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Mutation in a 17D-204 Vaccine Substrain-Specific Envelope Protein Epitope Alters the Pathogenesis of Yellow Fever Virus in Mice

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The heterogeneous nature of the yellow fever (YF) 17D-204 vaccine virus population was exploited in this study to isolate virus variants able to escape neutralization by the 17D-204 vaccine-specific MAb 864. The conformational change on the virus surface that resulted in the loss of the MAb 864-defined epitope was effected in each variant by a single amino acid mutation in the envelope (E) protein at either position E-305 or E-325. Interestingly, both positions were mutated during attenuation of the 17D-204 vaccine substrain from the wildtype Asibi strain. The mutations in several of the variants represented reversion to the wildtype Asibi virus sequence consistent with loss of a 17D-204 substrain-specific epitope. The majority of the variant viruses were shown to have altered mouse neurovirulence phenotypes, ranging from complete avirulence through to increased virulence. The avirulent variants are the first flavivirus MAb-neutralization-resistant variants to be attenuated for neurovirulence in the adult mouse model. Overall, the results indicate that the E protein epitope recognized by MAb 864 defines a functionally important region that encodes major molecular determinants of YF virus pathogenesis *in vivo.*

Key Words: yellow fever virus; 17D-204 substrain-specific epitope; envelope protein.

INTRODUCTION

Yellow fever (YF) virus is the prototype member of the *Flavivirus* genus, family Flaviviridae. The flaviviruses are small, enveloped viruses with a single-stranded, positive-sense RNA genome. The membrane-associated membrane (M) and envelope (E) proteins constituting the viral "spikes" perform many important functions, including attachment of virus to specific host cell binding sites, fusion of the viral envelope with host cell membranes, virion assembly, and release. Accordingly, the conformation of the E protein may ultimately be expected to influence viral pathogenicity *in vivo.*

Studies of the E protein's role in the pathogenesis process are facilitated by the selection of single-site virus variants with altered ability to induce a fatal encephalitis in the mouse model. Neutralizing monoclonal antibodies (MAbs) can be used to select antigenic virus variants *in vitro,* which are termed MAb neutralization-resistant escape (MAb^R) variants. This approach has been used successfully to isolate MAb^R variants for several flaviviruses including tick-borne encephalitis (TBE) (Mandl *et al.,* 1989; Holzmann *et al.,* 1990), Japanese encephalitis (Cecilia and Gould, 1991; Hasegawa *et al.,* 1992), louping ill (Jiang *et al.,* 1993), dengue-2 (Lin *et al.,* 1994), Murray Valley encephalitis (McMinn *et al.,* 1995), and YF (Lobigs *et al.,* 1987; Ryman *et al.,* 1997b) viruses. Single amino acid mutations in the E protein reducing the neuroinvasiveness of the virus following peripheral inoculation of mice were observed in several cases (Holzmann *et al.,* 1990; Hasegawa *et al.,* 1992; Cecilia and Gould, 1991; McMinn *et al.,* 1995). However, to date no such MAb^R variants with altered neurovirulence when administered intracerebrally (ic) have been characterized.

In this study, a series of MAb^R variants was selected for resistance to neutralization by MAb 864, previously shown to be specific for a 17D-204 substrain vaccine virus E protein epitope (Buckley and Gould, 1985; Gould *et al.,* 1985). MAb 864 is considered to define an important functional site on the 17D-204 virus E protein, as exemplified by its high reactivity in neutralization, hemagglutination inhibition (HAI), and passive protection studies (Cammack and Gould, 1986). Moreover, the presence of the epitope recognized by MAb 864 exclusively on 17D-204 vaccine viruses may be indirectly relevant to the virulence of the YF vaccine viruses. Comparison of the corresponding parent and MAb 864^R variant viruses revealed differential biological and antigenic profiles. The molecular determinants of MAb neutralization escape were identified by sequence analysis of the M and E protein-encoding genes and the neurovirulence of the 864^R variant viruses was examined in the mouse model.

