Early restriction of alphavirus replication and dissemination contributes to age-dependent attenuation of systemic hyperinflammatory disease

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Severity of alphavirus infection in humans tends to be strongly age-dependent and several studies using laboratory-adapted Sindbis virus (SB) AR339 strains have indicated that SB-induced disease in mice is similarly contingent upon host developmental status. In the current studies, the consensus wild-type SB, TR339, and in vivo imaging technology have been utilized to examine virus replication and disease manifestations in mice infected subcutaneously at 5 days of age (5D) vs 11D. Initial virulence studies with TR339 indicated that this age range is coincident with rapid transition from fatal to non-fatal outcome. Fatal infection of 5D mice is characterized by high-titre serum viraemia, extensive virus replication in skin, fibroblast connective tissue, muscle and brain, and hyperinflammatory cytokine induction. In contrast, 11D-infected mice experience more limited virus replication and tissue damage and develop mild, immune-mediated pathologies including encephalitis. These results further establish the linkage between hyperinflammatory cytokine induction and fatal outcome of infection. In vivo imaging using luciferase-expressing viruses and non-propagative replicons revealed that host development results in a restriction of virus replication within individual infected cells that is manifested as a delay in reduction of virus replication in the younger mice. Thus, an important contributing factor in age-dependent resistance to alphavirus infection is restriction of replication within first infected cells in peripheral tissues, which may augment other developmentally regulated attenuating effects, such as increasing neuronal resistance to virus infection and apoptotic death.

INTRODUCTION

Several significant human pathogens are found among the mosquito-borne viruses of the genus Alphavirus, family Togaviridae, associated in the Old World with arthritides and haemorrhagic manifestations of varying severity (Laine et al., 2004) and in the New World with potentially fatal encephalitic disease (Smith et al., 1997). Although serological surveys indicate that human exposure to these viruses is not uncommon, fortunately most infections are either asymptomatic or resolve without complication after an acute, febrile illness. Disturbingly, however, the severity of prodromal illness, frequency of central nervous system (CNS) involvement, case fatality rates and incidence of permanent neurological sequelae in survivors are disproportionately high in children (Smith et al., 1997; Valero et al., 2001).

Age dependence of disease severity can be reproduced by infection of suckling mice with Sindbis virus (SB), the prototypic alphavirus. Most SB strains cause acute, fatal disease in newborn animals, regardless of inoculation route. However, mice rapidly develop resistance to disease during their first 2 weeks of life, developing only subclinical infection and protective immunity as adults (Griffin, 1976; Johnson et al., 1972; Trgovcich et al., 1999). Many host-encoded factors influence SB virulence and disease, including the type I interferon (IFN-α/β) response (Labrada et al., 2002; Ryman et al., 2000, 2005), regulators of apoptosis (Johnston et al., 2001; Levine et al., 1996; Lewis et al., 1999; Liang et al., 1998) and components of the adaptive immune response (Griffin & Johnson, 1977; Griffin et al., 1983). Furthermore, neuronal differentiation has been identified as a critical factor in the concurrent development of resistance to SB replication, virus-mediated apoptosis and fatal encephalomyelitis (Griffin, 2005). However, attempts to link ontogeny of these factors causally to the molecular underpinnings of age-dependent resistance to fatal SB infection have been largely inconclusive.
Historically, SB infections of mice have been studied as acute encephalomyelitis of newborn animals (Johnson et al., 1972), focusing on neurological aspects of disease and often isolating these events by administering the virus intracerebrally. However, recent studies illustrate that intracerebral inoculation combined with the inadvertent use of partially attenuated, cell culture-adapted SB strains has biased disease towards encephalopathy (Klimstra et al., 1999; Trgovcich et al., 1996, 1997). Exacerbated neurological signs associated with these laboratory SB strains are linked to the accumulation of positively charged amino acid mutations in the E2 attachment protein and coincident acquisition of heparan sulfate (HS) proteoglycan-binding capability (Klimstra et al., 1998, 1999), which appears to promote non-productive binding and sequestration by HS structures exposed to virus in serum, limiting viraemic potential and dissemination. In contrast, when cDNA clone-derived, wild-type SB strain TR339 is administered to newborn mice by subcutaneous inoculation to mimic the bite of an infected mosquito vector, the rapidly fatal infection resembles systemic inflammatory-response syndrome (SIRS) closely, with uncontrolled pathogen replication, systemic hyper-induction of inflammatory cytokines and little or no evidence of encephalomyelitis (Klimstra et al., 1999), more consistent with Old World alphavirus disease manifestations in very young humans. Compared with commonly used laboratory strains, TR339 does not interact efficiently with HS or cell surfaces (Klimstra et al., 1998) and probably reflects the cell-attachment and replication characteristics of the original SB AR339 isolate.

