Drug Targets in Infections with Ebola and Marburg Viruses

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Abstract: The development of antiviral drugs for Ebola and Marburg viruses has been slow. To date, beyond supportive care, no effective treatments, prophylactic measures, therapies, or vaccines are approved to treat or prevent filovirus infections. In this review, we examine the current treatments available to administer care for filovirus infection, the potential therapeutic targets that can be used for filovirus drug development, and the various drug targeting techniques used against filoviruses.

Keywords: Filoviridae, Ebola virus, Marburg virus, drug targets, therapeutics, pathogenesis, pathology, immunotherapy

INTRODUCTION

Due to high morbidity and mortality rates, filoviruses are considered among the deadliest of human pathogens. *Ebolavirus* and *Marburgvirus*, the two genera of the family *Filoviridae*, pose a significant threat to military personnel, global security, and public health [1]. Clinical symptoms appear suddenly after an incubation period of 2 to 21 days. Patients often present with complaints of high fever, chills, malaise, and myalgia. As the clinical disease progresses, there is evidence of multisystemic involvement, and manifestations include prostration, anorexia, vomiting, nausea, abdominal pain, diarrhea, shortness of breath, edema, confusion, and coma. Case fatalities often range from 23-90% depending on strain or species [2]. Fatal filovirus infections are usually associated with high viremia, increased endothelial cell permeability, widespread focal tissue destruction, severe coagulation abnormalities, and lymphopenia [3]. Great apes are now recognized as accidental hosts with outcomes from infections as pathogenic, if not more so, than in humans. Filoviral infections among the great apes have had devastating effects on the primate population; in some areas almost potentially eliminating the species [4, 5]. More than 40 years of effort have been focused on the search for the reservoir of these viruses in Central Africa. The culmination of this work recently implicated three species of fruit bats: *Hypsignathus monstrosus* (hammer-headed fruit bats), *Epomops franqueti* (singing fruit bats), and *Myonycteris torquata* (little collared fruit bats) as the reservoirs of *Ebolavirus* [6]. Likewise, Towner *et al.*, have demonstrated *Marburgvirus*-specific RNA and serological evidence in the fruit bat *Rousettus aegyptiacus* in Gabon, Africa [7].

Our understanding of filovirus pathogenesis in humans has been hampered by the geographical locations where many of the outbreaks occur, such as remote areas of Africa. Medical equipment for rapid diagnosis, clinical monitoring, and management is often in short supply or absent. To date, the majority of the work performed to characterize disease course and pathogenesis has been performed in animals. While a tremendous amount of work has been accomplished in the last decade, there remains much to be accomplished to identify effective therapeutics. Critical to this process will be not only identifying and optimizing therapeutics but also confirming that the models being used in the laboratory are predictive or reflective of human disease.

Filoviridae

The *Filoviridae* family contains the two genera, *Marburgvirus* and *Ebolavirus*. The *Marburgvirus* genus contains a single species: Lake Victoria Marburg virus (LVMARV). The *Ebolavirus* genus consists of the four species of Ebola virus (EBOV): Zaire EBOV (ZEBOV), Sudan EBOV (SEBOV), Reston EBOV (REBOV) and Ivory Coast EBOV (ICEBOV). After a recent outbreak in Uganda, a fifth species of EBOV has been proposed [8].

The first outbreak of filovirus hemorrhagic fever occurred during simultaneous outbreaks in Marburg and Frankfurt, Germany, and later in Belgrade, Yugoslavia during 1967 [9]. The agent responsible for the outbreak was named Marburg virus for the German town where illness was initially observed [9]. This newly emergent virus would later be classified as the first recognized member of the family *Filoviridae* [10]. Since 1967, MARV has only surfaced in sporadic outbreaks in South Africa [11], Kenya [12, 13], the Democratic Republic of Congo [formerly Zaire [14]], Uganda [15], and most recently in Angola [16]. EBOV (named after Ebola River in the Democratic Republic of Congo) was first recognized during nearly simultaneous outbreaks in Sudan and Democratic Republic of Congo in 1976 by serologically distinct viral species of EBOV [17, 18]. Since that time, outbreaks of EBOV hemorrhagic fever have been largely confined to the African continent. The one exception to this is REBOV and the outbreaks in non-human

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1871-5265/09 $55.00+.00

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
23 OCT 2009	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Drug targets in infections with Ebola and Marburg viruses. Infectious Disorders - Drug Targets 9:191-200	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
Olinger, GG Biggins, JE Melanson, V Wahl-Jenson, V Geisbert, TW Hensley, LE	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD	**TR-09-014**
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The development of antiviral drugs for Ebola and Marburg viruses has been slow. To date, beyond supportive care, no effective treatments, prophylactic measures, therapies or vaccines are approved to treat or prevent filovirus infections. In this review, we examine the current treatments available to administer care for filovirus infection, the potential therapeutic targets that can be used for filovirus drug development, and the various drug targeting techniques used against filoviruses.

