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Emerging Infections of CNS: Avian Influenza A, Rift Valley Fever and Human Parecho Viruses

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Abstract

History is replete with emergent pandemic infections that have decimated the human population. Given the shear mass of humans that now crowd the earth, there is every reason to suspect history will repeat itself. We describe three RNA viruses that have recently emerged in the human population to mediate severe neurological disease. These new diseases are results of new mutations in the infectious agents or new exposure pathways to the agents or both. To appreciate their pathogenesis, we summarize the essential virology and immune response to each agent. Infection is described in the context of known host defenses. Once the viruses evade immune defenses and enter CNS cells, they rapidly co-opt host RNA processing to a cataclysmic extent. It is not clear why the brain is particularly susceptible to RNA viruses; but perhaps because of its tremendous dependence on RNA processing for physiological functioning, classical mechanisms of host defense (e.g. interferon disruption of viral replication) are diminished or not available. Effectiveness of immunity, immunization and pharmacological therapies is reviewed to contextualize the scope of the public health challenge. Unfortunately, vaccines that confer protection from systemic disease do not necessarily confer protection for the brain after exposure through unconventional routes.

Introduction

There are no “new world” Native Americans who can recall smallpox because those indigenous civilizations were destroyed by novel Eurasian pathogens such as small pox, measles and influenza. No one reading this article has any personal memory of the “Spanish Flu”, yet without it the course of human history might have been quite different. A few readers may remember a much lesser epidemic of polio, but even here it stretches our

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imagination to think that in the 1950s more people knew a scientist named Jonas Salk and the polio field trials than knew the full name of the President of the United States (90).

With memory of these devastating pandemics behind us, it would be fair to say that most citizens of the modern, developed world have grown complacent about the potential of infectious agents to lay low our civilization. Perhaps the panic in the early years of the AIDS epidemic, the fear over the brief SARS outbreak or the more recent Ebola outbreak in West Africa might foretell the type of hysteria society could face with the next plague, but developed nations have mostly dodged those bullets. Examining the AIDS epidemic in the developed world shows it primarily caused panic in a defined segment of society that was particularly susceptible to infection. The virus was only modestly successful in spreading through sexual contact and it killed slowly. Even today there is no effective HIV vaccine, but we were fortunate in developing combination drug therapy that has significantly abated morbidity and mortality and diminished spread of the virus at the same time. Nevertheless, the denizens of Sub-Saharan Africa have a much different perspective on the scourge of AIDS, and if HIV did not have to stand in line behind a number of other lethal infectious diseases, it might have had an even greater cultural and historical impact.

The goal of this brief review is to focus on three emerging infectious agents that have a particular propensity to damage the brain. It is probably not a coincidence that all three are RNA viruses. The human brain has evolved with a highly complex diversification of gene expression. Recent studies have suggested that aberrant RNA processing may underlie many neurodegenerative diseases (4). Indeed our entire understanding of brain functioning and physiology must be reexamined in the context of the role of RNA metabolism. This review presents the proverbial tip of the iceberg by highlighting how once RNA viruses evade immune defenses and enter CNS cells, they are able to rapidly co-opt the efficient host brain RNA processing to an unprecedented and cataclysmic extent.

To appreciate this common theme of the susceptibility of the brain to RNA virus infection, each of the three examples is described in a standard framework. The purpose of the review is not to comprehensively review the pathogenesis of the three emerging neurological diseases, as that will require further study. Rather our intent is to acquaint the reader with the threats and encourage broad collaboration with other specialists to gain insight into how we can combat emergence of these and similar infectious agents. The essential virology of each agent is presented first in order to appreciate the implications of molecular replicative strategies on the host/pathogen duel. Next the immunological response to infection is described in the context of known host defenses and means of evasion by the pathogen. After setting the molecular and immunological stage, a brief description of the natural history of infection follows. The effectiveness of immunity, immunization and pharmacological therapies is then described to contextualize the medical problem. Finally a pathological description of the disease and animal models is provided to highlight the severity of the disease processes and scope of the challenge.

Influenza A Virus

Virology

Influenza A virus (IAV) is a member of the *Orthomyxoviridae* family consisting of enveloped viruses with single-stranded, negative-sense, segmented RNA genomes. The family has six genera (Influenza A, B, C, Thogotovirus, Isavirus and Quaranjavirus) that do not exchange genetic segments (reassortment) between genera. Based upon comparative sequence analysis of hemagglutinin genes, Influenza A, B and C viruses were derived from a common ancestor diverging approximately 2,000, 4,000 and 8,000 years ago, respectively (109). Therefore even on a human historical scale they are relatively “new” agents. While both influenza A and B viruses are currently important human pathogens, the following discussion will be limited to IAV, a major cause of morbidity and mortality in man.

Since its first isolation in 1933, the molecular and physical ultrastructure of IAV has been thoroughly elucidated, offering numerous targets to disrupt viral pathogenesis (67). Historically, subtyping of IAV strains was accomplished serologically utilizing the immune system’s recognition of two of the main surface proteins: hemagglutinin (HA) and neuraminidase (NA). Current molecular analysis has shown there are 18 different HA and 11 different NA subtypes that can occur in any combination (*i.e.* 198 possible different subtypes of IAV) but tend to be selective to specific host species.

The molecular steps of IAV replication are known in exquisite detail (67) (Figure 1) and will not be comprehensively reviewed here other than to note key features that pertain to pathogenesis as an emerging threat to the CNS. Embedded in the surface envelope of IAV, HA binds to neuraminic acids (sialic acids) on the surface of host cell membranes. Sialic acids are universally expressed on the extracellular portions of a wide variety of cell membrane proteins from many animal species; however, subtle differences in HA amino acid sequence determine its capacity to be activated by different cellular proteases and to bind preferentially to different terminal sugar linkages. Cleavage of HA is critical to permitting effective binding and thus to overall virulence. IAVs with HAs requiring tissue or cellular specific extracellular proteases that are restricted to limited number of cells or tissue types such as trypsin-like proteases for cleavage activation of the surface receptor are of limited virulence (low pathogenic strains), while IAVs with HAs that can be cleaved by ubiquitous proteases (*e.g.* furin-like proteases) recognizing polybasic residues are readily activated and consequently more capable of virulence with spread to any body compartment (highly pathogenic strains) (65).

Since host cell surface protein glycosylation patterns are species and location specific, the type of HA also substantially determines in which species and organs the virus can efficiently replicate. Avian strains of IAV preferentially bind α2, 3-linked sialic acids prevalent in avian gut epithelium, while mammalian strains prefer α2, 6-linked sialic acids prevalent in human upper respiratory epithelium and α2, 3-linked sialic acids prevalent in human lower respiratory tract (25, 26). After binding the cell surface (Figure 1.2), endocytosis (Figures 1.2 and 1.3) and fusion with low pH (endosomal) compartment (Figure 1.4) is required for release of the viral ribonucleoprotein complex into host cell cytoplasm

(Figure 1.5 and 1.6) (67). Blocking acidification of the virion with small molecule drugs is one means of inhibiting viral replication.

After fusion, the virion's nucleocapsid is metabolized (uncoated), and viral RNA and associated viral proteins (including three polymerases) are transported to the nucleus (Figures 1.7). From here positive stranded mRNA templates with 5′ caps and poly(A) tails are synthesized (with and without host splicing) at the expense of most host protein synthesis and exported to the host cytoplasm for translation (Figure 1.8). Newly formed viral ribonucleoproteins are also released to the cytoplasm (Figure 1.9) for virion assembly and budding (Figure 1.10).

IAV assembles and buds from the plasma membrane (Figure 1.10) in a polarized fashion (*e.g.* from apical surface of epithelial cells). This polarized budding may account in part for the limited systemic spread of IAV and its concentration within the lung and gut (32, 120). While HA and NA are incorporated in the envelope in a ratio of 4:1 to 5:1, only the most abundant viral protein underlying the envelope, matrix protein 1 (M1), is required for viral particle formation (17, 43, 85).

Critical to evolution of IAV is packaging each of the eight viral RNA segments into the individual virion. If done randomly, less than 1% of virus particles would be infectious (31). While there is some degree of selection and sorting, this is not a perfect process and incorporation of genes from other subtypes of IAV superinfecting individual cells leads to genetic reassortment (and consequent "Shifts" see below). After virion assembly, NA is crucial for viral release and virion movement through an environment filled with mucous and replete with sialic acid binding sites (91, 92).

Immune response

Of course the host mounts a variety of innate and adaptive defenses to block viral infection and replication. Although we are unable to fully describe the immune response here, a general overview will be summarized. As the first barrier of defense, soluble proteins in mucosal secretions such as mucins, gp-340, pentraxins, collectins, natural IgM, complement and defensins promote IAV aggregation, clearance and reduce infectivity (126). At the onset of cellular infection, IAV is detected by pattern recognition receptors TLR3 or TLR7 located in endosomal compartments. Single-stranded viral RNA triggers TLR7 signaling and secretion of type I interferons (IFNα/β) (indeed the discovery of IFN was the result of examining heat inactivated IAV (54)) (29). Type I IFNs induce a variety of antiviral responses including augmenting innate and adaptive immunity (*e.g.* natural killer and B cell proliferation, dendritic cell maturation, T cell survival and activation), induction of antiviral genes such as activating protein kinase R (PKR), ADAR, OAS, RNase L and Mx proteins that inhibit various steps of viral replication, and initiation of a cascade of cytokine secretion (IL6, IL8, TNFα amongst others) (39, 96). Not surprisingly IAV has evolved a protein (nonstructural protein 1 (NS1)) that abrogates IFN synthesis in epithelial cells and conventional dendritic cells; however, plasmacytoid dendritic cells are resistant to the effects of NS1 (29).

