

## Development and characterization of new flavivirus-resistant mouse strains bearing *Flv<sup>r</sup>*-like and *Flv<sup>mr</sup>* alleles from wild or wild-derived mice

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A single genetic locus, flavivirus resistance (*Flv*), controls virus titres and severity of flavivirus infection in mouse brain. It has been mapped to mouse chromosome 5 and shown to include different allelic forms. While the majority of laboratory mouse strains are susceptible to flaviviruses and carry the *Flv<sup>s</sup>* allele, wild mice and laboratory mouse strains recently derived from them are resistant and carry flavivirus-resistance alleles including *Flv<sup>r</sup>*-like and *Flv<sup>mr</sup>* alleles. Although there is a mouse model of flavivirus resistance conferred by the *Flv<sup>r</sup>* allele, other resistance alleles have not been adequately studied due to a lack of appropriate animal models. In this paper we describe the development of new flavivirus-resistant mouse strains, C3H.*M.domesticus*-*Flv<sup>r</sup>* and C3H.MOLD-*Flv<sup>mr</sup>*, which carry the novel resistance alleles *Flv<sup>r</sup>*-like and *Flv<sup>mr</sup>* on the genetic background of flavivirus susceptible C3H/HeJ mice. The new strains were created by 10 to 11 generations of backcrossing followed by brother–sister matings resulting in a generation of homozygous founder stocks. Genome analysis of the newly developed mouse strains has revealed chromosomal regions of approximately 9 and 11 cM, respectively, encompassing *Flv* on chromosome 5, which are derived from resistant donor mice. These segments are much smaller than the segment of approximately 31 cM described in the congenic resistant mouse strain C3H.PRI-*Flv<sup>r</sup>* (also known as C3H/RV). The new congenic mouse strains, which were created to carry the *Flv<sup>r</sup>*-like and *Flv<sup>mr</sup>* alleles on the standardized genetic background of susceptible mice, represent new animal models of flavivirus resistance conferred by these novel resistance alleles.

### Introduction

Genetic studies of innate resistance to flaviviruses in mice have identified a single genetic locus (Sabin, 1952), the flavivirus resistance locus (*Flv*; Green, 1989), which controls the level of virus replication *in vivo* and in cell culture (Goodman & Koprowski, 1962; Groschel & Koprowski, 1965). This locus has been mapped to mouse chromosome 5 (Shellam *et al.*, 1993; Sangster *et al.*, 1994) and a low-resolution genetic map of the chromosomal region around it has been produced

as a prelude to the positional cloning of alleles residing at this locus (Urosevic *et al.*, 1995; Shellam *et al.*, 1998).

Innate resistance to flaviviruses in laboratory mice is a very rare trait which has been identified in only a few mouse strains, namely Det (Lynch & Hughes, 1936), BSVR, BRVR (Webster, 1937) and PRI (Sabin, 1952). A single resistance allele was initially characterized at the *Flv* locus of outbred PRI mice (Sabin, 1952). The effect of this allele on flavivirus replication has been extensively studied revealing dominantly expressed resistance which is specific to flaviviruses. In contrast, Swiss outbred mice carry a recessive allele conferring susceptibility to flaviviruses at the same genetic locus (Sabin, 1952). Following nine generations of backcrossing and several brother–sister matings the resistance allele, designated *Flv<sup>r</sup>* (Green, 1989), of PRI mice was introduced into the genetic background of inbred flavivirus-susceptible C3H/He mice (*Flv<sup>s</sup>/Flv<sup>s</sup>*) and fixed in a homozygous state (*Flv<sup>r</sup>/Flv<sup>r</sup>*) in a congenic, flavivirus-resistant inbred strain, C3H.PRI-*Flv<sup>r</sup>* (pre-

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viously designated C3H/RV; Goodman & Koprowski, 1962; Groschel & Koprowski, 1965). For years these mice have been used as the only source of flavivirus-resistance allele (Smith *et al.*, 1980; Brinton, 1983; Urosevic *et al.*, 1997), since the parental PRI mouse strain became extinct.

Recently, innate resistance to flaviviruses has been identified in several inbred mouse strains such as CASA/Rk, CAST/Ei and MOLD/Rk (Sangster *et al.*, 1993), which have been directly derived from either wild *Mus mus castaneus* or wild *M. m. molossinus* mice, respectively (Bonhomme & Guenet, 1996). While the CASA/Rk and CAST/Ei mice carry the *Flv<sup>r</sup>*-like alleles, MOLD/Rk mice were shown to carry a novel, significantly different resistance allele designated *Flv<sup>mr</sup>* (minor resistance) (Sangster *et al.*, 1993). The *Flv<sup>mr</sup>* allele protects mice from the less virulent yellow fever (YF) virus, although challenge with Murray Valley encephalitis (MVE) virus was shown to be lethal for those mice (Sangster *et al.*, 1993). In order to study further the properties of this allele derived from wild *M. m. molossinus* mice, one of the aims of this study was to introduce this allele on the genetic background of susceptible C3H/HeJ mice and study its effects on flavivirus replication.

The majority of inbred laboratory mouse strains characterized so far, including several strains of C3H mice, have been shown to be susceptible to flaviviruses and develop severe encephalitis (Darnell *et al.*, 1974). In contrast, wild *M. domesticus* trapped at different geographical locations generally resist flavivirus infection (Darnell *et al.*, 1974; Sangster & Shellam, 1986; Sangster *et al.*, 1998), suggesting that the majority of standard laboratory mouse strains represent a poor sampling of the flavivirus-resistance alleles that are widespread in nature. Accordingly, the second aim of this study was to develop a new flavivirus-resistant mouse strain carrying the *Flv<sup>r</sup>*-like allele, derived directly from wild *M. domesticus*, on the genetic background of susceptible C3H/HeJ mice.

In this paper we describe the development of these two new flavivirus-resistant mouse strains, one carrying the *Flv<sup>mr</sup>* allele of MOLD/Rk mice and the other carrying the *Flv<sup>r</sup>*-like allele from wild *M. domesticus*, on the standardized genetic background of susceptible C3H/HeJ mice. Their genetic constitution around *Flv* and their relative resistance to two different flaviviruses have been characterized and compared with congenic C3H/HeJ and C3H.PRI-*Flv<sup>r</sup>* mice.

