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## An update on the use of antibodies against the filoviruses

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### Abstract

Multiple recent, independent studies have confirmed that passively administered antibodies can provide effective postexposure therapy in nonhuman primates after exposure to an otherwise lethal dose of Ebola virus or Marburg virus. In this article, we review composition and performance of the antibody cocktails tested thus far, what is known about antibody epitopes on the viral glycoprotein target and ongoing research questions in further development of such cocktails for pre-exposure or emergency postexposure use.

### Keywords

antibody; antibody cocktail; Ebola virus; ebolavirus; filovirus; glycoprotein; GP; immunotherapeutic; Marburg virus; marburgvirus; postexposure

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The family *Filoviridae* are enveloped, negative-sense RNA viruses with a characteristic filamentous shape. The filovirus family can be divided into two major genera, *Ebolavirus* and *Marburg-virus*, as well as a new genus, *Cuevavirus* [1,2]. The sole known cuevavirus, Lloviu, was recently identified in bats in Spain [3], but is not known to cause disease among humans.

Most ebolaviruses and marburgviruses, however, can cause highly lethal hemorrhagic fever among humans. In the *ebolavirus* genus are five antigenically distinct viruses, each named after the location of the outbreak in which they were first identified. These include Ebola virus (EBOV; formerly known as *Zaire ebolavirus*), Sudan virus (formerly known as *Sudan ebolavirus*), Reston virus, Taï Forest virus (formerly known as *Cote d'Ivoire ebolavirus*) and Bundibugyo virus. The two ebolaviruses that most commonly cause disease in humans are Ebola and Sudan. Sudan virus caused the largest ever outbreak of Ebola hemorrhagic fever, in the Gulu district of Sudan in 2000, while EBOV carries the highest lethality (up to 90% in

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some outbreaks). Curiously, Reston virus is the only ebolavirus not currently known to be pathogenic in humans, although it is highly lethal to nonhuman primates. The reasons why it has not caused disease among humans are not yet understood.

Within the *marburgvirus* genus is a single species, the eponymously named Marburg virus (MARV). MARV was the first filovirus identified, when, in 1967, it infected laboratory workers in Germany and Yugoslavia handling primates imported from Uganda. Although those outbreaks conferred disease with 20–40% mortality, recent outbreaks of Marburg hemorrhagic fever brought approximately 90% lethality. Indeed, in animal models, the modern Angola strain is noted to progress more rapidly than historic strains of MARV [4].

In humans, filovirus infection is associated with rapid viral replication that pervades most tissues and widespread and severe focal necrosis. The incubation period ranges from 2 to 14 days and death typically occurs between day 6 and 16 [5].

The incidence of filovirus infection could be becoming more common, and indeed, three filovirus outbreaks occurred in 2012 [6–8]. One of these outbreaks was linked to a species of ebolavirus, termed Bundibugyo [9], identified when it emerged in 2007, leading to a hemorrhagic fever outbreak in Uganda [10,11]. Furthermore, in this decade, the ebolaviruses were discovered to infect a new host – domesticated swine being raised for human consumption in southeast Asia. In 2008 in the Philippines and in 2011 in China, Reston virus was found among domesticated pigs on multiple ranches [12–14]. The virus may have been introduced into the Asian pig farms by fruit bats [15,16], which are a possible host reservoir of the filoviruses [17–24]. These fruit bats have extensive geographic range, and thus, the potential exists for further viral dissemination. Of additional concern is that in swine, ebolavirus does not manifest as a hemorrhagic fever, but instead as a respiratory infection [25] from which it could spread pig-to-pig, or pig-to-human via respiratory secretions. Although Reston virus is not currently thought to be pathogenic to humans, it is not known how many mutations would be needed to confer human pathogenicity. Furthermore, it has been noted that EBOV (Zaire), which is highly lethal to humans and is carried by similar bat hosts, can also infect swine [25].

The high morbidity and mortality rates in multiple recent outbreaks, the lack of prophylactic and treatment options, the geographic range of potential reservoir species, the potential for aerosol transmission, and the demonstrated methods for weaponization of the filoviruses have caused them to be labeled as National Institute of Allergy and Infectious Diseases Category A priority pathogens and CDC Category A agents of bioterrorism. The increasing natural prevalence of the filoviruses, their expansion into new hosts, and the increasing possibility of occupational exposure to these viruses in laboratories and medical facilities here and in outbreak locations, necessitates immediate development and provision of therapies for pre-exposure prophylaxis or postexposure treatment. No such treatments are currently approved, and the current standard of care is limited to palliative care [26].