In addition to arresting primary IAV infection, the initial innate immune response induces an adaptive immune response to clear virus and more quickly and effectively block future IAV infection. The humoral arm of the adaptive immune response is principally focused on surface envelope proteins NA and especially HA (27). Resistance to subsequent infection and illness correlates with HA and NA antibody titers. Mutations and reassortment in HA or NA genes is thus key to IAV's capacity to reinfect host populations and periodically cause epidemics and pandemics. The role of cellular immunity in clearing viral infection is less clear, but it does limit severity and transmission of IAV infection. Anti-viral specific CD4 T cells help B cells produce neutralizing antibodies and secrete Th1-type cytokines such as IFN-γ, IL2, and TNFα to direct T cell responses, but may also directly control IAV infection (15, 71). The major targets of T cell immunity in IAV are epitopes in M1 and nucleoprotein. IAV-specific CD8 T cells contribute to IAV clearance and control through mechanisms such as cytotoxic effector delivery, proinflammatory cytokine secretion (*e.g.* TNFα and IFN-γ) and expression of FasL and TRAIL death domain receptors (71). T cell responses generally recognize more conserved IAV epitopes than the antibody response, but viral escape variants may exist.

Drift versus shift

Epidemic Flu—As with other infectious agents (*e.g.* malaria), the ability of HA and NA glycoproteins in the coat of influenza virus accounts for much of IAV's capacity to elude immune detection and clearance and guarantees the pathogen's survival in the host population. The diversity of HAs and NAs in nature is evidenced by the observation that all but two known HA (H1-H16) and NA (N1-N9) subtypes can be found in sylvatic avian species, with the other subtypes (H17-18 and N10-N11) found only in New World bats (84, 116, 117). Adapted to the aquatic bird host, IAV is under limited selective pressure from an immune response. When transmitted to land-based birds or mammals, adaptive immune pressure leads to selection of mutations within HA and NA genes and a slow antigenic drift. At any one time, multiple viral strains circulate in human and other mammalian populations. While self-sustaining, this ecosystem is characterized by periodic epidemics. Every few years the "drift" in amino acid mutations mediates enough antigenic change that the virus escapes neutralization by antibodies specific to previous strains and can mediate new infections. This leads to a new epidemic in the host population. Immunization against newly emergent strains is one attempt to mitigate the severity of these epidemics.

Pandemic Flu—More precarious to humans is introduction of a new IAV HA subtype (with or without a novel NA subtype) that the population has not seen before and for which the population has insufficient adaptive immunity. This much more rare event is termed antigenic shift and occurs when there is direct introduction of IAV from other mammal or avian hosts or when two IAV subtypes infect the same host (mixing vessel) at the same time in the same cell. In this setting, gene segments are exchanged between different subtypes (*e.g.* HA of one strain is exchanged with HA of another strain leading to a new HA-NA combinations) and entirely new viral subtypes or reassortants are generated that with some minor adaptations of transmission efficiency can mediate pandemics in populations immunologically naive to the new agent. While swine are frequently sited as the "mixing vessel" for this reassortment to occur, pigs and barnyard avian species (such as ducks or

geese) intermingle and can mix influenza viruses between the two species and create novel influenza viruses. This mechanism may lead to introduction of novel influenza subtypes into the human population.

Pandemics of IAV emerge killing significant percentages of the human population and even changing the course of history (112). Forty to fifty percent of the human population is infected in the course of a pandemic with an increase in the number of expected deaths (104). The severity of such a pandemic is not well appreciated by current citizens of the developed world. The “Spanish Flu” of 1918 to 1919 (H1N1) killed from 25–50 million people or more worldwide (58) and decreased average life expectancy in the US by 10 years (44). Infection mortality during the 1918 pandemic changed from the usual 0.1% of infected individuals to 2.5% (79). Perversely rather then afflicting infants and elderly, pandemic infections are particularly lethal to young adults (68). Subsequent pandemics in 1957, 1968 and 2009 have resulted from reassortment events generating novel H2N2, H3N2 and H1N1 viral strains. For a variety of reasons, including great efforts to immunize the world population, these pandemics were more benign than Spanish Flu was.

Avian Flu—The restriction of IAV to infecting lung with only rare reports of systemic spread would suggest this virus has little relevance to neurological disease. However in 1997 an outbreak of lethal avian flu (H5N1) occurred in humans in Hong Kong (19). This outbreak was the result of direct avian-to-human transmission without an intermediate host and because the virus showed only limited capacity to spread from human to human, was not able to achieve epidemic proportions and was halted relatively quickly. Since this initial outbreak, avian H5N1 IAV has become endemic in Asia, the Middle East, Europe and Africa with 826 confirmed human cases, 440 of which were fatal (128). Systemic spread, including CNS involvement, has been reported in humans infected with H5N1 IAV and detailed in mammalian models (described below). In addition, the past few years have seen an increase in the number of reports detailing direct transmission of avian influenza viruses to humans (42, 73, 107) including strains such as H9N2 and H7N9. To date none of these avian transmissions resulted in new strains of virus capable of spreading efficiently between human contacts; however, recent cases of H7N9 virus in China suggest human-to-human transmission can occur but so far is not sustained. In part human transmission requires capacity to replicate at lower temperatures of the upper airways and the ability of the HA molecule to bind to the human epithelial glycosylation pattern of sialic acid with $\alpha 2, 6$ linkage (51). Before taking solace in this barrier to pandemic spread, it should be noted that mutation of only 4 additional amino acids is required to effectively support mammal-to-mammal spread (51, 74).

Treatment

With detailed knowledge of IAV replication strategy and pathogenesis, it has been possible to block infection and transmission either through drug or immune therapy. Drug therapies have focused on two viral functions critical to replication and spread. The first involves blocking the viral ion channel M2 protein in the virion that is essential for acidification of the endocytosed viral compartment and shedding of the viral coat. Amantadine and Rimantadine inhibit acidification of the inner virion and thus prevent viral release into the

host cell cytoplasm. A second class of drugs competitively inhibits viral neuraminidase activity, preventing viral particle release and spread within the mucous rich environment. Other drugs in use in other countries target different aspects of the viral replication cycle such as inhibition of target membrane fusion of IAV and host cells (Arbidol) (72) and inhibition of the HA cleavage by inhibiting trypsin and related proteolytic enzymes (aprotinin).

Of course one of the major advances in modern medicine has been the introduction of vaccinations. The effectiveness of vaccinations to prevent severe illness and death is no more evident than in the case of IAV. Inactivated whole or subcomponent IAV vaccines are able to generate protective antibodies to HA and NA (21). Live attenuated vaccines that replicate at lower temperatures of the upper airways have the additional advantage of inducing local immunity and potentially priming cellular immunity (57). Due to constant antigenic drift and occasional antigenic shift in IAV's HA and NA, the tricky part to flu vaccination is creating a vaccine for the strain that will circulate when flu season begins.

Neurological disease associated with avian influenza

Autopsies of H5N1 virus infected humans have been very limited; but commensurate with clinical syndrome, H5N1 virus infection mediates more of a gastrointestinal illness and most importantly a systemic infection. This systemic infection is particularly notable for severe CNS symptoms and infection (*i.e.* encephalitis). Influenza outbreaks have long been associated with neurological sequelae. Influenza encephalopathies, encephalitis, encephalitis lethargica and Reye's syndrome are rare, but serious CNS diseases that manifest with influenza infection, especially with young children (118).

What is necessary to change a traditionally self-limited pulmonic disease into lethal encephalitis? At least three conditions need to be met in order for influenza to directly mediate neurological disease. First, the active virus needs to escape local control at the site of primary replication to reach the brain compartment. The selective polarity of viral budding to the epithelial surface weighs against viremia and systemic spread, but some zoonotic influenza strains have shown to be highly virulent in their non-natural human hosts and may even trigger an overcharged innate immune response (70). As discussed above, highly pathogenic strains generally have HAs that can be cleaved and activated by ubiquitously expressed proteases such as furin-like proteins and thus readily capable of systemic spread. IAV could enter the brain by direct infection of nerve endings or via hematogenous routes. Neurovirulent H5N1 IAV strains have been shown to spread to the CNS by infecting nerve endings of olfactory, vagus, trigeminal and sympathetic nerves (93). Other neurovirulent viruses such as poliovirus and rabies virus spread to the CNS by infecting motor neurons at neuromuscular junctions, while others such as HIV enter the CNS when infected leukocytes (*e.g.* monocytes/macrophages) traverse the blood brain barrier (60, 70, 127). Many RNA viruses (*e.g.* West Nile virus and Hepatitis C virus) can infect brain microvascular endothelium after loss of effective clearance in peripheral sites leads to viremia. In brains of ferrets infected with H5N1 IAV, we have observed patterns of both hematogenous spread and entry via olfactory epithelium (11). Once in the CNS, IAV needs to infect neurons and spread. Most CNS neurons have abundant expression of α2,3-

linked sialic acid glycosylation pattern (86, 129), so IAV is thus able to bind to neuronal cells. Given the ubiquity of glycosylated proteins on the surface of mammalian cells this is not a particularly high threshold to clear. While a map of neuroglial glycosylation linkages is not available, certainly neurons are infectable by either binding avian IAV and/or through endocytosis. If IAV directly binds neurons, there are plenty of furin-like proteases present in the brain to cleave IAV HA for neuronal entry. After entry, IAV particles must undergo retrograde transport to co-opt replication machinery in the neuron (70). The complex and unusual distribution of neuronal cellular machinery in axons, dendrites and the cell body might facilitate IAV replication. The virus must then bud and spread through synapses or intimate cell-cell processes. Finally, the neuropathogenic strain needs to be able to subvert intrinsic immune responses. Clearance of virus from infected neurons is a big hurdle since immune responses tend to be slower (*e.g.* IFNγ secretion and antibody-mediated clearance rather than cytolytic mechanisms). If IAV already escaped the antibody response to spread to the CNS, this final restriction also does not present a high barrier to disease.

