find that manipulating the age, gender, and genetic background of recipient mice alters the pathogenesis of WEEV infection independent of virus tropism or replication in the CNS. Studies using different inbred host strains and various routes of inoculation shed light on pathogenic mechanisms, while experimental conditions that replicate disease features in humans provide a platform to test novel therapeutics.

Certain host factors are already known to influence the outcome of WEE in both humans and non-human primates. In a Canadian WEE outbreak from 1965, fatal disease progressed more rapidly in very young children and individuals older than 60 years (Rozdilsky et al. 1968). Others studies have reported that the ratio of asymptomatic to clinically apparent WEEV infections is 1:1 in children below the age of 1 year, 58:1 in children between the age of 1 and 4 years, and >1000:1 in children older than 14 years (Zacks and Paessler 2010).

Long-term neurologic sequelae are likewise more frequent and severe in children younger than 1 year (Calisher 1994; Zacks and Paessler 2010). While fatality rates following natural infection can range from 3-15% (Steele and Twenhafel 2010; Zacks and Paessler 2010), mortality from the few accidental cases of WEE described amongst laboratory workers exposed to small amounts of virus via aerosols is 40% (Hanson et al. 1967). In rhesus macaques, early reports showed that intracranial (i.c.) or i.n. WEEV inoculation caused fatal disease, while s.c. viral challenge resulted in either asymptomatic infection or fatal encephalitis (Howitt 1932; Hurst 1936; Wyckoff and Tesar 1939). Inhaled WEEV caused acute encephalitis in cynomolgus macaques with overt neurological signs and death in 75% and 25% of infected animals, respectively (Reed et al. 2005). These data highlight the fact that age and route of virus exposure both influence disease susceptibility, and our findings show declining levels of WEEV-induced neuronal cell death as mice increase in age (Fig. 8B).

Gender and host genetic background are two additional host factors that we find influence WEEV susceptibility in mice. Prior studies in other viral encephalitis models have identified sex differences in disease outcome (Andersen and Hanson 1974; Barna et al. 1996), and more recent work implicates the effects of estradiol on both local CNS host immune responses as well as on the expression of pro-survival factors in neurons as possible underlying mechanisms (Soucy et al. 2005). Regarding host genetic factors, disease susceptibility differences between BALB/c and BALB/cBy mice have previously been identified in both neuroadapted SV and Theiler's murine encephalomyelitis virus (TMEV) infections (Nicholson et al. 1994; Thach et al. 2000), but the molecular basis for this difference remains unknown. The closest explanations to date include variability in the host immune response elicited in these two hosts (Nicholson et al. 1994), and a still unidentified quantitative trait locus on chromosome 2 (Thach et al. 2001). In our hands, the susceptibility difference between these two closely related strains lies broadly in target cell susceptibility during infection (Fig. 8A), rather than anything that alters virus invasion or replication within the CNS (Fig. 5). More work clearly needs to be done in order to better understand how the BALB/cBy substrain resists these neurotropic infections.

By adjusting experimental conditions, a disease that kills many, but not all, WEEV-infected mice over a period of 10-12 days can be reproducibly generated (Table 1 and Fig. 1). Important survival determinants in this setting include the dose of virus exposure, as well as the age, gender, and genetic background of the host (Table 1 and Fig. 1). In our hands, route of infection impacts the rate of disease onset, but not the eventual outcome (Table 1 and Fig. 2). From the standpoint of pathogenesis, relevant disease features in mice include: a) the presence of a transient viremia with low-level infection of visceral organs following s.c. inoculation (Fig. 3), b) an interval of several days between viral invasion of the CNS and the development of overt clinical disease (Figs. 2 and 3), c) the capacity of inhaled virus to induce lethal disease without any apparent replication in the periphery (Fig. 4), and d) highly efficient virus replication in neurons compared to other target cells (Figs. 3 and 6). Our data also correlate the improved survival of both older and genetically resistant hosts with reduced neuronal damage rather than impaired neuroinvasion or suppressed CNS virus replication (Figs. 5 and 8). The therapeutic implications of our findings are correspondingly broad: a) future study of WEEV infections in mice should both account for and seek to

better understand these host variables, b) the molecular determinants of neuronal damage should be one particular focus of study, c) a therapeutic window exists between viral neuroinvasion and the cumulative effects of neuronal damage leading to death, and d) effective therapies will need to surmount the blood-brain barrier (BBB) and act within the CNS. We envision that such therapies will include compounds that suppress virus replication as well as others that inhibit the relevant neuronal cell death stimuli and pathways.

