Neurovirulence and neuroinvasiveness of Murray Valley encephalitis virus mutants selected by passage in a monkey kidney cell line

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Variants of the prototype Murray Valley encephalitis virus (MVE-1-51) were selected by serial plaque purification and amplification in monkey kidney (Vero) cells. Four clones (C1–C4) at passage levels two and nine (P2 and P9) were examined in 21-day-old Swiss outbred mice for neuroinvasiveness (assessed from LD₅₀ values after intraperitoneal inoculation) and neurovirulence (LD₅₀ values after intracranial inoculation). The growth characteristics of the clones were determined in intracranially inoculated mouse brain and in mouse neuroblastoma, Vero and mosquito (C6/36) cell lines. Genomic RNA of the cloned virus stocks was sequenced through the structural protein genes (E, prM/M and C) and the 5' untranslated region. Clone C2P2 was of high

neuroinvasiveness whereas C2P9 was of low neuroinvasiveness; there were also decreased yields of C2P9 in C6/36 cells compared to C2P2 and MVE-1-51. These changes were associated with the substitution of valine for phenylalanine at amino acid position 141 of the C2P9 E protein. Clone C4P2 was of high neurovirulence and low neuroinvasiveness; C4P9 was of low neurovirulence, a change accompanied by a further reduction in neuroinvasiveness. Concomitantly, C4P9 showed a pronounced reduction in growth rates and yields in 21-day-old Swiss mouse brain, in mouse neuroblastoma cells and in C6/36 cells compared to parental virus. The phenotypic changes in clone 4 appear to be due to mutation(s) within non-structural protein genes.

Introduction

Flaviviruses are small, enveloped viruses with single-stranded positive sense RNA genomes, and are the causative agents of a number of clinically significant human diseases including yellow fever, dengue fever, Japanese encephalitis, St Louis encephalitis and Murray Valley encephalitis. The majority of flaviviruses are transmitted to vertebrate hosts via arthropod vectors, usually mosquitoes or ticks. Birds are believed to be the most important vertebrate hosts in the primary cycles of several encephalitogenic flaviviruses, including Murray Valley encephalitis virus (MVE) (Marshall, 1988).

Considerable interest has focused on the selection of attenuated flavivirus strains for use as vaccines and on understanding the molecular determinants of viral virulence. One successful strategy in such investigations has been to passage virus in cells derived from a vertebrate species not normally infected by the virus. The yellow fever virus 17D vaccine was derived by serial passage of the virulent Asibi strain in chick embryo tissues (Theiler & Smith, 1937). The Japanese encephalitis

virus SA14-14-2 vaccine was obtained by passage of the virus in primary hamster kidney cells (Huang, 1982).

We have previously shown that serial passage of the prototype MVE-1-51 virus in the SW13 human adenocarcinoma cell line rapidly leads to reduced virulence for mice, and, as five of six variants selected in this way were altered at amino acid 390 in the envelope protein (E), it was proposed that this amino acid is part of a domain which is crucial in the pathogenesis of MVE infection in mice (Lobigs *et al.*, 1990). By contrast, passage of MVE virus in cells derived from birds (chick embryo fibroblasts) and in *Aedes albopictus* (C6/36) mosquito cells did not lead to attenuation of the virus, nor to changes in the E gene (Lobigs *et al.*, 1990).

In the light of these results it was of interest to determine whether mouse-attenuated MVE mutants are also selected in Vero cells, a line of monkey kidney fibroblasts in common use for the assay, plaque purification and propagation of flaviviruses. Four individual plaques were selected from Vero cell monolayers inoculated with end-point dilutions of prototype MVE-1-51 mouse brain stock virus and passaged a total of nine times in Vero cells. We report here the effects of this procedure on viral virulence in 21-day-old Swiss mice. Variants with altered virulence were also tested at passage levels two and nine for growth rates and yields in

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mouse brain, and in Vero, C6/36 mosquito and mouse neuroblastoma (N18) cell lines *in vitro*. The structural protein genes and the 5' untranslated region were sequenced at passage levels two and nine to determine if genetic changes in these regions were associated with observed phenotypic changes.

Methods

Virus and cells. MVE-1-51, the prototype strain of MVE (Berge, 1975), had been passaged twice in embryonated eggs and 15 times in mouse brain (French, 1952). Working stocks were prepared as 10% suckling mouse brain (smb) homogenates in Hanks' balanced salt solution (HBSS, pH 8.0).

African Green Monkey kidney (Vero) cells (ATCC; CCL 81) were grown at 37 °C in medium 199 supplemented with lactalbumin hydrolysate and 8% newborn bovine serum in 5% CO₂/95% air; they were used between passage levels 122 and 137.

Aedes albopictus (C6/36) cells (ATCC; CRL 1660) were grown at 28 °C in Eagle's basal medium plus non-essential amino acids and 8% fetal calf serum (FCS) in 5% CO₂/air; they were used between passage levels 122 and 132.

