End-point disease investigation for virus strains of intermediate virulence as illustrated by flavivirus infections

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Viruses of intermediate virulence are defined as isolates causing an intermediate morbidity/mortality rate in a specific animal model system, involving specific host and inoculation parameters (e.g. dose and route). Therefore, variable disease phenotype may exist between animals that develop severe disease or die and those that are asymptomatic or survive after infection with these isolates. There may also be variability amongst animals within each of these subsets. Such potential variability may confound the use of time-point sacrifice experiments to investigate pathogenesis of this subset of virus strains, as uniformity in disease outcome is a fundamental assumption for time-course sacrifice experiments. In the current study, we examined the disease phenotype, neuropathology, neural infection and glial cell activity in moribund/dead and surviving Swiss white (CD-1) mice after intraperitoneal infection with various Australian flaviviruses, including West Nile virus (WNV) strains of intermediate virulence (WNV NSW2011 and WNV NSW2012), and highly virulent Murray Valley encephalitis virus (MVEV) isolates. We identified notable intragroup variation in the end-point disease in mice infected with either WNV NSW strain, but to a lesser extent in mice infected with MVEV strains. The variable outcomes associated with WNV NSW infection suggest that pathogenesis investigations using time-point sacrifice of WNV NSW-infected mice may not be the best approach, as the assumption of uniformity in outcomes is violated. Our study has therefore highlighted a previously unacknowledged challenge to investigating pathogenesis of virus isolates of intermediate virulence. We have also set a precedent for routine examination of the disease phenotype in moribund/dead and surviving mice during survival challenge experiments.

INTRODUCTION

Time-course sacrifice experiments have been the conventional approach for viral pathogenesis studies. Post-challenge with the virus of interest, a number of animals are sacrificed randomly at various time-points in order to establish the in vivo kinetics of pathology, virological and immunological outcomes associated with the infection (e.g. Cross et al., 2015b; Garcia-Tapia et al., 2007; Jubelt et al., 1980). The principle of this approach relies on the assumption that all infected animals would reach a similarly timed fate after infection. Hence, it has been an aim for many research groups to achieve a uniform outcome (e.g. uniformly lethal) in experimentally infected animals (e.g. Cross et al., 2015a; Mucker et al., 2015). This uniformity allows random samples at various time-points to reliably represent the pathogenic processes towards a particular end-point, which in turn helps establish the pathogenesis of the virus of interest.

However, it is common to observe incomplete morbidity and mortality in a group of virus-challenged animals, particularly in strains of intermediate virulence, where neither 100% mortality nor 100% survival occurs (e.g. Frost et al., 2012; Monath et al., 1980). Furthermore, it is also common to observe variability in the time from infection to death in
animals challenged with virus strains of intermediate virulence (e.g. Frost et al., 2012). This suggests a potential variability in the balance between host restriction and in vivo virus replication. However, thorough examination of pathological, virological and immunological outcomes in moribund or dead (also collectively designated ‘fatalities’ or ‘fatal/lethal end-points’ in this paper) and surviving mice during survival challenge experiments is not routinely performed. When investigated, the disease phenotypes were often pooled according to the two survival outcomes: moribund/dead versus survivors (e.g. Morrey et al., 2012). Variability in disease phenotype within fatal end-points and its implications for downstream pathogenesis investigations have not been discussed (Morrey et al., 2012).

In the current study, we investigated the spectrum of end-point disease associated with infection with two Australian West Nile virus (WNV) strains of intermediate virulence (WNVNSW2011 and WNVNSW2012) in an established intraperitoneal infection model in female young adult Swiss white (CD-1) mice. In this outbred mouse model, these two virus strains have been shown to produce intermediate levels of mortality (Frost et al., 2012; N. A. Prow and others, unpublished results). We examined the end-point histopathology, virus infection in the nervous system and glial cell activation in moribund/dead mice. Surviving mice at the end of the trial were also included as part of the disease spectrum assessment. As a comparison, we characterized the neuropathology in mixed gender weanling Swiss white mice infected with the highly virulent Australian Murray Valley encephalitis virus (MVEV), which typically causes a high degree of morbidity and mortality in this model (Lobigs et al., 1988; McMinn et al., 1996).

