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TNFα, Interferon, and Stress Response Induction as a Function of Age-Related Susceptibility to Fatal Sindbis Virus Infection of Mice

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The age-related acquisition of resistance to fatal Sindbis virus infection was examined using a molecularly cloned laboratory strain of the AR339 isolate designated TRSB. TRSB caused 100% mortality in mice up to 5 days of age. Resistance to fatal infection developed abruptly between 5 and 9 days of age. Lethal Sindbis virus infection of mice inoculated at 4 days of age was characterized by high levels of virus replication, induction of high levels of interferon-α/β and TNF-α and severe thymic involution indicative of a systemic stress response. These changes correlated with predominantly noninflammatory lesions. In contrast, TRSB infection of older mice was characterized by survival, more limited virus replication, reduced cytokine induction, and the development of inflammatory responses leading to encephalitis, myositis, and myocarditis. Previous studies utilized infections of neonatal mice with TRSB and an attenuated mutant of TRSB to compare fatal and nonfatal Sindbis infection (Trgovcich *et al.,* 1996. *Virology* 224, 73–83). The experiments reported here utilize mouse age at the time of infection to create conditions for examination of fatal and nonfatal TRSB infections. Both experiments suggest that fatal infection is associated with a shock-like syndrome and little or no inflammatory pathology, while survival is correlated with greatly reduced cytokine levels and inflammatory lesions.

INTRODUCTION

The development of resistance to lethal neurotropic viral infections during the first few weeks of life has been well known for over 50 years (O'Leary *et al.,* 1942; Lennette and Koprowski, 1944; Sigel, 1952). Host developmental changes implicated in the acquisition of resistance in murine models have included maturation of immune responses, development of anatomic barriers, potentiation of interferon responses, and changes in receptor availability (Sabin, 1941; Overman and Kilham, 1953; Kunin, 1962; Heineberg *et al.,* 1964; Weiner *et al.,* 1970; Johnson *et al.,* 1972). More recently, maturation of neuronal cells has been implicated as the primary factor in the development of resistance to several viruses (Levine *et al.,* 1993; Oliver and Fazakerly, 1998; Ogata *et al.,* 1991). Age-related susceptibility also has been described in human infections with alphaviruses commonly associated with encephalitis, such as Eastern, Western, and Venezuelan equine encephalitis viruses. Both the severity of clinical disease and the risk of sequelae are increased in children and infants compared to adults (Johnston and Peters, 1996).

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.

Sindbis virus pathogenesis in humans is usually associated with fever and arthropathy (Johnston and Peters, 1996). In mice, this virus has been utilized extensively to investigate viral infections of the central nervous system with death in neonatal animals often ascribed to a fatal encephalitis (Johnson, 1965; Sherman and Griffin, 1990). Previously, we described the pathogenesis in neonatal mice of a molecularly cloned laboratory strain of the Sindbis AR339 isolate (designated TRSB) and an isogenic single-site attenuated mutant (Trgovcich *et al.,* 1996, 1997). Compared to other laboratory Sindbis strains derived from AR339, TRSB is considerably closer to the consensus Sindbis AR339 sequence (McKnight *et al.,* 1996). Infection of neonates with the virulent TRSB caused 100% mortality but did not cause encephalitis. Rather, infection with this virus was characterized by high virus titers in serum and brain, induction of potentially toxic levels of interferon alpha/beta (IFNα/β) and TNF-alpha (TNFα), and a severe stress response, as indicated by thymic involution, loss of hematopoietic centers in liver, and high levels of corticosterone (CORT).

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These responses were absent or minimal in infection with the attenuated mutant, which was characterized by extended survival, decreased mortality, and the development of encephalitis and myositis. Therefore, in the case of TRSB and its closely related attenuated mutant, inflammatory pathology was associated not with fatal illness but with reduced virulence and survival.

Infection with other laboratory strains of Sindbis has been characterized in both neonatal and weanling mice. Infection with most laboratory strains of Sindbis is uniformly fatal in neonatal animals (Sherman and Griffin, 1990; Trgovcich *et al.,* 1996). In older mice, Sindbis virus infection causes a nonlethal subclinical encephalitis characterized by an inflammatory reaction and scattered focal necroses (Jackson *et al.,* 1987; Johnson and MacFarland, 1972). Resistance to lethal infection with Sindbis develops rapidly in the second week of life. Previous studies indicated that resistance to lethal infection in older animals is related to reduced efficiency of virus replication and spread in tissues which was independent of the maturation of either the interferon system or the classical immune response (Reinarz *et al.,* 1971; Johnson and MacFarland, 1972; Griffin, 1976). Lower levels of replication in older mice have been related to resistance to infection of fibroblasts derived from older compared with neonatal animals (Johnson and MacFarland, 1972). Increasing expression of the *bcl-2* family of cellular oncogenes in brain also has been postulated to account for survival of older mice by limiting virus-induced apoptosis of neuronal cells, leading to the establishment of a persistent neuronal infection (Levine *et al.,* 1991, 1993; Lewis *et al.,* 1996).

