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SHORT COMMUNICATION

Sindbis Virus Infection of Neonatal Mice Results in a Severe Stress Response

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Neonatal mice were infected with virus derived from a molecular clone of a laboratory strain of Sindbis virus, TRSB. The resulting acute fatal infection was typified by few if any of the classic hallmarks of encephalitis, very high levels of interferon-alpha/beta (IFN$\alpha\beta$), and lesions in the thymus and hematopoietic tissues usually associated with a severe stress response. Infection with an attenuated mutant of TRSB, which harbors a single amino acid change in the E2 surface glycoprotein (TRSBr114), was characterized by encephalitis, reduced mortality, low levels of IFN$\alpha\beta$, and no thymic pathology (J. Trgovcich, J. F. Aronson, and R. E. Johnston, 1996, *Virology* 224, 73–83). Here we report that infection of neonatal mice with TRSB, but not TRSBr114, resulted in induction of high levels of tumor necrosis factor-α as well as high and sustained levels of adrenalcorticotropin-releasing hormone and corticosterone. This syndrome of potentially toxic cytokine and stress hormone induction correlates with lethal Sindbis virus infection and constitutes a previously unrecognized aspect of Sindbis virus pathogenesis in mice.

Sindbis virus has been widely used as a model for alphavirus replication and pathogenesis (*1, 2*). The advent of molecular clones of this virus (*3*) has allowed comparative studies of viruses which differ from each other by as little as a single nucleotide (*4–12*). Previously, we compared the disease course and histopathological profiles of neonatal mice infected with molecularly cloned, isogenic virulent and attenuated laboratory strains of Sindbis isolate AR339, designated TRSB and TRSBr114, respectively (*12, 13*). TRSBr114 was derived by site-directed mutagenesis of the TRSB clone and harbors a single nucleotide change in the E2 glycoprotein gene at position 8972, resulting in a change from serine to arginine at amino acid 114 (*13*).

TRSB causes an acute infection in neonatal mice characterized by overwhelming virus replication, severe virus-associated lesions in peripheral tissues and organs, high levels of interferon-alpha/beta (IFN$\alpha\beta$), and death within 4–5 days (*12*). While high levels of virus were observed in the brains of infected mice, strikingly little pathology was noted. In particular, the diagnostic features of encephalitis, lymphocyte infiltrates, and perivascular cuffing were conspicuously absent. Instead, TRSB-infected mice showed evidence of a systemic physiological imbalance, including a decrease in hematopoiesis and severe thymic involution in the absence of virus replication in the thymus. These changes were not observed in TRSBr114 infection, which was characterized by lower virus titers and limited peripheral tissue damage. TRSBr114-infected mice progressed to develop clear signs of encephalomyelitis and myositis, associated with approximately 50% mortality and a mean day of death of 8.5 days. We report here that infection with the virulent TRSB, but not the attenuated TRSBr114, leads to a syndrome of cytokine and stress hormone induction. Specifically, we demonstrate that TRSB infection of newborn mice is associated with high levels of tumor necrosis factor-alpha (TNFα) and a stress response that is mediated by glucocorticoid hormones (corticosterone). These may be elements of a shock-like syndrome in Sindbis-infected neonates similar to that caused by lipopolysaccharide (LPS).

One of the prominent qualitative differences between the pathologies induced by virulent and attenuated Sindbis infections was the striking thymic involution seen with the more lethal virus. As glucocorticoid hormones mediate apoptosis of immature thymocytes, involution of the thymus, and depression of hematopoiesis (*14–16*), we wished to determine if corticosterone (CORT) levels were elevated in TRSB-infected mice, and whether levels of proinflammatory cytokines, represented by TNFα, also were elevated in these animals. Sera from infected animals were examined for CORT, adrenalcorticotropin-releasing hormone (ACTH), TNFα, and viable virus. In this

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series of experiments, 12- to 24-hr postpartum mice were inoculated subcutaneously (sc) with TRSB, TRSBr114, or diluent (phosphate-buffered saline with 1% donor calf serum). At the times indicated in Figs. 1 and 2, 5–10 mice in each group were anesthetized and decapitated. Blood was collected in microtainer serum separator tubes (Becton–Dickenson), and samples from individual mice were pooled. After centrifugation at 3000 rpm (1000 *g*) at room temperature for 15–20 min, multiple aliquots of pooled sera were frozen at −70° and subsequently assayed for virus, CORT, ACTH, and/or TNFα. Virus titer was determined by standard plaque assay on baby hamster kidney cells. CORT and ACTH levels were determined by radioimmune assay as described elsewhere (*17*). TNFα titers were determined by ELISA (Genzyme).

