Role of Interferon and Interferon Regulatory Factors in Early Protection against Venezuelan Equine Encephalitis Virus Infection

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To investigate the role of type I interferon (IFN) and its regulatory transacting proteins, interferon regulatory factors (IRF-1 and IRF-2), in early protection against infection with virulent Venezuelan equine encephalitis virus (VEE), we utilized mice with targeted mutations in the IFN-α/β receptor, IRF-1, or IRF-2 genes. IFN-α/β-receptor knockout mice are highly susceptible to peripheral infection with virulent or attenuated VEE, resulting in their death within 24 and 48 h, respectively. Treatment of normal macrophages with anti-IFN-α/β antibody prior to and during infection with molecularly cloned virulent VEE resulted in increased VEE replication. However, treatment with high doses of IFN or IFN-inducing agents failed to alter percentage mortality or average survival times in mice challenged with a low dose of virulent VEE. In IRF-1 and IRF-2 knockout mice (IRF-1⁻/⁻ and IRF-2⁻/⁻), the 100% protection against virulent VEE that is conferred by attenuated VEE within 24 h in control C57BL/6 mice was completely absent in IRF-2⁻/⁻ mice, whereas 50% of IRF-1⁻/⁻ mice were protected. IRF-2⁻/⁻ mice were deficient in clearing VEE virus from the spleen and the brain compared to the heterozygous IRF-2⁻/+ knockout or C57BL/6 (+/++) mice. Furthermore, a distinct pattern of histopathological changes was observed in brains of IRF-2⁻/⁻ mice after VEE exposure. Taken together, these findings imply that the altered immune response in IRF-1 and IRF-2 knockout mice results in altered virus dissemination, altered virus clearance, and altered virus-induced pathology. Thus, type I interferon, as well as IRF-1 and IRF-2, appears to play an important and necessary role in the pathogenesis of, and protection against, VEE infection.

The genus alphavirus, with its over 26 recognized members, is widely distributed throughout the Old and New Worlds (Watts et al., 1997). Venezuelan equine encephalitis virus (VEE) is one of the human alphavirus pathogens that is found exclusively in the Americas, causing epidemic outbreaks in South and Central America, with occasional outbreaks in southern North America (Bowen, 1976).

In contrast to some members of the alphavirus genus that cause persistent infections, VEE induces an acute, febrile disease with potential progression to the central nervous system (CNS) and the development of encephalitis. In the murine model, it has been shown that the initial immune response to this acute CNS infection is critical in the protection against lethal encephalitis (Grieder et al., 1997). These early responses against viral infections have been postulated to include innate immune mechanisms, such as activation of macrophages, because macrophages and other myeloid cells, such as dendritic cells, perform essential roles during the early immune response against virus infections of the CNS (Balter, 1996). The role of macrophages in viral CNS infections is very complex, mainly because their function appears to be highly virus-specific. For example, following Theiler's virus infection in mice, the number of macrophages increases significantly, and depletion of macrophages prior to infection results in a decreased inflammatory response and absence of persistent infection (Rossi et al., 1997). In contrast, West Nile virus infection in macrophage-depleted mice resulted in exacerbated disease (Ben-Nathan et al., 1996). Furthermore, macrophages can provide virus access to the brain (Chebloune et al., 1998), contribute to host cell death, and help the virus evade detection by the immune system (Balter, 1996).

The interaction between macrophages and VEE during infection is not clearly understood. In early comparative pathology studies, VEE's tropism for neuronal, myeloid, and lymphoid tissues was described in rodents, with pathological lesions in CNS and non-CNS sites (Victor et al., 1956). Significant myeloid cell death in the spleen, lymph nodes, and bone marrow was described (Victor et al., 1956). The involvement of myeloid cells in VEE pathogenesis was later confirmed in studies in which VEE pathogenesis was evaluated (Grieder and Nguyen, 1996; Jackson et al., 1991). Moreover, in recent studies using replication-deficient VEE replicons, dendritic cells were identified as the first targets in mice after peripheral infection with VEE (MacDonald et al., 1997). Finally, the activation or differentiation state of macrophages has been shown to be important in VEE replication (Grieder and Nguyen, 1996).

Protection against VEE infection has generally been correlated with the induction of a neutralizing antibody

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titer (Pittman et al., 1996; Rivas et al., 1997). In mice, neutralizing anti-VEE antibodies were observed in the serum, as was a mucosal response (Charles et al., 1997; Greenway et al., 1995), following subcutaneous infection, although protection was mouse strain-specific (Hart et al., 1997). Protection against lethal VEE challenge of mice was also observed when mice were treated with immune spleen cell culture supernatants that contained interleukin-1 and -2 (Huprikar et al., 1990) or when “vaccinated” with molecularly cloned, attenuated VEE 24 h prior to challenge with virulent VEE (Grieder et al., 1997). The mechanism of this nonspecific protection against VEE is not well understood at present, but macrophages and other soluble immune mediators may play an important role. Mice with a severe combined immunodeficiency, infected with virulent VEE, survived longer than did intact mice infected with the same virus (Dr. Peter Charles, personal communication). These results further support the hypothesis that the initial, nonspecific response to the infection is important because it allows the immune response time to develop and confer solid protection.