15. SUBJECT TERMS

filovirus, Ebola, Marburg, drug targets, antivirals, drug development

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**SAR**	**9**	

primate (NHP) housing facilities in Virginia, Texas, Italy, and the Philippines [2]. Despite the high mortality observed in the NHP housed in these facilities, to date there have been no clinical infections associated with this species. The ICEBOV species was identified in Cote d'Ivoire in chimpanzees. A single documented, non-fatal case occurred in an individual performing a necropsy on an infected chimpanzee. A second suspected case was identified based on the presence of antibody.

Filoviruses possess an approximately 19kb, single-stranded, non-segmented, negative-sense RNA genome that is encased within the ribonucleoprotein complex. The viral genome contains seven genes that lead to the synthesis of seven structural proteins. While the various viral structural protein functions and genomic structures are similar for EBOV and MARV, the homology at the amino acid level is less than 55% [2]. Four of the proteins comprise the ribonucleoprotein complex: virion proteins (VP) 30 and VP35, the nucleo-protein (NP), and the viral RNA-dependent RNA polymerase (L). Homotrimers of the viral glycoprotein (GP) cover the surface of the virion. These homotrimers are comprised of $GP_{1,2}$ which has been cleaved from its precursor by cellular proteases [19]. For EBOV, the GP has also been observed in a secreted form which may contribute to the pathogenesis of the virus by a mechanism that is not yet fully understood [20-26]. It is believed that the viral GP is the sole host cell attachment factor for filoviruses, but the viral receptor remains unknown as filoviruses exhibit a wide cellular tropism in infected individuals. After entry, filoviruses replicate their genomes and viral proteins in the cytoplasm. Particle assembly then occurs at late endosomal surfaces with the help of the viral matrix protein, VP40, and various cellular factors. VP40 consists of two domains: an N-terminal oligomerization domain and a C-terminal membrane-binding domain [27]. Interestingly, VP40, can self-oligomerize and, when co-expressed with the viral GP, forms virus-like particles [28, 29]. VP40 also contains highly conserved "late-domain" motifs that may enable the protein to interact with cellular components during assembly and budding. These domains consist of the short PT/SAP, PPxY, or YxxL amino acid sequences of which VP40 contains two overlapping PTAP and PPxY motifs [reviewed in [30]]. Therapeutically targeting potential interactions between VP40 and cellular components via these motifs will be discussed in this review.

Pathogenesis

EBOV infection of humans and NHP is characterized by marked lymphopenia and severe degeneration of lymphoid tissues and defects in the coagulation system. Upon filovirus infection, dendritic cells (DC) and macrophages (Mϕ) are early and sustained targets. Infection of the DC likely compromises the ability of the host to respond to infection by EBOV or MARV. *In vitro* infection of DC has been shown to inhibit the upregulation of co-stimulatory molecules such as B7-1 and B7-2 [31, 32]. The result is the inability of these infected cells to effectively stimulate T cells. While the impact of infection of DC is still under investigation *in vivo*, the apparent lack of activation of T cells *in vivo* is consistent with this theory. In addition, this subversion of the DC responses may contribute to the observed lymphocyte apoptosis through the lack of co-stimulatory molecules. While the lack of co-stimulatory molecules likely contributes to some of the observed apoptosis, it is likely that there are multiple etiologies.

Infection of monocytes/Mϕ sets in motion a number of events. Once infected, Mϕ will facilitate distribution by carrying the virus to lymphoid tissues, eventually leading to the seeding of all major organs. In addition, infection of Mϕ also likely elicits an initial response cascade contributing or initiating an overwhelming inflammatory response. The release of cytokines and chemokines has a profound impact at both the local and systemic levels. It is the release of these cytokines that likely induces many of the disease symptoms (e.g., fever, myalgia). When present in sufficiently high levels, these proteins can have toxic and/or lethal effects. In addition to inducing a proinflammatory state, infection of Mϕ has also been demonstrated to upregulate the pro-coagulant protein tissue factor (TF) [33]. Overexpression of TF will induce activation of the coagulation cascade predominantly through the extrinsic pathway contributing to the development of a consumptive coagulopathy and eventually disseminated intravascular coagulation (DIC).

While DIC does occur, hemorrhage, which is often thought of as another hallmark of the EBOV of MARV infections, is often atypical. Abnormalities in blood coagulation and fibrinolysis are often manifested as petechiae, ecchymoses, uncontrolled bleeding at venipuncture sites, or mucosal hemorrhages. The presence of a maculopapular rash is typical, but is not pathognomonic for EBOV hemorrhagic fevers. Fibrin deposition is prominent during EBOV-infection of NHP. In addition, consumption of clotting factors, increases in clotting times, as well as increases in levels of fibrin degradation products (a hallmark of DIC), are all observed in EBOV-infected NHP. The presence of fibrin degradation products has now been documented in human cases of EBOV hemorrhagic fever [34]. Recent studies confirmed that the coagulation abnormalities are not the direct result of damage to the endothelium but rather are likely due to a combination of factors such as the over-production of TF and proinflammatory cytokines in conjunction with substantial drops in protein C. Infection of endothelial cells appears to occur late in the disease course in EBOV-infected NHP, after the onset of coagulation abnormalities [35]. Ultrastructural examination of tissues collected from infected NHP demonstrated an activation and disruption of the endothelium. However, these changes did not appear to be directly associated with viral replication in endothelial cells.

