

# Infection of SCID mice with Montana *Myotis* leukoencephalitis virus as a model for flavivirus encephalitis

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We have established a convenient animal model for flavivirus encephalitis using Montana *Myotis* leukoencephalitis virus (MMLV), a bat flavivirus. This virus has the same genomic organization, and contains the same conserved motifs in genes that encode potential antiviral targets, as flaviviruses that cause disease in man (N. Charlier *et al.*, accompanying paper), and has a similar particle size (approximately 40 nm). MMLV replicates well in Vero cells and appears to be equally as sensitive as yellow fever virus and dengue fever virus to a selection of experimental antiviral agents. Cells infected with MMLV show dilation of the endoplasmic reticulum, a characteristic of flavivirus infection. Intraperitoneal, intranasal or direct intracerebral inoculation of SCID mice with MMLV resulted in encephalitis ultimately leading to death, whereas immunocompetent mice were refractory to either intranasal or intraperitoneal infection with MMLV. Viral RNA and/or antigens were detected in the brain and serum of MMLV-infected SCID mice, but not in any other organ examined: MMLV was detected in the olfactory lobes, the cerebral cortex, the limbic structures, the midbrain, cerebellum and medulla oblongata. Infection was confined to neurons. Treatment with the interferon- $\alpha/\beta$  inducer poly(I)·poly(C) protected SCID mice against MMLV-induced morbidity and mortality, and this protection correlated with a reduction in infectious virus titre and viral RNA load. This validates the MMLV model for use in antiviral drug studies. The MMLV SCID model may, therefore, be attractive for the study of chemoprophylactic or chemotherapeutic strategies against flavivirus infections causing encephalitis.

## Introduction

Several flaviviruses such as Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), West Nile virus (WNV), Murray Valley encephalitis virus (MVEV) (Hurrellbrink *et al.*, 1999) and others (Han *et al.*, 1999; Heinz & Mandl, 1993) cause life-threatening neurological illness in humans. To date, most of the genetic and epidemiological studies on flaviviruses have focused on arboviruses, due to their impact on human health. An outbreak of WNV encephalitis in New York in the autumn of 1999 and 2000 with 79 cases of laboratory-confirmed WNV infection, nine of which were fatal, received much public attention (Asnis *et al.*, 2000; Briese *et al.*, 1999).

Outbreaks of WNV encephalitis also occurred recently in southern Russia and Israel and previously in 1996 in Romania (Han *et al.*, 1999; Lvov *et al.*, 2000; Siegel-Itzkovich, 2000). These outbreaks not only highlighted the risk of the emergence of flaviviruses in new ecosystems, but also the absence of any specific antiviral therapy for flavivirus encephalitis. One important reason for the latter situation is the lack of simple and convenient animal models (Leyssen *et al.*, 2000). Experimental infection of mice with JEV or TBEV by intracerebral or peripheral inoculation has been reported to cause morbidity and mortality (Chiba *et al.*, 1999; Hase *et al.*, 1990). However, the study of these viruses, which are highly pathogenic towards humans [BSL-3 for JEV, louping ill virus (LIV), WNV and St Louis encephalitis virus (SLEV) and BSL-4 for the TBEV complex, according to the American Committee on Arthropod-borne Viruses (ACAV); Subcommittee on Arbovirus

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Laboratory Safety (SALS)] requires special laboratory facilities. Interestingly, a neuroadapted yellow fever virus (YFV 17D) has recently been reported (Chambers & Nickells, 2001).

We report here a convenient model for studying flavivirus encephalitis, and the therapy thereof, using the Montana *Myotis* leukoencephalitis virus (MMLV) (BSL-2 according to the ACAV; SALS). MMLV was first isolated in 1958 from a mouse bitten under laboratory conditions by a naturally infected little brown bat (*Myotis lucifugus*) captured in western Montana. The virus was subsequently isolated from saliva, brain and various other tissues from other bats of the same species. Serological studies suggested that the virus belonged to the flaviviruses (Bell & Thomas, 1964). However, the virus was subsequently 'forgotten' by the scientific community.

The genus *Flavivirus* contains both viruses that are transmitted by mosquitoes or ticks (arboviruses) and viruses with no known vector (NKV) (Chambers *et al.*, 1990). In the accompanying manuscript we have described the complete genomic sequence of MMLV. Phylogenetic analysis has confirmed the classification of MMLV in the cluster of the NKV flaviviruses, as was suggested previously by Kuno *et al.* (1998) based on the sequence of a fragment of the NS5 gene of approximately 1 kb. We have also demonstrated that: (i) MMLV has the same genomic organization as other flaviviruses, and (ii) in the genes that encode the serine protease/helicase and RNA-dependent RNA polymerase (which are important targets for antiviral therapy), the virus contains the same conserved motifs for enzymatic activity as those flaviviruses that are pathogenic to humans. The animal model presented here may be attractive for the study of antiviral strategies against flavivirus infections, in particular flavivirus encephalitis.

## Methods

**■ Virus propagation and RNA isolation.** The original MMLV strain (Montana, 1958) was purchased from the ATCC (ATCC VR-537) and grown in Vero cells at 37 °C in minimum essential medium (MEM; Gibco) supplemented with 10% inactivated fetal calf serum (FCS), 1% L-glutamine and 0.3% bicarbonate. The viral RNA was extracted using the QIAamp Viral RNA kit (Qiagen), according to the manufacturer's instructions.

**■ Animals.** Eight- to twelve-week-old SCID mice and immunocompetent NMRI (Naval Medical Research Institute) mice weighing 16–20 g were used for all experiments. All animals were bred at the Rega Institute under specific pathogen-free conditions. Experiments on animals have been approved by and were in accordance with the guidelines of the Ethical Committee on Vertebrate Animal Experiments of the Katholieke Universiteit Leuven.

**■ Compounds.** Mycophenolic acid (MPA) was purchased from Sigma and ribavirin [1-( $\beta$ -D-ribofuranosyl)-1,2,4-triazole-3-carboxamide] was from ICN. EICAR (5-ethynyl-1- $\beta$ -D-ribofuranosylimidazole-4-carboxamide) was synthesized by Dr A. Matsuda (Hokkaido University, Sapporo, Japan). Tiazofurin and selenazofurin were provided by Dr R. Cooney and Dr D. G. Johns (National Cancer Institute, NIH, Bethesda, MD). Polyinosinic-polycytidylic acid [poly(I)·poly(C)] was purchased from Sigma–Aldrich.