Key immune mediators in virus elimination include the interferons (IFN). In previous studies conducted with Sindbis (SB) virus infection in mice, an important correlation between SB virulence and the level of IFN-α/β induction was established (Trgovcich et al., 1996). The working hypothesis was that rapid spread of virulent SB virus led to high IFN-α/β levels and, possibly, those of other cytokines. Rapidly replicating, virulent SB virus evades the innate immune response by accelerated induction of specific proinflammatory cytokines and a systemic stress response (Trgovcich et al., 1997). In contrast, a single-site-attenuated SB virus, which exhibits a reduced replication capacity, induced IFN-α/β levels with different kinetics, resulting in extended survival and altered pathology. Further, Semliki Forest virus (SFV) infection in neonatal, type I interferon receptor knockout (IFNAR1) mice resulted in rapid death and high virus titers in organs (Hwang et al., 1995; Muller et al., 1994).

IFN-α/β induction and the establishment of an antiviral state is regulated by IFN-inducible genes or transcriptional regulators and their mechanism of activation. Two structurally related molecules, interferon regulatory factor-1 and -2 (IRF-1 and IRF-2), are transcriptional regulators of IFN that act to activate and suppress gene expression, respectively, by binding to DNA regulatory elements in the IFN-α/β promoter and in the promoters of many IFN-inducible genes (Pitha, 1997; Nguyen et al., 1997). Both IRF-1 and IRF-2 have been implicated in the induction of IFNs (Kimura et al., 1994) and of nitric oxide by macrophages (Kamijo et al., 1994) following virus infection in mice. Additionally, IRF-1 and IRF-2 were shown to be induced by other proinflammatory cytokines (Fujita et al., 1989; Harada et al., 1989; Lehtonen et al., 1997).

In the studies reported here, we utilized mice with targeted mutations in the IFN-α/β receptor (IFNAR1), as well as mice with targeted mutations in the IRF-1 and IRF-2 genes. All three mouse strains exhibit increased susceptibility to infection with virulent VEE, and both IFNAR1 and IRF-2 knockout mice exhibit increased susceptibility to the attenuated VEE strain. Further, we show that treatment of normal macrophages with anti-IFN-α/β antibody resulted in increased replication of cloned virulent V3000. Administering exogenous IFN and IFN inducers failed to protect mice from VEE. Taken collectively, these findings imply that the defective immune response in the three knockout strains results in both increased initial virus dissemination and altered virus clearance. Thus, type I interferon appears to play an important and necessary, though not sufficient, role in the pathogenesis of, and protection against, VEE infection.

### RESULTS

To evaluate the role of IFN-α/β in VEE infection, the susceptibility of IFN-α/β-receptor knockout mice to VEE was tested. Groups of five adult IFNAR1 mice were infected in their left rear footpads with either 10^3 PFU attenuated V3032 alone or the combination of 10^3 PFU attenuated V3032 followed by 10^2 PFU virulent V3000 24 h later. 129Sv/Ev mice, the parental strain of the IFNAR1 mice, served as controls. The average survival times (AST) were calculated in days ± standard error (SE) after V3000 inoculation.

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>VEE challenge</th>
<th>AST ± SE (days)</th>
<th>Mortality %</th>
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<tr>
<td>129Sv/Ev (+/+)</td>
<td>V3032</td>
<td>—</td>
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<td></td>
<td>V3032/V3000</td>
<td>8.0 ± 0.5</td>
<td>20</td>
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<tr>
<td>IFNAR1 (IFN-α/β R^−/−)</td>
<td>V3032</td>
<td>1.0 ± 0.0</td>
<td>100</td>
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<tr>
<td></td>
<td>V3032/V3000</td>
<td>0.4 ± 0.5</td>
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*Note. Groups of five adult IFNAR1 mice were infected in their rear footpads with either 10^3 PFU attenuated V3032 alone or the combination of 10^3 PFU attenuated V3032 followed by 10^2 PFU virulent V3000 24 h later. 129Sv/Ev mice, the parental strain of the IFNAR1 mice, served as controls. The average survival times (AST) were calculated in days ± standard error (SE) after V3000 inoculation.*
role in very early VEE pathogenesis and, specifically, in preventing disease by attenuated V3032.

