MECHANISMS CONTROLLING VIRULENCE THRESHOLDS OF MIXED VIRAL POPULATIONS AND IDENTIFICATION OF THREE HOST BARRIERS TO POLIOVIRUS NEUROPATHOGENESIS

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DEDICATION

To my Mom, Dad, and Claire for all your love, support and encouragement, you have been my stronghold and inspiration throughout graduate school and I could not have done this without you.

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MECHANISMS CONTROLLING VIRULENCE THRESHOLDS OF MIXED VIRAL POPULATIONS AND IDENTIFICATION OF NOVEL HOST BARRIERS TO POLIOVIRUS NEUROPATHOGENESIS

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MECHANISMS CONTROLLING VIRULENCE THRESHOLDS OF MIXED VIRAL POPULATIONS AND IDENTIFICATION OF NOVEL HOST BARRIERS TO POLIOVIRUS NEUROPATHOGENESIS

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Neurotropic viruses comprise some of the worlds most widespread and deadly pathogens, including West Nile virus, rabies virus, and poliovirus. Poliovirus, as a model neurotropic virus, is also an RNA virus. RNA viruses have high mutation rates and a propensity to revert attenuating mutations, contributing to disease and complicating treatment and vaccine development. Despite worldwide epidemics in the early nineteenth century, paralysis from poliovirus is

a rare event occurring in less than 1% of poliovirus infections. This suggests the presence of viral and host barriers limiting disease. Here we examined viral barriers by exploring the concept of virulence thresholds using mixtures of virulent and attenuated viruses in a transgenic mouse model of poliovirus infection. We determined that 1000-fold excess of an attenuated strain of poliovirus was protective against disease induced by the virulent strain. Protection was induced locally, was a poliovirus specific effect, and inactivated virus conferred protection. Treatment with a poliovirus receptor-blocking antibody phenocopied the protective effect of inactivated viruses *in vitro* and *in vivo*, suggesting virulence thresholds may be modulated by competition for viral receptor. Furthermore, we found the attenuated virus became virulent in immune-deficient mice due to enhanced replication and reversion of attenuating mutations. We also identified additional host barriers limiting pathogenesis using a novel hybridization-based viral diversity assay to quantify the efficiency of poliovirus transport from the periphery to the central nervous system. We found viral replication in peripheral axons is limited and the type I interferon response limits viral replication in peripheral tissues, protecting against disease. Significantly, we discovered that retrograde axonal transport of poliovirus in the sciatic nerve was inefficient and only 20% of viral pool members reaching the brain. The efficiency of viral transport increased upon muscle damage, leading to increased viral diversity and pathogenesis. In summary, we identified a viral induced mechanism

controlling virulence of mixed viral populations, and characterized three host barriers that restrict poliovirus pathogenesis in the nervous system. The identification of these barriers restricting virulence may help explain the rare incidence of neurological complications following poliovirus infection and aid in our understanding of viral population dynamics and pathogenesis.

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PRIOR PUBLICATIONS

1. **Lancaster K**, Dietz DM, Moran TH, Pletnikov MV. Abnormal social behaviors in young and adult rats neonatally infected with Borna disease virus. Behav Brain Res. 2007;176(1):141-8

2. **Lancaster KZ**, Pfeiffer JK. Limited trafficking of a neurotropic virus through inefficient retrograde axonal transport and the type I interferon response. PLoS Pathog. 2010;6(3):e1000791

3. **Lancaster KZ**, Pfeiffer JK. Mechanisms controlling virulence thresholds of mixed viral populations. J Virol. 2011;85(19):9778–88.

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ABBREVIATIONS

BBB – blood brain barrier

CNS – central nervous system

DI particles – defective interfering particles

dpi – day post infection

HIV – human immunodeficiency virus

hpi - hour post infection

hPVR – human poliovirus receptor

IFN - interferon

IPV – inactivated poliovirus vaccine

IRES – internal ribosome entry site

MOI – multiplicity of infection

NCR – non-coding region

OPV – oral poliovirus vaccine

ORF – open reading frame

PFU – plaque forming units

PNS – peripheral nervous system

PVR-IFNAR$^{-/-}$ – PVR expressing, interferon alpha beta receptor knock out mice

VAPP – vaccine associated paralytic poliomyelitis

VP (VP0, VP1, VP2, VP3, VP4) – viral protein

WGA –wheat germ agglutinin

CHAPTER ONE

LITERATURE REVIEW

Neurotropic Viruses

Neurotropic viruses include some of the most widespread and lethal pathogens impacting human health such as West Nile virus, poliovirus, rabies virus, and human immunodeficiency virus (HIV). While most viral infections occur in the periphery, neurotropic viruses have the ability to invade the central nervous system (CNS) and replicate causing complications including meningitis, encephalitis, neurodegeneration, or neuronal cell death. Viral infection in the CNS can be severe as damage and disease is often irreversible due to the post-mitotic nature of the majority of neurons in the brain and spinal cord.

The non-renewable characteristics of the CNS make it uniquely vulnerable to damage and, therefore, three distinctive features protect it. First, similar to in the periphery, the CNS has a pathogen surveillance system comprised of macrophages, dendritic cells, and CNS specific microglial cells (3, 4). Second, the anatomical architecture of the capillaries in the CNS prevent circulating blood, and as a result pathogens, from entering the brain and spinal cord. This distinct

architecture has been termed the blood brain barrier (BBB) and encompasses capillaries that lack the pores that ordinarily permit solutes to exit into organs. Additionally, the BBB capillaries are lined by endothelial cells that are non-fenestrated and joined by tight junctions (5). Third, the CNS is immune privileged due to limited access to peripheral immune cells and a decreased inflammatory response, which is thought to help limit irreversible neuronal loss (6).

Despite the immune privileged nature of the CNS, neurotropic viruses have evolved strategies to gain access and can either cause an acute infection, a persistent infection, or latent 'infection'(6). During acute infection robust replication generates large amounts of infectious progeny damaging neurons either from destruction of the cells or an exaggerated inflammatory response (7-9). Persistent infection occurs when the virus is unable to be cleared by the host immune system, and continues to produce progeny over a long period of time. Latency involves minimal to no production of infectious progeny; however, the viral genomic material is maintained in cells and can be reactivated upon immunosuppression (10).

The three main strategies used by neurotropic viruses to enter and infect the CNS exploit weaknesses in the barriers protecting the CNS. First, viruses can cross the BBB either through disruption of the BBB, entry where the BBB is

considered to be leaky, or infection of the microvascular endothelial cells (11-13). Second, viruses may enter the CNS by what has been termed a 'Trojan horse' mechanism whereby viruses infect leukocytes in the periphery and the leukocytes, for example macrophages, enter the CNS and inadvertently deliver the virus (6, 14). Finally, viruses, such as poliovirus, may enter the central nervous system via neurons that have axonal projections with nerve terminals in the periphery.

Poliovirus Background and History

Poliovirus is an enterovirus of the *Picornaviridae* family, is spread via the fecal-oral route, and neurological complications occur in approximately 1% of infections. The most common outcome of poliovirus infection in the nervous system is asymmetric flaccid paralysis, termed paralytic poliomyelitis or infantile paralysis. Poliovirus was initially indentified as the causative agent of paralytic poliomyelitis by Landsteiner and Popper in 1909 (15). By then the virus had already begun to cause epidemic outbreaks in Europe, subsequently spreading to the United States and causing widespread panic and fear as the epidemics worsened. The incidence continued to escalate with more than 21,000 cases of paralysis reported in the United States in 1952 (16). The outbreaks, public awareness, and human toll drove the launching of a massive research initiative

culminating in the development of two excellent vaccines. The incidence of infection and paralysis decreased dramatically after the introduction of Jonas Salk's inactivated poliovirus vaccine (IPV) in 1954 (17-19) and further decreased after switching to the live attenuated oral poliovirus vaccine (OPV) developed by Albert Sabin in 1956 (20).

The success of the vaccines lead the World Health Organization to begin a worldwide poliovirus eradication campaign in 1988, with the goal of complete eradication by the year 2000. Despite missing the deadline, the overall results have been successful with the global cases of polio falling from 350,000 in 1988 to 791 in 2000 (21). One of the biggest setbacks to eradication came with the realization that the Sabin OPV vaccine strains could revert and cause disease (22-24). The propensity of the live attenuated vaccine to revert can partially be explained by some of the unique features of poliovirus as a prototypical RNA virus.

RNA viruses have extreme diversity and evolvability due to their high replicative yields, and high mutation rates due to an error-prone polymerase. Poliovirus is an excellent model to study viral mutation rates, vaccine development and RNA virus population dynamics. However, much about

poliovirus pathogenesis and host barriers to viral infection is still unknown due to the previous lack of a tractable small animal model system.

Only certain primates, including humans and old world monkeys, are naturally susceptible to poliovirus infection; therefore, research into transmission and pathogenesis was limited until the generation of transgenic mice expressing the human poliovirus receptor (hPVR) (25, 26). Generation of these mice was facilitated by studies demonstrating that receptors determine host and tissue tropism and are required for viruses to bind and enter cells (27-29), and was made possible by the identification of CD155/PVR as the receptor required for poliovirus binding and entry (30, 31). Two groups generated mouse strains that express hPVR (PVR-trangenic mice) and are susceptible to infection via intravenous, intracranial, intraperitoneal and intramuscular inoculation (25, 32). Despite ubiquitous expression of the receptor, including expression in tissues that do not support viral replication, PVR-transgenic mice are still resistant to the more physiologically relevant oral route of infection, suggesting that additional host factors control susceptibility (25, 26, 31, 33, 34).

It was demonstrated that type I interferon (IFN) plays an important role in limiting viral pathogenesis due to infection-mediated induction of interferon stimulated genes that confer an antiviral state (35-37). This lead to the generation

of PVR-transgenic mice lacking the interferon-αβ receptor (PVR-IFNAR$^{-/-}$ mice) that are orally susceptible to infection with poliovirus (37). The generation of PVR-transgenic mice, and PVR- IFNAR$^{-/-}$ mice has now opened the door for studying some of the fundamental unanswered questions about poliovirus pathogenesis. Alternatively, the molecular biology of poliovirus has been well characterized and has lead not only to advances in virology but also revealed novel insights into cell biology.

Molecular Biology

As the prototypical member of the *Picornaviridae* family, much that has been discovered about picornavirus structure and the viral cycle has been characterized using poliovirus. Poliovirus is a non-enveloped virus, with a single-stranded positive sense RNA genome, approximately 7500 nucleotides in length. The viral RNA contains a 5′ noncoding region (NCR), a single open reading frame (ORF), followed by a 3’ NCR with a polyadenylated tail (38, 39). The 5′ NCR has a highly ordered secondary structure, that confers cap-independent translation of the viral genome. A viral protein is linked to the 5′ end of the RNA terminus, followed by a cloverleaf and highly structured region of RNA that comprises the internal ribosome entry site (IRES) (40-47). The single ORF

encodes a polyprotein that is posttranslationally processed into structural and non-structural proteins that assist in viral replication, manipulation of the host cell, and formation of new viral progeny (39, 48-50). The 3′ end of the genome also contains complex secondary structure with two stem loops that are thought to interact to form a pseudoknot, followed by a tail of adenylate residues that varies in length (51-53).

The structure of the poliovirus virion was one of the first animal viruses to be solved (54). The particle is approximately 30 nm in diameter and the capsid is composed of four viral proteins (VP1, VP2, VP3, VP4). The capsid forms an icosahedron and contains 60 copies of each of the capsid proteins organized around a five-fold axis of symmetry (54-56). The five fold axis is surrounded by a depression, called a canyon, where the virus interacts with its receptor (55).

The Poliovirus Receptor

The poliovirus receptor (CD155/PVR) is a member of the immunoglobulin superfamily of glycoproteins and is expressed only in primate cells. CD155/PVR contains three domains with β strands, an N-terminal V-type domain that binds to canyon, and two C2-type domains (31, 57). Two membrane-

bound forms containing a transmembrane domain, and two secreted isoforms have been identified (58, 59). The secreted isoforms (CD155β and CD155γ) lack the transmembrane domain and are secreted from the cell. The function of these secreted isoforms is unknown, but it has been speculated that they, and other secreted immunoglobulin superfamily proteins, may have been pathogen driven precursors of the modern adaptive humoral immune system (60). The membrane bound isoforms (CD155α and CD155δ) are involved in cell-matrix contacts, tight junction formation and in natural killer cell activation (61-63). Additionally CD155 is highly expressed in the mammalian nervous system during development, with expression driven by sonic hedgehog and distribution localized to regions that give rise to eventual motor neurons (64, 65).

The distribution and expression of membrane bound CD155/PVR is one of the primary factors that determines poliovirus susceptibility and tissue tropism as it is necessary for viral entry into host cells (30, 66); however, there are also additional post-entry steps that control viral replication and permissivity of cells and tissues (33, 66). These post-entry blocks determine where viral replication can occur and influence the production and yield of infectious progeny within a host.

Poliovirus Replication Cycle

The viral replication cycle can be divided into an attachment and entry phase, replication phase, and assembly and exit phase. Since viruses are obligate intracellular parasites they require the presence of all susceptibility and permissivity factors for productive infection. When poliovirus encounters a cell, attachment is mediated through interaction of the virion canyon binding to the V-type domain of CD155 (56, 67, 68). Like other non-enveloped viruses, poliovirus must overcome the challenge of releasing viral RNA from the capsid. Poliovirus binding to PVR results in a conformational change from the native 160S virion to a 135S particle (69-71). The conformational change occurs in response to receptor docking and subsequently VP4 inserts into the membrane forming a pore for RNA release (72). Viral RNA is then released from the two-fold axis of symmetry, near the cell surface in epithelial cells, and is subsequently replicated leaving behind 80S particles (empty capsids) (73-77). Since the genomic material of poliovirus is positive-sense single-stranded RNA, the viral RNA can serve as messenger RNA and be directly translated by binding of ribosomes to the IRES (78). The viral ORF generates a single polyprotein that is then autocatalytically cleaved in a coordinated cascade generating structural and non-structural proteins (50, 79, 80). The incoming viral RNA is also used to generate, via the RNA polymerase $3D^{pol}$, a replicative intermediate that is the complimentary negative strand to the viral

RNA(80). The negative strand is then used to generate more positive strand RNA, which become the progeny virion genomes (80, 81).

Assembly of progeny virons via encapsidation of genomes is not completely understood, but viral replication occurs on membranous structures, perhaps aiding this process (82, 83). A final autocatalytic proteolysis event after encapsidation of RNA encompases the maturation step for formation of infectious poliovirus whereby VPO is cleaved to form VP4 and VP2, which stabilizes the capsid (84, 85). Virons begin accumulating in the cytoplasm and primarily exit the cell by lysis and disseminate in the host, although non-lytic pathways have been observed (86-89).

Dissemination in the host

Poliovirus is an enteric virus and spread via the fecal-oral route. After ingestion the virus replicates in the oropharynx and gastrointestinal tract (90-92). Transmission occurs primarily from virus shed in the feces from one host through direct contact with a secondary host. Virus can be shed for 2-8 weeks after ingestion and has an intrahost incubation period within the host ranging from 2-35 days (93-95). The primary sites of replication include the tonsils and Peyer's

patches, and from there the virus moves to the draining lymph nodes and enters the bloodstream via the thoracic duct (94, 96, 97). Three outcomes of infection with poliovirus can occur. In most infected individuals, a minor transient viremic phase occurs that can be absent of clinical symptoms or manifest as general symptoms of viral infection such as fever and sore throat. In the majority of individuals, the virus is cleared at this stage of infection. In 4-8% of infections a second major virema occurs as a result of viral replication in peripheral tissues such as muscle or reticuloendothelial tissues (98, 99). This secondary major viremia is a prerequisite for viral entry into the CNS, but neurological complications are rare. Less than 1% of individuals infected with poliovirus ultimately develop paralysis (100). Therefore, despite the prominence of neurological complications and paralysis associated with poliovirus infection, entry into the nervous system appears to be an accidental stochastic event, as this disease outcome does not appear to benefit the virus or aid in transmission between hosts.

Entry into the Nervous System – Poliovirus as a Neurotrope

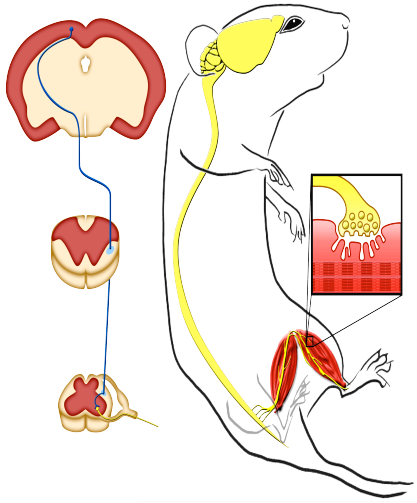
Poliovirus is thought to enter the nervous system by two, non-mutually exclusive pathways. The first is via a hematogenous route, whereby the virus

enters the CNS by crossing the blood brain barrier. The second pathway involves uptake and entry at peripheral nerve terminals, and trafficking via retrograde axonal transport to the neural cell bodies located in the spinal cord or brain. Additionally, the poliovirus receptor is expressed on monocytes, and monocytes differentiated into macrophages support poliovirus replication, suggesting that poliovirus may exploit all three points of CNS entry used by other neurotropic viruses (101, 102). Experimental data demonstrated that poliovirus can enter the CNS via crossing the BBB or via peripheral neurons, and that that viremia is a prerequisite for either route (103-105). Bodian was the first to hypothesize that poliovirus can enter the CNS via the BBB (106) and argued that the "provoking effect" of needle injections resulted in a localized leakiness of the blood brain barrier that permitted viral entry and resulted in a higher incidence of localized paralysis (106-109). Later work by Nomoto's group supported the theory that poliovirus can cross the BBB by demonstrating that [^{35}S]methionine-labeled virus accumulates in the brain of mice after entering the circulatory system (110). Poliovirus was inoculated by intravenous injection into the tail vein of mice and virus accumulated in the brain at a rate significantly above what would be expected for the vascular volume. Furthermore, the authors demonstrated that this was independent of the presence of poliovirus receptor because non-transgenic mice (not expressing the poliovirus receptor) still accumulated virus in the brain,

and pretreatment with an anti-hPVR antibody did not affect poliovirus accumulation (110).

Poliovirus can also invade the CNS via trafficking in neurons (98, 111-114). In PVR-transgenic mice, intramuscularly inoculated poliovirus invades the CNS only via a neural route as transection of the sciatic nerve limits disease (98, 112, 113, 115). Invasion via the neural route occurs after viremia. Poliovirus seeds and replicates in peripheral tissues, and from there the virus is taken up in peripheral neurons innervating the organs, or at the neuromuscular junction via receptor-mediated endocytosis (116, 117). The C-terminal portion of PVR interacts with the dynein light chain Tctext-1, and the virus in the endosome is transported via the fast retrograde axonal transport system from the nerve terminal to the soma (Figure 1-1) (113, 116, 118, 119).

A



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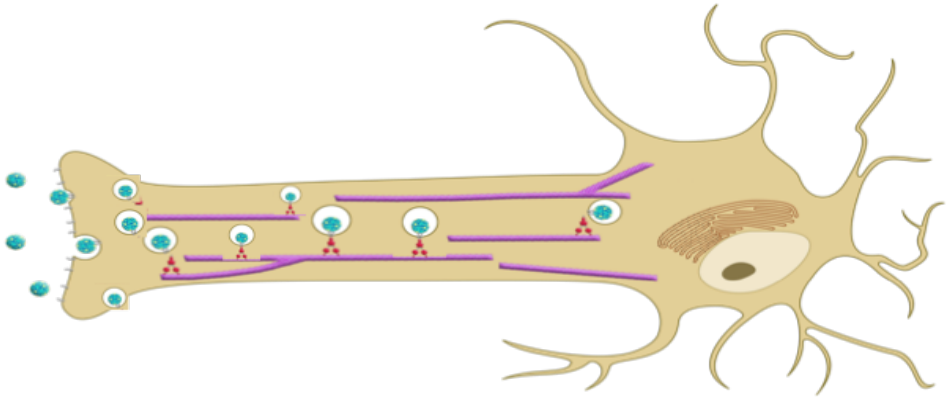

Figure 1-1. Poliovirus Trafficking in the Nervous system. (A) After primary viremia, or intramuscular inoculation, poliovirus seeds and replicates in peripheral tissue, for example muscle. Virions are taken up at the neuromuscular junction by receptor-mediated endocytosis (inset), and traffic along axons of neurons in the sciatic nerve to the cell bodies located in the spine. The virus then traffics up the spine along the corticospinal tract to the brain. (B) Model of viral transport within neurons. After receptor-mediated endocytosis at the nerve terminal, the C-terminal portion of the poliovirus receptor (PVR/CD155) attaches to the dynein light chain Tctext, and virus is trafficked along microtubules via retrograde axonal transport within the axon to the cell body (soma). Images adapted from schematics created by Chris Etheredge.

Viral infection and replication in neurons generates high viral titers and destruction of motor neurons, which presents as lesions in the ventral horn of the spinal cord (108, 109, 120-122). Disease symptoms are restricted to motor neurons in the absence of sensory neuron destruction. CD155/PVR is expressed at neuromuscular junctions and during development in regions that ultimately rise to motor neurons (64, 116). The acute flaccid paralysis caused by poliovirus invasion of the nervous system, in the absence of sensory neuron complications, is thought to be due to the restricted expression of CD155/PVR to motor neurons (123).

Viral Population Dynamics and Quasispecies

Viral barriers that limit disease can be elucidated by understanding the intricacies of viral population dynamics. As previously mentioned, the acute flaccid paralysis from poliovirus infection is a rare event, occurring in less than 1% of infections, suggesting that there are viral or host barriers that limit entry and disease in the nervous system. A population-based framework for understanding RNA viral evolution was adapted from a mathematical theory

initially developed to explain genetic evolution (124). Poliovirus as a model RNA virus exists as a viral quasispecies, which is a swarm of related but genetically distinct pool members that differ from each other by one or more point mutations but interact in unique ways (124-126). The extensive diversity inherent to RNA virus populations arises from high replicative yields and high mutation rates due to low fidelity RNA dependent RNA polymerases (127-131). This diversity allows the viral population to overcome selective pressures and disseminate in the host, and thus influences viral fitness (132-134). Fitness encompasses the ability of a virus to replicate and survive in a given environment, which ultimately determines the virulence and pathogenesis of the viral population (126).

