

Recovery from viral encephalomyelitis: immune-mediated noncytolytic virus clearance from neurons

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Abstract Viral encephalomyelitis is caused by virus infections of neurons in the brain and spinal cord. Recovery is dependent on immune-mediated control and clearance of virus from these terminally differentiated essential cells. Preservation of neuronal function is essential for prevention of neurologic sequelae such as paralysis, seizures and cognitive deficits. Using the model system of Sindbis virus-induced encephalomyelitis in mice, we have shown that immune-mediated clearance of infectious virus from neurons is a non-cytolytic process. The major effectors are antibody to the E2 surface glycoprotein produced by B cells, and interferon- γ produced by T cells. These effectors work in synergy, but neuronal populations differ in their responses to each. Virus is least likely to be cleared from brain neurons and most likely to be cleared from motor neurons in the cervical and thoracic regions of the spinal cord. Because the infected neurons are not eliminated, viral RNA persists and long-term control is needed to prevent virus reactivation. Virus-specific antibody-secreting cells residing in the nervous system after recovery from infection are likely to be important for long-term control.

Keywords Noncytolytic virus clearance · Interferon- γ · Antiviral antibody · Neuronal virus infection · Alphavirus encephalitis

Introduction

Viral encephalitis is an important cause of morbidity and mortality worldwide. During encephalomyelitis, the cells in the nervous system targeted for infection are neurons of the brain and spinal cord. Several types of viruses can infect neurons. Important human pathogens include herpes simplex virus, a DNA virus, and several RNA viruses in the enterovirus (e.g. poliovirus, enterovirus 71), flavivirus (e.g. West Nile, Japanese encephalitis, Murray Valley and tick-borne encephalitis viruses) and alphavirus (e.g. eastern

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equine, Venezuelan equine and western equine encephalitis viruses) families. Many of these viruses are emerging as increasing problems worldwide due to changes in virulence and spread to new geographic regions.

Particularly important are the arthropod-borne flaviviruses and alphaviruses that have caused explosive regional outbreaks of disease as demonstrated by Chikungunya in the Indian Ocean and by the spread of West Nile virus to North America. Some alphaviruses cause encephalitis, while others cause rash and arthritis. The encephalitic alphaviruses are particularly associated with the outbreaks of encephalomyelitis in the Americas [1]. Eastern equine encephalitis (EEE) is endemic on the Gulf and Atlantic coasts and has a high mortality in all age groups [2]. Numbers of cases are increasing, and in 2005 the most cases of EEE occurred in the US since 1964 [3]. Western equine encephalitis (WEE) is endemic in the western portions of North America and in South America, but has not been recently associated with human disease. In 1995, Venezuelan equine encephalitis (VEE) re-emerged in South America causing an epidemic of 75–100,000 cases [4]. As with other viral causes of encephalitis, encephalitic alphaviruses infect neurons, terminally differentiated, irreplaceable cells essential to function of the nervous system. Because mature neurons have limited capacity for regeneration, recovery that does not result in neuronal damage requires noncytolytic, rather than cytolytic, immune mechanisms for virus clearance.

The Sindbis virus model of acute alphavirus encephalomyelitis

Alphaviruses are enveloped, plus-strand, mosquito-borne RNA viruses in the *Togaviridae* family. The genome is approximately 11,700 nucleotides long, capped and polyadenylated. The structural proteins (C, PE2, 6 K and E1) are translated from a subgenomic RNA as a large polyprotein [5]. C is autoproteolytically cleaved from the developing nascent chain and rapidly assembled with genomic RNA into nucleocapsids. Precursor of E2 (PE2) and E1 are transported with 6 K as a noncovalently associated heterooligomeric complex through the cell secretory pathway to the plasma membrane. Later in the pathway, PE2 is processed to E2 and a small glycopeptide, E3, which is shed from the cell surface. At the plasma membrane, the specific association of E2 tails with nucleocapsids initiates a budding process that leads to the release of mature virions [6, 7]. E1 and E2 heterodimers trimerize to form the spikes on the virion surface [8]. E2 protrudes from the virion surface and is involved in attachment, while E1 forms a relatively flat skirt-like structure and is important for fusion of the virus and cell membranes to initiate infection [9].

Sindbis virus (SINV), the prototype alphavirus, is the most widespread of the alphaviruses and causes summertime outbreaks of arthritis and rash in Northern Europe (e.g. Ockelbo, Pogosta, Karelian fevers) and southern Africa [10–12]. SINV is closely related to WEE virus [13] and Mayaro virus, an emerging cause of rash and arthritis in South America [14]. SINV causes neuronal infection in mice and serves as a model system for the study of the pathogenesis of alphavirus-induced encephalomyelitis and the mechanisms and consequences of virus clearance from neurons [15, 16].

