

Virulence attenuation of *Dengue virus* due to augmented glycosaminoglycan-binding affinity and restriction in extraneural dissemination

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To gain insight into the role of cell surface glycosaminoglycans (GAG) in dengue virus (DEN) cell tropism and virulence, DEN-2 mouse brain-adapted vaccine candidate, neurovirulent prototype strain (NGC) and low-passaged strain, PUO-218, were passaged in BHK-21 and SW13 cells to isolate variants with high affinity for GAG. Sequence comparisons of parent and passage variants revealed five GAG-binding determinants, which all cluster in a surface-exposed region in domain II of the three-dimensional structure of the DEN envelope protein. Using an infectious cDNA clone of NGC and an NGC/PUO-218 prM–E chimeric clone, it was demonstrated that the GAG-binding determinants augment the specific infectivity for BHK-21 and/or SW13 cells by 10- to 170-fold and in some cases marginally reduce that for Vero cells. This altered cell tropism was due to a greater dependence of the variants on cell surface GAG for attachment/entry, given their increased susceptibility to heparin inhibition. The effect of the GAG-binding determinants on virulence was examined in mice deficient in alpha/beta/gamma interferon responses. High GAG affinity strongly correlated with low neuroinvasiveness due to rapid virus clearance from the blood. It was speculated that this mechanism accounts for the attenuation in primates of some DEN vaccine candidates. Interestingly, the GAG-binding variants did not display marked attenuation of neurovirulence and the opposing effect of enhanced neurovirulence was associated with one determinant (Lys¹²⁶) already present in mouse brain-adapted NGC. This discrepancy of attenuated neuroinvasiveness and augmented neurovirulence may be reconciled by the existence of different mechanisms of virus dissemination in the brain and in extraneural tissues.

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INTRODUCTION

Glycosaminoglycans (GAG) are complex, linear, sulfated polysaccharides found abundantly on cellular surfaces and in the extracellular matrix of most multicellular organisms (reviewed by Bernfield *et al.*, 1999; Esko & Selleck, 2002). They display an enormous structural diversity and have a critical role in a wide range of biological processes (see Bernfield *et al.*, 1999; Esko & Selleck, 2002; Handel *et al.*, 2005; Lindahl *et al.*, 1998; Perrimon & Bernfield, 2000; Sasisekharan *et al.*, 2002). Numerous viruses utilize GAG for attachment to host cells, although subsequent infection may require virus association with additional cellular receptors (reviewed by Rostand & Esko, 1997; Spillmann, 2001). Virus binding to GAG involves primarily the electrostatic interaction of clusters of basic amino acids on the virion surface with negatively charged sulfate groups on the

polysaccharide. The crystal structure of a foot-and-mouth disease virus–heparin complex (Fry *et al.*, 1999) and computer simulation of heparin docking to the adeno-associated virus capsid protein (Kern *et al.*, 2003) suggest that GAG binds along shallow depressions on the virions in which the GAG-binding determinants are situated, whilst a cryo-electron microscopy investigation on heparin association with a GAG-binding mutant of *Ross River virus* showed that binding was mediated by the distal end of the E2 spike proteins (Zhang *et al.*, 2005).

Virus affinity for GAG is a determinant of cell tropism, and the acquisition of additional positive charge at GAG-binding sites is a common adaptation of viruses grown in cell culture (reviewed by Spillmann, 2001). However, our studies on the encephalitic flaviviruses, *Murray Valley encephalitis virus* (MVE), *Japanese encephalitis virus* (JEV) and *West Nile virus* (WNV) (Lee *et al.*, 2004; Lee & Lobigs, 2002), and the work of others on *Foot-and-mouth disease virus* (Sa-Carvalho *et al.*, 1997), *Sindbis virus* (Byrnes &

Griffin, 2000), *Venezuelan equine encephalitis virus* (Bernard *et al.*, 2000) and *Classical swine fever virus* (Van Gennip *et al.*, 2004) have demonstrated that the increased infectivity in cell culture of host cell-adapted viruses is a disadvantage for virus growth *in vivo*, because the high GAG-binding affinity prevents efficient virus spread through the circulation. The loss of virulence due to high GAG-binding affinity raises the question as to whether, and to what extent, this mechanism also accounts for the virulence attenuation of live vaccines or vaccine candidates. In case of a live JEV vaccine widely used in China (strain SA14-14-2), high GAG-binding affinity contributes, at least in part, to its attenuated virulence phenotype (Lee & Lobigs, 2002).

The interaction of *Dengue virus* (DEN) with GAG was first shown by Chen *et al.* (1997) who also identified two putative GAG-binding regions, one spanning domains I and III and a second on domain III on the crystal structure of the viral envelope (E) protein (Modis *et al.*, 2003; Rey *et al.*, 1995). DEN is a mosquito-borne flavivirus causing a high incidence of human disease, ranging from fever to life-threatening haemorrhagic fever and shock, in tropical and subtropical countries (reviewed by Burke & Monath, 2001). Others have confirmed the putative involvement of cell surface GAG in attachment to and infection of host cells by DEN for each of the four serotypes (DEN-1–DEN-4) (Germi *et al.*, 2002; Hilgard & Stockert, 2000; Hung *et al.*, 1999, 2004; Lee *et al.*, 2006; Lin *et al.*, 2002; Thullier *et al.*, 2001) and the antiviral activity against DEN of heparan sulfate mimetics has been shown *in vitro* and *in vivo* (Lee *et al.*, 2006; Marks *et al.*, 2001; Ono *et al.*, 2003; Talarico *et al.*, 2005). The molecular GAG-binding determinants of DEN and the role of GAG-binding affinity in DEN virulence have, so far, not been defined and these topics have been investigated in this study.

METHODS

Cells and viruses. African green monkey kidney (Vero), baby hamster kidney (BHK-21), human adenocarcinoma (SW13) and *Aedes albopictus* (C6/36) mosquito cells were grown as described previously (Lee *et al.*, 2004, 2006). Working stocks of DEN-2 strains NGC, isolated in 1944, passaged 24 times in suckling mouse brain, four times in equine kidney (EK) cells and twice in C6/36 cells (Gruenberg *et al.*, 1988), and PUO-218, isolated from Thailand in 1988 from patient sera, passaged once in *Toxorhynchites splendens* mosquitoes, once in monkey kidney (LLC-MK) cells and twice in C6/36 cells, were C6/36 cell supernatants.

Serial passage of DEN-2 viruses in SW13 and BHK-21 cells. SW13 or BHK-21 cell monolayers in 60 mm dishes (1×10^6 and 5×10^5 cells, respectively) were infected with DEN-2 NGC or PUO-218 strains at an m.o.i. of 0.1 Vero p.f.u., and supernatants were collected and supplemented with HEPES (pH 8.0; 20 mM final concentration) at 5 days post-infection (p.i.). Four subsequent passages were performed for each passage series using 0.2 ml of the corresponding supernatants and harvesting culture medium following appearance of cytopathic effects (cpe) or at 5 days p.i. After five passages, plaque-purified stocks were prepared as described previously (Lee *et al.*, 2004) by amplification in BHK-21 cells.

Sequence analysis. Total cellular RNA from infected BHK-21 cells (for passage variants) or infected Vero cells (for parent viruses)

was extracted using Trizol (GibcoBRL) as described previously (Lee *et al.*, 2004). RT-PCR was performed as described previously (Lee & Lobigs, 2002) using random hexamers in the reverse transcription (RT) step and the DEN-2-specific primer pair, p2425: 5'-CAGCTC-ACAACGCAACCACT-3' and p400: 5'-GCAGGCATGATCATTATGCT-3', for PCR. PCR-amplified cDNA fragments were gel-purified and used in cycle sequencing with ABI Big-Dye v3 chemistry (Applied Biosys). Additional primers for sequencing of DEN-2 prM and E genes were P787: 5'-TGGAAACATGCCAGAGAAT-3', P1567: 5'-CAATGGTTCTAGACCTGCC-3' and P1669: 5'-ACAACATCCTGTTTCTTCGC-3'.