The importance of understanding viral populations dynamics was demonstrated for poliovirus infection where the researchers exploited two features of viral quasispecies behavior, fidelity and diversity. Fidelity refers to the intrinsic error-rate rate that is ultimately determined by the replicase. For RNA viruses the error-prone RNA dependent RNA polymerase that lacks proof reading and therefore has low fidelity creates a range of genetic variation in the viral pool members. RNA viruses must maintain a balance between sequence space exploration and maintenance of the parental genomic sequence.

Thus, RNA viruses have evolved a “perfect” error-rate accumulating mutations on the threshold of “error catastrophe”, able to maintain the parental genome but also able to adapt (126). The accumulation of too many deleterious and inactivating mutations would ultimately lead to collapse of the genome. However, too few mutations would reduce fitness due to loss of adaptability (126, 135). For poliovirus and other RNA viruses the error rate is roughly 1 nucleotide per 10,000 nucleotide copied. The poliovirus genome is less than 10,000 nucleotides; therefore, the viral pool members differ from each other by at least 1 mutation on average.

Since RNA viruses exist on the threshold of error catastrophe, the low-fidelity of the 3Dpol of poliovirus was exploited using a nucleoside analog, ribavirin, to push the virus over the threshold leading to an enhanced mutation accumulation and subsequent extinction (136-138). Crotty et al. demonstrated that by understanding the error threshold and fidelity of the poliovirus quasispecies that the population could be manipulated to decrease infectious virus production to less than 0.00001% of the initial population. Therefore, by understanding and manipulating the error rate of viral quasispecies, the authors uncovered a novel mechanism by which mutagens can be used as antiviral therapy. The converse is also true. Using a high fidelity variant that generated a more homogenous viral quasispecies, two separate studies demonstrated that a diverse quasispecies is

required for full virulence of poliovirus in a transgenic mouse model of infection (134, 139, 140). The authors generated a high fidelity variant by passaging poliovirus in the presence of increasing concentrations of ribavirin until a resistant variant could be isolated. Sequence analysis revealed a single mutation, G64S, in the RNA-dependent RNA polymerase that conferred enhanced fidelity (139, 140). The resultant viral population had increased resistance to guanidine, indicating a decrease in error frequency (138). Subsequent introduction of poliovirus, carrying a high fidelity polymerase, into mice showed reduced virulence and pathogenesis compared to wild-type poliovirus (134, 140). This finding was extrapolated to test a novel approach to live attenuated vaccine design. Because of the high mutation rates, the risk of reversion of an attenuated RNA virus strain back to the original pathogenic parental strain is high. Vignuzzi et al. used the high fidelity G64S poliovirus strain to demonstrate a more stably attenuated vaccine strain that still produced neutralizing antibody and conferred protection against a subsequent viral challenge with wild-type virus (141). Therefore, understanding the dynamics of diverse RNA virus populations is essential for treatment and control of these pathogens as well as for rational vaccine design.

Vaccine Reversion, Mixed Populations and Virulence Thresholds

Despite the success of the oral live attenuated Sabin poliovirus vaccine, circulating vaccine-derived polioviruses pose a serious threat to re-emergence of the pathogen. A diverse viral population reaches the central nervous system and virulent virus is excreted after administration of the attenuated vaccine due to a high frequency of reversion and recombination events (142-147). However, disease is rare, as only 1 case of vaccine associated paralytic poliomyelitis (VAPP) occurs for every 2.5 million vaccine recipients after immunization with the oral Sabin poliovirus (148). This suggests that, despite the presence of virulent viruses in mixed viral populations, there exists a virulence threshold within the viral population controlling the development of disease.

The term virulence threshold is used here to describe the dynamics between the propensity of a viral population to cause disease, and the actual condition in which the population will cause disease. For example, virulent virus must be present above a defined proportion of the mixed population to cause disease, but this number may vary depending on the environment within the host. In an immunocompromised host, the absence of an effective response to viral infection may lower the threshold for disease. Conversely, an individual challenged with a pathogen that they have recently encountered may have a ready,

primed immune response and therefore the threshold for disease may be higher (Figure 1-2). In other words, are there may be viral and host barriers controlling viral pathogenesis, setting the virulence threshold of mixed viral populations at a higher limit than the functional infectious dose.

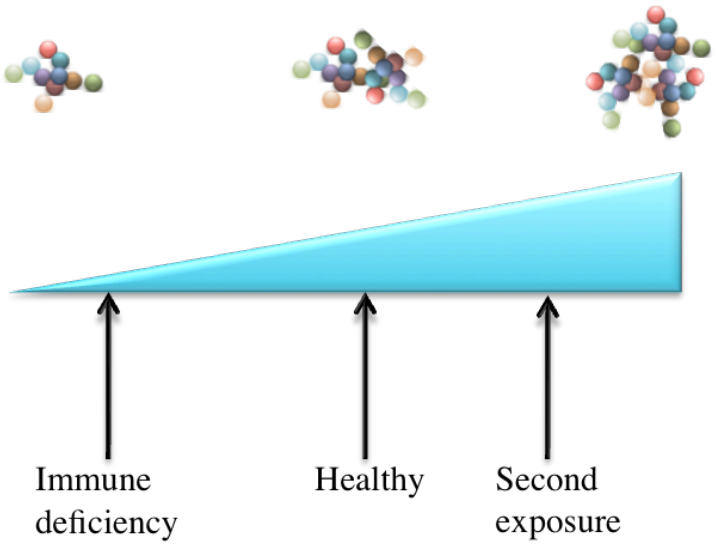

Figure 1-2. Virulence Thresholds. In mixed viral populations such as an RNA virus quasispecies, a certain portion of the viral population has a virulent phenotype. In healthy individuals the amount of virulent virus must be above a certain amount or threshold. This threshold can vary depending on the condition of the host. The virulence threshold may be lower in individuals with immune deficiency, and higher in individuals with prior exposure to the pathogen.

An example of a viral barrier that controls virulence thresholds in mixed viral populations is defective interfering (DI) particles. DI particles are spontaneously generated during viral replication, typically due to replicase errors, and are sub-genomic deletion mutants that are incapable of producing infectious virions independently (149). DI particles have been well characterized and have been shown to limit virulent phenotypes by multiple mechanisms (149-151). For example, direct competition for cellular resources between DI particles and intact virons could limit availability of essential factors required for virulent virus production (152, 153). Therefore, the virulent phenotype could be masked, essentially requiring more virulent virus to cause disease.

Host Barriers to Viral Infection

In addition to viral barriers controlling disease, host barriers may limit poliovirus pathogenesis en route to the nervous system. Host barriers include physical barriers, immune-mediated barriers, and barriers specific to viral infection in the nervous system (Figure 1-3). Physical barriers that limit disease from pathogens include the skin or acidic pH of the stomach. Work by Kuss et al. demonstrated that the gut epithelium limits poliovirus infection (1). Using a pool of ten tagged viruses in a hybridization-based diversity assay (Figure 1-4), they

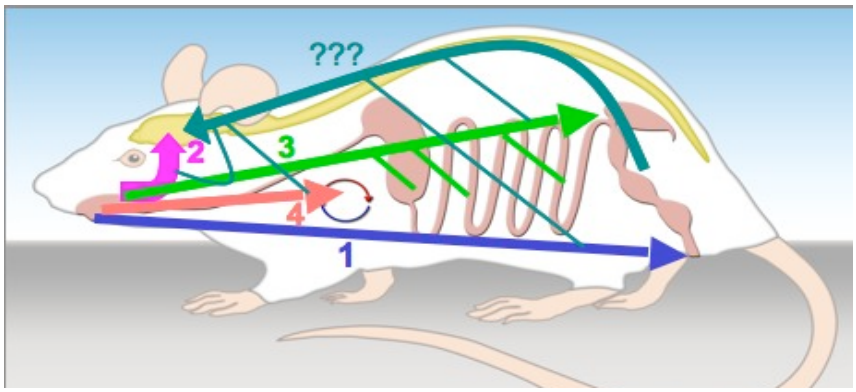

Figure 1-3. Host barriers to poliovirus infection. Potential and previously characterized host barriers to poliovirus infection as identified by decreases in viral diversity after oral inoculation. 1- Decreases in diversity were observed between ingestion and excretion (1). 2- A robust mouth to brain bottleneck effect was observed suggesting multiple host barriers limiting infection in the CNS (1, 2). 3- Acidic stomach environment and gut barriers limit diversity (1). 4- Host barriers, such as type I interferon response, limiting viral access to the blood. 5-Additional unidentified barriers limiting access to the CNS. Image created by Chris Etheredge.

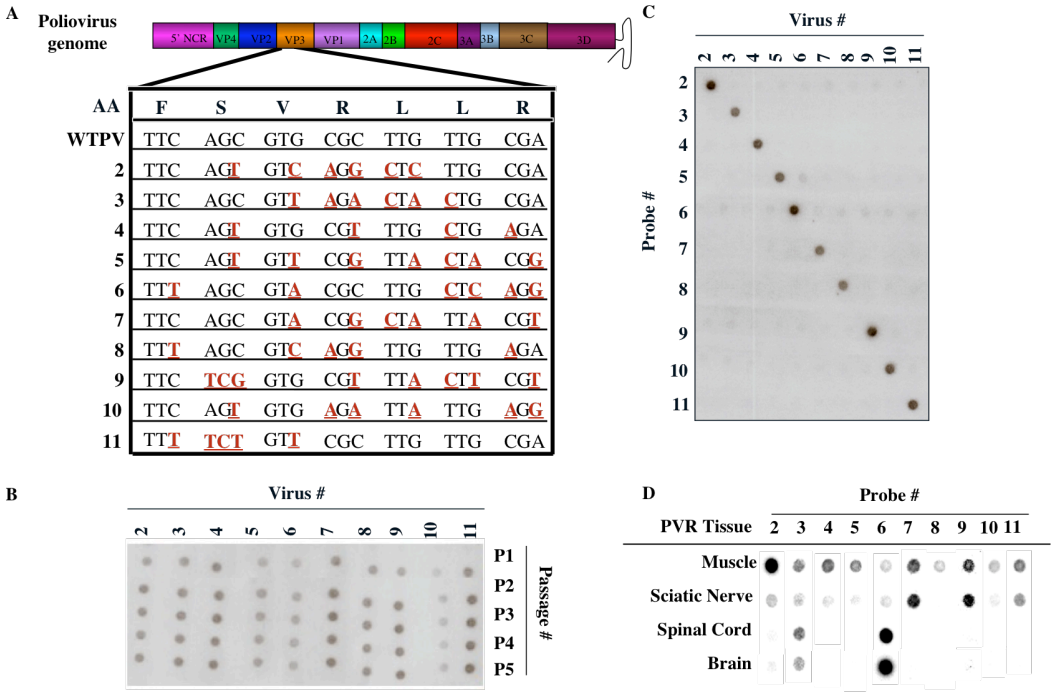

Figure 1-4. Hybridization-based artificial quasispecies assay for monitoring viral diversity and identifying host barriers. (A) Poliovirus genome and location of silent point mutations in the capsid coding region. The amino acid sequence is indicated as well as the nucleotide for the wild type strain of type 1 Mahoney poliovirus. The combinations of point mutations are indicated in red and bold for each of the "tagged" viral pool member. (B) Growth of the individual viral pool members. Each pool member is represented after multiple passages indicating no growth defects; furthermore, the point mutations confer no selective advantage or disadvantage *in vivo* as each pool member has equivalent representation in the CNS (1). (C) Specificity of the radiolabeled probes. Each probe has specific hybridization for its cognate viral pool member with very low background or cross-hybridization. (D) A representative blot from one mouse after intramuscular inoculation of the 10 tagged viruses. Each pool member present in the tissues along the neural trafficking route is represented by a radiolabeled hybridization signal matching the pool member's cognate probe. Images adapted from schematics created by Chris Etheredge.

demonstrated that the intestinal tract poses a major barrier to viral infection as shown by a reduction in viral diversity from the starting inoculum compared to what was observed in the blood or brain. They further demonstrated the significance of this barrier by disrupting it with dextran sulfate sodium, which increased viral diversity and titers in the blood, but not the brain, suggesting additional barriers may be playing a role after the viremic phase (1).

The innate immune system responds rapidly to invasion by pathogens and is an essential host barrier against viral infection. A primary component of the innate immune system against viral infection is the type I interferon response. Type I interferons are produced in many cell types and can activate macrophages and natural killer cells as well as stimulate an intracellular antiviral response (154-156). The presence of viral nucleic acid is sensed via pattern recognition receptors, starting a signaling cascade driving the production of interferon. IFN can then signal for the recruitment of leukocytes or set up a feedback loop by binding to its receptor and enhancing the intracellular antiviral state (155). Peripheral tissues express the poliovirus receptor and are vulnerable to infection once poliovirus has reached the blood (66). For poliovirus, West Nile virus and influenza virus, the type I interferon response is essential for controlling viral infection and determining tissue tropism (35, 37). Specifically, poliovirus can replicate to high titers in the absence of an efficient interferon response (PVR-

IFNAR$^{-/-}$ mice), and subsequently can replicate in tissues not previously able to support viral infection (37, 157). Visceral organs are primed for a fast response to infection and susceptibility to infection corresponds to the amount of interferon induction. The impact of this barrier at the post-viremic phase of infection was illustrated using a pool of ten tagged polioviruses to monitor viral diversity. In immune competent mice about 10% of pool members reached the brain after intraperitoneal inoculation and 10-30% of pool members reached the brain after intramuscular inoculation (1). Using PVR-IFNAR$^{-/-}$ mice, the authors demonstrated a dramatic increase in viral diversity in the brain in both intraperitoneally and intramuscularly inoculated mice suggesting an important role for type I interferon in controlling poliovirus pathogenesis in the post-viremic phase of infection. Taken together these studies implicate the type I interferon response as a barrier to viral infection, limiting poliovirus replication and controlling tissue tropism in the periphery.

The adaptive immune response is also a major host barrier to poliovirus infection, primarily mediating viral clearance and preventing subsequent infection. Serum IgG against poliovirus is sufficient to prevent paralysis (105, 158-161); however, a robust IgA response is required to prevent primary replication and infection of poliovirus in the gut (20, 92, 97). This protection was shown to be incomplete in infants and even adults receiving the oral Sabin

vaccine as infection and viral replication of the vaccine strains was shown to occur after ingestion - yet paralysis following vaccination rarely occurred (91, 104). Additionally, despite the importance of antibody-mediated clearance of poliovirus infection, there are cases of chronic asymptomatic replication of neurovirulent poliovirus strains (162). As of 2011, a patient with hypogammaglobulinaemia has been excreting neurovirulent type 2 poliovirus without apparent symptoms for almost 30 years (162). This has important implications not only for transmission of the virus in the community but also for the global eradication campaign. Yet in these cases, despite the absence of this important antiviral host barrier, the absence of paralytic disease argues that there are additional host barriers controlling poliovirus pathogenesis.

Additional host barriers limit viral access to the CNS. In cases of vaccine-associated paralytic poliomyelitis, research has shown that the viral populations found in the CNS represent only a subset of viruses isolated from the gut, and often the more neurovirulent variants that were in the gut were absent from the CNS (111, 147, 163). Viruses isolated from the brains of PVR-mice inoculated intraperitoneally and intramuscularly also represent only a small portion of the inoculum (1, 2). These barriers can be partially overcome by increasing the dose of the inoculum, but this is not sufficient to ensure representation of all pool

members (1, 2). Again this suggests the presence of host barriers limiting infection in the nervous system.

Identifying Host Barriers

An effective way of characterizing barriers that limit viral infection is to examine conditions in which the barriers are absent. “Provocation poliomyelitis” refers to the increased incidence of paralytic poliomyelitis that occurs after tissue trauma and has been observed both epidemiologically and experimentally (91, 164). The first example of provocation poliomyelitis occurred in what is termed the “Cutter incident”, where batches of polio vaccine that had been incompletely inactivated were administered via intramuscular injection. In the vaccine recipients given the incompletely inactivated virus, there was an increased incidence of paralysis and paralysis most often occurred in the inoculated limb (165, 166). Correlation with increased localized limb paralysis was also observed for vaccine recipients of the oral live attenuated vaccine who had concurrent tissue trauma in the form of exercise-induced or disparate muscle injury (93). Additionally, inflammation and tissue damage caused by unrelated vitamin injections administered within 30 days of receiving the oral live attenuated vaccine led to a higher incidence of vaccine-associated paralytic poliomyelitis,

with the initial onset of paralysis often occurring in the limb damaged by multiple injections (167-173). Tonsillectomies also increased the incidence of brainstem and bulbar poliomyelitis, suggesting a common mechanism whereby tissue damage increases poliovirus pathogenesis in the nervous system (164).

The mechanism to explain the increase in CNS invasion upon injury was investigated experimentally to with the goal of distinguishing between increased blood brain barrier permeability from muscle damage and increased retrograde axonal transport from muscle damage (108, 112). Work by Gromeier and Wimmer demonstrated that poliovirus is taken up in peripheral neurons, as sciatic nerve transection limited poliovirus induced paralysis, and the incidence of paralysis increased in the presence of tissue trauma(112). This suggests a barrier to viral infection in the central nervous system involving retrograde axonal transport.

The interferon response also limits viral replication of other neurotropic viruses and poliovirus in neural culture, suggesting this may play a role in limiting poliovirus infection in the nervous system (174-177). Furthermore, work by Daley et al. suggests that there may be additional intracellular barriers to poliovirus infection in neurons as replication in cultured neurons is delayed and viral yield is 100-fold lower in than non-neuronal cells (178).

Concluding Remarks and Hypothesis

The goal of the work described in this thesis was to identify additional barriers that restrict neurotropic viruses and their access to the central nervous system, as well as to uncover and characterize host factors limiting viral dissemination within the nervous system, thereby limiting disease. My hypothesis was that the rare incidence of paralysis following poliovirus infection is due to the presence of viral and host barriers limiting disease. I identified a replication-independent viral barrier to infection controlling virulence thresholds, as well as a role for type I interferon in limiting replication and reversion of the attenuated Sabin poliovirus strain. Additionally I identified three major host barriers that effect pathogenesis in the central nervous system. First, I demonstrated that limited viral replication occurs in the peripheral nervous system. Second, I found that the type I interferon response dramatically reduced virulence by limiting viral replication in peripheral tissues. Third, I found that poliovirus underwent inefficient retrograde axonal transport within the nervous system. From here we hope to further characterize the effect of these barriers on pathogenesis, fine-tune the mechanism by which the barriers act on viruses, and identify any additional barriers to viral trafficking in the nervous system.

CHAPTER TWO

METHODOLOGY

Cells and Viruses

HeLa cells were grown in Dulbecco's modified Eagle's medium with 10% calf serum as previously described (139). The virulent type 1 Mahoney strain of poliovirus, and its corresponding type 1 attenuated Sabin strain, containing 57 mutations, were propagated and quantified by plaque assay in HeLa cells as previously described (117, 139). In order to maintain the attenuating mutations in the type 1 Sabin strain, any propagation of virus was carried out as previously described at 32°C (179). Furthermore, we confirmed that the inoculum contained most of the major mutations attributed to attenuation via sequencing (See Figure 3-9). Light-sensitive virus was generated by infecting HeLa cells with type 1 Sabin poliovirus or type 1 Mahoney poliovirus in the presence of 10 mg/ml of neutral red dye (Sigma) as previously described (117). The light-sensitive virus stocks were stored and used in the dark to preserve viral integrity for viral replication studies (see below). A portion of this viral stock was used to generate inactivated virus. A pre-titered aliquot was removed from the viral stock in the

dark and the aliquot was then exposed to a fluorescent light bulb for 30 minutes (vortexing every 5 minutes) to inactivate the virus (71, 180). The ratio of culturable light-insensitive to light-sensitive plaque-forming units (PFU) in the inactivated neutral red-poliovirus stock was 1 to $1x10^7$. The T3SA+ strain of reovirus was a generous gift from Terry Dermody (Vanderbilt University), and was generated as previously described (181).

Hybridization-Based Viral Population Diversity Assay

For identification of host barriers virulent Mahoney type 1 poliovirus was propagated and titered in HeLa cells as previously described (1, 139). The ten marked viruses for the viral diversity assay each contain groups of 4-8 silent point mutations that allow specific probe annealing following RT-PCR and dot blotting (see Figure 1-4, 4-1 and (1) for more details). These viruses exhibit no detectable fitness differences (1). Detection of the 10 marked polioviruses was performed as previously described (1). Briefly, viruses from homogenized tissues with low viral titers (stomach, colon, small intestine, vagus, and sciatic nerve) were amplified in HeLa cells, followed by TRIZOL (Invitrogen, Carlsbad, CA) extraction of RNA. Tissues with high viral titers (brain, spine, muscle) were directly extracted with TRIZOL because there was no difference between results from amplifying viruses

in HeLa cells and direct TRIZOL extraction of high titer tissues (data not shown). RT-PCR for the tagged region of the virus was performed as previously described (1). After blotting equivalent concentrations of PCR products on Hybond N+ membranes (GE Healthcare, Buckinghamshire, UK) individual membranes were pre-hybridized and hybridized at 59°C. Primers specific for each of the 10 viruses were kinased with [γ-^{32}P]ATP to serve as probes (1). Following hybridization, membranes were exposed to PhosphorImager screens and specific signal was determined by normalizing blots to perfectly matched and mismatched control PCR product dots and image intensity was uniformly adjusted until mismatched sample was no longer visible in order to eliminate low low-level cross reactive signal (see (1) for more details). Light sensitive tagged poliovirus was prepared and analyzed as described above (1, 71, 180, 182). Briefly, HeLa cells were infected with each marked virus in the presence of 10 μg/ml neutral red dye. Work with neutral red viruses was preformed in the dark, using a red photography light. Inactivation of neutral red viruses was achieved by exposure to a fluorescent light for 10 min. Samples were processed in the dark and supernatant from each tissue was divided in half (half was then exposed to light and the other half was always kept in the dark). For titer analysis, the ratio of PFU in light exposed versus non-light exposed samples were compared to determine the percent replicated virus (1). For the neutral red diversity assay in Figure 4-5, signal from light exposed samples was compared to signal from non-light exposed samples

from the same tissue. Any viral pool members present only in the dark sample were scored as 'non-replicated' virus, and viral pool members present in both the dark and the light sample were scored as 'replicated + non-replicated' (see Figure 4-5 for more details).