Age is an important determinant of outcome from SINV infection. Neonatal mice die within the first few days after infection, while older mice clear SINV from the central nervous system (CNS) within 6–8 days without signs of paralysis or neurological damage [17, 18]. Age-dependent susceptibility is not associated with the maturation of the immune response, but rather with the changing intrinsic susceptibility of immature and mature neurons to infection [15]. Maturity of the infected neuron determines the level of virus

replication and the susceptibility to SINV-induced cell death independent of the immune response [19–22]. Immature neurons replicate SINV to higher titers and are susceptible to virus-induced apoptosis, while mature neurons are intrinsically more resistant to SINV replication and survive virus infection [21, 23, 24]. Recovery from infection in mature mice requires immune-mediated clearance of virus from these surviving infected neurons.

Because uncontrolled CNS inflammation can be damaging or even fatal, immune responses in the nervous system are highly regulated [25, 26]. The immunologically quiescent state of the uninfected CNS is maintained by ongoing interactions between neurons and glial cells, the presence of the blood brain barrier (BBB) and constitutive production of regulatory factors such as gangliosides, transforming growth factor (TGF)- β and interleukin (IL)-10 [25, 27–29]. Microglial cells, the resident macrophage lineage cells in the CNS, are closely apposed to neurons that express surface molecules such as CD200, CD47, HMGB1 and fractalkine/CX3CL1 that inhibit microglial cell activation [26]. Astrocytes maintain the endothelial tight junctions that form the BBB. Activated T cells routinely cross the BBB as part of normal immunologic surveillance of the CNS but leave or die if antigen is not encountered [15, 30–34].

When neurons become infected, changes in the CNS quickly occur to initiate a protective response [25]. The signaling pathway by which neurons sense most virus infections is not known, but neurons possess toll-like receptors (TLRs) and intracellular RNA helicases likely to be involved in alerting the CNS to virus infection [35, 36]. Neurons can respond to infection by producing IFN- β , IFN- γ , IL-6 and chemokines, such as CXCL10/IP-10, CCL21/SLC and CX3CL1 [37–41]. Macrophages and glial cells become activated in early phases of the response and rapidly produce an array of cytokines (e.g. IL-1, tumor necrosis factor (TNF)- α , IL-6) and chemokines (e.g. CXCL10, CCL2, CCL5) [29, 42] that up regulate major histocompatibility complex (MHC) molecules on microglial cells and increase adhesion molecule expression on cerebral capillary endothelial cells (e.g. intercellular adhesion molecule [ICAM]-1, vascular cell adhesion molecule [VCAM]-1) to enhance the entry of activated cells into the CNS [43].

Virus clearance

Clearance of acute virus infections requires elimination of cell-free virus to prevent continued infection of new cells and elimination of virus-infected cells to prevent continued production of virus. Only antibody can recognize the native antigen on virions. Neutralizing antibody that is generally directed to a viral surface protein and can prevent virus infection of new cells is important for preventing continued spread of infection. For viruses that bud from the plasma membrane, antibodies may also be able to interact with viral proteins on the surface of infected cells.

Elimination of virus-infected cells from tissue requires elimination of all cells in which the virus is replicating. This can occur by either virus-induced or immune-mediated cytolysis. T cells are ideal for this purpose because they recognize viral antigen as processed antigen only in the context of MHC class I (CD8⁺ T cells) or class II (CD4⁺ T cells) expressed on the cell surface and CD8⁺ T cells can possess cytotoxic properties. If the immune clearance mechanism is damaging to the infected neuron, then the function of that neuron will be lost, and the outcome for the host will be the same as if the virus infection had caused neuronal death. If infected cells are allowed to survive, the clearance of virus must include mechanisms for inhibiting intracellular synthesis of virus nucleic acid and protein, and for removing virus genomes from cells or preventing their replacement after

degradation. If the clearance process is not complete, then mechanisms for preventing resumption of virus replication must be in place to avoid progressive or relapsing disease [44–46].

Clearance of virus from cells in the brain parenchyma and recovery from infection is a multistep process. First, there is inhibition of virus spread to new cells, then clearance of cell-free infectious virus. Subsequently, virus-infected cells must be eliminated or intracellular virus replication must be permanently suppressed. The most straightforward measurement of virus clearance is the amount of infectious virus that is present in CNS tissue. However, as antibody specific for the virus is produced, the ability to detect infectious virus by standard assays is compromised by the presence of neutralizing antibody in the tissue homogenates. Therefore, quantitative measurement of virus nucleic acid provides the best indicator of whether the virus has truly been eliminated.