The current studies represent the first characterization of age-related resistance to SB disease in mice infected subcutaneously with the wild-type TR339 virus, known to be free of cell culture-acquired HS-binding mutations (Klimstra et al., 1998). We have focused upon the age-dependent alteration in the systemic inflammatory disease caused by TR339 and examined the replication of TR339-based viruses and non-propagative replicas by using in vivo imaging technology, thereby elucidating and quantifying the earliest events in the infection of extraneural sites after subcutaneous inoculation. Our results indicate that, when virus is inoculated subcutaneously, a dramatic restriction of peripheral replication and systemic inflammatory response occurs in older mice prior to significant neuroinvasion or encephalitic disease. Moreover, the age-dependent resistance to infection can be observed at the level of the first infected cell, consistent with potentiation of the efficacy of induced innate immune-response components that restrict genome replication.

**METHODS**

**Cell culture.** Baby hamster kidney cells (BHK-21; ATCC CCL-10) were maintained in alpha minimal essential medium supplemented with 10% donor calf serum (DCS), 2.9 mg tryptose phosphate ml⁻¹, 0.29 mg l-glutamine ml⁻¹, 100 U penicillin ml⁻¹ and 0.05 mg streptomycin ml⁻¹ (37 °C; 5% CO₂).

**Construction of virus cDNA clones and stock production.** Klimstra et al. (1998) previously described construction of the consensus wild-type SB cDNA clone, pTR339. A pTR339-based cDNA clone encoding firefly luciferase (LUC; p39MCS-LUC) was constructed by introducing a second copy of the 26S subgenomic promoter downstream of the E1 gene driving LUC expression and confirmed by DNA sequencing (Retrogen). Infectious virus RNA was generated by *in vitro* transcription (mMessage Machine; Ambion) from Xhol-linearized pTR339 or p39MCS-LUC DNA templates and electroporated into BHK-21 cells. Virus particles were harvested from the supernatant 18–20 h after electroporation, clarified by centrifugation and titrated as p.f.u. ml⁻¹ on BHK-21 cells.

**Construction of replicon cDNAs and replicon-particle production.** TR339-based replicon particles expressing LUC (39REP-LUC) were produced by packaging infectious replicon RNA transcripts in virus structural proteins provided *in trans* by helper RNAs as described previously for green fluorescent protein-expressing replications (Ryman et al., 2002). A replicon genome plasmid expressing LUC was constructed (p39REP-LUC), encoding the TR339 non-structural protein genes and LUC downstream of the 26S subgenomic promoter in place of the virus structural protein genes. *In vitro*-transcribed RNA from p39REP-LUC, glycopolypeptide (pINT) and capsid (pCH) helpers were co-electroporated into BHK-21 cells and 39REP-LUC replicon particles were harvested 24 h post-electroporation. From each preparation, 10% was evaluated by serial passage on BHK-21 cells for the presence of cytopathic effect inducing, propagation-competent virus recombinants or contaminants.

**Morbidity and mortality studies.** Pups born to outbred CD-1 mice (Charles River Laboratories) within a 12 h period were pooled and randomized as 10–12 pup litters. Virus inocula, diluted to contain 100 p.f.u. TR339 in a 50 μl volume (2 × 10⁶ p.f.u. ml⁻¹), were administered subcutaneously in the ventral thorax. Mock-infected mice received 50 μl virus diluent. Virus-infected and corresponding mock-infected mice were weighed at 24 h intervals where appropriate. Mean survival time (MST) and percentage mortality were calculated.