Mouse Experiments

C57/BL6 mice expressing the human poliovirus receptor (CD155/PVR) and C57/BL6 PVR mice deficient in the interferon-α/β receptor (PVR-IFNAR$^{-/-}$ mice) were a generous gift from S.Koike (Tokyo, Japan)(26, 37). Oral inoculations were performed by pipetting 2 x 10^7 PFU of each marked virus (2 X 10^8 total PFU in 15μl) into the mouth (1). For intramuscular injections, 2 x 10^6 PFU of each marked virus (2 x 10^7 total PFU in 50μl) was injected into the lower left gastrocnemius muscle (1). Needle sticks were given by inserting a 28-gauge needle into the leg 4 or 5 times twice daily (112). For all poliovirus experiments, mice were monitored twice a day (at ~10-14 hour intervals) and euthanized at the first sign of disease, which is typically paralysis of one hind limb. Upon onset of symptoms, mice fail to recover and typically succumb to disease within 12 hours (data not shown). Therefore, time of disease onset correlates with time of death, and can be used as a more humane alternative to death as an endpoint.

Tissue Harvest and Processing

Whole sciatic nerve was removed by lifting the biceps femoris and removing the nerve segment between the spine and ankle. The nerve was then sectioned into three equal pieces to generate upper, middle and lower sciatic sections. The vagus nerve was removed as a segment from the heart-lung junction to the diaphragm. Muscle included all non-bone tissue below the hip. Tissues (whole spine, brain, stomach, small intestine, colon and muscle) were weighed and resuspended in three volumes PBS+ (1 X PBS with 100μg/ml $MgCl_2$ and $CaCl_2$), and homogenized in liquid nitrogen with a mortar and pestle (2) or with a Bullet Blender tissue homogenizer (Next Advanced Inc, Averill Park, NY) as per manufacturers instructions, followed by freeze-thawing three times to release virus and chloroform extraction of gut samples to inactivate bacteria (1). Vagus and sciatic nerve tissue were dounce homogenized. All samples and tissues were stored at -80°C.

Wheat Germ Agglutinin Experiments

Tissues were collected from mice 6 hours after injecting 5μg WGA into the lower gastrocnemius muscle. Muscle was weighed and resuspended in 2 volumes of RIPA buffer (10 mM Tris,150 mM NaCl, 0.02% NaN_3, 1% Na-deoxycholate, 1% Triton X-100, 0.1% SDS) and sciatic nerve was resuspended in 200μl of RIPA buffer. Samples were then homogenized with the Bullet Blender, and 10μl/ml of a protease inhibitor cocktail (Sigma, St Louis, MO) and 10μl/ml of a phosphatase inhibitor (Calbiochem, San Diego, CA) were added to the supernatants. Dot blot westerns were performed in place of typical gel-based westerns due to multimerization of WGA; therefore, five microliters of each sample was pipetted directly onto a nitrocellulose membrane (GE Water & Process Technologies), which was probed with rabbit anti-lectin (triticum vulgaris) primary antibody (Sigma, St Louis, MO) and goat anti-rabbit HRP secondary antibody. Signal was visualized with ECL reagent (GE Healthcare, Buckinghamshire, UK) and quantified by densitometry (183). Specific WGA signal was distinguished from background by normalizing to a sciatic nerve sample that was not exposed to WGA. WGA signal was within the linear range of detection based on loading and quantification of purified WGA dilutions (data not shown).

Plaque assay and tissue titers

Tissues were harvested from mice at disease onset and processed by a Bullet Blender tissue homogenizer (Next Advanced Inc, Averill Park, NY) as per manufacturer's instructions, followed by freeze-thawing three times to release virus (117). Viral yield was determined by titer analysis of tissue supernatant on HeLa cells. The type 1 Sabin strain of poliovirus is temperature sensitive, yielding small plaques at 37°C. Therefore, in order to distinguish between the Sabin and Mahoney poliovirus strains, we used a plaque size assay performed at 37°C for 2 days. We compared plaque size of control Sabin virus and Mahoney virus to the plaques obtained from tissue samples to distinguish between the virus strains.

Viral replication assay

Mice were infected intramuscularly with light-sensitive virus, in the dark, using a red safety light. As described above, after infection tissues were harvested from mice at various time points and processed by a Bullet Blender tissue homogenizer (Next Advanced Inc, Averill Park, NY) followed by freeze-thawing three times to release virus. All processing and-freeze thaw steps were carried out in the dark (117). Tissue supernatant was then collected and each sample was split

in half: one half of the sample was kept in the dark, and the other half of the sample was exposed to a fluorescent light bulb for 30 min to inactivate any virus that had not replicated. The "light" and "dark" aliquot from each sample was then titered on HeLa cells, and titers were compared to determine the percentage of virus in each muscle sample that had undergone replication. Replication percentage was calculated by dividing the titer from "light" aliquot by titer from the "dark" aliquot, and multiplying by 100.

In vitro infection and viral yield assays

1 x 10^6 HeLa cells were infected with 1x10^7 PFU Mahoney virus (MOI of 10) in the presence or absence of inactivated Sabin virus at 1:0, 1:1, 1:3, 1:10, 1:30, 1:100, 1:300, and 1:1000 ratios. At 5 hours post infection cell-associated virus was harvested and quantified by plaque assay. Reduction in viral yield was calculated as percentage of titer reduction compared to cells infected with the Mahoney virus alone. Similarly, for the infection assays performed in the presence of antibody, 1 x 10^4 HeLa cells were pre-incubated with 0.1, 1, 10, or 100 μg of either a mouse anti-CD155/PVR antibody (Santa Cruz), or a mouse anti-IgG1 isotype control antibody (BioLegend), washed 3 times in PBS, then incubated with 1 x 10^5 PFU Mahoney poliovirus (MOI of 10) for 10 minutes at

37^{o}C. Non-bound virus was removed by washing 3 times in PBS, infection was allowed to proceed for 5 hours, and cell-associated virus was harvested and quantified via plaque assay. To determine viral yield HeLa cells were infected at at MOI of 10 with virulent virus, or virulent virus in the presence of increasing ratio's of excess attenuated virus (1:1, 1:10, 1:100, 1:1000, 1:10000). The infection was allowed to proceed for 5 hours in order to allow one round of viral replication for any virons able to enter and productively replicate in the cells. The viral yield of the virulent strain was then quantified via plaque assay where plaques from the experimental sample were compared to tissue culture derived control viral samples in order to distinguish between the virulent and attenuated strain. The virulent strain generally produces larger plaques while the attenuated strain has a slight temperature sensitivity and growth defect resulting in smaller plaque sizes (184). By comparing the tissue culture derived virus plaques to the sample plaques, viral yield of the virulent strain can be determined.

Viral replication in the presence of interferon

Cells were pretreated with 100 units/ml of IFN-2α (a gift from M. Gale, University of Washington, Seattle, WA) for 48 hours and then infected with Sabin or Mahoney poliovirus for 6 hours, and subsequent cell-associated viral yield was

determined by plaque assay and compared to cells infected in the absence of interferon (175).

^{35}S-poliovirus binding experiments

^{35}S-radiolabled poliovirus particles were produced by growing virus in medium lacking L-methionine and L-cysteine with the addition of ^{35}S-labeled methionine/cysteine to the culture 3.5 h postinfection. At 6 hours post-infection, cytopathic effect was observed, cell-associated virus was harvested by freeze thawing, and the virus-containing lysate was purified using a cesium chloride gradient (10 ml 1.2g/cm^3 CsCl in PBS gently layered on 10 ml 1.4g/cm^3 CsCl in PBS, and ultracentrifugation for 4 hours at 25,000 rpm). The fraction containing radiolabeled virus was determined via titer analysis and scintillation counting. Amicon filters (Millipore) were used to concentrate and desalt virus by adding the sample to the column and spinning at 5000 rpm, followed by 3 wash and spin steps in PBS. This purified virus had a specific activity of 243 PFU/counts per minute (CPM). Viral binding experiments in HeLa and L929 cells were obtained from triplicate infections. Note virus can bind non-specifically to membranes from both cell types; therefore, there is signal from L929 cells despite a lack of PVR on this cell type, this is considered background.

Sequencing of the 5' non-coding region

Viral RNA was purified from tissue homogenates from mice that had succumbed to disease after infection, via TRIZOL extraction (Invitrogen, Carlsbad, CA). Reverse transcription was performed with SuperScript II RT (Invitrogen) using an antisense primer in the capsid coding region (5'-CGAAGCCGCGTTACTAGC -3'). The 5' non-coding region was then amplified using PCR with primers 5'- GGTGGTGTAATTAATGGTAG-3' and 5'-TTAAAACAGCTCTGGGGTTG -3'. PCR products were sequenced by the UT Southwestern McDermott Sequencing Center using an antisense primer (5'-AAAGTCGACTCCAGCAAACAGATAGGGCC-3').

CHAPTER THREE

VIRAL BARRIERS LIMITING PATHOGENESIS

INTRODUCTION

RNA viruses pose a unique challenge to therapy and prevention of viral disease in that they exist as extremely diverse populations of related but genetically distinct pool members (126, 185, 186). These mixed viral populations are due to a low fidelity RNA dependent RNA polymerases and high replicative yields and have been modeled as quasispecies (127-131). In addition to generating diversity, the high mutation rates confer rapid evolution of RNA viruses, complicating antiviral therapy and vaccine design. The importance of understanding RNA virus quasispecies dynamics has been demonstrated recently for some of the most medically important RNA viruses. Influenza and ebola evade the immune system by rapid evolution, HIV frequently develops mutations that confer drug resistance, and hepatitis C virus evolution has been linked to development of chronic infection (187-193). Therefore understanding the dynamics of diverse RNA viral populations is essential for treatment and control of these pathogens as well as for and rational vaccine design.

Despite a worldwide eradication campaign, poliovirus still infects hundreds of thousands of people each year resulting in outbreaks of paralytic, and occasionally fatal, poliomyelitis (194, 195). Poliovirus is spread by the fecal-oral route, causing paralysis and/or death in approximately 1% of infected humans due to invasion of the central nervous system (91). In the early 20th century, the incidence of poliovirus disease continued to escalate until the development of the Salk and Sabin vaccines. Both vaccines were highly effective at dramatically decreasing the incidence of poliomyelitis. The oral live-attenuated Sabin poliovirus vaccine is still used in many countries, due to its low cost, oral delivery, and long-term immunity.

The Sabin poliovirus vaccine contains a mixture of three types of poliovirus attenuated by repeated serial passage, resulting in viral strains with reduced virulence (196). Each vaccine strain carries multiple point mutations, conferring temperature sensitivity and reduced neurovirulence. Not all mutations in the vaccine strains are attenuating. For example, although the type I Sabin strain contains 57 mutations, only 3 are thought to be major attenuating mutations (179, 197-200). The mutations in the vaccine strains can revert to virulence via nucleotide substitution and/or genetic recombination between different poliovirus strains or other enteroviruses (142, 143, 145, 201, 202). This is a special problem

in immunodeficient vaccine recipients, who have long-term viral replication and excretion, thus increasing the chances for reversion and excretion of virulent virus (203, 204).

Despite the success of the oral live-attenuated Sabin poliovirus vaccine, there is virulent virus present at low levels in the vaccine lots due to reversion during vaccine virus expansion (205-207). Furthermore, as mentioned above, attenuating mutations in the vaccine strains frequently revert to virulence following oral administration of the vaccine and virulent virus is excreted in feces due to a high frequency of reversion and recombination events in the gastrointestinal tract (130, 144, 145, 208). These virulent vaccine-derived polioviruses can circulate and pose a serious threat for re-emergence of the pathogen. However, disease is rare, as immunization with the oral Sabin poliovirus vaccine only causes one case of vaccine associated paralytic poliomyelitis (VAPP) occurring for every 2.5 million doses (23, 144, 148, 209, 210). This suggests that, despite the presence of virulent viruses in the vaccine and within the host, a virulence threshold controls the development of disease.

The term virulence threshold is used to describe the dynamics between the propensity of a viral population to cause disease, and the actual condition in which the population will cause disease. For example, virulent virus must be

present above a defined proportion of the mixed population to cause disease, but this number may vary depending on the environment within the host. In other words, this suggests there may be viral or host barriers controlling viral pathogenesis, setting the virulence threshold of mixed viral populations at a higher limit than the functional infectious dose.

Interestingly, Sabin vaccine strain reversion and recombination events are frequent in healthy vaccinees, but alone do not account for the development of disease. For example, up to 80% of viral genomes from healthy vaccinees are recombinant (143). Additionally, a high percentage of healthy vaccinated infants excrete the type 3 Sabin virus, and nearly all of these isolates had undergone reversion of the single major attenuating mutation in this virus, yet these infants did not develop disease (211) (212) (213). However, this small proportion of virulent viruses was unable to cause disease in monkeys unless the virulent revertants were present above approximately 1% of the total (206).

These examples demonstrate that virulent polioviruses likely exist in most individuals vaccinated with the live attenuated Sabin vaccine; however, unless virulent viruses are present above a certain proportion of the population, no disease develops. Understanding this “virulence threshold” is likely to be important for the rational design of many live-attenuated vaccines and will aid our

understanding of viral pathogenesis. The amount of virulent virus required for virulence is partly understood but the mechanisms controlling development of disease in the presence of mixed viral populations is unclear. Here we model the mixed viral populations characteristic of RNA viruses by inoculating mice with different ratios of attenuated and virulent viruses.

Intracellular dominant interference of defective viral genomes in mixed populations has been demonstrated as a mechanism controlling virulence (151, 214, 215); however, the role of attenuated viruses in limiting disease induced by virulent viruses has not been examined extensively in animal models. Teng et al. demonstrated that lymphocytic choriomeningitis virus virulence in mice was reduced if the virus was co-inoculated with an avirulent strain in 10-fold excess (216). In addition, Sanz-Ramos el al. uncovered hidden virulence determinants in mice infected with foot-and-mouth disease virus (217).

The goal of this study was to determine whether virulence can be masked in a mixed viral population and to determine mechanisms controlling virulence using a transgenic mouse model susceptible to poliovirus (25). Mice are not susceptible to poliovirus unless they express the human poliovirus receptor (PVR/CD155) (25, 26). When PVR-transgenic mice are injected intramuscularly in the leg, poliovirus enters neurons of the sciatic nerve and is transported by fast

retrograde axonal transport to the spinal cord and brain (98, 113, 119). Poliovirus replicates to high titer in the central nervous system, and mice succumb to disease within 3-12 days (98). Here, we used PVR-transgenic mice and determined that 1000-fold excess of the attenuated type 1 Sabin strain of poliovirus was protective against disease induced by the virulent type 1 Mahoney strain following intramuscular injection. We determined that protection was induced locally and could be conferred independently of viral replication, possibly through viral receptor competition. Additionally, we demonstrated that the type I interferon response also limits disease induced by the attenuated strain by reducing replication and reversion of attenuating mutations. Our results may explain the rare incidence of vaccine-associated paralytic poliomyelitis, despite the high propensity for reversion of attenuating mutations and the presence of virulent virus in the vaccine, as well as contribute to our understanding of the dynamics of mixed viral populations.

RESULTS

Virulence can be masked in mixed viral populations

In order to study potential virulence thresholds, we created a mixed pool of viruses to mimic a viral population containing an attenuated viral strain (type 1 Sabin poliovirus) in excess of its parental virulent strain (type 1 Mahoney poliovirus). Intramuscular injection of up to $1x10^8$ plaque forming units (PFU) of attenuated Sabin 1 poliovirus did not induce disease in PVR mice. When $1x10^5$ PFU of virulent Mahoney poliovirus was inoculated alone intramuscularly into PVR mice, 76% of the mice succumbed to disease (Figure 3-1A; black line). However, when PVR mice were intramuscularly inoculated with the mixed population of $1x10^5$ PFU of Mahoney poliovirus and $1x10^7$ PFU Sabin poliovirus, only 57% of mice succumbed to disease (Figure 3-1A; dashed blue line). These results suggest that 100-fold excess of the attenuated virus conferred mild protection against disease induced by virulent virus and delayed disease onset; therefore, we increased the amount of attenuated virus to 1000-fold excess.

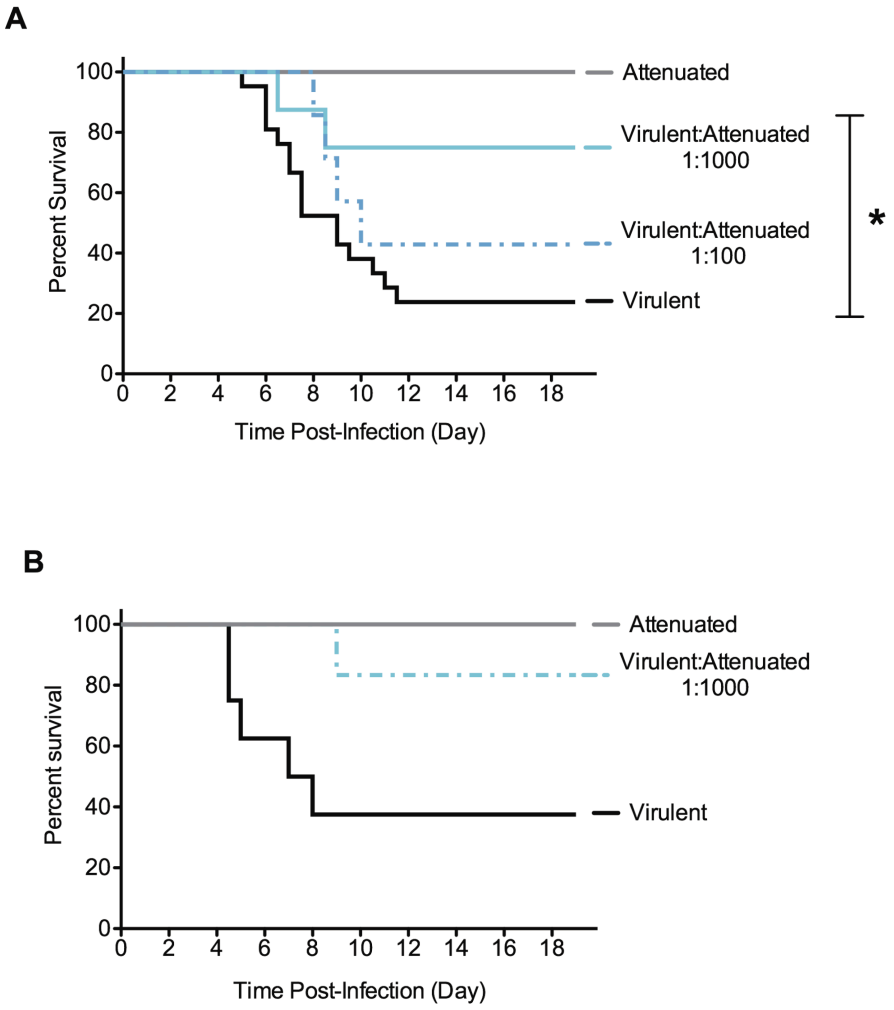

Figure 3-1. Virulence thresholds in mixed populations. (A) Survival curve of adult PVR mice infected with various virus strain combinations. PVR mice were intramuscularly inoculated with $1x10^5$ PFU virulent Mahoney virus (black line), $1x10^8$ PFU attenuated Sabin virus (dark gray line), $1x10^5$ PFU virulent Mahoney virus and $1x10^7$ PFU attenuated Sabin virus (dashed blue line), or $1x10^5$ PFU virulent Mahoney virus and $1x10^8$ PFU attenuated Sabin virus (turquoise line) in the left leg. Mice were euthanized after both legs were paralyzed. Data represent 6-21 mice per group. Data were pooled from all experiments for control mice inoculated with virulent Mahoney virus or attenuated Sabin virus. Statistically significant differences between groups are indicated by asterisks ($p<0.05$, Log-

rank test). (B) Survival curves of three-week old PVR mice inoculated with low amounts of virus. Three-week old PVR mice were intramuscularly inoculated with $1x10^3$ PFU virulent Mahoney virus (black line), $1x10^6$ PFU attenuated Sabin virus (dark gray line), or $1x10^3$ PFU virulent Mahoney virus and $1x10^6$ PFU attenuated Sabin virus (dashed turquoise line) in the left leg. Mice were euthanized after both legs were paralyzed. Only 6-8 of the three-week old mice per group were used; therefore, despite the absence of a statistically significant difference between groups, the survival curve of the three-week old mice had the same trend as the adult mice.

When PVR mice were intramuscularly inoculated with $1x10^5$ PFU Mahoney poliovirus and $1x10^8$ PFU Sabin poliovirus we found significant protection with only 25% of mice succumbing to disease (Figure 3-1A; turquoise line; $p<0.05$; Log-rank test). The 75% survival rate was impressive considering that mice in this group received the same amount of virulent virus as the group receiving the virulent Mahoney virus alone inoculum, where only 24% of mice survived. These results suggest that the attenuated Sabin strain was able to mask virulence of the Mahoney strain, provided that the attenuated strain was present in 1000-fold excess.

To test whether the observed virulence threshold exists with lower amounts of virus, comparable to the live-attenuated vaccine dose of ~$1x10^5$ PFU, we examined Sabin virus-mediated protection in hyper-susceptible young mice (218). When we inoculated three-week old PVR mice intramuscularly with $1x10^3$ PFU Mahoney virus, 63% of mice succumbed to disease. However, when 1000-fold excess of attenuated virus ($1x10^6$ PFU Sabin virus) was co-inoculated, only 17% of mice succumbed to disease (Figure 3-1B; dashed turquoise line), suggesting that the 1000:1 ratio holds true even when less virus is inoculated. Taken together, these results demonstrate that virulence can be masked in mixed viral populations when attenuated virus is present in 1000-fold excess over the virulent virus.