**■ Plaque reduction assay.** Serial dilutions of the test compounds were added to confluent Vero cell cultures grown in 96-well microtitre plates. Cells were infected with approximately 50 p.f.u. of either YFV, dengue fever virus (DENV), or MMLV. Cultures were further incubated at 37 °C for 7 days, after which they were fixed with 70% ethanol and stained with 2% Giemsa solution. Plaques were then counted under an inverted microscope. The antiviral activity of the compounds was expressed as the 50% effective concentration (EC<sub>50</sub>), i.e. the concentration required to reduce plaque formation by 50%.

**■ Titration for infectious virus content.** MMLV-infected mice were sacrificed at various days after infection by ether anaesthesia. Brains were dissected aseptically, and 10% (w/v) tissue homogenates were prepared in MEM supplemented with 2% FCS. Confluent Vero cell cultures grown in 96-well plates were infected with 10-fold serial dilutions of the tissue homogenates and incubated at 37 °C for 1 h, after which the inoculum was removed. Cultures were then washed twice with warm medium and further incubated at 37 °C for 7–9 days, after which the cultures were evaluated for virus-induced CPE. The titre was expressed as the 50% cell culture infective dose (CCID<sub>50</sub>), i.e. the infectious dose required to infect 50% of the cells.

**■ RT-PCR.** Viral RNA was extracted from 140  $\mu$ l of either the cell culture supernatant, or the serum, lymphocytes or macrophages of infected animals using the QIAamp Viral RNA kit (Qiagen). For the isolation of RNA from cell cultures, cell debris was first spun down with two consecutive centrifugation steps at 1500 g in a Minifuge-T. For the isolation of RNA from tissue samples, the RNeasy Mini kit (Qiagen) was used. Prior to reverse transcription (RT), 31.5  $\mu$ l purified viral RNA was mixed with 10  $\mu$ l 5  $\times$  RT buffer (Amersham Pharmacia Biotech), 0.5 mM each of dATP, dTTP, dGTP and dCTP, and 1.2  $\mu$ M of reverse primer (5' GGGAGGCTGAGTAAGCATACACAAGC 3', nt 3834–3859) and denatured at 92 °C for 1 min followed by chilling on ice. RT reaction mixtures contained this denatured RNA plus 95 U human placenta RNase inhibitor (HPRI; Amersham Pharmacia Biotech), 2.4 U RAV-2 reverse transcriptase (Amersham Pharmacia Biotech) and RNase-free water added to give a final volume of 50  $\mu$ l. The reaction mixture was incubated at 45 °C for 1.5 h. The PCR reaction mixture was prepared as follows: 5  $\mu$ l cDNA, 5  $\mu$ l 10  $\times$  PCR buffer, 2  $\mu$ l dNTP mix (100  $\mu$ M of each dNTP), 1.2  $\mu$ M forward primer (5' CGAGGTCAAAAACCCAAG-GAGG 3'), 1.2  $\mu$ M reverse primer (5' CCACAGCATGGGCTCAGAT-GTT 3') and 3.5 U Super *Taq* (HT Biotechnology). The following PCR programme was run: 10 min at 95 °C, 30 cycles of 30 s at 94 °C, 30 s at 60 °C, 1 min at 72 °C and, for the final extension, 10 min at 72 °C. The primers were specific MMLV primers that anneal in the NS1–NS2A genes (forward primer at nt 3281–3302 and reverse primer at nt 3639–3660).

**■ Semi-quantitative RT-PCR.** A calculated amount of 5 ng of total RNA isolated from tissue fragments was used in a 50  $\mu$ l semi-quantitative RT-PCR assay using the OneStep RT-PCR kit (Qiagen). GAPDH (glyceraldehyde phosphate dehydrogenase) served as an internal control and a primer ratio MMLV/GAPDH of 2/1 was used. Amplification primers were GAPDH forward primer: 5' GGTGAAGGTCGGTGTG-AACG 3'; GAPDH reverse primer: 5' ATGTTCTGGACAACCCACG 3'; MMLV forward primer: 5' GCACGAAGCTGCTGAGAGTGCC 3'; MMLV reverse primer: 5' TGAAAGCTGTGTAGGCGCTCCA 3'. After amplification (annealing at 60 °C, 30 cycles) and separation of the amplicons (GAPDH 609 bp, MMLV 978 bp) by agarose gel electrophoresis, the gel was scanned and the two bands were quantified using the ImageMaster software (Hoeffer Pharmacia Biotech).

**■ Quantification of MMLV RNA by 5' nuclease real-time RT-PCR.** RNA preparation was performed using the QIAamp Viral

RNA kit (Qiagen), according to the manufacturer's instructions. For elution of RNA, the columns were incubated with 50 µl of RNase-free water at 80 °C. Primers MMLVS2 (5' TCCGTAGGAAGCGTTGGTGT 3') and MMLVAs1 (5' ATTCCATTACTTCTGCCACTTCGA 3') were used to amplify a 170 bp segment in the 5' non-coding/core region of MMLV. For detection of PCR products, probe MMP (5' AAGAGCGA-CGTGGTTCCAGCC 3') was used. It was labelled with FAM (6-carboxy-fluorescein) at the 5' end and TAMRA (6-carboxy-*N,N,N',N'*-tetramethylrhodamine) at the 3' end. The probe was phosphorylated at its 3' end to prevent elongation during PCR. RT-PCR was carried out using the Superscript One-Step RT-PCR System with Platinum *Taq* (Life Technology). A 20 µl reaction volume contained 10 µl of 2 × reaction buffer, 2.5 mM additional magnesium sulfate, 300 nM each of primers MMLVS2 and MMLVAs1, 200 nM of probe MMP, 0.8 µg BSA (Sigma-Aldrich) and 0.4 µl of Superscript Reverse Transcriptase/Platinum *Taq* enzyme mix. Prepared RNA (2 µl) was added to each reaction. Thermal cycling in a LightCycler (Roche Molecular Biochemicals) involved reverse transcription at 45 °C for 20 min, denaturation at 95 °C for 5 min, followed by 45 cycles of 95 °C for 5 s and 57 °C for 35 s. (Wittwer *et al.*, 1997).