The outcome of our studies showed that despite challenge with the same virus strain at the same dose and route, there is considerable intragroup variability in the end-point disease phenotype in WNVNSW-infected mice and, to a lesser degree, in MVEV-infected mice. This difference in variability between WNVNSW- and MVEV-infected mice may be correlated with the difference in the levels of mortality (‘virulence phenotype’) and the variability of time to mortality between the two model systems. This comparison highlights the importance of various virus (family, strain), host (species, strain, age, gender, immune status and homogeneity of genetic background) and experimental factors (dose and route of inoculation) in contributing to the ‘virulence phenotype’ and its associated disease phenotypic spectrum. Therefore, it is paramount to characterize the end-point disease spectrum for new or existing virus isolates in any animal model system to be employed for pathogenesis studies. Furthermore, we propose that it may be inappropriate to investigate pathogenesis using time-point sacrifice in a model system that produces notable variability in the end-point disease phenotype of interest, as the assumption of uniformity in outcomes is violated. The present study therefore sets a precedent for routine evaluation of end-point disease variability, which in turn can help determine the experimental design of downstream pathogenesis investigations.

RESULTS

Time to fatal end-point

Table 1 summarizes the mortality rate and the day post-infection (p.i.) on which fatal end-points were observed for each virus-challenge group. The variance of the time from infection to fatal end-point was significantly larger in WNVNSW groups as compared with that of the MVEV groups at the low dose (Fig. 1a; P = 0.0103). However, the mean time to fatal end-point was not significantly different between the two WNVNSW strains at either dose. As expected, the mean time to fatal end-point in the MVEV groups was generally earlier than in the WNVNSW groups (Fig. 1a; low dose, P < 0.0001; high dose, P = 0.0009). Between the MVEV challenge groups, the survival curves differed significantly at both doses, with the more contemporary 2012 Western Australian strain (MVEK68838) producing a lower degree of mortality (Fig. S1a, b, available in the online Supplementary Material; 10 TCID50, P = 0.0003; 1000 TCID50, P = 0.0450). All survivors, except for one challenged with 1000 TCID50 MVE1-S1, seroconverted at the end of the trial. The seronegative MVE1-S1-challenged mouse was excluded from further analyses.

Histopathology

The severity and incidence of WNVNSW2012-induced lethal neuropathology were not significantly different to those of WNVNSW2011, regardless of dose (Fig. 1b–d, Table S1). Of the three aspects of neuropathology investigated, lesions in the neuropil of the brain (encephalitis) were generally more severe than those in the neuropil of the spinal cord (myelitis; Fig. 1c, d).

There was no significant dose-dependent effect on the severity of lethal neuropathology in WNVNSW-infected mice (Fig. 1b–d). However, within each of the WNVNSW-challenge groups, notable variability was observed. The variance in the brain pathology scores of the WNVNSW groups was significantly larger than that of the MVEV challenge groups (Fig. 1b, c; meningitis: at low dose, P = 0.0059, and at high dose, P = 0.0207; encephalitis: at low dose, P = 0.0049, and at high dose, P = 0.0481). The variability of myelitis severity was not significantly different between mice infected with WNVNSW and MVEV, regardless of the dose inoculated (Fig. 1d).

The scatterplots of the lethal neuropathology scores over time showed that mild neuropathology (scores < 1) in the brain was only observed in fatal cases after day 9 p.i. (Fig. 2a–d). This trend was more evident in mice infected with WNVNSW2012 at either of the doses (Fig. 2a, c). A similar pattern was observed for the spinal cord of mice in these groups (Fig. 3a–d). The features of neuropathology observed ranged from severe neuronal degeneration and necrosis typically in the cerebral cortex and hippocampus (Fig. 4a, b) to milder lesions such as focal mononuclear leukocyte infiltrates in the thalamus or
Table 1. Mortality rate and day of fatal end-points (moribund/dead) in mice challenged with WNV_{NSW} and MVEV strains