Murine neuroblastoma (clone N18) cells (from Dr D. E. Griffin, Johns Hopkins University Medical School, Baltimore, Md., USA) were grown at 37 °C in Dulbecco's minimal essential medium plus 10% FCS in 5% CO₂/air; they were used between passage levels four and ten. N18 cells express surface markers indicative of neuronal origin and partially differentiate during culture (Amano *et al.*, 1972). To enhance adherence of N18 cells, culture wells were pre-coated for 2 h at ambient temperature with 0.5 mg/ml polyornithine (Sigma; 0.5 mg/ml) in borate buffer (pH 8.4).

Plaque assay. Virus was assayed by plaque formation on Vero cell monolayers grown in six-well plastic trays (TC grade; Linbro Scientific) following Newton *et al.* (1981). Monolayers were stained 2 days post-inoculation (p.i.) with 1 ml of agar stain (0.7% agar, 0.01% neutral red in water) and plaques were counted after overnight incubation. Infectivity titres are expressed as p.f.u./ml.

Passaging of MVE. Individual plaques (passage one) of parental MVE-1-51 stock virus were amplified on Vero cells (passage level two; P2). Four cloned MVE stocks prepared in this way (C1, C2, C3 and C4) were individually passaged on Vero cells. Passaging involved three further plaque selection steps, followed by amplification on Vero cells; only large plaques (2–3 mm) occurring singly on monolayers were selected. After the fourth plaque selection, stocks were amplified by two additional passages on Vero cells (passage level nine; P9). Amplified clones (C1, C2, C3 and C4) at P2 and P9 were used as low and high passage level stocks. At P2, stocks consisted of a mixed population of small (0.5 mm) plaques and large (3.0 mm) plaques in approximately equal proportion (data not shown). By P9, the C1, C2 and C4 stocks were 100% large plaques; C3P9 was a mixed small and large plaque population (data not shown).

Polyclonal antibody. Mouse hyperimmune anti-MVE-1-51 ascitic fluid (HIAF) was prepared by the method of Tikasingh *et al.* (1966). The preparation had a 90% neutralization titre of 1280 against MVE-1-51 in plaque reduction neutralization tests. Ascitic fluid from uninfected mice was used as non-immune mouse ascitic fluid (NMAF).

Virulence assay. Virulence assays were in Swiss outbred mice aged 21 days, following Monath *et al.* (1980). Mice were divided into groups of five and inoculated either intracranially (i.c.) or intraperitoneally (i.p.) with 10-fold dilutions of virus, from 10⁻¹ to 10⁻⁹. Mice were examined daily for 17 days for signs of illness, and deaths recorded. LD₅₀s were

calculated for each route of inoculation by the method of Reed & Muench (1938). LD₅₀s varied less than 5-fold in repeat assays with individual viruses. Based on i.c. LD₅₀, individual stocks were rated as high (LD₅₀ < 2 p.f.u.) or low (LD₅₀ > 20 p.f.u.) neurovirulence; based on i.p. LD₅₀, stocks were rated as high (LD₅₀ < 10 p.f.u.) or low (LD₅₀ > 10³ p.f.u.) neuroinvasiveness. Mouse experiments were conducted in accordance with the guidelines of the Australian National University Animal Ethics Committee.

Virus growth in mouse brain and cell culture. Mice (21-day-old) were inoculated i.c. with 100–200 p.f.u. of virus. At daily intervals, groups of three mice were anaesthetized and killed by exsanguination. Brains were dissected intact, weighed, snap-frozen on dry ice and stored at –80 °C. They were subsequently dispersed in a Dounce homogenizer and prepared as 10% suspensions in HBSS (pH 8.0) containing 2% bovine serum albumin; titration was by Vero plaque assay.

Vero, C6/36 and N18 cell monolayers were infected at an m.o.i. of approximately five. Virus was allowed to adsorb to the monolayers for 1 h at 37 °C (Vero, N18 cells) or 28 °C (C6/36 cells). Monolayers were then washed with phosphate-buffered saline, pH 7.4 (PBS) and growth medium was added. Growth medium was replaced (after washing with PBS) at 6, 12, 18, 24, 30, 36, 42 and 48 h p.i.; 1 h after replacement, growth samples were collected from duplicate cultures, stored at –80 °C and titrated by plaque assay. Titres are given as p.f.u./ml/h.

Immunofluorescence assay. Vero, C6/36 and N18 cell monolayers were infected (m.o.i. ~ 1) as above. At appropriate times, medium was removed and monolayers washed (× 2) with PBS, fixed for 1 min in ice-cold acetone-methanol (1:1), air-dried and incubated for 2 h at 37 °C with a 1:160 dilution of anti-MVE-1-51 HIAF. Monolayers were washed (× 2) with PBS and incubated (90 min) with FITC-conjugated rabbit anti-mouse immunoglobulin G (Miles-Yeda Ltd) at a dilution of 1:100 in PBS. Monolayers were washed with PBS, coated with PBS-glycerol (1:9) and examined under UV light with a Leitz Fluovert FS inverted fluorescence microscope.

