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CHAPTER 1

Overview of Rhabdo- and Filoviruses

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Summary

Enveloped viruses with a negative-sense, single-stranded monopartite RNA genome have been classified into the order *Mononegavirales*. Five families of viruses that constitute the order are: *Rhabdoviridae*, *Filoviridae*, *Paramyxoviridae*, *Bornaviridae* and *Nyamiviridae*. Members of these families possess a helical nucleocapsid core containing the viral genome and a host-derived lipid envelope containing viral proteins. This introductory chapter provides a brief overview of the *Rhabdoviridae* and the *Filoviridae,* the two families of viruses that are the subject of this book. Many members of these two families are highly significant human and animal pathogens. The rationale and goal of the book is to provide the reader with the most recent information on the structure, genome organization and replication strategy, epidemiology, evolution and emergence, host response to infection, viral countermeasures as well as vaccines and antivirals against these pathogens. More detailed descriptions of these topics are presented in individual chapters of this book.

1. Introduction and Rationale

Rhabdoviridae is one of the most diverse families of viruses with its members having been isolated from vertebrates, invertebrates, and plants. Of the eleven genera of viruses recognized in this family,[1] eight are known to infect vertebrates, two infect plants and one infects invertebrates. During the past several decades, understanding of the basic molecular biology and pathogenesis of *Rhabdoviridae* have been primarily derived from studies using vesicular stomatitis virus (VSV), a member of the *Vesiculovirus* genus and rabies virus (RABV), a member of the genus *Lyssavirus*. Both VSV and RABV share many characteristics including structure, genome organization, and replication strategy but differ significantly in their pathogenic attributes. Members of the other genera of the family also appear to have similar characteristics, although many of them differ in the number of additional accessory genes they encode, in addition to the typical set of primary genes common to all members of *Rhabdoviridae* family (see below).

The family *Filoviridae*, on the other hand, contains only three genera, *Ebola-*, *Marburg-* and *Cuevaviruses*, with *Ebolavirus* genus having five recognized species whereas the other two genera contain only one species each.[1] Members of *Filoviridae* cause highly lethal hemorrhagic fever disease in humans and non-human primates with case fatality rates of over 50%. They exhibit pleomorphic structures but their overall genome organization, and the basic replication strategy are similar to each other and to some extent similar to those of *Rhabdoviridae*, although significant differences have been noted.

The goal of this book is to provide an in-depth review of the most recent information that highlights advances in our understanding of the replication, assembly, epidemiology/evolution and pathogenesis of these two related virus families. We have solicited experts in the field to write chapters that cover these topic areas, as well as how our understanding of virus-host interactions has resulted in the development of novel vaccines and therapeutic strategies to combat infectious diseases (both viral and bacterial) and cancer. Progress in our understanding of virus replication has also led to the development of novel vectors used to study the complex circuitry of the central nervous system (CNS). It is our hope that

the information assembled in this book will be a resource for both students seeking advanced degrees as well as seasoned investigators who wish to exploit these viruses in their own research endeavors. The following sections outline the major topic areas found in the book and highlight the most recent advances in our understanding of rhabdovirus and filovirus biology. We hope readers find this book as useful for gaining advanced knowledge about these two important virus families as we found it rewarding to assemble.