Masking of virulence is induced at the site of inoculation

To mechanistically dissect how attenuated viruses protect against virulent virus-induced disease, we first determined whether the effect was induced systemically or locally. PVR mice were inoculated with $1x10^5$ PFU Mahoney virus in the left hind limb and $1x10^8$ PFU Sabin virus in the right hind limb, and mice were observed for disease. If the protective effect conferred by attenuated viruses occurs through a systemic response, such as humoral immunity, then protection in these mice should be maintained since the same total amount of virus was inoculated. However, if the protective effect occurs locally, requiring both strains of virus at the same site, then the previously observed protective effect would be lost. In mice inoculated with $1x10^5$ PFU Mahoney virus in the left hind limb and $1x10^8$ PFU Sabin virus in the right hind limb, the mortality was equivalent to mice inoculated with Mahoney virus alone (Figure 3-2), indicating that the protective effect was lost. These results suggest that virulence thresholds in this system were induced locally, and not through a systemic response.

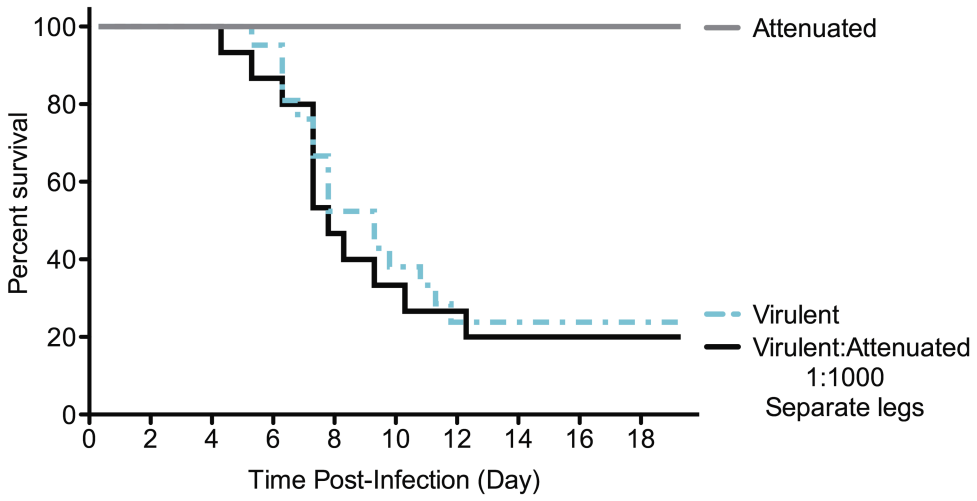

Figure 3-2. Pathogenesis of mixed viral populations in PVR mice inoculated in different limbs. Adult PVR mice were intramuscularly inoculated with $1x10^5$ PFU virulent Mahoney virus in the left leg (black line), $1x10^8$ PFU attenuated Sabin virus in the left leg (gray line), or $1x10^5$ PFU virulent Mahoney virus in the left leg and $1x10^8$ PFU attenuated Sabin virus in the right leg (dashed turquoise line). Mice were euthanized after both legs were paralyzed. Data represent 6-21 mice per group. The curves were not significantly different between mice inoculated in the same or different legs ($p>0.05$, Log-rank test).

Reducing viral transport barriers does not affect protection by attenuated viruses

Since the protection conferred by attenuated viruses occurred locally, and we previously found that local inefficient retrograde axonal transport of poliovirus in neurons limits pathogenesis (117), we tested whether excess attenuated virus exacerbates the retrograde axonal transport inefficiency thereby establishing the protection. Previously, in a mouse model of poliovirus infection in the nervous system, we demonstrated that a major barrier to viral dissemination in the nervous system is inefficient retrograde axonal transport (117). We showed that needle-stick mediated muscle damage increased retrograde axonal transport efficiency of poliovirus and a non-viral protein in the sciatic nerve. Furthermore, we found that increasing the transport efficiency of poliovirus via muscle damage increased the diversity of the viral population that reached the brain and increased viral pathogenesis to the same extent that a deficient type I interferon response contributed to disease (117). Therefore, since this local transport barrier has a substantial affect on pathogenesis, we wanted to examine whether it influences virulence thresholds of mixed viral populations. In order to determine whether increasing retrograde axonal transport efficiency impacts virulence thresholds, we intramuscularly injected PVR mice with $1x10^5$ PFU Mahoney virus, $1x10^8$ PFU Sabin virus, or both viral strains and a subset of each of these groups were given

needle stick-mediated muscle damage (112, 117). When mice inoculated with $1x10^5$ PFU Mahoney virus were given needle sticks to increase transport efficiency, 100% of mice succumbed to disease by day 10 post-infection (Figure 3-3; black dashed line). However, needle stick-mediated muscle damage had no effect on pathogenesis in mice inoculated with both viruses (Figure 3; turquoise line, and dashed turquoise line). Therefore, although the inefficient retrograde axonal transport barrier contributes to local protection against viral spread to the central nervous system, it does not appear to contribute to the protective effect conferred by excess attenuated viruses in our model used to test virulence thresholds.

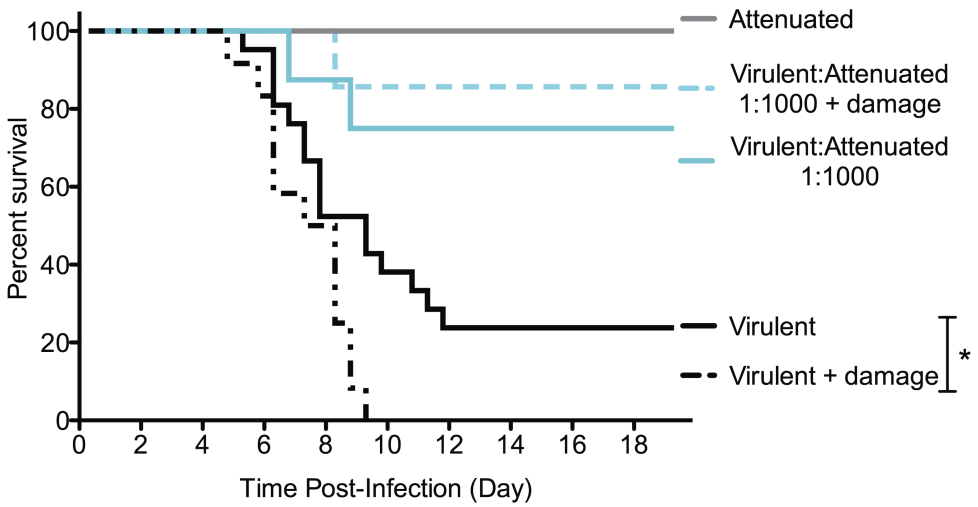

Figure 3-3. The effect of increased axonal transport on virulence thresholds. PVR mice were intramuscularly inoculated with $1x10^5$ PFU virulent Mahoney virus with (dashed black line) or without (black line) needle sticks, $1x10^8$ PFU attenuated Sabin virus (dark gray line), $1x10^5$ PFU virulent Mahoney virus and $1x10^8$ PFU attenuated Sabin virus with (dashed gray line) or without (light gray line) needle sticks. Mice were euthanized after both legs were paralyzed. Data represent 6-21 mice per group. Data were pooled from all experiments for control mice inoculated with virulent Mahoney virus or attenuated Sabin virus. The survival curves of mice receiving virulent Mahoney virus were significantly different than mice receiving virulent Mahoney virus and needle sticks ($p<0.05$, Log-rank test). The curves were not significantly different between the Mahoney and Sabin viruses versus Mahoney and Sabin viruses with needle sticks groups ($p>0.05$, Log-rank test).

Protection can be conferred by a replication-independent mechanism

Theoretically, attenuated viruses could protect against disease induced by virulent viruses through replication-dependent or replication-independent mechanisms; therefore, we evaluated whether the protection against virulence in mixed populations was dependent on replication using replication-incompetent inactivated viruses. Poliovirus propagated the presence of neutral red dye is light sensitive and exposing the stock to light inactivates the virus, generating intact but replication incompetent viruses (180). PVR mice were inoculated intramuscularly with $1x10^5$ PFU Mahoney virus, $1x10^8$ PFU inactivated Sabin virus, or both. Only 27% of mice succumbed to disease in the groups receiving Mahoney and inactivated Sabin viruses, comparable to the 25% mortality observed with replication-competent Sabin virus, indicating that protection can be conferred by a replication-independent mechanism (Figure 3-4A). Since the attenuated Sabin poliovirus strain may be immunochemically different and bind with different affinity to the receptor than the Mahoney poliovirus strain, we evaluated whether inactivated Mahoney poliovirus could protect against the virulent, replication competent Mahoney poliovirus strain. PVR mice were inoculated intramuscularly with $1x10^5$ PFU Mahoney virus, $1x10^8$ PFU inactivated Mahoney virus, or both. Only 13% of mice succumbed to disease in the group receiving both Mahoney and inactivated Mahoney viruses (Figure 3-4B), comparable to the 25% mortality

observed in the group that received replication-competent Sabin virus (Figure 1-1A). Taken together these data suggest that protection against virulence can be conferred by a replication independent mechanism.

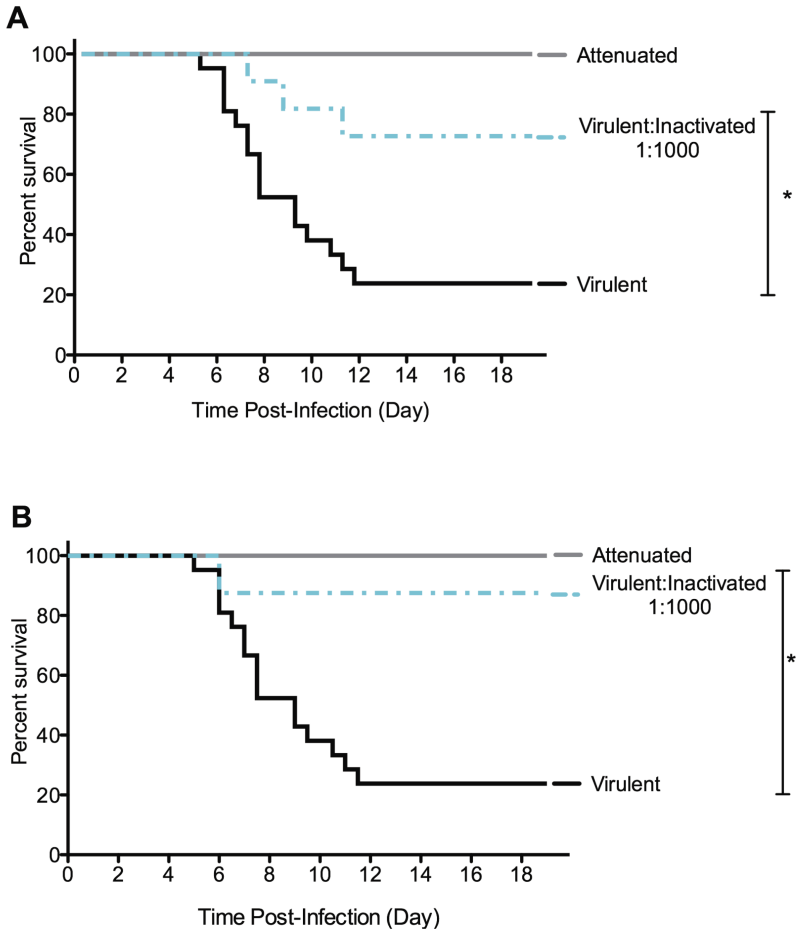

Figure 3-4. The effect of replication-incompetent viruses on virulence thresholds. (A) Replication-incompetent Sabin poliovirus. PVR mice were intramuscularly inoculated with $1x10^5$ PFU virulent Mahoney virus (black line), $1x10^8$ PFU attenuated Sabin virus (gray line), or $1x10^5$ PFU virulent Mahoney virus and $1x10^8$ PFU inactivated Sabin virus (dashed turquoise line). (B) Replication-incompetent Mahoney poliovirus. PVR mice were intramuscularly inoculated with $1x10^5$ PFU virulent Mahoney virus (black line), $1x10^8$ PFU attenuated Sabin virus (gray line), or $1x10^5$ PFU virulent Mahoney virus and $1x10^8$ PFU inactivated Mahoney virus (dashed turquoise line). Mice were euthanized after both hind limbs were paralyzed. Data represent 6-21 mice per group. The survival curves of mice inoculated with virulent Mahoney virus was significantly different than mice receiving virulent Mahoney virus and inactivated Sabin virus ($p<0.05$, Log-rank test).

Protection is not conferred by a heterologous virus, reovirus

After determining that the mechanism of protection is due to a local, replication independent mechanism, we next sought to determine whether the effect was due to the attenuated virions or a local immune response from the abundance of exogenous antigen. To distinguish between these two possibilities we infected mice with type 1 Mahoney poliovirus in the presence of a heterologous virus, reovirus strain T3SA+ (181), which does not cause disease in intramuscularly injected adult mice. If excess reovirus protected against Mahoney poliovirus-induced disease, then this would suggest a local immune response might be mediating protection. However, if excess reovirus did not protect against Mahoney poliovirus-induced disease, then this would suggest a virus-specific mechanism was mediating protection. Therefore, we infected mice with $1x10^8$ PFU of reovirus alone, $1x10^8$ PFU of reovirus and $1x10^5$ PFU of Mahoney poliovirus, or $1x10^5$ PFU of Mahoney poliovirus alone and monitored disease. We found that mice infected with reovirus alone showed no signs of disease, whereas mice infected with Mahoney poliovirus alone or Mahoney poliovirus with reovirus had equivalent disease (24% and 25% survival respectively; Figure 3-5).

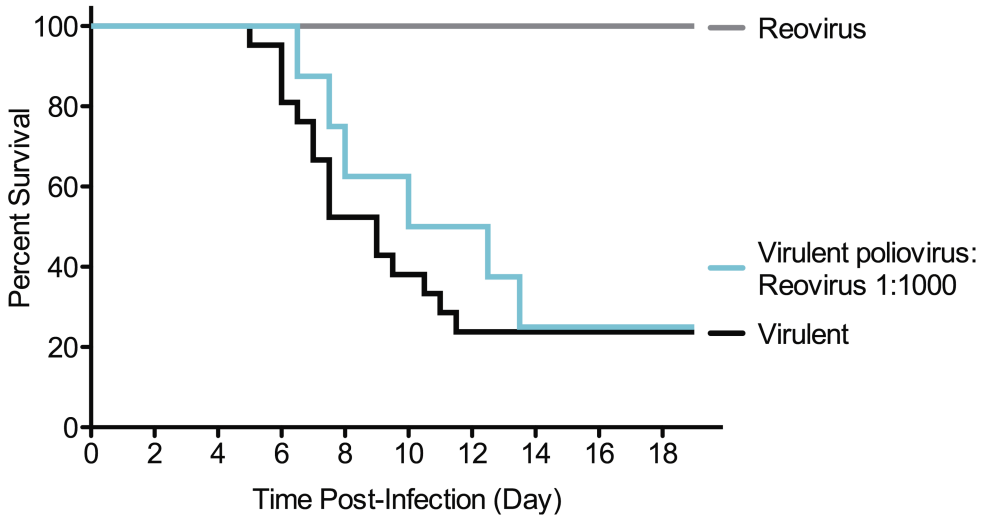

Figure 3-5. Virulence thresholds with a heterologous virus, reovirus. PVR mice were intramuscularly inoculated with $1x10^5$ PFU virulent Mahoney virus (black line), $1x10^8$ PFU T3SA+ reovirus (dark gray line), or $1x10^5$ PFU virulent Mahoney virus and $1x10^8$ PFU T3SA+ reovirus (turquoise line) and monitored for disease. Mice were euthanized after both hind limbs were paralyzed. Data represent 7-21 mice per group. Survival curves were not significantly different between the groups infected with the Mahoney poliovirus alone versus Mahoney poliovirus and T3SA+ reovirus ($p>0.05$, Log-rank test).

Protection can be conferred by blocking viral receptor

Since attenuated poliovirus can mask virulence in a virus specific, replication-independent manner, we considered whether competition for a limited resource, such as the viral receptor PVR, contributes to the protective effect. We began by examining whether the attenuated virus could reduce the viral yield of the virulent strain *in vitro* in order to determine if this correlates with the *in vivo* effects and molecularly dissect the mechanism of protection. HeLa cells were infected at an MOI of 10 with virulent virus, or virulent virus in the presence of increasing ratio's of excess attenuated virus (1:1, 1:10, 1:100, 1:1000, 1:10000). The infection was allowed to proceed for 5 hours in order to allow one round of viral replication for any virons able to enter and productively replicate in the cells. The viral yield of the virulent strain was then quantified via plaque assay where plaques from the experimental sample were compared to tissue culture derived control viral samples in order to distinguish between the virulent and attenuated strain. The virulent strain generally produces larger plaques while the attenuated strain has a slight temperature sensitivity and growth defect resulting in smaller plaque sizes (184). By comparing the tissue culture derived virus plaques to the sample plaques we can determine the viral yield of the virulent strain under our experimental conditions. The ratio of 100 PFU's of attenuated virus for every virulent virus was sufficient to begin decreasing viral yield of the virulent strain.

At 1:10000 the amount of virulent virus that was able to efficiently replicate in the presence of attenuated virus was decreased by over 90% compared to the virulent virus alone infection (Figure 3-6A). This suggested that the attenuated strain may be interfering with viral replication of the virulent strain (152, 219).

We hypothesized that the inhibition of viral replication of the virulent strain may be due to competition for a limited cellular resource; therefore, we tested whether decreasing the available cells had an effect on viral yield of the virulent strain. HeLa cells were infected at an MOI of 1, 0.5, and 0.1 with virulent virus, or virulent virus in the presence of increasing ratio's of excess attenuated virus (1:1, 1:10, 1:100, 1:1000, 1:10,000) and determined viral yield as described above. We found that by decreasing the amount of cells available less attenuated virus was required to decrease viral yield and effects were even observed at the 1:1 and the 1:10 ratio (Figure 3-6B).

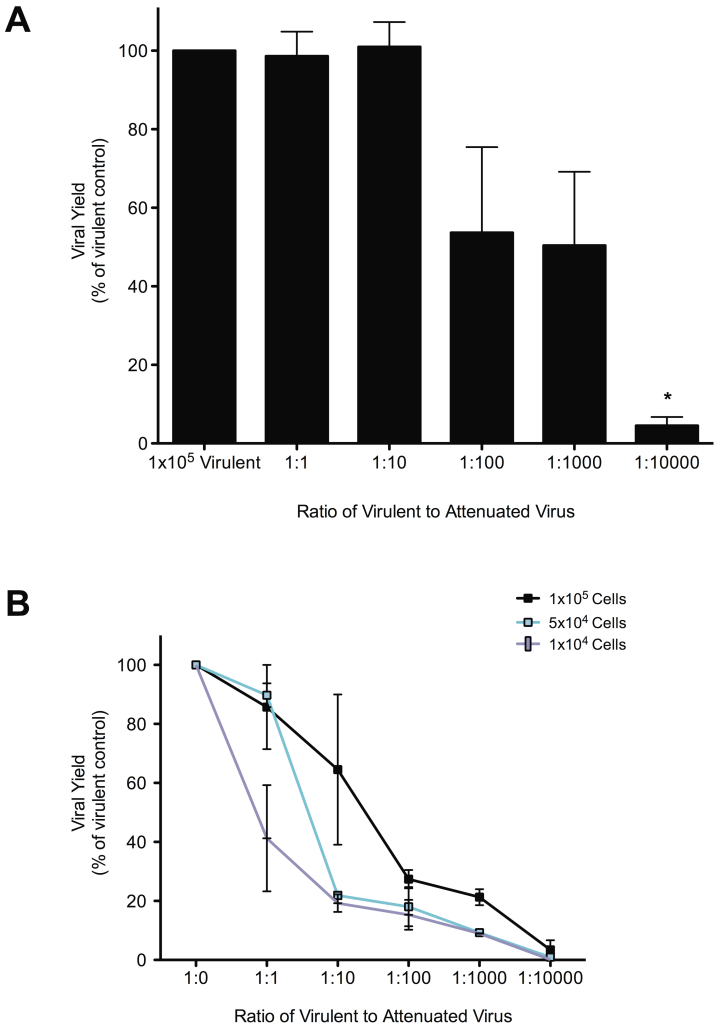

Figure 3-6. The effect of the presence of excess attenuated virus on viral yield of a virulent strain. (A) Increasing attenuate virus and virulent viral yield. HeLa cells were infected at an MOI of 10 with virulent virus, or virulent virus in the presence of increasing ratio's of excess attenuated virus (1:1, 1:10, 1:100, 1:1000, 1:10000). Cells were harvested after 5 hours of infection and viral yield of the virulent strain quantified via plaque assay. (B) The effect of limited cellular resources on viral yield. HeLa cells were infected at an MOI of 1 (black line), 0.5 (turquoise line), and 0.1 (purple line) with virulent virus, or virulent virus in the presence of increasing ratio's of excess attenuated virus (1:1, 1:10, 1:100, 1:1000, 1:10,000) and viral yield quantified via plaque assay.

Next we evaluated whether the effect of decreasing viral yield was independent of viral replication, as observed *in vivo*. We determined whether inactivated Sabin virus could reduce yield of Mahoney virus in cell culture infections and whether any yield reduction was PVR-dependent. We infected PVR-expressing HeLa cells with $1x10^5$ PFU Mahoney virus (MOI of 10) in the presence or absence of inactivated Sabin virus at 1:0, 1:1, 1:3, 1:10, 1:30 1:100, 1:300 and 1:1000 ratios, harvested cell-associated virus at 5 hours post infection, and quantified viral yield by plaque assay (Figure 3-7A; black line). We found that 30-fold excess inactivated Sabin virus significantly reduced virulent Mahoney virus yield in HeLa cells ($p<0.001$, Student's *t* test), suggesting that excess replication-incompetent virus limits replication of virulent virus *in vitro*.