Antibody is produced, and T cells begin to infiltrate the CNS 3–4 days after SINV infection and virus clearance begins shortly thereafter [34, 47, 48]. Type I interferon (IFN) is essential for initial control of virus replication [49–52], and both humoral [21, 49, 53] and cellular [23, 54] arms of the adaptive immune response play important roles in clearance. Mice deficient in all components of adaptive immunity (SCID or Rag^{-/-}) develop persistent nonfatal infection [55] and have been used as a test system to determine the contribution of different components of the immune response to virus clearance.

Role of antibody

Passive transfer of hyperimmune serum (HIS) containing polyclonal SINV antibody to persistently infected SCID mice results in clearance of infectious virus from the CNS (Fig. 1a). Clearance of SINV from the brain and spinal cord occurs within 48 h without apparent neurologic damage indicating an important role for antibody in noncytolytic clearance [55]. Analysis of the specificity of the effective antibody using a panel of monoclonal antibodies to the E1 and E2 glycoproteins has shown that clearance is dependent on the amount of antibody transferred and that antibody to the E2 glycoprotein is most potent [55]. In addition to clearance of infectious virus, viral RNA is decreased

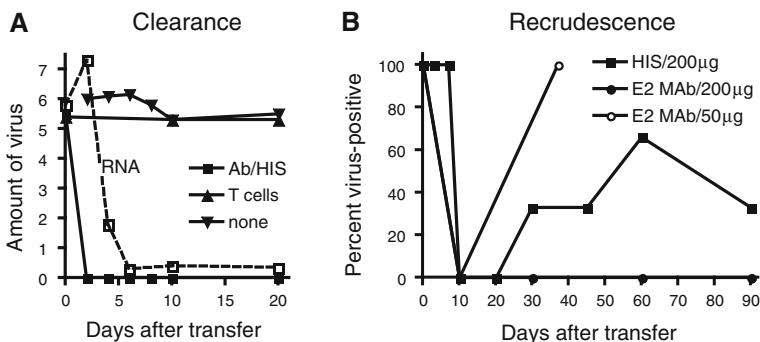


Fig. 1 Effect of passive transfer of antibody or T cells to SCID mice persistently infected with SINV. **a** Passive transfer of SINV-specific hyperimmune serum (HIS) on infectious virus in the brain (pfu/gram) and on levels of viral RNA (in situ hybridization/RT-PCR). Transfer of T cells had no effect. **b** Recrudescence of infectious virus in the brain after passive transfer of hyperimmune serum (200 µg IgG) or anti-E2 monoclonal antibody at two different doses (50 µg and 200 µg). Data from (21, 53, 56)

after passive transfer of antibody indicating inhibition of virus replication, not just neutralization of virus by the passively transferred antibody [21, 55]. However, RNA persists at a low level and virus replication resumes when antibody has decayed (Fig. 1b), indicating that replication competent viral RNA persisted [21, 56]. Investigation of virus clearance in immunologically normal mice has shown that viral RNA can be detected by RT-PCR in these mice for at least 12 months after infection. Therefore, failure to eliminate the infected neurons results in preservation of neuronal function, but persistence of viral RNA with the potential for recrudescence virus replication.

The mechanism by which antibody to a surface glycoprotein suppresses intracellular virus replication has been intensively studied using a variety of *in vitro* systems but is not yet completely understood. The E2 glycoprotein is expressed on the surface of virus-infected cells, and anti-E2 antibody binds to the cell surface. These studies have shown that clearance requires bivalent antibody but does not require complement or leukocytes and is independent of IgG isotype [21, 57]. Antibody treatment of infected cells blocks virus budding, restores Na⁺K⁺ATPase function, membrane potential, host protein synthesis and the ability of the cells to respond to IFN- α/β [58, 59]. These data imply that E2 cross-linking on the surface results in membrane alteration and transmission of a signal from the surface of the infected cell to intracellular sites of virus replication.