## Methods

■ **Mice.** Specific pathogen-free (SPF) inbred mice were obtained as follows; mice of the C3H/HeJ and C3H.PRI-*Flv<sup>r</sup>* strains were provided by the Animal Resources Centre, Murdoch, Western Australia, while mice of the MOLD/Rk strain were obtained from the Jackson Laboratory, Bar Harbor, Maine, USA. The strain designation C3H.PRI-*Flv<sup>r</sup>* uses current nomenclature and replaces the original term C3H/RV introduced by Groschel & Koprowski (1962). Mice were barrier-maintained under minimal disease conditions at the Department of Microbiology. Wild house mice, including the mouse which served as the donor of the *Flv<sup>r</sup>*-like gene, were trapped near Dubbo, New South Wales, Australia and were kindly provided by G. Saunders, New South Wales Department of

Agriculture. Since we have shown previously that flavivirus resistance does not vary significantly with age once mice have reached adulthood (M. Y. Sangster, unpublished), male and female mice which were used in breeding and for the virus infection experiments described below were between 8 and 40 weeks of age. Because of the unknown health status of the wild mice, backcross breeding for the creation of new congenic mouse strains was undertaken in a quarantine facility. However, the last generations of both backcross lines before brother-sister matings were caesarean-derived under SPF conditions at the Animal Resources Centre, Murdoch, and all further breeding was performed using barrier-maintained minimal disease conditions. All experiments on mice have been performed in accordance with the Animal Experimentation Ethics Guidance recommended by the National Health & Medical Research Council of Australia.

■ **Viruses.** Stocks of MVE virus strain OR2 (Liehne *et al.*, 1976) and YF virus vaccine strain 17-D (Commonwealth Serum Laboratories, Melbourne, Victoria, Australia) were stored in liquid nitrogen and were further propagated in Vero cells as described previously (Sangster *et al.*, 1993).

■ **Infection of mice.** Mice were inoculated with  $10^3$  LD<sub>50</sub> of either MVE or YF viruses intracerebrally (i.c.). The dose of either virus was calculated from the percentage of deaths of susceptible C3H/HeJ mice after i.c. infection with a serial dilution of the same virus and expressed as i.c. LD<sub>50</sub> titres (Sangster *et al.*, 1993). The same virus dose of  $10^3$  LD<sub>50</sub> when titrated in Vero cells by TCID<sub>50</sub> corresponded to  $10^{5.4}$  TCID<sub>50</sub> units for the MVE virus and  $10^{7.4}$  TCID<sub>50</sub> units for the YF virus. For selection of flavivirus-resistant mice in the backcrossing experiments, the outcome of infection was monitored for 28 days. Virus inoculation was performed under penthrane anaesthesia. Mice were observed twice daily and those mice showing signs of distress or at the end of experiment were euthanased by CO<sub>2</sub> inhalation followed by cervical dislocation.

■ **Organ collection and virus titration.** On various days post infection (p.i.) brains were collected and homogenized in cold Medium 199 containing 2% foetal calf serum. The homogenates were centrifuged at 2000 g for 15 min and supernatants stored at -70 °C before use (Sangster *et al.*, 1993). The virus contained in the brain homogenates was titrated in Vero cells by the TCID<sub>50</sub> assay as described previously (Sangster *et al.*, 1993).

■ **Genomic DNA preparation and Southern blot analysis.** Genomic DNA was extracted from livers and tails of mice and subjected to restriction enzyme digestion, agarose gel electrophoresis and nylon membrane hybridization using cDNA probes for either the *rd* or *Eta-1* loci as previously described (Sangster *et al.*, 1994).

■ **Microsatellite analysis.** The following mouse chromosome 5 microsatellite markers were used in this study: *D5Mit346*, -80, -304, -41, -175, -26, -211, -158, -209, -187, -210, -188, -136, -407, -425, -424, -68, -367, -408, -368, -159, -242, -118, -279, -137, -65, -369, -160, -95, -96, -138, -427, -215, -43 and -431 (Dietrich *et al.*, 1994). The microsatellites were analysed by PCR using specific primers followed by denaturing PAGE as described previously (Urosevic *et al.*, 1995). Microsatellite primers were either purchased from Research Genetics (Huntsville, AL, USA) and end-labelled by T4 polynucleotide kinase (Promega) in the presence of [ $\gamma$ -<sup>32</sup>P]ATP (Amersham), or synthesized on a 380B DNA synthesizer (Applied Biosystems) with the incorporation of an 5'-aminolink necessary for subsequent labelling with the fluorescent dyes 6-FAM-NHS and JOE-NHS (Applied Biosystems). Fluorescently labelled microsatellites were separated on denaturing polyacrylamide gels using a 373A automated sequencer (Applied Biosystems) and data collected using the Genescan software (Applied Biosystems).

■ **Statistical analysis.** Student's *t*-test was used to determine the statistical significance of the differences in virus titres among different mouse strains. The  $\chi^2$  test was used to compare the mortality rates in successive backcross generations of both lineages and to analyse the pattern of segregation of alleles at the *Flv* locus.

## Results

### Development of congenic strains

(a) **C3H. *M. domesticus* lineage.** In order to produce a congenic mouse strain bearing the novel *Flv<sup>r</sup>*-like allele, we have used a backcross breeding strategy (Flaherty, 1981) in which a flavivirus-resistant wild mouse (*M. domesticus*), trapped near Dubbo in New South Wales (DUB/3; Sangster *et al.*, 1998), was used as a donor, while a flavivirus-susceptible C3H/HeJ mouse strain served as an inbred recipient. Selection for the resistance allele in offspring mice of this backcross lineage has been performed by i.c. challenge with flaviviruses MVE and YF, which are lethal for susceptible mice. Backcross mice surviving the virus challenge were classified as resistant heterozygotes and used in further backcross mating to the susceptible C3H/HeJ strain (Table 1).

Initially, in the N1 to N3 backcross generations of the C3H.*M. domesticus* lineage, two strains of MVE virus, OR156 and OR155, and YF virus were used to discriminate between resistant and susceptible mice (Table 1; Sangster *et al.*, 1998). Since the MVE virus strains OR156 and OR155 caused a high mortality in heterozygous animals carrying *Flv<sup>r</sup>* (Sangster *et al.*, 1998), they were unsuitable for further use in discriminating the resistance allele in backcross mice of this lineage and accordingly were replaced with another strain of the same virus (Sangster *et al.*, 1998). The new MVE virus strain, OR2, has been used for the selection of resistant animals in further backcrossing from the generations N4 to N11 in the C3H.*M. domesticus* lineage as shown in Table 1.

