Lyssavirus infection activates interferon gene expression in the brain

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To investigate the innate immune response within the brain to lyssavirus infection, key transcripts indicative of innate defences were measured in a mouse model system. Following infection with Rabies virus, transcript levels for type 1 interferons (IFN-α and -β), the inflammatory mediator interleukin 6 (IL-6) and the antiviral protein Mx1 increased in the brains of mice. Intracranial inoculation resulted in the early detection of virus replication and rapid expression within the brain of the innate immune response genes. Transcripts for type 1 IFNs declined as the disease progressed. Peripherally, extraneural inoculation delayed the host response until virus entered the brain, but then resulted in a large increase in the level of IFN-β, IL-6 and Mx1 transcripts. Induction of this response was also observed following infection with the related European bat lyssaviruses, a group of zoonotic viruses capable of causing fatal, rabies-like disease in mammalian species.

Lyssaviruses are a group of negative-strand, non-segmented RNA viruses that are highly neurotropic and cause fatal encephalitis when the virus gains access to the central nervous system (CNS). Rabies virus (RABV) is the type species of the genus Lyssavirus and it is estimated that it causes over 35,000 human deaths throughout the world annually (Coleman et al., 2004). European bat lyssaviruses (EBLVs) are less common than RABV, are restricted geographically and cause rabies-like disease within their reservoir hosts, the Serotine bat (Eptesicus serotinus) for EBLV-1 and Daubenton’s bat (Myotis daubentonii) for EBLV-2. Human fatalities due to infection with European bat lyssaviruses (EBLVs) are rare, but have been reported (Fooks et al., 2003). Lyssavirus infections usually result from the bite of an infected animal. Subsequently, the virus infects local sensory and motor neurons (Tsang et al., 1986; Shankar et al., 1991) and rapidly ascends the nervous system to the brain. Uncontrolled replication leads to disease and ultimately death (Jackson, 2003). Infection can produce an adaptive immune response, often late in the clinical course, that fails to control disease. However, prophylactic and post-exposure vaccination provides effective immune protection through the induction of neutralizing antibodies (Hooper et al., 1998). In addition to the adaptive immune response, host defence against the early stages of infection is provided by the innate immune response (Haller et al., 2006). One of the most important groups of innate cytokines is the interferons (IFNs), which have potent antiviral effects against many viruses, including a number of negative-strand RNA viruses such as Measles virus, influenza viruses and vesicular stomatitis viruses (Meier et al., 1990; Schneider-Schaulies et al., 1994; Lobigs et al., 2003). High levels of IFN are detectable in the serum of mice inoculated with RABV by the peripheral or intracerebral routes (Stewart & Sulkin, 1966; Marcovizts et al., 1984, 1994). Inoculation of the IFN inducer poly(I : C), immediately after RABV challenge reduces mortality and improves RABV vaccine efficacy (Harmon & Janis, 1975). These results demonstrate the importance of innate immune responses in protection against RABV disease.

In mouse models of RABV infection, levels of a number of gene transcripts associated with innate defences, including STAT1, IFN-γ, tumour necrosis factor alpha, interleukin 6 (IL-6), IL-1β, T-cell growth factor β and Toll-like receptors (TLRs), have been shown to increase (Marquette et al., 1996; Prosnia et al., 2001; Baloul & Lafon, 2003; Saha & Rangarajan, 2003; McKimmie et al., 2005). A recent gene array-based comparison of mouse brain gene expression in...
response to infection with a highly neurovirulent RABV isolate and an attenuated laboratory strain demonstrated upregulation of many gene transcripts involved in innate antiviral and inflammatory responses (Wang et al., 2005). The magnitude of the response reflected the pathogenic properties of the virus. The innate response to infection with neurotropic lyssaviruses other than RABV has not been investigated. Given the differences in immune response gene activation between street RABV and attenuated laboratory strains of RABV (Wang et al., 2005), analysis of the activation of innate immune response genes by lyssaviruses could be informative as to their relative pathogenicity. The extent and temporal course of innate immune responses in the rodent brain during lyssavirus infection were determined by analysis of virus and a limited, but crucial, set of host genes known to be activated during RABV infection (Wang et al., 2005). Type 1 IFNs were selected for their pivotal role in the development of an antiviral state (Haller et al., 2006), IL-6 was selected as a marker for the inflammatory response to viral infection in the CNS (Frei et al., 1989) and Mx1 was selected as an IFN-inducible transcript with known antiviral properties for negative-strand RNA viruses (Haller et al., 1998).