Plasmids. Plasmid pDVWS601 contains the full-length cDNA of the DEN-2 NGC genome flanked by a T7 RNA polymerase promoter at the 5' end and an *XbaI* restriction site at the 3' end (Gualano *et al.*, 1998). Viral RNA from parental virus and passaged variants of DEN-2 PUO-218 and NGC strains was reverse-transcribed as described above and cDNA encoding the E protein was amplified by PCR using primers P787 and P2863: 5'-TTCCAGCGAATTCCAA-GCTCT-3'. To introduce changes at E protein residue 227 or 249, a 1 kb region flanked by *SphI* and *NheI* sites in pDVWS601 was replaced with *SphI*–*NheI* fragments derived from RT-PCR-amplified cDNA of SW13 cell-passaged or BHK-21 cell-passaged NGC, respectively. To replace the DEN-2 strain NGC prM and E protein genes in pDVWS601 with those from PUO-218, two fragments from pDVWS601, a 1.5 kb fragment flanked by *NotI* (upstream of the T7 RNA polymerase promoter) and *HindIII* (nt 1547) sites and a 1.3 kb fragment flanked by *SphI* (nt 1380) and *PstI* (nt 2731) sites were subcloned into vector plasmids pACdel, in which the *BamHI*–*BanI* fragment from pACYC174 (New England Biolabs) had been replaced with the polylinkers from pBluescriptKS+ (Stratagene) or pGEM-5Zf (Promega), respectively, to generate plasmids pACprME5' and pGEME3'. Regions flanked by *BclI* (nt 407) and *SphI* (nt 1380) or by *SphI* and *SpeI* (nt 2370) in pACprME5' and pGEME3', respectively, were replaced with the corresponding cDNA fragments derived from PUO-218 viral RNA, or those derived from SW13 or BHK-21 cell-passaged PUO-218 to introduce the PUO-218 prM and E genes with or without coding changes to the E aa 120, 124 or 202 into the DEN-2 NGC genome. Full-length DEN-2 plasmids were generated by ligating three DNA fragments: a *NotI*–*SphI* fragment from pACprME5' derivatives, an *SphI*–*NheI* fragment from pGEME3' derivatives and a *NotI*–*NheI* fragment from the pDVWS601 plasmid. All plasmid constructs were verified by sequencing the entire prM and E protein genes.

RNA transcription and electroporation of BHK-21 cells.

Plasmids containing the full-length DEN-2 cDNAs were digested with *XbaI*, transcribed using T7 RNA polymerase and full-length RNA transcripts electroporated into BHK-21 cells as described previously (Lee & Lobigs, 2000) with the following modifications. For determination of electroporation efficiency, 200, 20 or 2 μ l of electroporated cells ($25 \text{ ml at } 4 \times 10^5 \text{ cells ml}^{-1}$) were plated onto Vero cell monolayers in six-well trays (3.5×10^5 cells per well; Nunc), incubated for 1 h and agar overlay medium (M199 medium plus 1% agar and 2% fetal calf serum) added. Neutral red (0.02%) was added at 4 days p.i. and plaques counted after overnight incubation.

Heparin–Sepharose binding and heparin inhibition assays.

Binding of DEN-2 viruses (10^5 – 10^6 p.f.u.) to heparin–Sepharose beads was assayed as described previously (Lee *et al.*, 2004). Inhibition by heparin of infectivity of DEN-2 viruses was examined by incubating virus (1 – 5×10^6 p.f.u.) with heparin (0.2, 2, 20 or $200 \mu\text{g ml}^{-1}$) for 15 min prior to its addition to BHK-21 cell monolayers (4×10^5 cells per well in six-well plates) treated with similar concentrations of heparin. Mock-treated controls were performed by preincubation of the same amount of virus in Hanks' balanced salts solution (HBSS)–BSA in the absence of heparin. Cells were harvested

by trypsinization at 21 h p.i. and the percentage of infected cells determined by flow-cytometry as described previously (Lee *et al.*, 2006).

Specific infectivity determination. Specific infectivity of DEN-2 virus particles was determined as described previously (Lee *et al.*, 2004) with the following modifications. Infectivity of virus, released from infected BHK-21 cells over a 2 h incubation period, on BHK-21, Vero and SW13 cells was determined by TCID₅₀ assay in 96-well trays using NS1 protein-specific mAb 4G4 for the detection of virus-infected cell monolayers by ELISA (Licon Luna *et al.*, 2002). Viral RNA in the infected culture supernatant (100 µl) was extracted for quantification of virion RNA content by competitive RT-PCR. The internal standard was a DEN-2 competitor RNA (DEN2-cRNA). This was generated by *in vitro* transcription using T7 RNA polymerase (Promega) of a recombinant pBKS+ (Invitrogen) plasmid containing the DEN-2 NGC genome from nt 400 to 2444 (Gruenberg *et al.*, 1988), but with an insertion of a ~100 bp DNA fragment from the polylinker region of pBluescriptKS+ at the *MunI* site in the E protein gene (nt 2092). Plasmid DNA linearized at the *ApaI* site at the 3' end of the DEN-2 insert was used as template for T7 RNA polymerase and a trace amount of [³H]UTP was added for determination of the yield of DEN-2-cRNA transcript (Sambrook *et al.*, 1989). RT of mixtures of viral RNA and serially diluted DEN-2-cRNA was performed using the antisense primer P2425, followed by PCR using primer P2425 and the sense primer P1567 as described previously (Lee *et al.*, 2004). PCR products were electrophoresed in 1.3% agarose gels. The concentrations of DEN2-cRNA spiked into PCR reactions in the presence of a constant amount of virion RNA at which two PCR product DNA bands of 878 and 978 bp of similar intensity were visible allowed estimation of the concentration of the virion RNA.

Mouse experiments. Six-week-old alpha and gamma interferon receptor double knockout (IFN- α/γ -R^{-/-}) mice (van den Broek *et al.*, 1995) were used for virulence assays. Virus inocula (100 µl), diluted in HBSS-BSA, were injected by the intraperitoneal (i.p.) or intravenous (i.v.) routes. For intracerebral (i.c.) inoculation, the virus inoculum (30 µl) was given to mice anaesthetized with

ketamine/xylazol using a 26G needle. Mortality and morbidity were monitored over a period of 28 days. Clearance of virus from the circulation was assayed as described previously (Lee *et al.*, 2004).

RESULTS

Adaptation of DEN-2 NGC and PUO-218 strains to grow in SW13 and BHK-21 cells

To generate host cell-adapted variants of DEN-2 with high affinity GAG-binding phenotypes and to identify the molecular determinants for high affinity GAG binding of DEN-2, two virus strains, the mouse-passaged neurovirulent prototype strain, NGC (Meiklejohn *et al.*, 1952), and the natural isolate, PUO-218, were serially passaged five times in SW13 or BHK-21 cells. The same approach was employed previously for the selection of GAG-binding variants of JEV serotype flaviviruses and *Tick-borne encephalitis virus* (TBE) (Goto *et al.*, 2003; Lee *et al.*, 2004; Lee & Lobigs, 2002; Lobigs *et al.*, 1990; Mandl *et al.*, 2001). Tissue culture supernatants were harvested after each passage between 4 and 6 days p.i., or when cpe was detectable. By the fifth passage, significant cpe was observed at 3 days p.i. in both BHK-21 and SW13 cells. Sequence analysis was performed on virion RNA extracts from uncloned as well as four plaque-purified virus stocks from each passage series. There was at least 1 nt change in the E protein gene of each passage variant, which resulted in a non-conservative amino acid change (Table 1). Notably, the E protein changes in each of the passaged variants resulted in the net gain of positive charge consistent with enhanced GAG binding. The mutations appeared to be dominant in the fifth passage virus stocks, as sequence analysis of uncloned variant stocks did not show the parental sequence at the corresponding positions in the virus populations. BHK-21 cell-passaged PUO-218 comprised

Table 1. Host cell-adapted DEN-2 variants, genetic changes in E protein and heparin–Sepharose affinity

DEN-2 virus strain	Host cell selection	Nucleotide change*	Amino acid change†	Input virus bound to heparin (%)‡
NGC parent	–	–	–	96
NGC ^{R227}	SW13	A ⁶⁸⁰ →G	Gln ²²⁷ →Arg	99
NGC ^{N249}	BHK-21	G ⁷⁴⁵ →A	Asp ²⁴⁹ →Asn	98
PUO-218 parent	–	–	–	0
PUO ^{K202}	SW13	G ⁶⁰⁴ →A	Glu ²⁰² →Lys	100
PUO ^{D124/K202}	BHK-21	A ³⁷⁰ →G	Asn ¹²⁴ →Asp	100
		G ⁶⁰⁴ →A	Glu ²⁰² →Lys	
PUO ^{K120}	BHK-21	C ³⁵⁹ →A	Thr ¹²⁰ →Lys	98

*Nucleotide changes in plaque-purified fifth passage virus are numbered from the 5' terminal nucleotide in the E protein gene (Gruenberg *et al.*, 1988).