Very recent studies from our group have used this optimized mouse model to identify novel antiviral compounds capable of reducing disease symptoms and protecting mice against fatal WEEV infections *in vivo* (Delekta et al., 2014; Raveh et al. 2013). While the cumulative *in vivo* activity of lead compounds in both studies remains modest, these represent the first demonstration of clinical protection against WEEV when the treatment is initiated after viral challenge. Ongoing studies aim to optimize these antiviral molecules from the standpoint of solubility, metabolic stability and CNS penetration, and much work remains to be completed in order to improve dosing regimens and to better emulate potential outbreak scenarios such as pre- and post-exposure treatment as well as efficacy across different host ages, genders and strains. Still, our recent findings represent a notable achievement in preclinical drug development against neurotropic alphaviruses, and our continued use of this optimized mouse model capable of showing effects in clinical disease severity and survival will allow us to focus on those compounds with the highest probability of future clinical use. Such a screening program, albeit both time- and labor-intensive, would not be possible using *in vivo* models having either higher or more rapid lethality. Once further *in vivo* optimization is complete, we can then transition our future studies into systems using more virulent viral strains and even into non-human primate hosts. Finally, since bystander damage to uninfected neurons is an important feature of lethal SV encephalitis (Irani and Prow, 2007), and since blockade of pathogenic host responses prevents this bystander neuronal cell death and enhances host survival (Byrnes et al., 2000; Irani and Prow, 2007; Kimura and Griffin, 2000; Kimura and Griffin, 2003; Liang et al., 1999; Nargi-Aizenman et al., 2004; Rowell and Griffin, 2002; Ryman et al., 2000; Stanley et al., 1986; Tucker et al., 1996), our future studies will incorporate testing of neuroprotective agents in combination with antiviral compounds.

Conclusions

Host factors clearly have a substantial impact on the outcome of WEEV infection in mice. To further elucidate disease pathogenesis, s.c. or i.n. infection of 5-week-old, female C57BL/6 mice using 10^4 pfu of Cba 87 appears optimal since this causes reproducible disease with overt and stereotyped neurobehavioral symptoms coupled with high, but not necessarily complete, lethality over a period of 10-12 days. Indeed, these experimental conditions have allowed us to identify novel antiviral compounds that are active when given after virus inoculation, and they may eventually be useful even when treatment is delayed until the onset of clinically apparent disease (Delekta et al. 2014; Raveh et al. 2013). Use of these mice also means that the wide range of transgenic and gene knockout animals bred on this genetic background can be applied in this model. For the purpose of most closely reproducing human disease, s.c. or i.n. infection of BALB/cBy mice is more appropriate,

since mortality rates in these hosts are more in keeping with what has been described in both human and non-human primates. Regardless of the model being employed, investigators who seek to develop therapeutic interventions against WEEV are urged to maintain consistency in their selection of a murine host to avoid unintentionally confounding their experimental findings with host-derived variables.

Acknowledgements

This work was supported by NIH grant AI089417 to D.J.M.

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Fig. 1.

Outcomes in various strains of mice following WEEV challenge. (A) Cohorts of 5-week-old female mice ($n=10$ per group) were infected s.c. with 10^5 pfu of Cba 87 and monitored daily for weight loss and survival. The number of alive versus dead animals in each group on day 14 post-infection is shown. (B) Formal survival analysis between 5-week-old female BALB/c and BALB/cBy mice shows a significant advantage in favor of the BALB/cBy cohort ($p=0.006$ by log-rank (Mantel-Cox) analysis). (C) Animals showing a survival advantage also retained body weight better than more susceptible hosts, with the difference between BALB/c and BALB/cBy reaching significance ($p=0.0008$ by Student's t-test). (D) Formal survival analyses of 5-week-old female C57BL/6, A/J, DBA/2 and CD1 mice show that significant differences between C57BL/6 versus DBA/2 ($p=0.001$), A/J versus DBA/2 ($p=0.029$) and CD1 versus DBA/2 ($p=0.011$) were also observed (all by log-rank (Mantel-Cox) analyses). (E) Mean clinical disease scores on the day of death or on day 14 post-infection if alive were significantly different across host strains by one-way ANOVA ($p=0.003$).

