Replication of yellow fever virus in the mouse central nervous system: comparison of neuroadapted and non-neuroadapted virus and partial sequence analysis of the neuroadapted strain

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Serial passage of yellow fever virus (YF17D) in mouse brain enhances neurovirulence, causing a reduction in survival time after intracerebral inoculation of adult mice. To study the biological and genetic basis for this phenomenon, we compared neurovirulence properties of the neuroadapted Porterfield strain (PYF) to a YF17D strain generated from a full-length YF cDNA template (YF5.2iv). Adult mice were infected by olfactory bulb inoculation, which results in widespread distribution of virus throughout the central nervous system. Although PYF and YF5.2iv spread rapidly throughout the neuraxis, maximal titres of PYF in the brain and spinal cord were 1000- to 10,000-fold higher than those of YF5.2iv. Paralysis and death occurred earlier with the PYF strain. Several cDNA clones of the E/NS1 region of the PYF strain were sequenced. Three predicted amino acid changes were consistently observed in the envelope protein of the PYF strain compared to YF5.2iv. Common substitutions were also identified in NS1 and NS2A. The potential contribution of these genetic differences to neurovirulence was evaluated by generating recombinant, intertypic PYF/YF5.2iv viruses. Physical signs of disease and mean spinal cord titres after inoculation of one recombinant were not different from the YF5.2iv parent. Our data indicate that PYF and YF5.2iv differ significantly in their virulence properties, however, common amino acid substitutions in the E/NS1 region of the PYF strain do not determine its enhanced neurovirulence. Other regions of the viral genome may contribute dominant effects on the virulence properties of the PYF strain.

Introduction

Flaviviruses, of which yellow fever virus (YF) is the prototype, have in common the capacity to replicate in the central nervous system (CNS) of mice. This property, described first for YF by Theiler in 1930, can be modified by passage of the virus. Serial passage in mouse brain increases neurovirulence (Theiler, 1951; Meers, 1959; reviewed in Schlesinger, 1980), whereas passage in cell culture has been associated with reduction of virulence properties (Hardy, 1963; Hearn et al., 1965, 1966; Converse et al., 1971; Barrett et al., 1990). Although some biological markers associated with enhanced neurovirulence have been described (Schlesinger, 1980), molecular determinants of the virus-specific factors which account for this virulence remain unknown. Such determinants may potentially be encoded within multiple regions of the viral genome, as is true for other neurotropic RNA viruses (Agol et al., 1985; Omata et al., 1986; Tardy-Panit et al., 1993).

Flaviviruses contain a single positive-strand RNA genome of approximately 11 kb, encoding a single open reading frame that encodes at least 11 viral proteins (Rice et al., 1985, 1986). The gene order has been established as 5' C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-2K-NS4B-NS5 3' (Chambers et al., 1990; Lin et al., 1993). The C, prM and E proteins constitute the structural components of the virion and the remainder of
the genome encodes the non-structural proteins required for viral replication. The virion envelope protein, E, which subserves cell attachment, and the viral NS1 protein, which occurs as both cell surface and soluble forms, have been identified as critical targets of the protective antibody response against flavivirus infection, suggesting that these proteins may contain virulence determinants (Gould et al., 1986; Brandriss et al., 1986; Bray et al., 1989; Konishi et al., 1991; Schlesinger et al., 1985, 1986). The effect of amino acid substitutions in the E protein on virulence properties has been evaluated by sequence analysis of host-range mutants, monoclonal antibody-escape mutants and virulence revertants (Lobigs et al., 1990; Holzmann et al., 1990; Cecilia & Gould, 1991; Hasegawa et al., 1992; Jiang et al., 1993; Jennings et al., 1994; Sumiyoshi et al., 1995; McMinn et al., 1995a, b). These mutants often contain single mutations in the envelope protein, however, mechanisms responsible for the attenuated phenotype of these strains have not been identified. Since the mutations map to multiple distinct positions within the structure of the envelope protein, it is possible that the virulence may be associated with several different functions of this protein. Such studies have also indicated that the E protein contains determinants which affect neuroinvasiveness after peripheral inoculation of adult mice but the roles played by either E or NS1 in affecting neurovirulence have been less well characterized. Recent studies with engineered dengue viruses have revealed that the E protein contains determinants which affect neurovirulence in the mouse CNS (Kawano et al., 1993; Bray & Lai, 1991). It has also been shown that mutations in the viral NS1 protein, when examined in the context of a tick-borne encephalitis/dengue virus chimera can both increase and decrease neurovirulence (Pletnev et al., 1993).

