Role of type I and type II interferon responses in recovery from infection with an encephalitic flavivirus

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We have investigated the contribution of the interferon (IFN)-α/β system, IFN-γ and nitric oxide to recovery from infection with Murray Valley encephalitis virus, using a mouse model for flaviviral encephalitis where a small dose of virus was administered to 6-week-old wild-type and gene knockout animals by the intravenous route. We show that a defect in the IFN-α/β responses results in uncontrolled extraneural virus growth, rapid virus entry into the brain and 100% mortality. In contrast, mice deficient in IFN-γ or nitric oxide production display an only marginally increased susceptibility to infection with the neurotropic virus.

The encephalitic flaviviruses are the most important causative agents of viral encephalitis in humans. Japanese encephalitis virus (JEV) inflicts >35,000 fatal cases annually, and life-long debilitating neurological sequelae often persist in surviving patients (Burke & Monath, 2001; Tsai, 2000). Murray Valley encephalitis virus (MVE) is an Australian flavivirus genetically most closely related to JEV (Burke & Monath, 2001; Marshall, 1988). Other members of the JEV serocomplex include West Nile, St Louis encephalitis and Kunjin viruses (Calisher et al., 1989). Mice are an excellent animal model for flaviviral encephalitis. Similar to natural infection in humans with JEV or MVE, which is mostly subclinical, extraneural inoculation of adult mice produces rare mortality, low or undetectable viraemia, and poor virus growth in extraneural tissues (Eldadah et al., 1967; Grossberg & Scherer, 1966; Huang & Wong, 1963; MacDonald, 1952a, b). In contrast, intracranial inoculation with a low virus dose generally results in high virus titres in the brain and is mostly lethal. Thus, it appears that a rare stochastic event, perhaps that leading to virus entry into the brain, is key to disease severity. Given that neuroinvasion is most likely preceded by virus replication in extraneural tissues, the inhibition of virus growth in primary infected tissues probably plays an important role in preventing virus entry into the central nervous system (CNS). In contrast to adult mice, viraemia and virus replication in peripheral tissues is apparent in mice less than 3 to 4 weeks of age following infection by an extraneural route (Huang & Wong, 1963; McMinn et al., 1996; Monath et al., 1983). Factors that account for the age-dependent difference in growth of flaviviruses in extraneural tissues remain elusive.

The earliest host responses to viral infections are non-specific and involve among other cytokines the induction of interferons (IFNs) (Biron & Sen, 2001; Goodbourn et al., 2000). Type I IFNs, IFN-α/β, are produced by leukocytes and fibroblasts, respectively, in response to infection and activate the transcription of IFN-inducible genes which leads to the

Fig. 1. Susceptibility of mice deficient in type I or type II IFN responses to infection with MVE. (A) Groups of 6-week-old B6, IFN-γ−/− and IFN-α-R−/− mice, and (B) i-NOS+/+ and i-NOS−/− mice were infected i.v. with 10^2 p.f.u. of MVE. Mortality was recorded daily and surviving mice monitored for 28 days, at which time blood was taken to confirm sero-conversion by ELISA as described (Licon Luna et al., 2002). Difference in survival ratios of gene knockout mice in comparison to B6 (A) and i-NOS+/+(B) mice were assessed for significance using Fisher’s exact test and P values are given.
induction of antiviral pathways within hours. IFN-γ is made exclusively by natural killer and T cells and has important immunoregulatory functions (Boehm et al., 1997). Most of the antiviral activity of IFN-γ is mediated by nitric oxide (NO) synthesized by monocytes, following induction of the enzyme NO synthase by the cytokine (Guidotti & Chisari, 2000; MacMicking et al., 1997; Reiss & Komatsu, 1998). The contribution of IFN-α/β in recovery from infection with encephalitic flaviviruses has been shown in vivo by the therapeutic and prophylactic effects of administration of IFN-inducers (Haahr, 1971; Taylor et al., 1980; Vargin et al., 1977) or IFN (Brooks & Philipotts, 1999; Pinto et al., 1988). On the other hand, it remains unresolved whether IFN-γ, for instance via induction of NO, plays an exacerbating or protective role in encephalitic flavivirus infection. Whilst an immunopathological contribution of NO was noted in experimental infections with MVE and tick-borne encephalitis virus (Andrews et al., 1999; Kreil & Eibl, 1996), others have found increased mortality of JEV-infected mice when NO synthase was inhibited (Lin et al., 1997; Saxena et al., 2000, 2001). Here we have employed mice deficient in type I or type II IFN responses as a consequence of gene knockout in a model of flavivirus encephalitis to investigate the role of IFNs and NO in pathogenesis and recovery from infection with MVE.