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RESULTS AND DISCUSSION

Derivation and characterization of the 864^R variant viruses

Three series of MAb^R variant viruses, derived from 17D-204-WHO, 17D(−wt) or 17D(+wt) virus, were isolated by selection for neutralization resistance to the 17D-204 substrain-specific MAb 864 (Fig. 1). 17D-204-WHO was selected to represent 17D-204 vaccine viruses, while the other two viruses were selected for their unusual properties. The 17D(+wt) and 17D(−wt) variants are plaque isolates from the 17D-204-UK vaccine virus population, selected, respectively, for the presence or absence of the E protein wildtype-specific epitope recognized by MAb 117 (Gould *et al.,* 1989). The 17D(−wt) variant is attenuated for mouse neurovirulence due to a single mutation from 17D-204-UK at amino acid 240 in the E protein (E-240). The 17D(+wt) variant has an additional mutation at E-173, resulting in presentation of the MAb 117-defined wildtype-specific epitope and reversion to neurovirulence in mice (Ryman *et al.,* 1997a). In PRNTs, 17D-204 substrain-specific MAb 864 neutralized all three of these viruses greater than 10^4-fold. Seven MAb 864 neutralization-resistant (864^R) variant viruses were plaque-purified and characterized; three from the 17D(+wt) virus, two from the 17D(−wt) virus and two from the 17D-204-WHO vaccine virus. Loss of the MAb 864-defined epitope from the 864^R variant viruses was confirmed by indirect immunofluorescence (IIF). As expected, the wildtype-specific epitope recognized by MAb 117 was present on the 864^R(+wt) variants, but absent from the 864^R (−wt) and 864^R WHO variants. HAI assays with a panel of MAbs revealed that, although their epitopes remained physically present on the 864^R variants (by IIF), they no longer had HAI activity, indicating a subtle conformational change in the E protein.

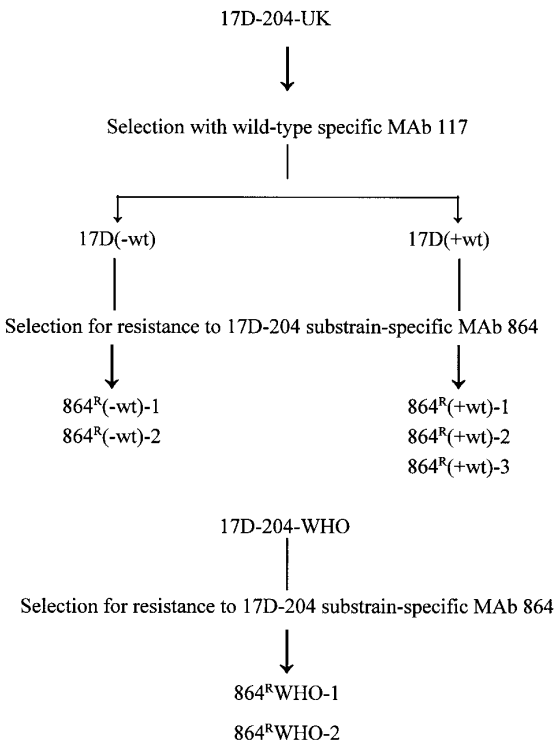

FIG. 1 Derivation of 864^R variant viruses.

TABLE 1

Amino Acid Differences Identified between the 864^R Viruses and Their Respective Parent Viruses in the E Protein

	E-153	E-173	E-240	E-305	E-325
Asibi	N	T	A	S	P
17DD	N	I	A	F	P
17D-204-UK	N	I	A	F	S
17D(−wt)	N	I	V	F	S
864^R(−wt)1	N	I	V	F	L
864^R(−wt)2	N	I	V	S	S
17D(+wt)	N	T	V	F	S
864^R(+wt)1	N	T	V	F	L
864^R(+wt)2	N	T	V	F	L
864^R(+wt)3	N	T	V	F	L
17D-204-WHO	T	I	A	F	S
864^{R}WHO-1	T	I	A	V	S
864^{R}WHO-2	T	I	A	F	L

Sequence analysis of the 864^R variant viruses

Comparison of the M and E protein gene sequence of the 864^R variant viruses with their respective parent virus revealed that, in each case, neutralization escape was effected by a single amino acid mutation in the E protein (Table 1). Substitutions of serine or valine for phenylalanine at E-305 or leucine for serine at E-325 each resulted in loss of the MAb 864-defined 17D-204 substrain-specific epitope, mapping the 17D-204 substrain-specific epitope recognized by MAb 864 in this region. It is interesting to note that amino acid substitutions have occurred at both E-305 and E-325 during the attenuation process of the wildtype strain Asibi virus to generate the 17D vaccine viruses. A serine to phenylalanine mutation at E-305 is conserved among the 17D vaccine strains, suggesting that this substitution may also have existed in the original 17D attenuated variant (Hahn *et al.,* 1987; Jennings *et al.,* 1993). Thus, the 864^R(−wt)-2 variant reverted to the wildtype amino acid at this position, while the 864^R WHO-1 variant exhibited a third variation. The 17D-204 substrain viruses also differed by a proline to serine amino acid substitution at E-325 from Asibi and 17DD substrain viruses. Therefore, 864^R(−wt)-1, 864^R WHO-2, and the 864^R(+wt) variants all had reverting mutations at this position. These observations were consistent with the 17D-204 substrain specificity of the MAb 864-defined epitope.