Haemagglutination assay. MVE-infected C6/36 cell supernatants were used as a source of haemagglutinin. Haemagglutination assays were performed by the method of Clark & Casals (1958). Titres were determined as the reciprocal of the highest dilution which gave complete haemagglutination. Haemagglutination activity was assayed over the pH range 5.6–7.2.

Low-pH-induced fusion of MVE-infected C6/36 cells. Fusion-from-within assays were performed following Randolph & Stollar (1990). Confluent monolayers of C6/36 cells in 48-well tissue culture plates (Costar) were mock-infected or infected with MVE (m.o.i. ~ 10) and incubated for 48 h (28 °C, 5% CO₂) in Eagle's minimal essential medium (EMEM) plus 8% FCS. Monolayers were then washed (× 2) in PBS and incubated in serum-free EMEM containing both 10 mM-MES and 25 mM-HEPES (adjusted to various pH values with 0.5 M-HCl or 0.5 M-NaOH) for 15 min at 37 °C. Cells were washed (× 2) in PBS, overlaid with EMEM-MES-HEPES (pH 7.4) and incubated for 30 min at room temperature. Cells were fixed and stained with Diff-Quik (Lab-Aids) according to the manufacturer's instructions, washed with PBS and covered with 100 µl PBS-glycerol (1:9). The number of nuclei and the number of cell bodies in at least three high-power fields (approximately 1500 nuclei) were counted to determine the extent of fusion.

Infected cell RNA extraction. Nucleic acids were extracted from MVE-infected Vero cell monolayers (at 48 h p.i.) with a mixture of 6% (w/v) sodium *p*-aminosalicylate and phenol-cresol-8-hydroxyquinoline (89%, 10%, 1%) followed by a second phenol-cresol-8-hydroxyquinoline extraction (Shine & Dalgarno, 1973). Nucleic acids were precipitated with ethanol and high molecular mass, single-stranded (ss) RNA was precipitated by addition of 4 M-LiCl to a

final concentration of 2 M. This was followed by further ethanol precipitation and resuspension of RNA in 10 µl TE buffer (5 mM-Tris-HCl, 0.5 mM-EDTA, pH 7.4).

Sequence analysis. MVE-infected Vero cell RNA was amplified for sequencing by RT-PCR following Lee *et al.* (1992). First strand cDNA synthesis was done with two HPLC-purified oligonucleotide primers of minus polarity (Bresatec) (P1: ACTTCATATTTGATG; P2: GTGTATGAATATTCCG) and avian myeloblastosis virus (AMV) reverse transcriptase (Pharmacia LKB). cDNA representing MVE genome nucleotides 3–1375 and 948–2529 (Dalgarno *et al.*, 1986) was generated by PCR using P1, P2 and two primers of plus polarity: P3 (ACGTTTCATCTGCGTGAGC) and P4 (GCTCCTGCTTGCTCC-TGC). cDNA was amplified using *Taq* DNA polymerase (Pharmacia LKB) and primer pairs P1/P3 or P2/P4 with 30 cycles at 95 °C (1 min), 45 °C (1 min) and 72 °C (1.5 min) in a Hybaid thermal reactor. One of the primers used in PCR was phosphorylated (Lee *et al.*, 1992). Amplified cDNA was treated with λ -exonuclease (Gibco-BRL) for 1 h at 37 °C to digest the phosphorylated cDNA strand (Higuchi & Oehman, 1989); ssDNA was extracted and resuspended in 10 µl TE buffer. A 100 µl volume of RT-PCR product generated sufficient ssDNA for four sets of sequencing reactions using the dideoxynucleotide method (Sanger *et al.*, 1973), T7 DNA polymerase (Pharmacia LKB) and [35 S]dATP (Biggin *et al.*, 1983). Eight minus-strand primers were used to sequence the genes for E, the membrane protein (M), its precursor (prM) and the capsid protein (C). The first 20 nucleotides of the 5' untranslated region were sequenced on an infected-cell RNA template using AMV reverse transcriptase, following the method of Fichot & Girard (1990). Primer sequences are available on request to P. C. M. Reaction products were electrophoresed on 6% acrylamide gradient buffer gels (Biggin *et al.*, 1983) at 40 mA for 1.5–3.5 h, dried under reduced pressure and exposed overnight to Fuji RX100 film for autoradiography.

Results

Neuroinvasiveness and neurovirulence of MVE passaged clones

Virus from four representative plaques (denoted C1, C2, C3 and C4) of a mouse brain stock of the laboratory-adapted prototype, MVE-1-51, were passaged on Vero cell monolayers (see Methods). The neuroinvasiveness

Table 1. Neurovirulence and neuroinvasiveness of MVE Vero passage variants in 21-day-old mice

Virus clone	i.p. LD ₅₀ *	Neuro-invasiveness†	i.c. LD ₅₀ *	Neuro-virulence†
MVE-1-51	0.4	High	0.6	High
C1P2	9.6	High	0.4	High
C1P9	8.5	High	0.1	High
C2P2	4.4	High	0.2	High
C2P9	5.2 × 10 ³	Low	0.1	High
C3P2	0.4	High	0.1	High
C3P9	1.2	High	0.1	High
C4P2	5.7 × 10 ³	Low	0.6	High
C4P9	5.0 × 10 ⁴	Low	26.0	Low

* LD₅₀s are calculated following the method of Reed & Muench (1938), and are expressed as Vero p.f.u.