<table>
<thead>
<tr>
<th>Inoculation dose</th>
<th>Virus strain</th>
<th>Mortality rate</th>
<th>Fatal end-points (day p.i.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 p.f.u.</td>
<td>WNV_{NSW}2012</td>
<td>4/10</td>
<td>8 (n=1), 9 (n=1), 10 (n=1), 14 (n=1)</td>
</tr>
<tr>
<td></td>
<td>WNV_{NSW}2011</td>
<td>8/10</td>
<td>8 (n=3), 10 (n=2), 11 (n=2), 12 (n=1)</td>
</tr>
<tr>
<td>1000 p.f.u.</td>
<td>WNV_{NSW}2012</td>
<td>7/10</td>
<td>6 (n=1), 7 (n=2), 8 (n=1), 9 (n=1), 10 (n=1), 11 (n=1)</td>
</tr>
<tr>
<td></td>
<td>WNV_{NSW}2011</td>
<td>7/10</td>
<td>7 (n=1), 8 (n=3), 10 (n=3)</td>
</tr>
<tr>
<td>10 TCID_{50}</td>
<td>MVE1-51</td>
<td>9/10</td>
<td>7 (n=3), 8 (n=4), 9 (n=1), 10 (n=1)</td>
</tr>
<tr>
<td>1000 TCID_{50}</td>
<td>MVE1-51</td>
<td>9/10</td>
<td>7 (n=7), 8 (n=1), 9 (n=1)</td>
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</tbody>
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*One mouse excluded from this group due to seronegativity at the end of the trial.

Fig. 1. (a) Time to fatal end-point and (b–d) severity of lethal neuropathology. Each data point represents one fatal case. Horizontal bars indicate the median and error bars represent the interquartile range. Low dose for WNV_{NSW} was 100 p.f.u. and for MVEV was 10 TCID_{50}. High dose for WNV_{NSW} was 1000 p.f.u. and for MVEV was 1000 TCID_{50}. The degree of variance between WNV_{NSW} and MVEV groups was compared using a modified Brown–Forsythe test of equality of variance. Statistical significance: *P < 0.05, **P < 0.01. Tables S3–S6 tabulate the corresponding neuropathology for each individual mouse, infected with either of the WNV_{NSW} or MVEV strains. For details of pathology score see Methods.
WNVNSW2011 at 1000 p.f.u., one of the two survivors from MVEV challenge groups had observable CNS lesions. None of the three survivors from the 100 p.f.u. WNVNSW2012 reached its lethal end-point on day 14 p.i. without any observable central nervous system (CNS) lesions. In contrast, a trend of low neuropathology ranging from focal mild endocarditis to focal mild-to-moderate vasculocentric myocarditis. No histopathological lesions were noted in any of the remaining extraneural tissues examined.

The heart was also examined in a subset of dead/moribund and surviving mice. Cardiac inflammation was consistently observed in one mouse per group. The severity and character ranged from focal mild endocarditis to focal mild-to-moderate vasculocentric myocarditis. No histopathological lesions were noted in any of the remaining extraneural tissues examined.

Virus infection

In order to explain the relatively large variability of lethal end-point neuropathology induced by the two strains of WNV, the severity and distribution of virus infection in the CNS were investigated by immunohistochemistry.

The presence of viral antigen in the CNS of fatal cases was predominantly observed in mice with concurrent encephalitis and myelitis scores of ≥2 (n=18/19 brains and n=16/16 spinal cords). There was a significant positive correlation between the severity of brain lesions and virus infection (encephalitis: r²=0.8270, P<0.0001; meningitis: r²=0.5254, P=0.0058). Only one virus-positive brain from a mouse infected with 1000 p.f.u. of WNVNSW2012 had a concurrent lethal encephalitis score of 1 on day 10 p.i. Similar to lethal neuropathology, low viral antigen load in the brain (<2) was only detected after day 9 p.i. (Fig. 2a–d). The variance in the severity of viral antigen load in the CNS was also large, similar to neuropathology (Fig. 5a, b). The median brain viral antigen scores were higher than those in the spinal cord for most groups, except for mice infected with 100 p.f.u. WNVNSW2012 (Fig. 5a, b). Notably, dead/moribund mice infected with 1000 p.f.u. WNVNSW2012 had a higher median brain viral antigen load than all other groups, although this difference was not statistically significant (Fig. 5a).

