Virulence variation among isolates of western equine encephalitis virus in an outbred mouse model

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Little is known about viral determinants of virulence associated with western equine encephalitis virus (WEEV). Here, we have analysed six North American WEEV isolates in an outbred CD1 mouse model. Full genome sequence analyses showed ≤2.7% divergence among the six WEEV isolates. However, the percentage mortality and mean time to death (MTD) varied significantly when mice received subcutaneous injections of 10^3 p.f.u. of each virus. Two WEEV strains, McMillan (McM) and Imperial 181 (IMP), were the most divergent of the six in genome sequence; McM caused 100% mortality by 5 days post-infection, whereas IMP caused no mortality. McM had significantly higher titres in the brain than IMP. Similar differences in virulence were observed when McM and IMP were administered by aerosol, intranasal or intravenous routes. McM was 100% lethal with an MTD of 1.9 days when 10^3 p.f.u. of each virus was administered by intracerebral inoculation; in contrast, IMP caused no mortality. The presence of IMP in the brains after infection by different routes and the lack of observed mortality confirmed that IMP is neuroinvasive but not neurovirulent. Based on morbidity, mortality, MTD, severity of brain lesions, virus distribution patterns, routes of infection and differences in infection of cultured cells, McM and IMP were identified as high- and low-virulence isolates, respectively.

INTRODUCTION

Western equine encephalitis virus (WEEV; genus Alphavirus, family Togaviridae) is an arthropod-borne, enveloped, positive-sense RNA virus (Calisher et al., 1988). WEEV is the prototype virus within the WEEV antigenic complex and is one of the few alphaviruses endemic to North America. WEEV is transmitted primarily among passerine birds by the mosquito Culex tarsalis. Humans and equines are incidental, dead-end hosts, as they typically do not develop viraemia sufficient to infect mosquitoes and propagate the cycle. However, WEEV has been responsible for periodic outbreaks of encephalitis in equines and humans and is a significant public-health concern in western/central North America (Calisher et al., 1988; Johnson & Pfeffer, 1999; Reisen & Monath, 1988). Numerous isolates of WEEV have been obtained from humans and equines during the many outbreaks, and mosquito isolates have been recovered from both epidemic and interepidemic periods. Furthermore, because WEEV has been shown to be infectious by aerosol, it is a threat as a potential bioterrorist (BT) weapon (Froeschle, 1964; Liu et al., 1970; Reed et al., 2005; Sidwell & Smee, 2003). WEEV is a recombinant virus, the descendant of a recombination event between an eastern equine enceph-
alitis (EEEV)-like ancestor and a Sindbis virus (SINV)-like ancestor (Hahn et al., 1988). Comparisons using the amino acid sequences of several alphaviruses has confirmed that the envelope glycoproteins were derived from the SINV-like ancestor, whilst the capsid protein was derived from the EEEV-like ancestor (Levinson et al., 1990). Further phylogenetic analyses and nucleotide sequence comparison data demonstrated that WEEV also acquired the genes for non-structural proteins from the EEEV-like ancestor (Weaver et al., 1993). Whilst several pathogenesis studies have been carried out for both SINV and EEEV (Del Piero et al., 2001; Griffin, 1989; Hirsch & Griffin, 1979; Polo et al., 1988; Sherman & Griffin, 1990), the pathogenicity of WEEV remains relatively undescribed (Forrester et al., 2008; Fraizer et al., 1985; Gardner et al., 2008; Hardy et al., 1997; Julander et al., 2007; Nagata et al., 2006; Zlotnik et al., 1972). Nagata et al. (2006) examined virulence of eight isolates of WEEV in adult female BALB/c mice infected via the intranasal route. All showed 100% mortality and little variation in rate of onset of mortality. Forrester et al. (2008) recently evaluated virulence of 10 WEEV isolates in subcutaneously infected Swiss–Webster mice. They observed notable virulence differences among isolates; however, only one isolate (CA46) caused >50% mortality, with an onset time of 8 days post-infection (days p.i.). We therefore wanted to determine the virulence patterns of a broad range of isolates with low passage histories, spanning a known temporal spectrum from vertebrate and invertebrate hosts. The investigation involved sequencing the full genomes of seven WEEV isolates for nucleotide and amino acid identity analysis, of which six were used for survival analyses. Two representative isolates were selected for detailed in vitro and in vivo pathogenicity studies, and five routes of infection (subcutaneous, intravenous, intranasal, aerosol and intracerebral) were utilized to determine the influence of route of infection on virulence.