† Virus stocks were rated for neuroinvasiveness and neurovirulence according to criteria outlined in Methods.

Fig. 1. Growth kinetics of MVE-1-51, C4P2 and C4P9 in the brain following i.c. inoculation of 21-day-old Swiss mice. Mice were inoculated with 100–200 p.f.u. of virus and at the indicated times p.i. mice were anaesthetized and the brains removed. The infectivity of 10% brain homogenates was titrated by plaque formation on Vero cells; titres are expressed as p.f.u./g. At each sample time, titres are the average of three individual mouse brains, which varied less than 4-fold. ○, MVE-1-51; □, C4P2; ■, C4P9.

and neurovirulence of these clones were assessed at Vero passage levels 2 (P2) and 9 (P9) (by measuring i.p. and i.c. LD₅₀s in 21-day-old Swiss outbred mice) and the data compared with those obtained for the uncloned parental MVE stock.

Parental MVE-1-51 had an i.p. LD₅₀ of 0.4 p.f.u. (Vero) and was taken as the criterion of high neuroinvasiveness in 21-day-old Swiss mice (Table 1). At P2, C1, C2 and C3 had i.p. LD₅₀ titres of < 10 p.f.u. and were thus of high neuroinvasiveness. In contrast, C4 had an i.p. LD₅₀ of 5.7 × 10³ p.f.u. at P2, and was rated as being of low neuroinvasiveness. Further passage in Vero cells to P9 resulted in little change in i.p. LD₅₀ for C1 (8.5 p.f.u.) and C3 (1.2 p.f.u.), a 10³-fold increase for C2 (to 5.2 × 10³ p.f.u.) and an additional 10-fold increase for C4 (to 5 × 10⁴ p.f.u.).

Parental MVE-1-51 had an i.c. LD₅₀ of 0.6 p.f.u. and was defined as being of high neurovirulence in 21-day-old mice (Table 1). At P2, the MVE clones had i.c. LD₅₀ values between 0.1–0.6 p.f.u. and were thus all of high neurovirulence. At P9, C4 showed a ~40-fold increase in i.c. LD₅₀ to 26 p.f.u. and was therefore assessed as being of low neurovirulence. The other three clones showed no significant changes in neurovirulence on passaging.

These data indicate that clonal populations of MVE derived from mouse brain-passaged MVE-1-51 may

Fig. 2

Fig. 3

Fig. 2. Growth kinetics of C4P2 and C4P9 in N18 neuroblastoma cells. Monolayers were infected with virus (m.o.i. ~ 10) and incubated at 37 °C. At the indicated times p.i., growth medium was removed, monolayers washed with PBS (pH 7.4) and fresh growth medium overlaid for 1 h prior to sampling. Extracellular virus (EV) was titrated by plaque formation on Vero cells; titres are expressed as p.f.u./ml/h. At each sample time, titres are the average of four individual cell monolayers (in 35 mm diameter wells), which varied less than 4-fold. $\square$, C4P2; $\blacksquare$, C4P9.

Fig. 3. Growth kinetics of (a) C2P2, C2P9, and of (b) C4P2, C4P9 in C6/36 mosquito cells. Monolayers were infected with C2P2 or C4P9 (m.o.i. ~ 10) and incubated at 28 °C. At the indicated times p.i., growth medium was removed, monolayers washed with PBS (pH 7.4) and fresh growth medium overlaid for 1 h prior to sampling. Extracellular virus (EV) was titrated by plaque formation on Vero cells; titres are expressed as p.f.u./ml/h. At each sample time, titres are the average of four individual cell monolayers (in 35 mm diameter wells), which varied less than 4-fold. $\triangle$, C2P2; $\blacktriangle$, C2P9; $\square$, C4P2; $\blacksquare$, C4P9.

change in neuroinvasiveness and in neurovirulence on passage in Vero cell cultures. The neuroinvasiveness of C2 changed considerably between P2 and P9 without a change in neurovirulence. In contrast to the other three clones, C4 at P2 was already of low neuroinvasiveness; C4 at P9 was the only clone to become attenuated in neurovirulence after Vero cell passage.

Growth in mouse brain

To determine whether the low neurovirulence of C4P9 was due to altered virus growth characteristics in mouse brain, 21-day-old Swiss mice were inoculated i.c. with 100–200 p.f.u. of MVE-1-51, C4P2 or C4P9. Three brains were collected daily and were assayed separately by plaque formation on Vero cells up to 6 days p.i. (Fig. 1).

From 2 days p.i., MVE-1-51 and C4P2 reached significantly higher titres in the brain than did C4P9. This differential decreased gradually during the course of infection, although at 6 days p.i. brain titres were still 10-fold higher for C4P2 than for C4P9. Thus, the increased i.c. LD₅₀ of C4P9 compared with C4P2 (~ 40 -fold) is accompanied by a marked reduction in initial virus growth rates and virus titres in mouse brain tissue.