In light of these findings, the age-related acquisition of resistance to TRSB infection in the mouse model was investigated. Specifically, histopathological correlates of virulence were examined as a function of mouse age at the time of inoculation in an effort to determine whether resistance to lethal infection correlated with attenuation of host cytokine and hormonal responses. Analogous to comparing infections with virulent and attenuated strains of Sindbis, examination of TRSB infection in mice of different ages allowed us to assess the pathological correlates of fatal versus nonfatal infection. The results presented here define the progression of disease as it varies from a fatal infection associated with high levels of cytokine induction and thymic involution to a benign infection characterized primarily by encephalitis and associated immune-mediated pathology.

RESULTS

Age-related susceptibility to TRSB infection

To assess age-related susceptibility to lethal infection with TRSB, groups of two litters of mice each were injected with 1000 plaque-forming units (PFU) of TRSB subcutaneously (sc) or intracerebrally (ic). Mice were inoculated on day 1 and every other day until they were 15 days of age. Consistent with previous findings (Trgovcich *et al.,* 1996), sc inoculation of TRSB induced 100% mortality in 1-day-old mice, with an average survival time (AST) of 3.5 ± 0.5 days (Table 1). Of mice inoculated up to 5 days of age 100% succumbed to infection, though ASTs were slightly extended. By 7 days of age, TRSB was no longer uniformly fatal; 70% mortality and an AST of 8.5 ± 1.4 days were observed. Resistance to lethal TRSB infection developed rapidly and only 5% mortality was observed in mice inoculated 9 days after birth. A similar pattern of resistance developed with regard to ic inoculation, but was slightly delayed compared to the sc route. Sixty-four percent mortality was observed in mice infected at 9 days of age, whereas all animals inoculated at 15 days of age survived infection.

TABLE 1

Mortality and Average Survival Time as a Function of Age at Inoculation[a]

Age at inoculation (days)	Subcutaneous inoculation		Intracerebral inoculation	
	Percent mortality	AST (days)	Percent mortality	AST (days)
1	100	3.5 ± 0.5	100	3.2 ± 0.4
3	100	5.8 ± 0.8	100	4.9 ± 1.0
5	100	6.7 ± 0.8	100	6.1 ± 1.3
7	70	8.5 ± 1.4	87	7.3 ± 2.1
9	5	7[b]	64	6.4 ± 1.6
11	0		21	8.6 ± 3.6
13	0		7	4[b]
15	0		0	

[a] Mice of the indicated age were inoculated with 1000 PFU of virus in 50 μl (subcutaneous) or 25 μl (intracerebral) and were observed for 21 days.

[b] Only one mouse died.

All survivors were challenged with S.A.AR86, an alphavirus closely related to Sindbis which elicits 100% mortality in adult mice inoculated ic. As expected, all mock-infected control mice succumbed to this infection, whereas all test animals which survived TRSB infection also survived challenge with S.A.AR86, indicating that they had been actively infected and had developed protective immunity (data not shown).

Virus replication *in vivo*

To begin to investigate the parameters of infection which distinguish lethal from nonlethal infection, virus replication was measured in mice inoculated sc at 4, 6, 8, and 10 days of age. In this experiment (Fig. 1), three mice were sacrificed at the indicated times, and virus titers were determined in serum and brain of individual mice.

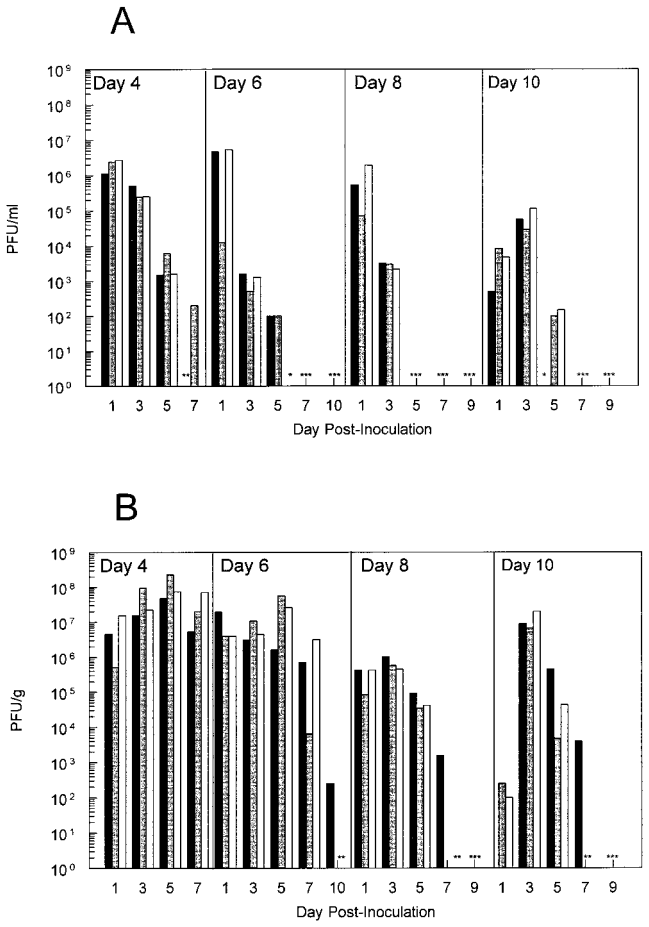

FIG. 1. Virus growth in CD-1 mice of increasing age. Mice were inoculated sc at 4, 6, 8, or 10 days after birth with 1000 PFU of TRSB in 50 μl. Each bar indicates data from one mouse (three mice per time point). (A) Serum; (B) brain. Asterices indicate that levels were below the limit of detection (ranging from 50 to 200 PFU/g or PFU/ml in individual assays).

