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Fatal Sindbis Virus Infection of Neonatal Mice in the Absence of Encephalitis

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A comparative pathogenesis study was performed in neonatal mice using a molecularly cloned laboratory variant of Sindbis strain AR339, designated TRSB, and a single-site attenuated mutant of TRSB derived by site-directed mutagenesis of the E2 glycoprotein from Ser to Arg at residue 114 (TRSBr114). TRSB caused 100% mortality with an average survival time of 3.0 ± 0.7 days, whereas mice inoculated with TRSBr114 exhibited an attenuated disease course with 46% mortality and an extended average survival time of 7.5 ± 3.4 days for those animals that died. Reduced virulence of TRSBr114 was characterized by delayed appearance of detectable virus, relative to TRSB, and by lower peak virus titers in both sera and brains of infected mice. TRSB infection induced very high peak serum titers of interferon alpha/beta (215,000 units/ml compared to 2100 units/ml for TRSBr114). *In situ* hybridization analysis demonstrated replication of TRSB in brain, but only minimal histopathological changes and no evidence of encephalitis were observed. However, extensive extraneural lesions and viral replication were found in skin, connective tissue, and muscle. Moreover, dramatic involution of the thymus and loss of hematopoietic tissues were observed in the absence of virus replication at these sites, suggesting the involvement of a systemic physiological stress response in TRSB infection. TRSBr114 infection did not cause thymic lesions. Otherwise, the attenuated mutant demonstrated a similar pattern of tissue and organ involvement, but lesions and positive *in situ* hybridization signal were much more limited in scope and intensity compared to TRSB. TRSBr114-infected mice developed myositis and encephalomyelitis approximately 6 days postinfection. Therefore, TRSB-infected animals may succumb to an early syndrome associated with the stress response, preventing their survival for a time sufficient for the development of encephalitis. Alternatively, a systemic stress response, as evidenced by thymic involution, may result in immunosuppression, thus contributing to the absence of encephalitis. In any event, the attenuating mutation in the E2 glycoprotein significantly altered the course of Sindbis-induced disease by limiting virus replication and associated damage early in infection. Mutant-infected animals survived beyond Day 4 and progressed to a classical encephalomyelitis from which about half recovered.

INTRODUCTION

Sindbis virus is the prototype member of the alphavirus genus (Strauss and Strauss, 1994). The Sindbis genome, a positive-sense RNA molecule of 11,703 nucleotides, is encapsidated within an icosahedral shell composed of 240 capsid protein monomers. The capsid is enveloped upon budding from host cell membranes. Two viral glycoproteins, E1 and E2, form heterodimeric spikes which associate as trimers within the envelope. The interactions of alphavirus glycoproteins with cells during attachment and entry, as well as their interactions with cellular elements during virion maturation, are major determinants of host range, cell tropism, and virulence in animals (Griffin, 1986; Johnston and Peters, 1996).

Experimental studies of Sindbis pathogenesis have focused primarily on central nervous system (CNS) involvement in both the adult and the neonatal mouse models of Sindbis virus infection. In adult mice inoculated intracerebrally (ic), Sindbis elicits a transient encephalitis, while in immunocompromised adult mice, a persistent neuronal infection is established. In neither case, however, is the infection lethal or even symptomatic (Taylor *et al.,* 1955; Jackson *et al.,* 1987; Reinarz *et al.,* 1971; Johnson and MacFarland, 1972; Levine *et al.,* 1991).

In contrast, Sindbis induces 100% mortality in neonatal mice inoculated at very low doses either ic or subcutaneously (sc). Death has been ascribed to encephalitis (Johnson, 1965; Sherman and Griffin, 1990), because the virus replicates to high titer in the brains of these animals, and signs of CNS involvement, such as hindlimb paralysis, are commonly observed (Taylor *et al.,* 1955). However, diagnostic parameters characteristic of encephalitis, such as extensive neuronal degeneration, perivascular cuffing, or inflammatory cell infiltrates have not been reported in several pathology studies of neonatal mouse infections with Sindbis or Sindbis-like viruses (Taylor *et al.,* 1955; Mahlerbe *et al.,* 1963; Johnson, 1965; Johnson and MacFarland, 1972). This suggests that neuronal pathology may be caused by the virus directly, perhaps by induction of apoptosis (Levine *et al.,* 1993), and/or that lesions in other tissues may contribute significantly to the proximal cause of death in neonatal mice. Human disease involving Old World alphaviruses rarely

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has been associated with encephalitis, in contrast to New World alphaviruses, which are associated with encephalitis more commonly (Johnston and Peters, 1996). Rather, viruses closely related to Sindbis, such as S.A. AR86, Girdwood S.A., and Ockelbo, induce a febrile illness characterized by arthralgia, skin rash, and headache. These considerations suggested one objective of the present study: to examine viral replication and pathological changes induced following Sindbis infection of neonatal mice not only in the CNS, but also in extraneural tissues.

The second objective of the experiments reported here was to define the pathogenesis of a laboratory strain of Sindbis isolate AR339 derived from a molecular clone and to determine the histopathological basis for attenuation mediated by a single-site mutation in the E2 glycoprotein. The advent of molecular clones of Sindbis and other alphavirus genomes (Rice *et al.,* 1987; Davis *et al.,* 1989; Kuhn *et al.,* 1991; Liljeström *et al.,* 1991) has facilitated the detailed genetic analysis of virulence. Such clones contain viral genomic sequences as a cDNA downstream of a phage promoter. *In vitro* transcription of linearized plasmids yields genomic replicas which are infectious when introduced into susceptible cells. Thus, studies of virulence and pathogenesis can be initiated with virus of defined sequence, and *in vivo* properties of parent and mutant strains differing by as little as a single nucleotide can be examined. Using these genetic systems, a number of alphavirus virulence mutations, primarily clustered in the glycoprotein genes, have been identified and reconstructed as defined mutations in otherwise isogenic cDNA clones, and their effect on virulence has been assessed in animal models (Polo *et al.,* 1988; Lustig *et al.,* 1988; Faragher *et al.,* 1988; Polo and Johnston, 1990; Sherman and Griffin, 1990; Davis *et al.,* 1991; Kuhn *et al.,* 1992; Schoepp and Johnston, 1993; Tucker *et al.,* 1993; Heidner *et al.,* 1994). One such attenuating mutation is an Arg for Ser substitution at E2 114, a cell-culture-adaptive mutation which arose during selection for efficient growth on baby hamster kidney (BHK) cells (Baric *et al.,* 1981). E2 Arg 114 is a potent attenuating mutation, as demonstrated in several different Sindbis biological and molecularly cloned genetic backgrounds (Olmsted *et al.,* 1984; Davis *et al.,* 1986; Olmsted, 1986; Polo *et al.,* 1988; McKnight *et al.,* 1996). While reduction in mortality and morbidity has been documented for the E2 Arg 114 mutation and for other alphavirus virulence mutations (Atkins *et al.,* 1982; Glasgow *et al.,* 1991; Tucker and Griffin, 1991; Grieder *et al.,* 1995), the mechanisms by which these mutations reduce virulence and alter disease course remain largely unknown.

