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What Kaplan-Meier Survival Curves Don't Tell Us About CNS Disease

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Abstract

Central nervous system consequences of viral infections are rare, but when they do occur, they are often serious and clinically challenging to manage. Our awareness of the perils of neuroinvasion by viruses is growing: the recently appreciated impact of Ebola and Zika virus infections on CNS integrity, decreases in vaccination coverage for potentially neurotropic viruses such as measles, and increased neurovirulence of some influenza strains collectively highlight the need for a better understanding of the viral-neural interaction. Defining these interactions and how they result in neuropathogenesis is paramount for the development of better clinical strategies, especially given the limited treatment options that are available due to the unique physiology of the brain that limits migration of blood-borne molecules into the CNS parenchyma. In this perspective, we discuss some unique aspects of neuronal viral infections and immune-mediated control that impact the pathogenic outcomes of these infections. Further, we draw attention to an often overlooked aspect of neuropathogenesis research: that lack of overt disease, which is often equated with survival post-infection, likely only scratches the surface of the myriad ways by which neurotropic infections can impair CNS function.

Introduction

All living things, from bacteria to humans, are potential hosts for viral infections. Though readers of this article have so far survived these challenges, viral infections are not without impact or lasting consequence. Pathogens cause a wide range of outcomes, from short-lived inconveniences such as congestion, body aches, and lethargy, to more serious manifestations, including diarrhea, high fever, liver cirrhosis, and immunosuppression. Why, then, do scientists, who use ever more precise and powerful mouse models, rely so often on survival curves as a catch-all surrogate in modeling “disease”?

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Development of a more incisive battery of approaches to evaluate non-lethal consequences should be considered, especially for those viruses that can infect resident brain cells. Beyond the brain's primacy as the master regulator of homeostasis, neurons within the brain possess unique features that may result in distinct outcomes following infection or interaction with anti-viral immune components. Here, we briefly summarize a growing literature focused on some newly appreciated aspects of the virus-neuron-immune cell axis, offer new data to underscore that "survival" is often too blunt a tool to effectively measure the pathogenic consequences of neurotropic infection, and conclude with some suggestions that may prove useful as the field moves forward. Beyond making such models more relevant to the human experience, a more precise set of tools may define the mechanisms by which pathogens cause disease, or provide evidence that a new treatment or intervention will be efficacious.

Interferon (IFN) signaling in neurons

Intrinsic immunity is defined as those processes within a cell that collectively prevent or limit pathogen replication and spread. These early responses are typically deployed before either the innate or adaptive players of the "professional" immune response become engaged, and thus represent the first-line of defense against unrestricted pathogen growth. Neurons appear to be pre-armed to fight infection at the level of intrinsic immunity, expressing higher basal levels of many classic interferon (IFN)-induced genes, such as ISG15 which plays a role in preventing ubiquitination of viral proteins, such as the human immunodeficiency virus type 1 Gag protein, thus preventing viral budding^{1,2}. In addition to possessing higher homeostatic levels of many canonical antiviral effectors, primary neurons can respond to IFN (as well as viral exposure) in a unique manner: studies have revealed the use of a nontraditional profile of signaling molecules activated upon IFN engagement that result in the induction of a unique set of gene products in neurons when compared to more classically studied cell types, such as fibroblasts²⁻⁴. For example, primary neurons express lower basal levels of the key IFN signaling molecules STAT1 and STAT3, and consequently the kinetics of induction of IFN signaling cascades are delayed in these cells^{2,3}. However, potent signal transducers such as ERK1/2 are rapidly activated in neurons upon IFN addition to primary cultures, resulting in the induction of chiefly pro-survival proteins⁴. Despite the decreased presence of key IFN signaling molecules (e.g., STATs) and the use of alternative signaling pathways, IFN signaling is crucial for successful resolution of many neurotropic viral challenges⁵⁻⁹. The availability of some antiviral effectors in uninfected or unstimulated neurons likely allows for a more immediate response upon infection, whereas the paucity of classical signaling pathways may delay or preclude the induction of apoptotic genes, allowing the neuron to survive while controlling a viral challenge.