Serum titers

At one day p.i., serum titers exceeded 10^6 PFU of virus/ml of serum in animals inoculated 4 days postpartum (p.p.), an age at which infection was invariably fatal. These levels fell only slightly by 3 days postinfection (p.i.), but viremia was undetectable in two of three mice by 7 days p.i. In this experiment, 40% mortality was noted in mice inoculated at 6 days of age. A distinguishing observation in this age group (mice inoculated at day 6 p.p.) was that serum virus titers dropped more rapidly and were cleared earlier than those in younger mice. At 3 days p.i., just over 10^3 PFU/ml of serum was observed, and serum virus titers were undetectable in all mice by 7 days p.i. Similar results were noted in animals inoculated at 8 days of age. However, consistent with a lower mortality (11%), serum titers fell even more abruptly and were below the limit of detection by 5 days p.i. Mice inoculated at 10 days of age uniformly survived infection and presented with a different serum viremia profile. Peak titers were observed at day 3 rather than day 1 p.i. and never exceeded 10^5 PFU/ml.

Brain titers

Brain virus titers generally correlated inversely with increasing age at time of inoculation. In mice inoculated at 4 days of age, levels in brain ranged from 5×10^5 to 2×10^7 PFU/g on day 1 p.i. In contrast to serum titers, brain titers continued to increase during the first 5 days of infection, reaching peak levels of 10^8 PFU/g. Mice inoculated at 6 days of age had titers similar to those of the younger animals on day 1 p.i. These levels did not increase, however, and began to decline by 7 days p.i. Virus titers in some samples fell below the detection limit at 10 days after inoculation. Brain titers in mice inoculated at 8 days of age were notably lower than those of younger mice. Starting below 10^6 PFU/g day 1, brain titers abated rapidly beginning day 5 p.i. and were undetectable in two of three mice by 7 days p.i. As observed in serum, mice inoculated 10 days p.p. presented with a strikingly different profile. At 1 day p.i., titers in brain were below 10^3 PFU/g. Peak levels were similar to those observed in younger mice at 3 days p.i., but diminished rapidly thereafter. In brain, then, the overall magnitude of replication as well as the persistence of high titers of virus appear linked with mortality in mice infected with TRSB at different ages.

TRSB-induced histopathological changes and virus spread as a function of age

Histopathological and *in situ* hybridization (ISH) analyses were performed on tissues from mice inoculated sc at 4, 6, or 8 days of age to investigate morphological changes and the extent of virus replication during infections which ranged from fatal to nonfatal in nature (Fig. 2). The results of these analyses demonstrate that as the animals age, the pathology changes from the shock-like stress response syndrome evident in fatally infected neonates to a nonfatal pathology characterized by extensive inflammatory infiltrates, encephalitis, and myositis.

Mice inoculated at 4 days of age. On day 1 p.i., no clear lesions in the CNS were detected despite the presence of sparse, but very intense, foci of virus-specific ISH signal. Changes in visceral organs included scattered microscopic foci of necrosis in heart, involving the interstitium of the myocardium with accompanying virus-specific ISH signal. Also, multifocal ISH signal was noted in gut, lung, and cortex of kidney of all mice without apparent lesion. Minor changes in skin were observed consisting of sporadic necrosis and necrobiosis of the dermis and coagulative necrosis of skeletal muscle. Virus-specific probe hybridized to a variety of mesenchymal tissues, especially dermis, skeletal muscle, connective tissue, brown fat, and tooth pulp.

