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[†]Author for Correspondence
 Virology Division, United States
 Army Medical Research Institute of
 Infectious Diseases, Fort Detrick,
 MD, 21702-5011, USA
 Tel.: +1 301 619 4803
 Fax: +1 301 619 2290
 tom.geisbert@amedd.army.mil

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Towards a vaccine against Ebola virus

Thomas W Geisbert[†] and Peter B Jahrling

Ebola virus infection causes hemorrhagic fever with high mortality rates in humans and nonhuman primates. Currently, there are no vaccines or therapies approved for human use. Outbreaks of Ebola virus have been infrequent, largely confined to remote locations in Africa and quarantine of sick patients has been effective in controlling epidemics. In the past, this small global market has generated little commercial interest for developing an Ebola virus vaccine. However, heightened awareness of bioterrorism advanced by the events surrounding September 11, 2001, concomitant with knowledge that the former Soviet Union was evaluating Ebola virus as a weapon, has dramatically changed perspectives regarding the need for a vaccine against Ebola virus. This review takes a brief historic look at attempts to develop an efficacious vaccine, provides an overview of current vaccine candidates and highlights strategies that have the greatest potential for commercial development.

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Ebola virus (EBOV) has gained public notoriety in the last decade largely as a result of the enormous interest and alarm generated by the news media. This attention is primarily a consequence of the highly publicized isolation of EBOV in a suburb of Washington, DC., in 1989 coupled with its high case-fatality rate (near 90% in some outbreaks), unusual and striking morphology and its dramatic clinical presentation and lack of effective specific treatment. Progress in understanding the origins of the pathophysiological changes that make EBOV infections of humans so devastating have been slow, primarily because these viruses require biosafety level (BSL)-4 containment for safe research.

EBOV infections are usually the most severe of the viruses that cause hemorrhagic fever (HF). Clinical symptoms appear suddenly after an incubation period of 2 to 21 days [1]. Common presenting complaints include high fever, chills, malaise and myalgia [2–7]. As the disease progresses, there is evidence of multi-systemic involvement and manifestations include prostration, anorexia, vomiting, nausea, abdominal pain, diarrhea, shortness of breath, sore throat, edema, confusion and

coma [2–7]. Petechiae, ecchymoses, mucosal hemorrhages and uncontrolled bleeding at venipuncture sites are notable observations [2–7]. The presence of a maculopapular rash is a prominent feature [2–7] but is not pathognomonic for EBOV HF. Fulminant EBOV infection typically evolves to shock, convulsions and, in most cases, diffuse coagulopathy ensues [2–7]. It should be noted that evidence of asymptomatic Ebola infection was documented in a small group of individuals during a recent outbreak [8] but the clinical and epidemiological relevance of this observation, at this time, is uncertain.

Genetics & viral proteins

The family *Filoviridae* is comprised of two genera: *Marburgvirus* (MARV) and *Ebolavirus* (EBOV). The *Ebolavirus* genus is further subdivided into four distinct species: *Ivory Coast ebolavirus* (ICEBOV), *Reston Ebolavirus* (REBOV), *Sudan Ebolavirus* (SEBOV) and *Zaire Ebolavirus* (ZEBOV). EBOV particles contain an approximately 19 kb single, negative-stranded, linear RNA genome that is noninfectious. The genome encodes seven structural and one nonstructural protein

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14. ABSTRACT Ebola virus infection causes hemorrhagic fever with high mortality rates in humans and nonhuman primates. Currently, there are no vaccines or therapies approved for human use. Outbreaks of Ebola virus have been infrequent, largely confined to remote locations in Africa and quarantine of sick patients has been effective in controlling epidemics. In the past, this small global market has generated little commercial interest for developing an Ebola virus vaccine. However, heightened awareness of bioterrorism advanced by the events surrounding September 11, 2001, concomitant with knowledge that the former Soviet Union was evaluating Ebola virus as a weapon, has dramatically changed perspectives regarding the need for a vaccine against Ebola virus. This review takes a brief historic look at attempts to develop an efficacious vaccine, provides an overview of current vaccine candidates and highlights strategies that have the greatest potential for commercial development.					
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with a gene order of: 3' leader, nucleoprotein (NP), virion protein (VP)35, VP40, glycoprotein (GP), VP30, VP24, polymerase L protein and 5' trailer [9]. Four of these proteins, NP, VP30, VP35 and L, associate with the genomic RNA in a ribonucleoprotein complex, while the three remaining proteins (GP, VP24, VP40) are associated with the membrane. GP is the surface GP that forms the spikes on the virion and is the effector for receptor binding and membrane fusion [10,11]. GP is synthesized as a precursor molecule, GP₀, which is postrationally cleaved by furin or a furin-like endoprotease into two subunits, GP₁ and GP₂; these subunits are linked by disulfide bonding to form a heterodimer [12,13]. Homotrimers of GP₁–GP₂ comprise the virion spikes and are the primary target of the host immune response. Interestingly, the primary product of the GP gene of EBOV is not the structural GP; rather it is a small nonstructural, soluble GP (sGP) that is expressed from unedited transcripts [14,15]. VP40 functions as a matrix protein and is responsible for the formation of the filamentous particles [16], while VP24 is a minor viral protein whose functions remain unknown.