Eventually, innate and adaptive cells of the host response are recruited to the infected brain. But here, too, the rules of engagement seem to be altered. Although cytotoxic T lymphocyte (CTL)-mediated lysis is an effective strategy to curtail viral replication in many cell populations, neurons, in some instances, appear to be resistant to such lysis. The reasons for this observation remain unclear, but various hypotheses have been put forward. Neurons were long believed to be incapable of expressing major histocompatibility (MHC) class I molecules, which are essential for T cell recognition and interaction with infected cells. Consequently, it was thought that this property made neurons "invisible" to antiviral CTL

that had crossed into the brain parenchyma. Subsequent studies showed that this is not universally true: while the homeostatic expression of MHC class I is lower in neurons in general, neurons can up-regulate MHC class I under inflammatory conditions, thus making them susceptible to cytotoxic T cell attack^{10–12}. This could be virus- or strain-specific however, as others have reported little to no detectable MHC expression on neurons following infection with noncytolytic viruses, such as lymphocytic choriomeningitis virus, which might contribute to the ability of these viruses to establish persistent infections^{13–15}.

Interestingly, clearance and control of many neuronal viral infections, while dependent on T cell receptor-MHC interaction, occurs in a non-cytolytic manner^{16–18}. For instance, in the case of measles virus (MV) infection of neurons, T cell-secreted interferon gamma (IFN γ) is crucial for control of MV replication, though infected neurons do not die^{4,17}. Moreover, after neuronal infection with Sindbis virus (SV), IFN γ and antiviral antibody are responsible for initial viral control and long-term suppression of viral replication, respectively, again in the absence of neuronal cell death^{16,18}. It may be that some neurons, especially those in the periphery, are inherently more resistant to perforin and granzyme-triggered killing¹⁹. This was shown in a series of studies in which primary herpes simplex virus (HSV)-infected dorsal root ganglionic neurons were monitored for cell death and viral reactivation after exposure to HSV-specific CD8⁺ T cells secreting type II IFN (IFN γ) and perforin/granzyme. These studies revealed that the molecules normally used to lyse infected cells were functioning to keep HSV in a latent state in neurons; in their absence, viral reactivation occurred^{19,20}. Thus, cellular intrinsic immune pathways and interactions with immune cells and their mediators is not “one size fits all”, but can be tailored, resulting in unique cell type-specific outcomes that ultimately can impact pathogen control and cellular fate. For neurons, potent IFNs and reduced cytopathic effects may permit viral control while maintaining the integrity of these generally non-renewable cells.

Increased risk of long-term infections within the CNS

Although non-lytic viral control may allow neurons to survive an antiviral immune assault, a consequence of such a strategy could be that non-cytolytic viruses are incompletely eradicated from the brain, as noted above for LCMV. In fact, many viruses, including those with RNA genomes, can be maintained long after infectious virus falls below detection limits, and in the absence of overt signs of CNS disease^{21–23}. Such long-lasting infections, while not lethal to the host, could nevertheless impair neuronal function and host metabolism^{24,25}. In some instances, viral persistence correlates with obvious neurological disease; for example, after infection with mouse hepatitis virus (MHV), mice develop a progressive demyelinating disease that shares many attributes with multiple sclerosis²⁶. Other CNS deficits associated with chronic infection are subtler. For example, infection of neonatal mice with lymphocytic choriomeningitis virus (LCMV) leads to a life-long infection associated with learning and memory defects²⁷. Moreover, in the same model system, persistence is associated with decreased neurotransmitter release (somatostatin)²⁸ without overt neuronal loss or other signs of neuropathology. Furthermore, age at time of infection is a pivotal factor in dictating later neurological dysfunction, as evidenced by an increased susceptibility of sub-acute sclerosing panencephalitis (SSPE) in children infected with measles virus before 2 years of age²⁹. Thus, non-cytolytic viral control in neurons may

be double-edged, enabling survival of crucial and generally irreplaceable cells, but providing a haven for non-cytopathic viruses to persist. If true, a correlative risk is that neurotropic viruses may reactivate or incite new immune responses later in life, temporally separated from the initial infection^{22,26,30–32}.