When the MVE virus strain OR2 was used to select for resistant mice in the C3H.*M. domesticus* backcross lineage, inheritance of resistance in offspring mice from backcross generations N4 to N8 followed a ratio of approximately 1:1 between the mice expressing resistance or susceptibility phenotypes. This ratio indicates that the segregation of alleles in backcross mice corresponds to the pattern of inheritance expected if a single dominant resistance gene was involved in the control of flavivirus resistance ( $\chi^2$  test,  $P \geq 0.1$ , no

**Table 1.** Inheritance of resistance to flaviviruses in successive backcross generations derived from wild mouse DUB/3

A male wild *M. domesticus* (designated DUB/3) trapped near Dubbo in New South Wales which survived challenge with MVE virus strain OR156 was used as a donor of the *Flv<sup>r</sup>*-like allele by initial mating with three female C3H/HeJ mice (Sangster *et al.*, 1998).

Backcross generations and mouse strains	Mortality (no. of dead/no. of infected mice)	% Mortality
N1*	21/42	50
N2†	35/48	73
N3‡	51/120	43
N4§	57/114	50
N5	32/60	53
N6	17/43	40
N7	19/39	49
N8	37/62	60
N9	53/78	68
N10	69/87	79
N11	65/124	52
C3H/HeJ	227/227	100
C3H.PRI- <i>Flv<sup>r</sup></i>	0/38	0
(C3H/HeJ) × C3H.PRI- <i>Flv<sup>r</sup></i> F1	0/10	0

\* The F1 hybrid progeny (backcross generation N1) of this mating was challenged i.c. with 50 LD<sub>50</sub> units of MVE virus strain OR156 for the selection of resistance (Sangster *et al.*, 1998).

† Mice of the backcross generation N2 were challenged i.c. with 50 LD<sub>50</sub> units of MVE virus strain OR155 (Sangster *et al.*, 1998).

‡ Mice of the backcross generation N3 were challenged with i.c. with 10<sup>3</sup> LD<sub>50</sub> units of YF virus.

§ Mice of the backcross generations N4 to N11 were challenged with MVE virus strain OR2. Parental mice C3H/HeJ, and resistant C3H.PRI-*Flv<sup>r</sup>* and (C3H/HeJ) × C3H.PRI-*Flv<sup>r</sup>*F1 mice were also challenged i.c. with 10<sup>3</sup> LD<sub>50</sub> units of MVE virus strain OR2.

**Table 2.** Inheritance of resistance to YF virus in successive backcross generations derived from a MOLD/Rk mouse

A male MOLD/Rk mouse was mated to three female C3H/HeJ mice and the F1 hybrid progeny (backcross generation N1) was selected for resistance by challenge i.c. with  $10^3$  LD<sub>50</sub> units of YF virus.

Backcross generations and mouse strains	Mortality (no. of dead/no. of infected mice)	% Mortality
N1	0/14	0
N2	25/59	42
N3	118/253	47
N4	73/152	48
N5	78/152	51
N6	39/83	47
N7	31/78	40
N8	33/78	42
N9	35/82	43
N10	47/98	48
MOLD/Rk	1/26	4
C3H/HeJ	405/424	96
C3H.PRI- <i>Flv</i> <sup>r</sup>	0/27	0

significant difference from the 50% expected values). Significantly higher than expected mortalities were observed in offspring mice of backcross generations N9 and N10 (Table 1;  $\chi^2$  test,  $0.025 > P > 0.01$  and  $P < 0.001$ ), which may have been caused by the incomplete penetrance of resistance conferred by this allelic variant sampled from the wild donor *M. domesticus*. In addition, a modifying effect of surrounding loci on the main locus under selection cannot be ruled out. However, the *Flv*-controlled resistance to flaviviruses seems to be fully restored in the last backcross generation (N11) as indicated by the 52% mortality (Table 1). Since no difference in mortality was observed between female and male mice (data not shown) and since the overall mortality was calculated to be 56%, this result is in agreement with a 1:1 segregation of the resistance and susceptibility alleles at a single autosomal genetic locus ( $\chi^2$  test).

Resistant heterozygous mice which survived challenge with MVE virus strain OR2 were further used to develop an inbred flavivirus-resistant mouse strain by brother–sister matings. Eight brother–sister breeding pairs of resistant heterozygous mice were established producing 86 offspring of which 32 (37%) succumbed to lethal challenge with this virus, a slightly higher mortality rate than the expected value of 25% ( $\chi^2$  test,  $0.025 > P > 0.01$ ). The hetero/homozygosity status at the *Flv* locus of some of the surviving resistant mice was estimated by backcrossing to C3H/HeJ mice and determination of mortality rates in their offspring after challenge with the same virus. Twelve progeny were heterozygous at the *Flv* locus as indicated by the 65% mortality (97/149) of their offspring after challenge with MVE virus strain OR2. Nine progeny (five females and four males) were homozygous

resistant mice (*Flv*<sup>r</sup>/*Flv*<sup>r</sup>) whose offspring were completely resistant to virus challenge (0/103). These homozygous resistant mice were further used as a breeding nucleus in the production of a new congenic flavivirus-resistant strain, C3H.*M.domesticus*-*Flv*<sup>r</sup>.

**(b) C3H.MOLD lineage.** In the C3H.MOLD backcross lineage a male MOLD/Rk mouse was used as donor of the *Flv*<sup>mr</sup> allele, which was introduced into the genetic background of flavivirus-susceptible C3H/HeJ mice. A challenge with YF virus was used to discriminate between resistant and susceptible mice since the more virulent MVE virus is lethal for the resistant mice of this lineage (Sangster *et al.*, 1993). Inbred MOLD/Rk mice are homozygous for *Flv*<sup>mr</sup> and, as a result of crossing with susceptible C3H/HeJ mice, produce offspring resistant to YF virus as shown by the absence of mortality in the N1 backcross generation (Table 2). The YF virus was successfully used for the selection of the *Flv*<sup>mr</sup> phenotype throughout the process of development of the C3H.MOLD-*Flv*<sup>mr</sup> strain, constantly producing mortalities in backcross mice from 40 to 51% which is not different from the 50% expected value ( $\chi^2$  test), indicating a single gene effect.