During PCR, the probe MMP was digested by the 5' exonuclease activity of *Taq* DNA polymerase when specifically annealed to the generated MMLV PCR product (Holland *et al.*, 1991). Probe digestion liberated the FAM from the TAMRA dye, causing an increase in FAM-specific fluorescence during PCR (Livak *et al.*, 1995). FAM fluorescence was measured on detection channel F1 (530 nm) and divided by fluorescence measured on channel F2 (640 nm) for normalization. As a quantification standard for real-time PCR, a strong positive MMLV stock was diluted in virus-negative human plasma prior to RNA preparation. One PCR unit (PCRU) of MMLV RNA was defined as the lowest possible dilution that could be amplified in five out of five replicate reactions. The quantification procedure using the LightCycler has been previously described (De Silva *et al.*, 1998).

■ **Animal experiments.** NMRI mice or SCID mice were inoculated with 10<sup>4</sup> p.f.u. of MMLV via either the intraperitoneal (i.p.; 200 µl), intracerebral (i.c.; 50 µl) or intranasal (i.n.; 20 µl) route. The animals were examined daily for signs of morbidity and mortality. The statistical significance of differences in the mean day of death was assessed by means of the Student's *t* test and differences in the number of survivals by means of the  $\chi^2$  test with Yates' correction. To study the progression of MMLV infection in NMRI mice and SCID mice, the animals were infected intraperitoneally with MMLV and two to four mice were sacrificed every 3 days. Blood samples were taken and total RNA was extracted from the serum and subsequently used for RT-PCR. Brain, salivary glands, lung, liver, kidney, pancreas, seminal vesicles and spleen were removed and subjected to immunostaining.

In some experiments, half of the infected animals were implanted on day 0 or on day 14 following infection with a mini-osmotic pump (Alzet Model 2002, Alza Corporation, California, USA) filled with ribavirin at a concentration of 83.3 µg/µl, resulting in a continuous subcutaneous calculated release of 50 mg/kg/day of ribavirin. Mice were monitored for 4 weeks after virus challenge.

■ **Electron microscopy.** Vero cells that had been infected with MMLV and that exhibited an extensive CPE were fixed in a buffered glutaraldehyde solution for 30 min, washed with isotonic phosphate buffer, post-fixed with 1% OsO<sub>4</sub> solution for 1 h, collected, dehydrated and embedded in Dow epoxy resin for electron microscopy. Sections (1 µm) stained with toluidine blue were examined with a light microscope. Sample sections (50 nm) of areas of interest were collected on grids, stained with uranyl acetate and lead citrate, and examined with a Philips CM10 electron microscope.

■ **Histopathology.** Moribund animals with severe signs of paralysis were euthanized with ether anaesthesia and were transcardially perfused with 20 ml of a buffered 4% formaldehyde solution for histology. Fixed tissue samples were embedded in paraffin and further processed using standard methods.

■ **Preparation of digoxigenin-labelled cRNA.** MMLV cDNA encompassing 655 nucleotides of the NS5 region of the MMLV genome (forward primer 5' TCATCCAGAGAAGAATTG 3'; reverse primer 5' TTCAAAAAGCCAAAGCTTTCAAATTC 3') was cloned into the transcription vector pGEM-T (Promega). To generate runoff transcripts, the plasmid was linearized with *NotI* (Promega) for 1.5 h at 37 °C. The linearized plasmid DNA was precipitated with 3 M sodium acetate (pH 4.6) and 2.5 vols ethanol. After centrifugation, the pellet was washed in 70% ethanol and dissolved in RNase-free water at a concentration of approximately 100 ng/µl.

Transcription reactions (20 µl) consisted of 2 µl water, 10 µl linearized cDNA (1 µg), 4 µl 5 × transcription buffer (Promega), 2 µl 10 × Dig Labelling Mix (Roche Diagnostics) and 2 µl T7 RNA polymerase (Promega). The reaction was incubated for 2 h at 37 °C. The cRNA derived by T7 polymerase *in vitro* transcription was isolated on a Mini Quick Spin RNA column (Boehringer Mannheim) and the eluate was frozen at -80 °C until use.

■ **In situ hybridization.** Paraffin-embedded tissue was sectioned, hydrated, fixed, denatured and acetylated according to standard procedures (Breitschopf *et al.*, 1992). The sections were permeabilized by digestion with proteinase K (100 µg/ml) (Boehringer Mannheim) for 20 min. Prehybridization was at 60 °C for 30 min. Hybridization solution was prepared by adding 200 ng of digoxigenin (DIG)-labelled cRNA per ml of hybridization buffer (EasyHyb, Roche Diagnostics). The hybridization solution (150 µl/section) was added, the section was heated for 4 min at 95 °C to denature the probe and hybridization was allowed to occur at 60 °C for 4–6 h in a humidified chamber. The sections were incubated in 2 × SSC (0.15 M NaCl, 0.015 M sodium citrate) for 12 h and washed with 1 × SSC. On completion of the hybridization step, the sections were incubated in buffer solution containing 0.5% (w/v) blocking reagent (Boehringer Mannheim) at room temperature for 15 min, followed by incubation with alkaline phosphatase-conjugated anti-DIG polyclonal antiserum (1:500 in blocking buffer) for 1 h and incubated overnight with the NBT/BICP reagent (Roche Molecular Biochemicals).

■ **Immunohistochemistry.** Primary antiserum was produced in New Zealand rabbits after immunization with a suspension of UV-inactivated MMLV and complete Freund's adjuvant. Tissue sections were rehydrated and blocked with 2% skimmed milk. The sections were then incubated with 200 µl of primary rabbit antiserum (dilution 1:100) for 1 h at 37 °C. The sections were rinsed in PBS and incubated with horseradish peroxidase-conjugated donkey anti-rabbit antibody diluted 1:500 in PBS for 30 min at 37 °C. After rinsing, endogenous peroxidase activity was quenched by incubation overnight in 70 ml buffer (0.1 M acetate and 0.15 ml of a 3% H<sub>2</sub>O<sub>2</sub> solution) containing 20 mg AEC (3-amino-9-ethyl-carbazole) (Sigma-Aldrich) in 5 ml *N,N*-dimethylformamide.