Viral control and pathogenesis in a mouse model of neuronal measles virus infection

To study viral infection of neurons in the central nervous system (CNS), our lab uses a transgenic mouse model in which CD46, one of the three identified human measles virus (MV) receptors, is expressed under the control of the neuron-specific enolase promoter (NSE-CD46 mice)^{33–35}. Mice are not naturally permissive to MV infection; therefore, this model allows exclusive infection of CNS neurons. As such, these mice can be used to investigate viral clearance and control *in vivo*, as well as viral spread and intrinsic immune responses *ex vivo*. We previously demonstrated the contributions of age, and key innate and adaptive immune components in MV control using select knockout mice backcrossed to NSE-CD46 mice^{2,17,36–38}. We showed that young mice (post-natal days 1–14) are highly susceptible to MV-induced disease, whereas immunocompetent NSE-CD46 adults are resistant³⁹. In these studies, we measured the pathogenic impact of depletion or deletion of a specific immune cell subset or protein by assessing death following infection, and typically considered survival as a surrogate for an absence of pathogenesis.

Analysis of multiple immune knockouts revealed that mice deficient in select signaling molecules, despite being infected with the same pathogen, display substantial variability in the kinetics and efficiency of viral control (Figure 1). For example, NSE-CD46 immunocompetent mice survive neuronal MV challenge, whereas all NSE-CD46/RAG2 KO mice (deficient in mature B and T cells), succumb to infection within 3 weeks. Moribund NSE-CD46/RAG2 KO mice progressively lose weight, develop a convex curvature of the spine (kyphosis) and display labored breathing². In contrast, infection of NSE-CD46/IFNAR KO mice (which lack the type I interferon receptor) results in approximately 80% mouse survival, though the consistent 20% that succumb do so within five days post-infection, and show signs of disease not typically observed in moribund RAG KO mice, including tremors and gait/balance problems (ataxia). The timing and nature of disease manifestations may offer valuable insight into how select immune modulators impact neurological disease and dysfunction.

To further illustrate this point, infection of NSE-CD46/STAT1 KO mice, deficient in the crucial type I and II IFN signal transducer, STAT1, results in the survival of approximately 75% of animals. Consistently, however, a small proportion (~25%) of these infected mice display grade 5 “popcorn” seizures that lead to death by 7 dpi (Video B)³⁸. The observed neurological disease in infected NSE-CD46/STAT1 KO animals is distinct from that observed in any other immunodeficient mouse, including those lacking the type I interferon receptor, or interferon gamma, both of which typically depend upon STAT1 for signal transduction.

A corollary to this observation is that survival following a neurotropic challenge does not imply an absence of pathogenicity. For example, infection of NSE-CD46/perforin KO mice results in 100% survival (Figure 1), which, coupled with our parallel observation that control of MV within neurons is non-cytolytic, led us to conclude that perforin, a molecule typically associated with CD8⁺ T cell lysis of infected targets, did not contribute to viral control and was dispensable for control of MV infection in the CNS^{17,40}. However, upon further examination, we found that this was not the case: NSE-CD46/perforin KO mice maintain appreciably higher viral loads in the brains of infected mice long after acute viral control (Figure 2). Whether such viral persistence has deleterious implications for infected neurons remains to be determined.

The detection of increased viral loads in NSE-CD46/perforin KO mice, despite an absence of pathogenesis, prompted us to re-assess viral loads in other immune deficient models in which some or all challenged mice survive, apparently disease-free. Similar to what we observed with MV-infected perforin-deficient mice, viral loads were higher in most all surviving mice on immunodeficient backgrounds (Figure 2). The increases in detectable viral RNA in surviving mice could indicate an inability to control the initial acute infection, or an inability to prevent viral replication long-term, and may possibly be explained by a paucity of neuronal MHC expression¹³. How these more subtle consequences of immune deficiency ultimately affect viral control and host health is an area of active investigation in our laboratory, but these early data prompted us to think more deeply about the impact of chronic CNS infections, and the missed opportunities in overlooking subtle neurological or viral events.