Virus was first detected in sera of TRSB-infected mice at 8 hr postinfection (p.i.) (Figs. 1 and 2). Serum titers rose rapidly, reaching over 10^8 plaque-forming units per milliliter (PFU/ml) by 72 hr p.i. Appearance of TRSBr114 in the serum was slightly delayed compared to TRSB, and virus was first detected at 16 hr p.i. In contrast to TRSB infection, TRSBr114 titers did not rise as high, and never exceeded 10^6 PFU/ml. These findings are consistent with those reported previously (*12*).

Elevated levels of CORT (30–50 ng/ml) were detected soon after inoculation in TRSB, TRSBr114, and diluent-only groups (Figs. 1 and 2). This was expected, as newborn mice experience a transient hypoglycemia, a well-documented inducer of CORT (*18*). Additionally, CORT is necessary for appropriate fetal and early postnatal development of several tissues including lung, adrenal gland, and pancreas (*19, 20*). By 48 hr p.i., the early CORT levels had subsided in the mock- and TRSBr114-infected groups. However, beginning at 24 to 48 hr p.i., a significant rise in CORT was observed in TRSB-infected mice only, although in the experiment depicted in Fig. 2, maximal levels of CORT were lower than in the previous experiments. Nevertheless, elevated CORT levels were maintained for 96 hr through the predicted mean day of death.

ACTH levels were examined to determine if CORT secretion in TRSB-infected mice resulted from activation of the hypothalamic–pituitary–adrenal (HPA) axis or was caused by direct activation of the adrenal cortex. A stressor, real or perceived, results in stimulation of neurons in the paraventricular nucleus of the hypothalamus to release corticotropin-releasing factor (CRF). CRF stimulates the pituitary to release ACTH, which in turn activates the adrenal cortex to synthesize and release CORT (*21*). During the first 24 hr p.i., a time when CORT levels were elevated in all three groups of mice, ACTH levels also were very similar in these three groups (Fig. 2). At 48 hr and beyond, a further elevation in ACTH levels occurred only in mice infected with virulent TRSB. This elevation in ACTH preceded the peak of CORT in TRSB-infected mice, suggesting that the HPA axis was functioning and that the activation of the HPA axis was sufficient to account partially, if not completely, for the rise in CORT observed following TRSB infection.

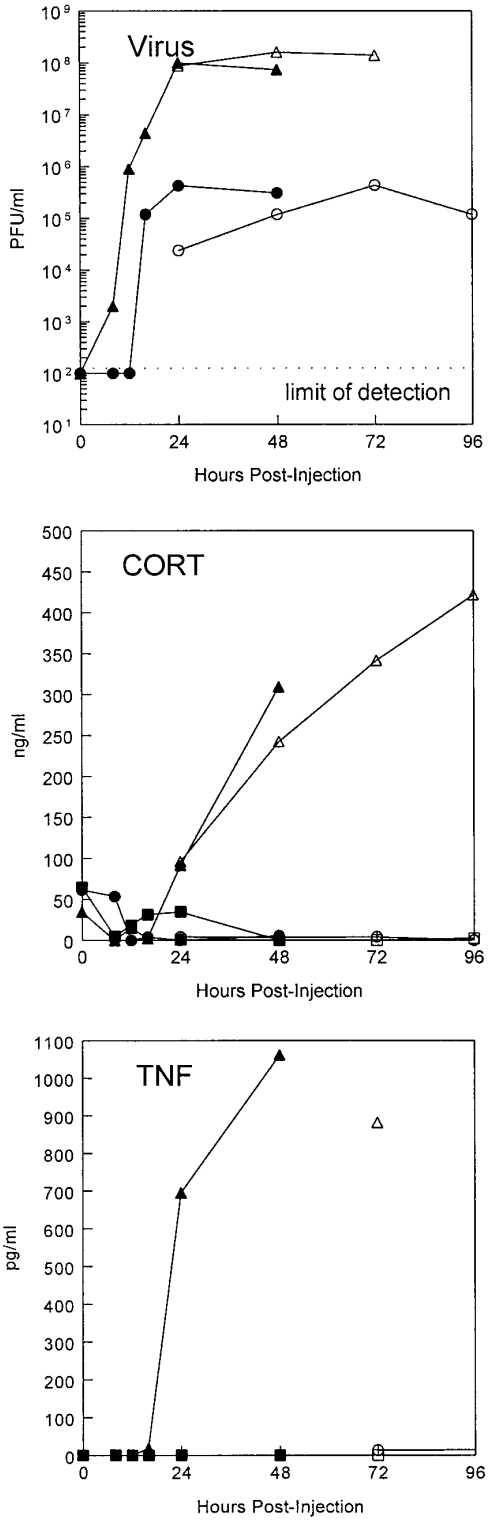

FIG. 1. Virus, CORT, and TNFα levels in infected and mock-infected neonatal mice. Mice were inoculated sc with 1000 PFU of TRSB (triangles), TRSBr114 (circles), or diluent (squares). At the indicated intervals, sera from 5 to 10 mice were pooled, and the pooled samples were assayed for virus titer, CORT, and TNFα. Closed symbols, Experiment 1; open symbols, Experiment 2.