In order to show that the inhibition by inactivated virus was specific to cells expressing PVR, we quantified inhibition of viral yield in murine L292 cells, which do not express PVR. We found that after infection with $1x10^5$ PFU Mahoney virus (MOI of 10), even 1000-fold excess inactivated virus was unable to reduce viral yield in L929 cells, in contrast to HeLa cells (Figure 3-7A; turquoise line). Therefore, inactivated virus-mediated inhibition of virulent virus replication required PVR expression. To confirm that poliovirus binds more efficiently to PVR-expressing HeLa cells than L929 cells, we quantified binding

using ^{35}S-labeled poliovirus. ^{35}S-labeled Mahoney poliovirus was incubated with cells at an MOI of 1000, in order to saturate receptors, for 10 minutes. Non-bound virus was removed by washing, and bound virus was quantified by scintillation counting of cell lysates. Significantly more ^{35}S-virus bound to HeLa cells compared with L929 cells, suggesting that PVR expression correlates with viral binding efficiency (Figure 3-7B, $p<0.0001$; Student's *t* test). Taken together, these results suggest that the ability of inactivated viruses to limit virulent virus yield correlates with the amount of PVR available on the cell surface and viral binding efficiency.

To further examine the effect of PVR availability on virulence thresholds, we assessed the effect of treatment with anti-PVR antibody on viral replication and pathogenesis. First, we incubated cells with increasing concentrations of anti-PVR antibody, infected cells with Mahoney poliovirus (MOI of 10), and quantified viral yield. HeLa cells were pre-incubated with 0.1, 1, 10, or 100 μg of either an anti-CD155/PVR antibody or an anti-IgG_1 isotype control antibody, washed, and then infected with virulent virus. After 10 minutes, non-bound virus was removed by washing, and after 5 hours cell associated virus was quantified. Viral yields in the presence of the anti-PVR antibody were comparable to those obtained with excess inactivated virus, with an inverse correlation between amount of anti-PVR antibody and viral yield (Figure 3-7C, black line; $p<0.001$,

Student's *t* test). Because PVR blockade phenocopied the yield reduction conferred by inactivated viruses *in vitro*, we examined whether anti-PVR antibody treatment would also limit Mahoney poliovirus pathogenesis *in vivo*. When PVR mice were intramuscularly inoculated with a mixture of $1x10^5$ PFU Mahoney poliovirus and 100 μg of the anti-IgG_1 isotype control antibody, 86% of mice succumbed to disease. However, when PVR mice were intramuscularly inoculated with a mixture of $1x10^5$ PFU Mahoney poliovirus and 100 μg of the anti-CD155/PVR antibody, we found significant protection with only 38% of mice succumbing to disease (Figure 3-7D; black line; $p<0.05$; Log-rank test). Therefore, both our *in vitro* and *in vivo* data indicate that reduced virulence and pathogenesis in mixed viral populations can be conferred by competition for PVR. Taken together, these results indicate that one mechanism of protection establishing the virulence threshold, conferred by attenuated and inactivated viruses, may occur through local PVR competition.

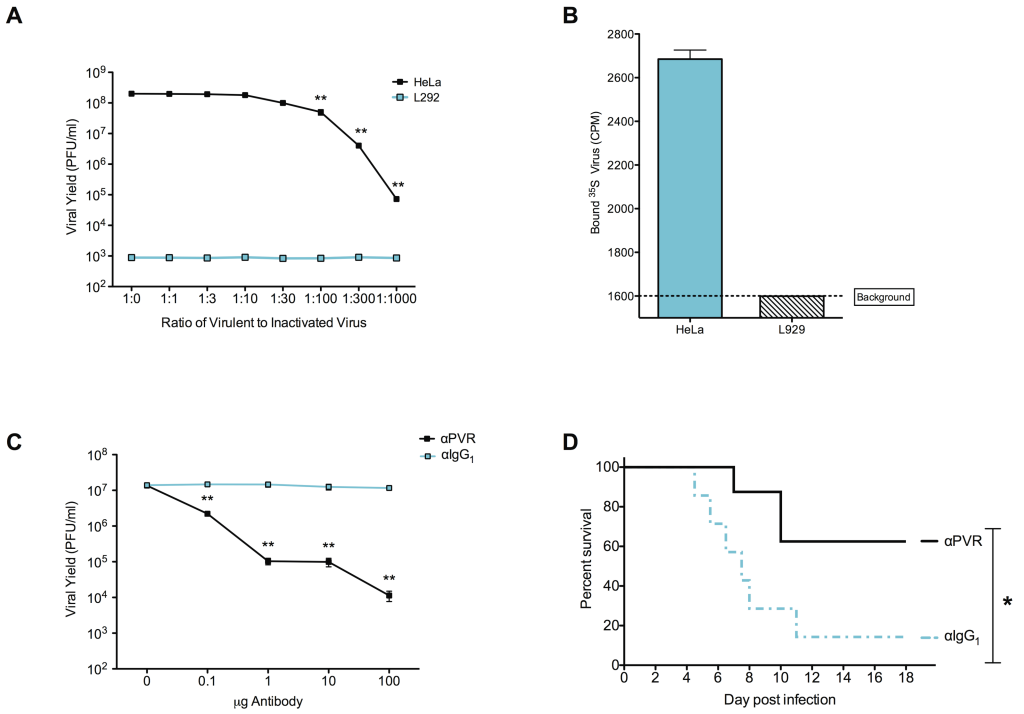

Figure 3-7. The effect of viral receptor availability on viral yield and pathogenesis. (A) Virulent virus yield in the presence of inactivated virus. HeLa and L292 cells were infected with 1x10^7 PFU Mahoney virus (MOI of 10) with inactivated Sabin virus at 1:0, 1:1, 1:3, 1:10, 1:30, 1:100, 1:300, and 1:1000 ratios, cell-associated virus was harvested at 5 hours post-infection, and yield was quantified by plaque assay. Boxes indicate mean and error bars represent SEM of duplicates. Asterisks denote statistically significant differences ($p<0.001$, Student's *t* test). (B) Receptor-specific viral binding. HeLa and L292 cells were incubated with ^{35}S-labled virus for 10 minutes at 4°C, washed 3 times with PBS, and cell pellet was harvested. Bound virus was quantified by scintillation count of the pellet, and L292 cells lacking PVR were used to detect non-specific viral binding and set as a baseline for signal. (C) Viral yield in the presence of antibody. HeLa cells were pre-incubated with 0.1, 1, 10, or 100 μg of anti-CD155/PVR antibody, or anti-IgG$_1$ isotype control antibody, washed, then infected with 1 x 10^5 PFU of virulent virus (MOI of 10). Non-bound virus was removed after 10 minutes by washing, and viral yield was quantified by plaque assay of cell-associated virus. Boxes indicate mean and error bars represent SEM of duplicates. Asterisks denote statistically significant differences ($p<0.001$, Student's *t* test). (D) Survival curve in the presence of antibody. PVR mice were intramuscularly inoculated with 1x10^5 PFU Mahoney poliovirus containing 100

μg of the anti-IgG_1 isotype control antibody or $1x10^5$ PFU Mahoney poliovirus containing 100 μg of an the anti-CD155/PVR antibody. The survival curves of mice inoculated with virulent Mahoney and anti-CD155/PVR was significantly different than mice receiving virulent Mahoney and anti-IgG_1 ($p<0.05$, Log-rank test).

The type I interferon response contributes to protection.

Although inactivated virus was sufficient to induce protection from virulent virus-induced disease, we wondered whether any additional host barriers, such as the innate immune response, could contribute to protection conferred by attenuated viruses. By demonstrating that the protective effect required virus to be inoculated at the same site, we excluded systemic adaptive immunity as a major mechanism controlling virulence thresholds in this system. Previously, the type I interferon response has been shown to control poliovirus pathogenesis (37); therefore, we examined whether the type I interferon response impacts virulence thresholds. We used PVR transgenic mice lacking the interferon-α/β receptor (PVR-IFNAR$^{-/-}$) to determine the role of interferon in controlling virulence (157). PVR-IFNAR$^{-/-}$ mice were inoculated intramuscularly with 1x10^5 PFU Mahoney virus, 1x10^8 PFU Sabin virus, or both. Unlike in our previous experiments with immune-competent mice, 100% of mice in all groups succumbed to disease (Figure 3-8A). To determine which viruses reached the brain, thereby contributing to disease, we initially used a plaque phenotype plaque assay to differentiate between Mahoney and Sabin viruses based on plaque size. The attenuated Sabin strain is temperature sensitive and has a small plaque phenotype at 37°C, so we used this feature to distinguish between Sabin and Mahoney strains.

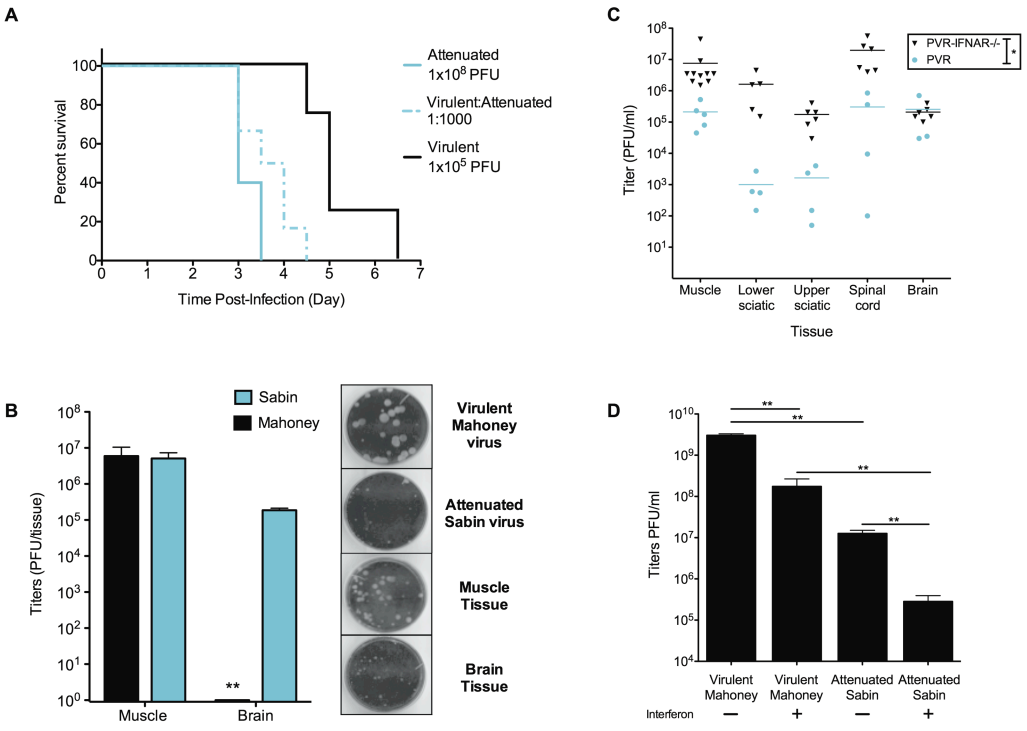

Figure 3-8. Impact of the type I interferon response on virulence and viral replication. (A) Survival curve of PVR-IFNAR$^{-/-}$ mice inoculated with virulent Mahoney virus, attenuated Sabin virus or both. PVR-IFNAR$^{-/-}$ mice were intramuscularly inoculated with 1x10^5 PFU virulent Mahoney virus (black line), 1x10^8 PFU attenuated Sabin virus (turquoise line), or 1x10^5 PFU virulent Mahoney virus and 1x10^8 PFU attenuated Sabin virus (dashed turquoise line). Mice were euthanized after both legs were paralyzed. Data represent 4-6 mice per group. (B) Plaque phenotypes and viral titers in tissues harvested from PVR-IFNAR$^{-/-}$ mice inoculated with virulent and attenuated viruses. Left: Viral titers from PVR-IFNAR$^{-/-}$ mice intramuscularly inoculated with 1x10^5 PFU virulent Mahoney virus and 1x10^8 PFU attenuated Sabin virus were quantified by plaque assay and scored as Mahoney virus (black bars) or Sabin virus (turquoise bars) according to plaque size. Mean and SEM for 6 mice are shown. Right: Representative plaque assays for muscle and brain tissue are shown below HeLa cells infected with Mahoney virus alone (large plaques) or Sabin virus alone (small plaques) as controls. Statistically significant differences between virus strains in tissue are indicated by asterisks ($p<0.001$, Student's *t* test). (C) Viral titers in tissues from PVR-IFNAR$^{-/-}$ mice and PVR mice each infected with 1x10^5 PFU Mahoney virus and 1x10^8 PFU Sabin virus. Tissues from PVR and PVR-

IFNAR$^{-/-}$ mice were harvested upon disease onset and viral titer was quantified for each tissue along the viral trafficking route from muscle to brain. Titers from PVR mice are depicted as turquoise circles, and titers from PVR-IFNAR$^{-/-}$ mice are depicted as black triangles. Statistically significant difference in viral titers across all tissues of PVR-IFNAR$^{-/-}$ mice compared to PVR mice is indicated by asterisks ($p<0.01$ one-way ANOVA). (D) The effect of interferon treatment on viral replication. HeLa cells were pretreated with or without IFN2α for 48 hours, infected with 1 x 10^7 PFU of Sabin virus or Mahoney virus (MOI of 10), cell-associated virus was harvested at 5 hours post-infection, and yield was quantified by plaque assay. Statistically significant differences existed for both viral strains in the absence of interferon, and in the presence of interferon (Student's *t* test-$p<0.001$). Mean and SEM for three experiments are shown.

Tissue-derived viruses were examined by plaque assay along with cell-culture derived Mahoney or Sabin viruses as controls to facilitate comparison of plaque size. In muscle there was a combination of small and large plaques suggesting the presence of Sabin and Mahoney viruses, respectively. However, in brain only small plaques characteristic of the Sabin strain were present (Figure 3-8B). This suggested that in mice with a deficient type I interferon response the Sabin strain may have contributed to disease onset.

To determine whether the lack of a type I interferon response facilitated viral replication and Sabin virus virulence, several additional experiments were performed. First, we quantified viral titers in the tissues along the viral trafficking route to the brain and, not surprisingly, found higher viral titers in all tissues of PVR-IFNAR$^{-/-}$ mice compared to PVR mice (Figure 3-8C, black inverted triangles; $p<0.01$ one-way ANOVA). The interferon response primarily limited viral replication in the periphery since there was a 36-fold difference in virus muscle titers and a negligible difference in brain titers. This suggested that in the absence of an efficient type I interferon response, even the Sabin strain of poliovirus was able to replicate efficiently *in vivo*.

Next, we quantified viral replication *in vitro* in the presence or absence of IFN2a treatment. Cells were pretreated with or without IFN2α for 48 hours,

infected with Sabin or Mahoney virus, each at an MOI of 10, and cell-associated virus was harvested at 6 hours post-infection and quantified by plaque assay. Sabin virus titers were 44-fold higher in the absence of interferon, suggesting that the attenuated Sabin strain is highly sensitive to interferon, but is able to efficiently replicate in the absence of a type I interferon response (Figure 3-8D). Furthermore, Mahoney virus titers were only 17-fold higher in the absence of interferon, suggesting that interferon treatment exerts more of an effect on Sabin virus replication than Mahoney virus replication (Figure 3-8D).

The enhanced replication of the Sabin strain in the absence of type I interferon (Figure 3-8C, and 3-8D), and the plaque phenotype suggesting that the Sabin strain was present in brain (Figure 3-8B), led us to hypothesize that the attenuating mutations in the Sabin strain reverted in PVR-IFNAR$^{-/-}$ mice, and the revertant viruses induced disease. Therefore we performed sequence analysis of the 5' non-coding region to determine whether the major attenuating mutations responsible for protection from neurovirulence had reverted. Sequence analysis revealed that the Sabin strain was present in the brains of PVR-IFNAR$^{-/-}$ mice, and that the major attenuating mutation, G480A, had reverted (Figure 3-9A). In addition, we found a mutation at position 189, which frequently accompanies the G480A reversion and contributes to decreased temperature sensitivity and increased neurovirulence (22, 179, 197, 199, 200, 220, 221). The sequence at

position C355, reported as U in the published type I Sabin strain sequence, matched our inoculum strain, as well as two additional sites at positions 649 and 674, confirming that the brain virus was the reverted Sabin strain rather than the Mahoney strain.

Our data suggest that Sabin poliovirus replicates efficiently in PVR-IFNAR$^{-/-}$ mice, facilitating reversion of major attenuating mutations and the development of disease; however, the replication efficiency of Sabin poliovirus in immune competent PVR mice, which survive infection, is unknown. Perhaps Sabin poliovirus replication is so minimal in PVR mice that reversion of attenuating mutations is a rare event, thereby facilitating establishment of protection from Mahoney poliovirus-induced disease. Koike et al. have demonstrated that Sabin poliovirus has diminished replication in the CNS compared to Mahoney poliovirus (26). In our study the virulence threshold was established in muscle, so we determined whether Sabin poliovirus has limited replication in muscle of immune competent PVR mice. We quantified viral replication kinetics *in vivo* using light-sensitive viruses, as previously described (117). Poliovirus propagated in the presence of neutral red dye is light sensitive due to dye-mediated RNA inactivation; however, upon replication the dye is absent in progeny virions, making them light-insensitive. By quantifying the ratio of light-sensitive to light-insensitive viruses, replication can be quantified. PVR

mice were inoculated intramuscularly with $2x10^7$ PFU of light-sensitive Sabin poliovirus in the dark, and muscle was harvested at 6 hpi and 30 hpi and processed to determine the percentage of viral replication (see Methods). We found that for the Sabin poliovirus strain, only 6.3% of virus in muscle had undergone replication at 6 hpi and 28% had undergone replication at 30 hpi (Figure 3-9B). These replication kinetics are much slower than what we, and others (178), have previously observed for the Mahoney strain. Specifically we found about 25% of Mahoney virus in muscle had undergone replication at 6 hpi and 60% had undergone replication at 30 hpi (117). This delayed and reduced replication kinetics observed here suggests that the Sabin poliovirus strain has reduced replication *in vitro* and *in vivo* in the presence of an intact interferon response.

Taken together, these data suggest that Sabin virus replicates more efficiently in the periphery in the absence of a type I interferon response, and reverts major attenuating mutations culminating in disease. In immune competent mice, Sabin poliovirus replication is diminished. Therefore, the type I interferon response appears to play a major role in limiting the pathogenesis of the attenuated Sabin strain by limiting replication and the potential for reversion.

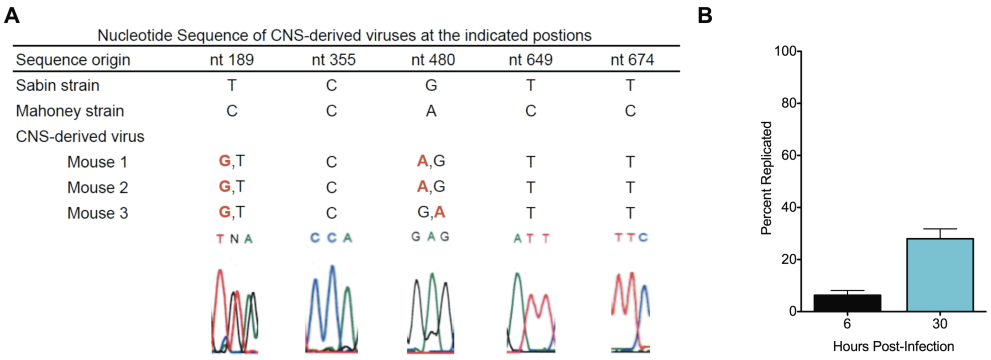

Figure 3-9. Nucleotide sequences of virus in the brain of PVR-IFNAR$^{-/-}$ mice intramuscularly co-inoculated with Sabin and Mahoney viruses and Sabin virus replication kinetics in muscle. (A) The sequence identity at nucleotide positions in the 5' non-coding region is indicated for the attenuated Sabin strain and the virulent Mahoney strain. The presence of the reported mutations in the attenuated Sabin strain were confirmed by sequencing the inoculum, and the sequence identity reported in the table reflects the inoculum used in the experiments. Sequences of viruses found in the brain of three PVR-IFNAR$^{-/-}$ mice were determined via sequencing RT-PCR products. A mixed peak indicates the presence of a mixed viral population in the brain. The nucleotide reported first was more prevalent, as indicated by relative peak size in the histogram, and mutations differing from the attenuated Sabin strain are indicated in bold and red. Below the reported nucleotide sequence is a representative histogram from each position to illustrate the sequence and mixed peaks. One nucleotide to the left and right of the nucleotide of interest is shown. (B) Sabin virus replication kinetics in muscle. PVR mice were intramuscularly inoculated with $2x10^7$ PFU of light-sensitive Sabin virus, and tissues were harvested at 6 and 30 hpi. Viral replication status was determined using light sensitive virus since any virus that undergoes replication releases the dye conferring light sensitivity. Percent of viral replication for each sample was determined by dividing titer obtained from the light inactivated half of the sample (replicated virus only), by titer from the half of the sample kept in the dark (overall titer). Data are from 10 tissues per time point and mean and SEM are shown.

DISCUSSION

Understanding the dynamics of diverse and highly evolvable RNA virus populations within animal hosts is essential to understanding pathogenesis of RNA viruses. These highly error-prone viruses are thought to have evolved the perfect mutation rate where too few mutations limits the ability of the virus to adapt and cope with selective pressure, while too many mutations can lead to error catastrophe and defective genomes. The resultant quasispecies creates a dynamic population capable of altered host and cell tropism, immune evasion, drug escape, and recombination (125).

It has previously been shown that the dynamics of an RNA virus quasispecies can be impacted by the host as well as other viruses in the population (222). Two well-characterized mechanisms by which the viral population can be influenced by the composition and diversity of the viral quasispecies include complementation and interference, both of which require viral replication (151, 219, 223). In this work, we focused on the interplay between attenuated and virulent viruses in mixed populations, and we describe a novel replication-independent mechanism that influences viral pathogenesis.