The 864^R(+wt) variant viruses were identical to the 17D(+wt) parental virus at amino acid position E-173,

TABLE 2

Neurovirulence of the 864^R Variant Viruses Compared with Their Parent Viruses 17D(−wt), 17D(+wt), and 17D-204-WHO

Virus	No. dead/ No. inoculated	% Mortality	Average survival time (AST) in days (±SEM)
17D-204-UK	10/10	100%	10.3 ± 0.6
17D(−wt)	8/45	17.7%	9.5 ± 0.5
864^R(−wt)-1	0/26	<4%	NA
864^R(−wt)-2	9/9	100%	9.2 ± 1.1
17D(+wt)	17/20	85%	10.1 ± 0.5
864^R(+wt)-1	0/8	<12.5%	NA
864^R(+wt)-2	1/9	11%	NA
864^R(+wt)-3	1/8	12.5%	NA
17D-204-WHO	8/8	100%	11.0 ± 0.7
864^{R}WHO-1	4/8	50%	9.5 ± 1.0
864^{R}WHO-2	8/8	100%	9.6 ± 0.5

Note. Adult mice were inoculated ic with 10^4 PFU of virus. N.A., not applicable.

consistent with the presence of the MAb 117-defined wildtype-specific epitope. Consequently, the 864^R(−wt)-1 variant virus differed from the 864^R(+wt) variant viruses only at position E-173; the difference observed between the 17D(−wt) and 17D(+wt) parent viruses (Ryman *et al.,* 1997a). The 864^R WHO variants lacked the E-240 substitution, but had a substitution at E-153 that led to a potential N-linked glycosylation site within the E protein (Post *et al.,* 1992).

Mouse neurovirulence of the 864^R variants

The single amino acid mutations in the E protein appeared to induce subtle conformational changes facilitating MAb 864 neutralization escape and were found to have profound effects on the virulence of the 864^R variant viruses *in vivo.* In adult mouse intracerebrally (ic) mortality studies the 864^R variants exhibited degrees of mouse neurovirulence ranging from completely avirulent through rapidly fatal (Table 2). In contrast to their 17D(+wt) parent virus (85% mortality), the three 864^R(+wt) variants were significantly attenuated for mouse neurovirulence with mortality rates ranging from 0 to 13%. Since these three viruses had an identical mutation this is unlikely to be a significant difference. Similarly, the 864^R(−wt)-1 variant was attenuated even further than its parent virus, 17D(−wt). In contrast, the 864^R(−wt)-2 variant virus was found to be significantly more virulent than its parent 17D(−wt) virus, despite being antigenically very similar to the completely avirulent 864^R(−wt)-1 variant (data not shown). This represents the reversion of an attenuated variant to the neurovirulent phenotype of the parental 17D-204-UK vaccine virus. Like the 17D-204-WHO vaccine virus, 864^R WHO-2 was neurovirulent, while 864^R WHO-1 was significantly attenuated, killing only 50% of infected mice. The surviving mice developed clinical signs of infection, but subsequently recovered.

Mouse neuroinvasiveness of the 864^R(−wt)-2 variant virus

To further delineate the neurotropic properties of the 864^R(−wt)-2 variant virus the neuroinvasiveness of this virus was tested by intraperitoneal (ip) inoculation of 8-day-old mice. The 17D-204-UK vaccine virus, the 17D(−wt) parent virus, and the 864^R(−wt)-2 variant virus were avirulent when administered by this route, whereas wildtype Asibi virus was lethal (data not shown). Therefore, the 864^R(−wt)-2 variant virus had not reverted to the wildtype YF virus phenotype for neuroinvasiveness in suckling mice; rather it had retained the attenuated phenotype of the 17D vaccine viruses. Similarly, the 864^R(−wt)-2 variant virus was not virulent for mice following intranasal (i.n.) inoculation of weanling mice (data not shown).