**Pathogenesis studies.** At predetermined intervals post-infection (p.i.), groups of three mice per treatment were selected randomly from different litters. The thoracic cavity was opened under isoflurane anesthesia and blood was collected by cardiac puncture. Serum was separated from whole blood by using microtainer tubes (Becton Dickinson). Mice were perfused with PBS/1% DCS for 10 min before harvest of hindlimb muscle and brain tissues. Tissues were homogenized in PBS/1% DCS by one freeze–thaw and mechanical disruption, and clarified by centrifugation (13 000 g, 15 min, 4 °C). The supernatant was assayed for virus by plaque assay or for IFN-α/β.

**Cytokine and hormone assays.** Serum IFN-α/β was measured by standard biological assay on L929 cells (Trgovcich et al., 1996), using a commercially prepared IFN-α/β standard (Access Biomolecular) and encephalomyocarditis virus as indicator virus. Clarified muscle and brain homogenates were centrifuged to remove material precipitated during the acidification/neutralization procedure. Serum levels of tumour necrosis factor alpha (TNF-α), IFN-γ and interleukin-1 (IL-1) were determined by sandwich ELISA (Pharmering). Total (free and bound) serum corticosterone (CORT) levels were determined by using a radioimmunoassay kit according to the manufacturer’s instructions (ICN).

**Histopathology and in situ hybridization studies.** Three mice per treatment were selected randomly from different litters. Under anaesthesia, each mouse was perfused with 4% paraformaldehyde (PFA) in PBS (pH 7.4) for 10–15 min, fixed in 4% PFA (pH 7.4) for 1 week, then decalcified in 4% PFA, 8% EDTA (pH 6.8, 4 °C) for up to 4 weeks. Tissues were paraffin-embedded and sectioned. Hematoxylin and eosin (H&E)-stained sections were viewed by light
microscopy. *In situ* hybridization (ISH) analyses to detect virus genomic RNA in tissue sections were performed (Klimstra *et al.*, 1999). Radiolabelled riboprobes were generated by *in vitro* transcription from linearized plasmid DNA in the presence of $[^{35}S]$UTP (Amersham Biosciences). Riboprobe complementary to a region in the subgenomic virus RNA was generated from AflII-linearized pGSV.SS (Trgovcich *et al.*, 1996). Riboprobe complementary to a region in the EBER-2 protein gene of Epstein–Barr virus controlled for non-specific probe hybridization.

**Viable thymocyte counts.** The entire thymus was removed and dissociated into a single-cell suspension. Viable thymocytes were identified by trypan blue exclusion, counted on a haemocytometer and expressed as percentage of mean viable thymocyte number in parallel mocks.

**In vivo bioluminescence imaging and data quantification.** Inoculation with 39MCS-LUC virus or 39REP-LUC replicon particles was performed as described above. Imaging of luciferase activity in mice was performed on a charge-coupled device camera (Xenogen). Briefly, mice were anaesthetized by intraperitoneal injection of ketamine/xylazine (90 mg ketamine kg$^{-1}$ and 10 mg xylazine kg$^{-1}$) and 0.7 mg luciferin substrate kg$^{-1}$, approximately 10 min prior to imaging. Images were acquired for 2–10 s, depending on relative light emission from various sites of infection. Relative intensities of transmitted light from *in vivo* bioluminescence were represented as a pseudocolour image ranging from violet (least intense) to red (most intense) superimposed on the corresponding greyscale photograph by using LivingImage (Xenogen) image-analysis software. Data for photon flux from manually defined regions of interest were calculated.