†Amino acid changes are numbered from the N-terminal residue in the E protein.

‡Heparin–Sepharose-binding affinity was determined by the formula: [1–free virus (p.f.u. ml⁻¹) after incubation with heparin–Sepharose beads/virus input (p.f.u. ml⁻¹)] × 100. Results given are the mean of two determinations. Less than 10% of each virus was removed by protein A–Sepharose used as control in experiments performed in parallel with heparin–Sepharose-binding assays.

two genotypes: PUO^{K120}, which represented the majority population on the basis of sequenced uncloned virus and PUO^{D124/K202}, which showed a minor presence.

One of the key phenotypic changes associated with the adaptation of mosquito- and tick-borne flaviviruses to grow in SW13 and/or BHK-21 cells is the enhanced affinity of the passage variants for cell surface GAG relative to parental viruses (Goto *et al.*, 2003; Lee *et al.*, 2004; Lee & Lobigs, 2000, 2002; Mandl *et al.*, 2001). To investigate further the generality of altered virus–host receptor interaction(s) among the flaviviruses due to adaptation to the two cell lines, virus binding to heparin–Sepharose was measured. Heparin is a highly sulfated reference GAG. The PUO-218 strain did not show detectable binding to heparin–Sepharose beads, while SW13 and BHK-21 cell-passaged PUO-218 variants showed greater than 97% binding (Table 1). Interestingly, the parental DEN-2 NGC strain bound efficiently to heparin–Sepharose. Nevertheless, the binding of NGC^{R227} and NGC^{N249} was slightly increased relative to the parent (Table 1).

Heparin-binding phenotypes of DEN-2 NGC and NGC/PUO-218 prM–E chimeric infectious cDNA clone-derived viruses with or without host cell adaptive mutations

To test the impact of single amino acid changes at PUO-218 strain E protein residues 120, 124 and 202 on heparin-binding affinity, the prM and E protein coding regions of PUO-218 with or without these host cell adaptive mutations were inserted into the full-length infectious cDNA clone of NGC (Gualano *et al.*, 1998); the NGC/PUO-218 prM–E chimeric viruses are denoted rPUO, rPUO^{K120}, rPUO^{D124} and rPUO^{K202}. Virus rPUO closely resembled the PUO-218 strain in the lack of detectable binding to heparin–Sepharose (Fig. 1). Viruses rPUO^{K120} and rPUO^{K202}, which differ from rPUO by the gain of a single positive charge in the E protein, showed >99% binding to heparin–Sepharose. In contrast, the Asn¹²⁴→Asp substitution in the E protein of rPUO^{E124} did not increase binding to heparin–Sepharose relative to that of rPUO. This mutation was selected in addition to the Glu²⁰²→Lys change during passage of PUO-218 in BHK-21 cells.

Given the strong binding to heparin–Sepharose of NGC relative to the PUO-218 virus and the fact that the two strains show only one charge difference in the E protein at residue 126 (Lys/Glu) amongst a total of 8 aa differences in prM and E, it appeared likely that Lys at residue 126 accounted for the high GAG-binding affinity of NGC. To confirm this proposition, recombinant viruses rPUO^{K126} and rNGC^{E126} were generated. Virus rPUO^{K126} showed efficient binding to heparin–Sepharose (>95%) comparable to that of rNGC, while the binding of rNGC^{E126} was markedly reduced (~15%) relative to rNGC (Fig. 1). Accordingly, Lys at E protein residue 126 is also a determinant of GAG affinity.

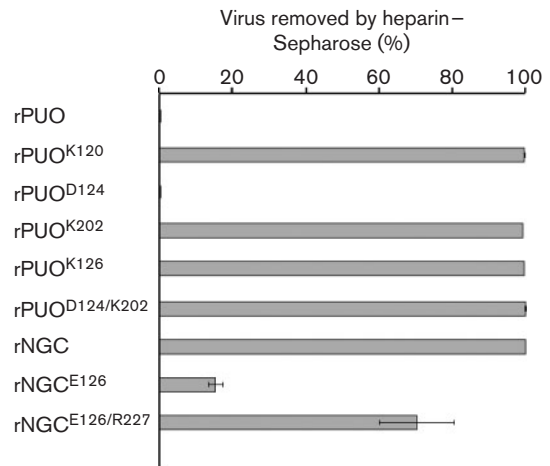


Fig. 1. Heparin–Sepharose-binding assays performed on infectious cDNA clone-derived DEN-2 viruses. Virus removed by heparin–Sepharose (grey bar) was calculated by the formula: $(1 - \text{titre of unbound virus in heparin–Sepharose mixture} / \text{titre of virus in HBSS-BSA}) \times 100\%$. Error bars represent standard error from two independent experiments. Less than 10% of each virus was removed by protein A–Sepharose in experiments performed in parallel with heparin–Sepharose-binding assays.

The already high GAG-binding affinity of NGC may obscure detection of further enhancement of GAG binding by other determinants. To corroborate that Arg at E protein residue 227, which was selected during passage of NGC in SW13 cells, is a major GAG-binding determinant, binding of rNGC^{E126} and rNGC^{E126/R227} to heparin–Sepharose was compared. The presence of a positive charge at residue 227 in variant rNGC^{E126/R227} significantly improved binding relative to rNGC^{E126} (Fig. 1).

Effect of GAG-binding determinants on susceptibility of virus infectivity to heparin

We next investigated whether the DEN-2 GAG-binding determinants influenced dependence on cell surface GAG during virus entry. For this assay, only DEN-2 NGC and NGC/PUO-218 prM–E chimeric infectious clone-derived viruses were used. Virus and BHK-21 cells were pre-incubated with heparin at given concentrations followed by infection and quantification of infected cells by flow-cytometry (Lee *et al.*, 2006). The infectivity of rNGC was reduced by more than 50% at heparin concentrations of $\geq 2 \mu\text{g ml}^{-1}$ (Fig. 2a), consistent with the previously determined 50% effective concentration value of heparin for DEN-2 NGC using this assay (Lee *et al.*, 2006). The presence of an additional positive charge in the E protein of rNGC^{R227} gave rise to a marked increase in heparin susceptibility, while the Asp²⁴⁹→Asn change in rNGC^{N249} consistently resulted in 10% greater inhibition compared

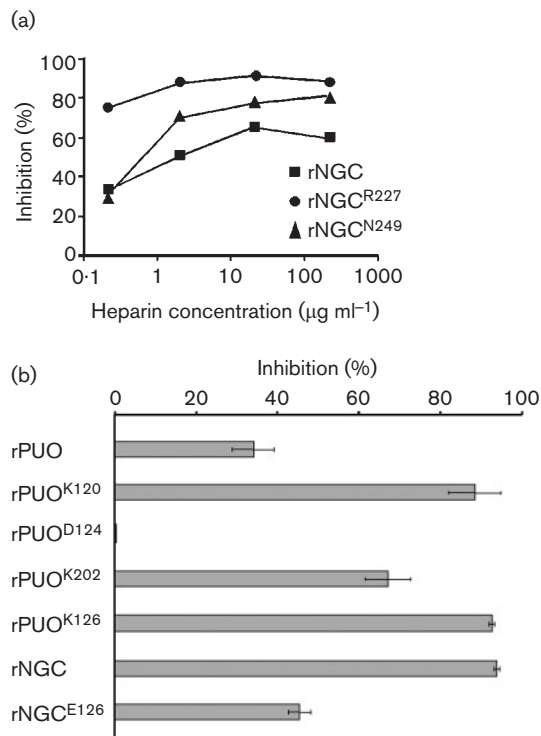


Fig. 2. Inhibition of BHK-21 infectivity by heparin. (a) Inhibition of DEN-2 viruses rNGC (solid rectangle), rNGC^{R227} (solid circle) and rNGC^{N249} (solid triangle) by heparin at 0.2–200 µg ml⁻¹ concentration. Results shown are representative of two independent experiments performed. (b) Inhibition of DEN-2 viruses by heparin at 200 µg ml⁻¹ (error bars represent standard error from two independent experiments).

with rNGC at heparin concentrations between 2 and 200 µg ml⁻¹ (Fig. 2a).