Our aims were (i) to identify virus isolates representing extremes in virulence phenotype for the purpose of determining whether recent isolates pose a significant health threat, (ii) to validate our animal model using different routes of infection, and (iii) to distinguish between neurovirulence and neuroinvasiveness in a low-virulence WEEV isolate. The work completed here will allow us to utilize infectious cDNA clones representing high- [McMillan (McM)] and low- [Imperial 181 (IMP)] virulence isolates to investigate, through the use of reciprocal chimeric viruses, molecular determinants of pathogenicity of WEEV by different routes of infection.

### METHODS

**Virus isolates.** All WEEV isolates came from the Arbovirus Reference Collection at the CDC, Fort Collins, CO, USA. Information on these isolates is summarized in Table 1. Seed stocks for these experiments were made by infection of Vero cells (ATCC) at an m.o.i. ≤ 0.01. Cell-culture supernatant was collected 48 h post-infection (h p.i.) and stored in aliquots at −80 °C. Virus titre was determined by plaque assay on Vero cells as described by Miller & Mitchell (1986).

**In vitro virus growth studies in vertebrate and invertebrate cells.** C6/36 (Aedes albopictus), Vero and baby hamster kidney (BHK) cells were grown to 90% confluence in 25 cm² flasks containing minimal essential medium (MEM) with 10% fetal calf serum. Cells were counted by using a haemocytometer and infected with McM or IMP at m.o.i. 0.1. Infected Vero and BHK cells were incubated at 37 °C, whereas C6/36 cells were incubated at 28 °C. Virus was removed after 1 h and monolayers were washed with PBS (Gibco BRL). MEM (6 ml) with 10% fetal calf serum was added to each flask and cells were incubated at either 37 °C (BHK21 and Vero cells) or 28 °C (C6/36 cells) for the duration of the experiment. Aliquots (300 µl) of supernatant were removed at 12–24 h intervals and stored at −80 °C every 12 h for subsequent plaque assay until the monolayers became detached. Virus titrations were carried out in duplicate and plaque assays were performed as described by Liu et al. (1970).

**Mouse virulence studies.** Groups of outbred CD1 mice (n=10 per group; The Jackson Laboratory), 6-8 weeks old, received subcutaneous injections on the inner left thigh with 200 µl of WEEV stock isolates diluted in PBS. Inocula were titrated by plaque assay on Vero cells to confirm dosage. For aerosol infections, mice were exposed to 10⁵ p.f.u. for 1 h in a Middlebrook Airborne Infection apparatus (Glas-Col), then returned to cages. To determine efficient infection and estimated inhalation dose, groups of three mice were killed immediately after exposure and their lungs were homogenized.

### Table 1. Mortality in CD1 mice challenged subcutaneously with 10⁵ p.f.u. of six different isolates of WEEV

Isolates in bold type represent the selected high-virulence (McM) and low-virulence (IMP) isolates. MP, Mouse; SM, suckling mouse; SMB, suckling mouse brain; DE, duck embryo cells; V, Vero cells; P, passage (? unknown passage number).

<table>
<thead>
<tr>
<th>WEEV isolate</th>
<th>Dose</th>
<th>Mortality</th>
<th>MTD (days)</th>
<th>Location and date of isolation</th>
<th>Host/passage history</th>
</tr>
</thead>
<tbody>
<tr>
<td>McMillan (McM)</td>
<td>10⁴</td>
<td>24/24</td>
<td>4.0</td>
<td>Ontario, Canada, 1941</td>
<td>Human brain/MP2, SMB1, V2</td>
</tr>
<tr>
<td>BFS-2005</td>
<td>10⁴</td>
<td>2/10</td>
<td>9.0</td>
<td>Kern County, CA, USA, 1974</td>
<td>Culex tarsalis/DE1</td>
</tr>
<tr>
<td>Imperial 181 (IMP)</td>
<td>10⁴</td>
<td>0/15</td>
<td>–</td>
<td>Imperial County, CA, USA, 2005</td>
<td>Culex tarsalis/V2</td>
</tr>
<tr>
<td>85-452NM</td>
<td>10⁴</td>
<td>1/10</td>
<td>8.0</td>
<td>New Mexico, USA, 1985</td>
<td>Culex tarsalis/P1, SM2</td>
</tr>
<tr>
<td>71V1658</td>
<td>10⁴</td>
<td>1/10</td>
<td>7.0</td>
<td>Oregon, USA, 1971</td>
<td>Horse brain/P2, SM1</td>
</tr>
<tr>
<td>Montana-64</td>
<td>10⁴</td>
<td>7/10</td>
<td>6.9</td>
<td>Montana, USA, 1967</td>
<td>Horse brain/DE1</td>
</tr>
<tr>
<td>AG80-646*</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Chaco Province, Argentina, 1980</td>
<td>Culex cossa/P1, V2, SM3</td>
</tr>
</tbody>
</table>