Growth of MVE clones in Vero, N18 and C6/36 cells

Growth of passaged MVE clones in cell culture was examined to determine if the observed alterations in virulence properties were associated with altered virus growth *in vitro*. Growth was assayed as virus released /h at eight time intervals up to 48 h p.i.

No significant differences in growth kinetics were observed between parental MVE-1-51 and the four clones at P2 in Vero, N18 or C6/36 cells (data not shown). MVE-1-51 and the four clones at P9 showed similar growth kinetics in Vero cells (data not shown). However, there was a > 100 -fold reduction in the growth of C4P9 (the clone of low neurovirulence) in N18 neuroblastoma cells relative to the growth of C4P2 (Fig. 2) and of parental MVE-1-51 (data not shown). In C6/36 mosquito cells, C2P9 grew poorly (~ 20 -fold reduction) compared to C2P2 (Fig. 3a). Similarly, C4P9 grew poorly in C6/36 cells (~ 20 -fold reduction) compared to C4P2 (Fig. 3b). Both C2P2 and C4P2 had similar growth rates in C6/36 cells compared to parental MVE-1-51 (data not shown).

Immunofluorescence studies were undertaken in N18 and C6/36 cells to determine if altered virus growth was reflected in a reduction in the proportion of cells infected.

Table 2. Percentage of N18 and C6/36 cells infected by MVE Vero passage variants

Virus clone	Time p.i. (h)...	Immunofluorescent cells (percentage of total cells)*			
		N18		C6/36	
		24	48	24	48
MVE-1-51		5	39	16	46
C1P2		7	46	21	46
C1P9		3	27	15	40
C2P2		2	39	13	58
C2P9		2	55	7	15
C3P2		7	36	14	51
C3P9		5	43	15	44
C4P2		3	15	15	53
C4P9		< 0.1	0.1	13	23

* Cell monolayers were inoculated (m.o.i. ~ 1) and incubated for the indicated times at 37 °C (N18) or 28 °C (C6/36). Anti-MVE-1-51 HIAF was used at a dilution of 1:100 in HBSS (pH 8.0). Controls were mock-inoculated cultures stained with anti-MVE-1-51 HIAF or MVE-1-51-inoculated cultures stained with NMAF. A total of ~ 600 cells in three separate fields were counted per virus-infected cell monolayer. All assays were performed in duplicate.

For N18 cells inoculated with C4P9 (the clone of low neurovirulence) the proportion of cells with detectable MVE antigen at 48 h p.i. was 150-fold less than for C4P2. N18 cells inoculated with MVE-1-51 or with C1, C2 and C3 at P2 and P9 showed much smaller differences in the percentage of cells showing MVE antigen whether compared at 24 h or 48 h p.i. (Table 2).

In C6/36 cells at 48 h p.i., the percentage of cells infected with C2P9 and C4P9 was 4-fold and 2-fold less, respectively, than for C2P2 and C4P2 as judged by immunofluorescent detection of MVE antigen (Table 2). Thus, the 20-fold reduction in growth of C2P9 and C4P9 in C6/36 cells compared to C2P2 and C4P2 (Fig. 3*a, b*) was accompanied by a 2- to 4-fold reduction in the percentage of C6/36 cells containing MVE antigen. The percentages of cells infected by clones C1 and C3 were essentially similar to those infected by MVE-1-51 at both passage levels.

Haemagglutination and low-pH-induced fusion of MVE-infected C6/36 cells

In other studies (P. C. McMinn, L. Dalgarno & R. C. Weir, unpublished data) we have characterized a neutralization escape variant of MVE with a single amino acid substitution in E which is of low neuroinvasiveness in mice. This mutant is unable to agglutinate goose erythrocytes at acidic pH and also shows decreased syncytium forming activity in a low-pH-dependent fusion-from-within assay (Randolph & Stollar, 1990).

Table 3. Nucleotide and amino acid substitutions of MVE Vero passage variants

Virus clone	Nucleotide mutation and position*	Amino acid substitution†
C1P2	No change	No change
C1P9	C1249 → U C1587 → U	No change E205 Thr → Ile
C2P2	No change	No change
C2P9	G1394 → U	E141 Val → Phe
C3P2	No change	No change
C3P9	G512 → U	prM14 Val → Leu
C4P2	U1101 → C	E43 Ile → Thr
C4P9	No change	No change

* Numbering is from the 5' nucleotide of the MVE RNA molecule (Dalgarno *et al.*, 1986).

† Numbering is from the N-terminal amino acid of E (Lobigs *et al.*, 1990) or prM (Dalgarno *et al.*, 1986).

To determine if the attenuation of C2P9 and C4P9 virulence was associated with changes in low-pH-induced haemagglutination and/or fusion, parental MVE-1-51 and clones C1–C4 at P2 and P9 were examined for haemagglutination and for syncytium formation (see Methods). No differences in the rate or extent of haemagglutination or of syncytium formation were observed between parental MVE-1-51 and clones C1–C4 at either passage level. Peak haemagglutination titres for parental MVE-1-51 and the Vero-passaged MVE variants were at pH 6.5–6.7 (data not shown). Similarly, the fusion pH threshold for parental and variant MVE was at pH 6.5 (data not shown).

