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Review

Ebola virus: the search for vaccines and treatments

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Abstract. Ebola viruses belong to the family Filoviridae, which are among the most virulent infectious agents known. These viruses cause acute, and frequently fatal, hemorrhagic fever in humans and nonhuman primates. Currently, no vaccines or treatments are available for human use. This review describes Ebola viruses, with a particular focus on the status of research efforts to develop vaccines and therapeutics and to identify the immune mechanisms of protection.

Key words. Ebola virus; filovirus; immunity.

Ebola viruses

Infection of humans and nonhuman primates with a member of the Filoviridae family of viruses causes a severe, potentially fatal illness. This virus family comprises the Marburg viruses, first identified in hemorrhagic fever outbreaks that occurred in 1967 [1, 2], and the Ebola viruses (EBOVs) that were discovered a decade later. Marburg and EBOV have similar appearances and structures, and are the focus of research efforts aimed at developing effective countermeasures for these agents. While experimental results similar to those described below have also been described for Marburg virus, this review will only discuss studies relating directly to the EBOVs.

The first identification of EBOV as the causative agent of viral hemorrhagic fever was from simultaneous outbreaks in Sudan and the Democratic Republic of the Congo (formerly Zaire) in 1976 [3, 4]. Since then, there have been intermittent outbreaks of EBOV which led to the identification of three different EBOV subtypes (Zaire: EBOV-Z; Sudan: EBOV-S; Ivory Coast: EBOV-IC) that are associated with human disease. Table 1 summarizes the known cases of infection with these EBOVs to date. The most recent EBOV epidemic, caused by EBOV-S, occurred in Uganda from August 2000 to January 2001 and resulted in 425 reported cases and 224 deaths [5]. A fourth subtype, EBOV Reston (EBOV-R), was initially isolated from cynomolgus macaques in a quarantine facility in Reston, Virginia, in 1989 [6] and was also introduced again into the United States, Italy, and the Philippines in the 1990s. Although EBOV-R is lethal in nonhuman primates, human infections have been asymptomatic [7].

The different EBOV subtypes possess both unique and conserved antigenic determinants. The genomes of these RNA viruses have shown remarkable stability between outbreaks (e. g., nucleotide divergence of less than 2% in the glycoprotein (GP) from EBOV-Z isolated in 1976 and 1995) as well as within individual outbreaks [8]. The Zaire subtype has caused the highest mortality in humans, with case fatality rates sometimes exceeding 80% [9, 10]. The observation that EBOV-Z is the most virulent of the three subtypes causing hemorrhagic fever in humans is also evident by the more extensive lesions caused by EBOV-Z in human cases and its increased lethality in experimentally infected animals [11–13].

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Epidemiology

The natural reservoir(s) and primary transmission route(s) of EBOV are not known. Experimental infection of plants and animals has led to the suggestion that bats may be a possible reservoir, because they support replication of EBOV without causing disease [14]. However, several ecological studies aimed at identifying the reservoir for EBOV failed to demonstrate evidence of EBOV infection in thousands of vertebrate and invertebrate animals examined, including bats [15–17]. When researchers used RT-PCR and Southern analysis to detect EBOV sequences in the organs of 242 small mammals captured during ecological studies in the Central African Republic, evidence of EBOV was found in seven animals, including one shrew and six rodents [18]. None of the bats examined in that study tested positive for EBOV [18]. Other research efforts have focused on the detection of early warning signs for EBOV outbreaks. When researchers at the World Health Organization and the U.S. National Aeronautics and Space Administration (NASA) used satellite imaging to examine rainfall data, an association was noted between rainfall after unusually dry weather and the onset of an EBOV outbreak [19].

The major route of EBOV transmission during outbreaks appears to be through direct contact with blood or infectious fluids from infected patients, and barrier nursing techniques appear to limit the spread of infection [20]. Caregivers are at an increased risk of infection and, because the virus is found in the skin [21], even touching cadavers may increase the risk for contacting the virus [20]. Transmission via large droplets, aerosolized particles, or fomites also cannot be excluded, but appears to be a minor route of infection during outbreaks [22]. However, nonhuman primates have been experimentally infected with EBOV by the aerosol [23], oral, and conjunctival routes [24].

Table 1. Cases of Ebola hemorrhagic fever.

Year	EBOV species	Location	Number of human cases	Case fatality %
1976	EBOV-Z	Zaire	318	88
1976	EBOV-S	Sudan	284	53
1976	EBOV-S	England	1	0
1979	EBOV-S	Sudan	34	65
1994	EBOV-Z	Gabon	44	63
1994	EBOV-IC	Ivory Coast	1	0
1995	EBOV-Z	Zaire	315	81
1996	EBOV-Z	Gabon	37	57
1996	EBOV-Z	Gabon	60	75
1996	EBOV-Z	South Africa	2	50
2000–2001	EBOV-S	Uganda	425	53

Information obtained from the Centers for Disease Control and Prevention.

Disease caused by EBOV

Detailed studies on EBOV pathogenesis in people have been difficult due to the infrequent and unpredictable outbreaks (table 1), the rapid course of infection, the remote areas in which infections occur, and the requirement to study infectious materials in a biosafety level 4 (BSL4) laboratory setting. The early clinical features of EBOV infection include nonspecific symptoms such as fever, headache, myalgia, abdominal pain, diarrhea, and vomiting. These initial symptoms are followed by hemorrhage, multiorgan involvement and necrosis, generalized shock, and death within 7–14 days after the initial onset of symptoms [3, 4].

Fatal EBOV infections usually end with high viremias and little evidence of acquired immune responses [25], even though (or because) viral replication occurs primarily in mononuclear phagocytes that normally present antigens to the immune system [24, 26–29]. EBOV infection severely damages the liver, kidneys, and spleen [29], possibly because anatomical structures of these organs allow the virus to gain access to large numbers of macrophages [30]. Although EBOV does not appear to infect lymphocytes [24, 27, 28, 31], a marked depletion of lymphocytes, which was subsequently attributed to apoptosis, has been observed in fatal human cases and in nonhuman primates infected with EBOV [25, 28, 29, 31–33]. At the time of death, serum antibodies to EBOV are either not detected or are lower than the antibody responses observed in survivors [25, 34]. The results of other recent research efforts indicate that virus-induced cytokine release [35] and cytotoxicity or immunosuppression mediated by specific viral proteins [36–39] may be responsible for the severe pathogenicity of EBOV.

Cytokine expression in EBOV infections

Identifying physiologic responses that predict the outcome of infection in people may provide insights for the development of effective therapies. The production of cytokines, which modulate immune responses, has been examined in EBOV fatalities, survivors, and asymptomatic people. A variety of cytokines have been studied, including interleukin (IL)-2, IL-6, and IL-10, interferon (IFN)-α and IFN-γ, and tumor necrosis factor-α (TNF-α).

One study compared samples from seven patients who died of Ebola hemorrhagic fever during the 1995 EBOV-Z outbreak to samples from two patients who recovered from EBOV and two patients who were on the same ward but died of unrelated infections [35]. Elevations in the concentrations of serum IL-2, IL-10, IFN-α, IFN-γ, and TNF-α, but not IL-6, were transiently observed in some of the fatal cases compared to survivors and controls, but

could not be correlated with mRNA levels. The inconsistent increases within groups resulted in high standard deviations in some assays, making it difficult to compare the responses between groups.