To explore the genetic basis for the neurovirulence of yellow fever virus, two YF17D strains that exhibit different rates of replication in mouse brain after intracerebral inoculation were studied. The first strain, YF17D-Porterfield (PYF), has been observed to be uniformly lethal following intracerebral inoculation and results in shortened survival time compared to its unpassaged vaccine parent (James Porterfield, personal communication). The second strain, YF5.2iv, is a cloned derivative of YF17D generated from infectious RNA transcribed from full-length cDNA templates (Rice et al., 1989). In the present studies, neurovirulence was assessed by determining the degree of viral replication and spread within the mouse CNS after intra-olfactory bulb inoculation. This route of challenge provides anatomically reproducible access to the CNS resulting in spread of virus to the hindbrain and spinal cord. It has also been proposed as the site of entry of some flaviviruses into the brain during the course of viraemia (Monath et al., 1983; Findlay & Clarke, 1935).

**Methods**

**Cells and viruses.** SW-13 and Vero cells were maintained as described previously (Chambers et al., 1989; Schlesinger et al., 1990). The PYF strain, originally obtained from Dr James Porterfield, was derived by more than 20 serial passages in suckling mouse brain. The strain was maintained by passage in suckling mouse brain and inocula prepared as 20% brain suspensions in phosphate-buffered saline. YF5.2iv was prepared as described previously (Rice et al., 1989; see below). YFS-3/232 was obtained from Dr Ernest Gould and passed once on SW-13 cells prior to further experiments.

**Animal experiments.** Female CD-1 white mice (3-4-week-old) were used for neurovirulence studies. For olfactory bulb inoculation, the naso-frontal bone of ketamine-anaesthetized mice was exposed, a burr hole created and 3 μl of virus suspension inoculated into the right olfactory bulb using a drawn-glass microinjector attached to a Hamilton syringe. At appropriate intervals following challenge, mice were anaesthetized with carbon dioxide and exsanguinated by cardiac puncture. Spinal fluid was collected by cisternal puncture (Carp et al., 1971) and was followed by removal of brain, spinal cord and adrenal glands. Fluids and tissues were stored in minimal essential medium at −80 °C before homogenization and plaque assay on Vero cells.

**Derivation of cDNA clones.** RNA was prepared from suckling mouse brain extracts containing approximately 10⁸ p.f.u./ml of the PYF strain using RNAzol (TelTest) and conditions recommended by the manufacturer. YF cDNA was derived using reverse transcriptase—polymerase chain reaction (RT-PCR). First-strand cDNA synthesis was carried out using negative-sense primers corresponding to YF nucleotides 2619–2639 or 2851–2870 (clones of series 67 and 68; see Table 1) and 3858–3877 (clones of series 72; see Table 1) and SuperScript reverse transcriptase (Gibco/BRL). PCR amplification of the cDNA was performed using primer sets containing the same negative-sense 3' primers and positive-sense 5' primers corresponding to YF nucleotides 1–18 (clones 67 and 68) or 773–789 (clones of series 72). PCR fragments were generated using AmpliTaq (Perkin-Elmer) using a program of 94 °C × 1 min for denaturation, 50 °C × 1 min for annealing and 72 °C × 3 min for polymerization for a total of 30 cycles. PCR fragments were isolated from low-melting temperature agarose gels. Fragments of the 67 and 68 series (see Table 1) were then cloned into the YFS-3'1IV plasmid (Rice et al., 1989) using the AvrI and NsiI restriction sites. Fragments of the 72 series were cloned into the YFM 5.2 plasmid (Rice et al., 1989) using the AvrII and NsiI restriction sites. Large-scale plasmid preparations were purified by caesium chloride centrifugation and the complete nucleotide sequence from position 537 (AvrI) to 3824 (AvrII) was derived for several clones using the dideoxynucleotide chain-termination method (Sequenase, USB). In some experiments, PCR fragments generated from the PYF and the YFS-3/232 cDNAs were analysed by cycle sequencing (Promega fmol cycle sequencing kit) using 32P-labelled primers specific for the YF envelope protein region.