There was a temporo-spatial trend in terms of the extensiveness of CNS infection and the timing of the fatal
event. Late fatal end-points typically had less widespread CNS infection than the earlier end-points (Fig. 6a–d). Common sites of lethal virus infection in the brain included the cerebral cortex, thalamus and pons (Fig. 5c). The medulla was also a common site to observe virus infection in WNVNSW2012-challenged mice (Fig. 5c).

WNV antigen was detected in the cytoplasm of either intact (Fig. 4e) or degenerating neurons (Fig. 4f). Lymphohistiocytic infiltrates were commonly associated with degenerating or necrotic infected neurons (Fig. 4f), whilst intact infected neurons were commonly seen in areas that were distant from neuropil- or perivascular-infiltrating leukocytes (Fig. 4e). For the former, phagocytosis of infected neurons (neuronophagia) was evident, suggestive of neuronal dropout in progress (Fig. 4f). No WNV antigen labelling was detected in any non-CNS tissues except for the inner ear of one day 9 p.i. fatal case infected with 100 p.f.u. WNVNSW2012. No viral antigen was detected in any of the survivors.

Astrocytic and microglial activation

In addition to the profile of end-point CNS infection, the activation state of glial cells in mice infected with WNVNSW2012 and WNVNSW2011 was also investigated, in order to assess whether a temporal trend corresponding to virus infection and pathology was present. Astrocytosis and microgliosis were assessed using glial fibrillary acidic protein (GFAP) and Iba1 immunohistochemical labelling of brain and spinal cord sections. The degree of cellular hypertrophy and density of these glial cells were used to assess the level of activation. Normal appearance of non-activated CNS is depicted in Fig. 7(a, d).

Glial activation (astrocytosis and/or microgliosis) was observed in most dead/moribund mice regardless of virus strain or dose inoculated (Fig. 7b, e). The overall degree of glial activation was not significantly different between the WNVNSW challenge groups (Fig. 5d–f). As for lethal neuropathology and CNS infection, the variability in the glial activity associated with lethality was high in all WNVNSW-challenged groups (Fig. 5d–f). However, contrary to pathology and infection, glial activity remained at notable levels in moribund/dead mice beyond day 9 p.i. (Figs 2a–d and 3a–d). The overall incidence of moribund/ dead mice that had undetectable CNS infection, low-grade neuropathology, but generally moderate-to-severe glial activation was 23% (6/26). Glial activation...
was also observed in a subset of survivors (Fig. 7c, f; WNVNSW2012 at 100 p.f.u., n = 3/6, and at 1000 p.f.u., n = 1/3; WNVNSW2011 at 100 p.f.u., n = 1/2, and at 1000 p.f.u., n = 1/3). Thus, astrocytic and/or microglial activity did not invariably correlate with virus infection and pathology in the CNS.

DISCUSSION

The present study used the examples of two Australian WNV strains of intermediate virulence (WNVNSW2011 and WNVNSW2012) in the established flavivirus virulence animal model, i.e. CD-1 Swiss white mice, to illustrate that intermediate levels of mortality and variable time from infection to severe morbidity/mortality are associated with different end-point disease outcomes. Notably, WNVNSW2011 and WNVNSW2012 infections induced different severity of neuropathology and CNS infection in a time-dependent manner in moribund/dead mice (Figs 2a–d and 3a–d). In particular, early lethal end-points typically had more profound neuropathology scores and CNS viral antigen load as compared with late end-points (Figs 2a–d and 3a–d). Glial activation did not vary in a notable temporal trend (Figs 2a–d and 3a–d). Hence, WNV strains of intermediate virulence induce a variable disease phenotype amongst outbred CD-1 Swiss white mice progressing to a clinically moribund state or death. The relatively narrower spectrum of lethal brain neuropathology in MVEV-infected weanling Swiss white mice (Fig. 1b, c) suggests that variability of lethal end-point disease in the target organ (i.e. brain) may differ with the ‘virulence phenotype’ (i.e. the proportion of morbidity/mortality in infected groups) of the isolate in a particular model system (host species/strain and experimental methods).