The studies reported here demonstrated that the single-site E2 Arg 114 mutation attenuates Sindbis virulence by drastically altering the disease course while targeting the same tissues as its virulent parent. Furthermore, the cloned parental strain did not cause encephalitis in neonatal mice. Rather, the histopathological profile of 1-day-old mice infected with this virulent strain suggested that replication in extraneural tissues may be far more important in disease pathogenesis than previously recognized.

MATERIALS AND METHODS

Cells, viruses, and virus clones

BHK cells used for virus production and titrations were obtained from the American Type Culture Collection and used between passages 55 and 65. Cells were maintained in Eagle's minimal essential medium (MEM) supplemented with 5 or 10% donor calf serum (DCS), 10% tryptose phosphate broth, 0.29 mg/ml L-glutamine, 100 U/ml penicillin, and 0.05 mg/ml streptomycin. Murine L929 fibroblasts and murine Swiss 3T3 albino fibroblasts were maintained in the same media used for BHK cells (10% DCS). Primary cardiac fibroblasts were a kind gift from Barbara Sherry (North Carolina State University) and were maintained in Dulbecco's MEM high glucose supplemented with 100 U/ml penicillin, 0.05 mg/ml streptomycin, and 7% fetal calf serum (Baty and Sherry, 1993). Cells were aged 5–7 days after seeding prior to infection. After infection primary fibroblasts were maintained in media without serum. Neuro 2A cells were maintained in MEM supplemented with 10% DCS. All cells were maintained at 37° in an atmosphere of 5% CO_2.

Sindbis virus strain AR339 was obtained from the laboratory of H. R. Bose of the University of Texas at Austin. This virus was biologically cloned in BHK cells and was designated SB. A full length cDNA clone (pTRSB) of SB was constructed by replacing the virus-specific sequences in pToto1101 (Rice *et al.,* 1987) with the homologous SB cDNAs downstream of an SP6 promoter (Polo *et al.,* 1988; McKnight *et al.,* 1996). A clone isogenic with pTRSB except for a change of cytidine to adenosine at nucleotide position 8972 also has been constructed (McKnight *et al.,* 1996). Briefly, the *Stu*I (in the E3 gene) to *Bss*HII (at the 3′ end of the E2 gene) region of pTRSB was replaced with an analogous fragment which contained the substitution at nt 8972. The resulting construct (pTRSBr114) was sequenced across the entire substituted fragment to demonstrate that pTRSBr114 differs from the parental pTRSB by a single codon change from Ser to Arg at E2 position 114.

Plasmids were linearized with *Xho*I and full length virus transcripts were produced in run-off transcription reactions using SP6 polymerase (Rice *et al.,* 1987; Polo *et al.,* 1988). BHK cells were transfected with viral RNA using cationic liposomes (Lipofectin, BRL) as described previously (Polo and Johnston, 1990) or electroporation (Liljeström *et al.,* 1991). Virus-containing supernatants were harvested approximately 36 hr posttransfection, clarified by low-speed centrifugation, and frozen at −70°. Virus

stocks derived from plasmids pTRSB and pTRSBr114 were designated TRSB and TRSBr114, respectively.

Virulence assay

All animal studies were performed in neonatal CD-1 mice (Charles River, specific pathogen free) inoculated 12–24 hr after birth. To assay virulence, litters of 8–19 mice were inoculated sc with 1000 plaque forming units (PFU) of TRSB or TRSBr114 in 0.05 ml. One or two mice per litter were inoculated with 0.05 ml diluent (phosphate-buffered saline, 1% DCS [PBS 1% DCS]) to serve as mock-infected controls. Animals were observed daily for up to 21 days, and average survival time (AST) and mortality were recorded.

Virus growth *in vivo*

Newborn CD-1 mice were inoculated with 1000 PFU of either TRSB or TRSBr114 as described above. Two or three animals inoculated with each virus were sacrificed at intervals indicated in the figures. Blood and whole brains were harvested from each animal. PBS 1% DCS was added to both samples to make 50% suspensions (w/v). Brain samples were homogenized, and both samples were clarified by low-speed centrifugation. Multiple aliquots of sera and clarified brain homogenates were frozen at −70°. Infectious virus in each sample was quantitated by standard plaque assay on BHK cells.

Viral growth in Swiss-3T3 albino fibroblasts, primary cardiac fibroblasts, and Neuro 2A cells

Growth in murine Swiss 3T3 fibroblasts and Neuro 2A cells was measured in 60-mm plate cultures. Virus diluted in PBS 1% DCS was adsorbed to the cells at the indicated m.o.i.'s for 1 hr at 37° in a volume of 0.2 ml. After two washes with media, monolayers were overlaid with 4 ml of media and incubated at 37°. Primary cardiac fibroblasts were infected in six-well plates in media without serum as described above. After infection, cells were washed and overlaid in media without serum and incubated at 37°. Samples of culture supernatants were harvested at intervals, stored at −70°, and quantitated for virus by standard plaque assay on BHK cells.