*This isolate was included for sequence analysis only (phylogenetic outgroup).
and plaque-assayed, with a resulting titre of 10^5 p.f.u. g⁻¹. Intranasally infected groups were infected with 20 µl WEEV with a titre of 10^5 p.f.u. ml⁻¹ per nostril, as described previously (Logue et al., 2008). Intravenously infected mice received 50 µl WEEV with a titre of 10^5 p.f.u. via the tail vein. Two groups of CD1 mice were infected intracerebrally as described by Liu et al. (1970) with 25 µl WEEV McM and IMP at a titre of 10^5 p.f.u. All mice were observed twice daily for signs of morbidity. The day that a moribund mouse was killed was considered the day of death for calculation of mean time to death (MTD). Survival was followed for a period of 14 days, at which point all survivors were reinfected with McM to ensure that initial doses of virus were adequate to induce at least partial immunity.

**Viraemic study.** Blood was collected from the tail vein of subcutaneously infected mice and serum from three mice was separated into heparinized tubes at 6 h, 24 h (day 1) and every 24 h after that until day 5.

**Tissue-titre study.** Tissues infected with McM and IMP were collected from three mice per group at 12, 24, 48, 72, 96 and 120 h p.i. In total, 17 tissues were extracted per mouse, including eye, brain, salivary gland, thymus, heart, lung, adrenal gland, kidney, spleen, pancreas, liver, skeletal muscle, popliteal lymph node, inguinal lymph node, duodenum, knee and nasal tract (in aerosol-infected mice only). Samples were removed after a 5 min PBS perfusion by cardiac puncture to ensure that all systemic blood was expelled. Tissues were placed in pre-weighed 1 ml green bead tubes (Roche) containing 0.5 ml MEM. The tubes were then reweighed upon addition of an organ and prior to homogenization using two pulses of 30 s at 1400 R/min at room temperature and the supernatant was removed and stored at −80 °C for RNA extraction. All mouse experiments were carried out in triplicate in Biological Safety Level 3 containment facilities.

**Viral nucleic acid extraction and TaqMan Real-Time PCR assay.** Viral RNA was isolated from homogenized mouse tissue, serum and virus seeds by using an RNeasy 96 Universal Tissue kit (Qiagen). Total RNA was extracted from 25 µl tissue homogenate that had been diluted 1:1 in MEM and eluted from the kit columns to a final volume of 100 µl elution buffer. WEEV TaqMan virus-specific primers and probe were designed against IMP and McM isolates with the Primer Select software program (DNASTAR; see Supplementary Table S1, available in JGV Online). The TaqMan probe was labelled with a 5'-end 6-carboxy-fluorescein (FAM) reporter dye and a 3'-end Black Hole Quencher 1 (BHQ1) quencher dye. A QuantiTect probe RT-PCR kit (Qiagen) was used for the TaqMan assay. A 50 µl total reaction volume consisted of kit components, 10 µl RNA, 0.4 µM primer and 0.15 µM probe. The reactions were subjected to 45 cycles of amplification in an iQ5 Real-Time PCR detection system (Bio-Rad) according to the recommended QuantiTect probe PCR kit RT-PCR conditions. A standard curve was used to quantify the viral nucleic acid in the mouse tissue samples. The standard curve was completed by serially diluting a WEEV stock, titrating each dilution and extracting the RNA according to the RNeasy protocol. A curve correlation coefficient of >0.950 and a PCR efficiency of 90–100% were used to validate each detection assay.

**Virus sequencing and phylogenetic analysis.** RT-PCR assays were performed by using a Titan One tube RT-PCR kit (Roche Molecular Biochemicals) according to the manufacturer’s protocol, using an MJ Research PTC-200 Peltier Thermal Cycler (Bio-Rad). Non-translated regions (NTRs) were amplified according to the manufacturer’s suggested protocol using a FirstChoice RLM-RACE kit (Ambion). A 5’-NTR antisense primer, WEEV 592(–), dT12, and a 3’-NTR sense primer, 11286(+), were used together with the primers included in the kit (see Supplementary Table S1). After amplification, a 10 µl volume of amplified product was analysed by gel electrophoresis on 1% agarose gels. Amplified DNA fragments were visualized by ethidium bromide staining and amplicons were purified from agarose gels prior to sequencing. Sequencing reactions were performed by using Applied Biosystems BigDye Terminator v3.1 Cycle Sequencing kits on an ABI 3130XL Genetic Analyzer automated capillary sequencer and the full genome sequences were submitted to GenBank under accession numbers GQ287640–GQ287647. Sequences were aligned by using the gap and pileup programs in the Genetics Computer Group package (Accelrys) and MUSCLE software (Corpet, 1988). Phylogenetic analyses included maximum-parsimony (heuristic algorithm), neighbour-joining distance matrix (Kimura two-parameter and F84 corrections) and maximum-likelihood models within the PAUP program (Sinauer Associates). Bootstrap resampling (1000 replicates) provided estimates of confidence for the groups generated in each analysis. The 48 nt 5’-NTR sequences of all seven WEEV isolates were analysed by using MFold version 3.0 using the default parameters (Zuker, 2003).