**Regeneration of infectious YF17D.** Plasmids containing the engineered PYF sequences (YF 5.2'TV-67.2 and YFM 5.2-72, 72.1, 72.11, 72.21 and 72.26; see Table 1) were used to generate full-length YF cDNA templates by in vitro ligation. RNA transcripts were synthesized and transfected into monolayers of SW-13 cells essentially as described
Table 1. *Amino acid substitutions in the E protein of PYF clones*

<table>
<thead>
<tr>
<th>Clone*</th>
<th>Virust</th>
<th>52</th>
<th>123</th>
<th>173</th>
<th>185</th>
<th>282</th>
<th>305</th>
<th>325</th>
<th>380</th>
<th>444</th>
<th>462</th>
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<tr>
<td>67.1</td>
<td>NT</td>
<td>G</td>
<td>T</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>G</td>
<td>T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>68.1</td>
<td>NT</td>
<td>G</td>
<td>T</td>
<td>R</td>
<td>T</td>
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</tr>
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<td>NT</td>
<td>G</td>
<td>T</td>
<td></td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
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<td>G</td>
<td>T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>72</td>
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<td>R</td>
<td>M</td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>72.1</td>
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<td>V</td>
<td>R</td>
<td>W</td>
<td>M</td>
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<td>72.11</td>
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<td>S</td>
<td>V</td>
<td>L</td>
<td>T</td>
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</tr>
<tr>
<td>72.21</td>
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<td>V</td>
<td>T</td>
<td>I</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>R</td>
<td>K</td>
<td>I</td>
<td>Q</td>
<td>R</td>
<td>F</td>
<td>S</td>
<td>R</td>
<td>G</td>
<td>I</td>
</tr>
</tbody>
</table>

* Clones denoted by 67 and 68 refer to sequences within the YF5'iv plasmid; 72 indicates the YFM5.2 plasmid. The breakpoint for the sequence of the E protein in these clones occurs at residue 229.

† Virus indicates whether infectious virus was recovered from the respective clone. NT, Clone not tested for recovery of virus.

: Blank spaces within columns indicate that the predicted residue matches the sequence of the YF5.2iv molecular clone.

Results

Comparison of replication and spread of PYF and YF5.2iv in mouse CNS

Fig. 1 compares replication and spread of PYF and YF5.2iv within the CNS of young adult mice following olfactory bulb inoculation. Both viruses exhibited a roughly similar time course of detection in the left and right hemispheres of the brain, as well as spinal cord and adrenal glands following infection of the olfactory bulb. Peak titres of virus in brain and spinal cord reached by PYF were 1000- to 10000-fold higher than YF5.2iv. Concordantly, PYF-challenged mice uniformly exhibited signs of hind leg paralysis within 6 days of inoculation and were uniformly moribund by 7 days, whereas YF5.2iv challenged mice developed hind leg paralysis later and survived longer. In fact we have observed that some mice escape mortality from YF5.2iv at this dose of virus. PYF was detected in cerebrospinal fluid 3 days earlier than YF5.2iv, but the viruses reached similar peak titres which were lower than those in the brain parenchyma. Both viruses spread to the adrenal glands, presumably by a neural route since no virus was detectable in blood at any time after inoculation. These results suggest that factors which control the replication efficiency rather than the rate of spread and distribution of the two viruses within the CNS may be more important in accounting for the shortened survival time after challenge with PYF.