Interferon alpha/beta (IFNα/β) assays

IFNα/β was measured by standard biological assay on L929 murine fibroblasts using encephalomyocarditis virus (EMCV) as the indicator virus (Meager, 1987). Briefly, L929 cells were seeded at 4×10^4 cells/well in 96-well plates and incubated for 24 hr prior to addition of test samples. The volumes of serum and brain homogenate samples were adjusted to 250 μl by addition of PBS 1% DCS, acidified to pH 2 with 1 *M* HCl, and stored at 4° for 24 hr. Samples were returned to pH 7 with 1 *M* NaOH, 100 μl of each sample was added to duplicate wells of a 96-well plate of L929 cells, and the samples were serially diluted in twofold steps. Sera and brain homogenates from mock-infected mice or PBS 1% DCS spiked with an IFNα/β standard (Lee Biomolecular) were treated identically to the test samples and included as standards on each plate for all assays performed. Plates were incubated for 24 hr at 37° at which time EMCV was added to all test and standard wells (2×10^5 PFU in a volume of 50 μl). After an additional 24 hr at 37°, cells were stained with crystal violet, and the end point was determined as the concentration of IFNα/β required to protect approximately 50% of the monolayer from cytopathic effect by EMCV. The values reported were normalized to the standard.

Histopathology

Neonatal CD-1 mice were inoculated with either TRSB or TRSBr114 as described above. Two to three infected mice (and two to three control mice) per time point were anesthetized and perfusion fixed with 4% paraformaldehyde in PBS, or immersion fixed (after opening peritoneal, thoracic, and cranial cavities) in 10% buffered formalin. TRSB-infected mice were sacrificed daily for 4 days. TRSBr114-infected and mock-infected mice were sacrificed daily for 6 days, and every other day until Day 14. In a second study, mice were sacrificed on Days 2, 4, 8, and 12. Mice sacrificed Days 1 through 6 were bisected midsagittally and embedded in paraffin, and whole-body sections were analyzed for histopathology. Animals sacrificed after Day 6 were eviscerated and individual organs were dissected prior to embedding in paraffin. The fixed heads of these older mice were decalcified at 4° in 8% EDTA, 4% paraformaldehyde, pH 6.6, for approximately 14 days prior to embedding and sectioning. Hematoxylin and eosin-stained tissue sections were examined by light microscopy.

In situ hybridization

Riboprobes for the detection of viral nucleic acid sequences were generated by *in vitro* transcription of linearized pGSV.SS plasmid with T7 polymerase in the presence of [α-^{35}S]UTP. pGSV.SS was constructed by inserting the *Sma*I to *Sac*I fragment from pTRSB (nucleotides 7497 to 8568 in the E2 gene) into the polylinker of pGEM3 (Promega). Upon linearization at the unique *Afl*II site (nucleotide 7968), *in vitro* transcription generated a 486-nucleotide transcript complementary to virus message-sense RNA. Riboprobe complementary to the influenza virus hemagglutinin (HA) gene (strain PR-8) was used as a negative control. Transcripts of the HA gene, approximately 500 nucleotides, were generated in a similar fashion using SP6 polymerase from a construct described previously (Grieder *et al.,* 1995). Probes were hybridized to serial 3-μm tissue sections mounted on ProbeOn Plus slides (Fisher Scientific) from the same

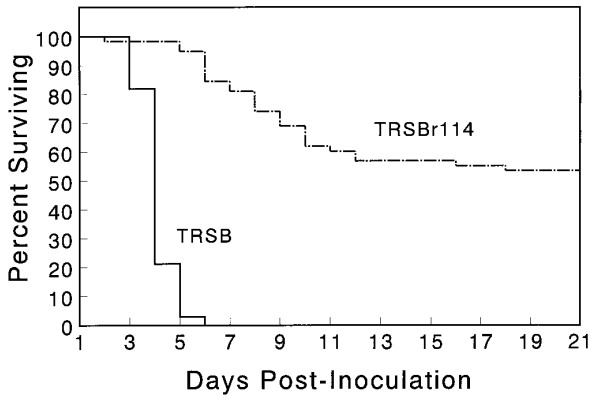


FIG. 1. Survival of neonatal CD-1 mice inoculated with TRSB or TRSBr114. Mice were inoculated sc with 1000 PFU of TRSB ($n = 33$; solid line) or TRSBr114 ($n = 58$; interrupted line) and observed for 21 days.

paraffin blocks sectioned for histopathological analysis. Hybridization and wash conditions were described previously (Grieder *et al.,* 1995). Slides were dipped in Kodak NTB-2 emulsion, dried at room temperature, and exposed at −20° for 3–5 days. Slides then were developed and counterstained with Gill's hematoxylin. Photomicrographs were taken on a Zeiss Axiovert-10 photomicroscope. As controls, tissues from virus-infected animals were incubated with HA-specific probe, and tissues from mock-infected animals were incubated with virus-specific probe. These controls were uniformly negative, indicating the specificity of the *in situ* hybridization (ISH) signal.

RESULTS

The pathogenesis of two Sindbis strains was examined in the neonatal mouse model. One virus was TRSB,