Despite the fact that virulent viruses are present in the live-attenuated Sabin poliovirus vaccine and attenuating mutations frequently revert during replication in vaccinated individuals, vaccine-associated paralytic poliomyelitis is very rare (148). This suggests that host or viral factors limit the spread of virulent viruses, thereby limiting disease (222). Through this process, a virulence threshold is established. In this study, we demonstrated that virulence was masked in mixed viral populations when the attenuated strain was at least 1000-fold in excess of the virulent strain (Figure 3-1). We found that the protection induced by excess attenuated viruses was conferred locally, requiring the virulent and attenuated viruses to be present at the same site within the host (Figure 3-2). Since our model is looking at barriers that might play a role after viremia, and which could prevent disease in the CNS, it would be interesting to determine if competition for receptors also occurs in the gut or lymphatic tissue after vaccination, and whether the ratio required for protection would be lower or higher.

We previously found that inefficient retrograde axonal transport limited poliovirus pathogenesis in a mouse model of viral infection in the nervous system (117), and since this was a local host barrier, we examined whether this contributed to protection conferred by the attenuated viruses in the mixture. However, increasing viral transport efficiency had no effect on the protection

conferred by attenuated viruses (Figure 3-3), suggesting an alternative mechanism of protection.

To further delineate the mechanism conferring protection, we determined whether attenuated viruses could protect against disease induced by virulent viruses through replication-dependent or replication-independent mechanisms. Using replication incompetent viruses we demonstrated that protection was induced independently of viral replication (Figure 3-4). Because inactivated viruses were sufficient for protection and protection was conferred locally, we hypothesized that excess inactivated or attenuated viruses may compete with virulent viruses for an extracellular factor, or replication-independent resource, such as viral receptor.

We began exploring this hypothesis by first determining whether protection was a virus-specific effect. If the mechanism modulating the virulence threshold involved competition for a specific cellular resource, such as viral receptor, then this would require co-inoculation with a virus that uses the same receptor. Therefore, we evaluated whether reovirus, which does not use CD155/PVR for entry, confers protection against Mahoney poliovirus (Figure 3-5). We found that the protective effect was absent in the presence of reovirus, suggesting that protection against poliovirus virulence is virus-specific.

Receptors control viral entry, influence cell and host tropism and are a requirement for virulence. Therefore, we determined whether competition for the poliovirus receptor plays a role in establishing virulence thresholds. We demonstrated that excess attenuated virus decreased yield of virulent virus in a cell/resource dependent manner (Figure 3-6). Furthermore we found that inactivated virus reduced virulent virus yield *in vitro*, and the yield reduction was dependent on PVR expression. Viral yield *in vitro* was similarly reduced in the presence of a PVR-blocking antibody (Figure 3-7). Furthermore, reducing receptor availability *in vivo* by infecting mice in the presence of PVR-blocking antibody reduced pathogenesis. This finding may explain why increasing viral transport in neurons had no effect on the virulence threshold (Figure 3-3), despite our previous findings that increasing retrograde axonal transport increases pathogenesis (117). In this instance the protective effect conferred by the presence of the attenuated strain was induced prior to viral entry into neurons; therefore, increasing the transport of virus in neurons had little to no effect on the protection. Because viral yield inhibition by inactivated virus was dependent on PVR expression, and blocking PVR by antibody treatment phenocopied the inactivated virus inhibition *in vitro* and *in vivo*, competition for viral receptor may play a role in establishing virulence thresholds. This finding is novel because

previously described mechanisms that modulate virulence within a viral population require viral replication (151).

While the protective effects of excess attenuated viruses were replication-independent, we wanted to determine whether other mechanisms could also contribute to virulence thresholds. The type I interferon response has been shown to play a role in limiting viral pathogenesis; therefore, we also examined whether the interferon response could limit virulence in our system. Using PVR-IFNAR$^{-/-}$ mice, we found that a deficient type I interferon response provided an environment where attenuated viruses could replicate efficiently, revert attenuating mutations, and induce disease (Figures 3-8 and 3-9). We demonstrated this effect by showing that the virus found in brain was the Sabin strain (small plaque phenotype shown in Figure 3-8B) that had undergone reversion of the major attenuating mutation, conferring neurovirulence (Figure 3-9B). Enhanced viral replication in the absence of a type I interferon response likely contributed to this effect since viral titers were higher in all tissues in PVR-IFNAR$^{-/-}$ mice compared to PVR mice (Figure 3-8C), and in *in vitro* infections with equal amounts of virus, the Sabin strain replicated less efficiently in the presence of interferon (Figure 3-8D).

These results mimic enhanced viral replication and reversion of attenuating mutations in immunocompromised individuals administered the attenuated poliovirus vaccine (22, 204). Perhaps the slower replication time renders the Sabin strain more susceptible to interferon as there is more time for gene induction after initial infection before the replication cycle is complete. In PVR mice with intact immune systems, Sabin poliovirus has reduced replication in the nervous system compared to the Mahoney strain (26). We demonstrated that the same is true in muscle (Figure 3-9B), suggesting that in the presence of a robust interferon response, replication of the Sabin strain is limited. This could explain why, despite inoculating $1x10^8$ PFU of Sabin poliovirus, PVR mice fail to succumb to disease. Therefore, the type I interferon response plays an important role in limiting virulence by reducing viral replication and subsequent reversion.

In this work, we demonstrated that the attenuated Sabin strain of poliovirus was able to protect against the neurovirulent Mahoney strain of poliovirus despite the enhanced replication capacity, temperature stability, and increased neurovirulence of the Mahoney strain (143, 179, 200, 224, 225). It has previously been shown that a diverse quasispecies can protect against viruses with a virulent or adapted phenotype (226-228). Our results suggest that attenuated viruses significantly reduce virulent virus-induced disease, as long as the attenuated viruses are present in at least 1000-fold excess. Teng et al.

demonstrated that a 10-fold excess of avirulent lymphocytic choriomeningitis virus was sufficient to limit disease induced by virulent virus, suggesting that virulence thresholds may vary depending on virus type (216). It is important to keep in mind that the particle:PFU ratio of poliovirus is greater than 100:1, meaning that only one of every 100 particles is infectious (91). The presence of these defective particles may have evolved in order to modulate virulence through multiple mechanisms, as previously characterized, DI particle interference limits virulence and as we have shown the presence of excess attenuated viruses modulate virulence within mixed viral populations.

The mixed viral population model used in this study, comprised of attenuated and virulent viral strains, could be used for future studies to elucidate additional mechanisms whereby virulence thresholds can be established. Overall, these results contribute to our understanding of viral dynamics and may explain the relative safety of the live attenuated oral Sabin poliovirus vaccine despite the presence of virulent revertant viruses in the host.

CHAPTER FOUR

HOST BARRIERS TO VIRAL INFECTION

INTRODUCTION

Many viruses are neurotropic, including West Nile virus, rabies virus, alpha herpesviruses, and poliovirus. To gain access and sustain infection in neurons, viruses must be able to efficiently traffic in axons, which can be up to one meter long. Therefore, viral trafficking in neurons requires an active transport system (229, 230). Poliovirus is thought to enter neurons via receptor-mediated endocytosis at the neuromuscular junction, followed by endocytic transport from the nerve terminal to the cell body using the host retrograde axonal transport system. Poliovirus and some herpesviruses are thought to hijack the host transport machinery via Tctex-1, a component of the dynein light chain involved in retrograde axonal transport (116, 118).

Poliovirus is an enteric virus that rarely causes disease; however, in the pre-vaccine era, ~1% of infected individuals developed paralytic poliomyelitis due to viral invasion of the central nervous system (CNS) and destruction of

motor neurons. It is still unclear whether poliovirus accesses the CNS via blood or neural routes, but it has been shown that viremia is a prerequisite for CNS invasion of humans and non-human primates (90, 108). In the 1990s, mice expressing the human poliovirus receptor (CD155/PVR) facilitated studies on poliovirus trafficking, although early models were limited in scope due to resistance of the mice to oral infection (25, 26). Ohka et al. recently developed PVR mice lacking the interferon α/β receptor ($IFNAR^{-/-}$), an important component of innate immunity, yielding PVR-$IFNAR^{-/-}$ mice that are orally susceptible to poliovirus, and can be used to study viral dissemination following the natural route of infection (157). While there is evidence for both blood and neural routes of poliovirus dissemination (92), recent *in vitro* studies with cultured neurons, and *in vivo* studies with PVR mice provide evidence for neural trafficking to the CNS (26, 98, 110, 113, 119). It is thought that viremic blood seeds peripheral tissues, virus enters neurons of the peripheral nervous system (PNS) that innervate peripheral tissues, and virus traffics to the CNS using retrograde axonal transport.

Sciatic nerve models of poliovirus trafficking further support CNS access via a neural route following peripheral infection, because sciatic nerve transsection prevented disease in PVR mice intramuscularly injected with poliovirus (98, 113). Similarly, sciatic nerve transsection prevented retrograde

axonal transport of Theiler's virus, a picornavirus related to poliovirus (231). Therefore, intramuscularly inoculated poliovirus traffics to the CNS in neurons via the sciatic nerve. The sciatic nerve contains a bundle of axons, each of which are single long cells that innervate the leg muscle and relay information from the periphery to their cell bodies in the spinal cord. Therefore, viral trafficking by this route requires viral uptake at the neuromuscular junction, active transport within the long axons of the sciatic nerve, viral release in the cell body within the spinal cord, and transport to the brain.

Here we use an artificial quasispecies to identify host barriers limiting viral trafficking from the periphery to the CNS. Previously, we uncovered a significant obstacle to viral trafficking between muscle and brain that severely bottlenecked the viral population (1, 2), and here we identify and characterize multiple barriers that contribute to this effect. By following viral population diversity, we discovered three distinct barriers the virus encounters between the periphery and the CNS: inefficient retrograde axonal transport in peripheral neurons, the type I interferon response, and limited viral replication in neurons of the PNS. To our knowledge, this is the first time that efficiency of viral retrograde axonal transport has been quantified, and identified as a major barrier limiting viral access to the CNS.

RESULTS

Poliovirus Trafficking from Peripheral Tissues to the CNS is Limited by a Barrier Between the Peripheral and Central Nervous Systems

Previously, using 10 marked viruses, we identified host barriers that limit poliovirus trafficking from the gut to the CNS (1). The marked viruses contain groups of 4-8 silent point mutations detectable by a hybridization-based assay, and constitute an artificial quasispecies that can be used to monitor viral population dynamics and identify host barriers that limit spread (Figure 4-1). Using this assay and another artificial quasispecies assay, a barrier was uncovered between a peripheral intramuscular injection site and the brain (1, 2); however, the specific nature of this barrier was unknown. The goal of this study was to legitimize viral trafficking in PNS neurons as a potential route to the CNS following oral inoculation, and to identify the specific host barriers limiting viral trafficking from peripheral tissues to the CNS.

To determine whether poliovirus is present in peripheral neurons following oral inoculation, orally susceptible PVR-IFNAR$^{-/-}$ mice were orally inoculated with 2 X 10^7 plaque forming units (PFU) of the 10-virus mixture,

tissues were harvested upon disease onset, and viruses were detected by RT-PCR and the viral diversity assay. We monitored poliovirus in two peripheral nerves: the vagus nerve, which innervates multiple organs and is part of the enteric nervous system, and the sciatic nerve, which innervates leg muscle. Importantly, orally inoculated virus was detected in the vagus nerve in 76% of mice and in the sciatic nerve in 71% of mice (Figures 4-2A and Figure 4-1).

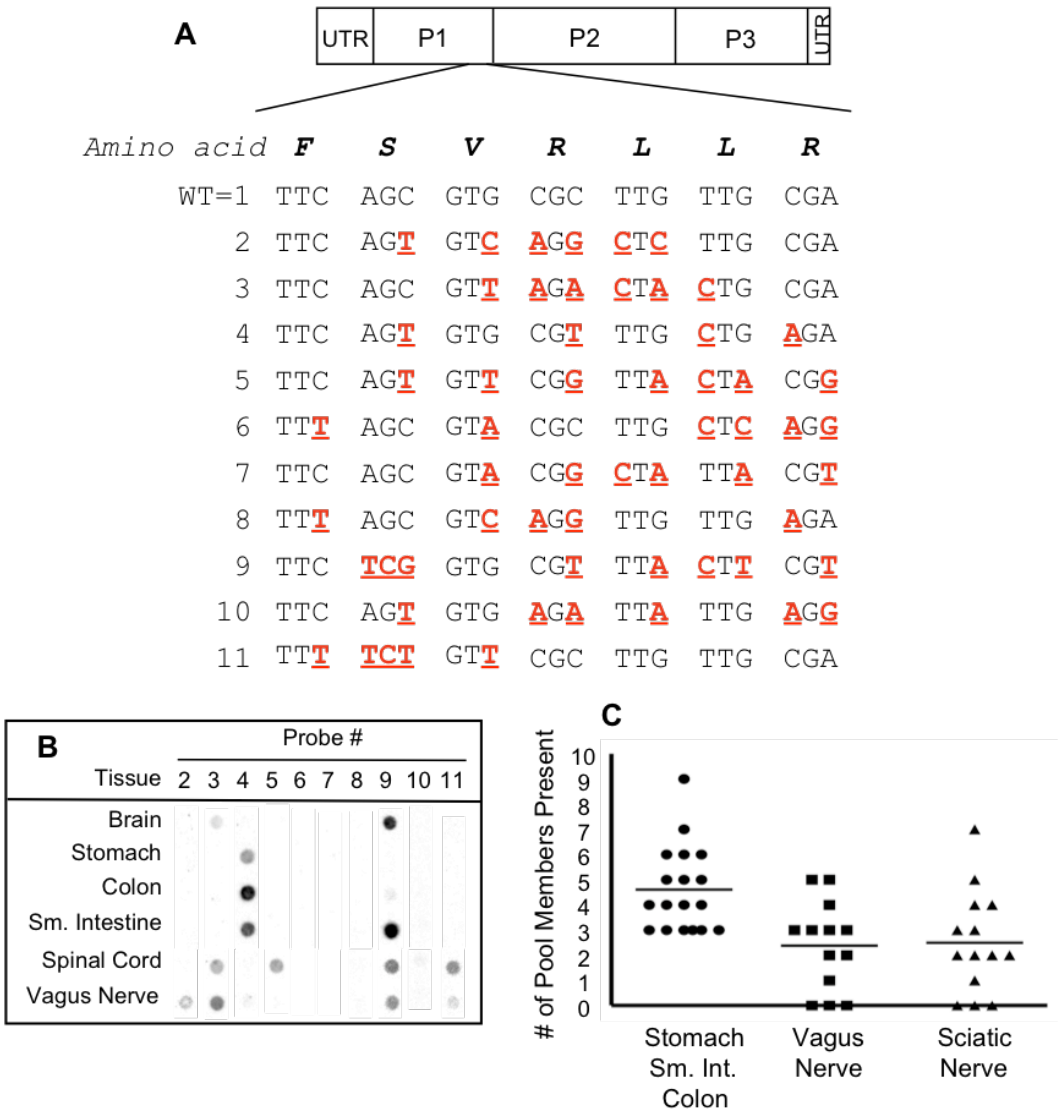

Figure 4-1: Artificial quasispecies system and poliovirus population dynamics following oral inoculation of PVR-IFNAR$^{-/-}$ mice. (A) Sequences of marked viruses, showing silent mutations (red, bold and underlined) in the poliovirus genome. (B) A representative blot from an orally inoculated PVR-IFNAR$^{-/-}$ mouse, and (C) results from all orally inoculated PVR-IFNAR$^{-/-}$ mice. Mice were orally inoculated with 2 x 10^7 PFU of the 10-marked viruses, and tissues were harvested upon disease onset. Horizontal lines represent mean diversity for each tissue.

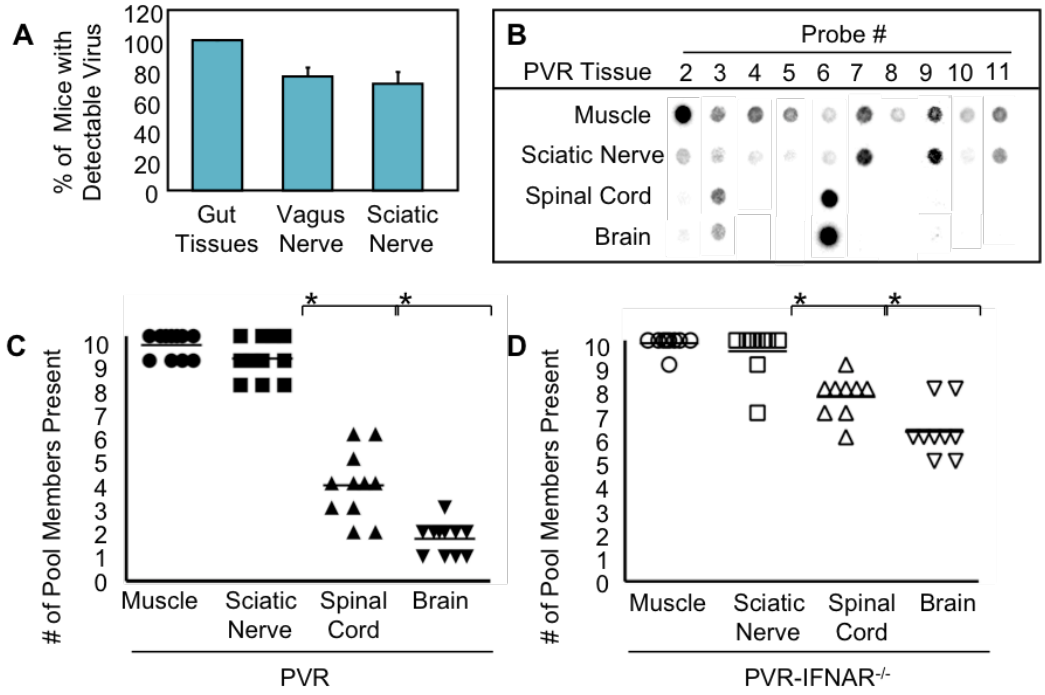

Figure 4-2. Identification of the barrier site between the periphery and CNS and the effect of type I interferon. (A) Detection of virus in peripheral nerves after oral infection. PVR-IFNAR$^{-/-}$ mice were orally inoculated with 2 x 10^7 PFU of poliovirus, tissues were harvested upon disease onset, and the percentage of mice with detectable virus was quantified by RT-PCR. Mean with SEM from 13-14 mice is shown; more details are shown in Figure 4-1. (B) A representative blot from the hybridization-based viral diversity assay. PVR mice were intramuscularly inoculated with 2 x 10^7 PFU of the 10-marked viruses, tissue were collected upon disease onset (ranging from 2 to 6 days post-inoculation, see Figure 6), and viral pool members were detected with the viral diversity assay. Results from one representative mouse are shown. Viral population diversity in intramuscularly inoculated (C) PVR mice or (D) PVR-IFNAR$^{-/-}$ mice. Mice were intramuscularly inoculated as in (B), and the number of pool members in each tissue for each mouse is shown. Horizontal lines represent mean diversity for each tissue. Asterisks denote statistically significant reductions in viral population diversity (Student's *t* test, $p<0.01$). Viral population diversity in PVR-IFNAR$^{-/-}$ mice was significantly higher than in PVR mice across all tissues ($p<0.0001$, 2 way ANOVA).

Because poliovirus was detectable in sciatic nerve following oral inoculation, we used the sciatic nerve as a relevant model peripheral neuron to identify barriers contributing to the bottleneck effect encountered by the virus between peripheral organs and the CNS. PVR mice were intramuscularly inoculated with 2 X 10^7 PFU of the 10-virus mixture, tissues were harvested upon disease onset, and viral population diversity was measured with the viral diversity assay (Figure 4-2B). Of the 10 original input viruses injected, we found an average of 9.5 viral pool members present in muscle, 9.0 in sciatic nerve, 4.2 in spinal cord, and 1.8 in brain (Figure 4-2C). A dramatic decrease in the number of viral population members occurred between sciatic nerve and spinal cord, suggesting that the viral population encountered a major barrier between these sites. Importantly, viral titers from tissues do not reflect the dramatic bottleneck encountered by the viral population, because viral titers in spinal cord were 10,000-fold higher than viral titers in sciatic nerve (Figure 4-3). Therefore, the viral population was limited by a host barrier between the PNS and CNS, but robust replication occurred post-barrier in the CNS. These results uncovered barriers to viral trafficking that would have been masked by analyzing titer alone, and suggest that a significant barrier to viral CNS access occurs between the sciatic nerve and spinal cord.

We devised three hypotheses to explain the barrier between the PNS and CNS. First, the interferon response may limit peripheral replication, reducing the amount of virus in the periphery. Second, viral replication in peripheral neurons may be minimal, limiting the number of viruses entering the CNS. Third, retrograde axonal transport may be inefficient for poliovirus. We tested each of these hypotheses to dissect the mechanism of the PNS-to-CNS barrier.

Host Innate Immunity Contributes to the Barrier

To determine whether the type I interferon response contributes to the sciatic-spinal cord barrier, we intramuscularly injected 2 X 10^7 PFU of the 10-marked viruses into PVR-IFNAR$^{-/-}$ mice, which lack the INFα/β receptor and are therefore deficient in generating a type I interferon response. Upon disease onset, tissues were harvested and viral population diversity was determined by the viral diversity assay. As shown in Figure 4-2D, 2.5-fold more viral pool members reached the brain in PVR-IFNAR$^{-/-}$ mice than in PVR mice. Not surprisingly, viral titers were 4-17-fold higher in PVR-IFNAR$^{-/-}$ mice than in PVR mice (Figure 4-3). Interestingly, the largest viral titer difference between PVR and PVR-IFNAR$^{-/-}$ mice was in the periphery, suggesting that the interferon response limited viral trafficking by reducing replication in peripheral tissue. In fact

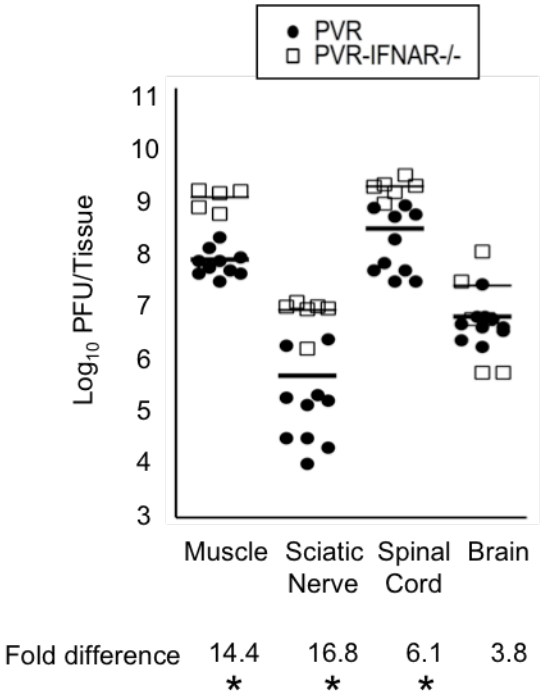

Figure 4-3. Viral titers from intramuscularly inoculated PVR and PVR-IFNAR$^{-/-}$ mice. Mice were inoculated with 2 x 10^7 PFU, tissues were harvested upon disease onset, and viral titers were determined by plaque assay. Titers from PVR mice are depicted as closed circles, and titers from PVR-IFNAR$^{-/-}$ mice are depicted as open squares, with mean indicated by horizontal lines. The magnitude of the titer difference between PVR and PVR-IFNAR$^{-/-}$ mice is indicated below each tissue, and asterisks denote statistically significant differences ($p<0.0001$, Student's *t* test).