Partial nucleotide sequence analysis of the PYF genome

The nucleotide sequences of several RT-PCR-derived clones of the PYF genome including the region encoding a portion of the prM protein (beginning at amino acid position 20), all of E and NS1 and a portion of the NS2A protein (amino acids 1–105) were determined (Table 1). Comparison of the predicted amino acid substitutions within the E, NS1 and NS2A proteins revealed both common substitutions and clone-specific substitutions. No predicted substitutions were observed in any clone within the region of the prM protein analysed (amino acid residues 20–164). Silent third codon position mutations were found in several clones (two in 67.1, one each in 68 and 68.1, one each in 72, 72.1 and 72.21) within the E, NS1 and NS2A proteins (data not shown). Only two of these occurred at the same position in separate clones suggesting that the differences observed do indeed represent independent clones. Clone-specific substitutions may represent PCR artifacts, especially since templates which failed to generate infectious virus (72.11 and 72.26; see below) contained the most substitutions. Within the E protein, substitutions common to all clones included R25 → G, I173 → T, and F305 → V. Heterogeneity was observed at two other positions in the envelope protein (R380 → T and I162 → M) and no clone contained both of these latter substitutions. Within the NS1 and NS2A proteins, common substitutions previously (Rice et al., 1989). Medium from transfected monolayers was harvested between 60 and 72 h post-transfection and titres of infectious virus determined by plaque titration on SW-13 and Vero cells.

Statistical analysis. Statistical significance was determined using non-parametric methods for comparisons of multiple group samples (Zar, 1974).
Fig. 1. For legend see opposite page.
proteins of PYF clones.

The YF5.2iv molecular clone was used for vaccine strain parent, YFS/3-232, at these common positions (data not shown). Although we cannot exclude the possibility that variants within YFS/3-232 contain such substitutions, it is reasonable to assume that passage in mouse brain selects for viruses containing the predicted amino acid substitutions illustrated in Tables 1 and 2.

### Table 2. Amino acid substitutions in the NS1 and NS2A proteins of PYF clones.

<table>
<thead>
<tr>
<th>Protein and residue</th>
<th>NS1‡</th>
<th>NS2A‡</th>
</tr>
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<tbody>
<tr>
<td>Clone*</td>
<td>Virus</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>Yes</td>
<td>Q</td>
</tr>
<tr>
<td>72.1</td>
<td>Yes</td>
<td>Q</td>
</tr>
<tr>
<td>72.11</td>
<td>No</td>
<td>Q</td>
</tr>
<tr>
<td>72.21</td>
<td>Yes</td>
<td>Q</td>
</tr>
<tr>
<td>72.26</td>
<td>No</td>
<td>A</td>
</tr>
<tr>
<td>YF5.2iv</td>
<td>D</td>
<td>H</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M</td>
</tr>
</tbody>
</table>

* Clones are indicated in Table 1. The NS1 and NS2A proteins are encoded only within the YFM5.2 plasmid (72 series).
† Virus indicates whether infectious virus was recovered when the clone was used for in vitro ligation with clone 67.2 (Table 1)
‡ Blank spaces indicate that the predicted residue matches that in the YF5.2iv molecular clone.

Included L228 → Q and M17 → L, respectively (Table 2). To confirm that these substitutions in PYF resulted from neuroadaptation, we derived the sequence of its 17D vaccine strain parent, YFS/3-232, at these common positions by sequencing PCR fragments generated from virus-infected cells by RT–PCR. The nucleotide sequence was identical to the YF5.2iv cDNA clone at the relevant positions (data not shown). Although we cannot exclude the possibility that variants within YFS/3-232 contain such substitutions, it is reasonable to assume that passage in mouse brain selects for viruses containing the predicted amino acid substitutions illustrated in Tables 1 and 2.