Anti-IFN-α/β antibody affects the growth of virulent, but not avirulent, VEE in peritoneal macrophages

Treatment of primary peritoneal macrophage cultures with IFN-α/β antibody resulted in increased levels of virulent V3000 replication (Fig. 1a). At 24 h postinfection, there was a >10-fold increase in VEE titer in the culture supernatants of antibody-treated cells compared to control antibody-treated cells. Peak virulent V3000 titers reached almost $1 \times 10^8$ PFU/ml at 24 h, 24 h before peak titers were reached in control-treated cells. Over the course of the next several days, the virulent V3000 titer in the anti-IFN-α/β antibody-treated cultures remained elevated (~8-fold) compared to the titers in control cultures. In contrast, treatment of primary macrophage cultures with identical concentrations of neutralizing IFN-α/β antibody failed to result in increased attenuated V3032 titers compared to titers in control cultures (Fig. 1b). Growth kinetics of the attenuated VEE in either treated or untreated macrophage cultures were comparable over the entire observation period.

IFN-β and IFN inducers fail to protect mice against low-level virulent V3000 infection

Groups of 8-week-old C57BL/6 mice were treated intraperitoneally with IFN-β. Poly I:C, or LPS 24 h prior to challenge with $1 \times 10^5$ PFU of virulent V3000 into the left rear footpad. Serum IFN levels of mice treated with IFN, Poly I:C, LPS, or PBS were measured by standard biological assay on L929 cells (Trgovcich et al., 1996) in control mice 24 h posttreatment and were 1600, 6400, 200, and 20 U/ml, respectively. Mice pretreated with IFN-β, Poly I:C, or LPS all died, with average survival times similar to those of mice pretreated with diluent (Table 2). Further, mice infected in the right rear footpad with $1 \times 10^5$ PFU of attenuated V3032 24 h prior to challenge with the same dose of virulent VEE survived the challenge without any symptoms of disease (data not shown). These results indicate that elevated serum IFN titers alone are not sufficient to confer protection against low-dose, peripheral virulent VEE challenge.

Virulent VEE mortality in mice with targeted mutations in their IRF-1 or IRF-2 genes

Mice between 8 and 20 weeks of age were tested in three independent experiments (compiled in Figs. 2a–2f). Groups of mice ranged from 8 to 27 animals, with both sexes equally represented. Molecularly cloned virulent V3000 caused 100% mortality in control C57BL/6, homo- and heterozygous knockout mice for the IRF-2 gene (IRF-2+/− or IRF-2−/−), and heterozygous IRF-1+/− mice (Fig. 2). Surprisingly, two IRF-1−/− mice injected with either 10 or $10^2$ PFU of virulent V3000 survived. These two mice were subsequently protected against intraperitoneal challenge with $1 \times 10^4$ PFU virulent VEE 1 week later. Survival of the second V3000 challenge confirmed that the two surviving mice had been exposed to VEE and developed a protective immune response. Therefore, the mortality rate was 96% for IRF-1−/− mice. Thus, the overall mortality rate of virulent V3000 for adult mice in these experiments was 99.2%. AST following rear footpad infection of V3000 ranged from 6.1 to 9.7 days p.i. Some trends in the survival data were observed in terms of infectious dose, age, and sex, but none were significantly different by statistical analysis. Specifically, higher doses decreased average survival time and younger adult mice died from V3000 infection earlier than did older mice, while male mice perished from VEE infection somewhat faster compared to age-matched females.

Interestingly, while the homozygous (−/−) IRF-1 and IRF-2 knockout mice died sooner compared to either the C57BL/6 or the heterozygous (+/−) controls, a trend that clearly increased with increased virus dose, at the lowest V3000 dose of 10 PFU, both IRF-1−/− and IRF-2−/− heterozygous mouse strains appeared to survive longer than either the homozygous knockout or the C57BL/6 control strains. Since neither the IRF-1 nor the IRF-2 mice are completely inbred onto the C57BL/6 background, this suggests that genes other than IRF-1 and IRF-2 that are present on the heterozygotic background or C57BL/6 mouse may contribute to resistance or susceptibility, respectively.

Failure of attenuated V3032 to protect IRF-1 or IRF-2 knockout mice challenged with virulent V3000

As seen in Fig. 2, V3000 kills virtually all mice; homozygous IRF-1 and IRF-2 knockout mice die more rapidly than do C57BL/6 or heterozygous IRF-1−/− or IRF-2−/− control mice. Rear footpad inoculation of $1 \times 10^3$ PFU of attenuated V3032 24 h prior to challenge with a lethal dose of virulent V3000 (1 × $10^3$ PFU into the opposite rear footpad) resulted in 100% survival of adult C57BL/6 mice, as has been previously demonstrated (Grieder et al., 1997) (Fig. 3). Groups of age- and sex-matched homozygous and heterozygous IRF-1 or IRF-2 knockout mice were inoculated either with the same two virus inoculation regimes (attenuated V3032 followed 24 h later by virulent V3000) or with virulent V3000 only (1 × $10^3$ PFU) into the rear footpad(s). In contrast to the control C57BL/6 mice, homozygous knockout mice exhibited either 44%((IRF-1−/−) or no (IRF-2−/−) protection when injected with both viruses. IRF-1 and IRF-2 heterozygous mice exhibited intermediate phenotypes with 89 and 50% protection, respectively. These results indicate that deletion of IRF-2 eliminates a critical factor (or factors) that is involved in the early protection against lethal peripheral VEE infection induced within 24 h by inoculation with the attenuated V3032 strain. IRF-1 also appears to play an important role in this induction of early protection, because heterozy-
FIG. 1. Growth kinetics of virulent and attenuated VEE in peritoneal macrophages treated with anti-IFN-α/β antibody. Thioglycollate-elicited peritoneal macrophages from C57BL/6 mice were treated with sheep anti-IFN-α/β antibody prior to and following infection with virulent or attenuated, molecularly cloned VEE, V3000 or V3032, respectively. Cell culture medium was supplemented with a 1:10 dilution anti-IFN-α/β antibody (neutralizing titer of $10^6$ against 8 U of IFN-α/β) and cultures were infected at a multiplicity of infection of 1.0. Culture supernatants were harvested and standard plaque assays performed on BHK-21 cells.
Virulent and attenuated VEE growth kinetics in primary peritoneal macrophages from homozygous IRF-2<sup>+/−</sup> and heterozygous IRF-2<sup>+/−</sup> mice