The histopathological profile changed dramatically by

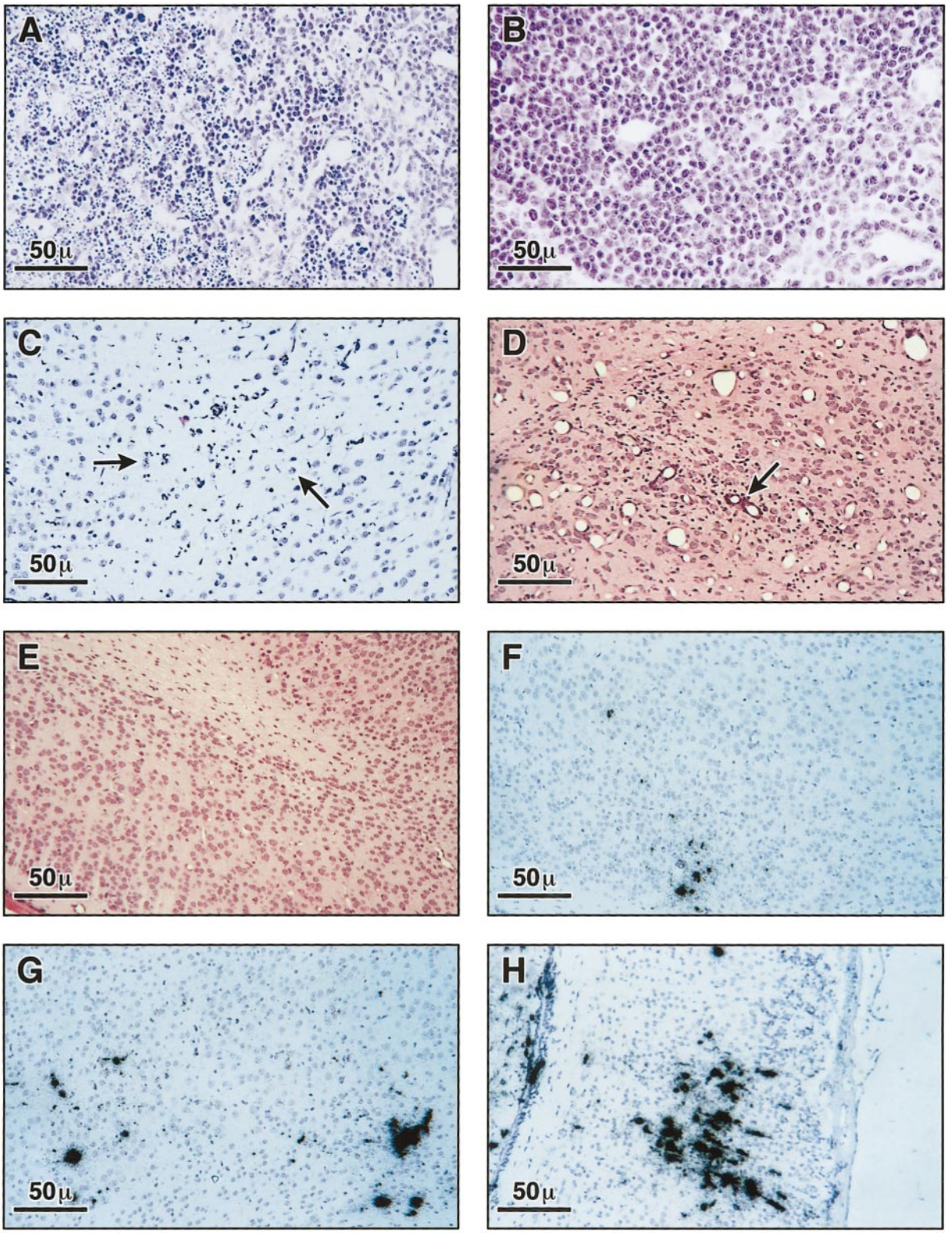

FIG. 2. Histopathological and *in situ* hybridization analyses of mice inoculated with TRSB at 4, 6, and 8 days of age. (A) H&E-stained section from a thymus of a mouse inoculated at 4 days of age and sacrificed 7 days p.i. showing extensive nuclear debris and depletion within the cortex characteristic of severe thymic involution. (B) H&E-stained section from a thymus of an age-matched mock-infected mouse showing normal thymic architecture. (C) H&E-stained section through the cerebral cortex of a mouse inoculated at 4 days of age and sacrificed 7 days p.i. Despite sufficient time for specific immune responses to develop, typical lesions were associated with either minimal or no cellular infiltrates. Arrows indicate neuronal dropout and neuronal pyknosis. (D) H&E-stained section through the midbrain of a mouse inoculated at 8 days of age and sacrificed 7 days p.i. with

3 days p.i. A distinguishing feature consistent in all tissues examined at this time was the lack of inflammatory changes. Necrotizing lesions without conspicuous accompanying inflammation were scattered throughout the brain. These changes corresponded with a dramatic increase in positive ISH signal (Fig. 2H).

Lesions in the visceral organs also had progressed considerably by 3 days p.i. In heart, changes ranged from individual necrotic cells to larger foci of coagulative necrosis. Again, this correlated with multiple positive foci of ISH signal. Additionally, focal lesions in the muscularis of the intestinal tract were observed, along with occasional positive signal by ISH. Of particular note was moderate to severe atrophy of the thymus cortex which was not associated with virus replication by ISH analysis (Figs. 2A and 2B).

Extensive necrosis in skin and muscle at 3 days p.i. was also observed. Changes in these tissues tended to be more severe at the snout and perianal regions of the mice. This was also described in the neonate and suggested a temperature-mediated effect on virus replication *in vivo.* Skeletal muscle lesions ranged from individual necrotic cells and degeneration of interstitium to severe, confluent necrotic lesions. ISH signal in these tissues also had evolved from day 1 p.i. and manifested as diffuse, confluent signal, multifocal punctate signal, and limited areas with large, intense foci. Mild, focal lesions of coagulative necrosis were noted in brown fat, as were limited positive foci of ISH signal.