New neuropathology of avian influenza

H5N1 virus-infected cells can be detected in the brains of infected birds, mice, ferrets and humans (10, 11, 37, 38, 45, 77, 95, 125). But before the virus can infect the brain, it first has to escape the immune response of respiratory epithelium. Animal models of H5N1 virus infection have been employed to chronicle this systemic spread. Because of similarities between ferret and human respiratory system glycosylation patterns, ferrets are the animal model of choice for pathogenesis studies (3, 80). However, immunological reagents to study the ferret model are more limited than traditional mouse models; therefore depending upon the experimental question, either rodent model has been used. Initial infection with either high or low pathogenic strains of IAV follows a similar course. Inhalation of the virus is followed by rapid binding to epithelial cell surface receptors, endocytosis and explosive replication. Different animal hosts express different glycosylation patterns in different regions of their respiratory and GI system. Similar glycosylation patterns in the human and ferret respiratory system leads to binding of α2–6 linkage-tropic viruses in the upper respiratory system (nasal epithelium and bronchi) and binding of α2–3 linkage-tropic viruses in lower respiratory system (terminal bronchioles and alveoli). Early innate immune response evolves to a cytokine “storm” that accounts for most of the early clinical symptoms of fever and malaise but is presumably crucial for limiting viral spread prior to the development of adaptive immunity (113). Within the first day, viral replication is robust and the host rapidly disseminates the virus through coughs and sneezes. Peak viral replication in the respiratory system occurs within 48 to 72 hours and then is rapidly suppressed such that by 7 to 8 days infected cells are difficult to detect (10, 11).

Five days after infection the clinical course of highly pathogenic and low pathogenic strains diverge. Low pathogenic strains are cleared from the lung and physiological function returns. With high pathogenic strains, lung damage is more severe and complicated by secondary bacterial infections, which mediate the majority of the morbidity and mortality (59).

The recent IAV pandemic in 2009 was the result of introduction of a novel strain of H1N1 (H1N1pdm09) to the human population that replaced the circulating H1N1 seasonal strains (12). This novel strain disproportionately affected children and young adults (22) but was not associated with widespread systemic dissemination. Humans infected with H1N1pdm09 virus exhibit severe bronchopneumonia (Figures 2A and B). Ferrets also show bronchopneumonia (Figure 2C) with infected cells found mostly in bronchi with occasional alveolar cells (Figure 2D) and prominent submucosal gland involvement (Figure 2E). H1N1pdm09 virus is able to spread to the gastrointestinal tract where it infects cells in the lamina propria (Figure 2F). Thus, compared to avian H5N1 viruses, H1N1pdm09 is an IAV with restricted systemic spread. Comparing the systemic spread of different IAV strains in ferrets shows all strains (H5N1, H1N1pdm09 and H3N2 viruses) infect the lung and gut lamina propria, whereas only H5N1 virus is able to spread to the brain and liver (Figure 3). Interestingly, prior infection with H1N1pdm09 virus protects against H5N1 virus infection, while prior infection with H3N2 virus protects against lung infection but leads to systemic infection of the CNS and liver.

But some highly pathogenic strains have an additional attribute that can contribute to worse clinical outcome: the capacity to spread systemically. Much of this capacity to spread systemically is associated with enzymatic stability of the hemagglutinin molecule (78) and the ability to escape containment by the host immune response. As described above, maturation of newly synthesized virus into an infectious agent requires cleavage of the virion HA. The HA of highly pathogenic strains is more readily cleaved by ubiquitous furin-like proteases and thus capable of maturing in any body compartment rather than being limited to extracellular spaces like those in the respiratory and gastrointestinal system. With systemic spread, infectious loci are distributed throughout the host contributing to a secondary viremia. Prior to the capacity of the adaptive immune system to control this systemic spread, IAV finds its way into the CNS, which for multiple known and unknown reasons is susceptible to infection.

Direct indication of IAV-infected neurons or CNS support cells in humans is limited (38, 45), but in ferret and murine models the neuron is the predominant infected cell type observed in the CNS (10, 11, 37, 56, 77). Macrophages are thought to be infected by influenza virus (88), and there are reports suggesting microglia are infected (37, 56), but others have not observed microglia infection (10, 11, 77). In mice, IAV is first detected in the brain stem and olfactory cortex at 4 days post-infection (10). Within a few days, it spreads throughout the cortex, largely sparing the striatum. Interestingly, the infection does not appear to be uniform, but rather occurs in small foci throughout the brain suggesting hematogenous dissemination (Figure 4C). A similar distribution is seen in ferrets (11). Some but not all H5N1 virus-infected ferrets show a severe bronchopneumonia (Figure 4A) characterized by abundant lower lung alveoli infection (Figure 4B). The virus spreads to the liver, spleen, gut and brain (Figures 4C–4F). The brain shows infection throughout the CNS in olfactory cortex, cerebral cortex, deep gray nuclei and brainstem (Figure 4C). Neurons are the predominant infected cell (Figure 4F). Occasional ferrets show infection of ependymal cells lining the ventricles or cells within the leptomeninges, suggesting dissemination through the cerebrospinal fluid.

It has been reported that C57B6 mice infected with a strain of H5N1 isolated from the CSF of a Vietnamese boy showed evidence of chronic microglial activation and loss of dopaminergic neurons approximately 50 days following viral clearance (56). This suggests that H5N1 brain infection can lead to long-lasting effects that could contribute to pathological processes observed in diseases such as Parkinson's disease. Recently, this same group has reported microglial activation in the CNS of mice infected with pandemic 2009 strains of H1N1 IAV in absence of CNS IAV infection (101). This may be a common outcome after systemic infection with any virus without neurotropism. Whether the microglial activation is neuroprotective or contributes to neurodegenerative processes is a matter of debate.

Why do RNA viruses devastate the brain once they obtain access to the CNS? As discussed above, the brain generally does not mount the full armamentarium of viral clearance strategies upon infection. This limited immune response has been thought to curtail death of a cell population with limited regenerative capacity. However, recent studies have revealed that the brain is unique not only in its massive expression of numerous genes but also in its overall processing of RNA (4, 131). Critical to the brain's very function is the capacity to modulate RNA processing in an intricate and spatially refined way. While systemic organs like the gut and lung can utilize generic innate immune responses that entail shutting down RNA processing, such a defense might not be available to the CNS. The inability of the brain to defend itself from viral infection may reside in the importance of meticulous control of RNA processing necessary for brain functionality. In the absence of an effective means of suppressing viral RNA replication, the brain rapidly becomes a sea of virus unparalleled in other organs.

Fortunately, humoral immunity provides an effective barrier to CNS invasion by RNA viruses. But this immunity is fine grained and needs to be approached comprehensively when designing immunization strategies. The immune response following natural infection is broad-based and recognizes numerous viral epitopes. Infection with one strain of IAV can generate an immune response that protects from modestly different strains of IAV. Called heterosubtypic immunity, this is the product of a robust immune response to an active infection. Immune response to most vaccines is more limited and focused on antigens chosen in the vaccine design and generated in body compartments distal from the site of natural exposure. Vaccines may provide enough immunity to block severe acute systemic disease without providing sterilizing immunity. This could open the possibility of spread to the CNS. We have observed precisely this scenario in examining vaccines to Rift Valley Fever Virus.

Rift Valley fever virus (RVFV)

Virology

The taxonomic family of *Bunyaviridae* was established in 1975 (87). Four genera of the family infect animals after transmission from arthropods. An important exception to that mode is Hantavirus, which is transmitted by rodent excreta. Infection of arthropod tissue varies depending on the arthropod and virus, but infected tissues include salivary glands and gonads that permit the viruses to be transmitted horizontally, vertically or venereally (8).