Shock is a primary activator of the HPA axis and the

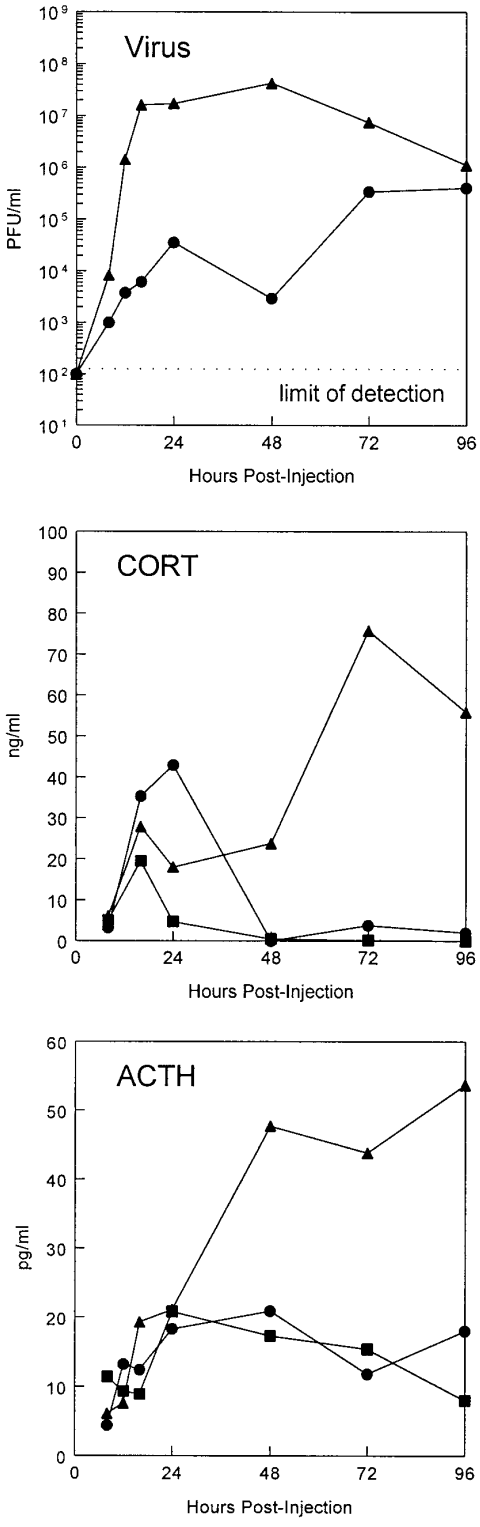

FIG. 2. Virus, CORT, and ACTH levels in infected and mock-infected neonatal mice. Mice were inoculated sc with 1000 PFU of TRSB (triangles), TRSBr114 (circles), or diluent (squares). At the indicated intervals, sera from 5 to 10 mice were pooled, and the pooled samples were assayed for virus titer, ACTH, and CORT.

glucocorticoid stress response through the activities of proinflammatory cytokines such as TNFα, IL-1, and IL-6. All three of these cytokines can activate the HPA axis (*22–24*). TNFα levels rose rapidly in TRSB-infected mice (Fig. 1). TNFα was first detected at 16 hr p.i. and peaked at 48 hr p.i. at over 1000 pg/ml, levels on the same order as those observed in models of LPS-induced shock (*25*). These measurements were made on pooled samples from groups of mice sacrificed at the indicated times postinfection. Therefore, it was possible that most of the cytokine detected in the TRSB pooled samples could have been contributed by only one or a few individuals in the group. To rule out this possibility, peak TNFα levels were determined for individual TRSB- or TRSBr114-infected animals (Fig. 3). TRSB uniformly induced high levels of TNFα in each individual animal.