Epidemiology

Outbreaks of EBOV documented by virus isolation are shown in TABLE 1. EBOV was first recognized during near-simultaneous explosive outbreaks in 1976 in small communities in the former Zaire (now the Democratic Republic of the Congo [DRC]) [6] and Sudan [5]. There was significant secondary transmission through reuse of unsterilized needles and syringes and nosocomial contacts. These independent outbreaks involved serologically distinct viral species, ZEBOV and SEBOV. The ZEBOV outbreak involved 318 cases and 280 deaths (88% mortality), while the SEBOV outbreak involved 284 cases and 151 deaths (53% mortality). Since 1976, EBOV has appeared sporadically in Africa, causing several small- to mid-size outbreaks between 1976 and 1979. In 1995, there was a large epidemic of ZEBOV HF involving 315 cases, with an 81% case-fatality rate, in Kikwit, a community in the former Zaire [1]. Meanwhile, between 1994 and 1996, there were smaller outbreaks caused by ZEBOV in Gabon [17]. More recently, Uganda, Gabon and the DRC have suffered large epidemics of viral HF attributed to EBOV. The current outbreak in the DRC has also involved a catastrophic decline in populations of great apes, which are thought to have a role in transmission to humans [18].

In 1989, a third species of EBOV, REBOV, appeared in Reston, Virginia, in association with an outbreak of viral HF among cynomolgus monkeys (*Macaca fascicularis*) imported to the USA from the Philippine Islands [19]. Hundreds of monkeys were infected (with high mortality) in this episode but no human cases occurred, although four animal caretakers seroconverted without overt disease. Epizootics in cynomolgus monkeys recurred at other facilities in the USA and Europe through 1992 and again in 1996. A fourth species of EBOV, ICEBOV, was identified in Côte d'Ivoire in 1994; this

species was associated with chimpanzees and only one nonfatal human infection was identified [20]. Very little is known about the natural history of filoviruses. Implication of animal reservoirs and arthropod vectors has been aggressively sought without success.

Pathogenesis

Human Ebola HF

The pathophysiology of human EBOV HF has not been clearly defined because of the limited number of cases being managed in a medical setting equipped for both safe and exhaustive clinical laboratory evaluations. Despite over 1200 known fatal cases of EBOV infection, only a very limited number of tissues from two cases of SEBOV in 1976, three cases of ZEBOV in 1976 and 18 cases of ZEBOV in 1996, have been examined [21–24]. Thus, much of what is known about EBOV pathogenesis has been inferred from using animal models (discussed below).

EBOV infection is characterized by lymphopenia with depletion of lymphoid tissue among the main features of the disease. Recent studies of ZEBOV outbreaks in Kikwit and Gabon have provided some new information on the inflammatory responses during filoviral infections [25–27]. Markedly elevated levels of interferon (IFN)- α , IFN- γ , interleukin (IL)-2, IL-10 and tumor necrosis factor (TNF)- α were reported in fatal cases in Kikwit [26]. In Gabon, the presence of IL-1 β and elevated concentrations of IL-6 in plasma during the symptomatic phase of infection were associated with survival while release of IL-10 and high levels of neopterin and IL-1RA were associated with a fatal outcome [27]. In addition, massive intravascular apoptosis developed rapidly after infection and persisted until death [25]. Available data suggest that T-lymphocytes are deleted mainly by apoptosis in peripheral blood mononuclear cells of fatal cases [25].

Animal models of Ebola HF

The use of animal models has been invaluable for studying the pathogenesis of numerous infectious diseases as well as for testing the efficacy of experimental prophylactic and therapeutic vaccine and/or drug regimens. Guinea-pigs and mice have been the primary rodent models employed to study EBOV HF [28–31]. While rodents clearly have utility as models of filoviral disease, we recently showed that rodent models of EBOV HF are not ideal for studying human EBOV HF [32]; others have suggested that guinea-pigs are inadequate for analyzing the pathogenesis of human EBOV HF [28]. More specifically, mice do not exhibit the coagulation abnormalities that characterize primate EBOV infections [32,33]. The development of coagulopathy in EBOV-infected guinea-pigs is uncertain with findings varying among studies [29–33]. Furthermore, bystander lymphocyte apoptosis, which is associated with human and nonhuman primate EBOV infections [34], has not been reported in EBOV-infected mice or guinea-pigs. As expected, clinical disease and related pathology in nonhuman primates infected with EBOV appear to more closely resemble features described for human EBOV HF.

Table 1. Ebola virus outbreaks documented by virus isolation.

Location	Year	Species	Human cases (mortality)	Origin, epidemiology
Southern Sudan	1976	SEBOV	284 (53%)	Unknown, close contacts
Northern DRC	1976	ZEBOV	318 (88%)	Unknown, iatrogenic
Tandanala, DRC	1977	ZEBOV	1 (100%)	Unknown
Southern Sudan	1979	SEBOV	34 (65%)	Unknown, same site as 1976
Virginia, USA	1989–1990	REBOV	4 (0%)	Imported monkeys
Sienna, Italy	1992	REBOV	0 (0%)	Imported monkeys
Minkouka, Gabon	1994	ZEBOV	49 (59%)	Unknown
Côte d'Ivoire	1994	ICEBOV	1 (0%)	Chimpanzee contact
Kikwit, DRC	1995	ZEBOV	315 (81%)	Unknown, close contacts
Texas, USA	1996	REBOV	0 (0%)	Imported monkeys
Mayibout, Gabon	1996	ZEBOV	31 (68%)	Chimpanzee consumption?
Booué, Gabon	1996–1997	ZEBOV	61 (74%)	Unknown
Uganda	2000–2001	SEBOV	428 (53%)	Unknown
Gabon/DRC	2001–2002	ZEBOV	92 (75%)	Gorilla consumption?
Northern DRC	2003	?	143 (90%)	Gorilla consumption?

DRC: Democratic Republic of the Congo; IC EBOV: Ivory Coast ebolavirus; REBOV: Reston ebolavirus; SEBOV: Sudan ebolavirus; ZEBOV: Zaire ebolavirus.