Overall, the histopathological profile on days 5 and 7 p.i. was similar to that at 3 days p.i. in that severe, predominantly noninflammatory lesions were observed. Generally, lesions tended to be more severe and confluent in nature than those on day 3. In brains, these lesions were characterized by focal areas of pyknotic neuronal nuclei and neuronal dropout (Fig. 2C). Although widespread cellular infiltrates were not seen, occasional mononuclear inflammatory cells were apparent in the CNS and muscle.

Necrosis of skeletal muscle was especially severe by this time. By ISH analysis, essentially all muscle in the trunk of these mice was intensely positive. Signal was strikingly intense in the thoracic area and in the perianal area and tended toward a more diffuse pattern closer to the core of these mice. Coagulative necrosis of brown fat was also dramatic, in some cases involving an entire lobe of tissue.

In summary, morphological changes in animals inoculated at 4 days of age are very similar to those described previously in 1-day-old mice. Also as described previously (Trgovcich *et al.,* 1997), lesions were characterized by bland necrosis, and manifestations of a severe stress response were evident, such as thymic involution and loss of hematopoietic centers from liver. The lack of widespread inflammatory cell infiltrates even at 7 days p.i. suggests that some degree of immunosuppression may be a feature of TRSB infection in these mice.

Mice inoculated at 8 days of age. In contrast to mice inoculated at 4 days of age, mice inoculated at 8 days of age developed clear inflammatory lesions, especially in brain, heart, and skeletal muscle. Generally, in tissues other than muscle and the CNS, tissue damage and virus replication were more limited in scope than those described in younger mice. This corresponded with the absence of thymic lesions in mice inoculated at this age.

Similar to that described in younger mice on day 1 p.i., positive ISH signal was observed in numerous tissues and organs of the body and head, though no convincing lesions were evident. On days 3 and 5 p.i., a range of CNS lesions were observed. Some were clearly inflammatory in nature and were characterized by perivascular cuffing, with focal extrusion of inflammatory infiltrate into the parenchyma of the brain and necrosis. Some lesions, however, were characteristic of those seen in mice inoculated at 4 days of age and were associated with liquefactive necrosis and mild degenerative changes. By 5 days p.i., mild myelitis was noted in all spinal cords, and inflammatory changes of the meninges were apparent. ISH signal was primarily in foci and patches of foci and was noted in most areas of the brain and cord. Positive ISH signal was most prevalent in the cerebral cortex and presented as both focal and diffuse in this region. However, a clear restriction in virus distribution was observed by ISH analysis compared to mice injected at 4 days of age (Figs. 2F, 2G, and 2H).

Virus replication and lesions in extraneural sites were significantly reduced compared to those in mice inoculated at 4 days of age. However, mild myocarditis was observed in all animals examined at day 5 p.i. Other than minimal focal lesions of smooth muscle of the gut, no discernible lesions of visceral organs were apparent at this time. There was virus replication in the dermis of the skin and brown fat but no accompanying necrosis or inflammation. An interstitial infiltrate composed of lymphocytes and monocytes was associated with skeletal muscle.

In contrast to that observed in mice inoculated at 4 days of age, mild to frank encephalitis was evident in all brains examined 7 and 9 days p.i. (Fig. 2D). Lesions, both discreet and confluent in nature, were present through-

evidence of mild encephalitis. Arrow indicates reactive endothelial cells associated with cellular infiltration. (E) H&E-stained section of a mock-infected control mouse (inoculated at 4 days of age and sacrificed 7 days p.i.) showing no histopathological change. (F–H) *In situ* hybridization of virus-specific probe (black grains above tissue) in sections through the cerebral cortex of mice inoculated at 8, 6, and 4 days of age, respectively, and sacrificed 3 days p.i. This series depicts the restriction in distribution of viral nucleic acid in older mice at early times p.i.

out (with the possible exception of the olfactory bulb), but were most severe in the cerebral cortex. ISH signal in all brains ranged from sparse foci to confluent patches of foci. The diffuse ISH signal observed in younger mice was minimal or absent in these older animals.

Other than myocarditis associated with multifocal positive ISH signal, no other visceral organs were involved at 7 or 9 days p.i. Very few changes were noted in the dermis, but severe inflammatory lesions of coagulative necrosis were noted in muscle of all mice, sometimes involving entire muscle tracts. ISH signal manifested primarily as sparse foci of positive signal. Brown fat was minimally involved at this time. Thymus appeared normal by hematoxylin and eosin (H&E) staining, and no virus replication was evident by ISH.

The most consistent histopathological features of mice inoculated at 8 days of age were the development of encephalitis, myositis, and myocarditis beginning day 3 p.i. and increasing in severity during the course of infection. With the notable exceptions of the thymic and hematopoietic lesions observed in younger mice, identical tissues and organs were involved in the older animals. The pattern of ISH signal tended to be more focal and less extensive at later time points compared to that of mice inoculated at 4 days of age. This may reflect a fundamental difference in the ability of TRSB to spread in tissues of older mice. Thus, limited replication and the development of encephalitis and inflammatory responses correlated with survival in mice inoculated at 8 days of age.