In fact, the difference between PVR and PVR-IFNAR$^{-/-}$ viral titers in the brain was minimal (less than 4-fold) and not statistically significant. One interpretation of these results is that the type I interferon response exerts its effects in the periphery and may contribute to the viral bottleneck by limiting viral replication in peripheral tissues.

Poliovirus Replication is Limited in Peripheral Neurons

Lack of replication in peripheral neurons could limit viral diversity and contribute to inefficient trafficking to the CNS. To quantify viral replication *in vivo*, we used light-sensitive polioviruses (1). Poliovirus propagated in the presence of neutral red dye becomes light sensitive due to dye incorporation into the virion (71, 180, 182). Exposure to light inactivates neutral red-containing virions, likely due to cross-linking of virion RNA; however, neutral red viruses maintain viability if not exposed to light. Upon replication, neutral red dye is diluted and viruses lose light sensitivity. Therefore, viral replication can be quantified by measuring the ratio of light-sensitive to light-insensitive virus. We have adapted this assay for *in vivo* studies by injecting mice with a pool of 10-marked neutral red viruses in the dark and comparing light-exposed versus non light-exposed tissue virus samples by viral titer analysis or the viral diversity assay (Figure 4-5).

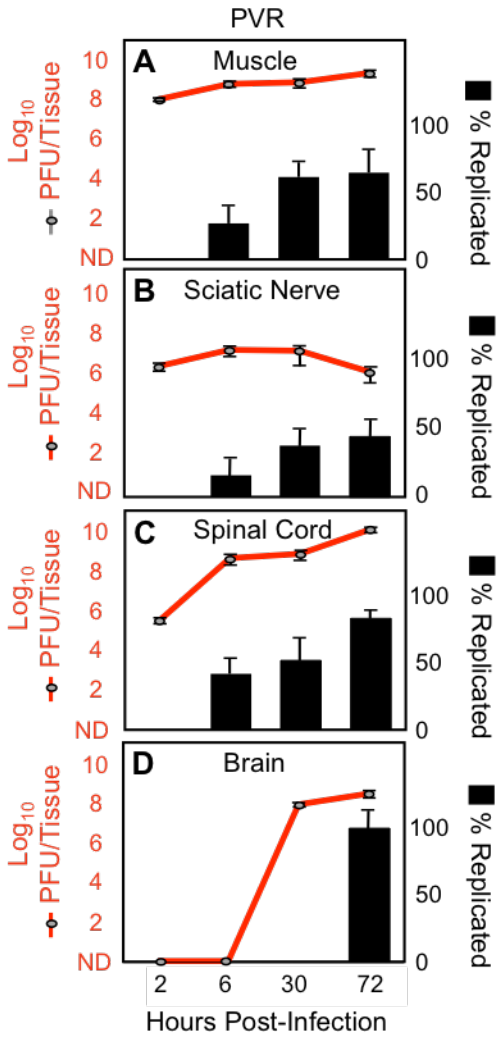

Figure 4-4. Viral replication kinetics. Mice were intramuscularly inoculated with 2 x 10^7 PFU of light-sensitive/neutral red poliovirus in the dark, tissues were harvested at 2, 6, 30 and 72 hpi in the dark, and virus was extracted in the dark. Half of the virus sample for each tissue was exposed to light (inactivating non-replicated viruses, to quantify replicated viruses), and half was maintained in the dark (to quantify all viruses). Virus in light-exposed and non-exposed samples was quantified by plaque assay. Total titer represents titer from non-light-exposed sample and is expressed as PFU/tissue (red lines). Therefore, the grey lines represent titer from inoculum virus plus replicated virus. The percent of replicated viruses was obtained by dividing the light-exposed titer by the non-light-exposed titer for each individual sample (black bars). Mean with SEM from 3-7 mice is shown.

First, we performed neutral red viral titer analysis to measure the kinetics of viral replication in various tissues along the route to the CNS. PVR mice were intramuscularly injected with 2 x 10^7 PFU of the neutral red 10-virus pool, and muscle, sciatic nerve, spinal cord, and brain were harvested in the dark (using a red safety light) at 2, 6, 30 or 72 hours post infection (hpi). Tissues were processed in the dark, and samples of light-exposed virus and non-light exposed virus from each tissue were titered. Figure 4-4 shows the total titer (i.e., dark titer), and the percent of virus that was replicated (i.e., light titer/dark titer x 100) at each time point for different tissues, and the data indicate three key points. First, there was no evidence of viral replication in any tissue at 2 hpi, but there was evidence of viral replication in muscle, sciatic nerve and spinal cord at 6 hpi; therefore, viral replication is relatively fast *in vivo*.

Second, virus was detectable in the spinal cord by 6 hpi, indicating that virus moves very quickly from the muscle injection site to the CNS, in agreement with previous work demonstrating viral movement by fast retrograde axonal transport (113). Third, while titers in spinal cord increase over time by an average of 10,000-fold, titers in sciatic nerve remain relatively constant. Similarly, viral titers in muscle remain relatively constant despite viral replication at that site. Taken together these results suggest a model where virus in muscle is transported rapidly to the spinal cord via the sciatic nerve, but little or no replication occurs in

the PNS (sciatic nerve); however, robust replication occurs in the CNS (spinal cord and brain).

To further test the idea that poliovirus does not replicate in the sciatic nerve, we examined the replication status of individual viral population members using neutral red-virus in conjunction with the viral population diversity assay. PVR mice were intramuscularly injected with 2×10^7 PFU of the 10-marked neutral red virus pool and tissues were harvested in the dark at 72 hpi, near the time of disease onset. After processing tissues in the dark, we divided the virus sample and exposed half to light and kept half in the dark. Light exposed and non-light exposed virus samples were amplified for a single cycle in HeLa cells to expand surviving viruses and minimize the prevalence of inactivated viruses. Replication status of tagged pool members was analyzed with the viral diversity assay by comparing the signal of every viral pool member on the "light" *vs.* "dark" blots (see Figure 4-5A). Light-sensitive viruses were scored as 'non-replicated', and light-insensitive viruses were scored as 'replicated + non-replicated' because they may contain a sub-population of non-replicated viruses, which would be masked by the signal from replicated viruses. 'Non-replicated' viruses consist exclusively of viruses that did not replicate while in the mouse, due to the absence of signal from the light-exposed sample.

The results from 10 PVR mice are summarized in Figure 4-5B, which shows the overall number of viral pool members present in each tissue and the proportion of those pool members that were non-replicated viruses. In muscle, 30% of virus was non-replicated, in sciatic nerve, 64% of virus was non-replicated, in spinal cord, 13% of virus was non-replicated, and in brain, 0% of virus was non-replicated. These results reinforce the idea that robust replication occurs in CNS tissues, since 100% of brain viruses showed evidence of viral replication. Interestingly, even at 72 hpi, 30% of virus in muscle was non-replicated, indicating that this virus was stable and not cleared over a three day period, but remained viable since productive replication occurred in HeLa cells after tissue harvest. In sciatic nerve, the majority of viruses (64%) were non-replicated. Importantly, every single potentially replicated virus in sciatic nerve was also replicated in muscle (28/28), implying that "replicated" virus in sciatic nerve had undergone replication in muscle prior to sciatic nerve entry. Because the majority of viruses in sciatic nerve were non-replicated, and the minority of light-insensitive/replicated viruses had undergone replication in muscle, it is likely that poliovirus does not replicate in axons of the sciatic nerve.

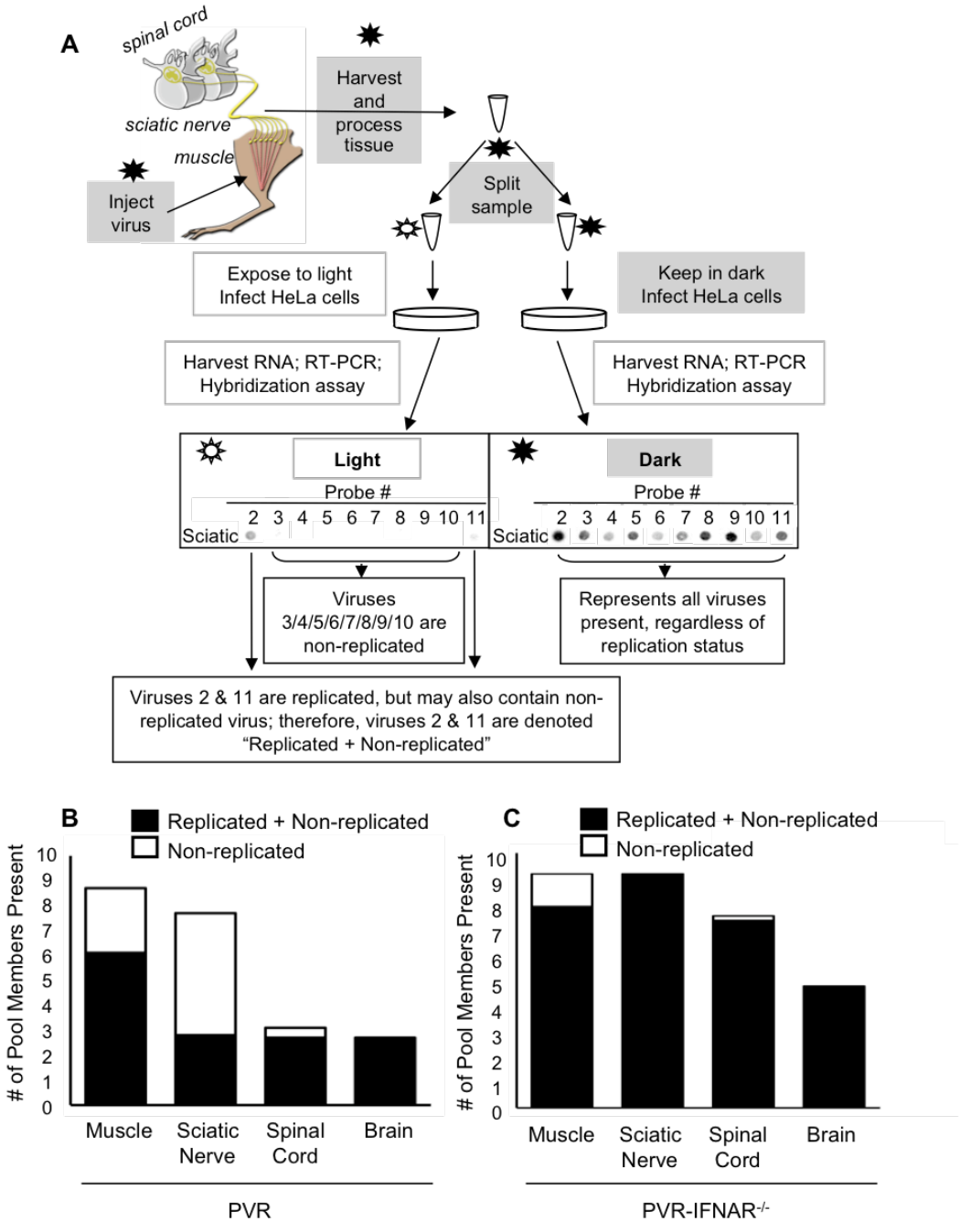
A
spinal cord
sciatic nerve
muscle
Inject virus
Harvest and process tissue
Split sample
Expose to light Infect HeLa cells
Keep in dark Infect HeLa cells
Harvest RNA; RT-PCR; Hybridization assay
Harvest RNA; RT-PCR Hybridization assay
Light
Dark
Probe #
2 3 4 5 6 7 8 9 10 11
Sciatic
Viruses 3/4/5/6/7/8/9/10 are non-replicated
Represents all viruses present, regardless of replication status
Viruses 2 & 11 are replicated, but may also contain non-replicated virus; therefore, viruses 2 & 11 are denoted "Replicated + Non-replicated"
B
Replicated + Non-replicated
Non-replicated
# of Pool Members Present
0 1 2 3 4 5 6 7 8 9 10
Muscle
Sciatic Nerve
Spinal Cord
Brain
PVR
C
Replicated + Non-replicated
Non-replicated
# of Pool Members Present
0 1 2 3 4 5 6 7 8 9 10
Muscle
Sciatic Nerve
Spinal Cord
Brain
PVR-IFNAR-/-

Figure 4-5. Replication status for individual viral population members. (A) Schematic of light-sensitive/neutral red poliovirus hybridization-based diversity assay for determining viral replication status. In the dark (using a red safety light; black star/grey boxes), the 10 marked viruses containing neutral red dye were intramuscularly injected into the left gastrocnemius muscle of PVR or PVR-IFNAR-/- mice. At 72 hpi, tissues were harvested and processed in the dark. The viral sample for each individual tissue was split, and half of the sample was exposed to light to inactivate non-replicated virus (unfilled star/unfilled boxes), and the other half of the sample was kept in the dark. Both virus samples were amplified by a single cycle of replication in HeLa cells to expand surviving virus and decrease the prevalence of light-inactivated virus. Total RNA was extracted, and the tagged region of the viral genome was amplified by RT-PCR. DNA was then spotted on a membrane, and the viral diversity assay was performed. Signal for each virus from the light-exposed and non-light exposed samples was compared for each tissue; sciatic nerve from a representative mouse is shown as an example. Light-sensitive viruses were scored as 'Non-replicated' (in this case, viruses 3/4/5/6/7/8/9/10), and light-insensitive viruses were scored as 'Replicated + Non-replicated' (in this case, viruses 2 and 11) because they may contain a sub-population of non-replicated viruses, which would be masked by the signal from replicated viruses. Pooled results from each tissue for 10 PVR mice (B) and 5 PVR-IFNAR-/- mice (C) are shown. Bars represent the mean diversity in each tissue, and the proportion of non-replicated virus is indicated (unfilled bar).

To test whether the interferon response limits viral replication, we repeated the experiment using PVR-IFNAR$^{-/-}$ mice. Not surprisingly, we observed high percentages of replicated virus in all tissues (Figure 4-5C). These data, in conjunction with titer data from PVR-IFNAR$^{-/-}$ mice (Figure 4-3), imply that in the absence of the interferon response, viral replication in muscle was so robust that nearly all viruses replicated prior to entering the PNS. Taken together, these results suggest that poliovirus does not replicate in axons of peripheral neurons, rather, virus moves quickly from the peripheral injection site to the CNS, and once in the CNS, undergoes robust replication.

Retrograde Axonal Transport of Poliovirus is Inefficient in Peripheral Neurons

Our data support previous work demonstrating that retrograde axonal transport of poliovirus is fast (112, 113, 116); however, the efficiency of viral axonal transport has never been quantified. To determine whether inefficient retrograde axonal transport contributes to the barrier observed between sciatic nerve and spinal cord, we monitored viral population diversity during viral ascension of the sciatic nerve by harvesting segments of the nerve. PVR mice were intramuscularly injected with 2 x 10^7 PFU of the 10-marked virus pool and

viral diversity was quantified in the peripheral injection site (muscle), in three sections of the sciatic nerve (lower, middle, upper), in spinal cord, and in brain. As shown in Figure 4-6A (black bars), the lower section of the sciatic nerve contained an average of 8.3 pool members, middle sciatic contained 4.9 pool members, and upper sciatic contained 2.3 pool members. Therefore, the entire barrier between the sciatic nerve and spinal cord was due to loss of viral population members between the lower sciatic nerve and upper sciatic nerve. Poliovirus entry into the sciatic nerve at the neuromuscular junction was efficient, since 87% of pool members present in muscle were present in lower sciatic nerve; however, retrograde axonal transport was inefficient, since only 28% of pool members were successfully transported from lower sciatic nerve to upper sciatic nerve.

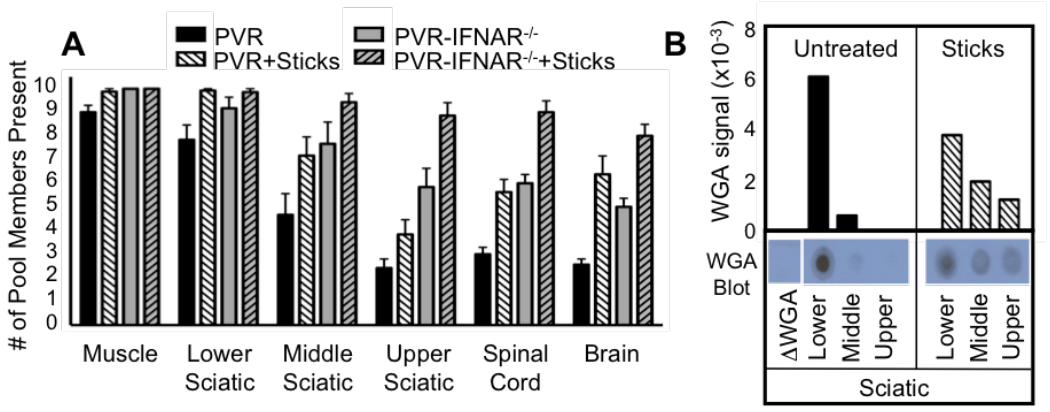

Figure 4-6. Quantification of retrograde axonal transport efficiency. (A) Efficiency of poliovirus transport and the effects of type I interferon and muscle damage. PVR and PVR-IFNAR$^{-/-}$ mice were intramuscularly inoculated with 2 x 10^7 PFU of the ten marked viruses, and were treated with or without needle sticks twice per day to induce muscle damage. Tissues were harvested at disease onset, and viral diversity was quantified. PVR mice, solid black bars; PVR mice with needle sticks, white hatched bars; PVR-IFNAR$^{-/-}$ mice, grey bars; PVR-IFNAR$^{-/-}$ mice with needle sticks, grey hatched bars. Results are expressed as mean with SEM, representing 4-13 mice per group. Statistically significant differences between groups are described in the text. (B) Efficiency of wheat germ agglutinin (WGA) transport and the effects of muscle damage. PVR mice were intramuscularly injected with 5 μg of WGA and treated with or without needle sticks. Tissues were harvested at 6 hours post-injection, and were processed for "dot-blot" protein immunoblot analysis. A WGA immunoblot, representative of four experiments, is shown under densitometry quantification (arbitrary units). Signal was normalized to sciatic nerve tissue from PVR mice not injected with WGA (denoted DWGA). Total WGA signal in the sciatic nerve represented approximately 1% of the WGA signal in muscle (data not shown).

Muscle Damage Increases Retrograde Axonal Transport and Poliovirus Trafficking to the CNS

While limited population diversity in the upper sciatic nerve suggests inefficient transport as a potential barrier to poliovirus trafficking, to formally demonstrate that transport inefficiency is the barrier, we determined whether increasing the efficiency of retrograde axonal transport would increase poliovirus population diversity in the CNS. Muscle damage via needle sticks is thought to enhance access to the CNS because Gromeier and Wimmer demonstrated enhanced poliovirus disease in mice subjected to needle sticks following intravenous inoculation (112). To test whether needle sticks increase the efficiency of poliovirus retrograde axonal transport, PVR mice were intramuscularly injected with the 10-marked virus pool, and mice received needle sticks twice per day to induce muscle damage. Upon disease onset, tissues were harvested and the viral population diversity assay was performed (Figure 4-6A). In mice that received needle sticks, the brain contained an average of 6.4 pool members, 3-fold more virus than untreated mice, suggesting that muscle damage increased poliovirus transport to the CNS ($p<0.01$, Students *t* test).

To verify that the muscle damage-mediated enhancement of poliovirus trafficking was due to increased efficiency of retrograde axonal transport, we

monitored trafficking of a non-viral protein, wheat germ agglutinin (WGA), which is commonly used as a neural tracer (232, 233). PVR mice were intramuscularly injected with 5μg WGA, and treated with or without needle sticks. Tissues were harvested at 6 hours post injection, and WGA was quantified by immunoblotting (Figure 4-6B). In support of the idea that muscle damage increased retrograde axonal transport, WGA signal in middle and upper sciatic nerve was >3-fold higher in mice given needle sticks compared with untreated mice. Interestingly, the combined total of WGA signal for all sciatic nerve segments was nearly identical in both treatment groups, suggesting that WGA uptake at the neuromuscular junction was comparable; however, WGA was transported more efficiently in mice with muscle damage since more WGA was present in the middle and upper sections of the sciatic nerve. Taken together, these data suggested that retrograde axonal transport of poliovirus is inefficient and constitutes a major barrier to viral access to the CNS, but that efficiency of transport to the CNS can be enhanced by muscle damage.

Overcoming Host Barriers Facilitates Efficient Viral Trafficking to the CNS and Accelerates Disease Onset

Having identified inefficient retrograde axonal transport and the interferon response as major barriers to viral trafficking, we sought to determine whether eliminating both barriers would facilitate efficient poliovirus trafficking to the CNS. PVR or PVR-IFNAR$^{-/-}$ mice were intramuscularly injected with the 10-marked virus pool in the presence or absence of needle sticks, and population diversity was monitored. As expected, tissues from PVR-IFNAR$^{-/-}$ mice contained significantly more population members in brain (2.5-fold) than PVR mice ($p<0.01$, Students *t* test)(Figure 4-6A). These numbers were comparable to the increased diversity in brain (3-fold) observed in PVR mice given needle sticks ($p<0.001$, Students *t* test). However, in PVR-IFNAR$^{-/-}$ mice given needle sticks, sciatic nerve, spinal cord, and brain contained nearly all ten viruses (average of 9.4 in upper sciatic, 9.0 in spinal cord, and 8.0 pool members in brain), with significantly more viral pool members trafficking to the brain than in PVR-IFNAR$^{-/-}$ mice or PVR mice given needle sticks ($p<0.001$, Students *t* test). Our results suggest that the type I interferon response and inefficient retrograde axonal transport are separate barriers and that overcoming both barriers facilitated efficient viral trafficking to the CNS.