Outbred CD1 mice were inoculated intracranially or peripherally (footpad) with 30 μl virus preparation [4·4 log10(MLD50)] in 30 μl; derived from an intracranial inoculation. Mice were sacrificed and brains were removed at each time point or upon development of overt disease. Total RNA was extracted by using TRIzol (Invitrogen) and treated with 0·3 units DNase μl−1 whilst bound to an RNeasy Mini column (Qiagen) for 15 min at room temperature. RNA (2 μg) was reverse-transcribed by using 8 pmol poly(dT) (Roche) and 200 U Moloney murine leukemia virus reverse transcriptase (Promega). Lyssavirus transcripts were analysed by end-point PCR and host transcripts were measured by quantitative (Q) PCR (Bustin & Nolan, 2004) using specific primers (see Supplementary Table S1, available in JGV Online). Amplifications were performed by using SyBr Green JumpStart Taq ReadyMix (Sigma) in an MX3000P thermal cycler (Stratagene), using an annealing temperature of 50 °C. All transcripts were normalized against β-actin and quantified by reference to a standard curve using threshold cycle (Ct) values as described previously (McKimmie et al., 2005).

The ability of RABV to enter the CNS is one of its most fundamental pathogenic characteristics (Coulon et al., 1998). Infection by the intracranial route avoids the requirement for virus to gain access to the CNS. To determine whether intracranial or peripheral inoculation with a fixed strain of RABV (CVS) induces an innate immune response in the brain, mice were inoculated with virus by these two routes and the levels of the RABV N gene transcript and host gene transcripts were measured. Intracranial inoculation resulted in the reproducible detection of replicating virus in the brain within 2 days (Fig. 1a) and the development of signs of disease, such as ruffled fur and reduced movement, by day 4. Peripheral inoculation, which matches natural transmission most closely, delayed the detection of virus replication in the brain until day 4 (Fig. 1a) and delayed clinical signs of disease until day 5. Significant increases in host innate immune gene transcript levels following intracranial inoculation of RABV were observed by day 4,
with levels of IFN-α and -β increasing by two- and eightfold, respectively (Fig. 1b). Gene transcript levels for type 1 IFNs declined at 6 days. Increases in IL-6 and Mx1 gene transcripts were first detected at day 4 and increased throughout the course of infection to approximately 150- and 240-fold, respectively, by day 6 (Fig. 1b). Virus inoculation at a peripheral site also led to an increase in host innate immune transcripts in the brain. However, in agreement with the delay in the detection of virus replication in the brain, these increases were delayed relative to those observed with direct intracranial inoculation. The increase in type 1 IFN transcript levels only became significant at day 6. IL-6 and Mx1 transcript increases were low but significant at day 4, but reached levels of 90- and 5000-fold, respectively, by day 6.

These observations demonstrate that both intracranial and peripheral infection with CVS induces innate immune responses in the brain. To compare this with the ability of other lyssaviruses to induce innate immune gene expression, a series of lyssavirus isolates (see Supplementary Table S2, available in JGV Online), including examples of street RABV, EBLV-1 and EBLV-2, were inoculated intracranially into mice and IFN-α gene expression was evaluated semi-quantitatively. Transcript levels for β-actin and lyssavirus nucleoprotein were also determined. Mock infection with uninfected mouse brain homogenate failed to induce detectable IFN-α transcripts (Fig. 2, M). RNA from all lyssavirus-infected brain samples contained detectable IFN-α gene transcript (Fig. 2), often producing a strong RT-PCR product. IFN-β and Mx1 transcripts were also observed consistently in all lyssavirus-infected, but not mock-infected, brain samples (data not shown). To investigate one isolate of EBLV in more detail, viral and host transcripts were measured in the brains of CD1 mice challenged intracranially or peripherally with EBLV-2 (isolate 1332) at the same titre as that used for RABV (Fig. 3). This virus was isolated from a Daubenton’s bat (M. daubentonii) in the UK (Johnson et al., 2003). Infection by the intracranial route caused reproducible disease in all mice inoculated (n=3), with signs of rabies-like disease first evident between days 8 and 9 (Fig. 3a). The time from first signs to development of limb paralysis was generally longer (more than 3 days) than for RABV (CVS strain). Challenge by the peripheral route (footpad) did not result in a reproducible outcome in all animals. In some animals, EBLV-2 replication was detectable in the brain by day 15 post-inoculation, with disease developing between days 16 and 18 (Fig. 3a); however, other animals failed to develop disease up to 90 days post-inoculation. Consequently, only those brain samples with detectable viral transcript were used to assess host brain transcriptional changes. Fig. 3(b) presents the transcript changes for type 1 IFNs, IL-6 and Mx1 in the brains of mice infected with EBLV-2 by the intracranial- and footpad-inoculation routes. In contrast to those observed for the fixed strain of RABV, changes were highly variable for both inoculation routes. This reflects the variable incubation and extended morbidity periods of this virus isolate. However, intracranial and peripheral inoculation both led to increases in all transcripts over mock-infected levels, although these were only significant for IFN-α and -β in those samples taken following footpad inoculation. The opposite situation was observed for IL-6 and Mx1 gene transcripts, where only intracranially infected mice produced significant increases.