A second study also observed increased IFN-γ, both as mRNA and in the plasma of patients who later died of EBOV infection [25]. In contrast to the first study, increased levels of IFN-γ mRNA were also detected in survivors, although only during the recovery period. However, no evidence of IL-2 production was observed in any of the EBOV-infected patients, regardless of whether or not they survived. Increased mRNA levels for proteins associated either with cytotoxic T cell (CTL) activity or cell death were observed in fatal cases from the onset of symptoms until a day or two before death. In contrast, survivors did not have similar evidence of CTL activation until their recovery phase [25]. Although the presence of serum IgG antibodies in the survivors during the symptomatic phase of disease might suggest that some helper T cells were activated in these patients, the CD3 and cytokine levels examined did not provide evidence for this.

Asymptomatic patients, who had evidence of EBOV exposure based on PCR amplification of EBOV genetic sequences from blood cells, had increased concentrations of pro-inflammatory cytokines including TNF-α and IL-6 a few days after infection [40]. However, no increases were observed in the levels of other cytokines, including IFN-α, IFN-γ, and IL-2.

Collectively, these studies indicate that the detection of elevations in cytokine expression is subject to large variations among individuals and studies. This may be due to several factors, including the half-lives of the cytokines, the need to transport samples from Africa to BSL4 laboratories, and the use of different kits for detecting the cytokines. It is tempting to speculate on the relationship between the observed cytokine levels and the outcome of infection, since using some of these cytokines to treat other diseases is associated with nausea, vomiting, fever, fatigue, and other clinical signs in patients [41–43]. However, at this time, the differing results suggest that conclusions regarding the pathogenesis observed in EBOV patients should be made with caution.

The interaction between EBOV and cytokines, particularly IFN, is also being evaluated in animal models and cell cultures. As with some of the human studies, increased serum concentrations of TNF-α and IFN-α were evident 3 days after subcutaneous infection of baboons with a guinea pig-adapted strain of EBOV [44]. These levels continued to increase until the animals died, approximately 7–8 days after infection.

In cell culture, endothelial cells were less responsive to the stimulatory effects of IFN-α and IFN-γ after EBOV infection compared to mock-infected controls. This prevented the induction of genes whose products, including the major histocompatibility class I molecules, are important for generating effective antiviral immune responses [45, 46]. The observation that all signaling pathways were not disrupted by EBOV infection suggested that one of the EBOV proteins might specifically interfere with this pathway. This function was recently attributed to the VP35 protein [36]. As a result, viral proteins that interfere with the induction of cytokines may contribute to the virulence of EBOV by facilitating viral replication, whereas increased expression of cytokines in response to increasing viral loads might exacerbate clinical illness.

Together, these observations indicate possible targets of therapeutic intervention for ameliorating disease and preventing the fatalities that would otherwise occur. Improved understanding of the roles of the other viral proteins may provide additional insights.

EBOV genes and proteins

EBOVs are enveloped, nonsegmented, negative-strand RNA viruses that have a characteristic filamentous morphology (fig. 1). EBOV virions are highly pleiomorphic, consisting primarily of '6-shaped' and long filamentous forms [47] with a uniform diameter of approximately 80 nm. Although the length of EBOV virions varies greatly, the average unit length is approximately 1000 nm [47]. The virions have a central core formed by a ribonucleocapsid (RNP) complex that is surrounded by a lipid envelope derived from the plasma membrane of the host cell.

The EBOV genome is composed of a single-stranded RNA molecule that is approximately 19 kb long (fig. 2). Transcription and replication of the virus occurs in the cytoplasm of infected cells, and virions are released through the plasma membrane by budding. The RNA is

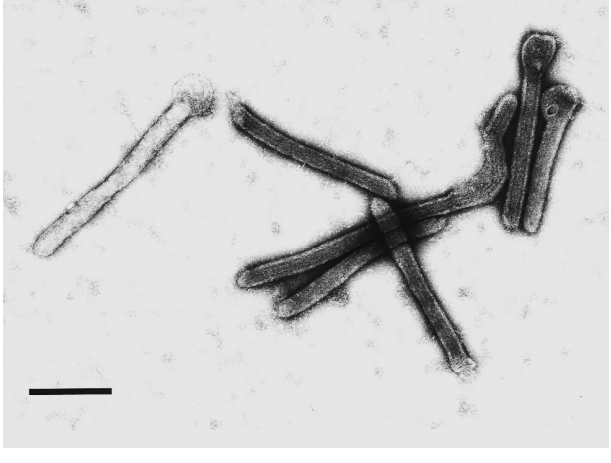

Figure 1. Negatively contrasted EBOV-Z (Mayinga) virions obtained from the serum of an experimentally infected macaque. The bar represents 385 nm. This picture was graciously provided by Tom Geisbert, USAMRIID, Ft. Detrick, Md.

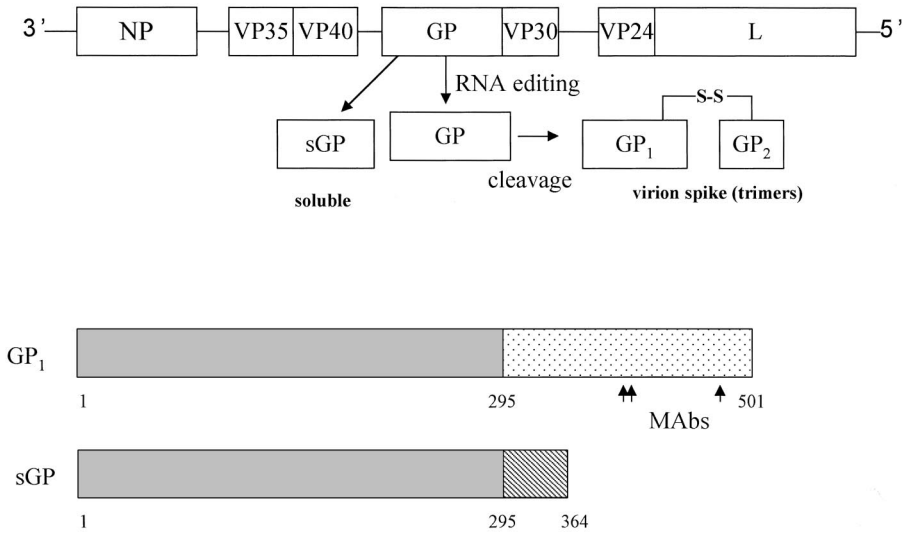

Figure 2. The EBOV genome. The upper scheme represents the organization of the EBOV genome. Unedited GP mRNA produces the non-structural soluble GP (sGP) whereas editing of the GP mRNA produces a protein that is cleaved into GP_1 and GP_2, which are disulfide-linked and present as trimers in the virion spike. As depicted in the lower diagram, the first 295 amino acids of GP_1 and sGP are identical (gray area) but the remaining amino acids are different (stippled and hatched areas). Arrows on GP_1 indicate the location of linear epitopes bound by protective mAbs [59]. Two conformational protective epitopes have also been identified in the region shared by GP_1 and sGP.

transcribed to produce eight subgenomic mRNAs that encode a type I transmembrane GP, a nucleoprotein (NP), four virion structural proteins (VP40, VP35, VP30, and VP24), an RNA-dependent RNA polymerase (L), and a non-structural soluble GP (sGP) [48–50].