While disseminated intravascular coagulation (DIC) is often viewed to be a prominent manifestation of EBOV infection in primates, the presence of DIC in human filoviral infections has been a controversial topic; cultural mores and logistical problems have hampered systematic studies. No single laboratory test is sufficient to permit a definitive diagnosis of DIC. In most instances, a diagnosis of DIC can be made by taking into consideration the underlying disease in conjunction with a combination of laboratory findings [35–37]. In human EBOV cases, clinical laboratory data suggest that DIC is likely to be an important feature of human disease [2,5,6]. The coagulation picture is clearer for nonhuman primates. Numerous studies showed histological and biochemical evidence of DIC syndromes in EBOV infection of a variety of nonhuman primate species [32,33,38–47].

Fibrin deposits in tissues of 15 of 15 rhesus monkeys and eight of eight cynomolgus monkeys, experimentally infected with ZEBOV at terminal stages of disease were demonstrated [32, TW GEISBERT, UNPUBLISHED DATA]. Moreover, the authors recently confirmed fibrin deposits in tissues of four of four monkeys euthanized on the fourth day after ZEBOV challenge in a model where all animals succumb to ZEBOV infection between the 6th and 8th day after challenge [47].

Some have argued that fibrin deposition is not ubiquitous in EBOV-infected primates citing studies reporting that both viral strain and nonhuman species can affect the prominence of fibrin deposits [44,45]. Unfortunately, these investigators failed to recognize that the appearance of fibrin deposits is

only one of several indicators of a dysregulated coagulation response. Other indicators of coagulopathy include consumption of clotting factors, increase in clotting times, increase in levels of fibrin degradation products and thrombocytopenia. A more extensive review of previous ZEBOV studies in non-human primates reveals evidence of coagulopathy in nearly every case, although those correlates may vary with species. For example, in ZEBOV-infected baboons, dramatic changes were noted in blood-clotting parameters including marked increases in fibrin degradation products but fibrin deposits were not a prominent feature. This finding conclusively shows that elevated levels of fibrin were being formed at some point during the course of infection. In contrast to nonhuman primates, Bray and colleagues convincingly reported that infection of BALB/c mice with mouse-adapted ZEBOV did not cause a progressive coagulation defect over the course of the illness [33] further corroborating previous observations.

While there is no definitive test for DIC, elevated levels of D-dimers are present in over 95% of diagnosed cases in humans [35]. We have observed elevated levels of D-dimers in all of the authors rhesus and cynomolgus monkeys experimentally infected with ZEBOV [47, TW GEISBERT, UNPUBLISHED OBSERVATION]. Regrettably, there are no reports of D-dimers being evaluated in human cases of EBOV HF; however, retrospective examination of historical samples may still be able to define the importance of DIC in human EBOV disease.

Monocytes/macrophages are primary cellular targets of EBOV in rodents and primates [24,30,31,42–44]. EBOV-infection

of mononuclear phagocytes triggers a cascade of events involving cytokines/chemokines and oxygen free radicals [48–49]; it is thought that the consequence of these events, rather than direct viral infection, causes much of the observed pathology [48–50]. Other recent work shows that ZEBOV infection induces over-expression of the procoagulant tissue factor in primate monocytes/macrophages, suggesting a potential triggering mechanism for the coagulation defects that characterize EBOV infections [47].

Lymphocytes do not support EBOV replication; however, EBOV infections induce apoptosis of bystander lymphocytes in nonhuman primate tissues and in cultures of human peripheral blood leukocytes [34]. More recently, in a temporal study of ZEBOV-infected monkeys, it was observed that apoptosis of bystander lymphocytes occurred relatively early in the disease course [46]. In addition, it was noted that dendritic cells (DCs) were early cellular targets of ZEBOV infection in these animals [46]. This finding is of particular importance as others have shown that EBOV infects human monocyte-derived DCs and impairs their function [51]. Specifically, these investigators demonstrated that monocyte-derived DCs exposed to EBOV failed to secrete pro-inflammatory cytokines, did not upregulate costimulatory molecules including B7-1 and B7-2 and stimulated T-lymphocytes poorly. Apoptosis may result from the lack of costimulatory signals or via the engagement of death receptors, such as Fas or TNF-related apoptosis-inducing ligand (TRAIL). As an example, DCs have been shown to prevent Fas-mediated T-lymphocyte apoptosis through costimulatory rescue signals [52]. Therefore, it is possible that EBOV-induced dysfunction of DCs impairs costimulatory signals important for both rescue of activated T-cells and/or for the proper development of T-lymphocyte responses. In addition, the rapid induction of TRAIL and possibly, Fas, in EBOV-infected macrophages and DCs [49] suggests that these may be key factors in the observed bystander apoptosis of lymphocytes in EBOV-infected non-human primates. Interestingly, we recently noted that EBOV induces antiapoptotic transcripts, neuronal apoptosis inhibitory protein (NAIP) and cellular inhibitor of apoptosis protein 2 (cIAP2), in cells that it infects [46]. Thus, regulation of host cell and bystander cell apoptosis by EBOV may be significant components of a strategy to evade immunity and enhance viral survival.

Historical perspective of Ebola vaccine development

The effort to develop an EBOV vaccine began after the initial identification of EBOV in 1976. Early attempts were based on classical approaches of using inactivated whole virion preparations as vaccines [53–55] (TABLE 2). Results from these studies were inconsistent. Lupton and colleagues showed partial protection of guinea-pigs using either heat- or formalin-inactivated whole virion preparations [53]. However, the guinea-pig model employed in these studies was not uniformly lethal as only 29% of the EBOV-positive control animals died. Mikhailov and colleagues were the first to demonstrate significant protection of

nonhuman primates against lethal filoviral challenge as they protected four of five hamadryas baboons (*Papio hamadryas*) in one study after vaccination with a formalin-inactivated purified whole virion ZEBOV vaccine [54]. Studies by other investigators in guinea-pigs, using a formalin-inactivated vaccine that was not purified, associated the protective effect of ZEBOV vaccination with the dose of challenge virus employed [55]. For example, when vaccinated guinea-pigs were challenged with a low infective dose (10 lethal dose [LD]₅₀) all vaccinated animals survived. However, all vaccinated animals in these dosing studies died after receiving higher infective doses (100 or 1000 LD₅₀) of ZEBOV.