The coagulation cascade and inflammatory pathways are intertwined. The process of coagulation and inflammation are entangled in many ways, making it difficult to determine the role of any particular factor. For example, interleukin (IL)-6, which has consistently been shown to be upregulated in filovirus infections, can upregulate expression of TF, thereby exacerbating activation of the coagulation cascade. Fibrin degradation products and thrombin can increase the production of pro-inflammatory cytokines such as IL-6. These feedbacks create a spiral of events that establishes what is referred to as a cytokine storm, hypo-cytokinemia or a severe inflammatory response syndrome (SIRS). To date,

there are over 150 inflammatory mediators, reactive oxygen species, and pro-coagulant proteins associated with this state. This syndrome is most often associated with bacterial septic shock. Similar to septic shock is this lack of homeostasis and uncontrolled host response to the invading pathogen that likely contributes to the disease pathology rather than the pathogen itself. Recently, intervention in ZEBOV-infected NHP with recombinant human-activated protein C, the only approved treatment of severe sepsis, increased survival from 0% to almost 20% and significantly increased the mean time to death in almost 60% of the treated animals [36].

THERAPEUTIC DEVELOPMENTS

Historically, the development of effective therapeutics or vaccines for filoviruses seemed unattainable. The recent successes with various vaccine candidates [reviewed in [37]] and the concurrent understanding of basic biology and pathogenesis of MARV and EBOV has permitted the filovirus research community the opportunity to consider a widening range of therapeutic approaches and brings the hope of an intervention within reach. Based on the experiences with other antiviral programs, the development of filovirus therapeutics will be tedious, requiring a large number of candidate compounds and a substantial investment. As a result of industry standards, the current anti-filoviral drug discovery is merely in the emergent stage of development. New funding initiatives and technology pushes may help to reduce the overall costs and time required to develop licensed drug therapies. Currently, supportive care, immunotherapy, and some emergency interventions have been used to treat filovirus infections with varying success. Despite these barriers, several promising drug candidates have emerged over the last few years.

Supportive Care

In the absence of a licensed vaccine and approved drug therapy, supportive care is the standard for treating filovirus infection. While supportive care may reduce the overall case fatality rate, the infection remains lethal in a high number of cases and the true impact of even simple interventions such as fluid management has yet to be evaluated [38]. Furthermore, the use of interferon-α2 (IFN-α2), heparin [39,40], and other measures to curtail infection reveal that these interventions are of little to no benefit.

Immunotherapy

Passive transfer of antibodies, either polyclonal or monoclonal, remains an attractive solution to preventing and treating EBOV and MARV. The history of immunotherapy for other infections, such as human respiratory syncytial virus, offers a direct scientific and regulatory pathway to human-use licensure [41]. Passive transfer of polyclonal antibody via hyperimmune serum or convalescent serum has been reported in filovirus infections [42-45]. However, the overall success of these therapies has been controversial and difficult to ascertain due to the conditions in which the studies were conducted (lack of adequate experimental controls, lack of appropriate medical equipment, etc.), and the outbreak was already well contained. These results have also been tempered by the conflicting results from studies in laboratory animal models.

Successes with passive therapy, both polyclonal and monoclonal antibodies, were demonstrated in rodents for both EBOV and MARV [46-48]. The monoclonal sources tested have ranged from murine monoclonal antibodies, some of which have been humanized, to recombinant-derived cloned human monoclonal antibodies from EBOV survivors [47, 49]. In contrast to these studies, administration of anti-EBOV antibodies has only delayed the onset of viremia and clinical signs in the macaque EBOV animal models. One product, hyperimmune horse serum, has been reported to be beneficial in a hamadryad baboon model for EBOV infection; however, this treatment failed to produce any significant reductions in morbidity and mortality in cynomolgus and rhesus macaque models of EBOV [50]. Further studies in the laboratory looking at passive transfer of convalescent blood, passive therapy using murine antibodies, or recombinant human monoclonal antibodies have all failed to increase survival in ZEBOV-infected NHP (unpublished observation, Olinger). Cross species differences between antibodies may limit the functional effectiveness in the various host species (Jarhling *et al.*, 2007). The success of the various vaccine platforms provides a tantalizing source of serum from a homologous source to evaluate the role of antibodies in protection and their eventual use as an immunotherapy. A definitive experiment utilizing exposed or convalescent NHP sera may resolve some of these important questions.