Like influenza, the *Bunyaviridae* are enveloped and have a segmented genome. The tripartite single-stranded RNA genome segments are named on the basis of their size (large, medium and small). Rather than being universally negative sense as in the case of influenza, *Bunyaviridae* are negative sense or a combination of negative sense and antisense (67) (*e.g.* the small RNA segment of *Phlebovirus* and *Tospovirus* genera have message transcripts generated in opposite orientations). For purposes here, other than the ambisense segments that necessitate two rounds of transcription to occur, *Bunyaviridae* replication is similar to that of IAV with the following exceptions:

1. Viral surface envelope attachment proteins (Gn and/or Gc) attach to host cell receptors (2, 30, 62), which for some viral strains are integrins or DC-SIGN but for most family members are not known (40, 75).
2. Cellular entry requires endocytosis and vesicle acidification as with IAV; however, RNA transcription occurs in the cytoplasm without the addition of poly(A) tails (47).
3. Most viral assembly occurs in the Golgi with transport and exocytosis at the plasma membrane (though some alternate assembly at the plasma membrane does occur).
4. Viral replication is generally cytolytic in vertebrate cells but not in arthropod cells (14).

Many members of the *Bunyaviridae* family are important human and livestock pathogens. Some of these like California encephalitis, La Crosse, etc. are notorious for mediating neurological disease; however, the following description will be limited to RVFV which was first isolated in 1930 (28).

Epidemiology and Natural History

As suggested by its name, members of the *Phlebovirus* family are transmitted by a diverse group of blood-sucking arthropods. RVFV is transmitted principally by the *Aedes* and *Culex* mosquitoes (94, 103, 119, 121). In 1977, an Egyptian outbreak of 200,000 cases (approximately 600 deaths) followed completion of dams that led to increased mosquito populations and greater livestock infection (52, 81).

After transmission of the virus through a mosquito bite, local replication takes place in regional draining lymph nodes, followed by viremia and secondary amplification in the reticuloendothelial system, liver, adrenals, lung and kidney tissue (67). Infection of livestock is signaled by increased incidence of abortions (not a feature of human disease) (7, 110). In humans the incubation period is approximately 3 to 6 days (99) and results in fever, headache and myalgia with complete recovery in most individuals. Less than 1% of infected individuals experience severe disease including fulminant hepatitis, retinitis, encephalitis and hemorrhagic syndromes (34, 41).

Alternate routes of transmission to humans via contact with tissues or fluids from infected animals can lead to particularly severe outcomes. Individuals involved in animal husbandry are at high risk of infection through the shearing or butchering of animals during epizootic infections. With such unconventional, usually aerosolized exposure, the resulting disease is

more severe consisting of hemorrhagic fever, encephalitis and retinitis. With dissemination via aerosolization, easy access to natural isolates during epizootics in endemic areas and easy amplification in cell culture or infected animals, there is concern that RVFV could be used as a bioterror agent (98).

Immunization—Vaccination of livestock is probably the most effective way of preventing human infections. A live attenuated vaccine is highly effective at controlling epizootic livestock infections; however, it too is associated with abortions in some pregnant animals (7). Formalin-inactivated vaccines are available but provide only short-lived immunity. Currently, there are no approved vaccines or therapeutics to ameliorate human disease. Attempts to create human vaccines depend upon developing reliable nonhuman primate models (48).

New Neuropathology

RVFV is highly infectious through the aerosol route with numerous reports of infections of laboratory personnel (16, 36, 52, 61). We have studied aerosol exposure of BALB/c mice to evaluate the pathogenesis of RVFV infection and the capacity of vaccines to protect mice from disease (98).

While most mammals are susceptible to RVFV infection, certainly the murine system is the most commonly used laboratory model. The natural route of infection via mosquito bite is readily mimicked by subcutaneous, intramuscular or intraperitoneal inoculation of mice. Comparison of live attenuated vaccine strain (MP12) versus wild type strain (ZH501) shows dramatic differences in survival and pathogenesis. Intraperitoneal infection of young BALB/c mice with the live attenuated vaccine strain MP12 readily reproduces most human mosquito-borne infections. Over the first 5 days post-infection (DPI), mice show acute signs of infection including ruffled fur and lethargy with mild weight loss. A low-level viremia is established but this is cleared within a week. Beginning at 3 DPI very low levels of virus appear in the liver (Figure 5A–C); but by 5 DPI, virus has been cleared from the liver and the animals are well on their way to recovery. No other organ shows evidence of infection.

In contrast, infection with the clinical isolate and pathogenic RVFV strain ZH501 is much more dramatic. Viral infection of the liver occurs earlier by 1 DPI and is massive by 2 DPI with abundant viral replication and hepatocyte necrosis (Figure 5D–F). At 2 DPI virus appears in the white pulp of the spleen and within another day rapidly inundates this organ with a pan-splenic infection. Because of liver failure most mice require sacrifice by 3 DPI. The brain does not show evidence of infection at this early time point. The value of immunization is clear by the observation that prior vaccination with MP12 completely protects mice from subsequent intraperitoneal challenge with ZH501.

Unconventional exposure to RVFV, such as that which might be experienced by abattoir workers or individuals potentially exposed to aerosolized biological weapons, has a very different clinical outcome. With aerosol exposure of BALB/c mice, death from ZH501 infection takes longer and is associated with widespread systemic dissemination. By 1 DPI, systemic infection has spread to many organs including the liver, spleen, lung, heart and gut. Compared to the liver (Figure 6A), the other organs show relatively low levels of virus

infection. The brain can show infection as early as 1 DPI, but by 6–7 DPI it is heavily involved. Mice develop neurological symptoms including circling the cage and hind limb paralysis. The pathogenesis of such broad dissemination is not known but is most likely hematogenous.

Unfortunately protection conferred by live attenuated vaccine strains or DNA/replicon RVFV GN protein vaccines is limited to conventional exposure (*i.e.* mosquito bite) as demonstrated in the mouse intraperitoneal challenge model (6). When challenged through the aerosol route, prior immunization provides protection of the heart and spleen and limited liver infection with clearance of virus by 8 DPI (Figure 6B). Infection of the gut epithelial crypt cells is more persistent (Figure 6C). RVFV aerosol challenge results in delayed infection kinetics of the brain. Around 10 DPI, RVFV mediates a lethal panencephalitis (Figure 6C and 6D). Protection from liver disease but death from late-developing encephalitis has been noted in RVFV-infected mice treated with ribavirin or infected with the M847-A strain (83).

How does RVFV get to the brain and why is the brain not protected from fulminant infection? The presence of neutralizing antibody in the serum would argue against hematogenous spread to the CNS (6). Infection of the olfactory bulb raises the possibility that virus could ascend directly from the nasal neuroepithelium; however, early olfactory bulb infection does not appear to be a feature of this disease. The frequent observation of gut epithelial cell infection raises the possibility that virus may pass through gut innervation to the brainstem followed by subsequent transsynaptic spread throughout the brain.

Regardless of how RVFV reaches the CNS, why does neuroinfection proceed unabated? The brain has been variably described as “immunologically privileged” (*i.e.* in some ways sequestered from systemic immune surveillance). Certainly immunoglobulin, a key component to neutralizing RVFV, does not normally pass into the CNS, but once infection appears in the CNS the blood brain barrier integrity is disrupted and immunoglobulin passes more freely. Additionally, astrocytes, microglia and endothelial cells in the CNS quickly release cytokines leading to rapid ingress of CD8 cytotoxic lymphocytes. Nevertheless, after aerosol exposure to RVFV, the brain is consumed by viral infection. It remains to be determined why RVFV replication cannot be suppressed in the CNS, but a potential hypothesis is that the CNS cannot respond to RNA viral infections like other organ systems. Because key brain functionality requires intricate RNA processing and transcription, perhaps immune response options in the CNS are more limited.

Human Parechovirus

Virology

Human Parechoviruses (HPeV) are a newly classified genus of the Picornavirus family. Picornaviruses are small (~30nm), non-enveloped, single-stranded RNA viruses of positive polarity that have been recognized as human and animal pathogens for over 100 years. The most notorious of these, poliovirus, was isolated at the turn of the previous century. There is a continually growing number of *Picornaviridae* genera (currently 26) (50, 69). Originally classified in the *Enterovirus* genus as echovirus 22 and 23, HPeV were later classified into

their own genus based upon their distinct biological and genomic sequence characteristics (sharing less that 30% amino acid identity with other Picornaviruses) and a unique capsid structure consisting of 3 rather than 4 capsid proteins (106). The enteric forms including parechoviruses are notable for significant acid stability (67). As a single-stranded RNA molecule, the picornaviruses have a complex 5′-non-coding region that facilitates ribosomal entry and translation of a single polypeptide protein. Cleavage of this polyprotein releases both enzymatic and structural proteins.

There are a wide variety of known (not to mention unknown) cellular receptors for picornavirus capsids. But not all cells possessing receptors are infectable because other host factors are required for viral replication or host factors in certain cells/tissues can inhibit viral replication. For example, tropism of poliovirus is further regulated by the capacity of the host cell to respond to type I IFN, and since the CNS lacks sufficient IFN responsiveness, it is targeted by poliovirus (53). After binding to the cell surface receptor, replication proceeds as described above for IAV with the following exceptions:

1. Only a subset of picornaviruses requires acidification to release the viral genome into the cytoplasm.
2. After uncoating, viral transcription depends upon binding of ribosomal subunits by a sequence in the 5′-noncoding region (76).
3. Following polypeptide synthesis and cleavage, virions are assembled in the cytoplasm around newly synthesized positive-stranded genomic RNA for release from the cell by cytolysis (67, 115).

Like other RNA viruses there is a substantially high virion-to-infectious virion ratio (2 to 3 orders of magnitude). HPeV are different to most Picornaviruses because they do not shut off host cell protein synthesis during replication and have limited proteolysis of their capsid proteins (105, 106).