**Neuropathology.** Three mice per study group (aerosol/subcutaneous, McM/IMP) were anaesthetized deeply with isoflurane and whole-body-perfused via the left ventricle with PBS for 5 min, followed by 10% formal saline for an additional 5 min. Perfused carcasses were left overnight in formal saline at 4 °C before the brains and olfactory bulbs were removed and processed for paraffin embedding (Colorado Histoprep). Paraffin sections (4–6 µm) were stained with haematoxylin and eosin (H&E). Coronal sections of brain at the level of the olfactory bulbs, frontal cortex, thalamus, optic tracts and pons were examined histologically.

**Statistical analyses.** Viraemia titres in mice were compared by using Kruskal–Wallace non-parametric ANOVA. Survival comparisons between isolates and routes of infection were done by the Kaplan–Meier method. Test statistics were generated by using the Mantel–Cox log-rank test in GraphPad Prism v.5.01 (GraphPad Software). Statistical significance of titres in organs from mice infected with McM and IMP via aerosol and subcutaneously was calculated by using a generalized linear model (S-Plus; Insightful Software). The model included log10(titre) as the response, and isolate, route, tissue and time as explanatory variables.

## RESULTS

### Nucleotide identity and phylogenetic analysis

A phylogram depicting the genetic relationships among the isolates used in this study, based on full genome sequences, is shown in Fig. 1. The divergent Argentinian WEEV isolate AG80-646 (Calisher et al., 1985) was used as an outgroup for clarity in the figure. There was no obvious clustering of isolates based on temporal, geographical or virulence characteristics. The nucleotide identity between McM and the other isolates tested here indicates that IMP (excluding the AG80 outgroup) is the most divergent from McM, with a nucleotide sequence identity of 97.3% over the entire genome. BFS-2005 had the closest (98.9%) nucleotide identity with McM. Supplementary Table S2 (available in JGV Online) illustrates the variation in numbers of amino acid changes unique to each isolate. The McM genome had 22 unique amino acid changes when compared with six other isolates. Of the 22 amino acid changes associated uniquely with McM, approximately one-third (seven of 22)
were in the E2 glycoprotein. IMP had no amino acids associated uniquely with it compared with all six isolates and McM alone. When comparing the amino acid sequences of only McM and IMP, 66 amino acid differences unique to McM were observed (Table 2). E2 contained 18 uniquely associated amino acid changes in McM, almost double the number in any other gene. Three of the major changes in E2 were clustered within a 68 aa stretch. Of the 66 unique amino acids associated with McM compared with IMP, 13 were major changes in the type of amino acid observed. These were noted in all genes, with the exception of 6K and E3. The NTRs of McM contained 10 nucleotide changes: one in the 5′ NTR, two in the junction region and seven in the 3′ NTR. No differences in secondary structure of the McM and IMP 5′ NTR stem–loops were observed (data not shown).

**Virus titres in cell culture**

McM titres in BHK cells were approximately $1 \log_{10}$ higher than IMP titres (Fig. 2a), whereas in the C6/36 mosquito cell line, IMP grew to $1 \log_{10}$ higher than McM (Fig. 2b). Growth in Vero cells varied between the two isolates, with 12 h intervals in peak titre times. Both isolates initially peaked at approximately the same titre between 18 and 24 h p.i.; however, IMP peaked again, approximately $0.5 \log_{10}$ higher than McM, at 48 h p.i. (Fig. 2c).

**Viraemia is brief and unassociated with virulence**

None of the infected mice (infected subcutaneously with $10^3$ p.f.u.) had detectable levels of virus in peripheral blood at 6 h p.i. At later time points, virus titres were quite variable. IMP had no detectable viraemia from day 2 onwards. By days 3 and 4, most groups had only a single individual with detectable viraemia and, by day 5, no sampled individuals had detectable viraemia. Mean viraemia showed no association with survival. Kruskal–Wallis non-parametric ANOVA of data from day 1 showed no significant difference among isolates in peak viraemia ($P=0.17$). Table 1 summarizes mortality data.