With the exception of the GP, each gene encodes a single polypeptide. The transmembrane GP, which is encoded by two overlapping reading frames, is expressed when the viral polymerase inserts a non-template-encoded adenosine residue during the transcription of seven uracil residues in the middle of the gene [49, 50]. GP is cleaved posttranslationally by pro-protein convertases such as furin, PC5, and PC7 into the amino-terminal 140-kDa GP_1 and the carboxy-terminal 26 kDa GP_2, which are disulfide-linked and present as a trimer in the mature spike of the virions [51–53]. In the absence of disulfide linkage with GP_2, GP_1 lacks a membrane anchor and is released from infected cells in a soluble form [54].

The majority of the GP mRNA is not edited and encodes a nonstructural, sGP that is present in infected cell cultures [49, 50] and in the serum of infected patients [55]. GP_1 and sGP are identical in their first 295 amino-terminal amino acids, whereas the remaining 69 amino acids of sGP and 206 amino acids of GP_1 are unique and are encoded by different reading frames (fig. 2). The processing of the GP and the potential roles of its different forms in pathogenesis have been addressed in two recent review articles [56, 57]. Secreted GP_1 or sGP have been suggested to effectively bind antibodies that might otherwise recognize membrane-bound GP and be protective [50, 54, 55, 58]. In support of this suggestion, antibodies capable of blocking infection of EBOV GP-pseudotyped vesicular stomatis viruses (VSVs) into 293 cells were inhibited by sGP [58]. However, we identified protective monoclonal antibodies (mAbs) that recognize two conformational epitopes present on both sGP and GP_1 [59], indicating that sequestration of protective antibodies by these soluble molecules in vivo in an animal model of Ebola hemorrhagic fever can be overcome.

Examination of the role of individual gene products in EBOV pathogenesis has been hampered by the lack of a reverse genetics system to enable recovery of full-length infectious virus from cloned cDNA. However, recent success in synthesizing an infectious EBOV from cloned cDNAs [60], and the subsequent ability to generate infectious EBOVs containing defined mutations should enable us to determine the contribution of individual EBOV proteins to viral pathogenesis.

Most research efforts aimed at elucidating the function of individual EBOV proteins have focused on the role of GP and sGP in viral pathogenesis. The EBOV GP is a highly glycosylated transmembrane protein that contains both N- and O-linked carbohydrates. This membrane-anchored GP is the only viral protein known to be on the surface of EBOV virions and infected cells and presumably mediates viral entry into host cells by a mechanism involving receptor-mediated endocytosis [47]. As a result, it is a possible target for the protective effects of antibodies as well as CTLs. The role of EBOV GP in receptor binding and fusion with host cells has been evaluated by examining the ability of VSV [61–63] and retroviruses [64–66] pseudotyped with EBOV GP to interact with and infect cultured cells. There has been no experimental evidence to suggest that cleavage of GP into GP_1

and GP_2 is necessary for infectivity of EBOV GP-pseudotyped viruses in vitro [58, 67]. However, variations in the cleavage site of the GP from different EBOVs, with EBOV-R having a sub-optimal cleavage site sequence compared to the EBOV-Z, EBOV-IC, and EBOV-S strains, have been speculated to account for the differences in pathogenicity of these viruses for humans [51]. If the pathogenesis or the ability of EBOV to infect host cells in vivo is affected by the cleavage of the EBOV GP, or differences in the cleavage site sequence, remains to be determined.

Recombinant GP can induce cell rounding and detachment of 293 human kidney cell lines in vitro [37, 38, 62]. The cellular detachment observed in these studies was induced by recombinant membrane-bound GP (not secreted GP_1 or sGP) and was attributed to a domain within GP_2. As the detached cells were still viable [37], the morphological changes were apparently not due to a cytotoxic effect of the GP, but may have resulted from downregulation of β1 integrin adhesion molecules by the GP [62]. In addition to the observed cellular detachment of the cultured cells, expression of EBOV GP in explanted blood vessels also resulted in endothelial cell loss and an increase in vascular instability [38]. Although recombinant GP from EBOV-Z was capable of inducing endothelial destruction in human and monkey blood vessels, expression of the EBOV-R GP did not have an effect on the human blood vessels. It is interesting to speculate that this difference may be correlated with the finding that EBOV-R does not appear to be pathogenic for humans. However, whether these mechanisms actually contribute to pathogenicity in vivo remains to be determined. In addition to these findings, a sequence at the amino-terminus of the GP suppresses mitogen-stimulated proliferation of human lymphocytes in vitro [39], and a region with homology to a putative retrovirus immunosuppressive domain has been identified on GP_2 [68]. Whether these sequences contribute to the downregulation of the immune responses that are observed in EBOV infections is not yet known [25].

Although sGP is produced during natural infection [55], it has no clear role in EBOV pathogenesis. sGP may bind to neutrophils and be involved in modulation of the immune response by inhibiting early neutrophil activation [66]. However, because the interaction of recombinant sGP with neutrophils was dependent on the Fc portion of the rabbit antibodies used to detect binding, the specificity of the interaction has been questioned [69]. An infectious EBOV clone that expresses little or no sGP was recently generated [60]. Because this mutant EBOV exhibited an increased cytopathic effect on Vero E6 cells compared to the wild-type virus that expresses sGP, the authors concluded that sGP downregulates the cytotoxicity caused by the full-length GP and may therefore enhance viral loads and promote the spread of infection in the host. If this mutant will also demonstrate altered virulence in an animal model of Ebola hemorrhagic fever remains to be determined. However, profound differences in pathogenicity were not observed in animals infected with either EBOV-Z variants that express only minute amounts of sGP, due to the addition of an extra U residue at the GP editing site, or EBOV-Z viruses that express wild-type levels of sGP [57, 70].

In contrast to the GP, the other EBOV proteins are internal virion proteins that are presumed to be inaccessible to the protective effects of antibodies. The functions of these proteins were initially proposed based on their location in EBOV virions and their similarity in genome organization to other nonsegmented, negative-strand RNA viruses. The NP, VP30, VP35, and L proteins associate with the genomic RNA in an RNP complex, with NP being the major component of this complex [22, 71].

An in vitro replication system was recently used to examine the function of individual EBOV proteins in transcription and replication [72]. The NP, VP35, and L proteins were found to be essential for replication and encapsidation of an EBOV minigenome which consisted of an antisense CAT reporter gene flanked by the leader and trailer regions of the EBOV genome [72]. Although VP30 was not essential for replication in this system, it appeared to be necessary for efficient transcription of the EBOV minigenomes. These findings are also supported by the observation that NP, VP35, VP30, and L are all essential for the recovery of infectious EBOV from cloned cDNAs [60]. In addition to being an essential component of the replication complex, VP35 was also recently implicated as an IFN antagonist [36]. VP35 may therefore facilitate viral replication in infected cells by blocking the induction of antiviral immune responses normally induced by the production of IFN.

In contrast, the VP40 and VP24 proteins do not appear to be associated with the virion RNP complex and are believed to be components of the virion membrane [71]. VP40 associates with cell membranes, where it is believed to be involved in maturation of the virus by inducing viral assembly at the plasma membrane of infected cells [73, 74]. The function of VP24 is not known but it may serve as a minor matrix protein, facilitating the interaction of VP40 and/or GP with the RNP complex, or it may function in the uncoating of the virion during infection [22].