During the initial 24 h p.i., virulent V3000 titers in IRF-2 knockout macrophages appeared similar to those in macrophages from control C57BL/6 mice. The typical exponential virus growth during the first 12 to 24 h of infection culminated in peak virus titers around 24 h p.i. Levels of peak titers depend on the original virus inoculum and the number of infected cells per culture dish, but usually persist for approximately 24 h in control C57BL/6 macrophages (Grieder and Nguyen, 1996). Thereafter, in both homozygous and heterozygous IRF-2 knockout macrophages, peak titers dropped significantly, resulting in 25- to 50-fold lower VEE titers at 48 and 72 h p.i. (Fig. 4a).

Similarly, attenuated V3032 growth kinetics in macrophages elicited from IRF-2<sup>−/−</sup> knockout mice was comparable to V3032 growth kinetics in IRF-2<sup>−/−</sup> macrophages (Fig. 4b). Identical attenuated and virulent VEE growth kinetics studies were also established in macrophages derived from IRF-1<sup>−/−</sup> and IRF-1<sup>+/−</sup> mice (data not shown). No significant differences in growth kinetics were observed between macrophages from the homozygous and the heterozygous knockout mice or in those from control mice. These results indicate the VEE growth kinetics in macrophages, as determined by <i>in vitro</i> growth pattern, do not appear to play a major role in the important differences of early VEE protection conferred by attenuated V3032 infection.

**VVE titers in IRF-2<sup>−/−</sup> mice following VEE infection**

Independent of genetic background, in all mice inoculated with virulent V3000 only, virus was recovered from the brain on days 7 and 8 p.i. This observation is consistent with the 100% mortality rate of virulent V3000 infection in mice. Titers were consistently high at 1 × 10<sup>7</sup> to 1 × 10<sup>8</sup> PFU/gram brain tissue (data not shown). In contrast, none of the mice inoculated with attenuated V3032 alone had a measurable VEE titer (data not shown). For the control C57BL/6<sup>+/+</sup> mice, this observation is consistent with previous reports characterizing attenuated V3032 dissemination; specifically, V3032 fails to invade the CNS (Grieder et al., 1995). However, it was somewhat surprising that no V3032 was recovered from the brains of V3032-infected IRF-2<sup>−/−</sup> mice, because in these knockout mice, V3032 caused 40% mortality.

VEE invasion of and clearance from the brain following dual VEE infection differed drastically between the C57BL/6<sup>+/+</sup> and the IRF-2<sup>−/−</sup> mouse strains (Table 3). In IRF-2<sup>−/−</sup> mice, which are not protected when peripherally primed with attenuated V3032 prior to challenge with 1 × 10<sup>7</sup> PFU of virulent V3000, 30, 60, and 80% of mice had VEE titers above the level of detection in their sera, spleen tissue, and brain tissue, respectively. The relatively low percentage of serum-positive mice implied that the observed VEE-positive tissues were not exclusively a result of contaminating serum (i.e., several mice with serum titers below 30 PFU/ml had positive spleen or brain titers). In contrast, 50% of the C57BL/6<sup>+/+</sup> mice tested positive in the brains, while all C57BL/6<sup>+/+</sup> serum and spleen titers were below the level of detection (i.e., 150 PFU/g fresh tissue). It was somewhat surprising that in the C57BL/6<sup>+/+</sup> mice, which usually survive virulent V3000 challenge when treated 24 h earlier with the attenuated V3032, 50% of the mice tested positive for VEE in their brains. Further, the average titer in the brains of the surviving C57BL/6<sup>+/+</sup> mice was significantly lower on days 7/8 p.i. compared to that measured in IRF-2<sup>−/−</sup> mice, e.g., 6 × 10<sup>5</sup> PFU versus 5 × 10<sup>6</sup> PFU per gram. These findings indicate that IRF-2 knockout mice were less efficient than the control mice in clearing VEE.