The Mouse Physical Exam

While there is little doubt that murine models of neuropathogenesis have proven extraordinarily valuable in understanding and defining how viral infections can cause CNS disease, simply reporting outcomes in a binary way (death or survival) is likely ignoring lessons that can be learned from these models. So how might we better assess non-fatal consequences of neurotropic infections? We propose three additions or alterations to the survival curves often used in mouse studies. First, basic vital functions should be collected on infected mice, including weight changes, blood pressure, temperature, and pulse oxygen saturation. A quick Google search will uncover multiple companies that sell equipment to reliably and quickly collect these data from mice, in some instances simultaneously. Inclusion of these data may aid in defining dis-regulated regions of the brain during infection or disease (for example: the hypothalamus for temperature, and the brainstem and medulla oblongata for breathing). Second, while detailed memory and behavior tests in mice require dedicated equipment and laboratories, many motor and cognitive tests to assess a mouse's balance and general awareness/cognition are easily done with limited time, expertise, and expense, even for investigators without access to highly sophisticated tools. For example, a cylinder test can quickly identify unilateral motor deficits. In this test, a mouse is placed into a clear cylinder and the number of load bearing contacts the animal makes with its limbs when rearing is quantified. A normal mouse will make an even number of load bearing contacts with left and right forelimbs; in contrast, favoring one limb over another may indicate damage in specific hemispheres of the brain leading to a loss of motor

control, as seen for example in human stroke patients^{41,42}. Simple motor tests have identified motor and rearing abnormalities associated with hippocampal dysfunction after infection with both influenza and dengue virus^{43,44}. Another straightforward motor phenotype analysis is to perform a footprint analysis, in which the mouse's front and rear paws are dipped in a dye that leaves footprints as the mouse walks across absorbent paper. By measuring the length and width of strides, gait disturbances can be identified and quantified⁴². Moreover, open field and grooming tests can also identify cognitive and processing phenotypes. In the open field test, a mouse is placed into an open space surrounded by walls; thereafter, the time spent in center quadrants is measured. A typical healthy mouse will immediately proceed to the wall of the field and explore around its edges until it feels comfortable to explore more open areas. Mice experiencing anxiety or motor deficits will show other behaviors, which can include a lack of movement from the open area, or a lack of curiosity about the new space⁴². Similarly, the grooming test is performed by observing a mouse in its home cage, and quantifying the time spent grooming over a 10 minute period. Healthy animals spend approximately 10% of their time grooming, while animals exhibiting repetitive behaviors may spend increased time on this task⁴⁵. For a more complete explanation of the potential benefits (and pitfalls) of using these approaches to identify motor and cognitive defects in mouse models, we refer the reader to an excellent 2009 review by Brooks and Dunnett⁴². Finally, we propose that instead of photographs to document a particular morbidity, investigators provide links to videos in their publications allowing the reader to have a more complete understanding of observed disease phenotypes; the ubiquitous presence of cell phones with high-quality video capacity makes this once-challenging task straightforward.

Certainly, many investigators have adopted strategies such as these in evaluating cognitive or motor deficits, but most of these studies are within the neurobiology literature, and are less prevalent in the neurovirology/neuroimmunology fields. We make the simple case here that such efforts could be time well spent, and could accelerate new discoveries or deeper insights into how neurotropic viruses might impact host biology.

Conclusions

We have made the case for a more thorough "exam" of mouse physiology, though a reasonable counterpoint is that these suggested ancillary tests could become time-consuming. To this point, we are reminded of Stanley Cohen's Nobel Prize lecture in 1986, in which he credited his discovery of epidermal growth factor to the "side effects" of precocious eyelid opening and tooth eruption⁴⁶. In his lecture, Cohen goes on to note that, had he not paused to watch his mice, this important clue would have gone unnoticed, and would have likely postponed the paradigm-shifting discoveries that followed.

Materials and Methods

Ethics statement

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. These studies were

reviewed and approved by the Fox Chase Cancer Center Institutional Animal Care and Use Committee (Office of Laboratory Animal Welfare assurance number A3285-01).