Replication of the 864^R variant viruses in mouse brain

The ability of the 864^R variants to replicate in mouse brain following ic inoculation was directly correlated with mortality (Table 3). Replication of the three 864^R(+wt) variants and the 864^R(−wt)-1 variant in neuronal tissue was clearly impeded as no infectious virus was detectable (<15 plaque-forming units (PFU)/brain) in the brains of mice infected with these viruses at any time point assayed. Virus titers in the brains of the 864^R(−wt)-2 virus-infected mice were variable, but higher than the 17D(−wt) parent virus (Ryman *et al.,* 1997a). Two days postinfection (pi), infection had been established in two of three mice with titers between 3×10^2 and 1×10^4 PFU/brain, increasing through 3 days pi Eight days pi, virus titers had fallen below 7×10^3 PFU/brain and in one mouse virus was no longer detectable, despite the clinical signs of encephalitis displayed by all three mice that were sampled. RT-PCR performed on a number of these mouse brain homogenates demonstrated that the presence of viral RNA correlated absolutely with infectious virus titer, thus confirming the sensitivity of the plaque assay (data not shown).

Histopathologic and immunohistochemical examination of brain sections

Brain sections from one mouse inoculated with 864^R(−wt)-1 and one inoculated with 864^R(−wt)-2, harvested 8 days pi, were examined. Routine hematoxin and eosin staining revealed very little pathology of the brain in either case (data not shown). Some focal meningeal, perivascular, and parenchymal inflammation was observed in brain sections from both mice. Additionally, possible ventricular inflammation and microglial nodules were found in the mouse infected with the 864^R(−wt)-2 variant virus. There was no evidence of demyelination and immunocytochemical staining was unable to detect

TABLE 3

Infectivity Titres in Infected Mouse Brain Homogenate

Virus	Day postinoculation	Infectivity titre (PFU/brain)
17D(−wt)		15
	2	3.0×10^2
		3.0×10^2
		1.0×10^4
	3	<15
		<15
		1.6×10^3
	8	<15
		<15
864^R(−wt)-1		<15
	2	<15
		<15
		<15
	3	<15
		<15
		<15
	8	<15
		<15
864^R(−wt)-2		15
	2	1.0×10^4
		3.0×10^2
		4.5×10^4
	3	3.0×10^3
		3.6×10^5
		<15
	8	7.0×10^3
		3.4×10^2
17D(+wt)		1.5×10^3
	2	2.4×10^4
		9.0×10^3
		7.5×10^3
	3	4.5×10^3
		2.0×10^4
		3.6×10^5
	8	2.0×10^6
		5.3×10^5
		<15
864^R(+wt)-1	11	<15
		<15
		<15
864^R(+wt)-2	11	<15
		<15
		<15
864^R(+wt)-2	11	<15
		<15

Note. RT-PCR results represent detection (+) or not (−) of 1331-bp amplicon from the E protein gene.

any viral antigen in either brain. In comparison, limited viral antigen was detectable in sections of a control mouse brain infected with 17D-204-UK virus.

Genetic correlates of pathogenesis

864^R(−wt)-1 and the 864^R(+wt) variants with combined mutations at E-240 and E-325 no longer induced lethal encephalitis in mice following ic inoculation and were highly attenuated with respect to their parent viruses and 17D-204-UK. This attenuation apparently resulted from the reduced ability of these viruses to replicate in mouse brain. However, in the background of the 17D-204-WHO vaccine strain the same E-325 amino acid substitution did not attenuate the virulence of the 864^R WHO-2 variant. This suggests either that the E-153 mutation negates the effects of the E-325 mutation,or that the E-240 change in the 17D(+wt) and 17D(−wt) variants is essential to the attenuated phenotype.

Reversion of the E-305 amino acid in variant 864^R(−wt)-2 to the serine observed in the Asibi wildtype virus led to the reemergence of the neurovirulent phenotype. In this instance the substituted amino acid led to more efficient replication of the virus in neuronal tissue. This mutation is able to reverse the effects of the E-240 mutation which attenuated the 17D(−wt) variant virus (Ryman *et al.,* 1997a) relative to 17D-204-UK vaccine virus. In contrast, the 864^R WHO-1 variant, with a unique valine substitution at E-305, was significantly attenuated for mouse neurovirulence. Overall, it appears that amino acid substitutions at E-240 and E-325 have a cooperative effect and that the molecular basis of mouse neurovirulence is multifactorial. These findings emphasize the critical role of the E protein in the mechanism of viral pathogenesis.