Mice inoculated at 6 days of age. The histopathological profile of mice infected with TRSB at 6 days of age appeared transitional in that the primarily necrotic, noninflammatory lesions described in younger mice were diminished, as were thymic and hematopoietic lesions associated with a severe stress response. Concurrently, immune-mediated pathology, primarily in muscle and brain, dominated the spectrum of changes observed in these animals. In mice inoculated at 6 days of age, the spectrum of histopathological changes involved the same tissues and organs associated with infection in animals inoculated at 4 days of age, and the progression of lesion development followed closely that described in younger animals. However, similar to mice inoculated at 8 days of age, inflammatory changes were increasingly associated with lesions in these mice, and in individual animals, this correlated with less severe or absent thymic lesions.

Cytokine and endocrine responses correlate with virus replication

The cytokine profiles in 4- and 8-day-infected animals reflected the histopathological findings. Peak virus titers decreased and virus was cleared more rapidly with increasing age at the time of inoculation (Fig. 3), and

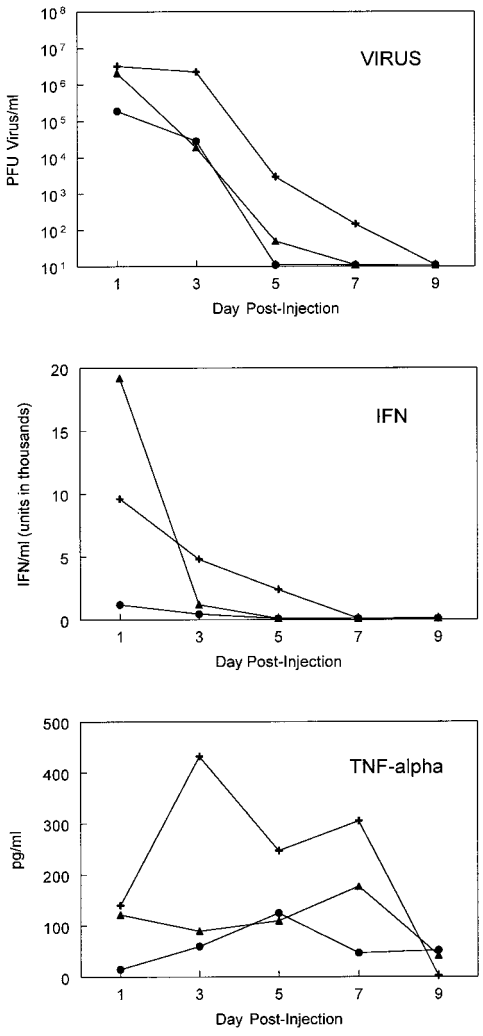

FIG. 3. Virus, IFNα/β, and TNFα in sera of TRSB-infected mice of increasing age. Titer analysis of pooled samples from mice inoculated at 4 (cross hairs), 6 (triangles), or 8 (circles) days of age. Mock-infected sera were below the following limits of detection for each assay: virus (50 PFU virus/ml); IFNα/β (271 IU/ml); and TNFα (15 pg/ml).

IFNα/β levels in serum correlated roughly with virus titers (Fig. 3). On day 1 p.i., IFNα/β titers in the serum of age-8-inoculated mice were strikingly lower than those of younger animals (by a factor of 8), consistent with reduced virus replication. The IFNα/β levels in the age-4-inoculated mice, however, were significantly lower than peak IFNα/β titers observed in neonatal mice infected with TRSB, which were over 200,000 IU/ml (Trgovcich *et al.,* 1996).

TNFα induction also varied with the age of the mouse at the time of inoculation. In the neonate, peak titers of over 1000 pg/ml of serum were reported in TRSB-infected mice (Trgovcich *et al.,* 1997) and were similar to those observed in lipopolysaccharide (LPS) models of endotoxic shock. Importantly, elevated levels of TNFα also were associated with lethal infection in the age-related model (Fig. 3). Peak TNFα titers of over 400 pg/ml of

serum were detected 3 days p.i. in mice inoculated at 4 days of age. While TNFα was detectable in sera of older animals, peak levels were 200 to 300 pg/ml lower than those in animals inoculated at 4 days of age, suggesting the association of TNFα with lethal TRSB infection in younger mice.

Corticosterone levels were elevated in neonatal mice fatally infected with TRSB (Trgovcich *et al.,* 1997) and in mice inoculated at 4 days of age [serum assayed 3 days postinfection: 60 and 35 ng/ml in two experiments with age 4-day mice vs <1 ng/ml in uninoculated controls compared with 75 ng/ml in animals inoculated as neonates (Trgovcich *et al.,* 1997)]. From 2 to 12 days of age, mice are in a stress hyporesponsive period (Sapolsky and Meaney, 1986; Cirulli *et al.,* 1994). Therefore, it is of interest that Sindbis infection at 4 days of age triggered increases in CORT levels at a time when these animals are normally unresponsive to other stress inducing stimuli. Elevated levels of CORT also were observed in animals inoculated at ages 6 and 8 days, but interpretation of these data was complicated by the dramatic developmental changes in CORT physiology over the first 10 to 12 days of life. These include the stress hyporesponsive period and the development of mature circadian rhythms characterized by increases in baseline CORT levels and daily fluctuations in CORT levels (Sapolsky and Meaney, 1986).

