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Mutation in NS5 protein attenuates mouse neurovirulence of yellow fever 17D vaccine virus

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The 17D-204 vaccine manufactured in South Africa (17D-204-SA) and a large plaque variant (17D-LP) derived from it were highly virulent in adult mice. The LD_{50} of 17D-LP virus was 0·2 p.f.u. for mice following intracerebral inoculation. In comparison, a medium plaque variant derived from 17D-LP, termed 17D-MP virus, was found to be attenuated in adult mice following the same route of inoculation ($> 10^4$ p.f.u./LD_{50}). Replication of 17D-MP virus was decreased in infected mouse brains compared to 17D-LP virus. Also, 17D-MP virus was slightly temperature sensitive at 39·5 °C. Compared to its parent viruses, 17D-204-SA and 17D-LP, 17D-MP virus had one unique mutation at nt 8045 in the genome which resulted in a single amino acid substitution (Pro → Ser) at residue 137 of the NS5 protein and appeared to be the mutation responsible for the attenuation of 17D-MP virus. This is the first time that altered virulence of a flavivirus caused by mutation in a non-structural protein gene, other than NS1, has been reported.

Yellow fever (YF) virus is a member of the genus *Flavivirus* of the family *Flaviviridae* (Chambers *et al.*, 1990; Monath, 1995). The RNA genome of YF virus is 10862 nt in length, single-stranded, positive-sense in polarity, capped at the 5′ terminus and lacks a poly(A) tract at the 3′ terminus (Deubel *et al.*, 1983; Rice *et al.*, 1985). A single large open reading frame encodes three structural proteins (core, membrane and envelope) and eight non-structural proteins (NS1–NS5) (Chambers *et al.*, 1990).

The live attenuated YF vaccine 17D is one of the safest and most efficacious viral vaccines in use today. The 17D vaccine has been shown to contain a mixture of virions which can be distinguished by a number of criteria. Virions of small, medium and large plaque morphologies have been identified in vaccine doses, although the large plaque phenotype is the predominant virion in the vaccine population (Liprandi, 1981). These plaque size variants can be distinguished biologically by mouse neurovirulence (Liprandi, 1981; Barrett & Gould, 1986) and replication in macrophages (Liprandi & Walder, 1983), and with monoclonal antibodies in neutralization, passive protection and haemagglutination–inhibition tests (Buckley & Gould, 1985; Gould *et al.*, 1986; Barrett *et al.*, 1989; Ledger *et al.*, 1992). Three plaque size variants were isolated from the 17D-204 vaccine manufactured in England and differentiated by virulence for adult mice following intracerebral inoculation (Liprandi, 1981). The small and large plaque variants were lethal for adult mice, whereas the medium plaque variant was attenuated and failed to kill mice at doses up to 10^6 p.f.u. The molecular basis for the attenuation of the medium plaque variant is unknown. In this paper, we report that a medium plaque variant derived from the 17D-204 vaccine manufactured in South Africa (17D-204-SA) is also attenuated for mice. To investigate the molecular basis of the attenuation of the medium plaque variant of 17D-204-SA virus, we have cloned and sequenced its entire genome, compared it to that of its parental viruses and demonstrated that the mutation is in the NS5 protein gene.

Vero cells and LLC-MK-2 cells were grown at 37 °C in Eagle's minimal essential medium (EMEM; Sigma) supplemented with 5% heat-inactivated foetal calf serum (Sigma), 2 mM L-glutamine (Sigma) and antibiotics. The 17D-204-SA virus, batch 10802, was kindly provided by Thomas Monath of the Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Colorado, USA. YF wild-type strains Peru-1 and -2 were isolated during an epidemic in Peru in 1995 (Wang *et al.*, 1996). Viruses were propagated in Vero cell monolayers infected at an m.o.i of approximately 0·1. Culture supernatant was harvested 6 days after infection at 37 °C. The virulence of viruses was investigated in two mouse strains: outbred NIH Swiss and inbred BALB/c (H-2^d). Female mice, 3–4 weeks of age, were inoculated intracerebrally with either 2000 or 10000 p.f.u. of virus. Following inoculation, the animals were monitored daily for signs of infection. Virus-infected NIH Swiss and BALB/c mice were sacrificed at days 8,

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The nucleotide accession numbers of the sequences reported in this paper are AF052444–AF052446.