EBOV-Z in animal models for vaccine and therapy studies

The pathogenesis of EBOV-Z infection has been evaluated in nonhuman primates including African green monkeys, baboons, rhesus monkeys, and cynomolgus monkeys [12, 31, 33, 75–81]. The course of disease progression in these primate species resembles that of human

cases in that the animals may experience weight loss, fever, hemorrhages, and skin rashes. In addition, EBOV replicates to high titers in the livers, kidneys, lungs, and spleens of infected nonhuman primates, and the pathologic changes observed in these animals resemble those observed in human cases [29].

To aid the testing of potential vaccine candidates, antibodies, and drugs, EBOV-Z has also been adapted in mice and guinea pigs. The benefits of using these animal models include the ability to conduct experiments with larger sample sizes for statistical relevance, a greater ease of performing the experiments in a BSL4 setting, and, for the mouse model, the availability of reagents for studying both cellular and humoral immune mechanisms. A drawback to using any animal host is that one cannot assume that the observed findings will necessarily extrapolate to humans.

Inoculation with EBOV-Z causes a nonlethal febrile infection in guinea pigs. However, uniform lethality of EBOV-Z has been achieved by serial passage in guinea pigs to increase its virulence. Similar to nonhuman primates and humans infected with nonadapted EBOV-Z, guinea pigs exposed to the adapted virus had infection of mononuclear phagocytes and extensive organ damage to the liver, spleen, adrenals, and kidneys [26, 76]. Viremias reached 5 $\log_{10}$ plaque-forming units (pfu)/ml by day 7 [70], and deaths usually occurred between days 8–11, without visible hemorrhages [26].

Similarly, although EBOV is virulent in newborn mice and adult mice with severe combined immunodeficiency, adult immunocompetent mice do not succumb to infection with EBOV-Z isolated from human infections [82, 83]. Serial passage of EBOV-Z in progressively older mice resulted in the isolation of a mouse-adapted variant that is uniformly lethal for adult, immunocompetent BALB/c, C57BL/6, and outbred ICR (CD-1) mice within a week after challenge. This virus is lethal when injected by the intraperitoneal (ip) route, but is not lethal when administered by the intramuscular (im) or subcutaneous (sc) routes unless IFN-α/β is inhibited [83, 84]. The LD_{50} of this mouse-adapted virus is approximately one virion, which is equivalent to between 0.025 and 0.04 pfu on Vero E6 cells [83]. Pathologic changes observed in the liver and spleen of infected mice were reported to resemble those found in EBOV-Z-infected primates, with virus titers approaching 10^9 pfu/g of tissue [83]. This mouse-adapted virus was also lethal for guinea pigs and caused a severe illness that was lethal for one of three rhesus macaques examined [85].

To better understand the changes that occur during the adaptation of EBOV-Z to guinea pigs and mice, the complete genomes of some of the adapted viruses were sequenced [70]. Comparing guinea pig-adapted variants with nonadapted EBOV-Z identified single amino acid changes in the NP and L proteins. In addition, three amino acid changes were detected in the VP24 protein, resulting in an altered electrophoretic mobility of VP24 and the suggestion that changes in VP24 occurring during the adaptation process may be responsible for the increased virulence of the guinea pig-adapted virus [70].

Similar to observations with the guinea pig-adapted EBOV-Z, very few sequence changes resulted from the adaptation of EBOV-Z in mice. Single amino acid changes were observed in the NP, VP35, and VP24 proteins, and two amino acid changes were observed in the L protein of the mouse-adapted virus compared to the precursor EBOV-Z used for the adaptation process [J. Wilson, J. Kondig, A. Kuehne and M. K. Hart, unpublished observations]. However, none of the changes identified in the mouse-adapted virus were conserved with the guinea pig-adapted virus, indicating that there is no single nucleic acid change resulting in the adaptation phenotype of increased virulence in these species. The use of reverse genetics [60] to identify the specific mutations resulting in increased virulence of EBOV in rodents will help determine whether adaptation is associated with the function of a particular EBOV protein.

Treatments and vaccines for EBOV

Analysis of patient cases and experimental infection of animals indicated that the rapid infection rate and extreme pathogenicity of EBOV infection allows little time for the production of effective antiviral immune mechanisms in nonimmune individuals. Efforts are therefore underway to develop effective antiviral therapeutics and vaccines for EBOV infection.

Antiviral drugs and therapies

Conventional methods of treating viral infections are limited to providing supportive measures that might reduce the severity of clinical symptoms, providing immune sera as a prophylactic or therapeutic measure, or administering available drug treatments. One report claimed that treating EBOV-infected patients with convalescent whole blood during the 1995 EBOV-Z outbreak in Kikwit protected seven of eight patients from death, although the provision of better supportive care may also have contributed to fewer fatalities [86]. When the survival of the transfused patients was compared statistically with that of other patients of a similar age and gender, and at a similar stage of infection, there was no observed benefit from the transfusion [87]. Identifying the source of any benefit from the transfusion is further complicated because, in addition to providing EBOV-specific antibodies, the transfusion would have also provided coagulation factors or other mediators which could have reduced shock symptoms, and donor cells that could have stimulated im-

mune responses in the patients [86, 88]. As human convalescent sera are not readily available for analysis, investigators have studied the efficacy of polyclonal or monoclonal antibodies in animals. The results of these studies are presented in the antibody section below.

Other investigators have tried to identify chemotherapeutic agents as antiviral therapies for EBOV. The status of drug treatments for hemorrhagic fevers was recently reviewed [89]. Targets for intervention by antiviral drugs include inhibiting steps in viral replication or pathogenesis, as well as finding compounds that induce specific components of the immune system, such as IFN. Inhibition of EBOV replication in vitro has been observed with the class of drugs known as S-adenosylhomocysteine hydrolase inhibitors, which prevent efficient translation of viral mRNAs [90]. These compounds have also demonstrated therapeutic efficacy in BALB/c mice treated 1 or 2 days after infection [90, 91]. However, when tested in cynomolgus macaques, efficacy was limited to a delay in death compared to controls [M. Bray and J. Huggins, personal communication]. More effective therapies may need to include combinations of drugs and antibodies to eliminate both intracellular and extracellular virus.

The ability of IFN-α to provide antiviral protection in mice was recently demonstrated by the observation that mice became susceptible to nonadapted EBOV after treatment with antibodies to IFN-α or if they lacked IFN-α/β receptors [84]. However, this did not extend to cynomolgus macaques, as animals treated with IFN-α before challenge succumbed to EBOV-Z infection, although viremia and death were delayed by a few days compared to controls [92].

Vaccines

There are obvious safety risks associated with using conventional vaccine approaches consisting of attenuated or inactivated virus preparations for the development of EBOV vaccines. Therefore, most of the vaccine approaches for EBOV have examined the ability of one or more EBOV proteins to serve as protective antigens. Because the manner in which an antigen is presented to the effector cells of the immune system can qualitatively and quantitatively affect the immune responses to it, the selection of a vaccine strategy can significantly impact the protective efficacy of the vaccine.

For EBOV, the subunit vaccine strategies published to date have used live vaccinia virus vectors, DNA vaccination, Venezuelan equine encephalitis virus (VEEV) replicons, and a DNA prime with adenovirus boost strategy. As observed with studies using inactivated EBOV [93], these different vaccine approaches have had varying successes with regard to the protection induced to some of the EBOV proteins and the ability of the vaccines to protect nonhuman primates from lethal disease. In addition, the use of different animal strains, vaccine schedules, and challenge viruses has complicated the identification of the criteria that resulted in the described successes or failures. The various EBOV vaccine strategies examined to date are described below. The specific animals, schedules, and doses used in the studies are shown in table 2.