DISCUSSION

The development of resistance to lethal Sindbis virus infection is a well-known phenomenon (Taylor *et al.,* 1955; Reinarz *et al.,* 1971; Johnson *et al.,* 1972; Hackbarth *et al.,* 1973; Griffin, 1976). We have described the histopathological and physiological correlates of disease which are linked to the acquisition of resistance to fatal infection. Specifically, we have demonstrated that both quantitative and qualitative disease manifestations of TRSB infection differ dramatically in mice inoculated at 4 and 8 days of age as the infection evolves from fatal to nonfatal in nature. Lethal TRSB infection of 4-day-old mice results in a syndrome characterized by high virus titers in sera and brains, thymic involution, and elevated levels of IFNα/β, TNFα, and CORT (this paper; Trgovcich *et al.,* 1997). These manifestations are similar in many respects to the induction of endotoxic shock and a physiological stress response. Significantly, mortality in these mice correlated with minimal inflammatory changes.

Within a very short time frame, developmental changes in the mouse profoundly alter these parameters of infection. By 8 days of age, TRSB infection is characterized by more limited virus replication and tissue damage and the development of immune-mediated pathology, including encephalomyelitis, myositis, and myocarditis. While encephalitis was previously considered the primary manifestation of Sindbis virus infection in both neonatal and adult mice, the studies presented here and previously (Trgovcich *et al.,* 1996, 1997) indicate that an encephalitic disease course is prominent only in mice inoculated at 6 days of age or older with the TRSB strain of the Sindbis AR339 isolate and is apparent in younger mice only when infection is with an attenuated mutant.

These findings, as well as previous studies of TRSB-associated pathogenesis (Trgovcich *et al.,* 1996, 1997), identify the skin, muscle, connective tissue, and CNS as the primary sites of TRSB replication in mice up to 4 days of age. The rapid spread of TRSB in these tissues likely drives the induction of very high levels of IFNα/β, TNFα, 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 induction of potentially toxic levels of cytokines and severe thymic involution. The neurotoxic effects of IFNα are well documented (Meyers *et al.,* 1991), and TNFα administration mediates hemodynamic and metabolic disturbances which may contribute to the disease syndrome (Remick and Kunkel, 1993). In 4-day-old mice, which survive long enough to have developed specific immune responses, the lack of encephalitis may suggest immunosuppressive effects of the host's cytokine response.

We hypothesize that at least two developmental changes contribute to the survival of mice inoculated at 8 days of age. First and of primary importance, early postnatal cellular changes restrict the capacity of the virus to replicate in target tissues, limiting the induction of potentially toxic levels of cytokines. Sindbis virus replicates in identical tissues in mice of different ages, and the reduction in virus replication in older animals occurs proportionately in all tissues. Therefore, it is the extent of virus replication throughout the animal, rather than an altered tissue tropism, which likely influences the magnitude of IFNα/β and TNFα induction in mice of different ages. Consistent with previous studies (Reinarz *et al.,* 1971; Johnson and MacFarland, 1972), restricted virus replication in mice inoculated at 8 days of age could not be explained by differences in the ability to induce IFNα/β, as the ratio of IFNα/β to virus titers was similar in mice of different ages. Also, previous studies have shown that differences in virus replication in the age-related model are detected within the first 24 h of infection and were not attributable to differential IFN induction or the development of specific immune responses (Reinarz *et al.,* 1971; Johnson and MacFarland, 1972). These data suggest that other age-related changes in susceptible tissues influence the magnitude of virus replication. The specific changes occurring between 4 and 8 days after birth which act to limit virus replication are unknown. Johnson and colleagues (1972) found that Sindbis replication in fibroblasts cultured from 4- or 8-week-old mice was far more limited and less cytopathic than that in fibroblasts from newborn mice. Another possibility

is that nonspecific antiviral responses play a role in determining the extent of virus replication. The activity of 2′–5′ oligoadenylate synthetase, an important antiviral protein upregulated in response to IFN, changes with age and does not reach maximal levels until early adulthood (Pfiefer *et al.,* 1993). Furthermore, the antiviral activity of human neutrophils has been found to be lower in neonates than in adults (Roberts *et al.,* 1994).

A second important developmental change which occurs concurrently with the acquisition of resistance is that mice develop mature circadian rhythms and the capacity to respond normally to cytokine stress through the activation of the HPA axis. Thus, in older mice, immune and inflammatory responses remain intact and controlled, while any toxic effects of the cytokine response are suppressed, and the classical specific immune response functions normally to clear the virus infection.