Variability in disease outcomes has been reported regularly for WNV infections in humans and certain mouse strains (Graham et al., 2015; Petersen et al., 2013). Particularly relevant in an Australian context, variable end-point disease outcomes have been reported for other Australian WNV strains of intermediate virulence in the Swiss white mouse model (Bingham et al., 2014). However, the significance and implications of this variability has rarely been discussed in the literature.

The variable outcomes in moribund/dead mice after infection with WNVNSW strains suggest that the mechanism of severe morbidity/death may differ depending on the time from infection to lethal end-point. Whilst the outcomes in early fatalities are consistent with the severe neuronal infection and neuropathology seen in most other mouse studies (Garcia-Tapia et al., 2007; Shrestha et al., 2003), the relatively benign neuropathology and CNS infection...
Fig. 5. Virus infection and glial activation in the CNS. (a, b) Overall viral antigen load score in the brain (a) and spinal cord (b) of moribund/dead mice infected with WNV_{NSW2012} and WNV_{NSW2011}. (c) Incidence of virus infection in different regions of the brain. (d) Astrocytosis in the brain. (e, f) Microgliosis in the brain (e) and spinal cord (f). For (a, b, d–f), each data point represents one fatal end-point. For (c), each point represents the incidence of virus infection in each region of the CNS for each virus challenge group. The number adjacent to these data points represents one challenge group: 1, WNV_{NSW2012} (100 p.f.u.); 2, WNV_{NSW2011} (100 p.f.u.); 3, WNV_{NSW2012} (1000 p.f.u.); 4, WNV_{NSW2011} (1000 p.f.u.). All horizontal bars indicate the median and the error bars, where used, indicate the interquartile range. Tables S3 and S4 tabulate the corresponding neuropathology, CNS infection and glial activation for each individual mouse, infected with either of the WNV_{NSW} strains. GFAP, Glial fibrillary acidic protein.
of the late lethal end-points have only been reported on a few occasions, but have not been a focus of the investigations (Bingham et al., 2014; Nagata et al., 2015). In general, there is a lack of research effort towards understanding the mechanism of severe morbidity/death associated with this surprisingly benign phenotype, which is actually the main presentation in fatal human and equine WNV infections (Angenvoort et al., 2013; Guarner et al., 2004). Given the observation of neuronophagia in earlier fatalities (Fig. 4f), it is possible that the late lethal end-points in our study captured a temporal snapshot after complete clearance of infected neurons. The cause of the severe symptoms/death in these late fatalities, therefore, may be due to drop out of essential neurons in vital areas of the CNS, such as the brainstem or spinal cord (Donadieu et al., 2013; Fratkin et al., 2004). Complete characterization of the distribution and degree of neuronal dropout is beyond the scope of the current study. However, these late lethal end-points do highlight that moribund/dead Swiss white mice infected with the same strain of WNV by the same route may not always progress through the same pathogenic pathways towards severe morbidity/death.

Variability in the end-point disease has significant implications on downstream pathogenesis investigations. Notably, conventional time-course sacrifice experiments may need to be used with caution, as they rely on the assumption that the experimental infection would produce a uniform outcome. This assumption allows the outcomes from randomly sacrificed animals at a particular time-point to accurately represent events along a timeline of pathogenesis towards a particular disease outcome, such as fatal encephalitis. Prior to the present study, our research group attempted to investigate the neuropathogenesis of fatal WNNSW2011 infection by random scheduled culling of infected female young adult Swiss white mice on day 3 and 7 p.i. Mice were intraperitoneally infected with 1000 p.f.u. WNNSW2011. This was performed alongside investigations using two other contemporary Australian