**Histopathological changes in brain sections from IRF-2<sup>−/−</sup> mice following VEE infection**

Coronal sections from mock-infected adult mice of either the C57BL/6 or the IRF-2<sup>−/−</sup> background showed very little histological difference when anatomically
Virulent VEE mortality in mice with mutated genes for interferon regulatory factors 1 and 2 (IRF-1\(^{-/-}\) and IRF-2\(^{-/-}\)). Groups of mice were injected into their left rear footpad with molecularly cloned virulent V3000 at a dose of 10 (a and b), 100 (c and d), or 1000 (e and f) PFU. Percentage survival was calculated for an observation period of 15 days. Survival of IRF-1\(^{-/-}\) and IRF-2\(^{-/-}\) was compared to age-matched groups of C57BL/6 control mice (data not shown) and heterozygous IRF-1\(^{+/+}\) and IRF-2\(^{+/+}\) littermate controls.
identical sections were compared (Figs. 5A and 5B). Minor differences were observed in the cerebellum (reduced ratio of the molecular to the granular layer in IRF-2<sup>−/−</sup> mice), while the appearance of the cerebrum in both the gray and the white matter was without significant differences.

On day 5 post-dual infection with attenuated and virulent VEE, mild to moderate signs of encephalitis were observed in the brain of one C57BL/6 mouse. This was the only one of four mice that had a significant VEE brain titer at that time (1 × 10<sup>6</sup> PFU/g tissue; data not shown). Coronal sections throughout the entire brain were characterized by mild neuronal degeneration with hyperchromatic cells, perivascular cuffing with lymphocyte infiltrate, and gliosis with activation of microglia and astrocytes (Fig. 5C). These histopathological changes were comparable to those in brains from mice infected with virulent V3000 alone (data not shown). In contrast, anatomically similar brain sections from age-matched IRF-2<sup>−/−</sup> mice infected with the same dual VEE infection were characterized by a moderate to severe degree of neuronal degeneration with much more significant vacuolization (Fig. 5D). Also, activation of glial cells was much more pronounced, resulting in moderate gliosis. However, there was a complete absence of perivascular cuffing with infiltrating lymphocytes. In 10 coronal brain sections obtained from infected IRF-2<sup>−/−</sup> mice, not a single blood vessel with infiltrating lymphocytes was observed, while 5 of 10 analyzed coronal brain sections from infected C57BL/6 mice had up to 32 blood vessels with the characteristic perivascular cuffing and cellular infiltrate. Moreover, microscopic analysis of the number of degenerative neurons in C57BL/6 infected brain sections resulted in a mean value of 5.6 ± 2.6 cells per high-power field. This is in contrast to a mean of 23.4 ± 5.8 degenerative neurons in similar brain sections from IRF-2<sup>−/−</sup> mice infected with the same two VEE strains (P < 0.001).

On day 8 post-dual infection, no significant histopathological changes were observed in any of four C57BL/6 mice analyzed. However, coronal sections from all five IRF-2<sup>−/−</sup> mice infected with the identical two viruses and sacrificed at 8 days postinfection presented with similar neuronal degeneration and vacuolization in the brain as described on day 5 postinfection. Further, there was no significant inflammatory response observed in any of the IRF-2<sup>−/−</sup> brain sections. These observations, together with the apparent failure of IRF-2<sup>−/−</sup> mice to clear the virus (see Table

![Graph showing percentage survival over days post-VEE inoculation for different groups of mice.](image)

**FIG. 3.** Attenuated VEE-induced protection against virulent VEE challenge in mice with deleted interferon regulatory factors-1 and -2 genes (IRF-1<sup>−/−</sup> and IRF-2<sup>−/−</sup>). Nine or ten adult C57BL/6 or homozygous or heterozygous IRF-1 or IRF-2 knockout mice were injected with 1 × 10<sup>3</sup> PFU of attenuated V3032 into the left rear footpad 24 h before challenge with 1 × 10<sup>2</sup> PFU of virulent V3000 into the right rear footpad. Percentage survival was calculated for an observation period of 14 days. All surviving mice were challenged intraperitoneally with 1 × 10<sup>4</sup> PFU of virulent V3000 at 3 weeks p.i. and survived without any clinical signs of encephalitis.
3) or to develop protection (see Fig. 3), indicate important differences in the IRF-2−/− mice’s neuropathogenesis of VEE infection. No significant histopathological changes were observed in brain sections from mice infected with the attenuated V3032 alone (data not shown).