Vaccine Status

Despite some pioneering efforts to develop potential therapeutics, the primary scientific focus has been on basic research and the development of a vaccine. While there is no licensed vaccine available, there are several promising vaccine candidates that have demonstrated immunogenicity and efficacy in animal models of disease. These platforms include the Venezuelan equine encephalitis (VEE) virus-like replicon (VRP), adenovirus 5 (Ad5), vesicular stomatitis virus (VSV)-based vaccines, and virus-like particles (VLPs) [reviewed in [51]]. To date, the candidate vaccines have demonstrated protection in rodent models of EBOV and MARV as well as protection in NHP models of disease. Each of the vaccines have advantages and disadvantages with respect to potency, anti-vector or pre-existing immunity, and safety.

At present, the vaccines are being evaluated to determine which platforms will be selected for advanced development. Given the priority of developing countermeasures for EBOV and MARV, it is likely that at least more than one candidate will be evaluated in Phase I and Phase II Food and Drug Administration (FDA) clinical trials. The current level of support increases the likelihood of obtaining a licensed vaccine within 5 to 10 years for EBOV and MARV. The primary hurdle will be focused on the refinement of the current animal models that will enable Phase II clinical studies. The development of these animal models and a better understanding of the human disease will further both the development of preventative vaccines and therapeutic countermeasures.

Postexposure vaccination is also being evaluated as a potential intervention for known or high-risk exposures. This approach has been used to prevent or modify disease for rabies, hepatitis B, and smallpox. In mice, administration of the rVSV vaccine expressing ZEBOV glycoprotein 30 min after a lethal exposure successfully protected eight out of ten mice. rVSV expressing SEBOV glycoprotein protected four out of four NHP from a lethal SEBOV challenge [52], while the rVSV expressing ZEBOV GP provided partial protection (50%) in the rhesus macaque model of ZEBOV when administered within 20-30 min after challenge. Moreover, the rVSV expressing MARV GP completely protected rhesus monkeys in a postexposure regimen [53]. While these results are encouraging, additional studies are needed to examine the postinfection window to determine how long treatment can be delayed after challenge. In addition, it is still unclear how postexposure vaccines are affording protection. Most likely, postexposure vaccination induces an innate and adaptive virus-specific response that can control viral infection as well as prevent the subversion of the host immune response. This then prevents the uncontrolled viral replication as well as the development of SIRS-like syndrome. Also, the utility of combination therapy of immunotherapy and vaccination has not been fully explored. For example, administration of immunoglobulin in conjunction with vaccination has been successful in preventing the spread of rabies virus in exposed individuals. Combinations with other therapeutic interventions will also need to be considered as the new treatments are evaluated.

POTENTIAL THERAPEUTIC TARGETS

In the past, filovirus-specific therapeutic research has aimed to promote postexposure recovery from the infection. In part, this has been due to the lack of available approaches that have shown efficacy after the onset of symptoms. A survey of the recent filovirus-based therapeutic discoveries reveals at least three areas of research currently being pursued to develop therapeutics to treat filovirus infections: 1) strategies that target the pathogenesis or clinical manifestations of the virus, 2) strategies that target the host immune response, and 3) strategies that seek to interrupt virus:host interactions within target cells.

Targeting Pathogenesis

Reversing or targeting the pathogenesis of disease represents an unusual yet potentially fruitful source of therapeutics. In fact, the first therapeutic identified for filoviruses was a drug that targeted the development of coagulation abnormalities and not the virus itself [54]. As discussed previously, infection leads to the development of what is termed a cytokine storm or a severe inflammatory response syndrome. Importantly, both activation of the coagulation systems and a profound inflammatory response are critical components of disease. As such, targeting of the coagulation abnormalities is an obvious first approach. Activation of the coagulation cascade may be triggered by a variety of factors. Depending on the stimuli, either the intrinsic or extrinsic arm of the coagulation cascade may be activated. Uncontrolled activation may lead to DIC. DIC is neither a disease nor symptom, rather it is a syndrome with both bleeding and thrombotic abnormalities characterized in part by the presence of histologically visible microthrombi in the microvasculature [55-57]. These microthrombi may hamper tissue perfusion and thereby contribute to multiple organ dysfunction and high mortality rates. In fact, there is ample experimental and pathological evidence that fibrin deposition contributes to multiple organ failure [56]. Before acute DIC can become apparent, there must be a sufficient stimulus to deplete or overwhelm the natural anticoagulant systems.

Clinically, there are increases in both pT and apTT indicating that the intrinsic and extrinsic arms of the coagulation cascade are involved. In addition, other studies have shown that there is a strong overexpression of tissue factor (TF). While TF predominantly activates the extrinsic pathway, activation of the intrinsic pathway also occurs. ZEBOV-infected NHP treated with recombinant nematode anticoagulant protein c2 (rNAPc2), which inhibits the FVIIa/TF complex activation of factor X, were protected ~ 33% of the time when the rNAPc2 was administered immediately or 24 hr postinfection [54]. Studies to further determine the window for intervention have not been reported. While this compound appeared to effectively target the TF-mediated activation of the extrinsic pathway, it would be expected to have no direct impact on the intrinsic arm of the coagulation cascade. As such, it is possible that other TF antagonists or compounds that target the common pathway may have additional effects.