To monitor MMLV infection in the brain, sections were stained with haematoxylin and eosin (H&E) according to standard procedures.

## Results

### Antiviral susceptibility

MMLV produces an obvious CPE in Vero cells. The susceptibility of MMLV to a selection of antiviral agents could therefore easily be monitored. We compared the inhibitory

**Table 1.** Susceptibility of MMLV, YFV and DENV to a selection of experimental antiviral agents

Antiviral agent	EC <sub>50</sub> (µg/ml)*		
	YFV	DENV	MMLV
Ribavirin	22 ± 14	38 ± 11	25 ± 8
EICAR	0.8 ± 0.6	1.6 ± 0.6	0.4 ± 0.2
Mycophenolic acid	0.06 ± 0.04	0.10 ± 0.06	0.03 ± 0.02
Tiazofurin	17 ± 9	73 ± 25	18 ± 7
Selenazofurin	2.6 ± 1.5	12 ± 5	5 ± 3

\* Concentration required to reduce virus-induced plaque formation in Vero cells by 50%.

effects of ribavirin, EICAR (the 5-ethynyl derivative of ribavirin) (De Clercq *et al.*, 1991), tiazofurin, selenazofurin (an oncolytic C-nucleoside) and MPA (an inhibitor of IMP dehydrogenase) on the replication of YFV, DENV and MMLV. As shown in Table 1, the antiviral efficacy of the different

compounds against all three viruses ranked as follows: mycophenolic acid > EICAR > selenazofurin > tiazofurin > ribavirin. Thus, the susceptibility of MMLV to these compounds proved to be more or less comparable with the susceptibility of the human flaviviruses YFV and DENV to these drugs.

#### Ultrastructural analysis

MMLV-infected Vero cell cultures were studied by means of electron microscopy. At 7 days post-infection, the subcellular pathology was characterized, as compared with uninfected cells, by extensive proliferation and dilation of the rough endoplasmic reticulum (RER) (Fig. 1), which is characteristic for cells infected with flaviviruses (Chambers *et al.*, 1990; Westaway, 1987). The size of the viral particles inside the RER was determined to be ~ 40 nm, which is very similar to the size of human flaviviruses (40–60 nm) (Monath & Heinz, 1996).

#### MMLV-induced encephalitis and mortality

The effect of different routes of inoculation with MMLV on morbidity and mortality in SCID and NMRI mice was studied.

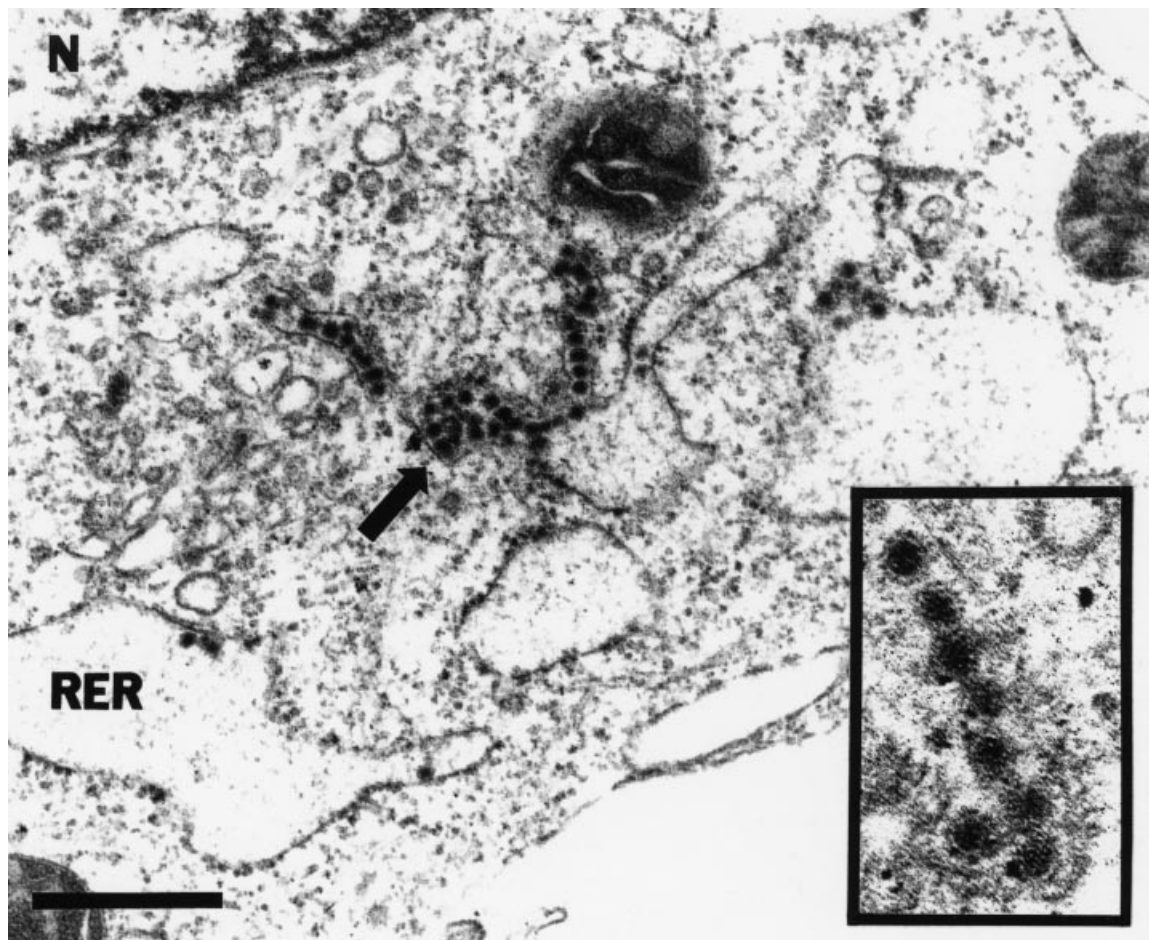


Fig. 1. Ultrastructural analysis of Vero cells 7 days post-infection, showing extensive proliferation and dilation of the RER (arrow, viral particles; N, nucleus; RER, rough endoplasmic reticulum) (inset: detail of particles). Scale bar, 0.5 µm.