Vaccinia virus-vectored vaccines

The use of live vaccinia virus as a vector to express the EBOV proteins has the advantages that vaccinia virus has previously been used as a human vaccine and the EBOV proteins will be expressed in the cells that are normally infected by the vaccinia virus. One disadvantage of this vector approach is that immunological competition may result from the production of the numerous vaccinia virus proteins in the host cell, lowering immune responses to the particular EBOV protein expressed by the vector. Another disadvantage to this approach is that pre-existing immunity in people previously vaccinated with vaccinia virus may clear the infected cells before a sufficient immune response can be generated to the EBOV protein. Furthermore, the use of vaccinia poses a health threat for persons who are immunocompromised.

The efficacy of the EBOV GP, sGP, NP, VP35, or VP40 proteins [94] or VP24 [95] expressed from recombinant vaccinia viruses was evaluated in guinea pigs. Only the GP-vaccinated animals exhibited any level of protection, with three of five surviving challenge with 10^4 pfu of guinea pig-adapted EBOV [94]. Although all the vaccinated groups had detectable antibodies to EBOV by ELISA, the titers to GP ($\leq 1:80$) were lower than those observed in the other vaccine strategies evaluated. Three of the five GP-vaccinated guinea pigs that survived the challenge did not have detectable viremias on day 7 after challenge, but a lack of viremia was also observed in one GP-vaccinated animal that did not survive challenge. The authors concluded that the vaccine-induced responses did not prevent EBOV infection, as the virus-specific antibody titers increased after challenge, even in the GP-vaccinated group with surviving animals [94]. When the efficacy of the recombinant vaccinia viruses expressing EBOV-Z GP was evaluated in cynomologous macaques, the animals did not survive a sc challenge with 1000 pfu of EBOV-Z [K. Anderson, K. J. Gilligan, J. B. Geisbert and P. Jahrling, personal communication].

VEEV replicons

In this vaccine strategy, the VEEV structural protein genes are replaced with a gene coding for one of the EBOV proteins, resulting in a self-replicating RNA molecule (replicon) that can be packaged into a virus-like particle with helper RNAs encoding the VEEV structural proteins [96]. As the packaged replicons do not contain RNAs encoding the VEEV structural proteins, only an

Table 2. Vaccine strategies for protection against Ebola virus.

EBOV protein*	Vaccine dose	Schedule (days)	Delivery†	Animal model	Challenge (months)‡	Survival	Ref.
DNA Vaccination ± adenovirus boost							
GP	100 µg	0, 14, 28	IM	Hartley guinea pigs	1×10^3 pfu IP (4)	4/4	104
GP	100 µg	1, 14, 28, 42	IM	Hartley guinea pigs	1×10^3 pfu IP (2)	6/6	102
GP	100 µg	1, 14, 42, 112	IM	Hartley guinea pigs	1×10^3 pfu IP (4)	3/5	102
GP	0.5 µg	0	gene gun	BALB/c mice	1 pfu = 30 LD_{50} IP (3)	1/10	103
GP	0.5 µg	0, 28	gene gun	BALB/c mice	1 pfu = 30 LD_{50} IP (3)	4/10	103
GP	0.5 µg	0, 28, 56	gene gun	BALB/c mice	1 pfu= 30 LD_{50} IP (3 or 5)	7/10	103
GP	0.5 µg	0, 28, 56	gene gun	BALB/c mice	1 pfu= 30 LD_{50} IP (8)	2/9	103
GP	0.5 µg	0, 28, 56	gene gun	BALB/c mice	1 pfu= 30 LD_{50} IP (11)	5/10	103
GP	0.5/1.5 µg	0, 28, 56	gene gun	BALB/c mice	1 pfu= 30 LD_{50} IP (4)	10/10	103
sGP	100 µg	1, 14, 28, 42	IM	Hartley guinea pigs	1×10^3 pfu IP (2)	5/6	102
sGP	100 µg	1, 14, 42, 112	IM	Hartley guinea pigs	1×10^3 pfu IP (4)	3/5	102
NP	100 µg	1, 14, 28, 42	IM	Hartley guinea pigs	1×10^3 pfu IP (2)	4/4	102
NP	100 µg	1, 14, 42, 112	IM	Hartley guinea pigs	1×10^3 pfu IP (4)	1/4	102
NP	0.5 µg	0, 28, 56	gene gun	BALB/c mice	1 pfu = 30 LD_{50} IP (3)	7/10	103
NP	3 µg	0, 28, 56	gene gun	BALB/c mice	1 pfu = 30 LD_{50} IP (3)	8/10	103
GP/NP	75 µg GP, 25 ug NP	0, 14, 28	IM	Hartley guinea pigs	1×10^3 pfu IP (4)	4/4	104
GP-Z,S,IC/NP	25 µg each	0, 14, 28	IM	Hartley guinea pigs	1×10^3 pfu IP (4)	4/4	104
GP-Z,S,IC/NP	1 mg each	0, 28, 56	IM (0, 28), biojector (56) followed by:				
GP	1×10^{10} pfu adenovirus	140	IM	*Macaca fascicularis*	6 pfu IP (8)	4/4	104
VEEV replicon vaccination							
GP	$1–2 \times 10^6$ ffu§	0, 28	SC	BALB/c mice	1 pfu = 30 LD_{50} IP (2)	18/20	98
GP	1×10^7 ffu	0, 28	SC	strain 2 guinea pigs	1×10^4 pfu = 1000 LD_{50} SC (2)	3/5	98
GP	5×10^6 ffu	0, 28	SC	strain 2 guinea pigs	2×10^4 pfu = 2000 LD_{50} SC (2)	3/5	101
GP	1×10^7 ffu	0, 28, 126	SC	strain 13 guinea pigs	1×10^4 pfu = 1000 LD_{50} SC (6)	5/5	98
NP	$1–2 \times 10^6$ ffu	0, 28	SC	BALB/c mice	1 pfu = 30 LD_{50} IP (2)	20/20	98
NP	2×10^6 ffu	0, 28	SC	C57BL/6 mice	10 pfu = 300 LD_{50} IP (2)	8/10	100
NP	2×10^6 ffu	0, 28, 56	SC	C57BL/6 mice	10 pfu = 300 LD_{50} IP (3)	15/20	100
NP	1×10^7 ffu	0, 28	SC	strain 2 guinea pigs	1×10^4 pfu = 1000 LD_{50} SC (2)	0/5	98
NP	1×10^7 ffu	0, 28	SC	strain 2 guinea pigs	2×10^4 pfu = 2000 LD_{50} SC (2)	0/5	101
NP	1×10^7 ffu	0, 28, 126	SC	strain 13 guinea pigs	1×10^4 pfu = 1000 LD_{50} SC (6)	1/5	98
GP/NP	$2–4 \times 10^6$ ffu	0, 28	SC	BALB/c mice	1 pfu = 30 LD_{50} IP (2)	20/20	98
GP/NP	2×10^7 ffu	0, 28, 126	SC	strain 13 guinea pigs	1×10^4 pfu = 1000 LD_{50} SC (6)	5/5	98
VP24	2×10^6 ffu	0, 28, 56	SC	BALB/c mice	10 pfu = 300 LD_{50} IP (3)	18/20	99
VP24	2×10^6 ffu	0, 28	SC	BALB/c mice	1×10^3 pfu = 3x 10^4 LD_{50} IP (2)	5/5	99
VP24	2×10^6 ffu	0, 28	SC	BALB/c mice	1×10^5 pfu = 3 x10^6 LD_{50} IP (2)	5/5	99
VP24	2×10^6 ffu	0, 28, 56	SC	C57BL/6 mice	10 pfu = 300 LD_{50} IP (3)	0/20	99
VP30	2×10^6 ffu	0, 28, 56	SC	BALB/c mice	10 pfu = 300 LD_{50} IP (3)	17/20	99
VP30	2×10^6 ffu	0, 28, 56	SC	C57BL/6 mice	10 pfu = 300 LD_{50} IP (3)	2/20	99
VP35	2×10^6 ffu	0, 28, 56	SC	BALB/c mice	10 pfu = 300 LD_{50} IP (3)	5/19	99
VP35	2×10^6 ffu	0, 28, 56	SC	C57BL/6 mice	10 pfu = 300 LD_{50} IP (3)	14/20	99
VP40	2×10^6 ffu	0, 28, 56	SC	BALB/c mice	10 pfu = 300 LD_{50} IP (3)	14/20	99
VP40	2×10^6 ffu	0, 28, 56	SC	C57BL/6 mice	10 pfu = 300 LD_{50} IP (3)	1/20	99