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**Table 2.** Positions and types of isolate-specific amino acid and non-translated region (NTR) nucleotide changes unique to representative high- and low-virulence WEEV isolates

<table>
<thead>
<tr>
<th>Gene/region</th>
<th>McM</th>
<th>IMP</th>
<th>Position(s)</th>
<th>Change(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amino acid</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nsP1</td>
<td>3</td>
<td>0</td>
<td>59, 222, 467</td>
<td>Q/E, N/K, K/R</td>
</tr>
<tr>
<td>Capsid</td>
<td>7</td>
<td>0</td>
<td>57, 74, 89, 156, 249, 250, 252</td>
<td>S/A, K/E, R/K, K/M, V/I, W/K, P/T</td>
</tr>
<tr>
<td>E3</td>
<td>1</td>
<td>0</td>
<td>315</td>
<td>H/R</td>
</tr>
<tr>
<td>6K</td>
<td>1</td>
<td>0</td>
<td>745</td>
<td>L/F</td>
</tr>
<tr>
<td>E1</td>
<td>5</td>
<td>0</td>
<td>780, 980, 1146, 1171, 1201</td>
<td>R/K, F/L, V/T, S/T, T/K</td>
</tr>
<tr>
<td><strong>Nucleotide</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTR</td>
<td>5′</td>
<td>1</td>
<td>33</td>
<td>G/A</td>
</tr>
<tr>
<td></td>
<td>Jxn</td>
<td>2</td>
<td>7489, 7495</td>
<td>T/C, T/A</td>
</tr>
<tr>
<td></td>
<td>3′</td>
<td>7</td>
<td>11218, 11238, 11291, 11354, 11366, 11394, 11463</td>
<td>A/C, A→C/T, C/T, T/C, A/G, G/A</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Phylogram based on full genome sequences, depicting the genetic relationships among seven WEEV isolates from Table 1. This figure was produced by using a distance algorithm; all three phylogenetic approaches used gave similar results. The numbers at nodes are bootstrap values (percentage of 1000 replicates) indicating the robustness of the analyses. NTID represents percentage identity of nucleotides over full genomes between McMillan (McM) and the other WEEV strains analysed. Bar, 1 % nucleotide divergence.
isolates varied from 100% mortality (McM) to no mortality (IMP) at a dose of $10^3$ p.f.u. The other isolates showed intermediate levels of virulence with respect to percentage mortality and MTD. Comparison of survival curves by the log-rank test identified three significantly different groups of isolates. The low-virulence group included IMP, 71V1658, BFS-2005 and 85-452NM. These isolates showed 0–20% mortality and an MTD of 7–9 days. Only IMP showed no mortality in mice at the $10^3$ p.f.u. dose throughout the time course. The intermediate group included Montana-64 with 70% mortality and an MTD of 6–7 days. The high-virulence isolate was McM, with 100% mortality and a short MTD of 4 days. Low-virulence (IMP) and the single high-virulence (McM) WEEV isolates were selected for further comparisons of virus titre in 17 distinct organs of CD1 mice infected by the subcutaneous and aerosol routes (Fig. 3a–e).

**Virus detection in 17 organs of mice infected subcutaneously with McM and IMP**

Of the 17 organs examined, eight showed statistically significant differences in titre between McM and IMP. McM titres in the brains of subcutaneously infected mice were between 3 and 5 log$_{10}$ higher than those infected by the same route with IMP (Fig. 3a). An increase of almost 3 log$_{10}$ in McM titre in the popliteal and inguinal lymph nodes was observed by 24 h p.i. (Fig. 3b, c). IMP showed a 1 log$_{10}$ titre increase in the spleen over the same time period (Fig. 3d). However, titres in the knees of infected mice were markedly higher (1–2 log$_{10}$) in mice infected with IMP compared with those infected with McM (Fig. 3e). In both isolates, virus titres in the brain ranged from 2 to 3 log$_{10}$ higher than those in other organs.

**Virus detection in 17 organs of mice infected with McM and IMP via the aerosol route**

A marked increase in the titres of both strains in the brains of aerosol-infected mice (compared with subcutaneously infected mice) was observed. Furthermore, titres observed in brains of McM-infected mice were 2–4 log$_{10}$ higher than in those infected with IMP (Fig. 3a). The increased virus titre in the brain was confirmed by the death of all McM-infected mice at the 120 h p.i. time point. Mice infected with IMP survived the full time course (Fig. 3a–e). With the exception of the brain, spleen, lung and salivary gland (results not shown for latter two organs), titres in all other organs were lower in mice infected by aerosol than in those infected subcutaneously.