2. Structure, Genome Organization, and Replication Strategy (Chapters 2, 3, 4, 5, 6, 8, 14, 17, 19, & 20)

Members of *Rhabdoviridae* possess a rod- or bullet-shaped morphology and package the five essential viral proteins: the glycoprotein (G), the matrix protein (M), the nucleocapsid protein (N; also called nucleoprotein, NP), the viral RNA polymerase consisting of the phosphoprotein (P) and the large (L) proteins in addition to the viral RNA genome. Both VSV and RABV exhibit bullet-shaped morphology with a flat base and a conical or dome-shaped tip. Studies suggest that the determinants of conical or dome-shape tip formation of VSV appear to reside primarily on the viral N protein.[2] The envelope of the virion is studded with the trimeric viral G protein and the virion core contains the viral nucleocapsid (NC) composed of the viral genomic RNA complexed with the N to which the viral RNA-dependent RNA polymerase (L+P) is associated. The M protein bridges the NC core with the G protein on the envelope and provides structural rigidity to the particles. Rhabdovirus genomes are organized in modular fashion with a minimum of five genes encoding the five essential proteins (N, P, M, G, and L) to as many as 15 genes that encode several small accessory proteins,[3] which are likely involved in modulating host response pathways and pathogenesis, although their specific role(s) remains to be determined. In recent years, not only the structure of the intact VSV virions, but also the atomic structures of full-length or fragments of individual viral proteins, as well as the nucleocapsid-like particles of VSV and RABV have been described. The structural studies coupled with reverse genetics approaches as well as biochemical and

mutagenesis studies have led to greater understanding of the overall architecture of the rhabdovirus particles, the viral NC structure, mechanisms of viral transcription and replication, virus particle assembly from individual components, and the role of viral and host proteins in virus life cycle. Each of these aspects has been described in individual chapters in this book.

Unlike the members of the *Rhabdoviridae* family, the members of *Filoviridae* adopt morphologically complex structures from six-shaped, spherical-shaped to long filamentous and flexible doughnut- or toroidal-shaped structures. They range in size from approximately 1μm to 22 μm in length and can package from one to 22 copies of the viral genome, aligned in an end-to-end manner within a single viral envelope.[4] This is unique to the members of *Filoviridae* and is not seen in any of the other families of *Mononegavirales*. Within the viral envelope, the NC is highly flexible and frequently bent to generate the complex structures that are typical for these viruses. The extremely long filamentous structure of virus particles has been suggested to be an adaptation that likely enhances their attachment to cell surfaces for efficient infection.[5] Filovirus entry mechanisms appear to be more complex than Rhabdoviruses and other families of *Mononegavirales*. Although the virus utilizes the human T cell Ig mucin 1 (TIM-1) protein on mucosal epithelial cells to enter, the receptor(s) in most other cell types remains unidentified. Attachment factors including lectins, signaling factors such as the Tyro3/Axl/Mer (TAM) family of receptor tyrosine kinases, integrins, and endo/lysosomal factors such as cathepsin B and L and Niemann-Pick C1 (NPC1) protein play critical roles in filovirus entry and genome uncoating processes.[6,7] The released viral genome in the form of NC undergoes transcription and replication in the cytoplasm. The viral genes are organized in a modular fashion, like those of *Rhabdoviridae* and are transcribed by similar mechanism(s). The steps in filovirus assembly are poorly understood. However, use of cryo-electron tomography and subtomogram averaging principles have revealed both vertical "rocket-like" as well as horizontal "submarine-like" mechanisms of virus budding that occur from infected cells.[8] Further details on the structure of filoviruses, processes of entry, uncoating, genome transcription, and replication have been detailed in individual chapters in the book.

Tremendous progress has been made in our understanding of the molecular biology of replication of Rhabdo- and Filoviruses in the past decades; however, many significant questions remain unanswered. As the viral genomes are coated with nucleocapsid proteins, it is still unclear how mechanistically the viral polymerase accesses the genomic RNA for transcription and replication. As challenging as it may appear, structural studies with actively transcribing/replicating templates coupled with biochemical analysis may reveal insights into structural alterations in the viral nucleocapsids induced by the polymerases that allow access to the RNA genome for transcription and replication. Future studies using advanced microscopy techniques and live-cell imaging approaches are likely to provide a clearer picture of the endocytic entry of these viruses and uncoating of their genomes. Identification of receptor(s) for filovirus entry and detailed investigation of the role of various cellular factors in the highly orchestrated pathway of the virus internalization may provide targets for development of therapeutics for filovirus infection and disease.