Sequence analysis of MVE clones

Sequence studies were carried out on clones C1–C4 at P2 and P9 to determine whether mutations in the structural protein genes were selected during passage. High molecular mass RNA was extracted from infected Vero cells at 48 h p.i. and the viral RNA amplified by RT-PCR (see Methods). Viral cDNA was sequenced through the C, prM and E genes and the 5' untranslated region.

At P2, no nucleotide or amino acid differences between parental MVE-1-51 and C1, C2 or C3 were detected; however, C4P2 differed from MVE-1-51 at codon 43 in the E gene (U1101 → C) resulting in the non-conservative amino acid substitution of isoleucine for threonine (Table 3). The sequence of parental MVE-1-51 in the C, prM and E genes did not differ from the published sequence (Dalgarno *et al.*, 1986).

Two mutations in the structural protein genes were identified in C1P9 (C1249 → U, C1587 → U) and one in each of C2P9 (G1394 → U) and C3P9 (G512 → U). Two of the four nucleotide substitutions observed at P9 led to changes in E and one led to a change in prM, the

precursor to M; no changes were observed in the C gene or in the 5' untranslated region. One silent change in the E gene was also identified. The nucleotide sequence data predict amino acid substitutions for C1P9 at E205 (Thr → Ile), for C2P9 at E141 (Val → Phe) and for C3P9 at prM14 (Val → Leu). The sequence of C4P9 was identical to the parental MVE-1-51 sequence and thus the E43 change identified in C4P2 had not survived subsequent rounds of plaque purification and amplification.

Discussion

Vero cells, a line of monkey kidney fibroblasts, are commonly used in the assay and propagation of flaviviruses. In this study we have examined the effect of serial passage in these cells on a mouse-brain-adapted MVE strain. Aspects of pathogenesis in 21-day-old mice, *in vitro* growth characteristics, and sequence analyses of the structural protein genes and 5' untranslated region of four clones of the virus were examined at Vero passage levels 2 and 9, and compared with the parent MVE-1-51.

Neither the neurovirulence, neuroinvasiveness nor the nucleotide sequences of clones 1, 2, and 3 at P2 could be distinguished from those of parental MVE-1-51. At P9, single mutations were detected in the structural protein genes of two of the clones (C2P9, C3P9) resulting in one amino acid substitution in each clone; the third clone (C1P9) had two nucleotide changes only one of which led to an amino acid change. Among these three virus stocks, phenotypic changes were detected only in C2P9, where the substitution at E141 (Val → Phe) was accompanied by a 1000-fold increase in the i.p. LD₅₀ in 21-day-old mice; i.e. there was a significant attenuation of neuroinvasiveness relative to MVE-1-51 and C2P2. There was also reduced infectivity and yield in C6/36 mosquito cells, but the neurovirulence of C2P9 was unimpaired. Amino acid 141 lies within a region (10 amino acids) of the ectodomain of E which is highly conserved between the MVE, Japanese encephalitis (JE) and West Nile (WN) viruses (Lobigs *et al.*, 1990). Although it is likely that changes in this region will induce changes in phenotype, we cannot exclude the possibility that changes in the non-structural protein genes or in the 3' untranslated region are involved in the loss of C2P9 neuroinvasiveness. Since, on passaging, clones C1 and C3 showed little change in neuroinvasiveness and neurovirulence, we conclude that the changes in E at amino acid 205 (C1P9) and in prM at amino acid 14 (C3P9) were phenotypically silent in relation to these particular properties.

Clone 4 produced results which were different in kind from the other three and is of particular interest. While retaining full neurovirulence, C4 at P2 differed from

parental MVE-1-51 in that it was only weakly neuroinvasive in 21-day-old mice. One mutation (U1101 → C) was detected in C4P2 leading to a non-conservative amino acid substitution at E43 (Ile → Thr). At P9 the neuroinvasiveness of C4 was further attenuated and, significantly, there was also a reduction in neurovirulence. Concomitantly and relative to MVE-1-51 and C4P2, there was a greatly reduced growth rate in i.c.-inoculated mouse brain and a 10-fold reduction in virus yield at 5 days p.i.; there was a 150-fold reduction in the proportion of N18 cells infected at 48 h p.i. and more than a 100-fold reduction in virus yield/h; the percentage of C6/36 cells infected at 48 h p.i. was halved and the virus yield/h reduced 10-fold; growth in Vero cells was unaltered.