FIG. 4. Virulent and attenuated VEE growth kinetics in primary peritoneal macrophages from mice with homozygous or heterozygous mutations in their interferon regulatory factor-2 gene (IRF-2−/− and IRF-2+/-). Thioglycollate-elicited peritoneal macrophages from control C57BL/6 or IRF-2−/− or IRF-2+/− mice were infected at a multiplicity of 1.0 with molecularly cloned attenuated V3032 or virulent V3000. Cell culture supernatants from two independent tissue culture wells were harvested and virus titers were determined by standard plaque assay using BHK-21 cells. IRF-2−/− or IRF-2+/− indicate peritoneal macrophages from thioglycollate-treated IRF-2−/− or IRF-2+/− mice, respectively.
DISCUSSION

The role of type I IFN in early VEE protection

VEE contains a single-stranded, positive-sense RNA genome in an icosahedral nucleocapsid that is enclosed in a host cell membrane-derived envelope (reviewed by Strauss and Strauss, 1994). Two virus-encoded glycoproteins, E1 and E2, are inserted into this envelope and present in icosahedral organization as spikes on the surface. Among other functions, the VEE spikes have been shown to play important roles in virus interaction with the host's immune cells (Bose and Sagik, 1970; Davis et al., 1986, 1995). In all experiments discussed in this communication, we used the two molecularly cloned VEE, which differed by only one amino acid in the glycoprotein spike (Davis et al., 1991; Grieder et al., 1995).

In this report, we showed that type I IFN is necessary in the initial response to VEE infection, as demonstrated by the accelerated, lethal course of attenuated VEE infection in IFNAR1 mice. Our observation is further supported by the findings that (i) in vivo anti-IFN-α/β antibody treatment of mice decreased average survival time of virulent V3000-infected animals (Grieder et al., 1997) and (ii) in vitro anti-IFN-α/β antibody treatment of primary peritoneal macrophage cultures prior to virulent VEE infection results in higher VEE titers than in control cultures. These findings parallel those in a different model, e.g., SFV infection in newborn mice, in which IFNAR1 mice died rapidly following SFV infection (Muller et al., 1994; Hwang et al., 1995).

However, in both the VEE and the SFV systems, IFN was not sufficient to protect against virus challenge. In this study we demonstrated that intraperitoneal administration of either 80,000 U of recombinant IFN-β or the IFN-inducing agent, Poly I:C, resulted in serum IFN levels 24 h after injection of 1600 and 6400 U/ml, respectively, yet failed to protect mice challenged with 100 PFU of virulent VEE. The average survival times of these mice were identical to those of mice treated with PBS prior to infection with virulent V3000. These findings are consistent with those in another study using the IFN-inducer Poly I:C to treat SFV infection in mice (Coppenhaver et al., 1995), in that Poly I:C alone failed to reduce mortality following SFV infection. In that study, the estimated measured IFN levels reached 1 × 10^8 U/ml serum, similar to those measured in sera of neonatal mice infected with attenuated SB virus (Trgovcich et al., 1996), while infection of neonatal mice with virulent SB virus resulted in serum IFN levels of 10^5 U/ml (Trgovcich et al., 1996). The higher IFN titers in the SB virus study were associated with reduced average survival times of the host. These results may be explained by different IFN sensitivity, different IFN-induction kinetics, or additive effects of different immunomodulators. We conclude from ours and these studies that IFN alone is insufficient to elicit a protective response to VEE.

The role of interferon regulatory factor in VEE infection

To understand the role of IFNs in VEE pathogenesis and protection, the potential role of IFN gene transcriptional regulators deserves evaluation. This group of transcription regulatory factors, called IRFs (Fujita et al., 1988; Miyamoto et al., 1988), functions as activator, repressor, and activation/repressor combinations for IFN gene transcription (Pitha, 1997). Harada and colleagues demonstrated that some are constitutively expressed (IRF-1 and -2) (Harada et al., 1989), while others (IRF-1) are induced following exposure to virus, to type I IFN, or to other cytokines (Fujita et al., 1989; Miyamoto et al., 1988). However, the role of IRFs in virus infections appears to be highly virus-specific. In initial reports, it was hypothesized the IRF-1^-/- mice should display decreased or absent IFN levels and therefore should evidence increased virus replication. Alternatively, IRF-2^-/- mice were predicted to have normal or increased IFN levels, which could result in reduced virus replication and extended survival times. Infection of IRF-1^-/- mice with encephalomyocarditis virus did indeed result in increased virus titers in the brain and heart of infected mice and in reduced average survival times, while infection with vesicular stomatitis virus or herpesvirus resulted in little or no change in the pathogenicity of such infected mice (Kimura et al., 1994).

In our experiments, we found that both IRF-1 and IRF-2...
knockout mice are highly susceptible to infection with molecularly cloned virulent and single-site-attenuated VEE. Although average survival times were not statistically different between the homozygous and the heterozygous mice in both the IRF-1 and the IRF-2 mouse strains, homozygous knockout mice always died before their heterozygous litter mates, at all levels of virus challenge (10, 100, and 1000 PFU of virulent V3000 per mouse).