Because the pathogenesis of enteroviral infection has been best established with polio, it will be used here to describe the paradigmatic infection. Fecal shedding of virus occurs over weeks supporting oral-oral route of transmission. With exposure, polio replicates initially in the tonsils or Peyer’s patches of the intestine (13). This leads to minor viremia followed by a more diffuse infection of the reticuloendothelial system and robust viremia. It is at this stage that the CNS can be invaded (though in the immune intact host, CNS invasion is distinctly uncommon occurring in less than 1:200 infections (13, 67,100). There is some controversy regarding the relative role of hematogenous spread to the CNS versus transaxonal retrograde spread to select sites such as anterior horn cells of spinal cord (89). Regardless of its systemic route of entry, polio binds to a ubiquitous host receptor, CD155 (82). The principal neurological disease of anterior horn motor neurons and brainstem neurons that follows is thought to be a product of neural cells having a diminished type I IFN response (53) or inability to suppress viral-directed RNA processing. It is unclear why poliovirus infection rarely spreads to cerebral and cerebellar cortex (exception of motor cortex) and the basal ganglia.

Epidemiology

While most enteroviral infections are asymptomatic, there are estimates of 10–15 million symptomatic infections per year in the United States (108), suggesting that most humans are infected at least once a year. In the immune intact host, symptoms are mostly mild; however, as a group, enteroviruses are the most common cause of meningitis (49). The even more rare infection of CNS parenchyma was first appreciated by Charcot in his studies of spinal cord poliomyelitis (20), that today only afflicts non-immunized hosts.

Echoviruses were first isolated in 1951 and received their name because they were **E**nteric isolates that mediated a **C**ytopathic effect mostly in **H**uman cells but were unassociated with a human disease ("**O**rphans") (24). With improvements in molecular sequencing it became evident that echovirus 22 and 23 were genetically distinct from other enteroviruses and thus reclassified into a new genus Parechovirus, of which there are now 16 strains (50, 97). Serologic studies have shown that HPeV infections are ubiquitous, with seroconversion occurring the first year of life (1, 111). Even more recently, HPeV3 was first identified in a 1999 stool sample (55). In the United Kingdom, virological screening of 3,415 CSFs found that HPeV3 was the most prevalent picornavirus present (49). Phylogenetic analysis of HPeV members indicates the family may be a recently evolved (emergent) agent (18).

Immune response

Little has been reported about HPeV specific immunity, so a brief review of general immune responses to enterovirus infection will be described here. As with all viral infections, innate immunity is key to controlling viral replication and dissemination. After infection, enteroviruses are recognized by Toll-like receptors and retinoic acid-inducible gene I (RIG-I)-like receptors, which trigger cytokines, chemokines and proteins that can directly attack viral replication (*e.g.* PKR and type I IFNs). IFNs and virus specific IgA are increased in saliva and respiratory and gastrointestinal tracts during acute infection (130). Antibody responses are essential for protection against enteroviruses; without them, hosts are unable to clear the virus, even with an intact CD8 T-cell response (64, 67). In fact, many CD8 T-cell responses are weak or undetectable and can be inhibited by viral mediated suppression of MHC class I expression (63). This may explain why shedding of virus from the gut can persist for weeks (23) and why some level of chronic infection has been documented following enteroviral infection (*e.g.* CVB3 in mice (66)).

Treatment

Treatment of most enterovirus infections is generally limited to physiological support. However, there are a variety of potential therapies for picornavirus infections including: IFN, pooled immunoglobulin, and antiviral drugs (*e.g.* Pleconaril) that bind to hydrophobic sites in the viral capsid thus blocking cell attachment (35, 114). Vaccines for picornaviruses are limited to poliovirus and hepatitis A virus (35). Poliovirus vaccines have been available for over 50 years. Intramuscular inactivated polio vaccine is administered at 2, 4, 6–18 and 48–72 months and is associated with life long humoral immunity. Thus the role of oral live attenuated vaccine has diminished. For years we have been on the cusp of eliminating polio throughout the globe, and while a potential historical accomplishment, would eliminate only

1 of many picornavirus CNS pathogens. There are currently no vaccines in the pipeline for HPeVs.

Neurological disease associated with HPeV infection

HPeV infections are common but are usually asymptomatic. Nevertheless, HPeV isolates have been recovered from patients (most frequently neonates) presenting with a variety of nonspecific disorders including sepsis, gastrointestinal and respiratory infections and meningitis (1, 5, 124). CSF analyses of patients with CNS HPeV3 infection show an atypical non-inflammatory profile. This makes the range of clinical CNS HPeV disease difficult to define. A survey of 10 infants presenting with seizures and diagnosed with HPeV CNS infection identified viral RNA in the CSF of seven, in the blood of three and in one each from the nasopharynx and stool (123). Abnormal periventricular white matter was observed after evaluation with imaging methods in all 10 infants. None of the infants succumbed to infection, but clinical follow up revealed no to varying neurological sequelae including: cerebral palsy, epilepsy and learning disabilities that were worse in preterm versus term infants (123). Similar patients with white matter abnormalities have been reported more recently (46).

New Neuropathology

Autopsy descriptions of CNS HPeV infection are limited since death associated with this viral infection is rarely documented (9, 33, 102, 122). As predicted by the clinical and imaging findings (Figure 7A), HPeV3 infection of the neonatal brain is associated with histopathological findings of classical periventricular leukomalacia (Figures 7B and 7C) (9, 122). Tissue cavitation and severe reactive gliosis is observed in the absence of a cellular immune response (Figures 7A–7E). Additionally, the leptomeninges demonstrates a moderate cellular reaction. Localizing a role of HPeV3 for this pleomorphic and multifocal pathology was challenging. Probes to detect infected cells demonstrated an intriguing distribution of virus and pathogenesis of tissue damage (9). Rather than infecting CNS parenchymal cells, HPeV was localized to blood vessel smooth muscle cells in the leptomeninges and pulmonary vasculature (Figure 8). This suggests that much of the severe CNS tissue damage (PVL) is an indirect effect of vascular compromise to metabolically active regions.

Conclusions

Review of these 3 examples of emergent neurological infections defines four important themes to consider for the future:

1. As demonstrated by influenza and HPeV, there are abundant opportunities for infectious agents to genetically evolve in real time. It is not necessary to review history books to detect emergence of pathogenic viral strains. Some degree of vigilance is required to identify these new agents when they arise so that we can quickly respond with effective preventative and therapeutic strategies.
2. Vaccines have proven to be highly effective means of protecting the human population from infectious agents of high morbidity or mortality. But it is clear that

we have been fortunate in creating vaccines to what proved to be easy targets. Many of these vaccines were developed at a time when not only did we have little understanding of the immunology, but in the case of some diseases, when we did not even know they were infectious agents (*e.g.* Jenner's original cowpox vaccine). The original polio vaccine worked because humans were the only natural host and there were only 3 strains of pathogenic polio and all 3 were incorporated into an effective vaccine. Within the genera of enteroviruses alone, there are many more candidates that may emerge as important human pathogens for which a comprehensive analysis of strains will be necessary before creating a successful vaccine. Also as clearly demonstrated by RVFV, vaccines that confer protection from systemic disease do not necessarily confer protection for the brain when exposed through unconventional routes.

3. Something about aerosol transmission is associated with uncontrolled neurological infection for agents that are usually spread by other routes. Agents notorious for causing systemic disease (*e.g.* enteroviruses) when delivered through a "natural" route for unknown reasons become highly neurotropic when delivered by aerosol. Perhaps this relates to some selective hole in our natural immune response to inhaled agents that for whatever reason such exposure is not associated with effective immunity for the brain. Such a hole can be readily targeted, if not by nature, then by malign members of our own species.

4. The brain is a marvelously complex organ with phenomenal functionality but in part this complex functionality depends upon intricate RNA processing. Innate immune responses like those precipitated by IFN with shutting down of viral RNA processing may be incompatible with brain function. This implies that our best immune defense could come at the cost of mutual destruction of brain functionality.

Future

How do we protect ourselves in general, and our brains in particular, from viral infections? First we need to know what agents in our environment are potential threats to our brain. This requires vigilance in monitoring and detecting agents as they emerge in zoonotic populations and taking these threats seriously (*e.g.* henipahviruses in pigs and arboviruses in avian populations). Second we need to know what natural immunological mechanisms protect the CNS and how agents circumvent these barriers. With this knowledge in hand, the creation of vaccines and pharmacological therapies can be efficiently designed to target these weaknesses.

While we cannot prevent microbes from mutating and evolving into more lethal pathogens, we can stop trying to give them a helping hand by developing biological weapons and unique ways to expose our fellow man through evolutionarily novel routes. We should also give serious thought to our blithe disregard for evolutionarily ancient ecosystems we destroy on whim. There appears to be no part of this globe that is not subjected to dense human monoculture. We could not intentionally design a more precarious setting ripe for exploitation by highly infectious pathogens. Perhaps a segment of our outbred population

with its tremendous genetically diverse immunity will be protected from the next plague, but it is clear our species is toeing a thin line of survival with some highly lethal agents.