During activation of the clotting system, the host regulates the process through the production and activation of a variety of inhibitors of the clotting system. In this process, however, the inhibitors are consumed, and if the rate of consumption exceeds the rate synthesized by liver parenchymal cells, plasma levels of inhibitors will decline. A number of studies have found positive correlations between plasma levels of inhibitors and the degree of DIC during sepsis [57, 58]. Human protein C is a serine protease that is secreted as a zymogen. Cleavage of the pro-enzyme yields an active enzyme (activated protein C; APC) that reduces the production of thrombin by catalytically cleaving factors Va and VIII. APC also has pro-fibrinolytic activity due to its ability to bind and inactivate plasminogen activator inhibitor 1 (PAI-1). Thus, APC acts as an anti-inflammatory, anti-coagulant, and fibrinolytic agent. Protein C levels were observed to drop substantially during filovirus infections with levels reaching 40% of baseline by day 4 postinfection in cynomolgus macaque models. Recombinant human APC was tested as a candidate therapeutic for ZEBOV hemorrhagic fever and was shown to protect 20% of animals and significantly increase the mean time to death in ~ 66% of treated NHP [36]. Despite reports of increased bleeding in human sepsis cases as a complication of treatment, there was no evidence of this side effect in NHP.

In recent years, the importance of the interaction between coagulation and inflammation as a response to severe infection has become increasingly appreciated. Inflammatory mediators upregulate pro-coagulant factors (such as TF), inhibit fibrinolytic activity, and downregulate natural anticoagulant pathways, in particular, the protein C anticoagulant pathway. This interconnection was highlighted

in the rNAPc2 studies where animals that responded and survived not only had reductions in fibrin degradation products but also had substantial reductions in levels of circulating proinflammatory cytokines such as IL-6 and monocyte chemoattractant protein (MCP)-1 [54].

Cytokines are key mediators of inflammation and vascular dysfunction. They can induce changes in endothelial cell structure that affect permeability, and they can also play a role in regulating the inflammatory response. Tumor necrosis factor (TNF)-α has been shown in a number of studies to induce endothelial cell-surface changes. Notably, TNF-α can provoke acute pulmonary vascular endothelial cell injury *in vivo* and *in vitro* [59, 60]. TNF-α was found to act directly on cultured human vascular endothelium to induce a TF-like pro-coagulant activity [61]. It also reorganizes human vascular endothelial cell monolayers [62]. Furthermore, several studies have shown that anti-TNF-α treatment of diseases such as rheumatoid arthritis and anti-neutrophil cytoplasmic antibody-associated systemic vasculitis (AASV) improves endothelial function and endothelium-dependent vasomotor responses [63, 64]. Feldmann and colleagues demonstrated that mediator-release from MARV-infected target cells can negatively affect the integrity of the endothelium and may contribute to vascular instability *in vitro* [62]. The increase in endothelial permeability correlated with TNF-α release and was inhibited by a TNF-α-specific monoclonal antibody. This effect may be exasperated by the presence of hydrogen peroxide. Samples collected from humans and NHP have shown substantially increased systemic serum nitrate levels, indicating increased *in vivo* nitric oxide production [65]. These observations suggest that the impact of even low concentrations of TNF-α on vascular permeability and function cannot be discounted.

Other cytokines or chemokines may also be involved in modulating endothelial function during EBOV infections either directly or indirectly. For example, interferon (IFN)-α, IFN-γ, IL-6, and MCP-1 are upregulated during EBOV infection of humans and/or NHP [25, 66-70] and may have indirect effects on endothelial function. Increased mRNA transcripts of the chemokine IL-8 were detected in peripheral blood mononuclear cells of EBOV-infected NHP [70]. Notably, IL-8 was recently shown to contribute to dengue virus-induced modification of transendothelial permeability [71, 72].

To date there has only been limited work performed to evaluate the benefit of targeting the inflammatory pathway during filovirus infections. Studies in small animal models have suggested some benefit. Desferal, an immumodulator that is an IL-1/TNF-α antagonist, partially protected a small cohort of guinea pigs [73]. Similar results were reported when guinea pigs were administered IL-1 receptor antagonist (IL-1RA) or anti-TNF-α serum. Currently, there are several anti-cytokine therapies in use for treating human diseases including anti-TNF-α and anti-IL-6 for the treatment of rheumatoid arthritis select cancers.

Targeting of the Host Immune Response

Another primary theme for treatment of EBOV infections has been modulation of the host immune response. This area has mainly involved efforts to boost innate immunity, but more recently has turned to evaluating reversing the subversion of the host immune response.

Interferons

Treatment with exogenous type I IFNs has been evaluated by several groups. A combination of ridostin (an IFN inducer) and reaferon (IFN-α2a) prolonged the mean time to death of ZEBOV-infected guinea pigs [74]. However, studies in cynomolgus monkeys treated with high doses of recombinant human IFN-α2a immediately after exposure failed to produce any significant reductions in morbidity and mortality. Despite the failure of IFN-α2a, there exist a number of other potential IFN products that have yet to be evaluated. Future studies should focus on alternative IFN-α subtypes of other IFNs (e.g., IFN-β. IFN-λ, IFN-γ) or combinations of type I and type II INFs.