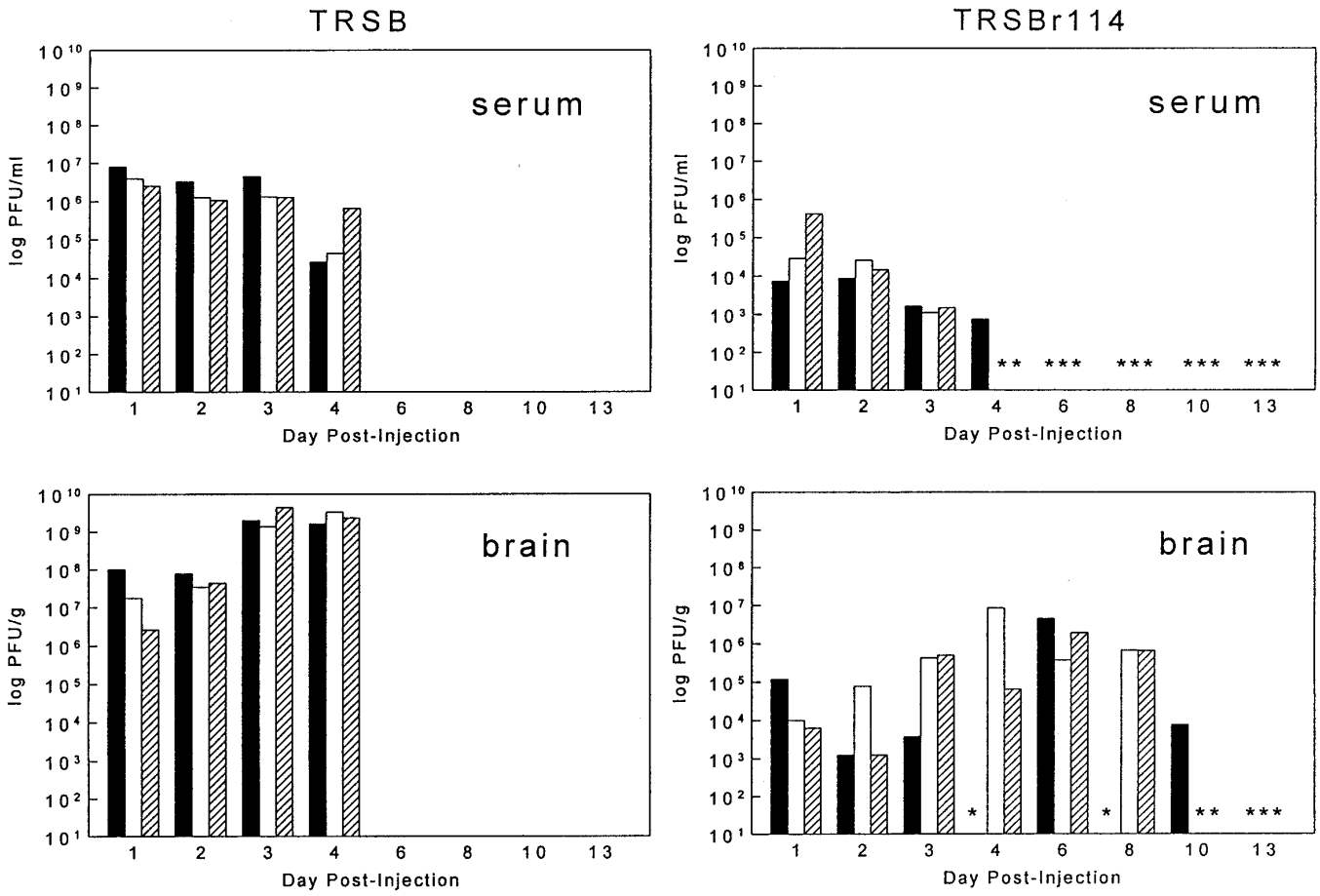
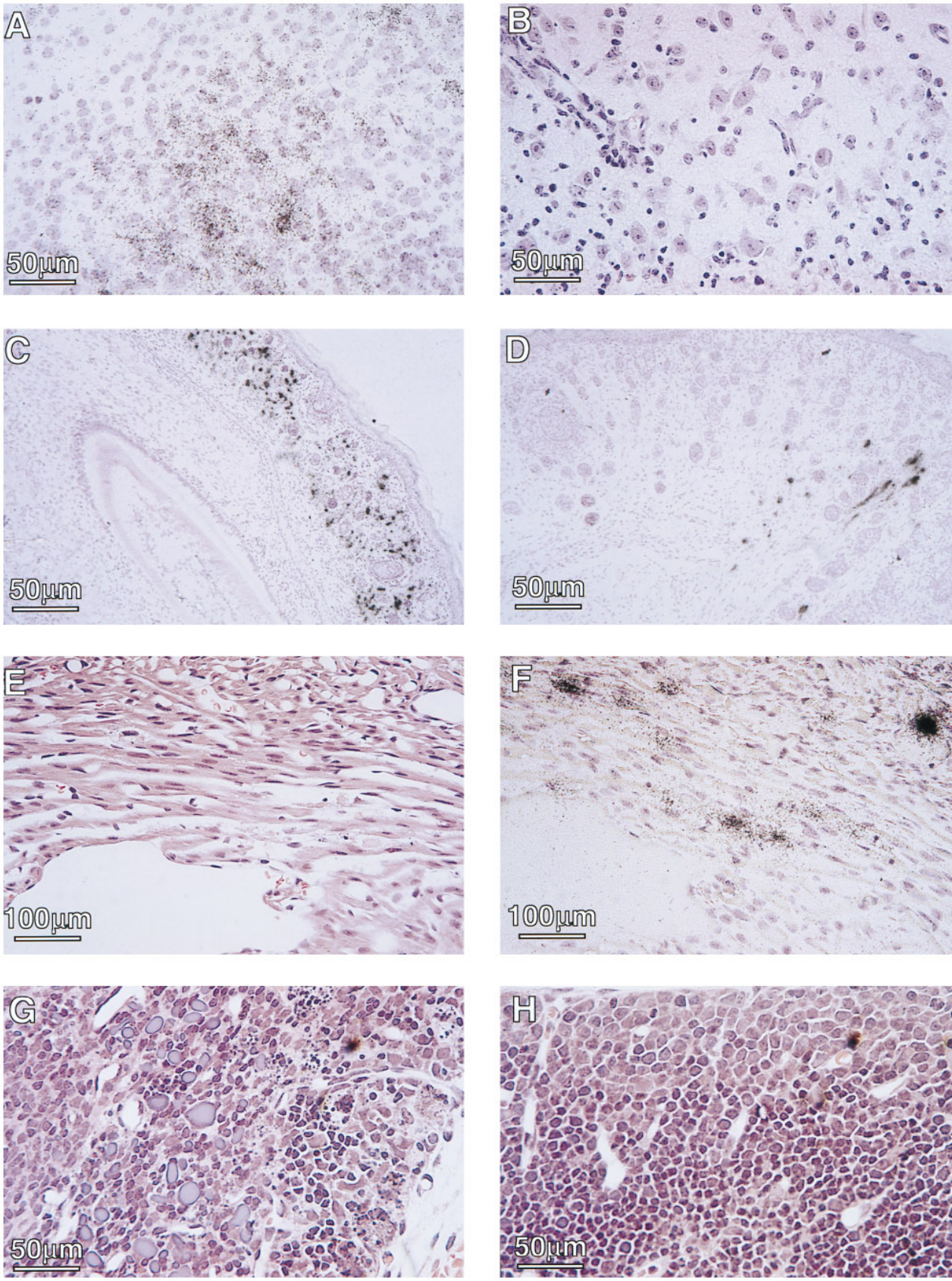


FIG. 2. Virus growth (PFU/ml) in neonatal CD-1 mice. Mice were inoculated sc with 1000 PFU of virus 12–24 hr after birth. Each bar indicates data from one mouse (three mice per time point). Asterisks indicate that levels were below the limit of detection (125–500 PFU/ml or PFU/g).