Flavivirus-resistant mice of the N10 backcross generation which survived challenge with YF virus were brother–sister mated (five breeding pairs) and their offspring challenged with the same virus. The majority of the surviving mice (21/30, 30% mortality, not significantly different from the expected 25%) were typed for the presence of the *Flv*<sup>mr</sup> allele in either the hetero- or homozygous state by further backcross breeding to C3H/HeJ mice and estimation of resistance in their offspring. Twelve progeny were determined to be hetero-

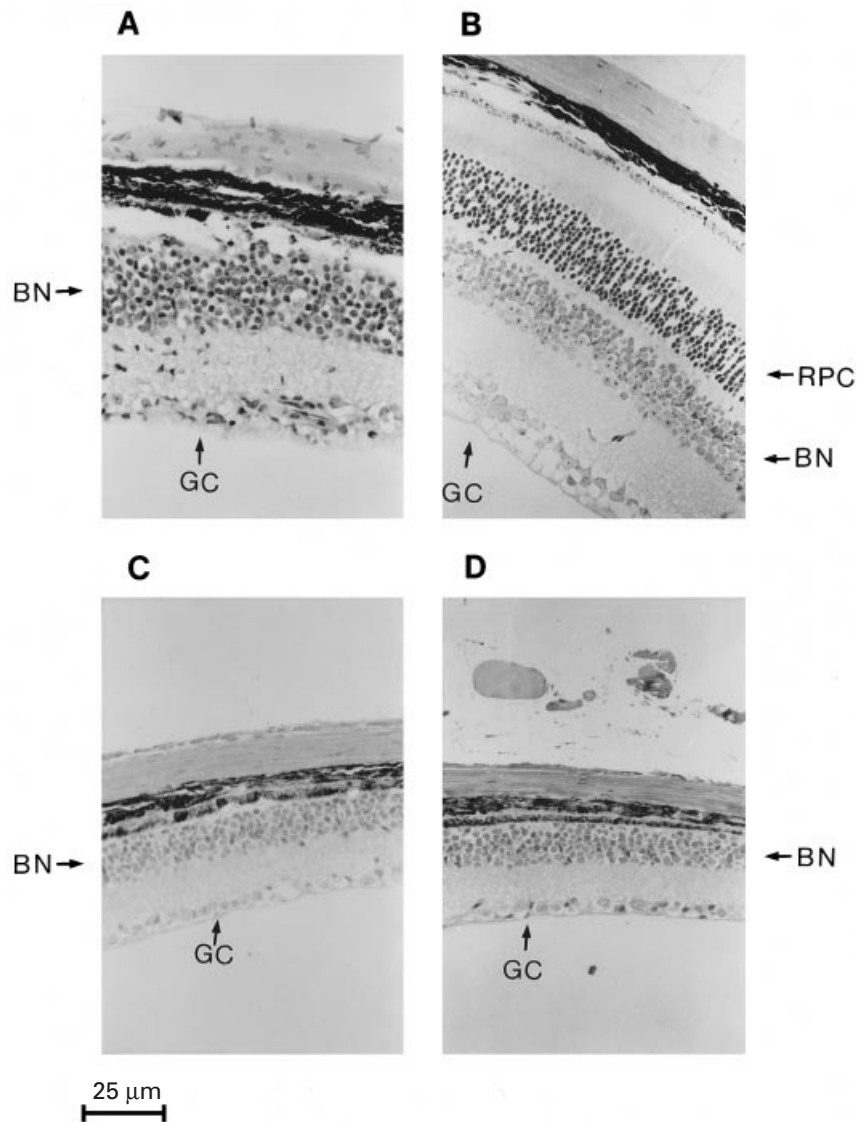


Fig. 1. Retinal histology of newly developed mouse strains ( $\times 400$ ). Flavivirus-susceptible C3H/HeJ mice (A), which served as inbred recipients of the *Flv<sup>r</sup>* and *Flv<sup>mr</sup>* genes during backcrossing, possess degenerate retinas consisting of only bipolar neuron (BN) and ganglion cell (GC) layers, while retinas of reference resistant C3H.PRI-*Flv<sup>r</sup>* mice contain all three layers of the normal retina including BN, GC and rod photoreceptor cell (RPC) layers (B). Newly developed flavivirus-resistant mouse strains, C3H.MOLD-*Flv<sup>mr</sup>* (C) and C3H.*M.domesticus-Flv<sup>r</sup>* (D), show the presence of only BN and GC layers in their retinas as observed in the C3H/HeJ parent, confirming the occurrence of a mutated allele at their *rd* locus.

zygous at the *Flv* locus since their offspring showed 42% mortality (34/82) after challenge with YF virus. Seven progeny (five females and two males) were homozygous resistant mice (*Flv<sup>mr</sup>/Flv<sup>mr</sup>*) whose offspring showed zero mortality (0/87) after the challenge with YF virus. The YF virus-resistant homozygous mice were used as a breeding nucleus for the production of the new inbred C3H/MOLD-*Flv<sup>mr</sup>* congenic mouse strain.