Table 1. Oligonucleotide primer pairs, expected sizes (bp) of PCR products, annealing temperatures (AT) for each reaction

Primer pair	Genomic position	Nucleotide composition (5′ → 3′)	Size of product (bp)	AT (°C)
B5	1–25	agtaaatcct gtgtgctaat tgaaa	678	65
YF300	678–652	tagcaccagc aatcaatgtc atctggc		
CAG	625–642	cgagttgcta ggcaataaac acatttgga	698	51
YF7	1312–1293	aatgctccct ttcccaaata		
AJ7up	1223–1245	gaagagaacg aagggacaat gc	1331	65
YF100	2554–2531	gtatgagtac ttgttcagcc agtc		
NS1a	2349–2415	ccatgagcat gatcttggta gg	685	61
NS1b	3079–3057	cccatttact tcatgacttc cca		
NS1c	2984–3006	gactgcgatg gatctatctt gggt	747	65
NS1d	3731–3710	gccatataca tggcgtctcc tcc		
YF200	3604–3624	tggaggagta gtgctcttgg g	615	61
YF201	4219–4199	tagaccagct gctgcgagtg		
Ns2b1	4138–4158	ggcctgtgtg catttctggc	531	59
NS2b2	4669–4646	caagaaggtt gactggaata tgcc		
SNS3a	4612–4633	cgaggaatgt gaacatctgg ag	620	52
ANS3b	5232–5210	agcgtctccg tgcgcactcg g		
SNS3c	5174–5195	gctgggaaga caagacgttt cc	702	57
ANS3d	5876–5855	gcttaaaagc cgtcctgcaa tc		
SNS3e	5834–5855	ctttgcgtgg agcgagtgct gg	709	53
ANS3f	6543–6522	ctcagagtgg aggaacacac tg		
NS4A1	6338–6359	ctgaaggtag gcggggagct gc	799	57
NS4A2	7234–7213	tatgccacag agcagaggca tc		
NS4B1	7165–7189	cataatgctg ctggtcagta gctgg	528	61
NS4B2	7693–7671	caacagattc agttccctct tcc		
SNS5a	7663–7684	tgaagtctgg aagagggaac tg	689	54
ANS5b	8352–8331	aggcgggatg tttggttcac ag		
FIG1	8270–8297	tccaggaatt ccactcatga aatgtac	668	54
ANS5d	8938–8917	ttcccagaac tttgggtctt tgg		
SNS5f	8895–8916	ggaagactgc caatgaggct gt	711	58
ANS5f	9606–9585	tcacatccgt gctcatgtag cc		
SNS5g	9557–9578	gaatcagttc tgaccaggct gg	673	56
ANS5g	10230–10209	gaggcccagg tggccctatt gg		
SNS5i	10167–10188	ccaagagaca agacaagctg tg	695	51
ANS5i	10862–10837	agtggtttgt gtttgtcatg gaaag		

11, 14, 23 and 30 post-infection. Virus infectivity titre in mouse brains was determined by plaque assay performed in Vero cell monolayers as described by Barrett *et al.* (1989). Specific oligonucleotide primers used in cDNA synthesis and PCR were synthesized according to the known sequence of 17D-204-ATCC (Rice *et al.*, 1985). RT–PCR, molecular cloning and sequencing were performed as described by Wang *et al.* (1995). Briefly, viruses were grown in Vero cell cultures and RNA was extracted from the cell culture fluid. Thirty-four oligonucleotide primers were designed based on the published sequence of 17D-204-ATCC vaccine virus (Rice *et al.*, 1985) (Table 1). Reverse transcription of viral RNA involved incubation at 55 °C for 1–2 h with one of the 17 antisense primers and 1·8 U reverse transcriptase (RAV-2; Amersham). The resulting cDNA was denatured by heating to 94 °C for 5 min followed by PCR amplification involving 35 cycles of 52 °C for 40 s, 72 °C for 4 min and 94 °C for 40 s. PCR products were either directly sequenced in an Applied Biosystems model 377 automated sequencer or cloned using the TA cloning kit (Invitrogen) prior to sequencing. Computer analyses of nucleotide sequences and deduced amino acid sequence were performed using PCGENE (Intelligenetics) program, including the Novotny method for protein secondary structure predictions.

Two plaque morphologies were identified after the 17D-204-SA vaccine virus was grown in LLC-MK-2 cells, a small plaque type (0·5–0·8 mm in diameter) and a large plaque type (1·8–2·0 mm in diameter), although the large plaque phenotype was predominant. A large plaque was selected from the vaccine population, amplified and plaque-purified twice in LLC-MK-2 cells as described by Ledger *et al.* (1992). This virus was termed 17D-LP virus (LP, large plaque). Subsequently, the 17D-LP

Table 2. Infectivity titres of 17D-LP and 17D-MP viruses in infected NIH Swiss or BALB/c mouse brains

Virus titres are given for individual mouse brains. Infectivity titres are shown in $\log_{10}$p.f.u. per brain. NA, Non-applicable (mice died at earlier times post-infection); NT, not tested.