Infection of 6-week-old B6 mice with 0·1 to 10⁵ p.f.u. of MVE (prototype strain MVE-1-51) intravenously (i.v.) gives mortality in up to 50% of animals, despite low or undetectable virus growth in extraneural tissues (Licon Luna et al., 2002). A lack of the IFN-α receptor dramatically increased the susceptibility of mice to MVE (Fig. 1A). Infection with 10⁵ p.f.u. i.v. of IFN-α receptor knockout mice (IFN-α-R⁻/⁻; B6 congeneic), which are completely unresponsive to type I IFNs (Müller et al., 1994), was lethal in 100% of animals. The average time to death (ATD) of these mice (5–6 days) was significantly shorter (P=0·004; Mann–Whitney test) relative to control B6 mice (13–2 days) infected with the same dose of MVE. In contrast, mice deficient in IFN-γ production (IFN-γ⁻/⁻; B6 congeneic) (Dalton et al., 1993) showed only a slight increase in mortality relative to the control group (P=0·045, Fisher’s exact test; Fig. 1A) after infection i.v. with 10⁵ p.f.u. of MVE. Mortality in a group of 28 infected IFN-γ⁻/⁻ mice was 60% in comparison to 30% mortality in a group of B6 mice infected in parallel. The ATD (11-6 and 13-2 days, respectively) was not significantly different between the two groups (P=0·14).

Given the conflicting reports on the effect of NO in disease outcome in flaviviral encephalitis (see above), we also tested the susceptibility to MVE of mice deficient in NO synthase-2 (i-NOS⁻/⁻) mice; MacMicking et al., 1995) backcrossed on B6 (Karupiah et al., 1998) (Fig. 1B). Infection of i-NOS⁻/⁻ mice with 10⁵ p.f.u. of MVE i.v. gave 60% mortality, which was slightly higher than that of a group of congeneic control i-NOS⁺/+ mice (35%), although the difference was not significant (P=0·205). The ATD in the groups of i-NOS⁻/⁻ and i-NOS⁺/+ mice was 12·7 and 14·3 days, respectively (P=0·07).

To analyse the role of IFNs in the control of virus growth in extraneural tissues and in turn, the rate of neuroinvasion, 6-week-old B6, IFN-γ⁻/⁻ and IFN-α-R⁻/⁻ mice were infected i.v. with 10⁵ p.f.u. of MVE and serum and tissues collected at the times p.i. shown in Fig. 2. Spleen and brain tissue was processed as described (Licon Luna et al., 2002); blood was taken from the tail vein of animals under anaesthesia or by heart puncture of animals that were sacrificed, allowed to

![Fig. 2. Growth of MVE in mice defective in type I or type II IFN responses.](Image)

Fig. 2. Growth of MVE in mice defective in type I or type II IFN responses. Mice (6-week-old) were infected i.v. with 10⁵ p.f.u. of MVE. At the indicated times blood was taken, or animals were sacrificed and blood and tissues collected. The lower limits of detection of virus in tissues and serum of infected mice were 10⁵ p.f.u. g⁻¹ and 10² p.f.u. ml⁻¹, respectively, and are indicated by the dashed lines. Each symbol represents an individual mouse.
clot at room temperature for 10 min, kept on ice for 20 min, centrifuged at 2000 r.p.m. for 10 min in an Eppendorf microfuge, and serum collected for storage at −70°C. Virus titres were determined by plaque formation on Vero cells (Licon Luna et al., 2002). Infection of B6 wild-type (wt) mice gave no detectable viraemia at 2, 4, 6, 8 and 9 days p.i.; only one mouse showed a low virus titre in serum (3\(^{\times}\)10\(^2\) p.f.u. ml\(^{-1}\)) at 24 h p.i. No virus was detected in all but one (2\(^{\times}\)10\(^3\) p.f.u. g\(^{-1}\) on day 4 p.i.) of the spleen samples harvested between days 2 to 9 p.i. In the absence of IFN-γ, virus growth in extraneural tissues was also poor. However, the observation that all spleen samples \((n = 5)\) taken at 4 days p.i. from IFN-γ\(^{-/-}\) mice had detectable virus (10\(^5\) to 10\(^7\) p.f.u. g\(^{-1}\)) provides evidence for an increased viral load in the knockout relative to wt mice. It should be noted that an inhibitory effect of spleen homogenates on growth of JEV serotype flaviviruses at low dilutions (\(\geq 0.5\) % homogenate) results in an underestimation of the true viral load by plaque assay (Lee & Lobigs, 2002). This is relevant for MVE titres in the spleen in the range of 10\(^3\) to 10\(^4\) p.f.u. g\(^{-1}\), which may be underestimated by up to 10-fold. No such inhibitory effect is apparent for virus detection in serum.