**RESULTS**

**Age-dependent resistance to fatal infection with wild-type SB strain TR339**

Neonatal mice [1 day old (1D)] inoculated subcutaneously with wild-type TR339 virus succumb to a fatal infection within 2–3 days (Klimstra *et al.*, 1999), whereas subcutaneous infection of adult mice (3–4 weeks old) with TR339 is subclinical, eliciting a protective immune response (Ryman *et al.*, 2000). Here, we have determined the precise age of transition from fatal to non-fatal SB infection under conditions of constant virus strain, dose and inoculation route. When randomized litters of outbred, suckling mice, aged between 1D and 15D, were inoculated subcutaneously with 100 BHK-p.f.u. TR339, infection was uniformly fatal for mice inoculated at 5D or younger, followed by a precipitous reduction in the percentage mortality such that all mice inoculated at 11D or older survived the infection (Fig. 1a). Between 5D and 11D, a strong inverse correlation between host age and mortality rate was observed ($r^2=0.98$). Increasingly extended MSTs observed from 1D onwards were correlated positively with host age ($r^2=0.85$). We infer that TR339 infection becomes gradually more attenuated until 5D, after which a critical threshold is reached, allowing some animals to survive the infection. By 11D, the virus infection is no longer fatal in any of the mice.

![Fig. 1. Morbidity and mortality caused by TR339 virus infection of suckling mice. Suckling CD-1 mice were infected subcutaneously with 100 BHK-p.f.u. TR339 SB. (a) Percentage mortality (●; line) was determined for mice infected at 1 day old (1D), 3D, 5D, 7D, 9D, 11D, 13D and 15D where $n=12–36$ mice. Mean survival times (MST; bars) of infected animals are shown ±SD. (b) Weight-for-age (WA) of mice infected at 5D, 8D or 11D was calculated as (weight of infected animal/expected weight) x 100 %, where the expected weight is determined from the growth of mock-infected animals. WA for individual mice (squares) and the mean for the group (bars) are shown.](image-url)
Clinical correlates of age-dependent virulence

We reasoned that characterization of TR339 infection in mice at 5D (susceptible), 8D (transitional) and 11D (resistant) would reveal pathogenetic and pathological correlates of the rapid acquisition of resistance to wild-type SB fatality. Weight-for-age (WA) determinations were used to calculate an expected weight for mice in virus-infected groups, providing a quantitative indicator for disease severity (Fig. 1b). For example, by 5 days p.i., 11D-infected mice were a mean of 96.4±12.5 % WA, 8D-infected mice were 79.0±15.9 % WA and 5D-infected mice were 56.9±8.5 % WA. Thus, compared with uninfected controls, 5D-infected mice did not thrive and became cachexic, with wasting of the muscles, rigidity and/or paresis of fore- and hindlimbs. In contrast, 11D-infected mice exhibited limited growth retardation, with mild, sporadic signs of paresis or flaccid paralysis of the hindlimbs. The 8D-infected mice exhibited intermediate weight gain and other clinical signs of infection, with greater variability between individual animals.

Proinflammatory-cytokine induction correlates with severe infection

We have previously characterized the fatal infection of TR339-infected neonatal animals as a SIRS-like disease with dysregulated proinflammatory-cytokine induction. Here, serum-cytokine levels were measured in 5D- and 11D-infected mice to determine whether a correlation existed between induction of the proinflammatory-cytokine cascade and the progressive attenuation of TR339 in mice of increasing age. Preliminary experiments evaluating serum samples collected at 24 h intervals p.i. indicated that cytokine induction occurred early in infection, but diminished to background levels by 72 h p.i. (data not shown). Consequently, serum cytokines were assayed at 6 h intervals until 48 h p.i. (Fig. 2). Low levels of proinflammatory cytokines were measured in the serum of 11D-infected mice, suggestive of homeostasis and an appropriately controlled response to the pathogen. In contrast, a dysregulated, hyperinflammatory cytokine response was observed in 5D-infected mice. IFN-γ levels peaked at 24 h p.i. at approximately 2000 pg ml\(^{-1}\) (Fig. 2a), TNF-α was first detectable in serum at 18 h p.i., peaking at 30 h p.i. in excess of 300 pg ml\(^{-1}\) (Fig. 2b), and the induction of IL-6 reached peak levels of 6000 pg ml\(^{-1}\) by 30 h p.i. (Fig. 2c). Overall, the cytokine profile in the 5D-infected mice was reminiscent of the SIRS-like disease described for TR339-infected neonatal animals (Klimstra et al., 1999), although peak levels were generally somewhat lower, whereas little or no inflammatory-cytokine response was observed in 11D-infected mice.