Time post-infection (days)	Mouse strain: NIH Swiss		BALB/c	
	17D-LP	17D-MP	17D-LP	17D-MP
8	5·3	3·0	5·3	4·1
	5·6	4·3	5·4	4·3
	5·7	5·0		4·5
		5·3		4·6
11	NA	4·3	NA	4·0
		4·4		4·0
		4·7		
14	NA	< 2·0	NA	< 2·0
23	NA	< 2·0	NA	< 2·0
		< 2·0		
30	NA	< 2·0	NA	NT
		< 2·0		

virus was cloned in LLC-MK-2 cells by limited dilution and a variant, termed 17D-MP virus (MP, medium plaque), was identified on the basis that its plaque size (1·2 mm in diameter) was smaller than the parent 17D-LP virus but larger than the small plaque virus.

The 17D-LP virus was lethal for mice following intracerebral inoculation and had a LD_{50} of 0·2 p.f.u. in NIH Swiss mice and < 10 p.f.u. in BALB/c mice. In contrast, the majority of mice survived infection by 10^4 p.f.u. of 17D-MP virus: 93·3% (42/45) for NIH Swiss mice and 89·1% (26/29) for BALB/c mice. Also, both strains of mice infected with 17D-LP virus died by day 9 post-infection, whereas 17D-MP virus-infected mice died only after day 11 post-inoculation. Approximately one-third of the surviving mice (13/42 NIH Swiss and 10/26 BALB/c) had hind-limb paralysis which appeared permanent as the same clinical signs were observed in mice at 1 year post-inoculation.

At day 8 post-inoculation, virus was detected in the brains of NIH Swiss and BALB/c mice infected with 17D-LP virus at a mean infectivity titre of $5{\cdot}5 \pm 0{\cdot}1$ $\log_{10}$p.f.u. per brain (Table 2). For 17D-MP virus-infected NIH Swiss and BALB/c mice, infectious virus was detected at a significantly lower mean titre, $4{\cdot}4 \pm 0{\cdot}2$ $\log_{10}$p.f.u. per brain (Table 2). Virus was detected in the brains of all 17D-MP virus-infected mice sampled at day 11 post-infection but not at later times.

17D-MP virus was temperature sensitive at 39·5 °C [efficiency of plaquing (e.o.p.) at 39·5 °C versus 37 °C was 0·06], whereas neither 17D-204-SA nor 17D-LP virus was temperature sensitive at 39·5 °C (e.o.p. = 0·2). No difference in the replication kinetics was observed between 17D-LP and 17D-MP viruses when they were grown in Vero cell monolayers at 37 °C (data not shown).

All strains of YF virus tested so far, including 17D vaccine strains, are lethal for adult mice following intracerebral inoculation (Fitzgeorge & Bradish, 1980; Barrett & Gould, 1986). However, two medium plaque variants, originating from different seeds of 17D-204 vaccines [England (Liprandi, 1981) and South Africa (this paper)], were attenuated and only killed a minority of mice at doses up to 10^4 p.f.u. In contrast, the large plaque variants of these two vaccine strains were lethal for mice following the same route of inoculation. Compared to 17D-204-SA vaccine virus, its medium plaque variant was at least 5×10^4-fold more attenuated than the large plaque variant. Therefore, from these limited observations, the medium plaque variants of 17D-204 vaccine appear to be more attenuated for mice than the large plaque variants.

Comparison of the entire genomic nucleotide sequence, other than the 25 nt at the termini, of the 17D-MP virus with those of 17D-LP and 17D-204-SA viruses revealed one unique nucleotide difference at position 8045 (C → U), which was confirmed by an independent RT–PCR followed by sequencing. Furthermore, three subsequent passages of 17D-MP resulted in the virus maintaining the medium plaque morphology, attenuation of mouse neurovirulence and the mutation at nt 8045. Thus, the phenotype and genotype appear stable. This nucleotide change resulted in a proline to serine substitution at residue 137 of the NS5 protein. This substitution altered the predicted secondary structure of the NS5 protein. Alignment of the amino acid sequences surrounding NS5-137 for several wild-type YF viruses, 17D vaccine viruses and several other flaviviruses revealed that this amino acid substitution was not seen in any 17D vaccine viruses nor in two wild-type YF strains [Asibi and French viscerotropic virus (FVV)] (Fig. 1). Most flaviviruses (8 out of 13) also had a proline at this position while three flaviviruses had a serine and two had a threonine at this position. This indicates that proline is highly conserved among flaviviruses. Interestingly, two wild-type YF viruses (Peru-1 and -2) had a serine residue at NS5-137. However, these two strains also had three other amino acid substitutions at NS5-79 (Ile → Thr), NS5-108 (Arg → Lys) and NS5-109 (Asp → Glu) which were not seen in 17D-MP or 17D-LP virus.