Innate Immune Response Interference

Among the many factors contributing to the pathology of filoviruses is subversion of the innate immune system by hindering the ability of the host to develop an adaptive immune response. Data from both *in vitro* and *in vivo* experiments show that EBOV initially infects DC and Mϕ [33, 67]. Infection of Mϕ are thought to act as key triggers for the uncontrolled and rapid secretion of pro-inflammatory mediators [75, 76]. However, despite the systemic release of inflammatory mediators after infection with EBOV, fatal or severe disease is often linked to a generalized suppression of adaptive immunity. This phenomenon is evidenced by the fact that markers for the early innate and adaptive immune responses are lacking in patients fatally afflicted with EBOV infection, whereas survivors have detectable virus-specific IgM antibodies along with transient elevation of pro-inflammatory cytokines [68, 77, 78]. As mentioned previously, filovirus infection of DC hinders activation of these cells and limits the ability to initiate an adaptive immune response. Transcriptional profiling of filovirus infected human DC will facilitate identification of genes differentially expressed upon infection. These genes can then be mapped to cellular pathways that are involved in DC maturation. Panels of small molecule inhibitors can then be screened against filovirus infected DC with a normal DC phenotype being used as a benchmark for success. Focusing research efforts on a single target cell type within the host immune system, such as DC, will help to narrow the search for filovirus specific small molecule inhibitors.

Inhibition of Apoptosis

It is likely that the marked apoptosis of natural killer (NK) and T-cells seen early in filovirus infections contributes to the observed immunosuppression. In addition, it is likely that the microparticles, which are formed as a natural part of the programmed death cycle, exasperate the coagulation abnormalities. Recent studies have shown that shed microparticles from T lymphocytes impair endothelial function and regulated endothelial protein expression [79]. As such, targeting apoptosis may have multiple beneficial impacts. While there have been no reports about targeting apoptosis during filovirus infections, there are a number of strategies under evaluation for other diseases that could be translated to filoviruses. Currently, therapies that inhibit Fas and/or TRAIL function are being evaluated in HIV. Use of

an anti-TRAIL monoclonal antibody in HIV-infected mice significantly reduces the development of CD4+ T cells. In addition, there are a number of studies evaluating the use of anti-caspase therapies in models of sepsis.

Virus-Host Interactions

While the use of therapeutics targeted at the virus itself may prove to be a very effective way to clear or prevent virus infection, an inherent flaw in this method does exist for several viruses. As the HIV literature recounts, there are numerous examples of viral escape mutants which have evolved resistance against not only HIV-specific antibodies, but also anti-retroviral drugs targeted at various aspects of the viral replication cycle [reviewed in [80, 81]]. There has been some success targeting filovirus proteins, but it may be only a matter of time before these targets are rendered ineffective as well. Therefore, an alternate therapeutic approach must target important molecules or pathways within the host cell itself that the virus appears to require for efficient replication.

Assembly and Budding

The cellular components required for filovirus assembly and release are becoming well-characterized. One such component is Tsg101, an ubiquitin-conjugating E2 enzyme variant, which is part of the endosomal sorting complexes required for transport (ESCRTs) within the vacuolar protein sorting pathway. The ESCRT complexes are used to sort various cellular proteins into internal vesicles that bud into the lumen of the endosome. It has been suggested that this endosomal invagination is highly similar to the plasma membrane vesicularization that occurs during filovirus budding [82]. Previously, it was demonstrated that the matrix protein of HIV has the ability to recruit Tsg101 to the plasma membrane via late domain motif interactions [83]. Similarly, the filovirus VP40 contains two overlapping late domain motifs and actively recruits Tsg101 and other ESCRT components to the site of viral budding [84]. While mutation of these motifs reveals that they are not essential for viral budding, they are an important component of the budding machinery [83]. This study also demonstrated that only a small conserved peptide motif is required for viral interactions with Tsg101. Small molecule inhibitors that mimic these sequences could prove to be very effective therapeutic agents.

It has also been recently demonstrated that filovirus budding can occur independently of interactions with Tsg101. The ability of a mutated VP40 to redirect proteins of the vacuolar protein sorting (vps) pathway from endosomes to sites of particle budding has been characterized [82]. While these mutant VP40 proteins could no longer recruit Tsg101 to the plasma membrane, several vps proteins (VPS4, VPS28, and VPS37B) could still be redirected to the plasma membrane. A mutant VPS4 that lacks ATPase activity can still traffic to the plasma membrane, but now inhibits filovirus budding. Furthermore, mice were protected from EBOV infection when VPS4-specific phosphorodiamidite morpholino oligonucleotides (PMOs) were injected into the mice. This is yet another example of a promising cellular target.