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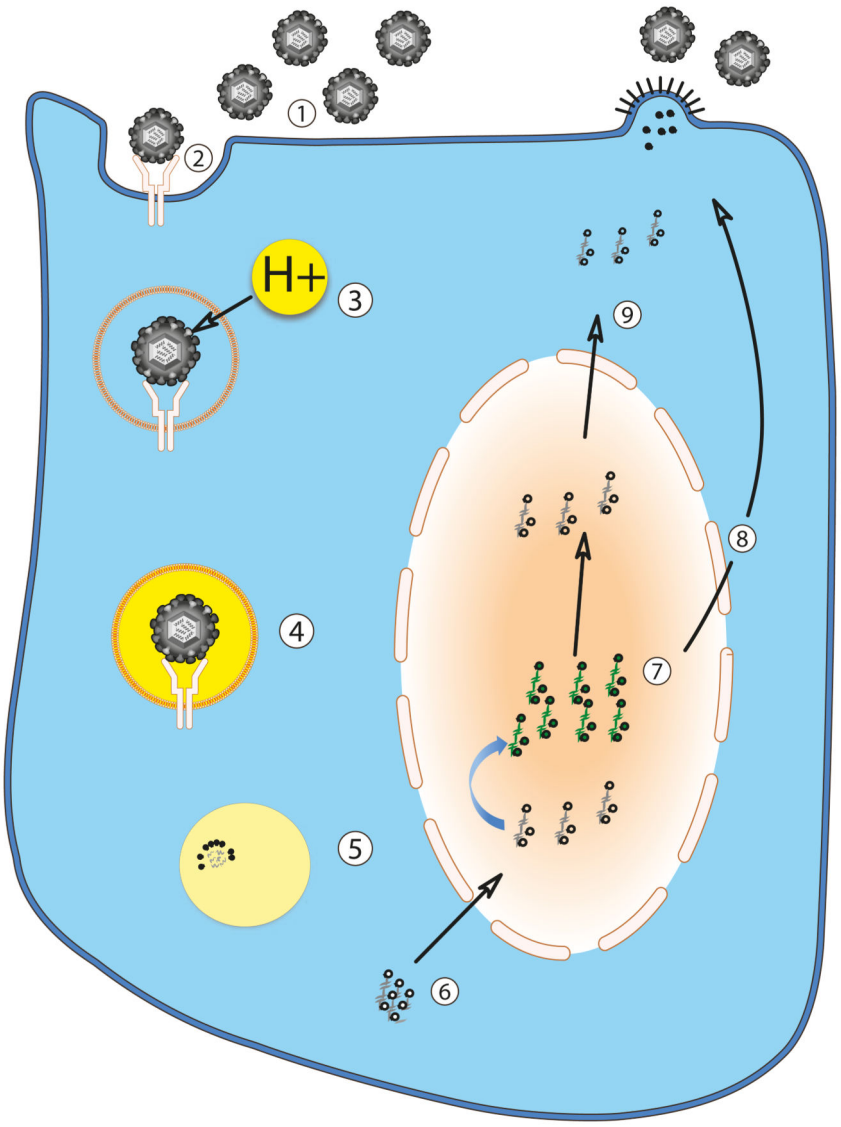

Figure 1. Diagram of influenza A virus infection of the cell

Free extracellular influenza A viruses **(1)** containing hemagglutinin on their envelopes bind sialylated glycoprotein receptors on the host-cell surface **(2)**. The virus enters the host cell by receptor-mediated endocytosis **(2 and 3)**. The resulting endosome becomes acidified by proton transport **(3)**, allowing fusion of the host and viral membranes **(4)**. Blocking acidification of the virion with small molecule drugs is one means of inhibiting viral replication. Fusion is required for metabolism of the nucleocapsid (uncoating) **(5)** and release of the viral ribonucleoprotein complex into host cell cytoplasm **(6)**. The viral RNA and associated viral proteins (including three polymerases) are transported to the nucleus **(7)**. From here positive stranded mRNA templates with poly(A) tails are synthesized (with and without host splicing) at the expense of most host protein synthesis and exported to the cytoplasm for translation **(8)**. Newly synthesized viral ribonucleoproteins are exported to the cytoplasm for eventual virion assembly **(9)**. Influenza A virus assembles and buds from the cell surface **(10)** in a polarized fashion (e.g. from apical surface of epithelial cells).

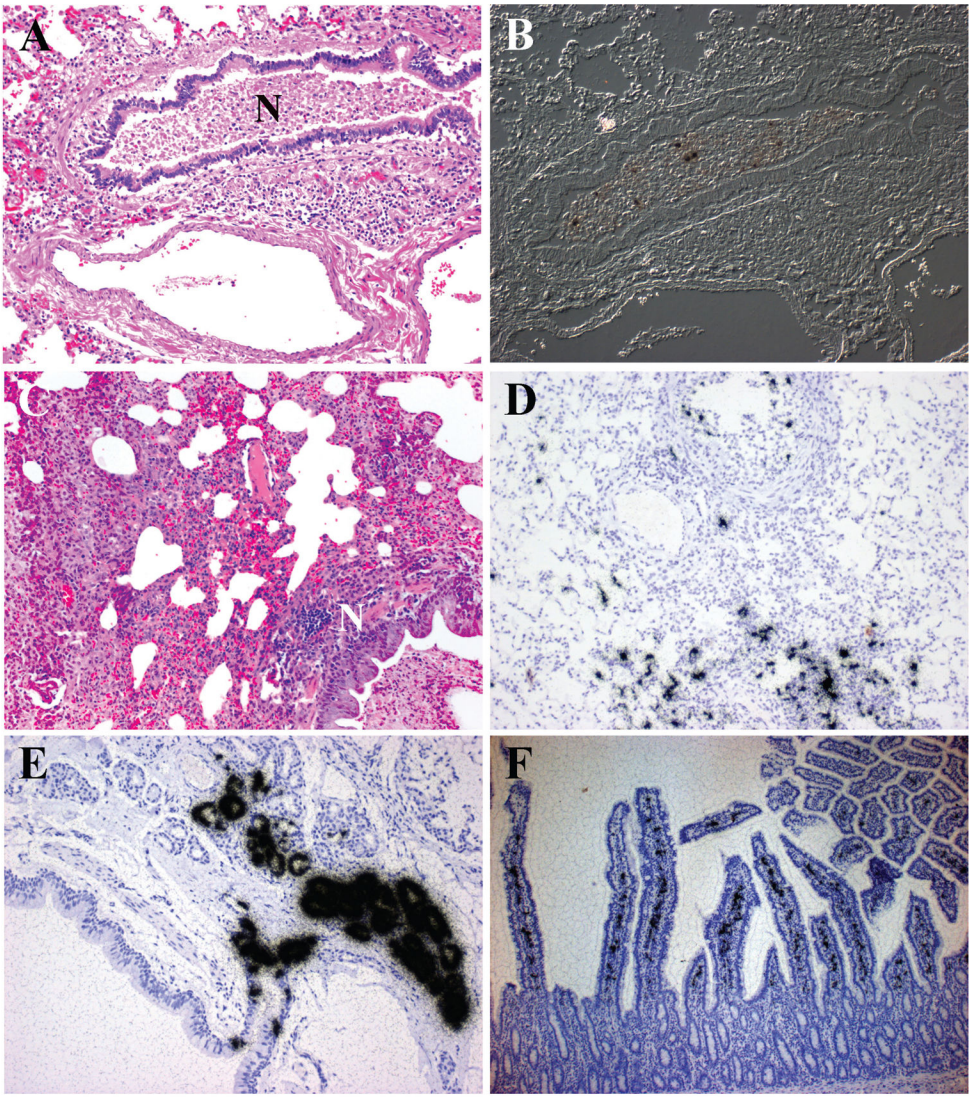

Figure 2. H1N1 virus infection of human and ferret

(A and B) Histopathology of the lung from a patient who succumbed to H1N1pdm09 virus infection. **(A)** H&E stained paraffin section demonstrates a severe bronchopneumonia. Necrotic debris (N) fills the lumen of a moderate size bronchiole. Surrounding alveolar tissue shows edema and severe inflammation. **(B)** Differential interference contrast and *in situ* hybridization and for influenza matrix protein RNA (black grains) demonstrates infected cells within the necrotic debris. At this late stage of infection, the immune response has cleared the majority of virus. **(C–E)** Histopathology of lungs from ferrets inoculated with H1N1 virus intranasally. **(C)** H&E stained paraffin section illustrates the severe bronchopneumonia at 5 days post-infection. Necrotic debris (N) fills the lumen of a moderate size bronchiole. Surrounding alveolar tissue shows edema and severe inflammation. **(D)** *In situ* hybridization for influenza matrix protein RNA (black grains) (counterstained with hematoxilyn) shows infected cells in epithelial cells of the bronchi and alveoli at 3DPI. More virus is detected in the ferret lung suggesting the animal was sacrificed at an earlier stage of infection than when the human case died. By 8 day post-

infection, no virus is detected in the ferret lung. **(E)** *In situ* hybridization for influenza matrix protein RNA (black grains) (counterstained with hematoxilyn) illustrates the severity of submucosal gland involvement as early as 1 day post-infection. **(F)** The histopathology of small bowel from a ferret infected with H1N1 virus 14 days previously. *In situ* hybridization for influenza matrix protein RNA (black grains) (counterstained with hematoxilyn) demonstrates infected cells within the lamina propria at a time when virus cannot be detected anywhere else systemically.