### Genetic analyses

All four congenic mouse strains of C3H background, including the flavivirus-susceptible C3H/HeJ strain and the three resistant strains, C3H.PRI-*Flv<sup>r</sup>*, C3H.*M.domesticus-Flv<sup>r</sup>* and C3H.MOLD-*Flv<sup>mr</sup>*, together with MOLD/Rk and BALB/c mice, were subjected to genotyping at 35 microsatellite and several genetic loci on the distal region of

chromosome 5. While C3H/HeJ and MOLD/Rk strains are parental, the BALB/c strain was included as an additional *Flv<sup>s</sup>*-bearing laboratory strain of a different genetic background to susceptible C3H/HeJ mice which has been previously used in the mapping of the *Flv* locus (Sangster *et al.*, 1994; Urosevic *et al.*, 1995). Genotyping of microsatellites was performed by PCR using either  $^{32}\text{P}$  or fluorescently labelled unique primers listed in The Mouse Genetic Database (The Whitehead Institute, MIT Centre for Genome Research, Massachusetts, USA), followed by electrophoretic separation of PCR products on denaturing polyacrylamide gels of high resolution, as described previously (Urosevic *et al.*, 1995). In addition, two genetic loci, one controlling the architecture of the retina (*rd*) and another controlling host response to *Rickettsia tsutsugamushi* (*Ric*), were analysed for the presence of different alleles using a Southern blot analysis (data not shown), while allelism at the *Gus* and *Flv* loci was determined by either PCR

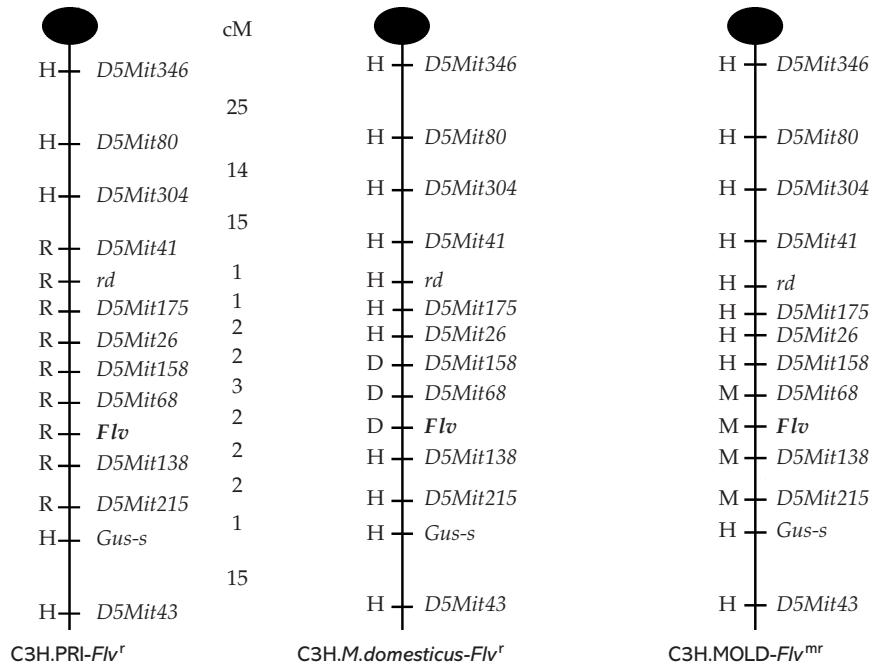


Fig. 2. Comparative genetic maps of chromosome 5 in different flavivirus-resistant mouse strains indicating the size of the genetic segment retained from the donor mice. H, alleles originating from C3H/HeJ; R, alleles derived from PRI; D, alleles derived from wild *M. domesticus*; M, alleles derived from MOLD/Rk. Genetic loci and distances between them in centiMorgans (cM) are deduced from Table 3, MGD column.

or virus titration analyses, respectively, as described elsewhere (Sangster *et al.*, 1994). Inheritance of *rd* alleles in the newly developed mouse strains was also examined by histological analysis of mouse retinas. This analysis revealed that C3H.PRI-*Flv*<sup>r</sup> mice, as previously reported (Shellam *et al.*, 1993), contain intact retinas, while C3H/HeJ, C3H.M.domesticus-*Flv*<sup>r</sup> and C3H.MOLD-*Flv*<sup>mr</sup> mice lack the photoreceptor rod cell layer in their retinas (Fig. 1). While both newly developed flavivirus-resistant mouse strains, unlike the reference resistant C3H.PRI-*Flv*<sup>r</sup> strain, retained C3H/HeJ-derived alleles at both *rd* and *Ric* genetic loci, the origin of the alleles at the remaining loci is variable depending on the size of the chromosome 5 segment inherited from the resistant donor mice (Fig. 2).

The results of this extensive study are summarized in Table 3 in which the genetic origins of the chromosome 5 loci analysed are listed for all mouse strains together with their map positions as defined by both the Whitehead Institute (MIT) and the Jackson Laboratory (MGD) Databases (Websites: [www-genome.wi.mit.edu](http://www-genome.wi.mit.edu) and [www.informatics.jax.org](http://www.informatics.jax.org), respectively). The symbols H, B, R, M or D denote the particular property of genetic markers such as the size of a PCR product, specific pattern of restriction fragments or particular histological feature, and correspond to the genetic background of C3H/HeJ, BALB/c, PRI, MOLD or *M. domesticus* parental mice, respectively. If two or more mouse strains under investigation carried an identical allele at the particular locus, the same symbol has been used to denote this identity regardless of their origin or ancestry, as described in the

footnotes to Table 3. As presented in Table 3 there are markers which are identical in all or only several mouse strains. A number of markers which were unique for the particular mouse strain were used later to construct a genetic map of chromosome 5 in different mouse strains, as shown in Fig. 2.

To further simplify the findings presented in Table 3, we have constructed partial genetic maps of chromosome 5 for the newly developed mouse strains and presented them in parallel with a genetic map of resistant C3H.PRI-*Flv*<sup>r</sup> mice (Fig. 2). These maps depict only those genetic markers which are relevant for the sizing of the genetic interval introduced from the genome of the resistant donor mice. As could be estimated from the genetic distances published for those markers (MIT and the Jackson Databases), mice of the C3H.PRI-*Flv*<sup>r</sup> background carry the largest chromosomal segment, originating from the resistant parent, of approximately 31 cM around *Flv*, while C3H.M.domesticus-*Flv*<sup>r</sup> or C3H.MOLD-*Flv*<sup>mr</sup> mice carry segments of about 9 and 11 cM, respectively.

### Virus replication studies

After a minimum of five generations of inbreeding, mice of both sexes from the newly developed congenic strains and the reference C3H/HeJ (susceptible) and C3H.PRI-*Flv*<sup>r</sup> (resistant) strains were evaluated for resistance to flaviviruses. A minimum of 30 mice per group was challenged i.c. with either MVE virus strain OR2 (Fig. 3 A) or YF virus strain 17D (Fig. 3 B), respectively. From days 1 to 10 p.i. three mice per group were sacrificed daily for determination of virus titres in the brain.