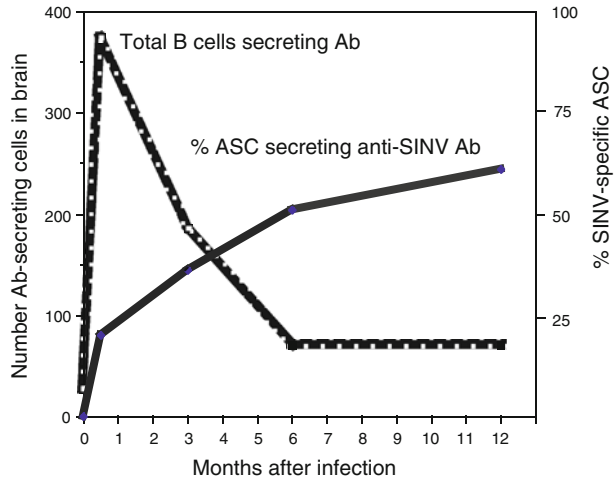
Because this process does not eliminate viral RNA, a mechanism for long-term immunologic control of virus replication is needed to prevent reactivation. Antibody is likely to participate in control, as well as initial clearance, and there are two mechanisms for maintaining antibody in the CNS: passage of immunoglobulin from the blood across the BBB into the brain parenchyma or local production of antibody by antibody-secreting cells that are resident in the CNS. Although the BBB normally restricts the entry of proteins from the blood into the CNS, this function is compromised by the inflammatory response during the acute phase of infection and plasma antibody enters the CNS in larger amounts than during recovery when the barrier has healed [60–62]. With a normally functioning BBB, interstitial brain levels of antibody are 1:100–1:200 of plasma levels, a level that may be inadequate for long-term control. Therefore, locally produced antibody may be needed to maintain levels in the CNS sufficient for continued suppression of virus replication.

Immunohistochemical studies have shown that B cells infiltrate the CNS as a prominent part of the inflammatory response to SINV infection [63, 64]. Small numbers of perivascular IgM-secreting cells are detected 3 days after infection, followed by IgG- and IgA-secreting cells beginning at 5 days after infection [53, 61, 63]. By day 10–14, 80% of the B cells in the CNS express either IgG or IgA. Antibody-secreting cells are maintained in the CNS for at least a year after recovery with continuous enrichment for cells secreting antibody to SINV (Fig. 2) [53]. The relative roles of plasma antibody and resident B cells in the antibody-mediated clearance process and in suppression of reactivation have not been defined.

Role of T cells in immune-mediated clearance

No mice deficient in mature B cells are able to clear virus from the brain or prevent persistent virus replication. However, antibody-deficient mice can clear infectious virus from the brain stem and spinal cord without apparent neuronal damage, indicating a role for T cells in noncytolytic clearance of SINV from some, but not all types of neurons [23, 52]. Lymphocyte depletion studies showed that both CD4⁺ and CD8⁺ T cells are

Fig. 2 Numbers of total B cells secreting immunoglobulin in the CNS after intracerebral infection of BALB/c mice with SINV and percent of those antibody-secreting cells (ASC) producing antibody specific for SINV (53)



involved in this clearance process suggesting a soluble factor. The potential role of different T cell cytokines was investigated by expression from recombinant SINV to provide a local source. These studies demonstrated that $\text{IFN-}\gamma$ is the main effector of T cell-mediated virus clearance [23].

During SINV-induced encephalomyelitis, $\text{IFN-}\gamma$ is produced as a part of the adaptive immune response that begins 3–4 days after infection [29]. Therefore, $\text{IFN-}\gamma$ must induce an antiviral response in neurons that are already infected, rather than protect neurons from becoming infected. The $\text{IFN-}\gamma$ receptor is expressed on many cells, including neurons, and has two subunits [65].

The cytoplasmic domains of both chains of the receptor are necessary for signal transduction and are constitutively associated with Janus tyrosine kinases (Jaks) 1 and 2 that phosphorylate signal transducer and activators of transcription (Stats) [65]. Stat-1 phosphorylated by Jak1 at Tyr₇₀₁, forms homodimers that are translocated to the nucleus where they bind gamma-activated site (GAS) elements to initiate expression of antiviral genes. Transcription is dependent on a C-terminal transcriptional activation domain in Stat-1 α and is regulated in a cell-type-dependent way by Ser₇₂₇ phosphorylation and recruitment of other co-regulators [66–71]. GAS regulatory elements have been identified in over 200 genes, suggesting the broad array of responses that can be initiated by $\text{IFN-}\gamma$ [65, 72].

To identify the mechanism of $\text{IFN-}\gamma$ -mediated clearance, we have studied CSM14.1 neuronal cells that can be differentiated *in vitro*. These cells become persistently infected with SINV, and treatment with $\text{IFN-}\gamma$ results in virus clearance and improved cell survival [24, 73]. $\text{IFN-}\gamma$ induces prolonged phosphorylation of Stat-1 at both Tyr₇₀₁ and Ser₇₂₇ and transient phosphorylation of Stat-5. This is accompanied by a transient increase in synthesis of viral RNA and protein followed by cessation of viral protein and RNA synthesis and restoration of host cell protein synthesis [24]. Prolonged activation of Stat-1 may be important for the shut down of virus replication that progresses over several days [24]. The importance of Stat-5 activation is unclear. Inhibition of the Jak/Stat pathway with Jak inhibitor 1 blocked the beneficial effects of $\text{IFN-}\gamma$ treatment on cell viability and virus clearance indicating an essential role of Jak/Stat signal transduction in the $\text{IFN-}\gamma$ -mediated noncytolytic control of SINV replication in neurons [74].