The effect of host cell adaptive mutations in the E protein of rPUO on the susceptibility of virus infectivity to heparin was tested at a concentration of 200 µg ml⁻¹ since there was negligible inhibition at 20 and 0.2 µg heparin ml⁻¹ (data not shown). Virus rPUO showed 35% inhibition of infectivity, whilst that of rPUO^{K120} and rPUO^{K202} was inhibited to a much greater extent (88 and 67%, respectively; Fig. 2b). Notably, rPUO^{D124} displayed reduced susceptibility to heparin inhibition relative to rPUO (Fig. 2b), consistent with its lack of binding to heparin–Sepharose. Thus, the increase in negative charge on the viral surface of this variant appears to reduce further the relatively low GAG-binding affinity of PUO-218.

To investigate the role in attachment/entry of E protein residue 126, the susceptibility of infectivity to heparin inhibition of rNGC, rNGC^{E126}, rPUO and rPUO^{K126} was compared. The results show that the rNGC^{E126} and rPUO, and rPUO^{K126} and rNGC pairs are comparable in regard to their moderate and high susceptibility to heparin inhibition,

respectively (Fig. 2b), and confirm the importance of residue 126 as a GAG-binding determinant.

Influence of GAG-binding determinants on specific infectivity of DEN-2 for BHK-21, SW13 and Vero cells

The impact of E protein substitutions at GAG-binding sites on specific infectivity of DEN-2 for BHK-21, SW13 and Vero cells was examined using infectious clone-derived viruses collected over a 2 h period at 28 h p.i. The virus harvests were used for infectivity titration in Vero, BHK-21 and SW13 cells by TCID₅₀ assay and RNA quantification by RT-PCR (Lee *et al.*, 2004), and specific infectivity was determined as genome equivalent/TCID₅₀ ratio. Gain-of-positive-charge mutations at E protein residues 120, 202, 227 and 249 increased infectivity for BHK-21 and SW13 cells by 10- to 170-fold, whereas that for Vero cells was comparable or slightly poorer (<10-fold) relative to the parental strains (Table 2). The Asn¹²⁴→Asp change, which contributed little to heparin binding (see above), resulted in a moderate enhancement (<10-fold) of specific infectivity for BHK-21 and SW13 cells, without markedly influencing the specific infectivity for Vero cells (Table 2).

The contribution of the GAG-binding determinant Lys¹²⁶ in the E protein of NGC to its significantly higher infectivity for BHK-21 cells relative to rPUO was examined. The specific infectivity of rPUO^{K126} for BHK-21 cells resembled that of rNGC, while that of rPUO and rNGC^{E126} was similar (Table 2), suggesting that Lys¹²⁶ was mainly responsible for the high infectivity of NGC for BHK-21 cells. Notably, a similar effect of residue 126 on the specific infectivity for SW13 cells (compare rNGC and rPUO; Table 2) was not observed.

Mouse virulence of DEN-2 NGC and host cell-adapted variants

Natural isolates of DEN do not cause mortality or overt morbidity in adult immunocompetent mice by extraneural

Table 2. Specific infectivity of DEN-2 variants

ND, Not determined.

Virus	Specific infectivity (genome equivalent/TCID ₅₀)		
	Vero	BHK-21	SW13
rNGC	1.9 × 10 ²	5.0 × 10 ²	1.1 × 10 ⁵
rNGC ^{R227}	1.4 × 10 ²	1.4 × 10 ²	2.0 × 10 ³
rNGC ^{N249}	1.2 × 10 ²	8.2 × 10 ¹	9.8 × 10 ³
rNGC ^{E126}	8.0 × 10 ²	5.5 × 10 ⁴	ND
rPUO	9.4 × 10 ¹	2.5 × 10 ⁴	9.0 × 10 ⁵
rPUO ^{K120}	7.9 × 10 ²	7.9 × 10 ²	5.8 × 10 ³
rPUO ^{D124}	2.1 × 10 ²	3.8 × 10 ³	1.8 × 10 ⁵
rPUO ^{K202}	6.9 × 10 ²	5.0 × 10 ²	5.3 × 10 ³
rPUO ^{K126}	8.0 × 10 ²	8.1 × 10 ²	ND

routes of inoculation. However, mice deficient in types I and II interferon responses (IFN- α/γ -R^{-/-} mice) provide a suitable mouse model for assessment of virulence properties of DEN, given that they are susceptible to DEN-2 NGC infection by the i.p. route (Johnson & Roehrig, 1999), as well as to natural DEN isolates when inoculated at a high dose i.v. route (Shrestha *et al.*, 2004). To compare mouse virulence of NGC parent and passage variants, groups of 6-week-old IFN- α/γ -R^{-/-} mice were inoculated via the i.c. or i.p. route with 10³ and 10⁶ p.f.u., respectively. While the parental NGC virus caused 100% mortality via both routes, NGC^{R227} was attenuated with no mortality observed by i.p. inoculation and significantly prolonged mean survival time (mst) relative to the parent following i.c. inoculation (Table 3a). NGC^{N249} was partially attenuated by the i.p. route, showing reduced mortality and prolonged mst but was equally virulent by the i.c. infection route in comparison to the parental NGC virus (Table 3a). All surviving mice were protected against i.p. challenge with 10⁶ p.f.u. NGC given 4 weeks after primary infection, whilst challenged naïve IFN- α/γ -R^{-/-} mice at 10 weeks of age showed 100% mortality (mst ~ 18 days).

Mouse virulence of NGC and NGC-PUO-218 chimeric infectious clone-derived parent and GAG-binding variants

The impact of GAG-binding determinants on DEN virulence in IFN- α/γ -R^{-/-} mice was further examined using NGC and NGC/PUO-218 prM-E chimeric infectious clone-derived viruses (Table 3b). Mice infected i.v. with 10⁶ p.f.u. of rPUO showed 100% mortality (mst = 12.6 days); rPUO^{D124}, which showed reduced susceptibility to heparin inhibition, was also uniformly lethal but slightly

more virulent than rPUO according to the shorter mst. All GAG-binding variants except rPUO^{K202} were significantly attenuated relative to rPUO. Unexpectedly, the GAG-binding variant rPUO^{K202} gave 100% mortality; however, with a significantly prolonged mst relative to rPUO. To test whether the apparent lack of virulence attenuation of rPUO^{K202} was the result of phenotypic instability, sequence analysis was performed on viral RNA derived from two individual rPUO^{K202}-infected mouse brains. This showed complete reversion of Lys²⁰²→Glu and implies that the second site mutation (Asp¹²⁴) in the E protein of rPUO^{D124/K202} stabilizes the GAG-binding and virulence attenuation phenotypes produced by Lys²⁰².