Considering that heat and/or formalin may alter the structure of potentially protective epitopes, other efforts evaluated whole-virion preparations of ZEBOV inactivated by γ -rays. In one study, BALB/c mice vaccinated with a γ -irradiated, purified ZEBOV preparation were partially protected when challenged with mouse-adapted ZEBOV [56]. The survival rate ranged from 40 to 70% and the outcome was associated with both the route of vaccination and the interval between the final vaccination and EBOV challenge. In another study, only one of four macaques vaccinated with this same γ -irradiated ZEBOV whole virion preparation survived lethal challenge [32].

Liposomes containing lipid A were evaluated as a delivery system for inactivated EBOV antigens in hopes that this method would elicit enhanced antibody and cellular immune responses [56]. Mice vaccinated with γ -irradiated, purified ZEBOV whole virions in liposomes containing lipid A developed a cytotoxic T-lymphocyte (CTL) response to two peptides present in the GP but did not develop neutralizing antibodies to ZEBOV. The level of protection observed in vaccinated mice was dependent on the route of administration with the animals vaccinated intravenously being uniformly protected. Moreover, this study showed that establishing protective immunity in these mice required the presence of CD4⁺ T-cells during the vaccination period as administration of anti-CD4 monoclonal antibodies before and during vaccination prevented the induction of a protective immune response. While these results using liposome-encapsulated irradiated ZEBOV in mice were encouraging, this same strategy failed to protect cynomolgus monkeys from lethal ZEBOV infection [32].

Current status of Ebola virus vaccines

The recent focus on EBOV vaccine development has been concentrated on various recombinant vectors for expression of EBOV-encoded proteins in various combinations to induce protective immunity and tested for protective efficacy in animal models of EBOV HF (TABLE 3). Many of these strategies have centered on GP, as it is the only structural protein exposed on the surface of viral particles and thus is the logical target for neutralizing antibody. Delivery systems used to express EBOV proteins for these purposes include naked DNA, adenovirus, baculovirus, vesicular stomatitis virus (VSV), vaccinia and Venezuelan equine encephalitis virus (VEEV) replicons.

Table 2. Comparison of different inactivated Ebola virus vaccines.

Vaccine	Inactivation method	Survivors/total challenged				Reference
		Animal model				
		<i>Mouse</i>	<i>Guinea-pig</i>	<i>Macaque</i>	<i>Baboon</i>	
Virions	Heat	NT	14/14 ^a	NT	NT	[53]
Virions	Formalin	NT	0–100% ^b	NR	6/9, 0/26	[53–55]
Virions	γ rays	40–70% ^c	NT	1/4	NT	[32,56]
Virions in liposomes	γ rays	50–100% ^c	NT	0/3	NT	[32,56]

a: The model used in this study was only 29% lethal (only 4 of 14 unvaccinated control animals died); b: 100% protection was only observed in the 29% lethal model, 0–64% protection was observed in a separate study using a 100% lethal model with protection correlating with the dose of challenge virus employed; c: Level of protection was dependent on route of vaccination; NT: No testing reported.

Recombinant vaccinia viruses

A number of EBOV proteins have been tested for immunogenicity and protective efficacy using the vaccinia virus system [32,57,58]. Low levels of Ebola virus-specific antibodies were elicited in guinea-pigs vaccinated with recombinant vaccinia viruses expressing ZEBOV sGP, GP, NP, VP24 and VP40 [57,58]. Although the guinea-pigs developed an immune response to these vaccines, the recombinant viruses did not usually confer protection from viremia and/or lethal infection. In some cases, vaccination prolonged survival and/or protected small percentages of animals from death. Of the EBOV proteins evaluated, GP was the most efficacious as guinea-pigs vaccinated with constructs expressing GP were partially protected (three of five) from lethal disease [58]. However, the recombinant vaccinia viruses expressing GP were unable to prolong survival or protect cynomolgus monkeys from lethal EBOV HF [32].

Although the results obtained thus far using recombinant vaccinia viruses in animal models of EBOV HF have not been encouraging, improvement may be possible. Currently, most vaccinia expression systems employ the modified vaccinia virus Ankara (MVA)-T7 RNA polymerase promoter. A recent study comparing two different recombinant vaccinia viruses, one generated using the MVA-T7 RNA polymerase promoter and the other using a different RNA polymerase promoter (phage T7), revealed that post-translational processing of Marburg virus (MARV) GP is impaired in the MVA-T7 but not in the vTF7-3 system [59]. At least one of the two approaches tested as candidate EBOV vaccines [32,58] did not use either of these systems [K. ANDERSON, PERSONAL COMMUNICATION]. Moreover, it is unknown whether EBOV GP is impaired in any of these systems but the possibility of incorrect protein processing may relate to the failure to generate an effective immune response.

Venezuelan equine encephalitis virus replicons

Perhaps the most extensive effort to develop an EBOV vaccine has been directed toward the Venezuelan equine encephalitis virus (VEEV) replicon platform. The potential for alphavirus replicon vectors as vaccines against microbial pathogens was

realized nearly a decade ago. A VEEV replicon vaccine vector system was first developed by Pushko and colleagues and was used to protect mice in a lethal model of influenza virus infection [60]. These investigators subsequently employed this system to evaluate ZEBOV proteins in murine and guinea-pig models of ZEBOV HF [61–63]. Specifically, VEEV replicons expressing either GP, NP, or both GP and NP, conferred nearly uniform protection to BALB/c mice from lethal ZEBOV challenge. Interestingly, while NP protected 20 of 20 mice, the same vaccine protected only one of ten guinea-pigs from lethal EBOV HF. VEEV replicons expressing GP protected three of five strain two guinea-pigs and five of five strain 13 guinea-pigs from lethal disease and vectors expressing both GP and NP also protected five of five strain 13 guinea-pigs.