**Table 3.** Genotyping of mouse chromosome 5 microsatellite and genetic loci in different mouse strains including newly developed C3H.*M.domesticus-Flv*<sup>r</sup> and C3H.MOLD-*Flv*<sup>mr</sup> strains

A minimum of two to ten mice of each mouse strain was used for the genetic analyses summarized in this table.

MGD*	MIT†	Locus	C3H/HeJ	BALB/C	MOLD/Rk	C3H.PRI- <i>Flv</i> <sup>r</sup>	C3H.MOLD- <i>Flv</i> <sup>mr</sup>	C3H. <i>M.domesticus-Flv</i> <sup>r</sup>
1	0	<i>D5Mit346</i>	H	B‡	M	H	H	H
26	17.5	<i>D5Mit80</i>	H	B	M	H	H	H
41	27.3	<i>D5Mit304</i>	H	B	M	H	H	H
56	38.3	<i>D5Mit41</i>	H	B	M	R	H	H
57	NA	<i>Pdeb(rd)</i>	H	NA	M	R	H	H
NA	NA	<i>Ric (Eta1)</i>	H	NA	M	R	H	H
58	40.4	<i>D5Mit175</i>	H	B	M	B	H	H
60	45.9	<i>D5Mit26</i>	H	B	M	B	H	H
65	NA	<i>D5Mit211</i>	H	H	M	H	H	H
62	48.1	<i>D5Mit158</i>	H	B	M	B	M	B
63	49.2	<i>D5Mit209</i>	H	H	M	H	M	D
63	49.2	<i>D5Mit187</i>	H	H	M	H	M	H
64	51.4	<i>D5Mit210</i>	H	H	M	H	M	D
64	51.4	<i>D5Mit188</i>	H	H	H	R	H	D
65	53.6	<i>D5Mit136</i>	H	B	M	H	M	D
65	53.6	<i>D5Mit407</i>	H	H	H	H	H	H
65	53.6	<i>D5Mit68</i>	H	B	M	R	M	H
65	54.6	<i>D5Mit425</i>	H	B	M	R	M	D
65	54.6	<i>D5Mit424</i>	H	B	M	R	M	D
65	54.6	<i>D5Mit367</i>	H	H	M	H	M	D
NA	55.7	<i>D5Mit431</i>	H	B	M	R	M	H
66	55.7	<i>D5Mit242</i>	H	B	M	R	M	B
67	56.8	<i>D5Mit408</i>	H	B	M	R	M	M
67	56.8	<i>D5Mit368</i>	H	H	M	R	M	H
67	56.8	<i>D5Mit159</i>	H	H	M	R	M	R
67	56.8	<i>Flv</i>	H	H	M	R	M	R
67	56.8	<i>D5Mit118</i>	H	H	M	R	M	M
68	57.9	<i>D5Mit279</i>	H	H	H	H	H	H
68	57.9	<i>D5Mit137</i>	H	B	M	H	M	D
68	57.9	<i>D5Mit65</i>	H	B	M	R	M	B
68	57.9	<i>D5Mit369</i>	H	H	M	R	M	H
68	57.9	<i>D5Mit160</i>	H	B	M	R	M	H
68	57.9	<i>D5Mit95</i>	H	B	M	R	M	H
68	57.9	<i>D5Mit96</i>	H	R	M	R	M	H
69	59	<i>D5Mit138</i>	H	B	M	R	M	H
71	61.2	<i>D5Mit215</i>	H	B	M	R	H	H
72	62.3	<i>D5Mit427</i>	H	B	M	H	M	H
72	NA	<i>Gus-s</i>	H	B	NA	H	H	H
89	77.6	<i>D5Mit43</i>	H	H	M	H	H	H

\*MGD, distances of the genetic markers from the centromere in cM as reported by The Mouse Genome Database, The Jackson Laboratory, Maine, USA. Website: [www.informatics.jax.org](http://www.informatics.jax.org) (November 1998).

†MIT, distances of the genetic markers from the centromere in cM as reported by The Mouse Genetic Database, The Whitehead Institute, MIT Centre for Genome Research, Massachusetts, USA. Website: [www-genome.wi.mit.edu](http://www-genome.wi.mit.edu) (November 1998).

‡Different allelic forms at a number of genetic loci in various mouse strains are symbolized with different capital letters in the following order: H denotes C3H/HeJ alleles and alleles of other strains identical to them; B denotes BALB/c alleles and alleles of other strains identical to them except C3H/HeJ alleles; R denotes PRI alleles and alleles of other strains identical to them except C3H/HeJ and BALB/c alleles; M denotes MOLD/Rk alleles and alleles of the new congenic mouse strains identical to them; D denotes alleles derived from a wild *M. domesticus*. NA, Not available.

The growth of MVE virus strain OR2 in susceptible C3H/HeJ mice was very rapid, plateauing at day 4 p.i. when the first signs of disease were observed, with deaths occurring from

day 6 p.i. However, a different pattern of virus growth was seen in mice of resistant strains (Fig. 3 A). Resistant C3H.PRI-*Flv*<sup>r</sup> mice exhibited much lower virus titres than the susceptible

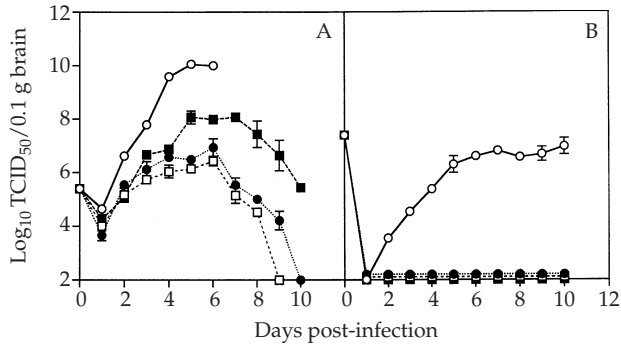


Fig. 3. Replication of MVE (A) and YF (B) viruses in the brains of C3H congenic susceptible and resistant mice. At various days p.i. the virus titre in the brains of C3H/HeJ (○), C3H.PRI-*Flv*<sup>r</sup> (●), C3H.*M.domesticus-Flv*<sup>r</sup> (□) and C3H.MOLD-*Flv*<sup>mr</sup> (■) mice was determined by TCID<sub>50</sub> assay. Three mice of each strain were used for each time-point. The assay sensitivity limit is 2 log<sub>10</sub> TCID<sub>50</sub>/0.1 g brain tissue.