FIG. 5. Histopathology analysis of coronal brain sections in IRF-2−/− mice following VEE infection. Groups of mice were inoculated with VEE as described in Table 3 and brain sections were subjected to staining with hematoxylin and eosin. Histological appearance of mice mock injected (A, C57BL/6, and B, IRF-2−/−) are shown in comparison to mice injected with attenuated V3032 followed by virulent V3000 24 h later (C, C57BL/6, and D, IRF-2−/−) at 6 days postinjection. Original magnification 200X.
One notable observation is that two IRF-1<sup>−/−</sup> mice inoculated with virulent V3000 at doses of 10 and 100 PFU, respectively, survived the initial infection and were protected against subsequent challenge with a more highly concentrated VEE dose. These two mice came from a cohort of 216 homozygous and heterozygous IRF knockout mice. This is significant, because in over 1000 C57BL/6 mice injected with virulent V3000 over several years, survival was never observed after infection with any dose higher than 1 PFU. This leads to the interesting speculation that disruption of one IRF-1 allele may affect expression of IRF-2, subsequently altering regulation of IFN and, perhaps, other factors. If confirmed, such improved resistance to virulent infection suggests that a potential new pathway might exist in early protection. There is further tantalizing evidence of a gene dosage effect and the relative contribution of IRF-1/2 to this system: heterozygous IRF-1<sup>−/−</sup> and IRF-2<sup>−/−</sup> mice always survived homozygous knockout mice; but IRF-1<sup>−/−</sup> mice were significantly more protected by attenuated V3032 than IRF-2<sup>−/−</sup> mice.

We generated supportive data for the hypothesis that deletion of the IRF-2 locus increases the severity of VEE infection by demonstrating that IRF-2<sup>−/−</sup> mice clear virus from the brain and the spleen more slowly than IRF-2<sup>−/−</sup> mice (data not shown). This slow or absent viral clearance was shown by an increasing virus brain titer in the IRF-2<sup>−/−</sup> mice by day 8 p.i. Similarly, slower clearance and increased tissue virus titers in IRF-2<sup>−/−</sup> mice were also found when they were compared to the C57BL/6 strain.

VEE infection in mice with genetically altered IRF-2 genes showed clear evidence of altered initial inflammatory response and subsequent neuronal degeneration. Five days postinfection with the combination of V3032 and V3000 24 h apart, one of four C57BL/6 mice had a virus brain titer of 1 × 10<sup>6</sup> PFU/g tissue. This mouse showed typical histopathological changes associated with VEE-induced encephalitis, i.e., perivascular cuffing with lymphocyte infiltrate and mild neuronal degeneration with glial activation. These histopathological changes contrasted with those observed in IRF-2<sup>−/−</sup> mice infected with the same virus stocks and doses. The IRF-2<sup>−/−</sup> mice showed a complete absence of perivascular cuffing or infiltrating lymphocytes and more moderate to severe neuronal degeneration with significant vacuolization. Additionally, there was significant glial cell activation resulting in pronounced gliosis. The absence of inflammatory cells indicates that the responses to peripheral VEE infection are altered in IRF-2<sup>−/−</sup> mice.

In summary, we demonstrated that type I IFN is necessary, but not sufficient, for early protection against virulent VEE infection. Further, we showed that early protection against VEE is dependent on IRF-2 and to a lesser extent on IRF-1. And finally, we found the IRF-2<sup>−/−</sup> mice show a reduced inflammatory response to VEE infection that may be linked to the reduced capacity to clear virus from both the periphery and the CNS. We speculate that this altered activation of proinflammatory cells is related to proinflammatory products such as reactive nitrogen intermediates, which have been shown to be down-regulated in IRF-1<sup>−/−</sup> and IRF-2<sup>−/−</sup> macrophages (Salkowski et al., 1996) and IRF-1<sup>−/−</sup> mice (Fujimura et al., 1997).

**MATERIALS AND METHODS**

**Animals**

Eight to 20-week-old IRF-1 and IRF-2 knockout mice (IRF-1<sup>−/−</sup> and IRF-2<sup>−/−</sup>) were obtained originally from Dr. Tak Mak (Amgen Institute, Toronto, Canada). IRF-1<sup>−/−</sup> and IRF-2<sup>−/−</sup> mice were generated by targeted disruption in the embryonic stem cell using the neomycin resistance gene (neo<sup>r</sup>) (Matsuyama et al., 1993). Mice were backcrossed to C57BL/6 mice before the establishment of IRF-1<sup>−/−</sup> and IRF-2<sup>−/−</sup> mouse colonies which were maintained at USUHS. The genotype of all IRF-1 and IRF-2 mice was determined by PCR using the primers and methods described previously (Salkowski et al., 1996). The IRF-1 and IRF-2 primers amplify a 300- and 100-bp sequence, respectively, from genomic DNA. For the backcross breeding, 10-week-old C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME), while for experimental controls, age-matched C57BL/6 (+/+) mice were obtained from the National Cancer Institute, NIH (Bethesda, MD). Mice with disrupted genes in the interferon-α/β receptor (IFNAR1) (Muller et al., 1994) were obtained originally from B&K Universal Ltd. (Aldbrough, UK) and bred at the Department of Molecular Microbiology and Immunology, The Johns Hopkins University School of Hygiene and Public Health (Baltimore, MD). Age-matched 129Sv/Ev mice used as controls for the IFNAR1 mice were obtained from Taconic Laboratories (Germantown, NY). At USUHS, all animals were maintained on standard laboratory feed and water ad libitum in AAA-LAC-accredited animal facilities.