Encouraged by these successful rodent data, this group vaccinated cynomolgus monkeys with VEEV replicons expressing either GP, NP, or both GP and NP. None of the nine vaccinated animals were protected from lethal ZEBOV infection [32]. Once again, vaccines that protected rodents failed in primates. In analogous studies to evaluate the VEE replicon vector expressing MARV GP, cynomolgus monkeys were protected from homologous MARV challenge despite the absence of neutralizing antibody titers in prechallenge sera [64]. As the T-cell responses were not measured in either of these VEEV replicon studies in macaques, we cannot assess the importance of the CTL response in conferring protection against MARV but not EBOV.

Subsequent studies by other groups showed some protective efficacy in mice using other ZEBOV proteins including VP24, VP30, VP35 and VP40, although vaccination failed to protect mice from viremia [65]. In these studies, protective efficacy was correlated with the strain of mouse employed. For example, vaccination with VEEV replicons expressing VP24 protected 37 of 40 BALB/c mice from lethal ZEBOV infection but failed to protect any of 20 C57BL/6 mice employed. Conversely, vaccination with VEEV replicons expressing VP35 protected 14 of 20 BALB/c mice but only conferred protection to 9 of 39 C57BL/6 mice. This group also demonstrated that C57BL/6 mice were somewhat more difficult to protect using VEEV replicons expressing NP; 23 of 30 mice were protected from

Table 3. Comparison of different Ebola virus genetic vaccines.

Vaccine	Gene Product	Survivors/total challenged			Reference
		Animal Model			
		Mouse	Guinea-pig	Macaque	
Vaccinia	GP	NT	3/5	0/3	[32,58]
Vaccinia	sGP	NT	0/5	NT	[58]
Vaccinia	VP24	NT	0/30	NT	[57]
Vaccinia	VP35	NT	0/5	NT	[58]
Vaccinia	VP40	NT	0/5	NT	[58]
VEEV replicon	GP	18/20	13/15 ^a	0/3	[32,62,63]
VEEV replicon	NP	20/20 ^b	1/10	0/3	[32,62,66]
VEEV replicon	GP+NP	20/20	5/5	0/3	[32,62]
VEEV replicon	VP24	37/60 ^c	NT	NT	[65]
VEEV replicon	VP30	30/60 ^c	NT	NT	[65]
VEEV replicon	VP35	23/59 ^c	NT	NT	[65]
VEEV replicon	VP40	32/60 ^c	NT	NT	[65]
VSV	GP _Δ	100% ^d	NT	NT	[82]
Baculovirus	GP	NT	3/6	NT	[70]
Baculovirus	GP	NT	1/6	NT	[70]
DNA	GP	50–100% ^e	14/21	NT	[67–70]
DNA	sGP	NT	8/11	NT	[68]
DNA	NP	70–80% ^e	5/8	NT	[67,68]
DNA	GP+NP	NT	8/8	NT	[69]
DNA + Adeno	GP+NP	NT	NT	4/4	[69]
DNA + Baculovirus	GP	NT	0/6	NT	[70]
DNA + Baculovirus	GP _Δ	NT	2/6	NT	[70]
Adeno	GP+NP	NT	NT	8/8	[72]
Adeno + Adeno	GP+NP	NT	NT	8/8	[72]

a: Total number represents the combined data of two published studies from the same group; b: Subsequent study using a different mouse strain protected 23 of 30 from lethal Ebola infection; c: Total number represents the combined data for two mouse strains; d: Total number of mice was not reported; e: Survival rate varied depending on dose of DNA administered; Adeno: Adenovirus; GP: Glycoprotein; GP Δ : Terminally deleted GP; NP: Nucleoprotein; NT: No testing reported; s: Soluble; VEEV: Venezuelan equine encephalitis virus; VP: Virion structural protein; VSV: Vesicular stomatitis virus.

lethal ZEBOV infection [66] versus the uniform protection seen in other studies using BALB/c mice [62].

The utility of the VEEV replicon system as a viable platform for an EBOV vaccine is unresolved and is the subject of much debate. It is possible that improvements to the vector itself and/or to the choice of proteins included in the vaccine may enhance the efficacy of this system. Conversely, there are concerns about the acceptability of this system for use in humans. As previously discussed by others, the presence of

copackaged and/or recombinant virus in replicon preparations, or the antigenicity of high doses of VEEV replicons, may induce immunity to the vector itself and limit its usefulness for subsequent vaccinations against other pathogens [60]. Although VEEV replicons are designed not to replicate vector structural proteins, an antivector response could occur. Moreover, there are concerns regarding replication-competent VEEV in a vaccine preparation and the likelihood that any such incident would cause disease.