\[ \text{Fig. 6. Temporo-spatial distribution of CNS infection. (a–d) Viral antigen load in different regions of the CNS in moribund/dead mice infected with } \text{WNV}_{\text{NSW2012}} [100 \text{ (a) and 1000 p.f.u. (c)}] \text{ and } \text{WNV}_{\text{NSW2011}} [100 \text{ (b) and 1000 p.f.u. (d)}]. \text{ The diagram above the graphs indicates the three regions of the CNS represented by the different symbols. Each data point represents the viral antigen load in one region of one animal. Tables S3 and S4 tabulate the corresponding neuropathology, CNS infection and glial activation for each individual mouse, infected with either of the WNV_{NSW} strains.} \]
WNV-Kunjin isolates (WNV_{K68967} and WNV_{Gu1009}). The time-course experiment did not detect neuropathology nor virus infection in the brain of WNV_{NSW2011}-infected mice, even on day 7 p.i. (Table S2). In light of the wide spectrum of end-point diseases associated with WNV strains of intermediate virulence, it was uncertain whether the sacrificed mice were progressing towards the early or late lethal end-point. It was also possible that the sacrificed mice were progressing towards survival. This illustrates the issue of loss-to-follow-up bias introduced by the time-course experimental design, the magnitude of which is more notable when a wide spectrum of end-point disease outcomes is associated with the infection.

Similar illustration of variable disease outcomes and loss-to-follow-up bias associated with time-point sacrifice experiments was observed in a previous study, involving an inbred mouse strain (BALB/c), a different virus strain (WNV_{NY99}), route (intravenous) and dose (10^6 p.f.u.) of inoculation (Nagata et al., 2015). After infection, only a
proportion of the moribund or dead adult BALB/c mice developed neuropathology and had detectable viral antigen in the brain, similar to the findings of the current study (Nagata et al., 2015). Furthermore, a notable range in the time to fatal end-point, ranging from day 6 to 14 p.i., was observed in this model system (Nagata et al., 2015). When the investigators performed scheduled time-point sacrifice of infected mice, they failed to observe neuropathology and brain infection at the three time-points (day 3, 5 and 7 p.i.), similar to our pathogenesis study (Nagata et al., 2015). Therefore, variability in disease outcomes as well as its associated implications for time-course experiments is not just a phenomenon in outbred mouse strains, such as the CD-1 Swiss white mouse model, or with more attenuated virus strains, such as WNVNSW, used in this study.

Other inbred mouse strains, such as C57BL6, also produce variable disease outcomes, such as brain viral load, in moribund or dead mice when infected with specific WNV strains at specific doses and routes (Donadieu et al., 2013). This highlights that the ‘virulence phenotype’ of a virus isolate and its associated disease spectrum are dependent on the model system used. Specifically, they are dependent on the in vivo infection, replication and dissemination capacity of a virus isolate within a host of a certain species, age, gender, breed/strain, immune status and homogeneity of genetic background. Experimental factors, such as route and dose of inoculation, also affect the ‘virulence phenotype’ in the model system.

Conventionally, CD-1 outbred Swiss white mice have been used for assessing virulence of WNV and other flaviviruses (Beasley et al., 2002; Monath et al., 1980), thus justifying the use of this mouse strain in the current study. However, the correlation between intermediate levels of morbidity/mortality (i.e. intermediate virulence) and the variability of disease outcomes likely applies to other non-flavivirus challenge models, as isolates of intermediate virulence have been reported in other virus families (e.g. Pol et al., 1989; Rautenschlein et al., 2003; Zhao et al., 2013). Therefore, we recommend that routine assessment of the end-point disease spectrum should be performed for any new virus isolate in an established model system, or for existing isolates in a previously uncharacterized model system, in order to determine the validity of time-course sacrifice experiments for downstream pathogenesis investigations. We also recommend individual recording of pre-mortem clinical parameters, such as that performed in (Morrey et al., 2012), so that disease outcomes may potentially be predicted pre-mortem.