Sequencing the 5' untranslated region and structural protein genes of C4P9 revealed that the mutation at nucleotide 1101 (U → C) in the E gene observed in C4P2 had reverted to the MVE-1-51 sequence, and that the sequenced regions of the genome were now identical to those of parental MVE-1-51. It is most likely that virus carrying the parental sequence at nucleotide 1101 was plaque purified between P2 and P9, and suggests that C4P2 probably represented a mutant that was not *per se* better adapted for growth in Vero cells than parental MVE-1-51. Alternatively, concurrent or subsequent mutations in the non-structural and/or 3' untranslated regions (which became phenotypically apparent by P9) may have imposed a selective advantage on the original configuration of E. Thus, it appears that the attenuation of neuroinvasiveness seen with C4P2 was the result of change in the non-structural or 3' untranslated regions rather than a result of the amino acid substitution at E43. The attenuation of neurovirulence by P9 would require a second mutation in the non-structural/3' untranslated region, and this could also account for the apparent further decline in neuroinvasiveness between P2 and P9 (the assay of both i.c. and i.p. LD₅₀ is based on lethal neuronal infection and if peripherally inoculated virus breaching the blood-brain barrier is not fully neurovirulent there will be an apparent attenuation of neuroinvasiveness).

Considered together, the data show that a small number of plaque purification and amplification steps in Vero cells allowed the selection of mutants attenuated in neuroinvasiveness (2/4 cases) or neurovirulence (1/4 cases) in mice. Lobigs *et al.* (1990) showed that serial passage of six individual clones of MVE virus in SW13 human adenocarcinoma cells resulted in the attenuation of neuroinvasiveness in mice by passage level 10 in 5/6 cases. When inoculated by the i.c. route there was no indication that these SW13 passaged strains were attenuated in neurovirulence (Lobigs *et al.*, 1990). This indicates that attenuation of neuroinvasiveness is a

common response to passaging MVE virus in cultured Vero and SW13 cells. Studies demonstrating attenuation of flavivirus neurovirulence have also been reported, but this appears to be a less frequent response to passaging. Dunster *et al.* (1990) demonstrated attenuation of WN virus neurovirulence after six passages in HeLa cells; the passaged virus showed altered reactivity to an E protein MAb compared to parental virus, suggesting that attenuation was due to mutation in the E protein. Recently, Ni *et al.* (1994) showed that the highly passaged JE vaccine strain (SA14-14-2), which is of low neurovirulence in adult mice, has two amino acid substitutions in the E protein at amino acids 138 (Glu → Lys) and 176 (Ile → Val). It was suggested that these amino acid substitutions may contribute to attenuation of neurovirulence in the JE vaccine strain. It is of interest that amino acid 138 (associated with altered neurovirulence in JE) is in the same conserved region of E as amino acid 141 (associated with altered neuroinvasiveness in MVE). Thus it is possible that the region 138–141 in E is a virulence determinant. In this context, it would be of interest to know whether the JE vaccine strain is also of low neuroinvasiveness. It would now be possible to address this question by the creation of single site mutants using the JE infectious cDNA clone (Sumiyoshi *et al.*, 1992).

Changes in the structural protein genes of SW13-passaged MVE variants were restricted to the E gene (Lobigs *et al.*, 1990). Similarly, with the Vero-passaged MVE variants, three of the four observed changes in the structural protein genes were in the E gene. Following passage in SW13 cells, changes were observed in E at amino acids 117, 118, 390, 423 and 460 but not in M; changes at E390 were frequent and appeared consistently in variants with decreased neuroinvasiveness (Lobigs *et al.*, 1990). Although passage in Vero cells most commonly led to changes in E, the changes followed a different pattern to those seen in SW13 cells. Changes were at amino acids 43, 141 and 205, with one at prM14, suggesting that selection pressures may have been different. Also, within the limits of the properties tested, at least two and probably three of the four changes in E and M were phenotypically silent. However, the passaging procedure used in this study differed substantially from that of Lobigs *et al.* (1990), and thus limited comparison of observed phenotypic and genetic differences can be made.

In other studies designed to identify any 'overlap' between MVE neutralization epitopes and virulence determinants, we have shown that certain MAb escape variants of MVE altered in the E-1c neutralization epitope (Hawkes *et al.*, 1988) at amino acids 276 or 277 were of reduced neuroinvasiveness in 21-day-old mice (P. C. McMinn & E. Lee, unpublished results). Attenuated MVE virus, derived by passage in Vero or SW13

cells, was not altered at these amino acids. We conclude that different selection strategies can be used to introduce apparently different classes of independent attenuating mutations into the E protein of MVE. These observations might ultimately be useful in the design of non-reverting flavivirus vaccines from infectious cDNA clones.