### Properties of intertypic PYF/YF5.2iv viruses

In preliminary experiments using small numbers of mice, the intertypic recombinant virus PYF/YF5.2iv clone 67/72 (derived from YF5'3'iv clone 67.2 and YFM5.2 clone 72 (see Table 1) appeared to reach higher spinal cord titres than YF5.2iv after olfactory bulb inoculation (data not shown). Survival after conventional intracebral challenge of young adult mice, was similar however, to that of the YF5.2iv parent [average survival time 10.7 ± 1.4 days (SEM); data not shown]. We then compared the virulence properties of PYF, YF5.2iv and the PYF/YF5.2iv-67/72 recombinant. As an index of neurovirulence in these experiments, levels of titratable virus in the spinal cord at 6 days following the inoculation of the olfactory bulb with approximately equivalent doses of virus were measured (see Table 3). As shown in Fig. 1, PYF and YF5.2iv can be differentiated by the amount of virus present in the cord on day 6, and neurological impairment occurs after infection with PYF group at this time. Table 3 summarizes results of an experiment that employed challenge doses of approximately 1000 p.f.u. for each virus. Mean levels of titratable virus in the spinal cord were approximately 100-fold higher after challenge with PYF than YF5.2iv or the PYF/YF5.2iv recombinant. Analysis of variance was used initially to determine whether significant differences existed among these three groups. The critical value (F statistic) for detecting a difference was significant at a P value of between 0.005 and 0.0025. Statistical tests were then used to determine pairwise differences among the three groups. A significant difference was detected between mean cord titres of PYF and either YF5.2iv [P < 0.005 (Neuman Keul's test); p < 0.05 (Scheffe's test)] or PYF/YF5.2iv-67/72 (P < 0.0025; P < 0.05, respectively)], but not between YF5.2iv and PYF/YF5.2iv-67/72 (p > 0.05). Hind leg weakness was a constant feature with the PYF group but not the other groups when animals were sacrificed. Virus was detected in the CSF and adrenal glands of most PYF-infected animals (5/7), but not from CSF or adrenal glands of YF5.2iv-infected animals (0/6), and less consistently from PYF/YF5.2iv challenged animals (1/7). These results are consistent with the data in Fig. 1 where similar intervals of time were required for detection of virus throughout the neuraxis. Taken together the data...
Table 3. Replication of yellow fever viruses in spinal cord, cerebrospinal fluid and adrenal glands of mice after olfactory bulb inoculation

Statistical significance was determined using Neuman Keul's test as described in Methods.

<table>
<thead>
<tr>
<th>Challenge virus</th>
<th>No. of mice</th>
<th>Spinal cord</th>
<th>CSF</th>
<th>Adrenal glands</th>
<th>Paralysis†</th>
</tr>
</thead>
<tbody>
<tr>
<td>YF5.2iv</td>
<td>6</td>
<td>5.05 ± 1.64,§ (6)</td>
<td>None</td>
<td>None</td>
<td>+ / - (4)</td>
</tr>
<tr>
<td>PYF/YF5.2iv</td>
<td>7</td>
<td>4.57 ± 0.79,§ (7)</td>
<td>3.3 (1)</td>
<td>3.5 (1)</td>
<td>+ / - (3)</td>
</tr>
<tr>
<td>PYF</td>
<td>7</td>
<td>7.09 ± 1.19,</td>
<td></td>
<td>(7)</td>
<td>4.32 ± 0.44 (5)</td>
</tr>
</tbody>
</table>

* Parentheses indicate number of mice for which virus was recovered or exhibiting symptoms of paralysis.
† Neurological impairment graded as: - (none) to + + (severe).
§ YF5.2iv compared to PYF: P < 0.01.
¶ YF5.2iv compared to PYF/YF5.2iv: P > 0.05.
|| PYF/YF5.2iv compared to PYF: P < 0.005.

indicate that significant differences exist between the neurovirulence properties of the PYF and YF5.2iv viruses, but that the PYF/YF5.2iv-67/72 virus exhibits properties similar to its YF5.2iv parent.