3. Epidemiology, Evolution, and Emerging Viruses (Chapters 12, 13, & 18)

As rhabdoviruses are a large and diverse family of viruses with a very wide spectrum of host species including plants and animals and can be transmitted (except for lyssaviruses) by arthropod vectors, the potential for zoonotic and epizootic diseases caused by these viruses always remain high. The *Rhabdoviridae* represents a viral family with the potential to jump species and cause disease in the newly acquired host. This has been exemplified by the isolation of many rhabdoviruses such as Chandipura virus (CHPV),[9] Bas Congo virus (BASV)[10] and other rhabdoviruses from humans with illnesses, although a direct link between the viruses and the diseases remains to be established. The members of genus *lyssavirus* include several related rhabdoviruses that are typically found in bats. The most clinically relevant lyssavirus is the RABV, which causes rabies disease in humans and animals although other lyssaviruses are also known to infect humans causing rabies-like illnesses.[11-13] The bovine ephemeral

fever virus (BEFV), a member of the *ephemerovirus* genus of *Rhabdoviridae* family causes an acute febrile illness in cattle with low mortality rates, which was first reported in early-to-mid 20th century in Africa. The virus continues to emerge and spread and has now been seen in many countries globally including Australia, Africa, Asia, and the Middle East.

Among the *Filoviridae* family members, the two most pathogenic viruses, the Marburg virus (MARV) and the Ebolavirus (EBOV) were recognized in 1967 and 1976, respectively. Since then, these viruses have caused several outbreaks of hemorrhagic fever (HF), mostly in the African nations, where these viruses are presumed to have been originated.[14] The number of HF outbreaks caused by EBOV in recent years is significantly higher than those caused by MARV. The reason(s) for this is unclear but could be related to the conditions that favor EBOV emergence.[15] In the early- to mid-2014, a deadly EBOV emerged with HF outbreaks of unprecedented proportions in West African nations of Guinea, Sierra Leone, and Liberia, resulting in over 5,300 reported cases with over 2,600 fatalities.[16,17] Although bats have been considered to be one of the major reservoirs for filoviruses, finding other host species, understanding their emergence and determining their mode of transmission will be important to control the disease outbreaks.

Like most RNA viruses, the members of *Rhabdoviridae* and *Filoviridae* are prone to mutations and thus have the potential for rapid evolution. Rhabdoviruses, and in particular, VSV have been used extensively to study RNA virus evolution in vitro and in vivo. Critical concepts in viral evolution such as *quasispecies theory*, *competitive exclusion principle*, *red queen hypothesis*, and *Muller's ratchet* were developed or experimental evidence provided by the use of VSV as a model system. The epidemiology, evolution, and emergence of rhabdo- and filoviruses have been elaborated in individual chapters in the book.

4. Host Response and Pathogenesis (Chapters 7, 14, 15, 18, & 21)

Despite similarities in structure and replication strategies, the response of the host to infection by different members of the *Rhabdoviridae*, and the ensuing pathogenic effects that result from infection, varies

widely. This variability is best exemplified by the very different diseases caused by most vesiculoviruses such as VSV in comparison to the lyssaviruses, such as RABV. VSV causes an acute illness in pigs, horses, cattle and other ruminants which is typified by vesicular lesions on the mouth, tongue, hooves and udder that generally resolve without significant sequelae. Infection of humans with VSV (Indiana serotype) is rare and healthy individuals are either asymptomatic or they may exhibit mild flulike symptoms. However, some vesiculoviruses can cause serious human morbidity, such as is seen with CHPV.[18] RABV, in contrast to the acute illness typified by vesiculoviruses, causes a slow progressive disease that manifests with entry of the virus into the CNS causing death due to massive encephalitis, dissemination to peripheral organs and eventual failure of the autonomic nervous system. VSV and other mammalian vesiculoviruses are transmitted to animals and humans by arthropod vectors, such as biting flies, whereas RABV is a mammalian-restricted virus. In cell culture, most vesiculoviruses, ephemeroviruses, and novirhabdoviruses cause extensive cytopathic effects (CPE) that are observed very soon after infection, whereas many lyssaviruses-infected cells exhibit little to no CPE. These differences are due to the way host cells respond to the respective virus infections. For example, VSV causes a rapid shut-down of host protein synthesis as the primary mechanism responsible for limiting the host's innate immune responses to the virus.[19] This effect is mediated by the matrix (M) protein and results in apoptosis.[20] In contrast, RABV relies on direct inhibition of the interferon response pathway though activities of the P protein.[21]