CORT release

Stress hormones, released from the adrenal gland in response to cytokine stimulation of the hypothalamic–pituitary–adrenal axis or direct stimulation of the adrenal gland itself by IL-6, provide a tightly regulated feedback-inhibition loop to dampen inflammatory-cytokine responses, maintain/restore homeostasis and protect the host from cytokine-mediated pathology (Elenkov et al., 2005; Silverman et al., 2004, 2005). The age dependence of CORT release in response to TR339 infection was assessed (Fig. 3a). Interestingly, no significant CORT release was observed until 5 days p.i. in 5D-infected mice, probably contributing to the unchecked cytokine response. CORT was also undetectable in their 11D-infected counterparts, characteristic of an appropriately controlled immune response in which homeostasis is restored rapidly. This was in contrast to mice infected with TR339 at 1D (Fig. 3a) or inoculated with a fatal dose of lipopolysaccharide, in which high levels of CORT (300–400 ng ml\(^{-1}\)) were present in serum without significant age-dependent differences (data not shown).
Immune-mediated pathology

Severe thymic involution, characteristic of SIRS, was shown previously to be a hallmark of neonatal TR339 infection (Klimstra et al., 1999). In the present study, the degree of inflammatory response-mediated thymic pathology correlated with the outcome of the infection. In 5D-infected mice, moderate to severe thymic atrophy, not associated directly with virus replication, was observed by H&E staining, whereas little or no damage was evident in 11D-infected animals (data not shown). The degree of thymic involution was quantified as the percentage of viable cells compared with mock-infected controls (Fig. 3b), demonstrating an almost 100-fold reduction in viable thymocytes of 5D-infected mice compared with approximately 2-fold loss in those infected at 11D.

Age-dependent restriction of systemic TR339 replication

To determine whether the development of SIRS-like disease and age-dependent attenuation of infection correlated with quantitative and/or qualitative differences in virus replication, TR339 replication was measured in serum, hindlimb muscle and brain tissues of suckling mice inoculated subcutaneously at 5D or 11D (Fig. 4). Overall, a similar pathogenetic sequence was observed in both age groups, consistent with early virus replication in peripheral tissues seeded by and sustaining a serum viraemia, with subsequent neuroinvasion and replication in the CNS. However, the onset of detectable viraemia was delayed by at least 12 h in
11D- vs 5D-infected mice and remained consistently at least 100-fold lower in magnitude (Fig. 4a). Early replication in muscle and appearance of virus in the brain were correspondingly delayed and reduced significantly with increasing age, peaking at approximately 100-fold lower levels (Fig. 4b, c, respectively). Importantly, this was true in serum and muscle early after subcutaneous inoculation, as well as in CNS tissues, indicating that suppressed virus production from early extraneural sites of infection in the older animals preceded the reduction in neuroinvasion and CNS replication. In both cases, virus clearance from serum began 3–4 days p.i., coincident with the appearance of TR339-specific antibody, which was not impaired significantly in the younger animals (data not shown). However, despite the degree of virus clearance from serum and the presence of TR339-specific antibody in 5D-infected mice, virus replication continued in muscle and brain without marked restriction or clearance. In mice infected at 11D, more limited replication and subsequent clearance of the virus from tissues between 6 and 8 days p.i. correlated with decreased proinflammatory-cytokine production and reduced morbidity, allowing survival.

**Quantitative differences in virus replication and histopathology**

Histopathological and ISH analyses of infected tissues were performed to confirm primary sites of infection. In 5D-infected mice, 2 days p.i., large areas of intense virus-specific ISH signal corresponding to productively infected cells were observed, particularly in the dermis of the skin, skeletal muscle, fibroblast connective tissue, periosteum and articular growth plates of the bones (Fig. 5a). An analogous section from an 11D-infected mouse, 2 days p.i., demonstrates that
replication was significantly more restricted in the older animal, but the tissue tropism appears similar (Fig. 5b). Virus replication in the spinal cord and CNS was also increasingly restricted with age, as early as 2 days p.i. (Fig. 5c, d). By 6 days p.i., numerous infected neurons were evident in 5D-infected mice, particularly in the outer cortex region of the brain (Fig. 5e), whereas only infrequent focal areas of virus-specific signal were observed in 11D-infected mouse brains (Fig. 5f). An H&E-stained adjacent section showed virus-associated pathology in the brain of the 5D-infected mouse (Fig. 5g); however, inflammatory lesions involving infiltrates of immune cells were limited, suggesting that immunosuppression may be a feature of fatal TR339 infection. In 11D-infected mouse brain, histopathological changes consistent with mild encephalitis were apparent, including perivascular congestion and cuffing, reactive endothelial cells and characteristic glial nodules (Fig. 5h). Thus, more restricted neuroinvasion and virus replication in the brain with the development of mild encephalomyelitis correlated with clearance of virus and survival in older mice.