The NS5 protein in flaviviruses is believed to have two enzymatic functions: RNA-dependent RNA polymerase in the C-terminal domain (Tan *et al.*, 1996), and methyltransferase in the N-terminal domain (Koonin, 1993). The latter enzyme is proposed to be involved in viral RNA capping, and subsequently in viral protein synthesis. The region containing NS5-137 is involved in the methyltransferase activity. The mutation at NS5-137 is predicted to cause a secondary structural change in the methyltransferase domain of the NS5 protein which may further induce a tertiary structural change in this protein and be in part responsible for the slight temperature sensitivity of 17D-MP virus at 39·5 °C. Consequently the enzymatic

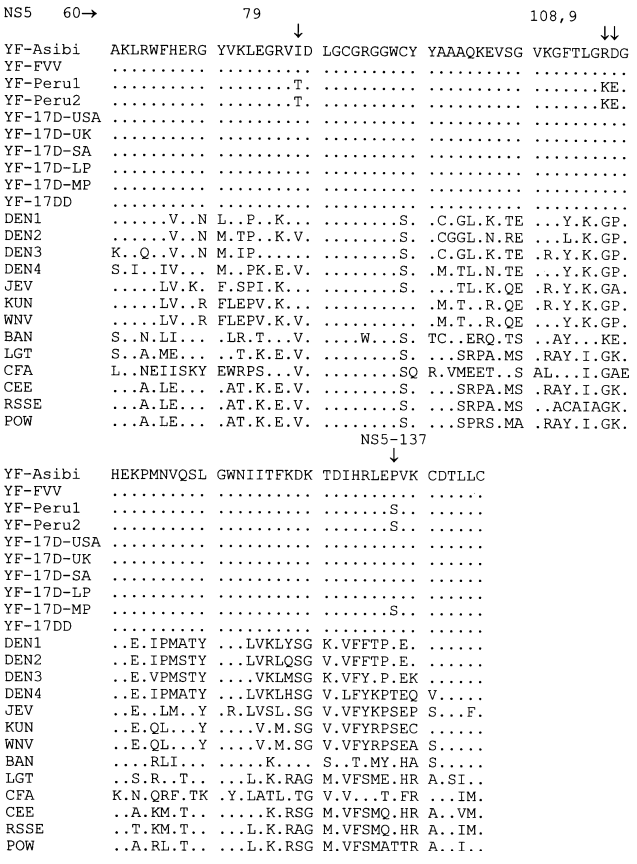

Fig. 1. Alignment of amino acid sequences around NS5-137 among strains of YF virus and several flaviviruses. DEN1–DEN4, dengue virus types 1–4; JEV, Japanese encephalitis virus; KUN, Kunjin virus; WNV, West Nile virus; LGT, Langat virus; CFA, cell fusing agent; BAN, Banzi virus; CEE, Central European encephalitis virus; RSSE, Russian spring–summer encephalitis virus; POW, Powassan virus. See Ni *et al.* (1995) for sources of flavivirus sequences.

function of the NS5 protein may be affected, resulting in impaired viral RNA capping and synthesis of viral proteins, and decreased replication of 17D-MP virus in mouse brains because the virus can not multiply efficiently. Reduced replication of 17D-MP virus (Table 2) may explain the hind-limb paralysis seen in many 17D-MP virus-infected mice. Although we can not measure the temperature in the mouse brain directly, support for this proposal comes from examination of rectal temperatures of virus-infected BALB/c and NIH Swiss mice. Both 17D-LP and 17D-MP virus-infected mice had peak rectal temperatures at days 5, 6 and 7 post-infection of 38·5 °C (data not shown). Other than reduced replication of 17D-MP virus at 39·5 °C, we have found no evidence for reduced replication of 17D-MP virus compared to 17D-LP virus in Vero cell culture. Increased temperature of the infected mouse is considered the most likely candidate responsible for the attenuated phenotype. However, we cannot exclude the possibility that the mutation in NS5 alters the interaction of virus and host cell components of the virus replication complex.

It is also possible that NS5-137 is a part of a T cell epitope. This mutation at NS5-137 may lead to altered interaction of viral antigen with T lymphocytes, although to date no flavivirus T cell epitopes have been mapped to the NS5 protein.

Finally, non-structural proteins in flaviviruses have very important functions in virus replication (Chambers *et al.*, 1990). However, prior to this study, only mutations in the NS1 protein gene of flaviviruses have been demonstrated to affect virus multiplication and subsequently viral pathogenesis (Pletnev *et al.*, 1992; Muylaert *et al.*, 1996). Mutations in the NS1 protein which ablated the first and both glycosylation sites impaired NS1 protein secretion, reduced mouse neurovirulence and decreased replication of YF virus (Muylaert *et al.*, 1996).

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