The rapid induction of TNFα suggests that proinflammatory cytokines, including IL-1 and IL-6, may activate the HPA axis and a cascade of cytokine and hormone upregulation induced by infection with TRSB. Moreover, these results raise the possibility that the severe metabolic and hemodynamic dysfunction attributed to TNFα during shock also may contribute to the demise of TRSB-infected mice (*26, 27*). In contrast to TRSB infection, TNFα was at significantly lower levels throughout infection with TRSBr114 (Figs. 1 and 3; data not shown).

Another possible mechanism of CORT upregulation involves IFN. High levels of IFN$\alpha\beta$ are observed in both sera and brain homogenates of TRSB-infected mice with peak serum titers on the order of 10^5 international units/ml at 24 hr postinfection (*12*). Such levels of IFN$\alpha\beta$ could account for primary activation of the HPA axis (*28, 29*). IFNα can bind and activate μ-opioid receptors in the central nervous system (CNS) and stimulate the release of CRF from hypothalamic organ cultures (*28–30*). Moreover, some studies have suggested a structural relatedness between ACTH and IFN (*31*), and IFNα may activate the adrenal cortex directly to release CORT (*30*).

The magnitude and duration of the CORT and TNFα responses were sufficient to account for the thymic and hematopoietic lesions characteristic of TRSB infection of neonatal mice (*12, 14–16*). Another characteristic of this infection is the lack of any histopathological evidence of encephalitis in the brains of animals which succumb (*12*). As high levels of CORT also are associated with suppression of immune and inflammatory responses (*32*), it is possible that TRSB infection resulted in immunosuppression of the host, consistent with the lack of inflammatory lesions in the brains of TRSB-infected mice (*12*). However, the absence of inflammatory lesions and other evidence of encephalitis also could have resulted from the animals dying before such lesions had a chance to develop. To distinguish between these two alternatives, mice were inoculated with TRSB at 4 days of age instead of at 12–24 hr postpartum. Mortality remained at 100% in the 4-day-old animals, but the average survival time was extended from 3.0 $\pm$ 0.7 to 7.4 $\pm$ 2.7 days. Like TRSB infection of neonates, thymic involution was associated with extensive nuclear debris, as well as loss of cortical mass and the corticomedullary junction (Fig. 4A).

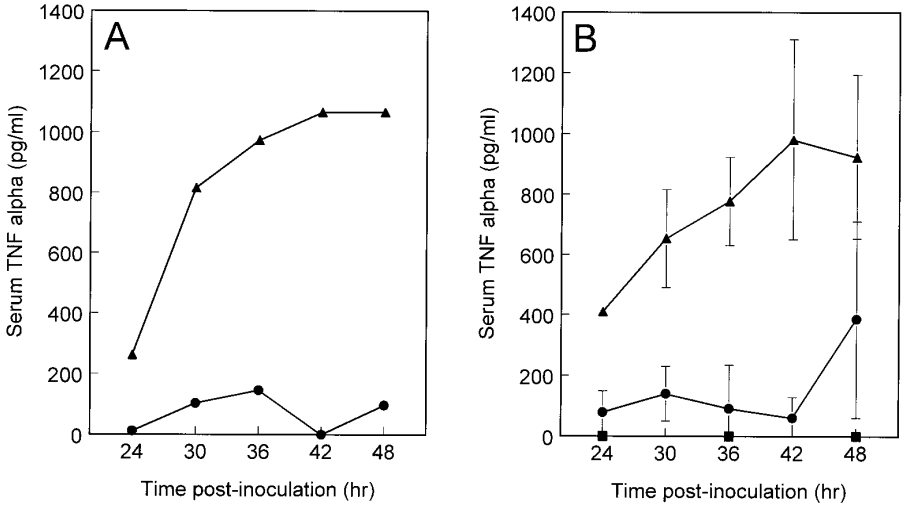

FIG. 3. TNFα levels in individual TRSB-infected, TRSBr114-infected, and mock-infected neonatal mice. Mice were inoculated sc with 1000 PFU of TRSB (triangles), TRSBr114 (circles), or diluent (squares). At the indicated times postinoculation, sera from six mice for each virus infection were obtained. (A) Samples from three mice for each virus were pooled, and the pooled sera were assayed for TNFα by ELISA. (B) Samples from three mice for each virus were assayed individually. Error bars indicate 1 standard error. Differences between TRSB and TRSBr114 by *t* test: $P < 0.01$ for 24–42 hr; $P < 0.1$ for 48 hr.

In the CNS, no evidence of encephalitis was observed at any time postinfection in the brains of mice inoculated at 4 days of age (Fig. 4B). CNS lesions were typical of liquefactive necrosis associated with nuclear debris and neuronal dropout. Therefore, TRSB did not induce encephalitis even under conditions where the survival time was sufficiently long for an encephalitic response (*12*). While the link between CORT induction and the lack of encephalitic lesions remains unproven, it is one plausible explanation for this *in vivo* TRSB phenotype.