Interestingly, rNGC was less virulent than rPUO when inoculated by the i.v. route in IFN- α/γ -R^{-/-} mice (Table 3b). This was surprising, given that the PUO-218 parental strain was not lethal for these mice when inoculated i.p. (10⁶ p.f.u.) and only gave 20% mortality when the same dose was inoculated i.v. (data not shown). Thus, it appears that the prM and E proteins of PUO-218 in combination with additional regions in the NGC polyprotein and/or non-coding regions of the NGC genome enhance neuro-invasiveness relative to the PUO-218 and NGC strains. Furthermore, we showed by i.c. inoculation of 10³ p.f.u. that the neurovirulence of rNGC and rPUO was comparable in IFN- α/γ -R^{-/-} mice: both strains gave 100% mortality with mst of 8.0 and 8.8 days, respectively (Table 3b). The conflicting virulence profiles of rNGC and rPUO in IFN- α/γ -R^{-/-} mice may be attributed to their differential capacity to spread in extraneural tissues and, in turn, be a consequence of the differential GAG-binding properties of the two viruses. This hypothesis is further supported by virulence data for the rPUO^{K126} and rNGC^{E126} pair: mortality

Table 3. Virulence in IFN- α/γ -R^{-/-} mice

Virus	Mortality (%; <i>P</i> value*)	Mst (days) ± SD (<i>P</i> value*)	Mortality (%; <i>P</i> value)	Mst (days) ± SD (<i>P</i> value)
(a)		i.p. (10⁶ p.f.u.)		i.c. (10³ p.f.u.)
NGC	5/5 (100%)	13.2 ± 0.4	5/5 (100)	8.4 ± 0.5
NGC ^{R227}	0/5 (0%; 0.008)	–	5/5 (100)	10.6 ± 1.5 (0.016)
NGC ^{N249}	2/5 (40%; 0.16)	23.5 ± 4.5 (0.05)	5/5 (100)	8.0 ± 1.0 (0.53)
(b)		i.v. (10⁶ p.f.u.)		i.c. (10³ p.f.u.)
rPUO	8/8 (100%)	12.6 ± 0.7	5/5 (100)	8.8 ± 1.0
rPUO ^{K120}	2/7 (29%; 0.007)	26.0 ± 0 (0.04)	NT	NT
rPUO ^{D124}	8/8 (100%; 1.0)	9.3 ± 3.6 (0.08)	NT	NT
rPUO ^{D124/K202}	1/6 (17%; 0.003)	22 ± 0	NT	NT
rPUO ^{K126}	3/8 (38%; 0.026)	22.7 ± 6.8 (0.026)	NT	NT
rPUO ^{K202}	5/5 (100%; 1.0)	17.6 ± 3.6 (0.002)	NT	NT
rNGC	6/7 (86%; 0.47)	24.5 ± 2.3 (0.002)	5/5 (100)	8.0 ± 0 (0.30)
rNGC ^{E126}	9/9 (100%; 1.0)	15.3 ± 3.0 (0.01)	5/5 (100)	8.4 ± 1.8 (0.84)
PUO-218	1/5 (20)	20 ± 0	NT	NT

NT, Not tested.

*Statistical analysis was performed in pairwise comparison relative to NGC strain in (a) and rPUO strain in (b) on mst values using the Mann-Whitney U test and on mortality values using the Fisher's exact test; differences considered significant (*P* ≤ 0.05) are in bold.

for the strongly GAG-binding virus, rPUO^{K126}, following i.v. infection was significantly reduced relative to rPUO, whilst it was slightly increased for rNGC^{E126}, which binds poorly to GAG, relative to rNGC. Notably, groups of mice infected with rNGC^{E126} showed significantly reduced mst relative to rNGC (15.3 versus 24.5 days; $P=0.0004$).

In addition to being a determinant of GAG-affinity and neuroinvasiveness, residue 126 also influences mouse neurovirulence (Gualano *et al.*, 1998), since new-born BALB/c mice inoculated i.c. with the 10^3 p.f.u. of rNGC or rNGC^{E126} showed mortality of 100 and 80 % and significantly different mst of 7.4 and 10.4 days, respectively ($P=0.008$), while mice similarly infected with rPUO or rPUO^{K126} showed mortality of 0 and 100 % (mst of 8 days) (our unpublished results, which confirm those of Gualano *et al.*, 1998). The neurovirulence phenotype mediated by the presence of a positive charge at residue 126 is unlikely to be due to increased GAG affinity because other GAG-binding variants (rPUO^{K120} and rPUO^{D124/K202}) did not show greater mouse neurovirulence than rPUO when tested similarly (data not shown).

Rapid clearance of DEN-2 GAG-binding variants from the circulation

Our mouse virulence data showed a remarkably consistent correlation between GAG-binding affinity of DEN-2 viruses and virulence attenuation in IFN- α/γ -R^{-/-} mice following inoculation by an extraneural route. This raised the possibility that virulence attenuation resulted from the rapid clearance of GAG-binding variants from the circulation by non-productive binding to surfaces enriched in GAG, as has been demonstrated for JEV serogroup flaviviruses (Lee *et al.*, 2004; Lee & Lobigs, 2002). To test this possibility, virus titres in serum samples collected over a 30 min period from mice inoculated, i.v., with a high dose of rPUO or rPUO^{K120} was determined. The rPUO virus titres did not show significant reduction over 30 min, while rPUO^{K120} showed ≥ 2 log reduction in serum titres over the same period (Fig. 3).

Predicted location of GAG-binding determinants on the DEN-2 E protein structure

The GAG-binding determinants, 120, 126, 202, 227 and 249, are located at sites that are highly conserved between DEN-2 isolates. When plotted on an alignment of E protein sequences from 33 DEN-2 isolates (Lewis *et al.*, 1993) representing five subtypes and isolated between 1944 and 1990, only substitutions that gave rise to an increase in positive charge were found in a small number of isolates at these residues, with most isolates displaying the low GAG-binding determinant. When mapped onto the crystal structure of DEN-2 E protein (Modis *et al.*, 2003), residues 120, 126, 202, 227 and 249, without exception, cluster in the central region of the E protein dimer and are exposed on the upper face of domain II (Fig. 4).

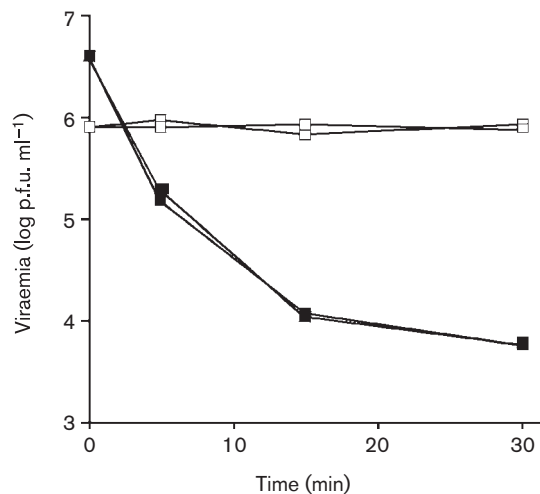


Fig. 3. Clearance of DEN-2 variant from the circulation. Virus titres in the serum over 30 min after i.v. inoculation of 8×10^5 p.f.u. DEN-2 rPUO (open symbols) or 3.6×10^6 p.f.u. rPUO^{K120} (solid symbols) are shown. Results from two mice are shown for each virus. Serum titre at 0 min is calculated as input virus/blood volume, assumed to be 15% of body weight.

DISCUSSION

This study describes five GAG-binding determinants on the E protein of DEN-2; four of these (residues 120, 202, 227 and 249) were identified as host cell adaptive mutations involving the acquisition of increased net positive charge on the viral surface, while one (residue 126) was found to be present in the mouse brain-adapted NGC strain, which is mouse neurovirulent and an early DEN-2 vaccine candidate along with other mouse brain-adapted DEN viruses (Meiklejohn *et al.*, 1952; Sabin, 1952, 1955; Sabin & Schlesinger, 1945; Schlesinger *et al.*, 1956). Consistent with recent studies by us and others on the encephalitic flaviviruses MVE, JEV, WNV and TBE, GAG-binding variants selected by serial passage of DEN-2 in BHK-21 and/or SW13 cells displayed reduced mouse virulence by peripheral infection routes (Goto *et al.*, 2003; Lee *et al.*, 2004; Lee & Lobigs, 2000, 2002; Mandl *et al.*, 2001). Accordingly, flaviviruses appear to universally engage with high affinity to cell surface GAG for efficient attachment/entry of some cell types, an adaptation that is detrimental for virus spread in the animal host.