Reverse transcriptase quantitative real-time PCR (RT-qPCR)

RNA was purified from homogenized whole brain tissue (see below) and quantified using a Nanodrop spectrophotometer. RNA was reverse-transcribed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems) with random hexamer priming. Gene-specific primers were used in combination with probes designed using the Universal Probe Library algorithm (Roche) and Universal Master mix (Roche); all reactions were run on a Mastercycler Realplex2 system (Eppendorf). Cycling conditions were 50°C, 2 min; 95°C, 10 min; followed by 40 (2-step) cycles (95°C, 15 sec; 60°C, 60 sec). Relative quantification to the control (cyclophilin B) was done using the comparative CT method. Individual sample PCR reactions were performed in triplicate. The following gene specific primers (Integrated DNA Technologies) were used: Cyclophilin B Forward – 5′-ttctcataaccacagtcagacc-3′, Cyclophilin B Reverse – 5′-accttcgtaccacatccat-3′, UPL 20; MV nucleoprotein Forward - 5′-ggaaactgcaccctacatgg-3′, MV nucleoprotein Reverse- 5′-gggtatgatcctgcactgaact-3′, UPL 80.

Mice and in vivo infections

Homozygous NSE-CD46⁺ transgenic mice (line 18; H-2^b)³⁵ were maintained in the closed breeding colony of the Fox Chase Cancer Center. Homozygous NSE-CD46⁺ and haplotype-matched homozygous immune knockout (KO) mice were intercrossed for three or more generations to obtain NSE-CD46⁺ mice on the desired KO background. STAT1 KO mice were obtained from Jackson Laboratory⁴⁷. IFNAR KO⁴⁸ mice were obtained from Luis Sigal (Fox Chase Cancer Center, Philadelphia PA.) RAG2 KO⁴⁹ mice were a gift from F. W. Alt (Howard Hughes Medical Institute, Boston, Mass). The genotypes of all mice, including perforin KO⁵⁰ and IFN γ KO⁵¹ mice was confirmed by PCR analysis of tail biopsy DNA.

Isoflurane-anesthetized mice were infected with MV-Edmonston via intracranial inoculation of 1 $\times$ 10⁴ PFU in a volume of 30 μ l, delivered along the midline using a 27g needle. Mice were monitored daily post-infection for signs of illness, including weight loss, ruffled fur, ataxia, and seizures. Moribund mice were euthanized in accordance with IACUC guidelines. RNA was isolated from individual mice at indicated times post infection using TriReagent (Sigma) and subjected to analysis as described above.

Statistical analysis and figure preparation

Data representation and statistical analysis were performed using Prism GraphPad. Statistical analysis was done using the Mann-Whitney U test or the Log Rank (Mantel Cox) test as indicated in the figure legend.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Survival of various immune knockout mice following neurotropic MV challenge
 NSE-CD46⁺ and NSE-CD46⁺ mice of the indicated immune KO (NSE-CD46⁺/STAT1 KO, NSE-CD46⁺/RAG2 KO, NSE-CD46⁺/Perforin KO, NSE-CD46⁺/IFNAR KO, and NSE/CD46⁺/IFN γ KO) were challenged intracerebrally with 1×10^4 PFU of MV-Edmonston. Mice were monitored daily. Percent survival of indicated genotypes over time are indicated. Data represent at least 2 individual experiments with the following number of animals in parentheses: NSE-CD46⁺ (14), NSE-CD46⁺/STAT1 KO (25), NSE-CD46⁺/RAG2 KO (8), NSE-CD46⁺/Perforin KO (10), NSE-CD46⁺/IFNAR KO (9), and NSE/CD46⁺/IFN γ KO (16).

Figure 2. Immune deficient mice maintain increased levels of viral RNA after survival of a neurotropic infection

NSE-CD46⁺ and NSE-CD46⁺ mice of the indicated immune KO background challenged intracerebrally with 1×10^4 PFU MV-Edmonston. RT-qPCR data were generated using random hexamer priming for cDNA generation followed by qPCR using primers specific for the MV nucleoprotein and cyclophilin B as a standard. Data analyzed using the $\Delta\Delta C_T$ method. # $P < 0.05$ Mann Whitney U test of NSE-CD46⁺ controls compared to all indicated immune KO mice. Data represent at least 2 individual experiments with the following number of animals in parentheses: NSE-CD46⁺ (34), NSE-CD46⁺/Perforin KO (10), NSE-CD46⁺/IFNAR KO (9), and NSE/CD46⁺/IFN γ KO (15).