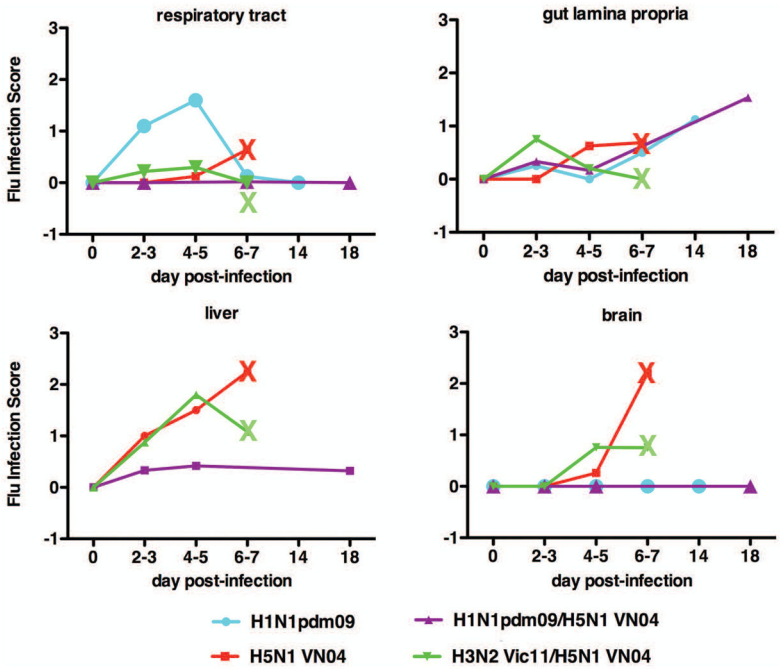

Figure 3. Distribution and quantitation of influenza infection in the ferret at different time points after infection

Throughout the time course of infection with H1N1pdm09 virus, viral infected cells are restricted to the respiratory tract except for a late chronic infection of the gut lamina propria. Infection with H5N1 virus (VN04) follows an entirely different course. While beginning in the lung, H5N1 virus infection quickly spreads to systemic organs. H5N1 virus can be detected in the liver by 2 days post-infection (DPI) and as early as 4DPI in the brain. At the terminal stage of infection (marked by X on the line chart) the vast majority of virus can be detected within the brain, while infection in the lung has begun to abate. Ferrets infected first with H1N1pdm09 or H3N2 virus (Vic11) followed by H5N1 virus (VN04) challenge three months later have different outcomes as well. Prior infection with H1N1pdm09 virus protects the ferret from H5N1 virus infection except for a late chronic infection of the gut lamina propria and liver. Prior infection with H3N2 virus leads to systemic spread of H5N1 virus to the brain and liver with lethal encephalitis by 6DPI (marked by X on line chart).

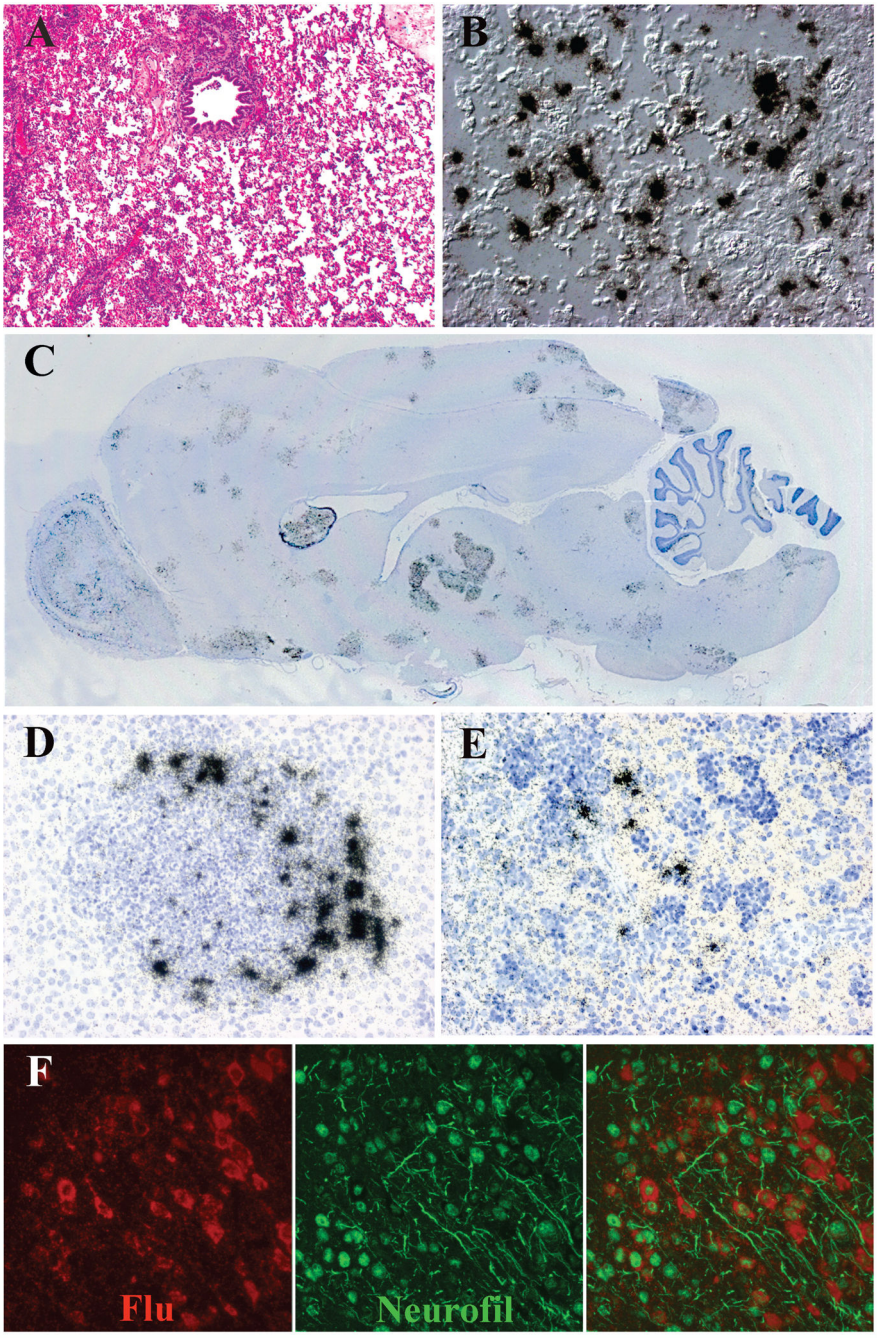

Figure 4. H5N1 virus infection of the ferret

(A and B) The histopathology of the lung from a ferret infected with H5N1 virus 5 days previously. **(A)** H&E stained paraffin section demonstrates a severe broncho- and alveolar pneumonia. **(B)** Differential interference contrast and *in situ* hybridization for influenza matrix protein RNA (black grains) demonstrates infected alveolar cells in lower airway at 2 days post-infection (DPI). **(C)** Whole mount of the ferret brain 6 DPI with H5N1 virus hybridized with radioactive probes to influenza matrix protein RNA (black grains) demonstrates multifocal infection in olfactory cortex, cerebral cortex, deep gray nuclei and

brainstem. **(D)** *In situ* hybridization for influenza matrix protein RNA (black grains) (counterstained with hematoxilyn) shows infected cells in liver surrounding intense inflammatory nodules. **(E)** *In situ* hybridization for influenza matrix protein RNA (black grains) (counterstained with hematoxilyn) illustrates infected cells in splenic red pulp at 18DPI. **(F)** Double label *in situ* hybridization for influenza matrix protein RNA (red) and immunohistochemistry for neurofilament (green) shows infection of neuronal elements.

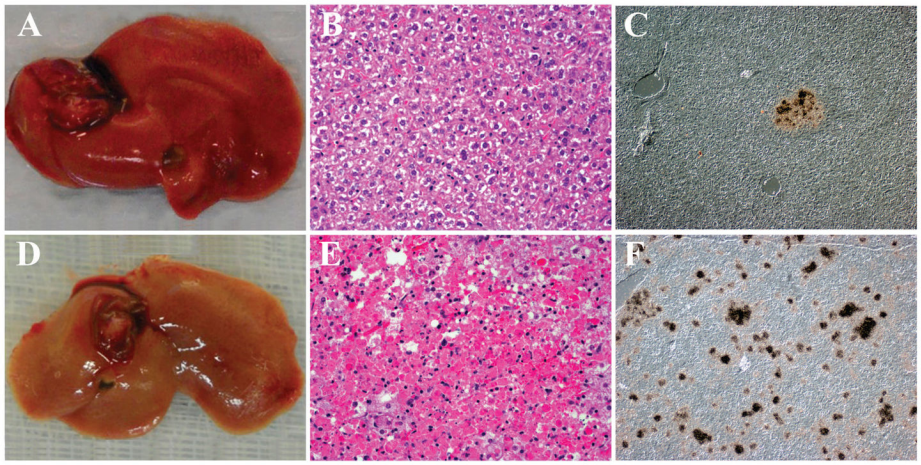

Figure 5. Intraperitoneal (IP) infection of the mouse with pathogenic and nonpathogenic RVFV

IP infection of mice with nonpathogenic (MP12) RVFV leads to no significant weight loss over 4 days, while infection with pathogenic RVFV (ZH501) leads to rapid weight loss and lethal infection within 4 days. **(A and D)** Gross photographs of livers of mice infected with MP12 and ZH501 strains of RVFV. **(A)** MP12 infection shows no significant gross pathology at 4 days post-infection (DPI), while livers of mice infected with pathogenic RVFV (ZH501) are pale in color **(D). (B and E)** H&E stained paraffin sections from livers of mice infected with MP12 and ZH501 strains of RVFV. **(B)** Mice infected with nonpathogenic RVFV (MP12) show normal histology, while sections from livers of mice infected with pathogenic RVFV (ZH501) demonstrate widespread necrosis **(E). (C and F)** Differential interference contrast and *in situ* hybridization for RVFV RNA (black grains) in liver. **(C)** Mice infected with nonpathogenic RVFV MP12 show rare foci of parenchymal infection, while similar studies of mice infected with pathogenic RVFV ZH501 show multifocal necrosis of abundant viral-infected hepatocytes **(F)**.