**Statistically significant differences in virus titre were observed in organs of mice infected with McM and IMP**

The two-way interaction between isolate and tissue was statistically significant ($P < 0.01$), suggesting that the difference in mean titre between the McM and IMP isolates is not constant across all tissues, but rather varies from one tissue to another. Multiple comparisons using Scheffe’s method indicated that the magnitude of the difference in mean titre between the McM and IMP isolates was significantly larger for adrenal tissue than it was for heart, kidney, knee, lung, salivary gland, spleen and thymus tissues. An examination of significantly different mean titres for adrenal, brain, inguinal lymph node, liver, pancreas and skeletal muscle tissue showed higher mean titres in organs from mice infected with McM than in those...
infected with IMP. The mean titre for knee tissue is lower for the McM isolate than it is for the IMP isolate (Fig. 4a).

**Statistically significant differences in virus titre were observed in organs of mice infected via aerosol and subcutaneous routes**

The mean titre in brain, lung and salivary gland tissue was significantly higher for the aerosol route than for the subcutaneous route. The mean titre in knee, popliteal lymph node and skeletal muscle tissue was significantly lower for the aerosol route than for the subcutaneous route. There were no tissues for which the difference in mean titre was significantly different by subcutaneous infection, but not by aerosol (Fig. 4b).

**Survival**

The isolates identified as high (McM) and low (IMP) virulence by the subcutaneous route showed similar survival trends for mice infected by the aerosol route. The two isolates showed highly divergent virulence phenotypes by the aerosol route, similar to those observed after subcutaneous inoculation (Fig. 5a). Aerosol infection with McM resulted in 100% mortality with an MTD of 4.6 days (n=10), with IMP causing 10% mortality and an MTD of 7 days (n=10). The survival curves were significantly different between isolates according to the Mantel–Cox log-rank test (P<0.0001). Survival curves were also significantly different between McM and IMP by the intranasal and intravenous routes (Fig. 5b, P=0.002), as well as the intracerebral route of infection (Fig. 5c, P<0.0001). IMP showed no significant differences (P=0.54) in a comparison of routes of virus administration. There were significant differences in survival among routes of infection with McM (P<0.0001), with intracerebral being the most rapidly lethal (1.9 days) and intravenous showing the longest MTD (6.1 days). The intranasal, aerosol and subcutaneous routes of infection were intermediate in MTD.
Neuropathology

Brain lesions occurred in all McM-infected animals sampled and were considerably more severe at 120 h p.i. than at 96 h p.i. Multifocal and laminar areas of neuronal necrosis and oedema were distributed randomly mostly in forebrain areas (Fig. 6a). Small blood vessels were prominent with margi-nated lymphocytes, reactive endothelium and apoptotic nuclei and oedema in perivascular spaces (Fig. 6b). Fibrin thrombi were an occasional finding. Small clusters of lymphocytes and apoptotic nuclei were located in the meninges (Fig. 6c) and in subependymal areas. In contrast, two of three IMP-infected animals at 7 days p.i. and two of three animals at 13 days p.i. appeared normal. Isolated necrotic foci and small blood vessels with apoptotic forms and perivascular oedema were present in one animal at 7 days p.i. Glial nodules (Fig. 6d) and thin perivascular cuffs were an occasional finding.

DISCUSSION

The WEEV isolates studied here displayed wide variation in mortality and MTD by the subcutaneous route. The mouse model was used to distinguish between higher-virulence epizootic isolates of WEEV and lower-virulence enzootic viruses (Weaver et al., 1999). The epizootic McM isolate, originally recovered from a fatal human case of encephalitis, was by far the most lethal isolate tested here. However, other isolates showed intermediate to low mortality whether recovered from mosquito vectors or horse brain. By using the intracranial and intraperitoneal routes, others have shown that enzootic isolates of WEEV from California were not uniformly of low pathogenicity and could show a range of virulence in adult mice (Hahn et al., 1988). Nagata et al. (2006) reported 100 % mortality in each of eight isolates of WEEV by the intranasal route in adult BALB/c mice, although there was variation in MTD. One would therefore expect a greater variation between isolates in outbred strains (Hardy et al., 1974). Forrester et al. (2008) demonstrated that only one of ten WEEV isolates (CR46) caused >50 % mortality in subcutaneously infected Swiss–Webster mice (age of mice not given). The MTD of IMP when infected via aerosol was 7 days, considerably longer than any of the isolates tested by Nagata et al. (2006), and the survival rate was 90 %. Bianchi et al. (1993) also infected outbred Swiss NIH mice (28–32 days old) with similar doses, but using the intracranial and intraperitoneal routes of infection, and showed that

Fig. 4. (a) Eight tissues for which the mean log_{10}(titre) is statistically significantly higher in one isolate. ●, IMP; ◊, McM. Means are taken over all time points and both routes of infection. (b) Six tissues for which the mean log_{10}(titre) is statistically significantly higher for one route of infection. ●, Aerosol route; ◊, subcutaneous route. Means are taken over all time points and both isolates.
**Fig. 5.** Mortality in CD1 mice groups (n=10) infected with the McM (solid lines) and IMP (dashed lines) isolates of WEEV by the (a) subcutaneous (𝘹) and aerosol (●), (b) intranasal (●) and intravenous (𝘹) and (c) intracerebral (■) routes.