DNA-based vaccines

Results evaluating the immunogenicity and protective efficacy of DNA vaccines using both GP and NP are equivocal. Vaccination of BALB/c mice with plasmids expressing either the ZEBOV GP or NP genes elicited both antibody responses and CTL responses to these viral proteins [67]. Challenge of the vaccinated mice resulted in partial protection against homologous virus depending on the dose of DNA administered [67]. Vaccination of guinea-pigs with plasmids expressing either ZEBOV GP, sGP, or NP elicited humoral immune responses against all three gene products and CTL responses against GP and sGP [68]. Protection against lethal ZEBOV challenge was incomplete and appeared to depend on the vaccine regimen [68]. However, results of this study were difficult to interpret because all guinea-pigs were euthanized 10 days after ZEBOV challenge, which is within the expected survival time for untreated animals (8–14 days). Subsequent studies using small groups of animals ($n = 4$) showed complete protection of guinea-pigs vaccinated with ZEBOV GP or both GP and NP [69]. However, other studies demonstrated little protection (one in six) of guinea-pigs vaccinated with ZEBOV GP against a lethal challenge with homologous virus [70].

Perhaps the greatest utility of DNA vaccination is when used as part of a prime boost vaccination strategy. Until very recently, the most successful EBOV vaccine strategy involved using a DNA prime followed by an adenovirus boost. This first success at completely protecting nonhuman primates from EBOV HF was demonstrated by Sullivan and colleagues [69]. In this study, cynomolgus monkeys were vaccinated three times with DNA expressing GPs of ZEBOV, SEBOV and ICEBOV and NP of ZEBOV followed 3 months later by a booster vaccination of adenovirus expressing the ZEBOV GP. All four vaccinated animals survived challenge at week 32 of the vaccination regimen when exposed to 6 plaque forming unit (PFU) of ZEBOV. The results of this study suggested that cell-mediated immunity was important but not an absolute requirement for protection, while concomitantly showing that antibody and T-memory helper cells were strongly associated with protection [69]. The significance of this study was diminished by the choice of low viral challenge dose [71]; nonetheless, all positive control animals succumbed to ZEBOV infection.

Recently, other prime-boost vaccine approaches have been pursued for EBOV. Specifically, a DNA prime-baculovirus-expressed ZEBOV GP boost regimen was tested in guinea-pigs [70]. Although animals developed antibody responses to EBOV GP, protection against homologous virus was incomplete with only two of 12 animals surviving lethal challenge.

Adenoviruses

Previous studies compared the immune response of mice vaccinated with plasmids encoding ZEBOV GP followed by boosting with adenovirus expressing the ZEBOV GP with adenovirus expressing ZEBOV GP alone [68,69]. These studies

showed that the antibody response to vaccination with the adenovirus vector encoding GP was induced more rapidly than with DNA priming and adenovirus boosting, but was of a lower magnitude. To determine whether this earlier immune response was sufficient for protection against disease, cynomolgus monkeys were vaccinated with adenovirus expressing both ZEBOV GP and NP and boosted 9 weeks later [72]. 1 week after the boost, the animals were challenged with either a low (13 PFU) or high (1500 PFU) dose of ZEBOV. All eight macaques (four challenged with 13 PFU, four challenged with 1500 PFU) were completely protected from viremia, clinical illness and death, while all five saline-injected control animals succumbed to the challenge (four challenged with 13 PFU, one challenged with 1500 PFU). Both humoral and CD8⁺ cellular immune responses were associated with protection. Antibody titers to the EBOV were elicited in the vaccinated macaques, which minimally increased after challenge. Significant increases were observed before exposure to EBOV in the CD8⁺ T-cell response to EBOV antigens by intracellular cytokine staining for IFN- γ versus unvaccinated control animals.

As the second vaccination in the adenovirus-expressing ZEBOV GP/NP regimen did not substantially increase the EBOV-specific immune responses, cynomolgus monkeys were vaccinated with a single dose of adenovirus expressing ZEBOV GP/NP and challenged with homologous virus 1 month later [72]. As in the initial study, all eight macaques were completely protected from viremia, clinical illness and death at both low ($n = 4$) and high ($n = 4$) challenge doses. In this study, antibody titers were detected at the time of viral challenge and were associated with protection. CD8⁺ T-cell responses were detected before ZEBOV challenge, or were observed shortly after challenge, in five of the eight animals, again correlating with protection against lethal infection. The results of this study are by far the most encouraging data demonstrating that adenovirus-based EBOV vaccines can accelerate protection against EBOV in primates.

There are several concerns about the use of adenoviral vectors in humans primarily in those with pre-existing immunity to adenoviruses. A significant percentage of the population has been exposed to natural adenovirus infection [73,74], which could potentially limit the efficacy of adenovirus-based vaccines. Of equal concern is the realization that the same vector may be utilized in a number of vaccines. In an attempt to overcome these limitations, Yang and colleagues, in a proof-of-concept study, recently showed that it is possible to counteract prior viral immunity by priming with a nonviral DNA vaccine [75]. Additional efforts are being directed toward identifying adenovirus serotypes that are less prevalent in the human population than the adenovirus 5 serotype currently used as the backbone for most adenovirus-based vaccines. For example, antibodies against adenovirus 35 are found in less than 5% of the global population and development of adenovirus 35 as a gene transfer vector was recently reported [76]. In the past, the presence of replication-competent adenoviruses in preparations of replication-defective adenoviral

vectors has been a major problem in the application of these vectors for use in humans. However, recent development of a new helper cell line, PER.C6 (patented by Crucell NV, Netherlands), has eliminated the problem of replication-competent adenovirus generation by homologous recombination, that plagued earlier helper cells such as 293 cells [77].

Other virus-based platforms

Baculoviruses

As noted above, baculovirus-expressed ZEBOV proteins were tested as part of a prime–boost approach to develop an efficacious EBOV vaccine. The same study also evaluated baculovirus-derived protein vaccines for ZEBOV in guinea-pigs in the absence of a DNA prime [70]. Specifically, guinea-pigs were vaccinated and boosted with recombinant baculovirus expressing either ZEBOV GP or a terminally deleted ZEBOV GP, and subsequently challenged with homologous virus. Interestingly, the ZEBOV GP regimen protected six of the six guinea-pigs from viremia but only three of the six from death; while the ZEBOV terminally deleted GP regimen did not protect animals from viremia or death.