Following i.v. infection of B6 mice with 10\(^2\) p.f.u. of MVE, virus is first detected in brain after day 6 p.i. (Licon Luna et al., 2002). This was also found in the experiment shown in Fig. 2. Three out of nine B6 brains harvested at days 8 and 9 p.i. were positive for MVE with titres in the range 10\(^5\) to 10\(^7\) p.f.u. g\(^{-1}\). This rate of neuroinvasion was identical to the mortality rate of MVE-infected B6 mice shown in Fig. 1; accordingly, virus entry into the brain is a strong correlate of lethal disease outcome. Consistent with the slightly increased mortality of MVE-infected IFN-γ\(^{-/-}\) relative to B6 wt mice, we also found an increase in the proportion of infected brains (70 %) in IFN-γ\(^{-/-}\) animals sacrificed on days 8 and 9 p.i. (Fig. 2). However, the range of virus titres did not differ from that found in infected B6 wt mice at the corresponding times p.i.
In 6-week-old mice deficient in IFN-α/β responses, injection of 10^7 p.f.u. of MVE i.v. resulted in a fulminant infection. At 2 days p.i., all animals were viraemic (mean titre = 8 × 10^3 p.f.u. ml⁻¹) and had virus in spleen (mean titre = 8 × 10^4 p.f.u. g⁻¹) but not brain. The viral load in the spleen increased massively in the following 2 days of infection (mean titre on day 4 p.i. = 7.5 × 10^6 p.f.u. g⁻¹) and exceeded viral titres in serum 1000-fold, suggesting that MVE grows efficiently in the spleen of IFN-α-R⁻/⁻ mice. Virus could be recovered from the brain at 4 days p.i. with viral titres ranging from 5 × 10^3 to 2 × 10^8 p.f.u. g⁻¹. Given that in each mouse the viral load in the brain was equal or greater than that in the serum, it is unlikely that virus detection in brain samples was due to viraemic blood contamination.

To evaluate the contribution of IFN-γ and NO to inflammation and pathology in the brains of 6-week-old mice infected with MVE (10^7 p.f.u., i.v.), brain tissue from mice sacrificed at 8 and 9 days p.i. was histologically examined (Fig. 3 and Table 1). Virus titres in the brain were determined in parallel. Detection of virus by plaque assay in brain samples was most indicative of the presence of inflammatory infiltrates and necrotic cells in the brain parenchyma and leptomeninges on days 8 and 9 p.i. in B6 wt, IFN-γ⁻/⁻ and i-NOS⁻/⁻ mice. However, the severity of inflammatory and histopathological manifestations, which were more prominent at 9 days p.i., showed no correlation with the magnitude of viral titres and was not markedly different between the mouse strains. Flavivirus-immune CD8⁺ T cells, which are found in the brains of infected mice as early as 5 days p.i. (Liu et al., 1989), secrete IFN-γ upon in vitro stimulation with viral antigens (Liu & Chambers, 2001; Regner et al., 2001), and IFN-γ is produced in the CNS of infected mice (Liu & Chambers, 2001), suggesting that the stimulation of the cytokine is not down-regulated in flaviviral infections.

In conclusion, we show that growth of MVE in extraneural tissues in adult B6 mice is strongly inhibited by IFN-α/β and that host survival is critically dependent on a functional type I IFN response. It appears that a high and persistent viraemia that host survival is critically dependent on a functional type I IFN response. However, IFN-α/β are not the sole determinants of resistance of older mice to flavivirus replication, given our recent report that growth of an attenuated variant of JEV was significantly more restricted in 6-week-old than 3-week-old IFN-α-R⁻/⁻ mice (Lee & Lobigs, 2002). A similar conclusion on the role of IFN-α/β in age-related permissiveness of mice to Sindbis virus was reached by others (Ryman et al., 2000). Finally, we show that mice with a targeted disruption of the IFN-γ or NO synthase-2 genes were only marginally more susceptible to CNS invasion and

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<th>Day p.i.</th>
<th>Strain</th>
<th>Virus titre*</th>
<th>Infiltration†</th>
<th>Necrosis‡</th>
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<tr>
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<tr>
<td>8</td>
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<td>i-NOS⁻/⁻</td>
<td>1.2 × 10⁶</td>
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*B: Brains from mice infected i.v. with 10^7 p.f.u. of MVE were harvested at the times p.i. shown and cut in half: one half was used for virus titration by plaque assay and the second for histological examination. Tissues were fixed in 10% buffered formalin, embedded in paraffin and following staining with haematoxylin and eosin, examined by light microscopy.† Evaluation based on the number of cell infiltrates/field from three serial fields of the 25× objective: −, no infiltrate; +, up to 5 infiltrates per field; + +, >5 infiltrates per field.‡ Evaluation of necrosis: −, no cell degeneration; +, up to 5 necrotic cells per field; + +, >5 necrotic cells per field.
mortality after i.v. infection with MVE than B6 mice. Importantly, a significant contribution of IFN-γ or NO to immunopathology was not noted. This questions the use of immunosuppressants in patients with MVE CNS illness (Hoke et al., 1992). A partial role of IFN-γ and NO in host defence has also been reported in a model of yellow fever encephalitis (Liu & Chambers, 2001) and JEV infection (Lin et al., 1997; Saxena et al., 2000, 2001) and may account for exacerbation of West Nile virus infection following macrophage (a major producer cell of NO) depletion (Ben-Nathan et al., 1996). Thus, this study suggests that the potentially deleterious inflammatory response resulting from the production of IFN-γ and NO is more than compensated for by their antiviral functions.

REFERENCES