Fig. 2.

Quantification of clinical disease severity in C57BL/6 mice following WEEV challenge.

Cohorts of mice were challenged with 10^5 pfu of Cba 87 via three different routes of infection and scored daily as follows: 0, normal; 1, slightly ruffled fur, no visible signs of infection; 2, very ruffled fur, definite signs of infection with reduced cage activity; 3, very ruffled fur, hunched posture, reduced mobility; 4, very ruffled fur, hunched posture, little to no mobility, rapid breathing (moribund); 5, dead. Data shown here are representative of experiments done three times with at least 5 mice per group in each replicate. Mean clinical scores across all three groups were not different from one another on day 3, day 6, day 7 or day 8 post-infection by one-way ANOVA ($p > 0.05$).

Fig. 3.

Quantification of WEEV replication in extraneural and CNS tissues following s.c. virus inoculation. Mice infected s.c. with 10^5 pfu of Cba 87 were euthanized at daily time points so that titers of infectious virus could be measured in individual organs or body fluids via plaque titration assays as described in Materials and Methods. Cerebrospinal fluid samples were obtained via cisternal puncture and peripheral blood by intra-cardiac puncture; all other tissues were then harvested following trans-cardiac perfusion with chilled PBS. Serum and cerebrospinal fluid viral titers were calculated based on pfu/ml of sample; all other values were determined as pfu/gm tissue examined.

Fig. 4.

Assessment of WEEV replication outside the CNS following i.n. inoculation. Cohorts of mice were challenged with 10^4 pfu of Cba 87 via three different routes of infection and the amount of infectious virus present in extraneural tissues was determined on day 3, day 6 and day 8 post-infection via plaque titration assays as described in Materials and Methods. Samples obtained from i.n.-challenged mice had no detectable virus present in any extraneural tissue at all three time points post-infection.

Fig. 5.

Comparison of WEEV replication in the CNS of BALB/c and BALB/cBy mice. (A) Mice infected s.c. with 10^4 pfu of Cba 87 were euthanized at two defined time points so that titers of infectious virus could be measured directly in tissue samples obtained from discrete CNS compartments via plaque titration assays as described in Materials and Methods. Statistical comparisons between samples at each time point ($n=3$ per group) were performed by unpaired Student's t-tests. None of the titers at either day 4 or day 7 post-infection were different between BALB/c and BALB/cBy animals ($p>0.05$). (B) Tissue viral RNA levels were measured in these same samples using quantitative reverse transcription-PCR (qRT-

PCR) as described in Materials and Methods. Statistical comparisons between samples at each time point (n=3 per group) were performed by unpaired Student's t-tests. None of the RNA levels at either day 4 or day 7 post-infection were different between BALB/c and BALB/cBy animals ($p>0.05$).

Fig. 6. WEEV tropism and target cell responses in the CNS of C57BL/6 mice infected s.c. with 10^4 pfu of Cba 87. (A) Immunohistochemical staining using polyclonal rabbit antisera raised against recombinant WEEV capsid protein shows cytoplasmic signal (brown) in hippocampal cells on day 6 post-infection having the morphological appearance of neurons. (B) Infected cells (arrow) were detected in a patchy distribution throughout multiple brain regions, including the cerebellum, on day 4 following s.c. viral challenge. (C) Using the established Fluoro-Jade staining method to identify damaged neurons (green), extensive

target cell injury was detected in the hippocampus on day 6 post-infection (bottom). Bar = 50 μm in both A and C, 100 μm in B.

Fig. 7.