Vesicular stomatitis virus

In the last few years, Rose and colleagues have pioneered the use of VSV, the prototypic member of the *Rhabdoviridae* family, as an expression and vaccine vector [78–80]. Notably, this group demonstrated that live attenuated VSV expressing the HIV envelope (env) and core (gag) proteins protected rhesus monkeys from AIDS after challenge with a pathogenic AIDS virus [80]. Similarly, these investigators developed VSV vectors expressing influenza hemagglutinin (HA) protein, which are completely attenuated for pathogenesis in the mouse model [79]. This nonpathogenic vector also completely protected mice from lethal influenza virus challenge. Using the strategy shown for developing nonpathogenic VSV vectors expressing influenza genes, Takada and colleagues have developed a recombinant VSV vaccine for EBOV [81]. The vector was modified to carry the ZEBOV GP in place of the VSV G-protein (chimeric VSV–ZEBOV GP). Initial results from studies in mice were presented recently [82]. Briefly, neither the wild type VSV nor the chimeric VSV–ZEBOV GP were pathogenic in mice. Importantly, mice receiving only a single injection of chimeric VSV–ZEBOV GP were uniformly protected from lethal ZEBOV infection (LD₅₀ 3000) when challenged 28 days after vaccination. Chimeric VSV–ZEBOV GP-vaccinated mice were aviremic and asymptomatic for the duration of the study (4 weeks), while mice receiving only wild type VSV rapidly succumbed to illness and all died within 7 days after ZEBOV challenge. Future development of this platform will depend on whether the success demonstrated in mice is achievable in nonhuman primates.

Newer technologies

Recent developments in using virus-like particles (VLPs) as delivery systems for vaccines has raised the possibility that

VLPs may have utility as an EBOV vaccine. In fact, EBOV-like particles have been demonstrated and can be efficiently produced through coexpression of the membrane proteins GP and VP40 [16]. In studies with other viruses, VLPs have been shown to elicit potent humoral and cellular immune responses [83]. The advantages of VLPs are that they are not infectious, which addresses a major safety concern associated with using live vaccine vectors and they are not subject to problems associated with pre-existing antivector immunity. Regarding their utility as potential EBOV vaccines, it should be re-emphasized that inactivated whole virion preparations have not yet proven to be completely efficacious in animal models of EBOV HF. The ability of VLPs to elicit a uniformly protective response in systems, where inactivated whole virions have failed, might relate to the inactivation procedure; for example, exposure to γ -rays might alter the conformation of an important protective epitope(s).

Perhaps the most significant breakthrough in filovirus research in the last decade was the development of infectious clones for ZEBOV [84,85]. Conventional strategies of attenuating viruses for use as EBOV vaccines for human use have not been developed because of concerns about reversion to a wild type form. However, the possibility of adopting this strategy using the newly developed infectious clones of EBOV may now be rational. Many of the effective vaccines currently used for RNA viruses are live-attenuated viruses such as Japanese encephalitis, yellow fever and poliovirus. Therefore, in addition to having utility in studying mechanisms of viral pathogenesis, the recent development of reverse genetics methods to manipulate viral genomes may provide a unique opportunity to generate highly attenuated filoviruses as vaccine candidates. Classic examples of the utility of this approach were recently demonstrated for respiratory syncytial virus and parainfluenza virus where reverse genetics systems were used to analyze virulence determinants and to produce attenuated chimeric viruses expressing proteins from different strains [86,87].

Expert opinion

The validation of rodents and nonhuman primates as accurate and reliable models of human EBOV HF will be critical to the final evaluation of candidate vaccines. A more thorough understanding of the pathogenesis of human EBOV HF is critically needed to fully assess and compare the available animal models. More effort needs to be directed toward evaluating the disease pathogenesis during the sporadic outbreaks in Africa using modern immunological and molecular techniques. Clearly, rodents have not been accurate in predicting the efficacy of EBOV vaccine candidates in nonhuman primates. This group and others, have demonstrated that EBOV HF in nonhuman primates is more representative of human disease than EBOV infection in rodents. No EBOV vaccine will be approved for human use if it cannot protect nonhuman primates from clinical illness, viremia and/or death.

There are essentially two different issues that must be addressed regarding the management of EBOV HF, that call for different clinical paradigms. First, in either a natural outbreak or an outbreak associated with bioterrorism, an immediate response is needed to contain the outbreak and prevent the spread of disease to other geographic regions. Thus far, quarantine practices have been effective in limiting EBOV outbreaks but infection and mortality have been devastating in the quarantined community and modern advances in global travel do not ensure that future outbreaks will be as easily contained. The availability of a vaccine that could be rapidly employed to create a ring of vaccination around an epidemic zone will be critical to controlling subsequent spread of EBOV. The recently published one-shot, 4 week, adenovirus-based ZEBOV vaccine regimen [72] demonstrates the plausibility of developing a product to meet this need. Whether this vaccine can confer protection in less than 28 days and might even have utility for postexposure prophylaxis, remains to be determined.

The second clinical paradigm that needs to be addressed is long-term immunity that would be needed for laboratory workers and first-responders including medical personnel and/or the armed forces. We are unsure whether a single-shot vaccination regimen will confer long-term immunity to EBOV. Moreover, issues regarding pre-existing vector immunity are a particular concern in this setting. Thus, employment of a longer DNA prime-viral vector boost strategy may be necessary to address this requirement. In addition, adjuvants may have utility in improving efficacy of the adenovirus-based system or any of the other vaccination strategies.