Discussion

In the present study, we have demonstrated that neuroadapted yellow fever virus (PYF), differs from unadapted virus (YF5.2iv) in certain aspects of neurovirulence in adult immunocompetent mice. The average survival time is shorter and maximal titres of virus throughout the brain and spinal cord were substantially higher after inoculation of the neuroadapted PYF strain than YF5.2iv. These data are consistent with original observations concerning fixation of flavivirus neurovirulence by multiple passages in suckling mouse brain (Theiler, 1951; Meers, 1959; Schlesinger, 1980). In our experiments, no consistent difference was observed between PYF and YF5.2iv in the distribution of virus throughout the neuraxis, although YF5.2iv tended to require longer to replicate maximally in distal sites (CSF and adrenal glands) based on titration of recovered virus by plaque assay. More subtle differences in rates of spread of the two strains may exist but are not apparent with our current techniques. The reduced duration of survival associated with more rapid replication of the PYF strain, as indicated by the higher titres achieved by this strain suggests that virus-specific factors are important in the pathogenesis of fatal encephalitis in the mouse. This may reflect either a higher level of cytopathic effects caused by a larger burden of replicating virus or possibly the induction of a more vigorous immune response, which has been implicated as deleterious during YF encephalitis in the mouse (Hirsch & Murphy, 1967). An alternative explanation is that the amino acid substitutions in the PYF E protein promote stability of this protein and enhance accumulation of virus, as has been proposed as a possible basis for differences in pathogenicity among wild-type and vaccine strains of YF (Cane & Gould, 1989).

As an approach to identifying the virus-specific factors associated with the difference in neurovirulence between PYF and YF5.2iv, the nucleotide sequences of several clones of the E/NS1 region of the PYF strain were determined. Although PCR artifacts complicated the analysis, true sequence heterogeneity involving at least two positions in the E protein of this strain was apparent (positions R389 and I160). This suggests that the evolution of variants occurs during the adaptation of YF vaccine strains to growth in suckling mouse brain and may reflect a quasispecies phenomenon. It is notable that except for I160 → M, all common predicted substitutions in the E protein (R35 → G, I173 → T, F190 → V, the clone-specific substitution R389 → T) and also the single substitution in NS1 (L228 → Q) occur at positions where YF17D differs from the virulent Asibi strain (Hahn et al., 1987) and the French viscerotropic (FVV) and neurotropic (FNV) strains of YF (Jennings et al., 1993). The appearance of these residues in the PYF E protein may represent selection of new mutational events or selection of rare viral variants present in the vaccine parent (YFS/232), which itself was originally derived from serial passage of the highly virulent Asibi strain. The observation that several substitutions present in PYF are common to virulent YF virus strains of different origins suggests that these residues might confer functional properties upon the E protein which are important for the pathogenesis of these respective strains in mammalian hosts. In this regard, residues 52, 173 and 305 map to clusters of high sequence variability which have been defined by alignment of the tick-borne flavivirus envelope protein (Gritsun et al., 1995) and which localize to sites of substitutions associated with monoclonal antibody neutralization escape and attenuation of virulence. According to the structural model of the E protein (Rey et al., 1995),
residue 52 occupies a position at the base of domain II believed to be involved in low pH conformational transitions. Residue 305 maps to the distal face of domain III, a proposed site of cell attachment, and residue 380 occurs within a conserved motif (RGD) which has been suggested to influence the receptor-binding properties of the mosquito-borne flaviviruses (Rey et al., 1995). Substitutions at the RGD motif are also associated with attenuation of virulence of Murray Valley encephalitis (MVE) for mice (Lobigs et al., 1990).