Table 2 (continued)

VP40	2×10^6 ffu	0, 28, 56	SC	BALB/c mice	10 pfu = 300 LD_{50} IP (3)	14/20	99
VP40	2×10^6 ffu	0, 28, 56	SC	C57BL/6 mice	10 pfu = 300 LD_{50} IP (3)	1/20	99
Vaccinia-vectored vaccination							
GP	1×10^7 pfu	0	SC	strain13 guinea pigs	1×10^4 pfu = 1000 LD_{50} SC (1)	3/5	94
NP	1×10^7 pfu	0	SC	strain13 guinea pigs	1×10^4 pfu = 1000 LD_{50} SC (1)	0/5	94
VP24	5×10^5 pfu	0	scarification	guinea pigs	1×10^4 LD_{50} IP (0.75)	0/30	95
VP35	1×10^7 pfu	0	SC	strain13 guinea pigs	1×10^4 pfu = 1000 LD_{50} SC (1)	0/5	94
VP40	1×10^7 pfu	0	SC	strain13 guinea pigs	1×10^4 pfu = 1000 LD_{50} SC (1)	0/5	94
sGP	1×10^7 pfu	0	SC	strain13 guinea pigs	1×10^4 pfu = 1000 LD_{50} SC (1)	0/5	94

* GP, glycoprotein; sGP, soluble glycoprotein; NP, nucleoprotein; VP, virion protein; Z, Zaire; S, Sudan; IC, Ivory Coast. Unless otherwise noted, all vaccinations were with the EBOV proteins from the Zaire strain.

† IM, intramuscular injection; SC, subcutaneous injection.

‡ Animals were challenged with the indicated dose of adapted EBOV (guinea pigs or mice) or nonadapted EBOV-Z (M. fascicularis). Dose is indicated by pfu (plaque forming units) or LD_{50} (dose lethal for 50% of the animals). Numbers in parentheses indicate the time of challenge, expressed in terms of the number of months since the first vaccination. SC, subcutaneous; IP, intraperitoneal.

§ FFU, focus-forming units

abortive infection occurs in vivo, during which the cloned EBOV genes are expressed and presented to the immune system. The advantages of this system include the tropism of the VEEV for antigen-presenting dendritic cells [97] and the low likelihood of recombination leading to the production of viable VEEV. A disadvantage of this vector is that it may also be susceptible to pre-existing immunity [M. K. Hart, unpublished observation], although fewer people are immune to VEEV than to vaccinia virus. VEEV replicons encoding EBOV GP, NP, VP24, VP30, VP35, and VP40 have been evaluated as vaccine components. Protection from challenge with 30 LD_{50} (1 pfu) of mouse-adapted EBOV was induced in BALB/c mice by two doses of VEEV replicons expressing EBOV GP and/or NP [98]. In addition, the majority (70% or more) of BALB/c mice were protected from a 300 LD_{50} (10 pfu) challenge of mouse-adapted EBOV after vaccination with two or three doses of VEEV replicons expressing EBOV GP, NP, VP24, VP30, or VP40, but not VP35 [99]. Vaccination with the VP24 construct also protected five of five BALB/c mice from challenge with 3×10^6 LD_{50} (1×10^5 pfu) of mouse-adapted EBOV-Z. The ability of the other constructs to protect against very high doses of EBOV has not been tested. In contrast, C57BL/6 mice which differ genetically from BALB/c mice, including at the major histocompatibility locus, were only protected after vaccination with EBOV NP [100], GP [J. Wilson, M. Bray, R. Bakken and M. K. Hart, unpublished observation] or VP35 [99]. All of the protected groups had reduced viremias compared to control animals. The different efficacy observed in the two inbred mouse strains after vaccination with the EBOV-Z VP proteins, together with a lack of protection afforded by VP-specific antisera [99] suggests a role for T cells in protection against EBOV.

When the VEEV replicons expressing the EBOV GP and NP proteins were evaluated for efficacy in guinea pigs, vaccination with EBOV GP protected 60% of the inbred strain 2 guinea pigs [98, 101] and all of the strain 13 guinea pigs tested [98]. The different level of efficacy may be due to strain differences in the guinea pigs. Alternatively, it may be due to the higher EBOV-neutralizing antibody titers present at the time of challenge, presumably as a result of the extra dose of vaccine received by the strain 13 animals [98]. Although vaccination with replicons expressing NP induced EBOV-specific antibody titers (2.8–3.9 $\log_{10}$ in ELISA), neither guinea pig strain was protected from challenge. Cynomolgus macaques also failed to survive a sc challenge with 1000 pfu of EBOV-Z after vaccination to GP or GP and NP expressed from the VEEV replicons [J. Smith and P. Jahrling, personal communication].

The reason for the different efficacies between species is not known. The suggestion has been made that mice are easier than primates to protect against a variety of pathogens, which would appear to be supported by the

EBOV GP and NP data in the replicon studies. However, the published mouse studies examined protection against lower challenge doses (30 or 300 LD_{50}, 1 or 10 pfu), whereas the guinea pigs (1000 LD_{50}, 10,000 pfu) and monkeys (unknown LD_{50}, 1000 pfu) received higher challenge doses. The difference between 30, 300, and 1000 LD_{50} may not seem significant given the ability of these viruses to replicate to high titers in animals; however, similar increases in challenge doses overcame the efficacy afforded to guinea pigs by inactivated virus preparations [93]. Thus, whether vaccine failures in different species occurred because the necessary protective responses were not induced in the tested animals, or because the magnitude of the responses did not meet the threshold needed for protection against higher challenge doses remains to be determined.

DNA vaccination

In this vaccine strategy, selected EBOV genes were cloned into plasmid vectors. The recombinant DNA was administered by im injection or coated onto gold particles that were bombarded into the skin using a gene gun. As with the vaccinia and VEEV replicon vectors, an advantage to this system is that both humoral and cell-mediated immunity are induced to the EBOV proteins. Unlike the other two approaches, DNA vaccination is not subjected to concerns about anti-vector immunity. However, this vaccine strategy may require a high number of injections, and the possibility of integration of the cloned DNA into the host genome is also a concern.