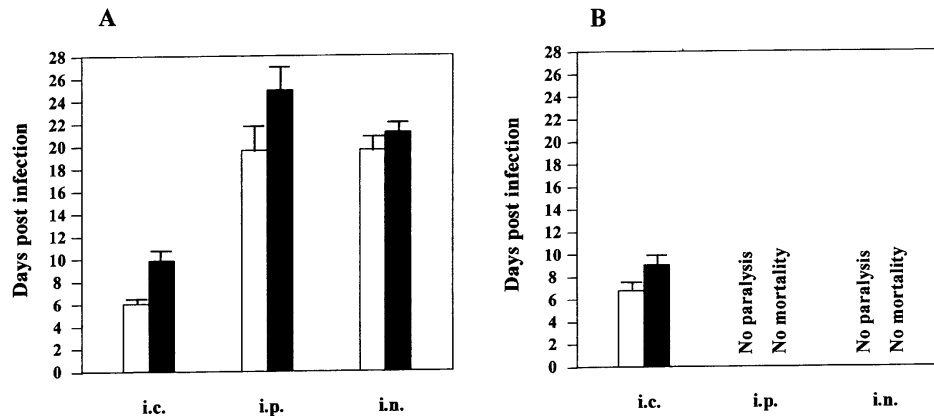


Fig. 2. Paralysis and mortality following intracerebral (i.c.), intraperitoneal (i.p.), or intranasal (i.n.) inoculation of SCID mice (A) or NMRI mice (B) with MMLV. Each group consisted of 10 to > 50 mice. The columns represent the mean day of paralysis (MDP; white columns) and the mean day of death (MDD; black columns)  $\pm$  SD.

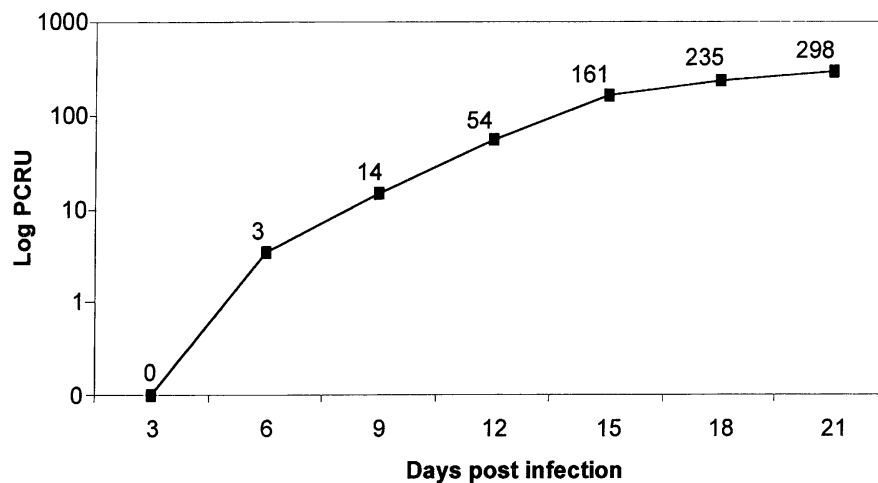


Fig. 3. Detection of viral RNA in the serum of SCID mice infected with MMLV via the i.p. route. Sera from two mice were collected every third day after infection until day 21 post-infection and were pooled. Quantification of the viral RNA load in the serum was carried out in triplicate by TaqMan RT-PCR (SD below 15%). One PCR unit (PCRU) is defined as the lowest template copy number detectable in three out of three replicate reactions as determined by a limiting dilution series.

Direct intracerebral (i.c.) inoculation of the virus ( $10^4$  p.f.u.) in both SCID and NMRI mice led to paralysis within 6 days and mortality within 9 days [Fig. 2A, B; mean day of paralysis (MDP):  $6.1 \pm 0.3$  (SCID) and  $7.1 \pm 0.6$  (NMRI); mean day of death (MDD):  $10.0 \pm 1.2$  (SCID) and  $9.6 \pm 0.7$  (NMRI); 10 NMRI mice and 15 SCID mice]. Morbidity was characterized by ruffled fur, paralysis of the hind legs and a wasting syndrome. Intraperitoneal (i.p.) or intranasal (i.n.) infection of SCID mice with MMLV resulted in morbidity (Fig. 2A; i.p. route: MDP  $19.8 \pm 2.6$ , > 50 animals; i.n. route: MDP  $20.2 \pm 1.1$ , > 15 animals) and mortality (Fig. 2; i.p. route: MDD  $25.1 \pm 2.0$ , > 50 animals; i.n. route; MDD  $21.6 \pm 0.5$ , > 15 animals). Immunocompetent NMRI mice infected with MMLV via the i.p. or i.n. route remained healthy throughout the experiment (Fig. 2B).

### Viral RNA in blood serum and white blood cells

Total RNA was extracted from the serum and white blood cells of i.p. MMLV-infected SCID mice that exhibited obvious signs of paralysis. Following RT-PCR (detection limit: 1.2 fg viral RNA; data not shown), viral RNA was detected in the serum starting at day 6 post-infection (Fig. 3). This was confirmed by real-time quantitative RT-PCR. The level of viral RNA in the serum increased continuously until the animals died. No viral RNA was detected in the white blood cell population (leucocytes and macrophages) of infected animals.