Ubiquitination

It is clear that ubiquitinated proteins are important for cellular endocytosis and exocytosis processes [reviewed in [85, 86]]. During normal cellular activities, monoubiquitination is a signal for delivery and internalization into vesicles of the multi-vesicular body complex. The proteins are then available for sorting by the vps pathway for ultimate delivery to the lysosome for degradation. The PPxY late domain motif of VP40 has been shown to interact with WW motifs of ubiquitin ligase enzymes such as Nedd4 and its yeast homolog, Rsp5, both of which play a role in the cellular ubiquitin enzyme cascade as E3 ubiquitin ligases. It has been shown that mutations in the active site of Nedd4 not only abolish ligase activity but also reduces the ability of Nedd4 to enhance filovirus budding [87]. Importantly, they demonstrated that the IFN-inducible ubiquitin-like protein ISG15 can inhibit filovirus budding via interactions with Nedd4 to inhibit ubiquitination of VP40 [88]. Although it remains unclear whether VP40 is ubiquitinated in infected cells, it has been shown that for several other viruses, ubiquitination is important for assembly and final separation of the newly formed particle from the host cell [89]. Nedd4 likely provides VP40 with the ubiquitination signal for delivery to the ESCRT complex of the vps pathway. Here, VP40 has the opportunity to interact with Tsg101 and recruit the cellular protein to the plasma membrane for viral assembly. Thus, Nedd4 could be a potential drug target candidate to combat filovirus infection.

Protein Transport

During the replication of filoviruses, viral components must be shuttled to and from several locations within the infected cell. While it remains unclear for filoviruses, it is well known that many viruses utilize host cytoskeleton scaffolding to accomplish various processes such as entry, transport of viral proteins throughout the cell, and assembly/budding [reviewed in [90]]. Although it would be challenging to therapeutically target proteins involved in the host cell cytoskeleton in a non-lethal manner, it is interesting that both EBOV and MARV viruses appear to interact with microtubules and actin filaments, respectively. EBOV has been shown to interact directly with microtubules via the VP40 protein, and this interaction seems to stabilize polymerization of microtubule bundles [91]. Furthermore, EBOV VLP release is dependent on interactions with microtubules [92]. However, MARV VP40 does not contain the tubulin binding motifs observed in the C-terminal domain of EBOV VP40 and cannot interact with microtubules. Rather, release of MARV VLPs is inhibited by depolymerization of actin but not microtubules [91]. As EBOV VLP budding also appears to require interactions with actin, the mechanism by which each virus interacts with its respective host cell cytoskeleton component remains unclear.

Another set of proteins involved in cellular transport is the Rab family of proteins. These small GTP binding proteins regulate vesicular transport by tethering donor vesicles to their respective target membranes. Specifically,

Rab9 is involved with transport between late endosomes and the trans-Golgi network [93]. The use of Rab9 siRNA resulted in decreased filovirus replication as demonstrated by immunofluorescence and ELISA. Rab9 siRNA also decreased replication of HIV and measles virus. This was not observed in non-enveloped viruses, which confirms the interaction of Rab9 with various cellular membrane components [93]. However, whether there is direct interaction between any viral proteins and Rab9 remains to be elucidated. An interaction between Rab9 and filovirus VP40 may be critical for the delivery of VP40 to the late endosome for subsequent ubiquitination by Nedd4 and recruitment of Tsg101 to the plasma membrane for particle assembly. Thus, several therapeutic targets exist during filovirus assembly alone. Further examination of the viral replication cycle will likely reveal many more promising targets to combat filovirus infection.

Entry Inhibitors

The viral entry process offers several potential targets for intervention. One particular area of recent interest is the development of fusion inhibitors. In large part this was due to the approval of the novel fusion inhibitor T-20, or Fuseon, for use in retroviral therapy. Fuseon blocks the structural rearrangements necessary for successful fusion of the virus with the cell. Recently there have been tremendous advances in our understanding of how filoviruses enter cells. Specifically, a model for EBOV GP2-mediated membrane fusion with pesudotype viruses has been developed [94]. Using this model, it has been demonstrated that an oligopeptide corresponding to the coiled-coil structure of GP2 competitively inhibited EBOV entry. Additionally, the crystal structure for EBOV GP in its trimeric, pre-fusion conformation has been solved [95]. These breakthroughs will facilitate the identification of novel small molecules that target filovirus entry.

Another area of interest is the GP-mediated attachment of filovirus virions to host cells. It has been well demonstrated that the cellular endosomal cysteine proteases cathepsin B (CatB) and cathepsin (CatL) may play an important role in preparing the viral GP for interactions with the target host cells by generating an 18-19KDa form of GP1 required for EBOV infection [96-98]. This truncated form of the GP allows for more efficient attachment to host cells but not necessarily a greater rate of infection, which would suggest a third endosomal factor may be involved. Moreover, complimentary studies by Sanchez and Schornberg demonstrate that inhibition of these cathepsins by drug treatment or siRNA knockdown, respectively, resulted in a significant decrease in viral infection [97, 99]. This suggests that both CatB and CatL and a possibly third unknown factor may be prime targets for inhibiting viral entry through the use of small molecule inhibitors.