**Virus, cells, and antibody**

Two molecularly cloned VEE strains, virulent V3000 and attenuated V3032, were used in all the experiments. The parental V3000 and the single-site-attenuated mutant V3032 differ in their genotype by one single nucleotide, resulting in a single amino acid change at E2 glycoprotein position 209, which replaces a glutamic acid in V3000 with a lysine in V3032 (Grieder et al., 1995). Virus stocks were stored in a Biosafety Level 3 laboratory at −80°C.

Primary peritoneal macrophages from C57BL/6 or IRF knockout mice were generated as previously described (Grieder and Nguyen, 1996). Macrophages were maintained in RPMI 1640 medium containing less than 0.005 U/ml endotoxin, supplemented with 2% fetal bovine serum, 30 mM HEPES buffer, 36 mM NaHCO<sub>3</sub>, 2 mM L-glutamine, and antibiotics (100 U/ml penicillin, 100 μg/ml.
streptomycin). Baby Hamster Kidney 21 (BHK) cells were obtained from the ATCC in passage 53 and maintained according to ATCC guidelines. All experiments including virus stock preparations and titrations were performed using BHK cells between passages 55 and 65.

Purified sheep anti-mouse IFN-α/β antibody was provided by Dr. Ion Gresser (Viral Oncology, Villejuif Cedex, France). Anti-IFN-α/β antibody had a neutralizing titer of 10^6 against 8 units of IFN-α/β and was added to cell cultures at a 1:10 dilution in regular growth medium. Control cells were treated with normal goat serum at the same concentration.

Virulence and early protection

Groups of age- and sex-matched mice were inoculated subcutaneously (sc) into their left rear footpad with 10, 1 × 10^3, or 1 × 10^4 plaque forming units (PFU) in a volume of 25 μl. Percentage mortality and average survival times ± standard error (AST ± SE) were calculated following an observation period of 14 or 15 days. Only dead animals were included in the AST calculation. Surviving mice were challenged intraperitoneally with 1 × 10^6 PFU of virulent V3000 to verify that they were truly infected initially. Control groups were injected with identical volumes of diluent (phosphate-buffered saline containing magnesium, calcium, and 0.1% donor calf serum; PBS–DCS) by the same route.

For early protection experiments, groups of adult control or knockout mice (IFNAR1 or IRF-1/2) were injected into their rear left footpads with 10^3 PFU of attenuated V3032 followed 24 h later by injection of 10^5 PFU virulent V3000 into their right rear footpad. The AST were calculated in days ± SE unless tissues from infected mice were harvested for virus titration.

Virus replication in vivo and histopathology

Eight-week-old C57BL/6, IRF-2^{-/-}, and IRF-2^{+/-} mice were inoculated sc in the left rear footpad with 1 × 10^3 PFU of attenuated V3032 only or with attenuated V3032 followed 24 h later with 1 × 10^5 PFU of virulent V3000 in the right rear footpad. Control mice were injected with 1 × 10^2 PFU V3000 in the right rear footpad only. On days 5, 7, or 8 postinfection, mice were anesthetized and exsanguinated. Sera, spleen, and brain tissues were harvested for viral titration and for histopathological analysis. Fresh tissues were homogenized to make a 20% (w/v) tissue suspension in PBS–DCS. The final suspension was clarified by centrifugation and frozen at −80°C, and standard plaque assay on BHK cells was used to determine virus titers per gram fresh tissue.

Tissue samples for histopathological analysis were fixed in 10% buffered formalin for at least 24 h, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Negative controls were tissues of control mice inoculated with PBS–DCS and processed in parallel.

Coronal brain sections from C57BL/6 and IRF-2^{-/-} mice infected with the attenuated V3032 followed 24 h later with virulent V3000 and sacrificed on day 5 postinfection were used to quantitate the numbers of degenerative neurons in both mouse strains. Forty randomly selected high-power fields in the gray matter in hippocampus sections were used to count the total number of degenerating cells located in vacular spaces. Only cells with the histological appearance of neurons were included in the counts. Low-power magnification was used to ensure that only areas with similar neuron density were used. Mean numbers of degenerative cells were calculated and standard Student's t test was performed.

Virus growth kinetics

Primary peritoneal macrophage cultures were grown in six-well tissue culture plates at 1 × 10^6 cells per well for 24 h as described above. Cell cultures treated with anti-IFN or control antibody were infected with either V3000 or V3032 at a multiplicity of infection of 1 PFU/cell and samples from the supernatant were collected over time. Virus titers in the supernatants were determined by standard plaque assay method on BHK cells (Grieder et al., 1995).

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