### Histopathology

In SCID mice that had been inoculated with MMLV via the i.p. route, the virus was first detected in the brain by *in situ*

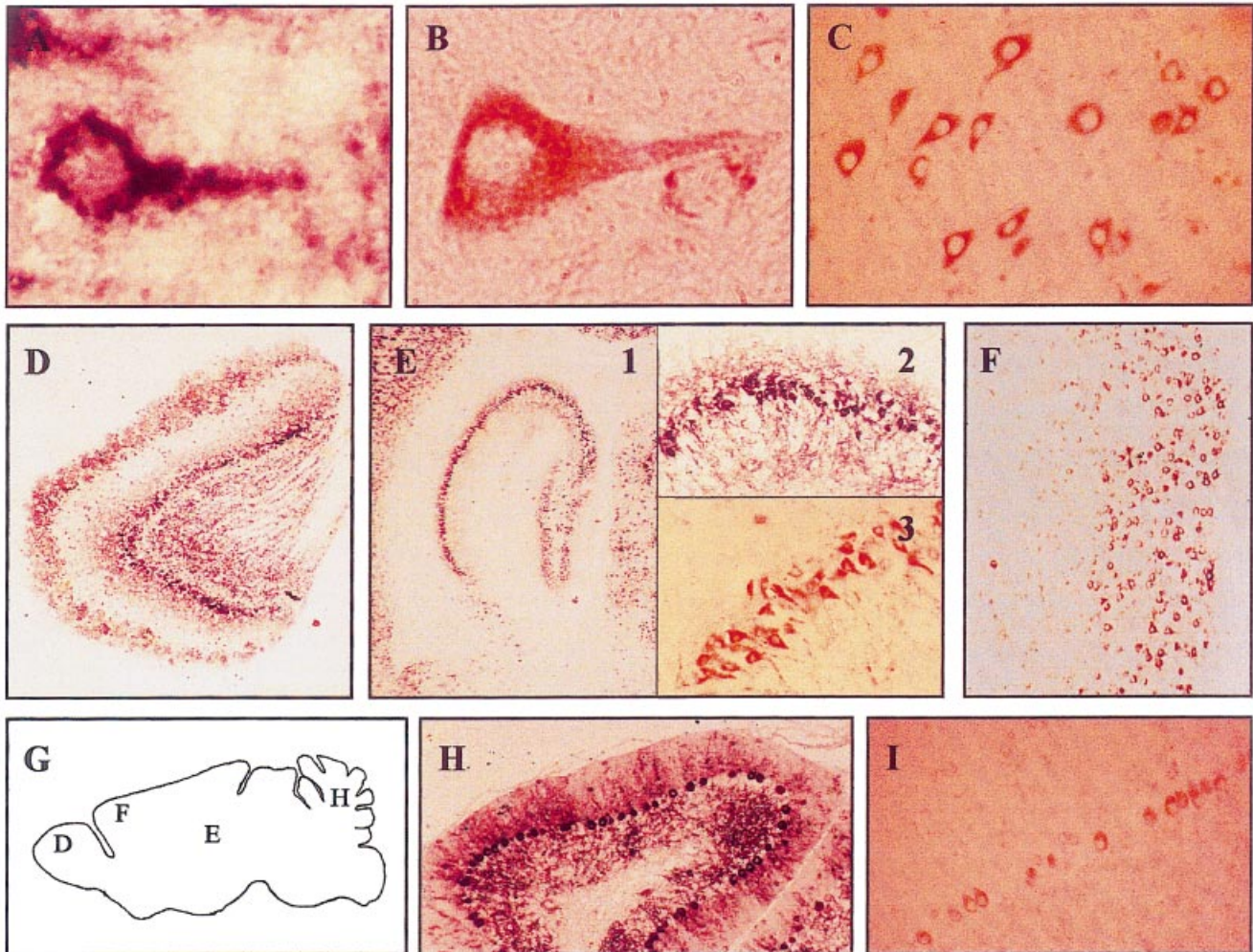
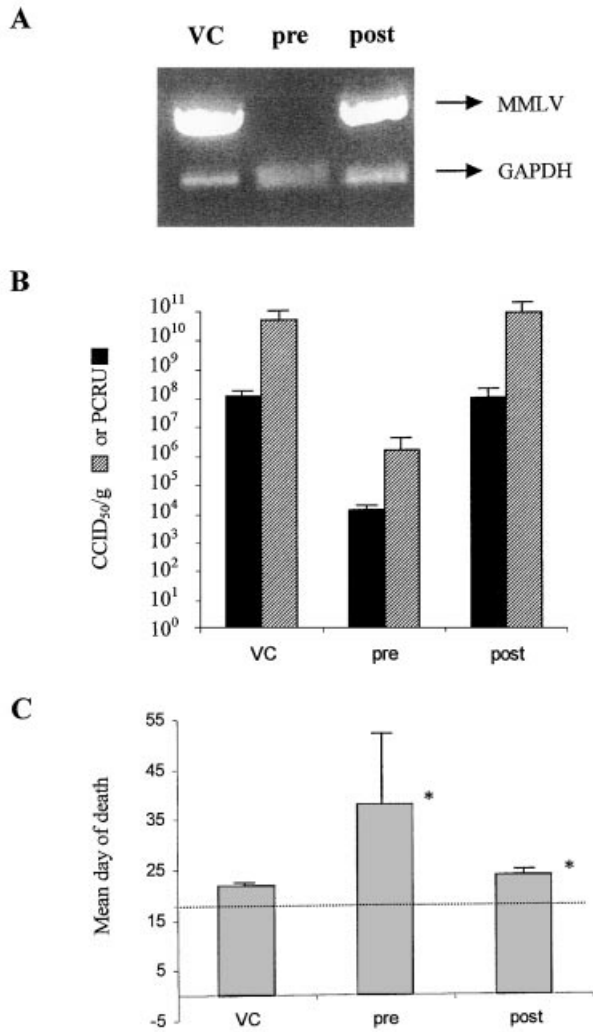


Fig. 4. Localization of MMLV RNA or antigens in the brains of SCID mice at the time of paralysis (day 18 following infection by i.p. route) by *in situ* hybridization [A, D, E (1, 2), F, H] and immunohistochemistry [B, C, E (3), I]. The presence of virus is shown in the neurons in the cortex (A; magnification  $\times 100$ ; B,  $\times 100$ ; C  $\times 40$ ), in the bulbus olfactorius (D,  $\times 2.5$ ), in the hippocampus [E, (1)  $\times 2.5$ , (2)  $\times 25$ , (3)  $\times 25$ ], in the temporal cortex (F,  $\times 10$ ), in the cerebellum (H,  $\times 10$ ), and in the Purkinje cells in the cerebellum (I,  $\times 25$ ). (G) Sagittal section through the mouse brain with indication of the position at which the microphotographs were taken.