Two of the major themes of this book are to explore how our understanding of virus-host interactions can be used to control infections and reduce mortality, and how we can employ this knowledge to develop novel therapeutic modalities using modified, recombinant viruses. In addition, it is clear that the interplay between the innate immune system, the molecules that recognize PAMPs to signal invasion by viruses, and previously unrecognized host factors that intersect these pathways will lead to new discoveries that could allow the development of new strategies for combating viral infections. This is needed no where more than in developing therapeutics for the filoviruses, which like RABV, are among the most deadly viruses known to man.

Filoviruses cause severe HF that can lead to shock, multi-organ failure, and death. While the pathogenesis of filovirus HF is not fully understood, early infection of phagocytic cells results in systemic spread of the virus that elicits a cytokine storm which contributes to the coagulation abnormalities and vascular leakage that are the hallmarks of filovirus HFs. A major contributing factor to the robust replication properties of filoviruses within an infected animal is the varied strategies these viruses use to evade the host immune response. Indeed, it is a race between the virus and the host as to whether the host can mount a sufficient immune response to limit the infection before abundant virus replication takes place leading to disease manifestations.

Several filoviral proteins have been shown to be antagonists of the innate immune response. These include VP35, which inhibits the RIG-I-like receptor signaling pathways that typically trigger interferon (IFN) production,[22] the VP24 protein which inhibits nuclear translocation of the STAT1 transcription factor that is critical for IFN-induced gene expression,[23] and for MARV, VP40 protein which has been shown to block IFN-induced activation of the kinase Jak1.[24] In addition, it has been shown that GP can antagonize tetherin/BST-2, which restricts the budding of several enveloped viruses by tethering them to the cell surface.[25] Thus targeting these proteins may offer a potential strategy for development of small molecule inhibitors to treat filovirus infections.

5. Vaccines and Antivirals (Chapters 9, 16, & 22)

One of the most remarkable landmarks in the treatment of viral diseases was development of the RABV vaccine by Louis Pasteur in 1885. Infection by RABV is unique in that the vaccine can be given after exposure, commonly known as post-exposure prophylaxis. This is due to the very slow replication of RABV in the periphery prior to entry into the CNS. Currently, there are no other vaccines used in humans for any of the other rhabdoviruses, although there is a vaccine that is used to prevent vesicular stomatitis in horses. However, there is tremendous interest in developing vaccines for a wide range of infectious diseases using recombinant rhabdovirus-based vectors. This became possible with the advent

of "reverse genetics system"[26] that led to recovery of fully replicating, recombinant rhabdoviruses, which was achieved first for RABV[27] and soon after for VSV.[28,29] Over fifty pre-clinical studies using VSV-based vaccine vectors have demonstrated effectiveness in animal models of HIV/AIDS, and in multiple other viral and bacterial disease models. For some, indications are that these vectors provide protective immunity after just a single dose. Remarkably, they can also be effective when given after exposure for some infections including both Ebola and Marburg viruses,[30] which is similar to the current post-exposure prophylaxis used for RABV. In addition, a VSV vector encoding the papilloma virus E proteins has been shown to act as a therapeutic vaccine against tumors caused by papilloma virus in a rabbit model.[31]

In addition to the very promising replication-competent VSV-based vaccines for the filoviruses, remarkable progress has been made in developing non-replicating preventive vaccines[32] and antivirals against Ebola and Marburg viruses. Included among these therapeutic modalities are antibody cocktails,[33] siRNAs,[34] and a nucleoside analogue that inhibits filovirus replication.[35] While efficacy for the filovirus vaccines and the various therapeutics has been shown in non-human primate models, it has not yet been determined whether the vaccines will provide broad-spectrum immunity against different species of Ebola and strains of Marburg viruses, or whether the various therapeutics will prove efficacious in humans. However, as data from the initial Phase I studies emerge, the path to developing these anti-filovirus modalities will become clearer.