mice, plateauing at days 4 to 6 p.i. The virus titres decreased from day 7 p.i. reaching values at the threshold of detection by day 10 p.i. The virus growth curve in resistant C3H.*M.domesticus-Flv*<sup>r</sup> mice was very similar to growth of the virus in brains of resistant C3H.PRI-*Flv*<sup>r</sup> mice throughout the course of infection, except that the titres were slightly lower in C3H.*M.domesticus-Flv*<sup>r</sup> than in C3H.PRI-*Flv*<sup>r</sup> mice (Fig. 3 A). Also, significantly lower titres were observed in the brains of C3H.*M.domesticus-Flv*<sup>r</sup> mice from day 8 p.i. onwards than in the brains of C3H.PRI-*Flv*<sup>r</sup> mice (Student's *t*-test, 0.02 > *P* > 0.01 and *P* ≤ 0.001, respectively), indicating that the C3H.*M.domesticus-Flv*<sup>r</sup> mice cleared the virus much quicker than resistant C3H.PRI-*Flv*<sup>r</sup> mice. Neither resistant mouse strain showed any outward signs of disease after infection with a high dose (10<sup>3</sup> LD<sub>50</sub>) of MVE virus strain OR2. In addition, both strains had virus titres 3 to 4 logs lower than susceptible C3H/HeJ mice and cleared the virus by days 9 to 10 p.i., respectively (Fig. 3 A), suggesting that they may carry slightly different variants of the flavivirus-resistance alleles.

The growth of MVE virus in resistant C3H.MOLD-*Flv*<sup>mr</sup> mice differed significantly from that in both congenic resistant mouse strains carrying the *Flv*<sup>r</sup> allele and susceptible C3H/HeJ mice carrying the *Flv*<sup>s</sup> allele (Student's *t*-test, 0.02 > *P* > 0.001). The virus titres plateaued at day 5 p.i. with the maximal virus titres 1 to 2 logs higher than in resistant C3H.PRI-*Flv*<sup>r</sup> and C3H.*M.domesticus-Flv*<sup>r</sup> mice, and 2 logs lower than in susceptible C3H/HeJ mice (Fig. 3 A). The virus titres remained high up to day 7 p.i. which coincided with the appearance of mild signs of disease. Although virus titres gradually declined starting from day 8, the signs of disease became more pronounced leading to severe symptoms of encephalitis and death at day 10 p.i. (Fig. 3 A).

When the same mouse strains were challenged with the less virulent YF virus, all susceptible C3H/HeJ mice succumbed to the infection, while none of the resistant mouse strains, including the congenic C3H.MOLD-*Flv*<sup>mr</sup> strain carrying the

minor resistance allele, showed any outward signs of disease. After the initial fall of virus titres at day 1 p.i., the virus grew well in the brains of susceptible C3H/HeJ mice, reaching a plateau at day 5 p.i. which coincided with the first signs of disease. Virus titres were maintained up to day 10 p.i. when deaths occurred (Fig. 3 B). In contrast, the virus titres in all resistant mouse strains were below or at the threshold level of 2 TCID<sub>50</sub> units/0.1 g brain (Fig. 3 B), which coincided with the lack of any outward disease signs.

## Discussion

In this paper we report on the development and characterization of new flavivirus-resistant mouse strains congenic to susceptible C3H/HeJ and resistant C3H.PRI-*Flv*<sup>r</sup> mice. The newly developed C3H.*M.domesticus-Flv*<sup>r</sup> mouse strain was obtained by backcrossing a single wild mouse, which survived a challenge with MVE virus strain OR156 (Sangster *et al.*, 1998), to susceptible inbred C3H/HeJ mice. This wild mouse, trapped in New South Wales near Dubbo, potentially carried a similar allele to C3H.PRI-*Flv*<sup>r</sup> mice in a heterozygous state as indicated by the 50% mortality after challenge with MVE virus strain OR156 in the first backcross generation (N1, Table 1). As the mortality of the second backcross generation, N2, after challenge with MVE virus strain OR155, was significantly higher than 50% ( $\chi^2$  test, 0.0005 > *P* > 0.001), it would appear that the penetrance of resistance conferred by this allelic variant may vary (Table 1). Similar incomplete penetrance of resistance was observed in the N9 and N10 backcross generations, while the final N11 generation of backcrossing showed 52% mortality, a value not significantly different from the expected 50% ( $\chi^2$  test), indicating that a single locus is responsible for control of resistance.

The virus replication studies, presented in Fig. 3, show a significant difference in the efficacy of the C3H.PRI-*Flv*<sup>r</sup> and C3H.*M.domesticus-Flv*<sup>r</sup> mice in clearing virus from the brain. In combination with the incomplete penetrance of resistance observed in the N9 and N10 generations, this may suggest that the newly developed flavivirus-resistant strain C3H.*M.domesticus-Flv*<sup>r</sup> carries a flavivirus-resistance allele slightly different from the one recovered in the previously produced C3H.PRI-*Flv*<sup>r</sup> mouse strain. However, this allele is carried on a much shorter chromosome 5 segment deriving from a resistant mouse of different genetic background than PRI mice (Fig. 2), and a possible modifying effect of some additional genetic loci in the vicinity of *Flv* cannot be ruled out at present. In any case, these two mouse strains represent an excellent model to study fine differences in the expression of resistance to different flaviviruses.

While the majority of laboratory mouse strains presumably carry the same susceptibility allele, *Flv*<sup>s</sup>, derived from a common ancestral allele in a relatively small founding population of wild *M. m. domesticus*, feral mice of the same taxonomic origin predominantly carry resistance alleles of the

*Flv<sup>r</sup>* type (Darnell *et al.*, 1974; Sangster *et al.*, 1998). Since mice carrying the *Flv<sup>r</sup>* allele have rarely been sampled and maintained under laboratory conditions, excluding several resistant mouse strains which may be extinct nowadays, the creation of another resistant mouse strain, C3H.*M.domesticus-Flv<sup>r</sup>*, is of great significance for providing a wild-derived resistance allele in a homozygous state on the very well-defined genetic background of susceptible mice.