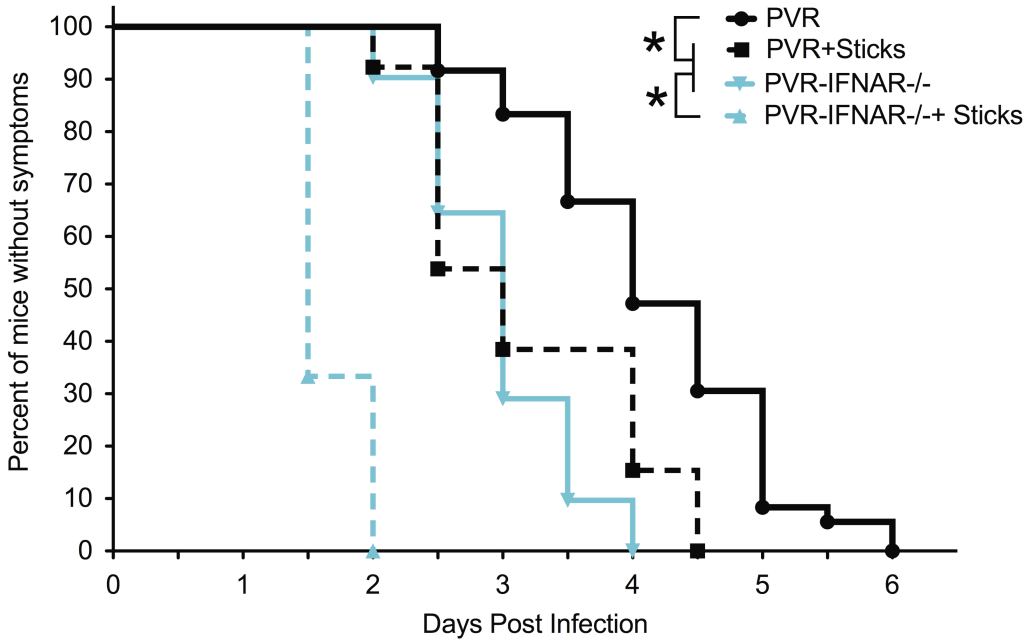

Figure 4-7. The effect of barriers on viral pathogenesis. PVR or PVR-IFNAR$^{-/-}$ mice were intramuscularly inoculated with 2 x 10^7 PFU of the 10-marked viruses, and were treated with or without needle sticks twice per day. Mice were euthanized upon disease onset; therefore, results are depicted as percent of mice without symptoms. Untreated PVR mice, solid black line with circles; PVR mice with needle sticks, dashed black line with squares; PVR-IFNAR$^{-/-}$ mice, turquoise line with inverted triangles; PVR-IFNAR$^{-/-}$ mice given needle sticks, dashed turquoise line with triangles. Data represent 12-36 mice per condition. Statistically significant differences between groups are indicated by asterisks (Mantel-Cox test, $p<0.0001$).

This result is further supported by the time of disease onset for each treatment group. As shown in Figure 4-7, using paralysis onset for 50% of mice per cohort as a measure of pathogenesis, untreated PVR mice developed disease on day 4.5 post infection, PVR mice given needle sticks and untreated PVR-IFNAR$^{-/-}$ mice developed disease on day 3 post infection, and PVR-IFNAR$^{-/-}$ mice given needle sticks developed disease on day 1.5 post infection. Therefore, overcoming one of the two barriers increased pathogenesis, as disease onset was 1.5-fold faster than mice with both barriers intact. Furthermore, eliminating two barriers dramatically enhanced pathogenesis, as disease onset was 3-fold faster than in untreated mice. Taken together, our results indicated that the type I interferon response and inefficient retrograde axonal transport are separate barriers of equivalent strength, and that these barriers reduce pathogenicity by limiting viral trafficking to the CNS.

DISCUSSION

Paralytic poliomyelitis due to viral CNS invasion and motor neuron destruction is very rare, occurring in less than 1% of unvaccinated individuals. A variety of hypotheses have been proposed to explain the rare poliovirus CNS

invasion, ranging from fatigue to recent injury (91, 112, 164). In this work, we identified three major barriers that may contribute to the rare incidence of paralytic poliomyelitis by limiting poliovirus trafficking from the periphery to the CNS: inefficient retrograde axonal transport, limited viral replication in the PNS, and the interferon response. Type I interferon has been shown to reduce replication of many viruses, and to limit the pathogenicity of neurotropic viruses (35, 37, 154, 157, 234, 235). Perhaps predictably, we demonstrated that the type I interferon response can limit poliovirus dissemination by limiting replication in peripheral tissues, such as muscle.

Surprisingly, we identified inefficient retrograde axonal transport as a major barrier limiting poliovirus trafficking in PNS neurons and viral access to the CNS. In peripheral neurons, retrograde axonal transport of poliovirus and other viruses is very fast (113, 115, 229), and transport can be increased by muscle injury (98, 112). While retrograde axonal transport may be fast, we demonstrate here that it is very inefficient for poliovirus, with only 28% of viral pool members successfully trafficking from lower to upper sciatic. By analogy, retrograde axonal transport of poliovirus can be thought of as a fast roller coaster without seatbelts, resulting in loss of passengers during the ride. The sciatic nerve consists of cells up to 5 cm long; therefore, active transport is required for viral trafficking. Although transport is inefficient, uptake of poliovirus at the

neuromuscular junction is efficient, because 87% of pool members present in muscle were present in lower sciatic nerve. The inverse has been observed for neurotrophins, a class of host proteins that are transported by retrograde axonal transport (236-239). For neurotrophins, retrograde axonal transport is thought to be efficient and processive; however, neurotrophin cellular entry at the neuromuscular junction is quite inefficient (233, 240, 241). Therefore, either viral transit mechanisms are not completely conserved with host transit mechanisms, or there are multiple host pathways that differ in retrograde axonal transport processivity (119). It is also possible that poliovirus overwhelms the transport system or is degraded during retrograde axonal transport, thus explaining inefficient transport despite efficient uptake at the neuromuscular junction.

While retrograde axonal transport of poliovirus in peripheral neurons was inefficient, the efficiency increased upon muscle damage. Gromeier and Wimmer suggested that muscle damage enhances poliovirus CNS access and contributes to some cases of paralytic poliomyelitis (98, 112), and nerve injury is known to increase retrograde axonal transport of neurotrophins (242). Provocation poliomyelitis occurs when physical trauma near the time of poliovirus infection coincides with increased incidence of paralytic poliomyelitis. This effect was observed during the Cutter incident, where batches of incompletely inactivated poliovirus vaccine caused paralysis preferentially in the inoculated limb (95, 243).

Additional cases of paralytic poliomyelitis occurred when the attenuated oral polio vaccine was administered near the time of multiple unrelated intramuscular injections (170). Our data demonstrating that muscle damage increases the efficiency of retrograde axonal transport may provide the mechanism for the increased incidence of paralytic poliomyelitis following muscle damage.

Several viruses traffic in PNS neurons to reach the CNS. For example, reovirus can traffic to the CNS via the vagus and sciatic nerves, and alpha herpesviruses traffic to the CNS in PNS neurons (244-248). Our results suggest that after oral infection, poliovirus may traffic through PNS neurons to the CNS because orally inoculated poliovirus was detected in peripheral neurons (vagus and sciatic). We also found that the viral pool members present in sciatic and vagus nerves were highly bottlenecked, and matched brain virus 63% of the time (Figure 4-1 and data not shown), suggesting that transport of virus from PNS neurons to the CNS may occur after natural oral infection.

In addition to inefficient retrograde axonal transport in neurons, we found that poliovirus replication was limited in peripheral neurons. Using light sensitive viruses, we found no evidence of viral replication in the sciatic nerve despite robust replication in the CNS. These results are supported by data from Ohka et al., showing intact 160S virions in sciatic nerve (113). Perhaps it is not surprising

that viral replication is limited in peripheral neurons, since substrates required for viral replication are likely to be limited in long axons. Nonetheless, if virions or virion-containing endosomes disassociate from the retrograde axonal transport machinery, viral replication may be impossible within the axon.

Taken together, our results support the neural route as a major pathway of poliovirus trafficking to the CNS; however, trafficking in neurons is difficult due to inefficient retrograde axonal transport. We propose that PNS barriers contribute to the low incidence of paralytic poliomyelitis in humans, and may contribute to inefficient trafficking of other neurotropic viruses.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

Novel Barriers Limiting Poliovirus Neuropathogenesis

Poliovirus is an enteric pathogen with the ability to invade and cause disease in the CNS; however, neurological complications are rare. We hypothesized that the rare incidence of paralytic poliomyelitis is due to viral and host barriers preventing disease. Owing to the previous lack of a tractable small animal model, factors limiting poliovirus pathogenesis were unknown. This work describes a novel replication-independent viral mechanism for reducing poliovirus pathogenesis, and characterizes two mechanisms by which type I interferon limits viral infection. Additionally, two previously uncharacterized host barriers were uncovered, limited viral replication in peripheral neurons and inefficient retrograde axonal transport. The role of type I interferon as an antiviral factor for other viruses has been well characterized. The finding that inefficient retrograde axonal transport is as robust at limiting infection as an IFN, and can work synergistically with IFN, illustrates the importance of this newly identified barrier

in limiting viral pathogenesis in the nervous system. Taken together, these viral and host factors contribute to our understanding of the stochastic nature and rare incidence of paralytic poliomyelitis.

Viral Population Dynamics and Modulation of Virulence

Modulation of virulence by the viral population impacts transmission and pathogenesis, and the idea of viral quasispecies can be used as a framework to characterize the dynamic nature of the viral population. It was hypothesized that RNA viruses need high error-rates to surmount the complex host environment (185, 249-251). The importance of a diverse population was demonstrated using a high fidelity poliovirus. The resultant quasispecies had lower error rates and as a result could not cope under selective pressure within the host (134, 139, 140). The converse is also true, in that high error rates leads to "error-catastrophe" and viral inactivation (136, 138, 139, 227, 252-256). Therefore, RNA viruses have evolved the "perfect" error rate, existing on the edge of error-catastrophe, but allowing for escape and propagation under selective pressure. This suggests the viral quasispecies is dynamic but highly modulated. It has been theorized that virulence can be modulated by the virions in the quasispecies by both negative and positive mechanism, interference and complementation (151, 186, 219, 223).

Experimental evidence of virulence modulation by interference has been well characterized by work with DI particles. Specifically, virulence phenotypes are altered by direct competition with DI particles for cellular resources, supporting the hypothesis that modulation of virulence can occur within the quasispecies (152, 214, 223, 257). Positive influence on viral population behavior occurs through complementation whereby weakened or defective particles can be rescued through sharing of viral proteins such as RNA-dependent RNA polymerase, of a more fit virus (150, 151, 223, 258-260). Both of these previously characterized mechanisms require viral replication; however, the novel viral barrier uncovered in this research is unique because the mechanism of virulence modulation is replication-independent.

I found that in mixed viral populations a virulence threshold is established that is controlled by different host and viral barriers. Significantly, I found that virulence can be masked if attenuated viruses are at least 1000-fold in excess, conferring protection against disease. This protection was a local, replication-independent, virus-specific effect and most likely occurred prior to entry into cells since increasing transport in neurons had no effect on pathogenesis. My data suggest that modulation of virulence in quasispecies can be replication-independent through competition between virions for limited resources, in this case viral receptor, essentially lowering the functional infectious dose. These

findings support one facet of viral dynamics described as “the trade off hypothesis” of viral adaptability, whereby the population evolves towards a less virulent phenotype to allow prolonged replication within a host, enhancing the probability of transmission between hosts (222, 261, 262). Since the virulent strain must be present above a certain percentage of the viral population within a host, the viral population contains a self-imposed limitation requiring that mutations conferring a virulent phenotype provide a selective advantage that would offset the cost of reduced transmission due to increased pathogenesis.

Other work has shown that in the context of quasispecies less virulent virions, and even less fit virons, can outcompete viruses that should have an advantage (222, 226, 261). This lends support for our model whereby virulence can be masked in diverse viral populations, and that perhaps the viral quasispecies has evolved this unique replication-independent barrier of competition for limited resources in order to modulate virulence and enhance transmission.

This work has important implications for antiviral therapeutics since quasispecies dynamics influence immune evasion, drug escape, and vaccine failure (135, 141, 253, 263, 264). This novel replication-independent host barrier and previously characterized features of viral quasispecies could be use to inform rational vaccine design. For example, despite the Sabin vaccine composition

existing primarily of attenuated viruses, the nature of this attenuation is not completely understood and the vaccine itself contains a small proportion of virulent virus. Furthermore, as we and others have demonstrated, the propensity for reversion and recombination in the host is high. Vignuzzi et al. propose that this is due to the inherent nature of error-prone RNA viruses whereby introduction of a live attenuated virus into a host will ultimately drive evolution of the viral population towards increased fitness and pathogenesis as well as increasing the probability of transmission of revertants with virulent characteristics (141). The authors propose using a high fidelity variant of poliovirus as an alternative to the current live attenuated Sabin vaccine. However, inoculating individuals with virulent poliovirus, despite the high fidelity and low reversion capability, is precarious. Therefore, taking into account that the attenuated viruses can mask virulence of revertants and mask the small proportion of virulent virus in the vaccine, combining the high fidelity mutant with the current Sabin vaccine strains would present a rational approach to enhancing the safety of the polio vaccine and reducing the transmission and disease from vaccine-associated reverted polioviruses.

Interferon as a Barrier to Poliovirus Pathogenesis

The interferon response has an essential role in limiting many neurotropic and non-neurotropic viral infections, for example, West Nile virus, measles virus, hepatitis C virus, dengue virus, ebola virus and influenza viruses (175, 183, 192, 234, 265). Type I interferon is important in controlling tissue tropism and oral susceptibility for poliovirus (37, 157); therefore, I wanted to determine the role of interferon in limiting poliovirus pathogenesis in nervous system. I found that IFN primarily limits poliovirus pathogenesis in the CNS by limiting replication in the periphery, and I demonstrated a difference in the impact of the type I interferon response on different viral strains. For the Mahoney strain, fewer virions reached the CNS and the viral population had less diversity due to the interferon response limiting viral replication. Immune competent PVR mice showed enhanced protection against disease compared to PVR-IFNAR$^{-/-}$ mice. For the Sabin poliovirus strain, type I interferon had an even more robust effect since IFN severely limited viral replication and fewer virions reached the CNS. Moreover, the probability for reversion to a neurovirulence phenotype was also reduced in the presence of a functional interferon response.

Novel Host Barriers to Poliovirus Neuropathogenesis

In addition to a role for IFN in limiting poliovirus-induced disease, the work presented here describes the identification of two previously uncharacterized host barriers, namely limited replication in peripheral axons and inefficient retrograde axonal transport. Previous work using an artificial viral quasispecies demonstrated a decrease in viral diversity from inoculation site to the brain (2). This "bottleneck effect" was indicative of host barriers limiting viral infection since it has been shown that a viral quasispecies has reduced diversity when a host pressure or barrier is encountered by the viral population (133, 250, 266, 267). Following this work, an expanded version of the artificial quasispecies was developed that utilized ten tagged viruses and a hybridization-based approach (1). Monitoring for decreases in diversity after inoculation of the ten tagged pool members, Kuss et al identified host barriers limiting poliovirus infection, including the intestinal epithelium and an efficient type I interferon response (1). The work presented here went on to further characterize how the type I interferon response plays a role in limiting disease in the CNS, and identified two novel host barriers to viral infection, limited replication in axons and inefficient retrograde axonal transport.

Limited viral replication in axons was not a completely unexpected result since the majority of cellular protein translation occurs in the soma of neurons; furthermore, viral replication in axons would result in non-productive virion production since PVR is required for transport of the virus. The site of poliovirus RNA release and viral replication has been well characterized in HeLa cells (73, 75-77, 85, 182, 268). However, it is unknown how uncoating and RNA release occurs in neurons. Studies using radiolabeled poliovirus indicate that the virions within the sciatic nerve are intact, implying that the virions have not yet released their RNA and that replication occurs in the cell body (113, 116, 269). Our data indicating that the majority of virus in the sciatic nerve was likely input virus and had not undergone replication supports this model.

Future studies to determine the site of poliovirus RNA release and replication in neurons will provide insight into the nature of poliovirus pathogenesis in neurons. We have set up an *in vitro* system to characterize poliovirus RNA release in primary motor neurons using a modified Campenot chamber to isolate the nerve terminals from the axons, and the majority of the length of the axons from the soma (229, 270). Using this system in addition to dissociated neuron culture, we can use a neutral red RNA release assay and infectious center assay, previously used to characterize RNA release in HeLa cells, to characterize poliovirus RNA release in neurons (75). Furthermore, the

site of poliovirus replication in neurons is currently unknown and identification and characterization of the replication site will lead to further elucidation of factors that limit pathogenesis in the nervous system. Using our transgenic mouse model in combination with our *in vitro* system we can determine the site of viral replication in neurons using imaging to monitor viral trafficking, as well as monitor the site of accumulation of viral proteins and viral replication intermediates.

Inefficient Retrograde Axonal Transport and Future Directions

The phenomenon of provocation poliomyelitis has been observed since before the introduction of the poliovirus vaccines but the mechanism for the increased incidence of paralytic disease after muscle damage was previously unknown (108, 112, 170). Work by Gromeier and Wimmer demonstrated that the effect was induced by muscle damage and involved induction of retrograde transport in neurons (112). Following up on this finding, the work presented here characterized and identified a conserved mechanism for an increase in transport efficiency for viral and non-viral proteins after muscle damage. I demonstrated that needle-stick induced muscle damage did not necessarily increase the uptake of virus into neurons but increased the transport efficiency of virus in neurons.

Muscle damage increased viral diversity in the CNS and increased viral pathogenesis and onset of disease. This inefficient retrograde axonal transport barrier was as robust as the previously characterized type I interferon response in limiting viral infection, and worked synergistically with the interferon response to reduce poliovirus invasion and pathogenesis in the nervous system.

Since this neural transport mechanism is likely to be conserved for other viruses that also hijack the retrograde transport machinery, uptake of neurotrophic factors and neurotoxins, and may have implications for neurodegeneration, our future directions will characterize the mechanism of the transport inefficiency and the mechanism by which muscle damage enhances transport efficiency. We have several hypothesis for the mechanism by which transport is inefficient in the absence of muscle damage.

Some of our preliminary data and research from other labs suggest that there are three more likely possible mechanisms that may explain viral transport inefficiency and the effect of muscle damage. First, PVR could be up-regulated after muscle damage, increasing the likelihood of virus binding and entry into cells (271). We plan to address this initially *in vivo* by imaging neurons in a mouse line expressing yellow fluorescent protein in neurons, and then quantifying the levels of counterstained PVR in the presence or absence of needle sticks. We

can also monitor PVR protein levels in our transgenic mice via western blot in the presence or absence of needle-sticks.

Second, viral RNA could be prematurely released at the nerve terminal, similar to in HeLa cells, resulting in the absence of viral progeny or viral progeny unable to be transported to the soma because of the lack of attachment to receptor. As mentioned above, preliminary data from our lab and others suggest that the majority of virus is transported intact (113, 116, 119). However, perhaps a portion of the viral population is releasing RNA at the terminal resulting in uptake but inefficient transport as only a portion of the viruses get productively transported. In addressing our third hypothesis, we will be able to concurrently evaluate this possibility.

Third, the virus might be differentially sorted based on uptake conditions. Receptor-ligand endocytosis at synapses has been well characterized and there are three general outcomes of internalized ligands; entry into the recycling pathway at the nerve terminal, transport to the soma and entry into the degradation pathway, or transport to the soma and release (272, 273). There is accumulating evidence that multiple endocytosed ligands and receptors as well as neurotransmitters are transported as a complex from the terminal to the cell body as "multivesicular bodies" (274-276). Our future directions to address this final hypothesis include

determining the nature of the endosomes or multivesicular bodies that include poliovirus and its receptor since other neurotransmitters or receptor-ligand vesicles trafficking with the virus may determine how it is sorted after internalization. We intend to use pull downs to evaluate what co-immunoprecipitates with the poliovirus-receptor vesicles, as well as utilize imaging to probe for specific receptors and ligands known to be involved in retrograde signaling during injury. Furthermore, we will evaluate how these vesicle populations differ in the presence of muscle damage, since it is known that retrograde signaling increases upon damage sensing (272, 277, 278). We speculate that in the presence of muscle damage, trophic support is increased, retrograde axonal transport is increased, and the poliovirus-receptor endosome is transported along with other factors into a productive pathway. Since the invasion of the CNS by poliovirus is an accidental event conferring no advantage to the virus, perhaps under basal conditions vesicles containing poliovirus are differentially sorted and some virions enter the recycling pathway at the terminal, some virions get degraded, and only a small proportion of virions reach the cell body and productively replicate. Upon muscle damage the increase in trophic factor transport may increase the likelihood of this final outcome for poliovirus, ultimately meaning more efficient transport, replication, and increased CNS disease and pathogenesis.

Concluding Remarks

Current barriers to viral infection in the CNS are well characterized but designing therapeutics against infection is challenging. Identifying novel barriers to infection in the CNS could open the door to new avenues of drug discovery, vaccine design and prevention of diseases caused by neurotropic viruses. The work presented here describes multiple barriers that could aid our understanding of viral pathogenesis. We identified a virally induced barrier, characterized two roles for interferon during poliovirus infection, and present two novel host barriers to poliovirus infection. These findings can be used to aid our understanding of RNA virus infections, viral population dynamics, host barriers to neurotropic viral infections and perhaps even expand our understanding of what it means to be immune-compromised.

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