Interpretation of amino acid substitutions in the 3D structure of the E protein

Rey *et al.* (1995) have recently determined the three-dimensional structure of the ectodomain of the TBE virus E protein. Due to the conservation of the disulfide bridges among flaviviruses, the TBE virus E protein model can be used to approximate the structure of the YF virus E protein. This model is shown in Fig. 2, viewed from above (Fig. 2A) and from the side (Fig. 2B), with the sites of amino acid substitutions reported in this paper highlighted. Although fairly distant on the linear protein sequence, on the predicted E protein structure and as demonstrated by the antibody footprint in Fig. 2A, the substitutions at E-305 and E-325 are closely oriented, consistent with the proposal that MAb 864 binds to a conformation-dependent epitope (Després et al., 1990). These amino acids map to domain III of the E protein, which has previously been implicated in the tissue tropism of flaviviruses (see Rey *et al.,* 1995, for an overview). It is proposed, therefore, that the altered neurovirulence phenotypes displayed by the 864^R variant viruses are a direct consequence of changes in the E protein affecting tissue tropism. Indeed, the presence of amino acid substitutions at E-305 in all 17D vaccine viruses and E-325 in the 17D-204 substrain suggests that changes in this region may have contributed to the attenuation of these viruses. In addition, the model predicts that E-173 and

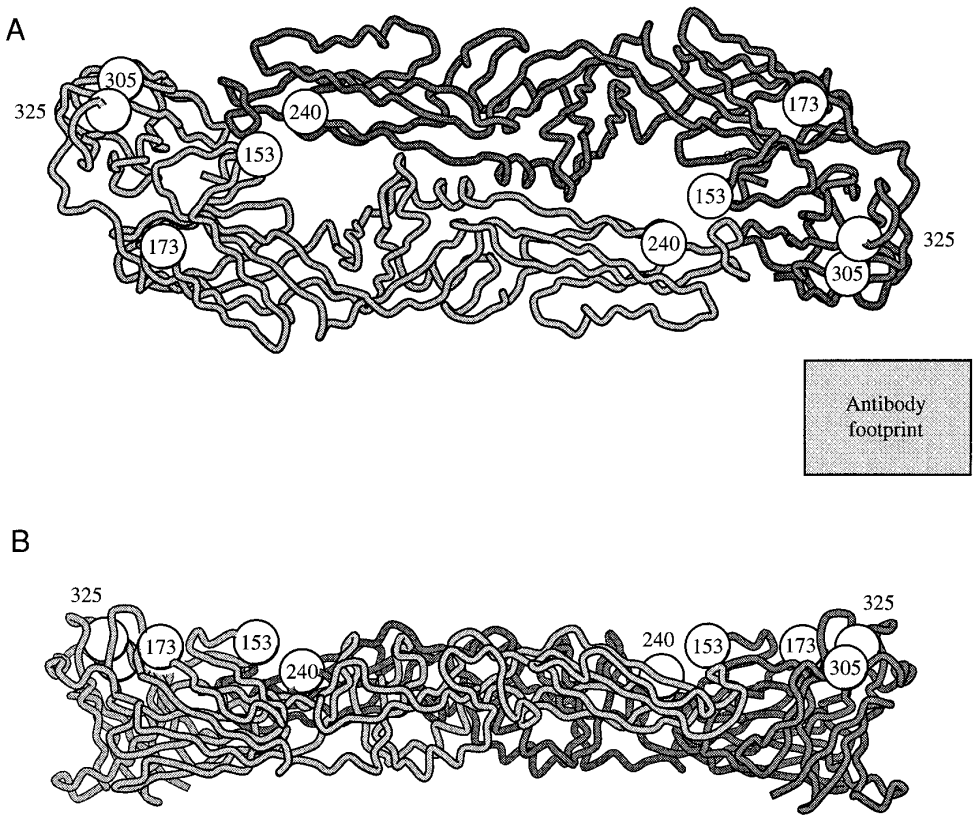

FIG. 2. Diagrammatic representation of the folded structure of the ectodomain of the E polypeptide in the dimer. (A) The dimer viewed looking down on the virus particle; (B) the dimer viewed from the side of the virus particle. Highlighted residues (E-153, E-173, E-240, E-305, and E-325) are variable amino acids in the YF virus E protein (see Table 1). The shaded box in (A) represents the approximate size of an antibody footprint.