Furthermore, for any virus challenge model that produce large variability in the disease of interest, a large sample size per time-point should be used, if pathogenesis is to be investigated by sacrifice experiments in that model. However, this only minimizes, and does not overcome, the problem of loss-to-follow-up bias associated with time-course experiments. Alternatively, the use of in vivo bioluminescence monitoring of host or virus gene expression will allow individual tracking of pathogenic events towards the different end-point outcomes (Contag & Bachmann, 2002). Similarly, intra-vital imaging of dye-labelled cells may also allow tracking of live virus-host interactions on an individual and cellular level (Mandl et al., 2012, 2014). These real-time monitoring tools, combined with transcriptomic and proteomic studies of the host immune response, may therefore be particularly ideal for pathogenesis investigation of virus strains that produce notable variability in disease outcomes in a particular model system.

In conclusion, variable disease outcomes are likely associated with virus isolates of intermediate virulence in a specific model system. We have demonstrated this, using the example of WNV strains of intermediate virulence in an established Swiss white mouse model. This variability challenges the assumption of uniform outcome that is crucial for time-course sacrifice experiments for pathogenesis investigation. Our study has therefore highlighted a previously unacknowledged challenge to investigating pathogenesis of virus strains of intermediate virulence, as well as the importance of characterizing the end-point disease spectrum during survival challenge studies.

**METHODS**

**Virus strains, animals and experimental design.** The 2012 Australian WNV strain WNVNSW2012 was isolated from *Culex annulirostris* mosquito pools sourced from the state of New South Wales, Australia (N. A. Prow et al., unpublished results). The isolation and propagation of the outbreak strain WNVNSW2011 has been described previously (Frost et al., 2012). These two strains were titrated by plaque assay on African green monkey kidney (Vero) cells (Prow et al., 2014) and the virus stock titres were expressed as p.f.u. Multiple survival challenge experiments involving female CD-1 Swiss outbred mice 24–28 days old were conducted, as described previously (Prow et al., 2014). Ten mice were randomly assigned to each virus challenge group. Mice were challenged intraperitoneally with either 100 or 1000 p.f.u. in 100 μl RPMI media, supplemented with 2 % FBS (Beasley et al., 2002). To investigate the end-point phenotype in a highly virulent Australian flavivirus strain, we used two strains of MVEV: a contemporary MVEV isolate (MVEK68838) and the prototype (MVEF1,51). Isolation of MVEF1,51 has been described previously (Lobigs et al., 1986). MVEK68838 was isolated from mosquitoes trapped in Western Australia in 2012 and was kindly provided by Dr Cheryl Johansen. MVEV strains were additionally passaged once on insect *Aedes albopictus* cells (C6/36) and titrated by a TCID50 assay on Vero cells prior to use (Prow et al., 2011). MVEV strains were inoculated intraperitoneally in the established 18-day-old mixed gender Swiss CD-1 outbred mouse model at doses of 10 and 1000 TCID50 with 10 mice randomly assigned to each challenge group (Lobigs et al., 1986; McMinn et al., 1996).

All animal experiments were conducted under Physical Containment Level 2 conditions with ethics approval from the University of Queensland Animal Ethics Committee [permit number SCMB/196/11/NHMRC(NF)]. Twice daily monitoring for clinical and neurological signs was conducted. A score of 0–4 was assigned to each mouse, where a score of 0 signifies normal behaviour and a score of 3 indicates a moribund state or exhibition of severe clinical signs, such as seizure and paralysis. Mice with a score of 3 were euthanized on...
humane grounds with an overdose of ketamine (100 mg kg\(^{-1}\)), xylazine (20 mg kg\(^{-1}\)) and PBS mixture. A score of 4 was assigned to mice that died naturally due to unexpected rapid deterioration. Fatal end-point was defined as a mouse that reached a clinical score of 3 or 4. Survivors at day 20–22 p.i. were humanely euthanized, as described above, following a cardiac bleed under deep anaesthesia for serum procurement.