**Fig. 6.** Neuropathology of WEEV in CD1 mice. (a) McM, 96 h p.i. Note multiple necrotic neurons in the hippocampus. (b) McM, 96 h p.i. Note focal leptomeningitis in the cerebrum. (c) McM, 120 h p.i. Note perivascular apoptotic nuclei and oedema in the frontal cortex. (d) IMP, 13 days p.i. Note focal gliosis in the cerebral cortex. All panels were stained with H&E. Magnification, ×400.
neuroinvasiveness was uniquely associated with epizootic strains of WEEV. This suggests that the strain and age of the mouse, the route of virus infection and virus titre are all paramount in designing a suitable animal model.

In our model, CD1 mice were infected by the subcutaneous route and were 6–8 weeks of age. The resulting range of mortality rates underlined the benefits of this mouse strain and age as a tool for investigating virus virulence. Our study also aimed to determine whether the selected WEEV isolates showed distinct phenotypes by an aerosol route of infection, as they are potential BT agents (Sidwell & Smee, 2003). McM was 100% lethal by aerosol, whereas IMP showed only 10% mortality. IMP is therefore a good low-virulence strain for aerosol-infection studies to understand the molecular determinants of WEEV pathogenesis, which should lead to targets for vaccine or therapeutic design. It is of particular note that IMP caused no mortality when administered intracerebrally. McM, conversely, caused 100% mortality within 2 days. This suggests that IMP is clearly neuroinvasive, given the titres of virus detected in the brains of subcutaneously infected mice and the presence of neuropathological lesions in the brains of one-third of IMP-infected mice. However, IMP is clearly less neurovirulent than McM, causing no intracerebellar mortality, considerably less neuropathology and having a titre at least 1 log₁₀ lower than McM in brains. These data, combined with the types of pathology observed in the brains of infected mice (Fig. 6), suggest that McM induces more extensive damage to neurons in the central nervous system (CNS) than IMP.

In vitro growth curves in two vertebrate cell lines (BHK and Vero) and a mosquito cell line (C6/36) showed higher titres (1 log₁₀) of McM in vertebrate cells than IMP (Fig. 2a). In contrast, IMP generated higher titres in C6/36 cells (Fig. 2b). This could be due to Mcm and IMP having adapted to their host types (human brain and insect cells, respectively). The growth of both isolates in Vero cells was more balanced, possibly due to both isolates having most recently been passaged in Vero cells (Fig. 2c). It is possible that additional unknown and undocumented McM passages occurred between 1941 (year of initial isolation) and 1973 (passage history first documented by the CDC). This gap in the passage history could possibly explain the high levels of in vitro and in vivo growth of McM.

Surprisingly few studies infecting adult mice subcutaneously with WEEV have been carried out, although it is a good representative route of the natural cycle of WEEV. Monath et al. (1978) followed viraemia and organ titres after subcutaneous infection of 5–6-week-old Swiss mice with WEEV. Peak viraemia of 7.5 log₁₀ p.f.u. ml⁻¹ was reached by 1 day p.i., in contrast to the present study, which found a similar length of viraemia but a lower peak viraemia of 3–5 log₁₀ p.f.u. ml⁻¹, depending on the isolate (Bowen & Calosher, 1976). We found no association between viraemia and virulence, as no statistically significant difference was observed among isolates in the magnitude of peak viraemia. IMP, however, differed significantly from other isolates in that no viraemia was detected in mice after 2 days p.i. In cases of Venezuelan equine encephalitis virus (VEEV) and EEEV infection, it has been reported that the apparent infection ratios are 11:1 and 3:1, respectively (Sabattini et al., 1991).

McM, the most virulent isolate, grew to the highest titre in the brain, whereas the least virulent isolate (IMP) had significantly lower titres in the brain. Liu et al. (1970) also reported that mortality in mice was associated with encephalitic signs after subcutaneous infection. It should be noted that Monath et al. (1978) documented pathological changes in the heart during WEEV infection and observed overall mortality of 40%, largely in the absence of neurological signs. Therefore, WEEV may be capable of causing death as a result of damage to organs other than the brain.