FIG. 3. Histopathological and ISH analysis of infected mice. (A) TRSB, ISH of brain, Day 4 p.i., showing extensive positive signal over intact immature neurons. (B) TRSBr114, H&E of brain Day 12 p.i., showing inflammatory cell infiltrates and perivascular cuffs characteristic of encephalitis. (C) TRSB, ISH of skin, Day 3 p.i., showing extensive virus replication in this tissue. (D) TRSBr114, ISH of skin, Day 3 p.i., showing restricted replication compared to TRSB in skin. (E) TRSB, H&E of heart, Day 4 p.i., showing characteristic lesions of coagulative necrosis. (F) TRSB, ISH of heart, Day 4 p.i., a tissue section adjacent to E, showing virus replication is associated with tissue damage. (G) TRSB, H&E of thymus, Day 3 p.i., showing severe thymic involution. (H) TRSBr114, H&E of thymus, Day 4 p.i., showing no histopathological changes.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our laboratory strain of Sindbis isolate AR339 derived from its cognate molecular clone, pTRSB (McKnight *et al.,* 1996). The other was TRSBr114, derived from a clone which differs from pTRSB only by an Arg for Ser substitution at the codon for E2 amino acid 114. Therefore, there are two major experimental variables in these studies: one, the genetic differences between TRSB and other laboratory strains of Sindbis used in previous pathogenesis experiments, and two, the specific E2 substitution which distinguishes TRSB from TRSBr114.

Virulence in neonatal CD-1 mice

The virulence of TRSB and TRSBr114 was determined in neonatal CD-1 mice at an sc dose of 1000 PFU, the dose used in the comparative pathogenesis studies reported below. The arginine at E2 position 114 had a dramatic effect on virulence. As shown in Fig. 1, none of the mice inoculated with the virulent TRSB survived infection, and the AST was 3.0 $\pm$ 0.7 days. In contrast, 57 of 58 mice inoculated with TRSBr114 survived beyond Day 4. The AST of the mice which succumbed to TRSBr114 infection was 7.5 $\pm$ 3.4 days, and the mortality was 46%, consistent with the previously reported attenuating effect of the E2 Arg 114 mutation at lower doses (Olmsted *et al.,* 1984; Davis *et al.,* 1986; Polo *et al.,* 1988; McKnight *et al.,* 1996).

Virus replication *in vivo*

Virus titers were compared in the sera and brains of neonatal CD-1 mice infected with TRSB or TRSBr114. Mice were inoculated sc with 1000 PFU of either virus. Three infected animals from each group were sacrificed at each time point, and brains and sera were collected for virus titration (Fig. 2). TRSB-infected animals were sacrificed daily for 4 days. TRSBr114-infected mice were taken daily for 4 days as well as on Days 6, 8, 10, and 13.

TRSB virus titers reached high levels in both sera (over 10^6 PFU/ml) and brains (10^6–10^8 PFU/g) by 24 hr postinoculation. Serum viremia persisted throughout the course of disease, and declined somewhat by Day 4. In the brains, however, virus titers continued to increase until the day of death, reaching peak titers of over 10^9 PFU/g.

In contrast, TRSBr114 titers were lower in both sera and brains compared to TRSB. Serum titers were 1–3 orders of magnitude lower than TRSB serum titers, with maximal levels of 10^5 PFU/ml on Day 1. Serum titers then declined, and virus was undetectable in serum by Day 6. TRSBr114 brain titers were 2–5 orders of magnitude lower than those observed in TRSB infection. TRSBr114 brain titers rarely exceeded 10^6 PFU/g (Days 4–6), and virus was undetectable by Day 13. Therefore, TRSBr114 was restricted for growth both in peripheral tissues (as indicated by serum titers) and in the CNS of newborn mice.

Histopathological and *in situ* hybridization analyses

Histopathological and *in situ* hybridization analyses were performed on tissues from TRSB- and TRSBr114-infected mice to determine (1) morphological changes during infection with each of these cloned viruses and (2) whether the arginine at E2 114 altered the targeting and/or extent of virus spread.

The central nervous system: Days 1–4. Consistent with high virus titers, ISH analysis of brains and spinal cords from TRSB-infected animals revealed extensive positive signal over intact immature neurons (Fig. 3A). However, compared to mock-infected control mice, very few morphological changes were observed in the CNS of either TRSB- or TRSBr114-infected mice during the first 4 days of infection, an interval in which most TRSB-infected mice died. Mild, nonspecific changes were observed including subtle neuronal dropout, mild meningeal inflammation, and scattered pyknotic cells. Occasional focal spongiform changes were detected in the cerebellum and spinal cord in both infections on Day 4 only. Apoptotic neurons, as judged by morphological criteria, were noted in infected brains. However, with the possible exception of individual cells in the hippocampus of TRSB-infected mice, the distribution and frequency of neurons showing morphological changes consistent with apoptosis (nuclear condensation/pyknosis without inflammation) were indistinguishable from mock-infected mice. No evidence of encephalitis or delay in brain development was observed during the first 4 days in either virus infection.

Peripheral tissues: Days 1–4. Although minimal lesions were observed in the CNS of TRSB-infected mice, severe damage was found in peripheral tissues, most notably in the dermis of the skin, skeletal muscle, and fibroblast connective tissue. Necrosis and necrobiosis of dermis and necrosis of subcutaneous muscle without inflammation (or associated with only occasional polymorphonuclear lymphocytes) was extensive and tended to be most severe at the snout and perianal regions. The severity of lesion and density of ISH signal in the extremities of the mouse may suggest the influence of temperature on the replication of Sindbis virus *in vivo.* Diffuse necrosis of muscle, especially the interstitium, and fibroblast connective tissue throughout the animal was observed, as was focal coagulative necrosis of brown fat. These lesions were always associated with positive virus signal by ISH analysis. Both lesion severity and ISH signal intensity tended to increase as the infection progressed, and ISH signal was often confluent in muscle and connective tissue by Day 4.

Identical tissues were affected in TRSBr114 infection, and these also were associated with virus-specific ISH signal. However, both lesion and ISH signal were consis-

tently more limited in scope and severity than those associated with TRSB (Figs. 3C and 3D).