**Histology.** At either time of end-point euthanasia or discovery of carcass, samples of the neural and extraneurial tissues were taken for histopathological and immunohistochemical analysis (Prow et al., 2014). The entire head, with skin and calvarium removed, along with the entire length of the vertebral column were fixed in 10 % neutral buffered formalin for 48–72 h, followed by de-calcification in 8 % formic acid for 3–4 days. The head, from the level of the cribiform plate to the foramen magnum, was sectioned to allow examination of the brain at the level of the olfactory bulb, two levels encompassing the hippocampus, thalamus and hypothalamus, and two levels encompassing the basal ganglia, cerebellum and brainstem (Paxinos & Franklin, 2004). The spinal cord was sectioned transversely into segments 2–3 mm thick and all processed in the same cassette. Extraneural tissues sampled with variable frequency included eyelids, salivary glands, bone marrow, blood vessels, thymus, spleen, lymph nodes, gastrointestinal tract, skeletal muscles, kidneys, heart and lungs. These samples were fixed in the same manner, followed by storage in 70 % ethanol until processing for histopathology examinations. All samples were processed and analysed, as described previously (Prow et al., 2014). Severity of histopathological lesions was scored semi-quantitatively on a scale of 0–5, by an observer blinded to the group and clinical outcome (H. B.-O.): 0, no apparent lesions; 1, minimal to mild, focally restricted leukocyte infiltration and/or microgliosis; 2, scattered foci of mild leukocyte infiltration with or without apparent neuronal changes (degeneration, apoptosis/necrosis or neuronophagia); 3, scattered foci of moderate leukocyte infiltration and neuronal changes; 4, multifocal, marked leukocyte infiltration and neuronal changes; 5, multifocal to diffuse, severe leukocyte infiltration and neuronal changes (representative illustrations are depicted in Fig. S3).

**Immunohistochemistry.** The immunohistochemistry protocol for detection of flaviviral non-structural protein 1 (NS1) antigen has been described previously in detail (Prow et al., 2014). Anti-flavivirus NS1-specific mAb 4G4 (Clark et al., 2007) was used for detection of WNV and MVEV infection in neural and extraneurial tissues. Rabbit anti-GFAP (Dako) and goat anti-Iba1 (Abcam) antibodies were used to assess astrocytic and microglial activation in brain and spinal cord sections, using a previously described protocol (Miura et al., 2008). The degree of viral antigen load and glial cell activation was scored semiquantitatively as described previously (Bielefeldt-Ohmann et al., 2012; Tolnay et al., 2010) by an observer (H. B.-O.) blinded to the group allocation. Viral antigen load was scored on a scale of 0–3: 0, no viral antigen apparent; 1, rare, scattered positive cells or a few positive cells limited to a single focus; 2, moderate numbers of scattered or positive cells or focally extensive occurrence; 3, widespread in all parts of brain/spinal cord. Glial cell activation was scored on a scale of 0–5: 0, normal GFAP/Iba1 pattern; 1, minimal to mild increase in cell sizes and signal intensity in a single region of the brain; 2, mild increase in cell sizes and signal intensity in several regions of the brain; 3, moderate increase in cell sizes and signal intensity in several regions of the brain; 4, marked increase in cell sizes and signal intensity in several regions of the brain; 5, diffuse, severe increase in cell sizes and signal intensity in several regions of the brain.

**Seroconversion.** At time of euthanasia, terminal cardiac bleed was performed on survivors at the end of the trial (day 20–22 p.i.) and sera were extracted. Antibody production against the WNV\(_{NSW}\) and MVEV strains was assessed in survivors by fixed-cell ELISA using WNV\(_{NSW}\) or MVEV-infected C6/36 cells, as described previously (Adams et al., 1995).

**Statistical analysis.** Times to fatal end-point have a right skewed distribution pattern and were log\(_{10}(y+1)\) transformed before performing parametric tests. All pathology and immunohistochemistry scores had a Gaussian distribution. Two-way ANOVA with post-test Sidak or Tukey was performed to assess group differences. For testing the equality of variance, we initially transformed the raw data to the absolute difference of the raw scores to the median of each group, before performing a two-way ANOVA to identify variance differences between groups (Segar et al., 2009). Post hoc tests (Sidak) also allowed identification of specific group differences in variance (Segar et al., 2009). This test is a modification of the Brown–Forsythe test of equality of variance (Segar et al., 2009). Correlation was performed by a two-tailed Spearman correlation test. Survival curve comparisons were performed by a log-rank (Mantel–Cox) test. All statistical analyses were performed in statistical software Prism 6 (GraphPad).

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