The altered host cell tropism of DEN-2 GAG-binding variants was confirmed by specific infectivity determinations: gain-of-positive-charge mutations at E protein residues 120, 202, 227 and 249 significantly increased infectivity for BHK-21 and SW13 cells, whereas that for Vero cells was comparable or slightly poorer relative to the parental strains. Thus, distinct cell surface receptors appear to be involved in attachment/entry of DEN-2 during infection of Vero, BHK-21 and SW13 cells; the contribution of virus binding to GAG seems to be minimal for the

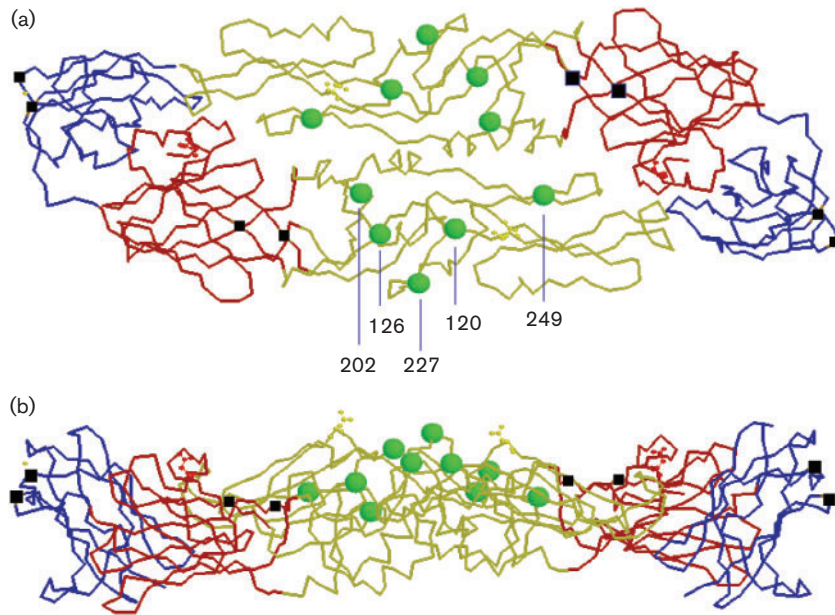


Fig. 4. Location of E protein changes in BHK-21 and SW13 cell-adapted DEN-2 variants. E residues 120, 124, 126, 227, 202 and 249 (circles) are plotted on the E protein ectodomain dimers (Modis *et al.*, 2003). Domains I, II and III are shown in red, yellow and blue, respectively. GAG-binding determinants mapped for MVE, JEV and WNV (Lee *et al.*, 2004; Lee & Lobigs, 2002) (squares) are shown. (a) Top view. (b) Side view.

efficient infection of Vero cells but is beneficial for that of BHK-21 and SW13 cells. The GAG-binding determinant at E protein residue 126 differed from the others in that a Lys at this residue markedly enhanced the specific infectivity for BHK-21 but not for SW13 cells. This may underscore the influence of the large structural diversity of cell surface GAG (Bernfield *et al.*, 1999) on the specificity of virus–host cell interaction. Given that specific quantitative and qualitative changes in expression of cell surface GAG are a hallmark of metastatic carcinoma cells (Sasisekharan *et al.*, 2002), it is possible that the marked change in cell tropism of DEN-2 and other flaviviruses selected for growth in SW13 cells (adrenal gland small cell carcinoma) relates to a characteristic cell surface GAG phenotype associated with malignant transformation. This raises the question whether the selective incorporation of GAG-binding determinants can improve the effectiveness of oncolytic viruses for clinical application.

The location of DEN-2 GAG-binding determinants on the E protein crystal structure was of interest, in particular in comparison to that of other flaviviruses belonging to different serocomplexes. Remarkably, residues 120, 126, 202, 227 and 249 are exclusively clustered on the exposed surface of domain II in the centre of the E protein dimer (Modis *et al.*, 2003, 2005). This contrasts with the location of key GAG-binding determinants of the JEV serotype flaviviruses in two clusters on domain III (residues 306 and 389/390) and domain I (residues 59 and 138) (Lee *et al.*, 2004; Lee & Lobigs, 2000, 2002), whilst adaptation of TBE to BHK-21 cells selected for GAG-binding variants altered at residues distributed throughout the upper surface across all three structural domains (Mandl *et al.*, 2001). It was surprising that none of the DEN-2 GAG-binding determinants were localized to E protein domain III, given that this part of the protein is thought to function in receptor binding (Lobigs *et al.*, 1990; Rey *et al.*, 1995) and that the

attachment of a soluble DEN-2 domain III to BHK-21 cells could be blocked by heparin (Hung *et al.*, 2004). Comparative analysis of the crystal structures of DEN-2 and -3 E protein ectodomains identified two clusters of positively charged residues predicted to function in GAG binding, one in domain I and a second in domain II, but could not confirm the existence of GAG-binding residues in domain III (Modis *et al.*, 2005). The GAG-binding determinants identified here cluster with the putative GAG-binding residues in domain II (Modis *et al.*, 2005), suggesting that the host cell adaptive changes enhance affinity of an existing GAG-binding site in the DEN E protein. Collectively, the spatial distribution of GAG-binding residues on the E protein from the different flaviviruses implies that multiple regions on the E proteins surface can engage with host cell receptors for virus attachment/entry, which is not inconsistent with the flat, elongated architecture of the E protein dimer.

We and others have reported that among the encephalitic flaviviruses an increase in GAG-binding affinity invariably gives rise to virulence attenuation (Goto *et al.*, 2003; Lee *et al.*, 2004; Lee & Lobigs, 2000, 2002; Mandl *et al.*, 2001) by a mechanism of rapid clearance of virus from the circulation (Lee *et al.*, 2004; Lee & Lobigs, 2002). Here, we show that this principle also applies to DEN encephalitis in mice. Although human DEN infection gives rise primarily to a febrile illness with arthralgia and occasional haemorrhagic manifestation, which cannot be reproduced in laboratory animals, the IFN- α/γ -R^{-/-} mouse model used in this study is pertinent to the understanding of DEN virulence in primates, where a reduction in viraemia titre and duration is considered the best marker of attenuation of DEN vaccine candidates (Saluzzo, 2003). In our mouse model, mortality as the result of encephalitis following virus inoculation by an extraneural route was used as virulence read-out and largely reflects the ability of a virus strain to produce viraemia of sufficient

magnitude and/or duration to cross the blood–brain barrier. A defect in the interferon responses was required for this to occur in adult mice. Therefore, the conclusions drawn here from studies in mice on the attenuation of DEN-2 GAG-binding variants most likely also apply to their pathogenicity in humans. Notably, DEN-2 vaccine candidates NGC and PR-159-S1 (Eckels *et al.*, 1976, 1980; Meiklejohn *et al.*, 1952; Sabin, 1955; Scott *et al.*, 1980) produced by serial passage in mouse brain and primary green monkey kidney cells display acidic to basic amino acid substitutions at GAG-binding determinants 126 (Gualano *et al.*, 1998) and 202 (Hahn *et al.*, 1988), respectively. We hypothesize that the mechanism of attenuation of these candidate DEN vaccines involves a defect in the ability of the viruses to spread in the infected host via the reticuloendothelial system due to high affinity GAG interaction.