**Early virus replication correlates with type I IFN induction**

Extremely high levels of IFN-α/β induction have previously been associated with fatal TR339 infection of neonatal mice (Klismstra et al., 1999), but paradoxically TR339, like other SB strains, is known to be acutely sensitive to the antiviral activity of IFN-α/β both *in vitro* and *in vivo* in adult mice (Ryman et al., 2000). As in previous studies, measurement of IFN-α/β levels in serum from 5D- and 11D-infected mice (Fig. 6a) demonstrated a positive correlation between IFN-α/β induction and serum viraemia. Indeed, levels of serum IFN-α/β in 5D-infected mice reached potentially harmful levels, abating slightly towards the day of death. In comparison, serum IFN-α/β peaked at significantly lower levels in 11D-infected mice. In both 5D- and 11D-infected mice, IFN-α/β appeared early in infection in the muscle where the primary sites of virus replication were also found (Fig. 6b) and was subsequently produced in the brain (Fig. 6c), again paralleling sites and levels of virus replication. However, it is interesting to note that, at 24 h p.i., when virus titres in serum and muscle were at least 100-fold higher in 5D-infected animals, corresponding levels of IFN-α/β exhibited little difference. Thus, we must consider that the failure of the type I IFN system to contain the replication of this IFN-α/β-sensitive virus in 1D- and 5D-infected mice may be due to insufficient rapidity of IFN-α/β production relative to virus replication and dissemination.

**In vivo imaging reveals dramatic differences in early virus dissemination**

Virus titration and ISH analyses suggested strongly that very early differences in virus replication contributed to disease outcome. In order to provide a comprehensive picture of early sites of virus replication in the whole animal, we have adapted Xenogen IVIS technology to evaluate more directly early events in the age-dependent restriction of TR339 replication, dissemination and neuroinvasion. By using IVIS, Cook & Griffin (2003) detected the presence of LUC expressed from a genetically modified SB in the tissues of subcutaneously and intracerebrally inoculated mice following intraperitoneal administration of the luciferin substrate. Furthermore, they demonstrated a direct correlation between relative light units (RLU) emitted, representing LUC activity, and virus replication. To facilitate our studies, we have generated TR339-based virus expressing LUC from a second downstream copy of the subgenomic promoter (39MCS-LUC) with which to analyse the replication and dissemination of the virus, and TR339-based replicon particles expressing LUC (39REP-LUC) to investigate relative permissiveness of first-round target cells.

![Fig. 6. IFN-α/β induction in serum and tissues of TR339-infected suckling mice. IFN-α/β levels were measured by bioassay of serum (a), muscle (b) and brain (c) homogenates from suckling mice infected with 100 BHK-p.f.u. TR339 virus at 5D (■) or 11D (□). Values represent the geometric mean IFN-α/β level \(\log_{10}(\text{IU ml}^{-1} \times \text{g}^{-1})\) where \(n = 3\) mice. Datum points are shown ± SD. The lower limit of detection is indicated (broken line). Asterisks on 5D datum points indicate significance of differences from 11D with confidence limits of \(P < 0.05\) (*) or \(P < 0.01\) (**). Levels in mock-infected animals were below the limits of detection for these assays.](Image)