Numerous visceral organs were capable of supporting Sindbis replication. Positive ISH signal associated with scattered focal necroses without inflammation was observed in the hearts of TRSB-infected animals (Figs. 3E and 3F). Similar morphological changes and virus distribution were detected in the muscularis and submucosae of the gastrointestinal tract (with relative sparing of the mucosae) as well as the muscular coat of the bladder and ureter. Occasional positive virus signal without apparent lesion was detected in lung and kidney.

The identical pattern of tissue and organ involvement characterized TRSBr114-infected mice, but again lesions were less severe, less extensive, and tended to be more focal in nature. Except for sparse positive signal in the capsular regions, the liver, spleen, and pancreas exhibited no apparent lesions and were not associated with replication of either virus as judged by ISH.

In addition to these quantitative differences, there were several striking qualitative differences between TRSB- and TRSBr114-infected mice which suggested the induction of a systemic stress response by the more virulent virus. Relative to control mice and TRSBr114-infected mice, a marked decrease in hematopoiesis in bone marrow and extramedullary hematopoiesis in liver were observed in TRSB-infected animals. Additionally, TRSB infection was characterized by severe thymic involution, primarily involving the cortex. Thymocyte depletion, acute lympholysis, and a loss of cortical–medullary demarcation were first observed on Day 2 postinoculation and persisted until death. The lesions were never associated with positive virus signal by ISH and were unique to TRSB infections (Figs. 3G and 3H). Such thymic changes are typical of steroid-mediated involution resulting from glucocorticoid (corticosterone) release during a severe stress response. In a separate study, lipid depletion of the adrenal cortex also was observed in TRSB-infected mice, and this may reflect metabolic changes consistent with glucocorticoid production. In preliminary studies (data not shown) thymic changes were limited in TRSB-infected mice treated with mifepristone, a receptor antagonist of glucocorticoids, indicating a glucocorticoid-mediated mechanism of thymic involution.

TRSBr114: Days 6–14. In contrast to TRSB infection, virtually all TRSBr114-infected mice survived beyond Day 4, and uniformly progressed to develop encephalomyelitis. Beginning as early as Day 4, reactive changes in capillary endothelial cells of the brain were noted. By Day 6, characteristic inflammatory changes were apparent including perivascular cuffing, infiltration of leukocytes into the parenchyma of the brain and spinal cord, degeneration of the neuropil, and neuronal necrosis. Prominent multifocal necrotic lesions associated with inflammatory cells were evident between Days 6 and 12 throughout the brain and spinal cord with lesions ranging from very mild to severe (Fig. 3B). CNS lesions began to resolve on approximately Day 13.

While virus could not be detected by titration in serum beyond Day 6, ISH analysis revealed positive foci, primarily in muscle, as late as Day 12. These foci were associated with leukocyte infiltrates, which were first noted in muscle 5 days postinoculation. The development of myositis and encephalitis corresponded to the onset of paralysis in TRSBr114-infected mice.

Virus replication in cell culture

Single-step growth experiments were performed in both established and primary cells to determine whether the more focal nature of TRSBr114-induced lesions and the lower virus titers observed reflected a general defect in replication in murine cells. The E2 Arg 114 mutation originally was observed in virus populations selected for rapid growth on BHK cells. In Sindbis genetic backgrounds other than TRSB, E2 Arg 114 specified a reduced latent period and growth to higher titer in BHK cells (Davis *et al.,* 1986; Polo and Johnston, 1990). Additional growth experiments were performed in murine Swiss 3T3 cells, primary murine cardiac fibroblasts, and Neuro 2A cells, a murine neuroblastoma cell line. The results are depicted in Fig. 4 and indicate that in contrast to *in vivo* virus titers, TRSBr114 replicated to similar or higher titers than did TRSB in both established and primary murine cells. This result appeared to be independent of m.o.i., as analogous results were obtained at m.o.i.s of 0.1 and 10 in cardiac fibroblasts and 10^{-4} in Neuro 2A cells (data not shown). These data suggest that the attenuated phenotype of TRSBr114 was not due to a general defect in the ability of TRSBr114 to replicate in cells of murine origin.

In cardiac fibroblasts, the production of IFNα/β was measured after infection with the two viruses. IFNα/β induction was proportional to virus growth regardless of virus or m.o.i. (data not shown), suggesting that neither virus was more efficient in the induction of IFNα/β in these cells.

Induction of IFN$\boldsymbol{\alpha/\beta}$ *in vivo*

One possible explanation for the discrepancy between relative virus titers observed *in vivo* and *in vitro* between TRSB and TRSBr114 is that these viruses may differ with respect to the establishment of, or sensitivity to, an IFN-induced antiviral state in the newborn mouse. To address this possibility, IFNα/β titers in sera and brains of infected animals were compared during infection. Mice were inoculated as described previously and sacrificed at the time points indicated in Fig. 5 (two mice per time point). Brains and sera were harvested and assayed for virus and IFNα/β by biological assay.

Peak serum IFNα/β titers were observed 24 to 48 hr

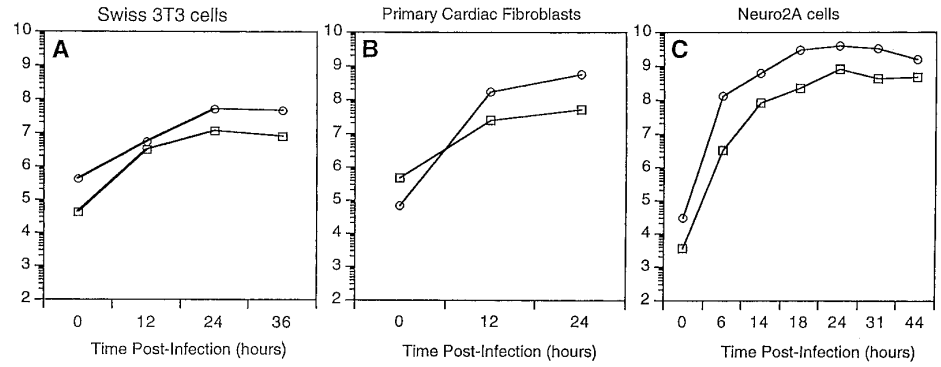

FIG. 4. Growth of TRSB (squares) or TRSBr114 (circles) in cultured cells. (A) Swiss 3T3 murine fibroblasts, m.o.i. = 5. (B) Primary murine cardiac fibroblasts, m.o.i. = 1. (C) Neuroblastoma cells, m.o.i. = 1.