There are significant pathological differences between fatal Sindbis TRSB infection, characterized by high cytokine levels, thymic involution, and conspicuous lack of inflammatory lesions, and nonfatal infections, in which the predominant pathologies are inflammatory infiltrates in muscle and brain. These differences have now been demonstrated in two model systems in which fatal and nonfatal infections have been examined. In the first instance, fatal and nonfatal infections of neonatal animals were induced by TRSB and an attenuated mutant of this virus (Trgovcich *et al.,* 1996, 1997). In the second model, reported here, TRSB infection of younger animals susceptible to fatal disease was compared to TRSB infection of mice only a few days older, in which the resulting disease was not lethal. The results derived from both of these systems agree and suggest that fatal Sindbis virus infection is concurrent with a high level cytokine response, including the induction of cytokines associated with a shock-like syndrome. On the other hand, infection of mice under conditions which render the virus less virulent results in classic encephalitis and myositis associated with a more benign outcome.

MATERIALS AND METHODS

Cells, viruses, and virus clones

Baby hamster kidney (BHK) cells used for virus production and titrations were obtained from the American Type Culture Collection and used between passages 55 and 65. BHK cells and murine L929 fibroblasts were maintained as described previously (Trgovcich *et al.,* 1996).

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). TRSB was derived by transfection of *in vitro* transcripts of pTRSB into BHK cells, as described previously (Trgovcich *et al.,* 1996). The sequence of TRSB differed from that of the deduced consensus AR339 sequence at three amino acid residues (Consensus > TRSB: nsP3 528 Arg > Gln, E2 1 Ser > Arg, E1 72 Ala > Val; McKnight *et al.,* 1996).

Virulence assay

Virulence determinations were performed in CD-1 mice (Charles River, specific pathogen free) inoculated 1, 2, 3, 4, 5, 7, 9, 11, 13, or 15 days after birth. Two litters (17–22 mice per group) were inoculated subcutaneously (sc) or intracerebrally (ic) with 1000 PFU of TRSB in 0.05 or 0.025 ml, respectively. One to two mice per litter were inoculated with an equal volume of diluent [phosphate-buffered saline, 1% donor calf serum (PBS 1% DCS)] to serve as mock-infected controls. Animals were observed daily for up to 21 days, and AST and mortality were recorded. Survivors were challenged with 1200 PFU of S.A.AR86 ic, an alphavirus closely related to Sindbis which is highly virulent in adult mice.

Virus growth *in vivo*

CD-1 mice were inoculated with 1000 PFU of TRSB at 4, 6, 8, or 10 days after birth as described above. Three animals were sacrificed at intervals as indicated in the figures. Serum and brain were dissected and processed as previously described for virus titration on BHK cells (Trgovcich *et al.,* 1996).

Histopathology

CD-1 mice were inoculated with TRSB as described above. Three infected mice (and one control mouse) per time point were anesthetized with Metofane (Pittman-Moore), perfusion-fixed with 4% paraformaldehyde in PBS, and immersed in 10% buffered Formalin (after exposing peritoneal, thoracic, and cranial cavities). Mice were sacrificed every other day until the mean day of death. One exception was that 6-day-old mice were sacrificed 1, 3, 5, 7, and 10 days pi. Mice under 8 days of age at time of sacrifice were bisected midsagittally and embedded in paraffin, and whole body sections were analyzed for histopathology. Older animals were eviscerated, and individual organs were dissected prior to embedding in paraffin. The fixed heads of these older mice were decalcified at 4°C in 8% EDTA, 4% paraformaldehyde, pH 6.6, for approximately 14 days prior to embedding and sectioning. H&E-stained tissue sections were examined by light microscopy.

In situ hybridization

TRSB sequences present in tissue sections were detected by ISH as previously described (Grieder *et al.,*

1995). Briefly, a TRSB-specific riboprobe, specific for a portion of the E2 gene, was used as a positive probe, and a riboprobe specific for influenza hemagglutinin (HA) was used as a negative control. Probes were hybridized to serial 3-μm tissue sections mounted on ProbeOn Plus slides (Fisher Scientific) from the same paraffin blocks sectioned for histopathological analysis. As controls, tissues from virus-infected animals were incubated with HA-specific probe, and tissues from mock-infected animals were incubated with Sindbis-specific probe. These controls were uniformly negative, indicating the specificity of the ISH signal.

Cytokine and hormone analyses

Pooled litters of 9–11 mice were inoculated 4, 6, or 8 days after birth with 1000 PFU of TRSB or PBS 1% DCS. At the times indicated in the figures, 9–10 mice in each group were anesthetized and decapitated, and blood was collected in Microtainer Serum Separator Tubes (Becton–Dickinson). After centrifugation at 3000 rpm at room temperature for 15–20 min, multiple aliquots of pooled sera were frozen at −70°C and subsequently assayed for virus, IFNα/β, TNFα, CORT, and adrenal corticotropin releasing hormone (ACTH). Brains of sacrificed animals were homogenized as 50% suspensions in PBS 1% DCS (w/v) and clarified by low-speed centrifugation. Equal volumes of supernatants were pooled for each group, subsequently frozen at −70°C as multiple aliquots, and assayed for virus and IFNα/β. IFNα/β titers were determined by biological assay using L929 cells and encephalomyocarditis virus (EMC) as the indicator virus, as described previously (Trgovcich *et al.,* 1996). TNFα levels were assayed by ELISA (Genzyme). CORT and ACTH levels were determined by radioimmune assay, as described elsewhere (Sonntag *et al.,* 1987).

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