The consequences of proinflammatory cytokine induction and a stress response may constitute previously unrecognized aspects of Sindbis-induced disease in neonatal mice which could lead to the mortality observed in this infection. In the brains of adult BALB/cJ mice infected with other Sindbis strains, estimated levels of mRNA for IL-1β, IL-4, IL-10, TNFα, and LIF increased after infection (*33*). However, no differences in cytokine mRNA levels were noted between strains of differing virulence. Also in adult mice, stress increases the mortality observed with experimental Sindbis virus, Semliki Forest virus, and West Nile virus infections (*34, 35*). In the absence of infection, abnormally high CORT levels during the postnatal period are detrimental to CNS development (*36*). While few histopathological changes were noted in the CNS during TRSB infection of neonates, and no gross abnormalities in development were observed (*12*), these results raise the possibility that CORT is mediating pathological changes in the brains of infected animals which were not detectable using conventional histological procedures.

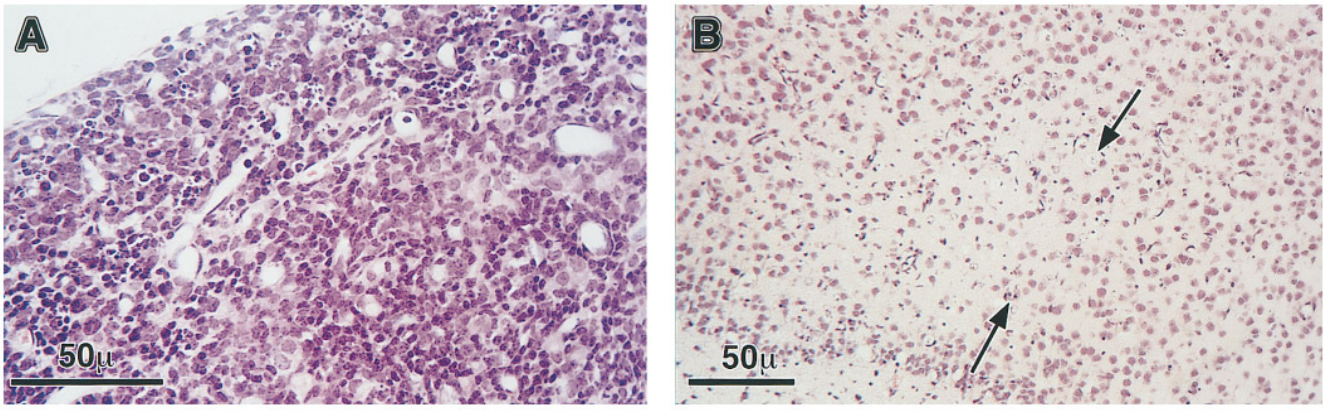

FIG. 4. Histopathological evaluation of brain and thymus after TRSB infection of mice at 4 days of age. Four-day-old CD-1 mice were inoculated sc with 1000 PFU of TRSB. At 7 days postinoculation, animals were anesthetized with Metofane (Pittman–Moore) and fixed by perfusion with 4% paraformaldehyde in PBS. The peritoneal, thoracic, and cranial cavities were exposed prior to immersion in 10% buffered formalin. Selected organs were removed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. (A) Thymus showing severe involution. (B) Brain exhibiting a noninflammatory lesion of the cerebral cortex. Arrows indicate neuronal dropout (top) and neuronal pyknosis (bottom).

It is interesting to note that neonatal mice exhibit a stress hyporesponsive period between 3 and 12 days after birth (*37*), in which they are incapable of generating a stress response to many classical stimuli. The stress response induced by Sindbis, then, may initiate before the onset of the hyporesponsive period, or Sindbis infection may fall into the category of stressors to which the neonatal mouse can respond during this period.

In summary, we report that infection of neonatal mice with TRSB, a virulent laboratory strain of Sindbis isolate AR339, was characterized by high levels of TNFα and a severe stress response mediated by glucocorticoid hormones, two hallmarks of endotoxin-induced shock. Infection with an attenuated mutant, TRSBr114, did not induce high levels of IFN$\alpha\beta$ or TNFα, a CORT-mediated stress response, or thymic involution. These parameters also were absent in preliminary experiments with TRSB infection of mice 8–10 days of age in which this infection produced low or no mortality (Trgovcich *et al.,* unpublished observations). Therefore, we suggest that the cytokine and hormonal responses described here are significant elements of lethal Sindbis virus pathogenesis in neonatal mice.

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