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All mice aged 5D, 8D and 11D, inoculated subcutaneously with 39MCS-LUC virus or 39REP-LUC replicon particles, had detectable LUC activity corresponding to virus replication in the ventral thorax (site of inoculation) when imaged 8 h p.i. (Fig. 7a, c, respectively). A small, but not consistently significant, reduction in the mean RLU was observed between age groups (quantified in Fig. 7g), suggesting that infection of the initially targeted cells was not greatly restricted with increasing age. By 24 h p.i., however, mean luciferase expression from 39MCS-LUC virus in the 5D-infected mice was significantly higher than in their 11D-infected counterparts and, whereas LUC signal had increased by over 10-fold in 5D-infected animals, no increase was observed in those infected at 11D. In the 39REP-LUC-inoculated animals, the reduction in mean LUC activity since 8 h p.i. was approximately 5-fold in 5D mice, compared with approximately 50-fold in 11D mice, from which we infer that non-specific antiviral mechanisms controlling replication may be more potent in the older animals.

In a parallel experiment, 5D, 8D and 11D mice were inoculated intracerebrally with either 39MCS-LUC virus or 39REP-LUC and imaged from the dorsal surface at 8 and 24 h p.i. (Fig. 7d–f, data not shown). Quantification of the LUC activity in the 39REP-LUC-infected animals (Fig. 7h) indicated comparable early permissivity of CNS tissues regardless of age but, unlike infection by the subcutaneous route, LUC activity was mitigated similarly in both 5D and 11D animals by 24 h p.i. Moreover, a similar increase in mean RLU was evident by 24 h p.i. in both 5D and 11D animals infected intracerebrally with 39MCS-LUC, suggesting that spread of the virus infection within the CNS was not restricted dramatically with host development.

**DISCUSSION**

The developmental status of the host strongly influences susceptibility to alphavirus infection and severity of disease. This phenomenon has been reproduced in the laboratory by SB infection of suckling mice, with the goal of elucidating molecular mechanisms of age-dependent attenuation. Previous studies have revealed that, although the host’s age is the predominant factor, the age of transition from fatal to non-fatal outcome can be influenced significantly by the virus genotype and/or inoculation route (Griffin, 1976; Hackbarth et al., 1973; Johnson et al., 1972; Lustig et al., 1988, 1992b; McKnight et al., 1996; Trgovcich et al., 1999; Tucker et al., 1993). Consequently, we have studied the phenomenon of age-dependent resistance to alphavirus infection with three innovations in experimental design: (i) instead of the partially attenuated, cell culture-adapted, HS-binding viruses studied in the past, we have used the wild-type (i.e. non-cell culture-adapted) SB strain, TR339; (ii) instead of inoculating mice intracerebrally, we have inoculated mice subcutaneously to mimic natural alphavirus delivery by an infected mosquito; and (iii) we have focused on early virus replication in extraneural tissues and the SIRS-like disease now known to be a major component of wild-type SB infections (Klimstra et al., 1999; Ryman et al., 2000), exploring the concept that developmentally regulated changes in the host alter the earliest events in infection and thereby determine the disease course.

In common with other SB strains (Griffin, 1976; Trgovcich et al., 1999), the outcome of TR339 infection was strongly dependent on the host’s developmental status at the time of virus exposure, demonstrating that age-related virulence is neither confined to encephalomyelitic disease nor determined by differential cell-surface receptor expression. Transition from symptomatic to asymptomatic infection occurred gradually from 1D onward and continued beyond 11D through adulthood, leading us to speculate that the precipitous increase in survival between 5D and 11D occurs because a critical pathogenetic threshold is reached.

We have shown previously that TR339-infected newborn mice succumb near the peak of the proinflammatory cytokine burst, possibly due to multiple-organ dysfunction (Klimstra et al., 1999). Interestingly, the magnitude of the inflammatory response appears sufficiently diminished even in 5D-infected animals for them to survive this phase of infection, dying 3–4 days after cytokine levels have returned to baseline, in keeping with the observed extension in MST. The major inflammatory mediators TNF-α, IFN-γ and IL-6 peaked at levels sufficiently high to cause extensive damage typically associated with SIRS (Conti et al., 2004). Probably as a consequence of the extended disease course, more widespread replication in the CNS and evidence of encephalopathy were also observed in 5D-infected mice as the disease began to evolve from systemic hyperinflammatory presentation towards encephalomyelitis. Furthermore, high-level circulating cytokines, particularly TNF-α, are known to facilitate virus neuroinvasion from the bloodstream by damaging the cerebral microvascular endothe- lium of the blood–brain barrier, damaging cells of the CNS directly and enhancing virus apoptotic potential (Brouckaert & Fiers, 1996; Lustig et al., 1992a; Wang et al., 2004).