IMP may replicate more slowly in the brain (lower neurovirulence) or affect the innate immune response differently, resulting in delayed pathogenesis. The data in Fig. 3(a) indicate clearly that IMP replicates or enters the brain at a much slower rate than McM when administered subcutaneously. Even when administered by aerosol, IMP titres in the brain (although significantly higher than those in mice infected by the subcutaneous route) are 2–5 log₁₀ lower than McM titres. The high titres (10⁸ p.f.u. g⁻¹) detected in the brains of infected mice are indicative of the efficiency of McM replication. This trend is seen in mice infected with virus by either route. The intracerebral route proved to be most neuroinvasive, with no McM-infected mice surviving beyond 48 h. These data, combined with the types of pathology observed in the brains of infected mice (Fig. 6), indicate that McM induces more extensive damage to neurons in the CNS than IMP, and the possible restriction of the ability of IMP to replicate in the neurons is responsible for the slower multiplication in the CNS, as seen with other alphaviruses such as Semliki Forest virus (Atkins et al., 1999). Thus, it is the difference in the rate of development of neuronal damage in the brains of mice infected with McM (compared with IMP) that could result in a lethal threshold before the immune system can intervene fully (Fig. 6).

The titres of both WEEV isolates in other organs in our investigation showed that virus administered by aerosol generated higher titres in the brain, spleen, lung and salivary gland than virus administered subcutaneously. As the inguinal and popliteal lymph nodes are found peripherally, it was not surprising that virus titres measured in those tissues were higher in subcutaneously infected mice than aerosol-infected mice (Fig. 3b, c). The virus titre of IMP was considerably higher in the knee joint of mice infected by either route than was observed with McM (Fig. 3e). This observation, combined with the virus titres in the brains and the absence of mortality in mice infected intracerebrally with IMP, suggests that IMP is a
more arthralgic isolate, whereas McM is more encephalitic (although still detectable in the knee-joint tissues). Future immunopathological and quantitative investigations will help to elucidate this issue.

Aguilar (1970) infected 1–2-day-old suckling mice and 3-week-old mice subcutaneously with WEEV isolated in Kern County, CA, USA, in 1957. In suckling mice, damage to mesodermally derived tissues resulted in death within 48 h. Three-week-old mice showed no clinical signs of disease although, beginning at 9 days p.i., encephalitis and changes including inflammatory infiltrates and haemorrhaging in some organs, such as lungs and liver, were noted. Aguilar (1970) used a different strain and age of mouse (Swiss Mice – Rockefeller strain), as well as a different virus isolate, which may explain this discrepancy. The change in tissue tropisms observed with ageing may continue, therefore, as mice mature past 3 weeks of age, to the point where WEEV does not replicate efficiently in many organs, but retains neuroinvasiveness. It was evident that both McM and IMP replicated in most organs; however, only the presence of virus in the brain, adrenal, inguinal lymph node, knee, liver, pancreas, skeletal muscle and spleen showed any significant difference between isolates (Fig. 4a). When analysing statistical significance between virus titres in mice inoculated by different routes (aerosol and subcutaneous), it was noted that only in the brain, lung and salivary gland were titres significantly higher via the aerosol route, and titres of both viruses in the knee, popliteal lymph node and skeletal muscle were significantly higher in mice infected subcutaneously (Fig. 4b). Given the routes of infection (respiratory tract and inner thigh) and the location of these organs with respect to the route of virus, these results are to be expected.

McM and IMP were the most genetically diverse isolates examined, having 66 amino acid changes (13 major changes in the type of amino acid observed) spanning their genomes, as illustrated in Table 2. Potential pathogenic determinants have been mapped to a region of the WEEV genome containing the carboxy-terminal 13 aa of the E2 gene, the 6K and E1 genes, and the 3’ NTR. Three of the major changes in E2 were clustered within a 68 aa stretch and could possibly play an important role in the efficiency with which WEEV binds/enters host cells. Previous studies have shown that the secondary structure of the stem–loops in the 5’ NTR of other alphavirus genomes plays a role in virus neurovirulence (Evans et al., 1985; Kinney et al., 1993; Logue et al., 2008; Netolitzky et al., 2000). The single nucleotide difference between McM and IMP at position 34, however, did not affect the secondary structure of the sole stem–loop of the WEEV 5’ NTR. Follow-up studies to determine the role of each of these virus elements in pathogenicity are under way.

This investigation demonstrated the effectiveness of our animal model in identifying two distinct WEEV isolates, representative of high and low virulence in mice. Through a series of in vivo and in vitro analyses, we have been able to show statistically significant differences in virus growth and survival between these isolates. Having selected two distinctly different WEEV isolates and mapped their amino acid and non-coding changes, we will identify which changes play an important role in virulence and neuro-pathogenesis and incorporate these data into the development of antiviral therapeutics for WEEV.

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