Repeated mouse brain passage of DEN and *Yellow fever virus* (YFV), which are not neurotropic in natural infections, yields variants that are attenuated in viscerotropism in humans and monkeys but have acquired high neurovirulence in mice (reviewed by Burke & Monath, 2001). For DEN-2 NGC, a Glu→Lys mutation at E protein residue 126 was implicated as a major determinant of mouse neurovirulence (Bray *et al.*, 1998; Gualano *et al.*, 1998). Notably, a YFV–DEN-2 chimera with the prM–E of PUO-218 is far less neurovirulent and replicates poorly in the brain relative to the YFV 17D vaccine and a YFV–DEN-4 chimera with the prM–E of a neurovirulent DEN-4 strain (Chambers *et al.*, 2003). Taken together with our investigation showing that Lys¹²⁶ is a GAG-binding determinant which attenuates neuroinvasiveness, it is apparent that a single mutation can reduce disease in extraneural tissues (viscerotropism) as well as increase neurovirulence.

Given that cell-to-cell spread in neurons is a route for virus dissemination in the central nervous system, it is not unexpected that the influence of GAG affinity on virulence can differ in virus infections of the brain and in extraneural tissues. Indeed, the greater neurovirulence associated with DEN-2 NGC as a consequence of Lys¹²⁶ relative to variants with low GAG-binding affinity suggests that the efficiency of interaction between the virus and GAG on the surface of neuronal cells contributes to virus pathogenicity in the brain. A correlation between high GAG-binding affinity of strains of *Murine leukemia virus* (Jinno-Oue *et al.*, 2001), Theiler's murine encephalomyelitis virus (Reddi *et al.*, 2004) and high neurovirulence has also been reported. With regard to the neurotropism of DEN-2 NGC in comparison to that of non-brain-adapted strains (e.g. PUO-218), we find that the prM and E proteins of the latter (when incorporated into the NGC infectious clone; rPUO) confer comparable neurovirulence in IFN- α/γ -R^{-/-} mice. However, this is not the case in i.c. infection of new-born mice where rPUO is attenuated but NGC and rPUO^{K126} are both virulent (Gualano *et al.*, 1998). This raises the possibility that Lys¹²⁶ in the E protein of NGC reduces the susceptibility of virus growth to interferons.

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REFERENCES

- Bernard, K. A., Klimstra, W. B. & Johnston, R. E. (2000). Mutations in the E2 glycoprotein of Venezuelan equine encephalitis virus confer heparan sulfate interaction, low morbidity, and rapid clearance from blood of mice. *Virology* **276**, 93–103.
- Bernfield, M., Götte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincecum, J. & Zako, M. (1999). Functions of cell surface heparan sulfate proteoglycans. *Annu Rev Biochem* **68**, 729–777.
- Bray, M., Men, R., Tokimatsu, I. & Lai, C. J. (1998). Genetic determinants responsible for acquisition of dengue type 2 virus mouse neurovirulence. *J Virol* **72**, 1647–1651.
- Burke, D. S. & Monath, T. P. (2001). Flaviviruses. In *Fields Virology*, 4th edn, pp. 1043–1126. Edited by D. M. Knipe & P. M. Howley. Philadelphia, PA: Lippincott Williams & Wilkins.
- Byrnes, A. P. & Griffin, D. E. (2000). Large-plaque mutants of Sindbis virus show reduced binding to heparan sulfate, heightened viremia, and slower clearance from the circulation. *J Virol* **74**, 644–651.
- Chambers, T. J., Liang, Y., Droll, D. A., Schlesinger, J. J., Davidson, A. D., Wright, P. J. & Jiang, X. (2003). Yellow fever virus/dengue-2 virus and yellow fever virus/dengue-4 virus chimeras: biological characterization, immunogenicity, and protection against dengue encephalitis in the mouse model. *J Virol* **77**, 3655–3668.
- Chen, Y., Maguire, T., Hileman, R. E., Fromm, J. R., Esko, J. D., Linhardt, R. J. & Marks, R. M. (1997). Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. *Nat Med* **3**, 866–871.
- Eckels, K. H., Brandt, W. E., Harrison, V. R., McCown, J. M. & Russell, P. K. (1976). Isolation of a temperature-sensitive dengue-2 virus under conditions suitable for vaccine development. *Infect Immun* **14**, 1221–1227.
- Eckels, K. H., Harrison, V. R., Summers, P. L. & Russell, P. K. (1980). Dengue-2 vaccine: preparation from a small-plaque virus clone. *Infect Immun* **27**, 175–180.
- Esko, J. D. & Selleck, S. B. (2002). Order out of chaos: assembly of ligand binding sites in heparan sulfate. *Annu Rev Biochem* **71**, 435–471.
- Fry, E. E., Lea, S. M., Jackson, T. & 7 other authors (1999). The structure and function of a foot-and-mouth disease virus-oligosaccharide receptor complex. *EMBO J* **18**, 543–554.
- Germi, R., Crance, J. M., Garin, D., Guimet, J., Lortat-Jacob, H., Ruigrok, R. W., Zarski, J. P. & Drouet, E. (2002). Heparan sulfate-mediated binding of infectious dengue virus type 2 and yellow fever virus. *Virology* **292**, 162–168.
- Goto, A., Hayasaka, D., Yoshii, K., Mizutani, T., Kariwa, H. & Takashima, I. (2003). A BHK-21 cell culture-adapted tick-borne encephalitis virus mutant is attenuated for neuroinvasiveness. *Vaccine* **21**, 4043–4051.
- Gruenberg, A., Woo, W. S., Biedrzycka, A. & Wright, P. J. (1988). Partial nucleotide sequence and deduced amino acid sequence of the structural proteins of dengue virus type 2, New Guinea C and PUO-218 strains. *J Gen Virol* **69**, 1391–1398.
- Gualano, R. C., Pryor, M. J., Cauchi, M. R., Wright, P. J. & Davidson, A. D. (1998). Identification of a major determinant of