Glial and inflammatory responses following WEEV invasion of the CNS of C57BL/6 mice infected s.c. with 10^4 pfu of Cba 87. (A) Immunohistochemical staining (brown) showed numerous GFAP-positive, reactive astrocytes in cortical grey matter, while hematoxylin counterstaining (purple) revealed inflammatory cell infiltration (arrowheads) of the submeningeal compartment on day 6 post-infection. (B) Similar staining shows reactive astrocytes in subcortical white matter adjacent to perivascular inflammatory cell infiltrates. Bar = 100 μ m in both A and B.

Fig. 8.

Quantification of neuronal damage in the brains of mice with differing susceptibility to WEEV. (A,B) Fluoro-Jade staining was performed on brain sections from 5-week-old BALB/c and BALB/cBy mice (A), and 5- or 15-week-old C57BL/6 mice (B), on day 4 or day 7 post-infection. For each set of samples, the number of positive cells per 20x microscope field was counted as described in Materials and Methods. Statistical comparisons between groups was performed via unpaired Student's t-tests and showed significantly reduced amounts of neuronal damage in BALB/cBy mice or older C57BL/6 mice at both time points examined (* $p < 0.01$).

Table 1

Outcomes in C57BL/6 mice infected with the Cba 87 strain of WEEV

Expt	Gender	Age (wks)	Virus Dose (log ₁₀ pfu)	Route	Alive/Total (day 14)	Wt. Loss (grams) *	MDD [†]	MCS ^{**}
1	F	5	2	i.p.	2/5	5.50±1.80	10.7±1.2	4.00±1.41
	F	5	3	i.p.	1/5	6.28±2.44	10.8±1.7	4.40±1.34
	F	5	4	i.p.	1/5	5.88±0.70	10.3±1.3	4.60±0.89
	F	5	5	i.p.	0/5	6.64±0.94	9.8±1.3	5.0
	F	5	6	i.p.	0/5	5.72±1.76	8.4±2.1	5.0
2	F	5	2	s.c.	4/5	3.18±3.97	14.0	2.80±1.48
	F	5	3	s.c.	1/5	4.92±2.68	11.8±1.7	4.40±1.34
	F	5	4	s.c.	1/5	6.30±1.78	12.0±0.8	4.60±0.89
	F	5	5	s.c.	1/5	6.02±3.01	10.3±1.3	4.60±0.89
	F	5	6	s.c.	0/5	7.18±1.01	10.8±1.1	5.0
3	F	5	2	i.n.	3/5	1.82±3.55	10.5	4.00±1.41
	F	5	3	i.n.	1/5	4.24±3.36	11.0±0.8	4.40±1.34
	F	5	4	i.n.	0/5	5.36±2.13	9.4±1.5	5.0
	F	5	5	i.n.	0/5	5.02±1.34	9.2±1.1 ^a	5.0
	F	5	5	i.p.	0/5	6.20±2.11	10.0±1.4 ^a	5.0
	F	5	5	s.c.	1/5	4.16±4.24	10.3±0.5 ^a	4.60±0.89
4	F	5	5	s.c.	2/8	5.71±2.45	10.2±1.2	4.62±0.74
	F	10	5	s.c.	1/8 ^b	8.86±1.84	11.8±0.9	4.75±0.71 ^c
	F	15	5	s.c.	6/8 ^b	5.92±3.65	11.5	3.00±1.31 ^c
5	F	5	4	s.c.	1/16 ^d	5.36±0.76	9.1±0.9	4.88±0.50 ^e
	M	5	4	s.c.	11/15 ^d	4.38±3.47	10.0±2.7	3.47±1.19 ^e

* Mean (± SD) weight loss from day 0 through day 14 or until the day prior to death

[†] MDD = mean day of death (± SD, where applicable)^{**} MCS = mean clinical score on the day of death or on day 14 if alive (± SD, where applicable)^a p=0.37 i.n. versus i.p., p=0.12 i.n. versus s.c., p=0.75 i.p. versus s.c. via Student's t-tests^b p=0.03 10-week-old versus 15-week-old via log-rank (Mantel-Cox) test^c p=0.005 10-week-old versus 15-week-old via Student's t-test^d p<0.0001 male versus female via log-rank (Mantel-Cox) test^e p=0.0002 male versus female via Student's t-test