In the context of bioterrorism, it is important to consider that biological agents may enter the body via several routes. Most vaccines are tested in animal models against a parenteral challenge; however, the inhalation or aerosol route is the most important to consider when planning defenses against biological attacks [88]. Stability as a respirable aerosol concomitant with the ability to induce infection by aerosol is one important criterion for weaponization [89]. While the role of aerogenic transmission in EBOV outbreaks is unknown and thought to be uncommon [90], EBOV is moderately stable in aerosol [91] and intercache transmission, suggesting mediation by small-particle aerosols, has been documented [92]. Notably, EBOV is highly infectious by aerosol exposure in rhesus macaques [93,94]. Thus, it will be important to prove the efficacy of any candidate EBOV vaccine against several routes of infection to include aerosol exposure.

Currently, there are no available therapies to treat EBOV infections. Immunoprophylaxis has been largely ineffective in animal models. While passive vaccination with neutralizing monoclonal antibodies and hyperimmune horse serum has protected rodents from lethal EBOV infection [95–96], these antibodies failed to protect nonhuman primates from challenge with ZEBOV [95,97]. Recently, there has been some discussion about the role of antibodies in enhancing EBOV infection and potentially exacerbating disease [98–100]. While

the significance of immunological enhancement has yet to be documented *in vivo*, any such demonstration would clearly require a re-evaluation of vaccination strategies. As with the various immunotherapies, antiviral drugs have also consistently failed to ameliorate the effects of EBOV HF and again, compounds that show some efficacy in rodents are ineffective in monkeys [101–102]. Current studies in our laboratory suggest that therapeutic regimens that target the disease process rather than, or in addition to, viral replication may be the most effective approach for reversing the disease course after exposure [47].

Five-year view

Recently, significant progress was made toward the development of an EBOV vaccine as a result of collaborative studies performed by the Vaccine Research Center, National Institutes of Allergy and Infectious Diseases (NIAID) and the US Army Medical Research Institute of Infectious Diseases (USAMRIID). An agreement was reached between NIAID and a vaccine production company, Crucell N.V., to develop an EBOV vaccine using Crucell's novel proprietary adenovirus vaccination platform AdVac™ that will be suitable for use in humans. Studies are being conducted to determine the duration of immunity conferred by this platform, demonstrate efficacy against different isolates and/or species of EBOV by different routes of exposure including aerosol and optimize the regimen accordingly; potential problems associated with pre-existing vector immunity may be the largest obstacle to overcome. It is likely that any EBOV vaccine approved for use in humans by the US Food and Drug Administration (FDA) will rely on a new bypass rule which allows companies to use preclinical test data showing efficacy in two relevant animal models combined with Phase I studies. Due to recent concerns regarding bioterrorism, the FDA has recognized that it may be difficult if not impossible to conduct Phase II and III studies to determine efficacy against rare and highly lethal agents, such as EBOV. Although the two-animal efficacy rule will facilitate approval of an EBOV vaccine, the regulatory requirements will be as rigorous as for a controlled human efficacy trial, were such a trial possible.

In addition, we expect that much will be learned about the molecular actions of EBOV in the host system during the next 5 years as a direct result of the development of the reverse genetics system and/or other plasmid-based systems. Moreover, these systems offer unique opportunities to evaluate previously unexplained findings, including the observation that mouse-adapted ZEBOV appears to be attenuated in nonhuman primates. A better understanding of EBOV pathogenesis should augment the development of additional vaccination strategies. Finally, testing of VLPs as EBOV vaccine candidates should be completed during this period and the issue of whether or not VLPs are viable filoviral vaccine candidates answered.

Key Issues

- Ebola virus (EBOV) in humans and nonhuman primates causes acute disease that leads to shock, hemorrhage, multiple organ failure and usually death with case fatality rates ranging from 53% to 90% in confirmed outbreaks. There are four different species of EBOV and two of these, *Zaire ebolavirus* (ZEBOV) and *Sudan Ebolavirus* (SEBOV), are important human pathogens. An EBOV vaccine will need to protect against both of these species.
- Heightened awareness of bioterrorism advanced by the events surrounding September 11, 2001, concomitant with knowledge that the former Soviet Union was evaluating EBOV as a weapon [103,105], has dramatically changed perspectives regarding the need for a vaccine against EBOV hemorrhagic fever (HF).
- Dramatic steps were recently taken toward the development of an efficacious EBOV vaccine. Specifically, uniform protection of cynomolgus monkeys from a high-dose lethal exposure to ZEBOV was demonstrated using a single dose of adenovirus expressing ZEBOV glycoprotein and nucleoprotein. Issues regarding pre-existing vector immunity and longevity of protection remain to be determined, although proof of concept studies have shown that prior vector immunity may be overcome by priming with a nonviral, DNA vaccine.
- Protection from EBOV in nonhuman primates was associated with the generation of EBOV-specific CD8⁺ T-lymphocyte and antibody responses; thus, it appears that adequate protection of primates requires both antibodies and cytotoxic T-lymphocytes.
- A ZEBOV minigenome-based reverse genetics system was recently developed. This infectious clone system will provide valuable information essential to understanding protein function, viral replication, pathogenesis and should also facilitate the development of vaccines and chemotherapeutic interventions.
- Validation of rodents and nonhuman primates as accurate and reliable models of human EBOV HF will be critical to the final evaluation of candidate vaccines. This is particularly important considering that any EBOV vaccine approved for use in humans by the FDA will likely rely on the new two-animal model bypass rule.
- A licensed EBOV vaccine using the adenovirus-based delivery system is currently being pursued by the US Government (Health and Human Services, NIAID) with Crucell NV (Netherlands) as a commercial partner.

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Affiliation:

- Thomas W Geisbert, Virology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD, 21702-5011, USA
Tel.: +1 301 619 4803 Fax: +1 301 619 2290
tom.geisbert@amedd.army.mil