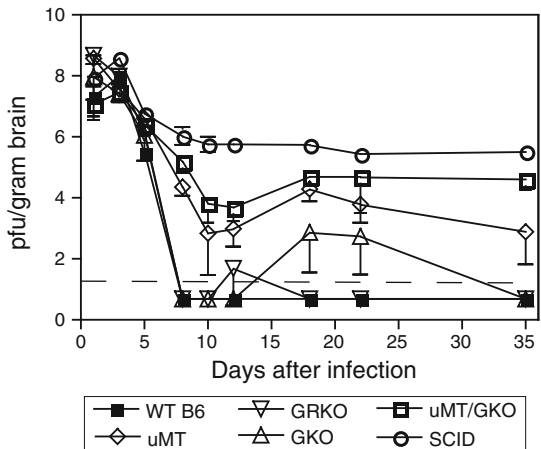
The antiviral proteins responsible for IFN- γ -mediated control of SINV replication are poorly characterized. As in other virus infections of the CNS, a large number of IFN-stimulated genes are expressed in response to infection [18, 75]. There is no evidence that the well-characterized PKR, Mx or RNase L pathways are essential for control of SINV replication [76]. IFN-induced antiviral proteins that have been implicated include ISG-15 and zinc-finger antiviral protein, but their importance in neurons and mechanisms of action are unclear [77–80]. Future studies will be required to determine the downstream effectors of Jak/Stat-induced suppression of virus replication in neurons.

Synergism between antibody and IFN- γ

Studies in mice deficient in production of both IFN- γ (IFN- γ -/-, GKO) and antibody (μ MT) indicate a synergistic role for these mediators in clearing SINV from the CNS [52]. Doubly deficient μ MT/GKO mice develop persistent infection in the brain and spinal cord that is intermediate in titer between that of μ MT and SCID mice (Fig. 3), suggesting that additional factors contribute to the complete clearance of SINV.

To investigate the roles of antibody and IFN- γ in the long-term control of SINV replication, CNS tissues were studied approximately 3 months after infection. SCID mice showed persistent virus replication at all sites. All μ MT and μ MT/GKO mice had persistent virus in brain, while GKO and GRKO mice did not. In the brain stem and spinal cord, virus was detectable in most μ MT/GKO mice, but in only a few μ MT or GKO mice. SINV is particularly likely to infect motor neurons of the anterior horn of the spinal cord [25, 81], and mechanisms of virus clearance from this site are of particular interest because of their importance for paralytic disease. To identify differences in clearance spinal cord regions of μ MT/GKO, mice were analyzed separately. Peak virus replication (day 3 pi) and the initial phases of clearance (day 5) were similar in the cervical, thoracic and lumbar regions. After day 8, SINV was no longer detected in the cervical or thoracic regions of most μ MT/GKO mice, while the majority had detectable virus in the lumbar spinal cord through day 81. However, levels of virus were lower than those observed in the lumbar spinal cords of SCID mice. These data indicate that IFN- γ is an important contributor to antibody-mediated control of SINV replication in the lumbar spinal cord and that virus can be cleared

Fig. 3 Clearance of infectious SINV from the brains of wild type (WT), IFN- β -/- (BKO), IFN- γ -/- (GKO), antibody-deficient (μ MT), antibody and INF- γ -deficient (GKO/ μ MT) and severe combined immunodeficiency (SCID) C57BL/6 (B6) mice. Dashed line indicates the limit of detection. Data from (52)



from cervical and thoracic motor neurons by an unidentified antibody and IFN- γ -independent mechanism [52].

These studies show that antibody and IFN- γ play synergistic roles in controlling SINV replication and preventing reactivation of infection in a site-specific manner in the CNS and that each individual arm of the immune response plays a role in virus clearance during the course of infection. Virus is least likely to be cleared from brain neurons and most likely to be cleared from motor neurons in the cervical and thoracic regions of the spinal cord. Neurons may differ either in expression of IFN- γ receptor subunits, in intracellular signaling pathways necessary for an antiviral response or in the ability to synergize with type I IFN [82–86].

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