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References

- AMANO, T., RICHELSON, E. & NIRENBERG, M. (1972). Neurotransmitter synthesis in neuroblastoma cells. *Proceedings of the National Academy of Sciences, USA* **69**, 258–263.
- BERGE, T. O. (1975). *International Catalogue of Arboviruses Including Certain Other Viruses of Vertebrates*, 2nd edn, pp. 504–505. Bethesda, Md.: Dept. of Health, Education and Welfare.
- BIGGIN, M. D., GIBSON, T. J. & HONG, G. F. (1983). Gradient buffer gels and ³⁵S label as an aid to rapid DNA sequence determination. *Proceedings of the National Academy of Sciences, USA* **80**, 3963–3965.
- CLARK, D. H. & CASALS, J. (1958). Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. *American Journal of Tropical Medicine and Hygiene* **7**, 561–573.
- DALGARNO, L., TRENT, D. W., STRAUSS, J. H. & RICE, C. M. (1986). Partial nucleotide sequence of Murray Valley encephalitis virus genome: comparison of the encoded polypeptides with yellow fever virus structural and non-structural proteins. *Journal of Molecular Biology* **187**, 309–323.
- DUNSTER, L. M., GIBSON, C. A., STEPHENSON, J. R., MINOR, P. D. & BARRETT, A. D. T. (1990). Attenuation of virulence of flaviviruses following passage in HeLa cells. *Journal of General Virology* **71**, 601–607.
- FICHOT, O. & GIRARD, M. (1990). An improved method for sequencing of RNA templates. *Nucleic Acids Research* **18**, 6162.
- FRENCH, E. L. (1952). Murray Valley encephalitis: isolation and characterisation of the aetiological agent. *Medical Journal of Australia* **1**, 100–103.
- HAWKES, R. A., ROHRIG, J. T., HUNT, A. R. & MOORE, G. A. (1988). Antigenic structure of Murray Valley encephalitis virus E glycoprotein. *Journal of General Virology* **69**, 1105–1109.
- HIGUCHI, R. G. & OCHMAN, H. (1989). Production of single stranded templates by exonuclease digestion following the polymerase chain reaction. *Nucleic Acids Research* **17**, 5685.
- HUANG, C. H. (1982). Studies of Japanese encephalitis in China. *Advances in Virus Research* **27**, 71–101.
- LEE, E., NESTOROWICZ, A., MARSHALL, I. D., WEIR, R. C. & DALGARNO, L. (1992). Direct sequence analysis of amplified dengue virus genomic RNA from cultured cells, mosquitoes and mouse brain. *Journal of Virological Methods* **37**, 275–288.
- LOBIGS, M., USHA, R., NESTOROWICZ, A., MARSHALL, I. D., WEIR, R. C. & DALGARNO, L. (1990). Host cell selection of Murray Valley encephalitis virus variants altered at an RGD sequence in the envelope protein and in mouse virulence. *Virology* **176**, 587–595.
- MARSHALL, I. D. (1988). Murray Valley and Kunjin encephalitis. In *The Arboviruses: Epidemiology and Ecology*, pp. 151–189. Edited by T. P. Monath. Boca Raton, Fla.: CRC Press.
- MONATH, T. P., CROPP, C. B., BOWEN, G. S., KEMP, G. E., MITCHELL, C. J. & GARDNER, J. J. (1980). Variation in virulence for mice and rhesus monkeys among St Louis encephalitis virus strains of different origin. *American Journal of Tropical Medicine and Hygiene* **29**, 948–962.
- NEWTON, S. E., SHORT, N. J. & DALGARNO, L. (1981). Bunyamwera virus replication in cultured *Aedes albopictus* (mosquito) cells:

- establishment of a persistent viral infection. *Journal of Virology* **38**, 1015–1024.
- NI, H., BURNS, N. J., CHANG, G.-J. J., ZHANG, M.-J., WILLS, M. R., TRENT, D. W., SANDERS, P. G. & BARRETT, A. D. T. (1994). Comparison of nucleotide and deduced amino acid sequence of the 5' non-coding region and structural protein genes of the wild-type Japanese encephalitis virus strain SA14 and its attenuated vaccine derivatives. *Journal of General Virology* **75**, 1505–1510.
- RANDOLPH, V. B. & STOLLAR, V. (1990). Low pH-induced fusion in flavivirus-infected *Aedes albopictus* cell cultures. *Journal of General Virology* **71**, 1845–1850.
- REED, L. J. & MUENCH, H. (1938). A simple method of estimating fifty percent endpoints. *American Journal of Hygiene* **27**, 493–497.
- SANGER, F., NICKLEN, S. & COULSON, A. R. (1973). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences, USA* **74**, 5463–5467.
- SHINE, J. & DALGARNO, L. (1973). Occurrence of heat-dissociable ribosomal RNA in insects: the presence of three polynucleotide chains in 26S RNA from cultured *Aedes aegypti* cells. *Journal of Molecular Biology* **75**, 57–72.
- SUMIYOSHI, H., HOKE, C. H. & TRENT, D. W. (1992). Infectious Japanese encephalitis virus RNA can be synthesized from *in vitro*-ligated cDNA templates. *Journal of Virology* **66**, 5425–5431.
- THEILER, M. & SMITH, H. H. (1937). The effect of prolonged cultivation *in vitro* upon the pathogenicity of yellow fever virus. *Journal of Experimental Medicine* **65**, 767–786.
- TIKASINGH, E. S., SPENCE, L. & DOWNS, W. G. (1966). The use of adjuvant and sarcoma 180 cells in the production of mouse hyperimmune ascitic fluids to arboviruses. *American Journal of Tropical Medicine and Hygiene* **15**, 219–226.

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