hybridization on day 12 post-infection (data not shown). In one animal (out of eight) a massive degeneration of the hippocampus was seen on day 18 (data not shown). The brains of the other seven animals showed a less pronounced degeneration. At the time of paralysis, viral RNA was detected in the grey matter of the olfactory lobes, the pyriform cerebral cortex, the temporal cerebral cortex, the limbic structures, the midbrain structures (thalamus, hypothalamus), and in the medulla oblongata. Viral RNA was also detected in the cerebellum with infection of virtually all of the Purkinje cells. Overall, MMLV RNA was present in the cytoplasm of infected cells and appeared to be confined to neurons. Neuron dysfunction may therefore be expected to be the cause of encephalitis and ultimately death. The pattern of MMLV infection, as assessed by *in situ* hybridization, was confirmed by immunohistochemistry (Fig. 4).

#### Effect of treatment with an interferon inducer on MMLV replication and associated mortality

To validate the MMLV model for future antiviral studies, we sought to assess whether there was a correlation between the protective effect on morbidity and mortality of a therapeutic or prophylactic agent, and inhibition of virus replication. Treatment with ribavirin (given as a continuous infusion at 50 mg/kg/day for 14 days) did not protect mice from MMLV-induced morbidity and mortality; nor did it cause a reduction in viral RNA in the infected organs (data not shown). We therefore studied the effect of poly(I)·poly(C), an inducer of interferon- $\alpha/\beta$  on virus replication. To this end, SCID mice were treated with a single dose of poly(I)·poly(C) at 15 mg/kg and were infected intraperitoneally with MMLV 24 h later. This pretreatment with poly(I)·poly(C) resulted in a



**Fig. 5.** MMLV RNA detected in the brain of MMLV-infected SCID mice that were treated with poly(I)·poly(C) either 24 h before infection (pretreatment) or starting at 2 h post-infection (post-treatment). (A) Gel electrophoresis of the amplicons obtained by One-Step RT-PCR (VC, untreated virus control group; pre, pretreatment group; post, post-treatment group). (B) Quantification of viral RNA load using TaqMan technology (black bar) or infectious virus content (grey bar). (C) Mean day of death (\*,  $P < 0.05$  compared with the untreated virus control group). Dashed line: day at which animals in a parallel group were sacrificed for the determination of infectious virus titre and RNA load as depicted in panels A and B.

significant reduction in the number of animals developing signs of paralysis (2/6 compared with 6/6 in the untreated control group) (data not shown) and also a 16 day delay in virus-induced mortality (Fig. 5C). When poly(I)·poly(C) was given 2 h post-infection, all protective activity was gone. In a parallel group of mice, the infection was monitored by real-time quantitative (Fig. 5B) and semi-quantitative (Fig. 5A) RT-PCR and by titration (Fig. 5B) for infectious virus content in the brain of MMLV-infected SCID mice that had received either mock or poly(I)·poly(C) pretreatment. Tissue samples were taken at a time at which untreated control animals had

developed signs of paralysis (i.e. 18 days post-infection). Levels of viral RNA detected in the brain of infected SCID mice that had been pretreated with poly(I)·poly(C) were markedly reduced ( $1.2 \times 10^4$  PCRU compared with  $1.18 \times 10^8$  PCRU in the untreated control group) (Fig. 5B). In addition, titration for infectious virus content (Fig. 5B) showed an important decrease in viral load in the brain of the pretreatment group ( $1.5 \times 10^7$  CCID<sub>50</sub>/g tissue compared with  $5 \times 10^{11}$  CCID<sub>50</sub>/g tissue in the control group). Thus, the protective effect of poly(I)·poly(C) on virus-induced morbidity and mortality was reflected by a marked reduction in the infectious virus titre and viral RNA load.

## Discussion

Several flaviviruses cause life-threatening neurological illnesses in man. There is currently no specific antiviral drug available for the treatment of infections caused by these viruses. The search for treatment strategies has been hampered, in part, by the absence of a convenient animal model. Several flaviviruses are pathogenic and lethal in mice, but only following direct intracerebral inoculation. Other flaviviruses can cause morbidity and mortality following peripheral inoculation; however, these viruses require special safety conditions for manipulation. Flaviviruses such as DENV, YFV or JEV can infect monkeys, but because of the costs involved and the restricted availability of monkeys, the number of studies using these animals is limited.

We have presented a model for the study of antiviral strategies against flavivirus encephalitis employing the Montana *Myotis* leukoencephalitis virus. MMLV is highly pathogenic to mice and has been classified as a biosafety level II pathogen by SALS (<http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4s71.htm>). As we report in the accompanying paper (Charlier *et al.*, 2002), MMLV has the same overall genome organization as flaviviruses of clinical importance, and in those genes that are considered to be interesting antiviral targets (i.e. the NS3 gene, which encodes an NTPase/helicase and serine protease, and the NS5 gene encoding an RNA-dependent RNA polymerase), it has the same conserved motifs as flaviviruses that are infectious to humans.

MMLV can be readily propagated in cell culture and the virus has a susceptibility to a selection of antiviral drugs, including ribavirin and its 5-ethynyl analogue, EICAR (Leyssen *et al.*, 2000), that is comparable with that of YFV and DENV, which further points to the relevance of MMLV for the study of antiviral strategies against flaviviruses.