- mouse neurovirulence of dengue virus type 2 using stably cloned genomic-length cDNA. *J Gen Virol* **79**, 437–446.
- Hahn, Y. S., Galler, R., Hunkapiller, T., Dalrymple, J. M., Strauss, J. H. & Strauss, E. G. (1988). Nucleotide sequence of dengue 2 RNA and comparison of the encoded proteins with those of other flaviviruses. *Virology* **162**, 167–180.
- Handel, T. M., Johnson, Z., Crown, S. E., Lau, E. K. & Proudfoot, A. E. (2005). Regulation of protein function by glycosaminoglycans – as exemplified by chemokines. *Annu Rev Biochem* **74**, 385–410.
- Hilgard, P. & Stockert, R. (2000). Heparan sulfate proteoglycans initiate dengue virus infection of hepatocytes. *Hepatology* **32**, 1069–1077.
- Hung, S. L., Lee, P. L., Chen, H. W., Chen, L. K., Kao, C. L. & King, C. C. (1999). Analysis of the steps involved in Dengue virus entry into host cells. *Virology* **257**, 156–167.
- Hung, J. J., Hsieh, M. T., Young, M. J., Kao, C. L., King, C. C. & Chang, W. (2004). An external loop region of domain III of dengue virus type 2 envelope protein is involved in serotype-specific binding to mosquito but not mammalian cells. *J Virol* **78**, 378–388.
- Jinno-Oue, A., Oue, M. & Ruscetti, S. K. (2001). A unique heparin-binding domain in the envelope protein of the neuropathogenic PVC-211 murine leukemia virus may contribute to its brain capillary endothelial cell tropism. *J Virol* **75**, 12439–12445.
- Johnson, A. J. & Roehrig, J. T. (1999). New mouse model for dengue virus vaccine testing. *J Virol* **73**, 783–786.
- Kern, A., Schmidt, K., Leder, C., Muller, O. J., Wobus, C. E., Bettinger, K., Von der Lieth, C. W., King, J. A. & Kleinschmidt, J. A. (2003). Identification of a heparin-binding motif on adeno-associated virus type 2 capsids. *J Virol* **77**, 11072–11081.
- Lee, E. & Lobigs, M. (2000). Substitutions at the putative receptor-binding site of an encephalitic flavivirus alter virulence and host cell tropism and reveal a role for glycosaminoglycans in entry. *J Virol* **74**, 8867–8875.
- Lee, E. & Lobigs, M. (2002). Mechanism of virulence attenuation of glycosaminoglycan-binding variants of Japanese encephalitis virus and Murray Valley encephalitis virus. *J Virol* **76**, 4901–4911.
- Lee, E., Hall, R. A. & Lobigs, M. (2004). Common E protein determinants for attenuation of glycosaminoglycan-binding variants of Japanese encephalitis and West Nile viruses. *J Virol* **78**, 8271–8280.
- Lee, E., Pavy, M., Young, N., Freeman, C. & Lobigs, M. (2006). Antiviral effect of the heparan sulfate mimetic, PI-88, against dengue and encephalitic flaviviruses. *Antiviral Res* **69**, 31–38.
- Lewis, J. A., Chang, G. J., Lanciotti, R. S., Kinney, R. M., Mayer, L. W. & Trent, D. W. (1993). Phylogenetic relationships of dengue-2 viruses. *Virology* **197**, 216–224.
- Licon Luna, R. M., Lee, E., Mullbacher, A., Blanden, R. V., Langman, R. & Lobigs, M. (2002). Lack of both Fas ligand and perforin protects from flavivirus-mediated encephalitis in mice. *J Virol* **76**, 3202–3211.
- Lin, Y. L., Lei, H. Y., Lin, Y. S., Yeh, T. M., Chen, S. H. & Liu, H. S. (2002). Heparin inhibits dengue-2 virus infection of five human liver cell lines. *Antiviral Res* **56**, 93–96.
- Lindahl, U., Kusche-Gullberg, M. & Kjellen, L. (1998). Regulated diversity of heparan sulfate. *J Biol Chem* **273**, 24979–24982.
- Lobigs, M., Usha, R., Nestorowicz, A., Marshall, I. D., Weir, R. C. & Dalgarno, L. (1990). Host cell selection of Murray Valley encephalitis virus variants altered at an RGD sequence in the envelope protein and in mouse virulence. *Virology* **176**, 587–595.
- Mandl, C. W., Kroschewski, H., Allison, S. L., Kofler, R., Holzmann, H., Meixner, T. & Heinz, F. X. (2001). Adaptation of tick-borne encephalitis virus to BHK-21 cells results in the formation of multiple heparan sulfate binding sites in the envelope protein and attenuation in vivo. *J Virol* **75**, 5627–5637.
- Marks, R. M., Lu, H., Sundaresan, R., Toida, T., Suzuki, A., Imanari, T., Hernaiz, M. J. & Linhardt, R. J. (2001). Probing the interaction of dengue virus envelope protein with heparin: assessment of glycosaminoglycan-derived inhibitors. *J Med Chem* **44**, 2178–2187.
- Meiklejohn, G., England, B. & Lennette, E. T. (1952). Propagation of dengue virus strains in unweaned mice. *Am J Trop Med Hyg* **1**, 51–58.
- Modis, Y., Ogata, S., Clements, D. & Harrison, S. C. (2003). A ligand-binding pocket in the dengue virus envelope glycoprotein. *Proc Natl Acad Sci U S A* **100**, 6986–6991.
- Modis, Y., Ogata, S., Clements, D. & Harrison, S. C. (2005). Variable surface epitopes in the crystal structure of dengue virus type 3 envelope glycoprotein. *J Virol* **79**, 1223–1231.
- Ono, L., Wollinger, W., Rocco, I. M., Coimbra, T. L., Gorin, P. A. & Sierakowski, M. R. (2003). In vitro and in vivo antiviral properties of sulfated galactomannans against yellow fever virus (BeH111 strain) and dengue 1 virus (Hawaii strain). *Antiviral Res* **60**, 201–208.
- Perrimon, N. & Bernfield, M. (2000). Specificities of heparan sulphate proteoglycans in developmental processes. *Nature* **404**, 725–728.
- Reddi, H. V., Kumar, A. S., Kung, A. Y., Kallio, P. D., Schlitt, B. P. & Lipton, H. L. (2004). Heparan sulfate-independent infection attenuates high-neurovirulence GDVII virus-induced encephalitis. *J Virol* **78**, 8909–8916.
- Rey, F. A., Heinz, F. X., Mandl, C., Kunz, C. & Harrison, S. C. (1995). The envelope glycoprotein from tick-borne encephalitis virus at 2 Å resolution. *Nature* **375**, 291–298.
- Rostand, K. S. & Esko, J. D. (1997). Microbial adherence to and invasion through proteoglycans. *Infect Immun* **65**, 1–8.
- Sabin, A. B. (1952). Research on dengue during World War II. *Am J Trop Med Hyg* **1**, 30–50.
- Sabin, A. B. (1955). Recent advances in our knowledge of dengue and sandfly fever. *Am J Trop Med Hyg* **4**, 198–207.
- Sabin, A. B. & Schlesinger, R. W. (1945). Production of immunity to dengue with virus modified by propagation in mice. *Science* **101**, 640–642.
- Sa-Carvalho, D., Rieder, E., Baxt, B., Rodarte, R., Tanuri, A. & Mason, P. W. (1997). Tissue culture adaptation of foot-and-mouth disease virus selects viruses that bind to heparin and are attenuated in cattle. *J Virol* **71**, 5115–5123.
- Saluzzo, J. F. (2003). Empirically derived live-attenuated vaccines against dengue and Japanese encephalitis. *Adv Virus Res* **61**, 419–443.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual, 2nd edn*. Cold Spring Harbor: Cold Spring Harbor Laboratory.
- Sasisekharan, R., Shriver, Z., Venkataraman, G. & Narayanasami, U. (2002). Roles of heparan-sulphate glycosaminoglycans in cancer. *Nat Rev Cancer* **2**, 521–528.
- Schlesinger, R. W., Gordon, I., Frankel, J. W., Winter, W., Patterson, P. R. & Dorrance, W. R. (1956). Clinical and serologic response of man to immunization with attenuated dengue and yellow fever viruses. *J Immunol* **77**, 352–364.
- Scott, R. M., Nisalak, A., Eckels, K. H., Tingpalapong, M., Harrison, V. R., Gould, D. J., Chapple, F. E. & Russell, P. K. (1980). Dengue-2 vaccine: viremia and immune responses in rhesus monkeys. *Infect Immun* **27**, 181–186.
- Shresta, S., Kyle, J. L., Snider, H. M., Basavapatna, M., Beatty, P. R. & Harris, E. (2004). Interferon-dependent immunity is essential for

resistance to primary dengue virus infection in mice, whereas T- and B-cell-dependent immunity are less critical. *J Virol* **78**, 2701–2710.

Spillmann, D. (2001). Heparan sulfate: anchor for viral intruders? *Biochimie* **83**, 811–817.

Talarico, L. B., Pujol, C. A., Zibetti, R. G., Faria, P. C., Nosedá, M. D., Duarte, M. E. & Damonte, E. B. (2005). The antiviral activity of sulfated polysaccharides against dengue virus is dependent on virus serotype and host cell. *Antiviral Res* **66**, 103–110.

Thullier, P., Demangel, C., Bedouelle, H., Megret, F., Jouan, A., Deubel, V., Mazie, J. C. & Lafaye, P. (2001). Mapping of a dengue virus neutralizing epitope critical for the infectivity of all

serotypes: insight into the neutralization mechanism. *J Gen Virol* **82**, 1885–1892.

van den Broek, M. F., Muller, U., Huang, S., Aguet, M. & Zinkernagel, R. M. (1995). Antiviral defense in mice lacking both alpha/beta and gamma interferon receptors. *J Virol* **69**, 4792–4796.

Van Gennip, H. G., Vlot, A. C., Hulst, M. M., De Smit, A. J. & Moormann, R. J. (2004). Determinants of virulence of classical swine fever virus strain Brescia. *J Virol* **78**, 8812–8823.

Zhang, W., Heil, M., Kuhn, R. J. & Baker, T. S. (2005). Heparin binding sites on Ross River virus revealed by electron cryo-microscopy. *Virology* **332**, 511–518.