As observed previously for neonatal infection (Klimstra et al., 1999), little evidence of inflammatory-cell infiltration was observed in peripheral or CNS tissues in 5D-infected mice, coinciding with dramatic reduction in thymocytes. This may be similar to the profound, apoptosis-induced loss of lymphocytes caused by the dysregulated inflammatory responses of SIRS and sepsis, which creates a hypoinflammatory phase in animals surviving the initial cytokine burst (Hotchkiss et al., 2003). The proximal cause of death in the 5D-infected animals is attributed to virus- and cytokine-mediated pathologies coupled with a generalized cachexic retardation of development.

As virus replication becomes increasingly restricted beyond 5D, TR339 infection attenuates still further until all 11D-infected animals survive and clear the virus. A regulated,
**TH1-biased cytokine response was elicited with early induction of IL-12 (data not shown) and IFN-γ, desirable for control and clearance of virus replication without permanent damage to the host. Inevitably, lower viraemic titres and circulating inflammatory-cytokine levels would result in reduced neuroinvasion and seeding of the CNS, augmenting the attenuating effects of advancing neuronal maturation. Interestingly, these mice developed mild, immune-mediated pathologies with occasionally pronounced paresis/paralysis, probably due to encephalomyelitis. The appearance of inflammatory infiltrates in 11D-infected animals coincided with dramatically lower levels of TNF-α and lymphocyte loss, but not with improved activation of these cells (K. D. Ryman, D. L. Browning & W. B. Klimstra, unpublished data). From the observation that mice lacking T and B cells develop age-dependent resistance to fatal SB infection similarly to normal animals (Griffin, 1976), it is inferred that adaptive immune responses are not responsible for age-dependent survival, but they probably contribute to the evolution of the disease phenotype and clearance of the virus infection in survivors.

*In vivo* imaging studies demonstrated dramatic age-dependent restriction of SB replication and dissemination in peripheral tissues prior to neuroinvasion. Furthermore, as evidenced by the greater reduction in luciferase expression between 8 and 24 h p.i. in 11D-replicon-infected mice, an autocrine activity is involved that does not require virus spread. This is the first evidence that virus replication-inhibiting activities increase at the single-cell level with increasing age. Together, these data imply that the activity of one or more powerful mechanisms of host antiviral defence potentiated during this developmental phase, regardless of the disease phenotype, and that factors in addition to neuronal maturation impact the accrual of resistance to fatal SB infection.

Type I IFN is a primary antiviral defence mechanism against SB infection *in vitro* and *in vivo* (Ryman *et al.*, 2000, 2002, 2005), but the maturation of innate immune-mediated antiviral responses has been largely discounted as a mechanism controlling age-dependent disease attenuation because induction of IFN-α/β and IFN-stimulated genes parallels levels of virus replication, being most vigorous in neonatal animals (Griffin *et al.*, 1994; Labrada *et al.*, 2002; Lewis *et al.*, 1999; Trgovcich *et al.*, 1999; Vilcek, 1964). However, changes in induction kinetics or the efficacy of innate immune mediators have not been examined. There is superficial similarity between the pathogenesis of TR339 in adult IFNAR1−/− mice, which are unable to respond to induced IFN, and neonatal outbred mice, including extensive virus replication, induction of a SIRS-like cytokine profile and rapid mortality. However, an age-dependent reduction in the permissiveness of certain key tissues (e.g. skeletal muscle) clearly occurs in adults even in the absence of an effective IFN-α/β response (Ryman *et al.*, 2000). Data from the current studies are consistent with an increase in the effectiveness of induced IFN-α/β across the transition from fatal to subclinical infection. However, we conclude that additional factors, which remain to be identified, act in concert with a more effective innate immune response to restrict the replication potential of TR339 (and other SB strains) with increasing host age.

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