6. Applications Using Recombinant Viruses (Chapters 9, 10, & 11)

The development of both replicating and single-cycle vaccine vectors based on recombinant VSV and RABV is just one of the high-impact outcomes that have resulted from years of extensive research into the understanding of the requirements for rhabdovirus replication, assembly and virus-host interactions. The two other areas that will likely have a significant impact on human health and that are currently being developed is the use of VSV-based oncolytic vectors that preferentially replicate in and kill tumor cells without adversely affecting healthy cells, and the use

of rhabdovirus-vectors to dissect the interconnections within the CNS.

The recognition that many different types of viruses can target and kill tumor cells has resulted in an expansive body of work over the last 10-15 years evaluating the mechanisms responsible for the oncolytic activities of these viruses. VSV and the related Maraba virus have emerged as some of the best oncolytic viruses and a recombinant VSV encoding interferon-β (IFN-β) is currently being evaluated in a Phase I clinical trial for hepatocellular carcinoma (ClinicalTrials.gov, 2014, trial IDNCT01628640; http://clinicaltrials.gov/ct2/show/NCT01628640). These rhabdoviruses are naturally oncolytic due to their exquisite sensitivity to IFN, the robust growth characteristics of the vesiculoviruses, and the fact that most people are immunologically naïve to these viruses, thus reducing the concern of pre-existing antibody that would limit the efficacy of these viruses when given to patients. All oncolytic vesiculoviruses contain attenuating mutations, mostly in the M protein, which increases their sensitivity to IFN.[36] Because many tumor cells lack the ability to mount an IFN-induced antiviral response, this provides the tumor selectivity needed for a successful oncolytic agent. In addition to these IFN-sensitizing mutations in M protein, a wide-range of transgenes that are encoded by recombinant oncolytic VSV are being evaluated. These include various microRNAs, inhibitors of the host antiviral response, suicide genes, cytokines and tumor-associated antigens to promote induction of an anti-tumor response by the host. The potential for oncolytic rhabdoviruses to benefit patients with cancer is very high and the fact that both rhabdovirus-based oncolytic and vaccine vectors are moving from the bench to the clinic is truly exciting and a huge milestone for the field.

Another exciting application is the use of rhabdoviruses to map the neural circuitry within the brain. The Brain Initiative aims at revolutionizing our understanding of the human brain, and mapping all synaptic connections (i.e. the connectome) of the brain is an important and critical component of the initiative. One of the approaches to achieve this goal is to use recombinant neurotropic viruses, such as VSV and RABV to trace synaptically connected neurons. Strategies using recombinant viruses in which the glycoprotein gene is deleted allow for monosynaptic tracing, while others that have alternative or modified envelope proteins that allow more restricted targeting to defined cell types are also being used. While the use of recombinant VSV and RABV is proving to be an

extremely powerful approach, there are a number of caveats and limitations that remain. However, the development of rhabdovirus variants that express calcium sensors,[37] glutamate sensors,[38] ChR2,[39] sub-cellular markers[40] and Cre recombinase[41] will undoubtedly provide the tools that, when combined with appropriate optogenetic, electrophysiological, and imaging methods, will result in a more complete understanding of how the neuronal connectome makes us who and what we are. It is truly an exciting time for virology and we are only now beginning to reap the fruits of our labor to move beyond asking fundamental questions in cell biology and molecular virology, to providing therapeutics and tools that will have a broad impact on human health.

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