MMLV is neuroinvasive in SCID mice, but not in immunocompetent mice, in which it is only neurovirulent. In SCID mice infected via the peripheral route with MMLV, the virus is detected in the brain and serum; no viral antigens or viral RNA are detectable in solid organs (by means of immunohistochemistry or *in situ* hybridization) nor in macrophages or other blood-borne cells (by means of RT-PCR) (data

not shown). Virus titres in the serum of MMLV-infected SCID mice start to rise well before the virus is detected in the brain. It may be assumed that this viraemia is the result of low-level virus replication in peripheral organs, a level that is, however, too low to be detected by *in situ* hybridization or immunohistochemistry. Virus replication in these organs was not detectable by means of RT-PCR or titration for infectious virus content, since, despite extensive perfusion of the animals, traces of blood resulted in false positive signals for all organs. M. Halevy and colleagues concluded that WNV replicates in as yet unidentified target tissues following peripheral inoculation of SCID mice with the virus (Halevy *et al.*, 1994). Modoc virus (MODV), a flavivirus isolated from the white-footed deer mouse (Johnson, 1967), is, like MMLV, neuroinvasive in SCID mice, but, unlike MMLV, replicates in peripheral organs such as the salivary glands and in the spleen (Leyssen *et al.*, 2001). In a recent study, a neuroadapted strain of YFV, 17D, derived from a multiple mouse brain-passaged virus, proved neuroinvasive in SCID mice. Unlike MMLV, this virus exhibited an efficient growth in peripheral tissues of SCID mice (Chambers & Nickells, 2001). In addition to isolation from the brain, JEV was also isolated from the liver and spleen of newborn mice infected via transplacental transmission of intraperitoneally infected pregnant mice (Mathur *et al.*, 1981). It remains largely unclear which determinants influence the organ tropism of flaviviruses in peripheral organs. As is the case for the murine MMLV and WNV models, in patients with WNV encephalitis, virus was not detected in major organs such as lung, liver, spleen and kidney (Nash *et al.*, 2001; Sampson *et al.*, 2000; Shieh *et al.*, 2000).

It has been demonstrated for YFV, WNV, JEV, MVEV, TBEV and LIV that specific amino acid substitutions within the E protein are associated with loss of neuroinvasiveness in mice (Cecilia & Gould, 1991; Hasegawa *et al.*, 1992; Sumiyoshi *et al.*, 1995; McMinn *et al.*, 1995a, b; Holzmann *et al.*, 1990; Jiang *et al.*, 1993; Chambers *et al.*, 1998; Chambers & Nickells, 2001). Obviously the E protein plays a prominent role in neuropathogenesis of flaviviruses and the particular characteristics of the E protein of MMLV may thus determine the tropism of this bat virus.

MMLV-induced encephalitis in SCID mice is characterized by the presence of viral RNA in virtually all regions of the brain and infection is clearly confined to neurons. We did not detect other cells of the CNS that were infected with MMLV. In the brain of mice infected with JEV, major cytopathological changes were observed in neurons and developing neurons (Ogata *et al.*, 1991; Hase, 1993; Hase *et al.*, 1993; Wang *et al.*, 1998). Also, in rodent infection models with viruses such as MVEV, YFV and WNV, virus replication in the brain was mainly confined to neurons (Matthews *et al.*, 2000; Schlesinger *et al.*, 1996; Weiner *et al.*, 1970; Eldadah & Nathanson, 1967). Presence of virus in all regions of the brain has also been observed in patients with Japanese encephalitis (Johnson *et al.*, 1985). In patients with West Nile (meningo)encephalitis, virus

was detectable in neurons throughout the brain (Nash *et al.*, 2001; Nichter *et al.*, 2000; Sampson *et al.*, 2000; Shieh *et al.*, 2000) and, as seen in the MMLV model, also in the medulla oblongata. TBEV was detected in the thalamus, substantia nigra and cerebellum of the brain of patients with tick-borne encephalitis (Mazlo & Szanto, 1978).

In the CNS of MMLV-infected SCID mice, no or little cell infiltration was observed. Since SCID mice do not carry (functional) B and T cells, infiltration of the brain by lymphocytes is not possible. Yet, SCID mice do harbour functional macrophages as well as natural killer (NK) cells; however, no or little infiltration of these cells in the brain of infected animals was observed. In WNV-infected mice, two populations of inflammatory cells were detected in the central nervous system: NK cells and cytotoxic T cells (Liu *et al.*, 1989). In normal mice, JEV infection caused prominent inflammatory changes with leukocytic infiltration and perivascular cuffing (Hase *et al.*, 1990). The fact that no or little inflammation was observed in the brain of MMLV-infected SCID mice points to the fact that direct viral damage is the main (or sole) reason for brain dysfunction and virus-induced mortality. This may make the MMLV model particularly attractive for the study of antiviral strategies against flaviviruses. Indeed, a protective effect in the MMLV model will probably be solely attributable to a direct inhibitory effect of the compound on virus replication in the brain. If such a molecule can prove sufficiently protective in the MMLV model, it may be interesting to study its effect in animal (including monkey) models with more pathogenic flaviviruses, in which an inflammatory response is also involved in the pathology of the disease.

To validate the MMLV model for future use in antiviral drug studies, we sought to prove that protection against MMLV disease progression correlates with an inhibitory effect on virus replication. Treatment with ribavirin had a weak inhibitory effect on the replication of MMLV *in vitro* (as for YFV and DENV) and did not protect mice from MMLV-induced morbidity and mortality, nor did it cause a reduction in viral RNA in the brain. This is in line with the finding that ribavirin does not result in any protective effect in rhesus monkeys infected with dengue virus type 1 (Malinoski *et al.*, 1990). Ribavirin has also never been shown to be protective against flaviviruses in man. Ribavirin, therefore, did not prove useful to validate the MMLV model for antiviral drug studies. For this reason, we made use of the interferon- $\alpha/\beta$  inducer poly(I)·poly(C). This molecule has previously been shown to elicit a protective effect against JEV and MODV infection in mice and/or monkeys (Leyssen *et al.*, 2001; Harrington *et al.*, 1977; Singh & Postic, 1970; Worthington *et al.*, 1973). The interferon inducer delayed MMLV-induced mortality and reduced virus titres, which further demonstrates that the MMLV model may be useful in both prophylactic and therapeutic studies.

In conclusion, the MMLV mouse model has several clinical, histopathological and virological features reminiscent of

flavivirus infections, in particular flavivirus encephalitis, in humans. Therefore, the MMLV model may be valuable for the study of antiviral strategies against infections with flaviviruses, in particular those causing encephalitis.

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