We also have reported here on the development of another flavivirus-resistant mouse strain, C3H.MOLD-*Flv<sup>mr</sup>*, which carries the *Flv<sup>mr</sup>* allele of MOLD/Rk mice, a different allelic variant of the *Flv* gene (Fig. 3; Sangster *et al.*, 1993) derived from a different mouse subspecies (Fig. 2). The donor MOLD/Rk mouse strain is an inbred strain recently derived from Japanese wild mice of the *M. m. molossinus* subspecies (Festing, 1996). Mice of this subspecies have been recently shown, by analysis of mitochondrial DNA polymorphism, to be descendants of hybrids between the *M. m. musculus* and *M. m. castaneus* subspecies (Yonekawa *et al.*, 1994). While the minor resistance allele *Flv<sup>mr</sup>* has only been characterized in MOLD/Rk (Sangster *et al.*, 1993) and MOLO mouse strains (Sangster *et al.*, 1998), also recently derived from the *M. m. molossinus* subspecies, flavivirus-resistance alleles similar to *Flv<sup>r</sup>* have been described in different mouse species and subspecies including *M. m. domesticus* (Sabin, 1952; Darnell *et al.*, 1974; Sangster & Shellam, 1986), *M. m. castaneus* (Sangster *et al.*, 1993) and *M. spretus* (Sangster *et al.*, 1998). Wild mice of the *M. m. musculus* subspecies have been shown to carry either resistance (Skive) or susceptibility (CZI-O strain) alleles which confer either lesser resistance than *Flv<sup>r</sup>* or greater susceptibility than *Flv<sup>s</sup>* alleles to the mice of *M. m. domesticus* subspecies (Sangster *et al.*, 1998). Although resistance conferred in Skive mice seems to resemble that of MOLD/Rk mice, the *Flv* alleles of the *M. m. musculus* subspecies have not been studied in great detail (Sangster *et al.*, 1998). Since limited numbers of mice and mouse strains of other subspecies have been tested, the possibility still exists that the *Flv<sup>mr</sup>* allele may be found among other mouse taxonomic categories. Alternatively, the *Flv<sup>mr</sup>* allele may represent a unique allelic variant found only in hybrid *M. m. molossinus* mice.

The level of genetic polymorphism at the chromosome 5 loci between mouse strains C3H/HeJ and BALB/c, both presumably derived from the common ancestral stock of *M. m. domesticus*, has been determined in this study to be 62% (23/37), which is much lower than the 92% polymorphism rate (35/38) determined between C3H/HeJ and MOLD/Rk mouse strains derived from different mouse subspecies (Table 3). The flavivirus-resistant mouse strains, C3H.PRI-*Flv<sup>r</sup>* and C3H.MOLD-*Flv<sup>mr</sup>*, show a similar polymorphism rate of 62% when compared to congenic C3H/HeJ mice, although they are much more polymorphic between each other (79%; 31/39; Table 3). In contrast, the new resistant C3H.*M.domesticus-Flv<sup>r</sup>* mouse strain, obtained by the more advanced backcrossing of the resistant wild mouse of the same taxonomic category to

susceptible C3H/HeJ mice, shows the lowest polymorphism rate of only 38% (15/39) to susceptible C3H/HeJ mice (Table 3).

In this report we present evidence that the new resistant mouse strains, C3H.*M.domesticus-Flv<sup>r</sup>* and C3H.MOLD-*Flv<sup>mr</sup>*, developed in our laboratory, carry novel resistance alleles at the *Flv* locus on a much shorter and less polymorphic chromosomal segment than resistant C3H/PRI-*Flv<sup>r</sup>* mice. These new mouse strains will provide excellent models to study the effects of different allelic variants of *Flv* on infection with different flaviviruses. In addition, study of the effect of the surrounding polymorphic loci on *Flv*-controlled resistance to flaviviruses and further analyses aimed at identifying structural and functional differences between the allelic variants of *Flv* will also be facilitated.

The resistance conferred by *Flv* has been shown to operate by reducing virus titres in target organs and accordingly by protecting mice from the lethal effects of virus infection (Sabin, 1952; Goodman & Koprowski, 1962). There is some evidence suggesting that certain flavivirus strains, including the 'French neurotropic' strain of YF (Sabin, 1952), a replication-efficient mutant of WN (Brinton & Fernandez, 1983) and two strains of MVE virus, OR155 and OR156 (Sangster *et al.*, 1998), have acquired the ability to evade such resistance expressed in either homozygous or heterozygous mice. Those virus strains may not necessarily be highly virulent for the susceptible host, as determined for MVE virus strains OR155 and OR156 (Lawson, 1988), or they may not replicate to high titres (Sabin, 1952), although they still could be lethal for the *Flv* resistant mice. Here we suggest that certain discrete structural alterations in either coding or non-coding regions of those unusual virus strains may have occurred resulting in lack of interaction with the host cellular factor(s) mediating resistance. This could have been induced in the laboratory by long-term propagation of the wild-type virus (Sabin, 1952; Brinton & Fernandez, 1983), or may have occurred in nature as determined for strain OR156, a very unusual strain of MVE virus (Lawson, 1988; Poidinger *et al.*, 1996). This MVE virus strain, isolated in 1973 from infected mosquitoes in the Ord River area of Western Australia (Liehne *et al.*, 1976), was shown to have a very low i.c./i.p. LD<sub>50</sub> ratio in susceptible mice, to produce low titres of infectious virus in cell culture and to have 9% sequence divergence from the MVE virus prototype strain 1-51 in the 5' portion of the genome (M. Lawson, personal communication). A 62 nucleotide deletion immediately downstream of the stop codon in the 3' noncoding region has also been determined for the same MVE virus strain (Poidinger *et al.*, 1996).

Spontaneous and engineered deletions in the similar 3' noncoding region of tick-borne encephalitis virus have been recently reported to result in a high degree of attenuation of the virus without affecting its capacity to replicate and induce protective immunity in mice (Mandl *et al.*, 1998). Although valuable for the development of live vaccines such viral constructs may cause unwanted effects on naturally resistant

hosts. Therefore, the newly developed resistant mouse strains, in addition to their intended use in gene cloning, candidate gene analysis and natural resistance studies, may provide an excellent model for testing virulence of both naturally emerging new virus strains and those attenuated in the process of vaccine development.

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