Two groups used DNA vaccination to evaluate the protective efficacy of EBOV GP and NP. One group [102] injected 50 μg of DNA encoding either EBOV GP, sGP, or NP three times into BALB/c mice, or 100 μg of the same DNAs four times into outbred Hartley guinea pigs, using different vaccine schedules (see table 2). Vaccinating mice to sGP and GP induced detectable antibody responses and CTLs, but only detectable antibody responses were induced to NP by vaccination. Similarly, guinea pigs had antibody responses to the EBOV immunogens, and T cell proliferative responses were induced by vaccination to GP and sGP, but not NP. The protective efficacy of these vaccine candidates was only evaluated in the guinea pigs. Survival was defined as living for 10 days after challenge, at which time the animals were killed for pathologic examination. Control animals died within a week after intraperitoneal challenge. None of the animals vaccinated to either GP or NP on a shortened schedule (days 1, 14, 28, and 42) and challenged on day 62 died within 10 days after challenge, although one guinea pig vaccinated to sGP failed to survive challenge. In contrast, only three of five animals vaccinated to GP, and one of four vaccinated to NP survived 10 days beyond challenge when the vaccine schedule was changed to days 1, 14, 42, and 112, with challenge on day 122.

The early termination of the study by Xu et al. [102] has been questioned [81, 103]. Because unvaccinated guinea pigs may not die until 10 days after challenge, killing the vaccinated animals on day 10 did not allow sufficient observation time to determine if the animals were truly protected from challenge or if they would have had delayed deaths compared to controls. However, in general, the animals defined as survivors also tended not to have detectable viral antigen in their tissues. The finding, in one of two experiments, that vaccination to EBOV NP induced protection in guinea pigs differs from the studies performed using vaccinia or VEEV replicons. Whether this is due to the vaccine approach or if it is the result of the different challenge doses, routes, guinea pigs, and guinea pig-adapted viruses used in this experiment is not clear.

As serum from protected guinea pigs did not confer protection on three animals or inhibit virus replication in vitro, the authors concluded that antibody titers serve as a surrogate marker of immunity but effective immunity required a productive T cell response. However, as previously noted [103], this conclusion does not explain the protection attributed to the NP DNA vaccine, which induced detectable antibodies but not T cell responses.

When recombinant DNA was delivered by gene gun [103], the most effective schedule required four vaccinations of mice with EBOV GP DNA, the first at 0.5 μg and the subsequent ones at 1.5 μg. This is significantly less DNA than that used by Xu et al. [102]. Although only partial protection was observed in BALB/c mice given repeated doses of 0.5 μg of the GP DNA, some protective efficacy persisted for at least 9 months. As in the previous study [102], vaccination induced both GP-specific antibodies and GP-specific CTLs.

The study by Vanderzanden et al. [103] also evaluated EBOV NP as a protective antigen, and demonstrated that gene gun vaccination induced antibodies to NP and provided protection to the majority of mice tested. In contrast to the study by Xu et al. [102], CTLs were also induced in mice vaccinated to NP using the gene gun [103]. As both studies used BALB/c mice, this discrepancy can most likely be attributed to the efficiency of the vaccination strategy used or to differences in the methodology used to re-stimulate and test the cytolytic activity of spleen cells from vaccinated mice.

Studies evaluating the efficacy of gene gun vaccination in nonhuman primates have not been published. However, Sullivan et al. [104] recently extended the DNA injection studies to demonstrate protection in cynomolgus macaques after challenge with 6 pfu of EBOV-Z. The vaccination strategy included three 4-mg doses of DNA encoding the NP of EBOV-Z and the GPs of EBOV-Z, EBOV-S, and EBOV-IC at 1-month intervals, followed by a booster vaccination at 5 months with 1×10^{10} pfu of recombinant adenovirus expressing EBOV-Z GP. Mice

primed with DNA and boosted once with the recombinant adenovirus had approximately tenfold higher antibody titers than those induced by two vaccinations with DNA, and twofold higher titers than those induced by two vaccinations with recombinant adenovirus [104]. This suggests that the use of the adenovirus vector afforded a significant advantage to the vaccine protocol. However, a disadvantage is that the recombinant adenovirus vector may also be susceptible to pre-existing immunity.

The vaccine strategy used by Sullivan et al. [104] is the first known to successfully afford protection from EBOV to nonhuman primates and, as such, indicates that the development of an efficacious vaccine for human use may be feasible. This is a significant step forward. However, the study used high doses of DNA and recombinant adenovirus to induce immunity to a low challenge dose, which was estimated to be the equivalent of a few nanoliters of blood [105], or less than that which might be received from an accidental needlestick. Whether this vaccine strategy is capable of protecting against higher challenge doses of EBOV, and whether other vaccine approaches that failed to protect against a 1000 pfu challenge would protect against the lower dose remains to be determined.

The study by Sullivan et al. [104] also claimed that vaccination achieved sterile immunity in three of four macaques and that surrogate markers for protection were identified. Sterile immunity is a term used to indicate that the challenge virus was completely inactivated by the onboard immune responses before the virus was capable of infecting host cells and, as such, would not induce an anamnestic immune response. However, this was not evaluated in this study. Most vaccines do not achieve sterile immunity, but induce immune responses that control infections before the onset of illness. In addition, to determine statistically which immune responses correlate with protection, there should be protected and unprotected animals within the vaccinated groups. The statistical significance cited [104] actually represents a comparison between vaccinated and unvaccinated animals. Although this indicates that vaccination induced significant antibody and proliferative responses relative to the unvaccinated control animals, the analysis does not identify the responses or the magnitudes of responses that correlate with protection. Therefore, the surrogate markers of protection have yet to be identified for EBOV.

Immune mechanisms of protection

Numerous vaccine studies have suggested that the optimal EBOV vaccine should induce both antibody and CTL responses. The observation that both humoral and cellular responses are induced to EBOV proteins makes it difficult to determine whether both are necessary for protection. The examination of CTL responses is further complicated by the difficulty in examining these responses in the guinea pig and nonhuman primate models because of the need to know the MHC background of the tested animal. However, the development of the mouse-adapted EBOV-Z [83] has provided an important tool for identifying and characterizing the immune responses to specific EBOV proteins and for evaluating their contribution to protection.

Antibodies

The role of antibodies in mediating protection to EBOV has been questioned because of the low levels of virus-inhibiting antibodies detected in the sera of convalescent patients and because polyclonal sera to EBOV have inconsistently protected recipient animals from challenge. Similar questions were raised in a recent study that indicated that antibodies specific for the EBOV GP enhanced infection by a pseudotyped virus in vitro [106].

Both positive and negative reports of the ability of antibodies to mediate protection have been published. As convalescent human plasma is not readily available, one group demonstrated that a goat EBOV-specific immunoglobulin preparation provided protection to guinea pigs when administered within a day of challenge [107]. This preparation was later co-administered with IFN-α to four people who may have been accidentally exposed to EBOV. Only one of these, who sustained a needlestick wound from a syringe containing blood from an EBOV-infected monkey, developed signs that were suggestive of EBOV disease. However, although blood work was performed daily, the report does not mention looking for EBOV until after recovery, at which time neither virus nor EBOV-specific antibodies were found.