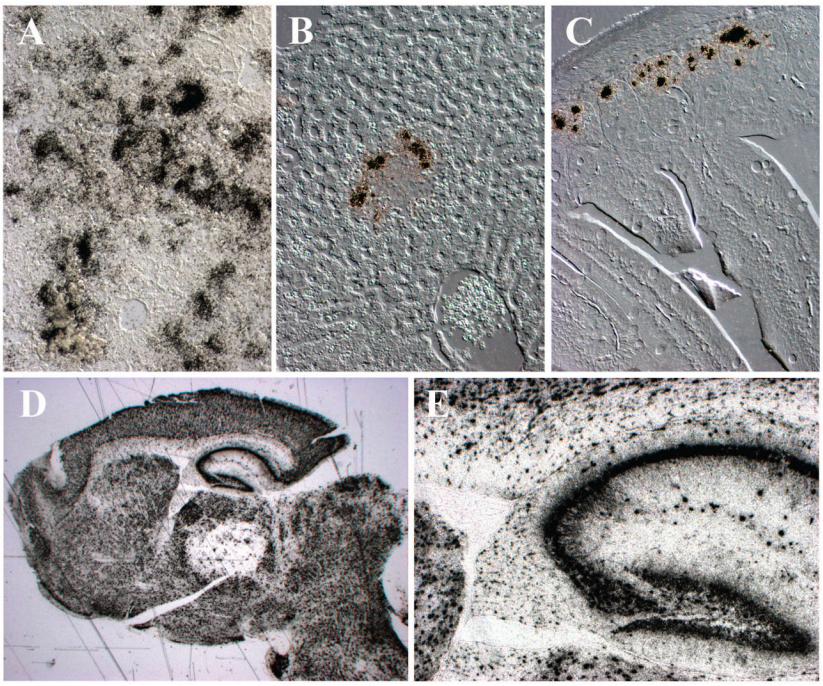

Figure 6. Aerosol infection of non-immunized and previously immunized mice with pathogenic RVFV

Non-immunized mice show a severe hepatitis after aerosol exposure to ZH501 RVFV, while previously immunized mice show only mild hepatitis after aerosol exposure. **(A–F)** Differential interference contrast and *in situ* hybridization for RVFV RNA (black grains). **(A)** Non-immunized mouse shows severe hepatic infection at 6 days post-infection (DPI). Infection of the liver is delayed with aerosol infection compared to intraperitoneal infection. **(B)** Mice immunized with alphavirus replicons expressing the Gn glycoprotein of RVFV show occasional infected foci in the liver 3DPI. Virus is cleared from the liver by 6DPI. **(C)** 3DPI after aerosol exposure to RVFV, enteric infection is observed in epithelial cells at the depths of small bowel crypts in mice immunized with alphavirus replicons expressing the Gn glycoprotein of RVFV. **(D& E)** Despite modest systemic infection in mice previously immunized with DNA plasmids expressing Gn glycoprotein of RVFV fused to three copies of complement protein (C3d), aerosol exposure to RVFV ZH501 leads to lethal encephalitis 7 to 10 days later. **(D)** The brain of terminally ill mice illustrates that the vast majority of neurons are infected. **(E)** High power image of the hippocampus of (D).

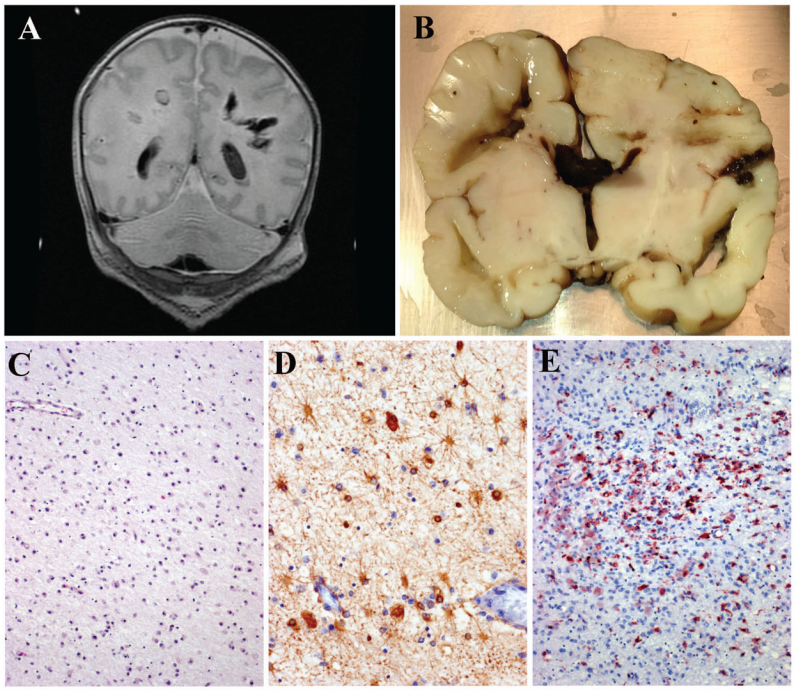

Figure 7. HPeV3 infection of neonates

(A) T1-weighted non-contrast MRI from an HPeV3-infected infant. The child was healthy at birth but developed “neonatal sepsis” after exposure to an ill adult 30 days after delivery. Initial radiologic studies were normal; but after developing seizures, subsequent scans demonstrated cavitary deep white mater lesions. The infant died the following day. **(B)** Gross coronal section of the infant’s brain confirms the presence of deep-seated periventricular cavitary lesions with associated hemorrhage. **(C)** H&E stained sections adjacent to the cavitary lesions demonstrate a bland gliosis with mineralization and no adaptive immune cell infiltration. **(D)** Immunohistochemistry for GFAP confirms perilesional astrocytosis, while immunohistochemistry for CD68 confirms perilesional microglial activation **(E).**

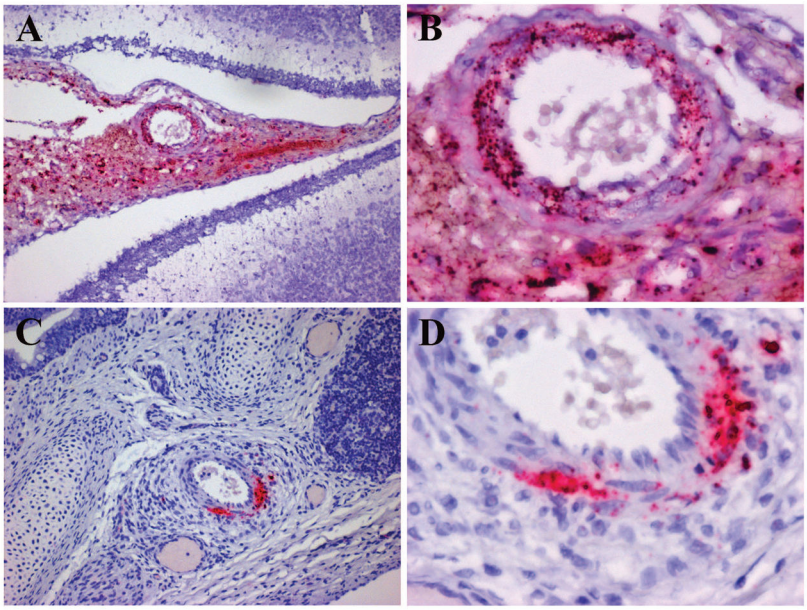

Figure 8. HPeV3 infects blood vessel smooth muscle cells in leptomeninges and pulmonary vasculature

(A and B) Paraffin embedded cerebellum and overlying leptomeninges probed for HPeV3 RNA using in situ hybridization (red) (counterstained with hematoxilyn). Abundant HPeV3 viral RNA is confined to the modestly hypercellular leptomeninges with no evidence of infection of the brain parenchyma. Higher power **(B)** image of (A) confirms presence of HPeV3 RNA in leptomeningeal cells and particularly in smooth muscle cells of blood vessel walls. **(C and D)** Paraffin embedded lung probed for HPeV3 RNA using in situ hybridization (red) (counterstained with hematoxilyn). HPeV3 RNA are confined to the modestly hypercellular pulmonary arteries without evidence of lung parenchymal infection. Higher power **(D)** of (C) confirms presence of HPeV3 RNA in smooth muscle cells of blood vessel walls. These observations suggest that damage noted in severe periventricular leukoencephalopathy damage is an indirect effect of vascular compromise to metabolically active regions.