The relatively high rate of amino acid change among the E proteins of virulent flavivirus strains and their attenuated derivatives (Hahn et al., 1987; Nitayaphan et al., 1990; Blok et al., 1992) suggests that this protein is important for viral virulence. Substitutions within the E protein can attenuate neuroinvasiveness in mice after peripheral inoculation (references included in the Introduction), however determinants on this protein which affect replication efficiency after entry into the CNS are less well characterized. The available studies indicate that substitutions which reduce or eliminate neuroinvasiveness do not necessarily attenuate neurovirulence. Recent studies with engineered dengue and tick-borne encephalitis (TBE)/dengue chimeric viruses however, have implicated the structural proteins as determinants of neurovirulence. The observation that structural proteins from a neuroadapted virus confer neurovirulence upon the non-neuroviral dengue-4 virus derived from a molecular clone indicates an important biological role for structural proteins in the enhanced virulence of neuroadapted virus (Bray & Lai, 1991; Kawano et al., 1993). Furthermore, using either dengue-4 or chimeric TBE/dengue-4 (DEN-4) clones, different mutations engineered within the prM and E proteins have been shown to have either enhancing or attenuating effects on neurovirulence (Pletnev et al., 1993). However, these studies were conducted in suckling mice and the role of flavivirus structural protein modifications in the context of viral replication in a mature nervous system in a fully immunocompetent mouse remains undetermined. Age-dependent differences in the susceptibility of rodents to neurotropic viruses have been well-documented and may reflect differences in the level of immunocompetence or of host factors involved in replication efficiency (Sabin, 1952; Ogata et al., 1991). Both factors may be important during flavivirus pathogenesis.

We addressed whether the E/NS1 region derived from a clone of the PYF strain would confer enhanced neurovirulence on the non-neuroadapted YF5.2iv virus. One such engineered virus, containing three amino acid substitutions common to virulent YF strains failed to exhibit significantly higher virulence properties than the parental YF5.2iv molecular clone. Several explanations are possible for this result. First, the sequence of the E/NS1 region included in the engineered intertypic virus may not be representative of the true neuroadapted parent. This problem has also been encountered with reconstruction of neuroviral dengue clones (Bray & Lai, 1991) and is believed to reflect the heterogeneity of viruses present in the parental virus population. A second explanation is that the structural protein modifications are important for virulence but require the presence of specific substitutions in the non-structural proteins (NS2A-NS5) or the 5' and 3' untranslated regions, to exert their full effects. A third potential explanation is that sequences within the non-structural region of the YF5.2iv genome may actually negate effects of the E/NS1 substitutions, by independently attenuating replication efficiency in the mouse nervous system. This is particularly interesting because mutations in flavivirus non-structural proteins have recently been found to have varying effects on neurovirulence in the mouse model. For instance elimination of the conserved glycosylation sites on the dengue 4 NS1 protein in the context of a TBE/DEN-4 chimera can modulate neurovirulence, suggesting potential functions of this flavivirus non-structural protein in pathogenesis (Pletnev et al., 1993). In addition, elimination of glycosylation sites on the YF NS1 protein also has attenuating effects on the neurovirulence of the YF5.2iv virus for adult mice (I. R. Muylaert, T. J. Chambers & C. M. Rice, unpublished data). Working with Vero-passaged MVE virus clones, McMinn et al. (1995a) have obtained evidence that attenuation of neurovirulence may depend on sequence determinants outside of the structural region. This raises the possibility that other non-structural proteins, such as the RNA replicase components NS3 and NS5, or the untranslated regions of the genome may also contain determinants which influence neurovirulence. Further investigation of such potential virus-specific determinants will lead to better understanding of virulence mechanisms and host factors which restrict replication and prevent progressive fatal disease. Our studies indicate the utility of an RT-PCR-based strategy in conjunction with a full-length infectious cDNA clone for rapid mapping of viral determinants associated with virulence properties. Use of high-fidelity polymerases and careful analysis of multiple clones are essential however, to avoid problems with potential PCR errors associated with this method.

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