A similar preparation made from hyperimmunization of a horse with live EBOV-Z [108] had higher virus-neutralizing antibody titers and protected two baboons from lethal EBOV infection (30 LD_{50} injected im) when 6 ml of immune serum was administered 2 h before challenge [109]. Reduced efficacy was observed when the administered serum was diluted or administered more than 1 h after challenge. Similarly, the immune serum was efficacious in strain 13 guinea pigs when administered early, but not if treatment was delayed for 4 days [92].

In contrast, cynomolgus macaques that received 1–2 ml/kg of the equine immune serum did not survive challenge with 1000 pfu of EBOV-Z, regardless of when the antiserum was administered. However, these macaques exhibited delayed viremias and deaths relative to control animals [92]. Similarly, the immune serum did not protect most mice from challenge with 30 LD_{50} of mouse-adapted EBOV when up to 3 ml/kg of immune serum was administered within 30 min of challenge [92]. The transferred serum may not have been able to overcome the increased viral loads observed in cynomolgus macaques and mice compared with baboons, or the lack of efficacy

may have been due to other species-related differences (e.g., interactions with Fc receptors) or the different challenge doses used [107, 110].

Studies that examined polyclonal serum transfers within the same species have not clarified the role of antibodies in protection. Polyclonal EBOV GP-specific sera from inbred or outbred guinea pigs vaccinated with VEEV replicons [98] or DNA injection [102] were not efficacious against lethal challenge, although they also had much lower titers of virus-neutralizing antibodies than the horse serum. Transfer of polyclonal sera obtained from EBOV GP-vaccinated mice also gave inconsistent results. Mice were protected by GP-immune serum in one study [99] but not in another [98], in which both the dose of replicon-vectored vaccine and the amount of antiserum administered were lower. The previous successes observed with the horse serum may have been due to the presence of additional protective antibodies reactive with EBOV proteins other than GP. However, protective efficacy was not observed in mice that received murine polyclonal sera to the NP [98, 100] or any of the VP proteins [99].

Examining the protective capacity of polyclonal immune sera has certain limitations that may affect both the outcome and the conclusions regarding the role of antibodies in protection. While the titer of virus-specific antibodies can be measured in ELISA, the proportion of the response due to protective antibodies and to non-protective antibodies to EBOV cannot be determined. Therefore, deciding what dose should be administered to protect recipient animals can be difficult. If different animal models exhibit different viremias, then the protective amount of antibody needed is likely to vary as well. Furthermore, the immune responses of the recipient animals to xenogeneic serum will accelerate the clearance of the transferred serum. The effector functions of the Fc portion of the antibody molecule may also be affected, such as when the Fc receptor of the host fails to bind the Fc region of antibodies derived from another species, thereby reducing the ability of the antibody to clear the virus or virus-infected cells.

To eliminate these issues when examining the therapeutic and protective capacity of EBOV-specific antibodies, murine mAbs to the EBOV GP were produced and screened for protective efficacy in mice [59]. mAbs specific for five unique epitopes on the EBOV GP, one of which is conserved on all EBOVs that are pathogenic in humans, were protective when administered within 24 h of infection. Three of the epitopes were mapped to contiguous regions on the GP (fig. 2). Some of the mAbs were also effective when administered therapeutically up to 2 days after challenge, after substantial viral replication had occurred in the mice. Pooled mAbs to GP were also efficacious when tested against a 6000 LD_{50} (200 pfu) challenge dose [J. Wilson and M. K. Hart, unpublished observation]. However, reducing the amount of transferred mAb also reduced efficacy against the lower challenge dose [59]. This suggests that there may be a critical threshold for the amount of antibody needed to survive EBOV infection, especially in animals in which the EBOV replicates to high titers, and may explain some of the failures of polyclonal sera.

As previously observed for alphaviruses [111], some protective mAbs to EBOV did not inhibit plaque formation by EBOV, even when complement was present [59]. This negatively impacts the reliability of using the plaque reduction test to predict the role of antibodies in protection, and indicates that the low titers of plaque-inhibiting antibodies observed in convalescent people and animals cannot be used as a basis for questioning the relevance of antibodies in protection against EBOV.

Using another approach, researchers produced recombinant human antibodies from phage display libraries constructed from the bone marrow of two survivors of the 1995 EBOV-Z outbreak [112]. Although it has not been shown that these antibodies were actually produced in people during the infection, these results demonstrate that humans do have the potential to produce such antibodies. The antibodies were affinity selected using EBOV virions, and the anti-GP Fab fragments of the antibodies were engineered into IgG1 molecules. One EBOV GP-specific antibody, KZ52, inhibited EBOV in a plaque reduction neutralization assay (90% at 2.6 μg/ml as the complete IgG1 molecule) [113] and is being evaluated for its protective capacity. Whether this mAb or the murine mAbs [59] will be protective against nonadapted EBOV in a nonhuman primate model of EBOV infection remains to be seen.

Cytotoxic T lymphocytes (CTLs)

CTLs recognize peptides that are generated during intracellular degradation of viral proteins, and are then bound by a major histocompatibility complex class I or class II molecule and transported to the cell surface. The binding of the CTL to a cell bearing a specific peptide and major histocompatibility complex molecule induces intracellular signals that eventually result in lysis of the infected cell. The activity of CTLs is controlled, in part, by the fine specificity of the recognition process, which can be ablated by changes in the peptide sequence or the class I or II molecule presenting the peptide. It is this recognition that can make the role of CTLs in mediating protection hard to define, and which complicates vaccine development.

Murine CTLs to EBOV GP have been induced by DNA vaccines [102, 103] and liposome-encapsulated irradiated EBOV [114]. In addition, vaccination with DNA [103] or VEEV replicons [100] expressing NP also induced CTLs to EBOV NP. Viral sequences recognized by the murine CTLs have been identified in the carboxy-terminal end of GP [114] and the amino-terminal end of NP

[100]. CTLs were shown to contribute to protection from EBOV by the demonstration that unvaccinated mice survived lethal challenge if EBOV-specific CTLs had been adoptively transferred to them [100]. However, because serum antibodies to EBOV were also detected in these mice after challenge, it is not known whether the CTLs were solely responsible for the observed protection. This lack of sterile immunity was not unexpected, because CTLs do not prevent viral infection, and the lysis of EBOV-infected cells would have exposed the humoral immune system to the viral antigens.

CTL activity to EBOV has not yet been demonstrated in the guinea pig or nonhuman primate models. However, it is possible that the inability of the EBOV NP and VP proteins to protect inbred guinea pig strains from lethal EBOV challenge [94, 98, 101] may have been due to an inability of the guinea pigs to generate CTL responses to these proteins.

The demonstration that both antibodies and CTLs contribute to protection in the murine model of Ebola hemorrhagic fever suggests that the optimal EBOV vaccine for human use may need to be capable of inducing both responses. Although CTLs to EBOV NP were necessary for protecting mice from lethal challenge [100], protective efficacy mediated by CTLs specific for the other viral proteins has not been demonstrated. To better ensure the likelihood of inducing CTLs in humans with different major histocompatibility complex backgrounds, the development of an optimal EBOV vaccine may require the inclusion of several EBOV proteins.

Summary

Significant advances have recently been made in our understanding of the possible role of individual EBOV proteins in pathogenesis. The development of a reverse genetics system to generate infectious EBOV should enable better characterization of the role of these proteins in their native context. In addition, the current studies attempting to identify the immune mechanisms of protection and targets for therapeutic intervention should help facilitate the development of efficacious vaccines and/or treatments for EBOV that are suitable for human use.

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