E-240 are spatially distinct regions of the E protein from E-305 and E-325 (Fig. 2). Therefore, it is proposed that amino acid substitutions at E-173 and E-240 alter the conformation of the protein, thereby altering the virulence phenotype of the virus.

It is possible to speculate on the mechanism of altered neurovirulence. In radioimmunoprecipitation and Western blotting analyses, MAb 864 recognizes the E and prM proteins (Gould *et al.,* 1985, 1989). This suggests that the 17D-204 substrain-specific epitope is directly or indirectly associated with the prM–E interaction and that mutation in this epitope alters the prM–E interaction. This proposal is supported by Guirakhoo *et al.* (1992), who have found that prM interacts with the E protein between E-200 and E-327 for MVE virus.

Conclusions

Although virus virulence is likely to be multigenic, this study presents the first indication that the pathogenesis of YF virus *in vivo* can be directly linked to specific epitopes on the surface of the E protein. As mentioned previously, the 17D-204 substrain-specific epitope recognized by MAb 864 is involved in a number of biological activities: neutralization (Buckley and Gould, 1985), HAI (Gould *et al.,* 1986), hemolysis (Cammack and Gould, 1985), and passive protection (Gould *et al.,* 1986). This study has revealed that loss of the MAb 864-defined epitope may result in the expression of a significantly altered mouse neurovirulence phenotype. Clearly, therefore, this epitope is also important in determining the neurovirulence of YF virus in mice and, as suggested by Cammack and Gould (1985), is a critical epitope on the E protein of YF virus.

MATERIALS AND METHODS

Virus strains and cell culture

YF vaccine viruses 17D-204-UK (Wellcome Biotechnology, UK; Batch No. YF/1/274) and 17D-204-WHO (World Health Organization; avian leukosis virus-free secondary seed) were obtained as freeze-dried doses of commercial vaccine. Two plaque-purified strains of 17D-204-UK, one with and one without the wildtype specific epitope recognized by MAb 117, termed 17D(+wt) and 17D(−wt), respectively (Gould *et al.,* 1989; Ryman *et al.,* 1997a), were kindly provided by Dr. E. A. Gould. Viruses were passed once through monkey kidney Vero cells to provide working stocks and for extraction of viral RNA for molecular analysis. Virus was titered by plaque assay in Vero cells and expressed as PFU/ml.

Monoclonal antibodies, indirect immunofluorescence, and hemagglutination inhibition

YF wildtype-specific MAb 117 and 17D-204 vaccine substrain-specific MAb 864 were provided by Dr. E. A. Gould and were used as dilutions of MAb-containing mouse ascitic fluid (Gould *et al.,* 1985, 1989). IIF tests and HAI assays were performed as described previously (Barrett *et al.,* 1989).

Selection of MAb^R variant viruses

Potential MAb^R variant viruses, which appeared as plaques resistant to neutralization by MAb 864, were plaque-purified according to the method of Wiktor and Koprowski (1980) and passed once on Vero cell monolayers to provide working stocks. The loss of the MAb 864-defined E protein epitope from these candidate 864^R variants was confirmed by IIF and PRNTs.

Nucleotide sequence analysis

Viral genomic RNA was extracted from infected Vero cell culture supernatant as described by Ni *et al.* (1995). For each 864^R variant virus the region of the genome encoding the M and E proteins was amplified by RT-PCR in two overlapping fragments as described by Lewis *et al.* (1990) and modified by Jennings *et al.* (1993). Gel-purified cDNA fragments were cloned and sequenced in duplicate.

Animal experiments

Groups of 3- to 4-week-old, female, outbred strain T0 (Harlan Olac, UK) or NIH Swiss (Harlan, CA) mice were inoculated ic or i.n. with 10^4 PFU virus in a 20-μl volume. Litters of 8-day-old, outbred NIH Swiss mice (Harlan, CA) were inoculated by the ip route with 10^4 PFU virus in 20 μl. The morbidity and/or mortality of infected mice were monitored daily for up to 37 days. Average survival time and percentage mortality were calculated where applicable. In parallel experiments, 3- to 4-week-old mice were sacrificed by cervical spinal dislocation at various time points pi. Mouse brain tissue was processed for virus titration or histopathological examination as described in Ryman *et al.* (1997a).

Histopathology and immunohistochemistry

Formalin-fixed whole mouse brains were sliced into five blocks of uniform thickness and location using standard landmarks on the base of the brain, except where an inoculation site was evident, in which case the slicing was modified to include that site in one section. Sectioning and staining were performed as described by Cao *et al.* (1995).

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