postinoculation (p.i.) in both TRSB- and TRSBr114-infected mice, but much higher levels were induced by the virulent virus (215,000 IU/ml vs 1600–2100 IU/ml for TRSB and TRSBr114, respectively). The two infections also differed in the kinetic relationship between virus growth and induction of IFNα/β. In TRSB-infected animals, significant serum virus titers were first detected at 8 hr p.i., substantially preceding first detection of IFNα/β at 16 hr. By 16 hr, TRSB already had attained a titer close to its peak, in excess of 10^7 PFU/ml. Therefore, TRSB may simply outrun the IFNα/β response by seeding most infectable cells before they can enter an antiviral

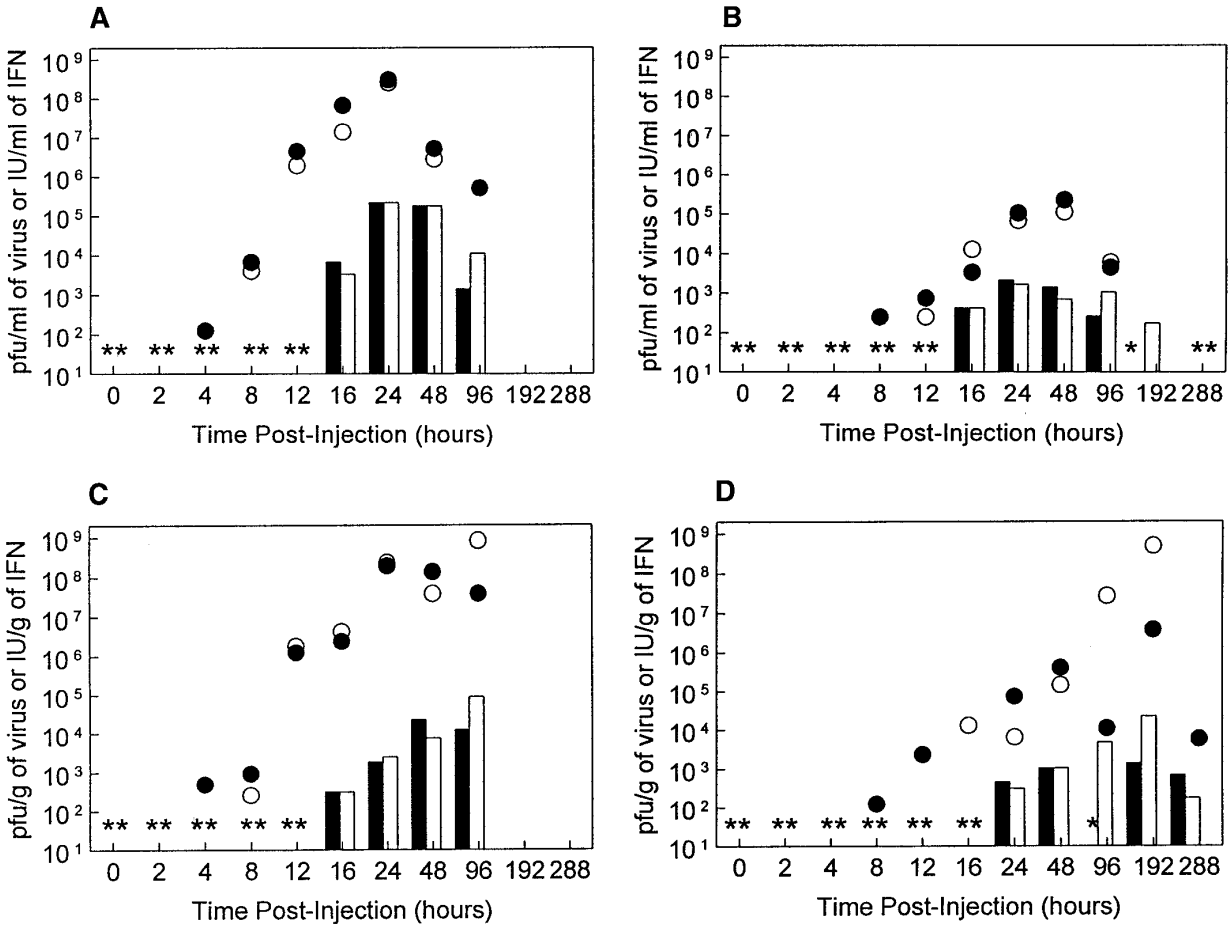

FIG. 5. Virus and IFNα/β titers in sera (A and B) and brains (C and D) of mice inoculated with TRSB (A and C) and TRSBr114 (B and D). Levels of virus (circles) and IFNα/β (bars) were determined from the same animal (two per time point). Replicate mice are distinguished by open or filled circles and bars. Asterisks indicate IFNα/β levels were below the limit of detection (200–400 IU/ml or IU/g). The limit of detection for virus was 125 PFU/ml or PFU/g. These data are from one of three replicate experiments.

refractory state induced by IFNα/β. This hypothesis would explain the subsequent induction of very high levels of IFNα/β and the failure of even this massive response to prevent lethal infection. In contrast, the mutation resident in TRSBr114 slowed its replication *in vivo* and delayed its appearance as a serum viremia. Consequently, the timing of the IFNα/β response relative to virus growth was more appropriate and effective in the case of TRSBr114. In animals treated with antibody to IFNα/β, 100% mortality was observed after TRSBr114 infection (data not shown).

DISCUSSION

Alphaviruses closely related to Sindbis strain AR339 cause an acute febrile illness in humans which is characterized by severe headache, skin rash, and a polyarthralgic syndrome that can linger from months to years (Johnston and Peters, 1996). In the neonatal mouse model, Sindbis infection is lethal, accompanied by growth of virus to high titers in the brain. Because of the permissiveness of murine CNS tissue for Sindbis growth, it is natural that experimental studies of Sindbis pathogenesis should focus on the brain. However, as the present results emphasize, the pathogenesis of Sindbis is far more complex and includes important changes peripheral to the CNS which influence the ultimate pathogenesis of the infection.