The ability of different RABV strains to invade the CNS has been viewed as a variable feature of these viruses (Dietzschold et al., 2005). Experimentally, this is manifested by the titre of virus required to induce disease, the incubation period required to reach the CNS and the level of innate immune transcripts induced in response to infection (Morimoto et al., 1996; Wang et al., 2005). Previous studies have noted delayed onset of morbidity and mortality when comparing EBLVs directly with RABV (Badrane et al., 2001; Brookes et al., 2005). This was also observed in the present study. Three recent reports demonstrate that RABV invasion and replication in the CNS induce an innate immune response characterized by an upregulation of type 1 IFN and IFN-inducible genes, particularly the Mx1 antiviral proteins (McKimmie et al., 2005; Préhaud et al., 2005; Wang et al., 2005). Here, we demonstrate that induction of innate immune responses in the brain can be extended to the EBLVs. For the limited but important innate immune gene transcripts measured in this study, there were no major differences between responses to RABV and to EBLVs. The IFN-α transcript increases of between two- and tenfold for CVS and EBLVs are consistent with previous in vivo studies (Wang et al., 2005). Transcript increases of up to 20-fold for

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**Fig. 2.** Detection of lyssavirus nucleoprotein, β-actin and IFN-α transcripts within the murine brain following mock intracranial inoculation (M), intracranial inoculation with RABV isolates 56, 64 and 61, intracranial inoculation with EBLV-1 isolates 19, 66 and 264 or intracranial inoculation with EBLV-2 isolates 8, 29, 594, 621 and 1333.
IFN-β are greater than in previous studies; however, they are considerably lower than the 150-fold increase observed in a human neuronal cell line infected with RABV (Préhaud et al., 2005). The greater fold increase observed in the present study relative to other in vivo studies may reflect the well-acknowledged increased sensitivity of QPCR over microarrays in the detection of small changes in transcript levels.

The variability in neuroinvasion observed with EBLVs contrasts with the efficient and reproducible neuroinvasion observed with CVS, which consistently established brain infection between 2 and 4 days after footpad inoculation. Relative to intracerebral inoculation, the delay of brain infection following peripheral inoculation of CVS resulted in a delay in innate immune gene transcription in the brain. Strikingly, whilst IFN-α and -β transcripts were detectable in this and other studies (Wang et al., 2005), fold increases over mock-infected brains were very low and considerably lower than the 20- to 200-fold increase that we measured to another neurotropic virus, Semliki Forest virus, by using the same QPCR technology and primers (McKimmie et al., 2005). CVS may antagonize IFN transcription in a manner analogous to the related vesicular stomatitis viruses (Ferran & Lucas-Lenard, 1997). Indeed, a recent report has suggested that, in vitro, the RABV phosphoprotein is capable of inhibiting the action of IFNs by blocking phosphorylation of IFN-regulatory factor 3 (Brzózka et al., 2005). This would have a direct negative impact on IFN transcription and would be consistent with the low levels of transcripts observed. An alternative explanation could be the rapid death or dysfunction of brain cells following virus infection, resulting in a reduction in transcript level. In particular, the reduction in the levels of type I IFN transcripts from days 4 to 6 following intracerebral inoculation with CVS could reflect this, although again, antagonism of IFN gene transcription could also explain this reduction and we cannot distinguish between these possibilities.

For both CVS and EBLV, despite the low levels of type I IFN transcripts, there were substantial increases in the levels of Mx1 gene transcripts in the brain. Mx transcripts are IFN-inducible and their upregulation in this and other studies in the absence of strong IFN-α transcription is intriguing and could indicate that IFN-independent pathways also lead to transcription of these genes; one possibility is that these genes are activated by TLRs independently of IFN signalling. TLR genes are expressed in the brain and are upregulated in response to RABV infection (McKimmie et al., 2005; Préhaud et al., 2005). Following peripheral inoculation and CVS invasion of the brain, there is a particularly large (>5000-fold) increase in Mx1 gene transcription. IFNs can cross the blood–brain barrier and it is possible that this large Mx1 response results from the priming of CNS cells by peripherally induced IFN prior to virus neuroinvasion, which results in the observed dramatic response upon subsequent brain infection. Recent observations suggest that Mx proteins are capable of inhibiting RABV (Leroy et al., 2006); nevertheless, the large response observed here was not sufficient to protect these mice. Greater understanding of the interactions between lyssaviruses and host innate defences is required and could eventually lead to antiviral therapies that augment innate immune responses and help prevent fatal disease.
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References