DRUG TARGETING DIRECTED AGAINST FILOVIRUSES

The viral lifecycles of EBOV and MARV are relatively similar and offer a variety of targets to which broad-spectrum and agent-specific drugs may be developed. Screening antiviral drug compounds has been hampered by the constraints of biosafety level (BSL)-4 laboratory conditions and the lack of high-throughput assays with these viruses. However, there have been recent advancements that have helped overcome these limitations. Two such advancements, mini-genome reporter systems and "pseudotyped" virus assays, have enabled the development of high-throughput assays and drug screening methods that can be performed outside of high containment. These methods allow screening of multiple compounds followed by verification of antiviral effectiveness. Currently, several promising antiviral compounds have been discovered using these types of technologies. These compounds are now being assessed as to their effectiveness to combat filovirus infections *in vitro* as well as *in vivo*.

While the results from these drug screens are exciting, the greatest challenge remains with replicating virus under BSL-4 conditions. The development of reverse genetics for filoviruses and the green fluorescent protein (GFP) expressing EBOV [100] has provided the means to develop the first true high-throughput assay for drug screening, which utilizes replication-competent EBOV in relevant cell systems in high containment. Before this work, the traditional plaque assay was laborious and could take up to 14 days for assay completion. Currently, high-throughput assays in 96-well formats can produce results within 48 hr. As a result, large drug compound library screens can be accomplished under high containment. This assay can potentially identify multiple compounds capable of disrupting filovirus replication in a single screen. Once a promising compound is identified, it is assessed against other viruses to determine if the drug is agent specific or broad spectrum, and is then tested for effectiveness *in vitro* and *in vivo* assays. Unfortunately, a viable high-throughput system for drug screening of MARV has yet to be developed, but the current construction of MARV-GFP will allow for new possibilities.

Although initial screening using compound libraries may yield potential antiviral drug candidates, the actual number of candidates displaying antiviral efficacy with low toxicity for *in vitro* and *in vivo* model systems are few. Therefore, to increase the probability of successful drug identification, future efforts should focus on increasing the number of compounds that can be screened (i.e., 384 well), identifying biologically relevant compound libraries, and implementing technology learned from more advanced high-throughput screening programs. Although the discovery of a drug for EBOV and MARV remains the primary objective of these screens, the data obtained from these screens provide basic research information on virus-virus and virus-host interactions. Ultimately, the data generated from screens directed against not only EBOV and MARV, but also other viruses, can be used to determine if there are common pathways that can be targeted for multiple viruses and other pathogens.

In addition, there have been some success *in vitro* and *in vivo* using anti-sense technologies. To date, the best results have been obtained with phosphorodiamidate morpholino oligomers (PMOs) or small interfering RNAs (siRNAs). PMOs are uncharged single-strand DNA analogs that bind to complementary sequences of mRNA, while siRNAs are short double-stranded RNA molecules that interfere with

specific gene expressions. Both PMO and siRNAs can effectively inhibit filoviruses in cell culture [101, 102]. While the greatest barriers to the use of these technologies are the availability of effective delivery systems and the ability to overcome or reduce toxicity and off target effects, tremendous progress has been made. Efficacy has been demonstrated in small animal models and is currently being evaluated in NHP models of filoviruses.

CONCLUSION

During the last decade, the filovirus field has experienced an influx of interest and support that has helped to advance the applied and basic science directed against EBOV and MARV. Despite these advances, there remains a critical gap in the availability of effective therapeutics or postexposure interventions. Moreover, while there are a number of candidate interventions under investigation, supportive care remains the primary method to treat infected patients. The use of reporter viruses in high-throughput assays to screen drug compound libraries is just beginning to offer new drug compound candidates that can be evaluated for use in humans. Concurrent studies conducted to dissect the pathogenesis are offering new insight into not only how the clinical picture develops, but also into virus host interactions. Understanding how the virus modulates host gene expression to its own advantage potentially provides new targets for therapeutic interventions.

Given the aggressive nature of filovirus infections, the overwhelming viral burdens, subversion of the host immune response, and induction of a cytokine storm, early diagnosis and rapid initiation will be critical to the success of any intervention strategy. As such, it is likely that any successful intervention, when implemented after the onset of significant clinical symptoms, will require a combination of approaches/ compounds that directly target the virus as well as clinical disease. Clearly, studies that continue to increase our basic understanding of filovirus pathogenesis in conjunction with high-throughput screening using new reporter assays will only facilitate our ability to develop effective counter-measures.

ACKNOWLEDGEMENTS AND FUNDING

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army. We thank the peer reviewers of this article for their comments. The authors' work on Ebola and Marburg viruses was supported by the Medical Chemical/Biological Defense Research Program, US Army Medical Research and Material Command. This review was prepared while Dr. Julia Biggins held a National Research Council Fellowship.

POTENTIAL CONFLICTS OF INTEREST

None Reported.

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Received January 21, 2009 Accepted January 26, 2009