TRSB infection in neonatal mice inoculated sc was characterized by minimal pathological changes in the brain; extensive replication and lesions in skin, muscle, and connective tissue; massive interferon induction; and dramatic involution of the thymus in the absence of virus replication in this organ. Even though TRSB replicated extensively in the brain, as evidenced by high titer and *in situ* hybridization signal, there was no evidence of encephalitis. Very few histopathological changes were evident in the brain, even at times shortly preceding death. The absence of lesions in the CNS, or mild nonspecific changes, has also been noted in previous studies in neonatal mice with related Sindbis isolates (Taylor *et al.,* 1955; Mahlerbe *et al.,* 1963; Johnson, 1965; Johnson *et al.,* 1972). It is certainly possible, however, that TRSB induces alterations in neuronal function without apparent changes in cell morphology at the level of light microscopy. Virus-induced alterations of homeostasis in the absence of morphological changes have been described in the lymphocytic choriomeningitis virus (LCMV) system. LCMV infection of the anterior pituitary led to an alteration in growth hormone levels without lysis or cytopathology of the infected cells (Oldstone *et al.,* 1984).

One must also consider the possibility that direct toxic effects of the very high levels of IFNα/β (or other cytokines) induced during TRSB infection could contribute to the disease. Neurotoxic effects have been observed in humans during IFN therapy (Adams *et al.,* 1984; Meyers *et al.,* 1991), as well as in LCMV-infected (Riviere *et al.,* 1977, 1980) and uninfected newborn mice (Gresser *et al.,* 1975). Furthermore, IFN-α alters single-cell activity in neurons of several regions of the brain when administered microiontophoretically (Dafny *et al.,* 1985; Saphier *et al.,* 1994) and can exhibit opiod activity (Blalock and Smith, 1981; Saphier *et al.,* 1994).

TRSB-infected neonatal mice showed clear evidence of a systemic stress response characterized by a marked decrease in bone marrow and extramedullary hematopoesis as well as a dramatic thymic involution in the absence of viral replication in this organ. These changes represented a major qualitative pathological difference between infections initiated by TRSB and the attenuated mutant. In other disease models, such a response is most often mediated by glucocorticoid release, and preliminary measurements of corticosterone levels in sera of TRSB-infected mice were consistent with induction of a systemic stress response (Trgovcich *et al.,* manuscript submitted). Similar histopathological findings have been noted previously in studies of Sindbis, S.A. AR86, and eastern equine encephalitis virus infections (Mahlerbe *et al.,* 1963; Yang, 1985; Guy *et al.,* 1993), and artificial induction of a stress response in adult mice exacerbates Sindbis-induced disease (Ben-Nathan *et al.,* 1991). These observations support the hypothesis that peripheral replication and tissue damage may be a primary factor in the virulence of Sindbis strains and that host cytokine and hormonal responses may contribute significantly as proximal causes of early TRSB lethality.

While TRSBr114 replicated in the same tissues as TRSB, replication of this mutant was much more focal, and the associated pathology was much less severe. These quantitative differences were clearly a consequence of the E2 Arg 114 mutation, as this is the only difference between the pTRSB and the pTRSBr114 clones (McKnight *et al.,* 1996). Involvement of the same tissues and organs in both infections suggests that the impairment of TRSBr114 replication was not due to altered tissue targeting mediated by the mutant E2 glycoprotein, but rather an effect that limited the rate of virus maturation and/or spread of the mutant within the affected tissue. Equivalent or increased growth of TRSBr114 relative to TRSB in cultured cells of murine origin suggested that the TRSBr114 defect did not cause a restriction for growth in murine cells generally, but may relate to a growth restriction in specific cells within the animal. In addition to restriction of TRSBr114 *in vivo* due to the mutation, the timely induction of IFNα/β, relative to the kinetics of TRSBr114 replication, also could have acted to limit virus growth and spread.

The single amino acid substitution at E2 114 not only reduced virulence significantly, but also completely altered the course of disease. Animals infected with the virulent virus were characterized by early thymic involution and the absence of encephalitis, whereas mutant-

infected animals evidenced no thymic changes but showed classic features of encephalitis, presumably due to appropriate immune responses to virus replication in the brain. As glucocorticoid release and thymic lesions are often associated with immunosuppression (Munck *et al.,* 1984), the absence of encephalitis in TRSB-infected mice could be explained by the failure to mount a virus-specific cellular immune response. Alternatively, early TRSB-induced lethality may have precluded survival of infected animals for a time sufficient to develop encephalitis. In the case of TRSBr114, thymic involution was not observed, mortality was reduced, and survival was extended for those animals which did die. Consequently, TRSBr114-infected mice may have developed encephalitis because they remained immunocompetent and/or the mutation allowed survival until a significant encephalitis could be established.

Ironically, these considerations suggest that encephalitis may be characteristic of Sindbis strains carrying partially attenuating mutations. Only in the presence of such mutations would the early pathology be abrogated, allowing infected animals to progress to the later development of encephalitis. Cell-culture-propagated laboratory derivatives of Sindbis strain AR339, and clones derived from them, contain a variable number and pattern of attenuating mutations relative to the Sindbis consensus sequence (McKnight *et al.,* 1996). TRSB contains fewer of these changes than other laboratory strains, based on the available sequence information, and therefore, it may retain sufficient virulence to invoke the early pathology we have observed. This may explain the discrepancies between the results presented here and those reported by others (Sherman and Griffin, 1990; Levine *et al.,* 1993).

In summary, we present the following as a working hypothesis to explain TRSB pathogenesis in neonatal mice. The skin, muscle, and connective tissues are the primary extraneural sites of TRSB replication and exhibit severe virus-associated damage. The rapid spread of TRSB in these tissues leads to the induction of very high levels of IFNα/β and presumably other cytokines. The cytokine response, however, is unable to contain the infection, as the virus appears to outrun the nonspecific host responses to it, leading to even more vigorous cytokine induction. A systemic stress response mediated by glucocorticoids is triggered and results in the severe thymic involution observed. The lack of encephalitis or significant CNS pathology may be explained by the death of the animal before encephalitis can develop and/or by immunosuppression due to the stress response which prevents the development of encephalitis. The attenuating mutation at E2 114 reduces the capacity of the virus to replicate in target tissues, thus allowing a more timely and effective IFNα/β response relative to the kinetics of virus replication. With TRSBr114 replication restricted, the potentially fatal manifestations associated with the induction of high cytokine levels and the stress response are prevented. The resulting extended survival and/or preservation of an intact specific immune response allow the later development of encephalitis. While encephalitis is associated with survival in most of TRSBr114-infected animals, it is likely the cause of death in those animals which do succumb to this infection.

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