## The need for antibodies against the filoviruses

In 2012 and 2013, a series of interagency workshops were held to identify medical countermeasures that would be potentially available for treatment of accidental biosafety

level 4 laboratory exposure. Postexposure vaccines, siRNA, small molecules, phosphorodiamidate morpholino oligomers and monoclonal antibodies (mAbs) have all been vetted. Consensus was reached that immediate efforts to get a potentially promising compound into the hands of treating physicians should focus on mAbs as lead candidates. This decision was based upon the observed efficacy in animal studies, the ability of mAbs to confer protection when given even 1–2 days after exposure, as well as the established track record of safety with similar mAb products in other diseases, as well as what was envisioned to be an easier, or perhaps more clearly defined, pathway to advanced development. Antibodies are generally safe, effective, bioavailable, able to be lyophilized and stockpiled, and can be produced relatively inexpensively.

Importantly, antibodies could be used for pre- or post-exposure use. They could be offered prophylactically to medical workers and scientific personnel before traveling to and working in outbreak areas, or could be given after acute accidental exposure. Furthermore, new research suggests that antibodies can successfully treat an established filovirus infection. Importantly, protection occurs even when antibody was administered only when symptoms of hemorrhagic fever had already developed, several days after exposure [27].

### **Historical studies of polyclonal antibodies in filovirus treatment**

Historical use of polyclonal antibodies to treat filovirus infection has shown some limited success. During the Kikwit, Zaire outbreak in 1995, eight humans with Ebola hemorrhagic fever were administered convalescent sera containing ebolavirus-reactive antibody, but free of ebolavirus antigen. Prior to treatment, EBOV antigen was detected in all eight patients and all eight had symptoms of Ebola hemorrhagic fever (fever, asthenia and hemorrhage) similar to other patients in the epidemic. Two had even become comatose as their disease progressed. After administration of convalescent sera, only one of these patients died (12.5%) [28], which is a significant improvement over the near 90% lethality of other patients in the epidemic. Whether administration of antibody was the sole factor in their survival is unknown, as the patients received whole blood, not just purified antibody, and also received supportive hospital care. Furthermore, the patients may have been at the early stage of a recovery phase of the disease [28].

In a separate study, equine IgG, from horses hyperimmunized with EBOV, was administered to cynomolgous macaques immediately after experimental EBOV infection. Macaques treated with this IgG showed a delay in onset of viremia, symptoms and death, but no ultimate increase in survival [29]. The study authors suggested that passively administered antibody could control the viral burden when present, but when antibody was depleted by complexing with the virus or by immune clearance because of its heterologous species, the virus would continue to replicate. Study authors predicted that effective treatment of humans would require antibodies with more favorable pharmacokinetic properties than the equine IgG [29]. These historical studies provided some optimism for further analysis, ideally, of species-matched IgG in nonhuman primates, for which a control group could be included.

## Unsuccessful treatment with one single mAb

Prior to 2012, only one mAb had been tested for postexposure protection in nonhuman primates. This antibody, termed KZ52, was identified in a phage display library constructed from the RNA of survivors of the 1995 Kikwit, Zaire EBOV outbreak [30]. KZ52 was found to strongly neutralize EBOV *in vitro*, and to confer complete, dose-dependent protection in guinea pigs when administered 1 h before or after lethal EBOV challenge. Interestingly, although protected, some animals still exhibited high-level viremia, suggesting that the mechanism of protection could extend beyond simple viremia reduction [31]. Subsequent studies of KZ52 in rhesus macaques, however, yielded disappointing results. Four animals were administered one intravenous dose of KZ52 50 mg/kg 24 h before EBOV challenge, followed by a second dose at 4 days after challenge. Disappointingly, KZ52 did not reduce viral replication following infection, and failed to protect the macaques against EBOV challenge. The lack of protection could not be explained by neutralization escape [32]. Instead, it could have been the result of insufficient quantities of antibody delivered, insufficient efficacy of KZ52, or the fact that only one single mAb was delivered.

Indeed, it is unknown if any single mAb administered alone is enough for protection. The filoviruses are nearly 1  $\mu\text{m}$  long, are thickly studded with glycoprotein (GP) spikes and enter the cells through macropinocytosis. Multiple different attachment factors may assist cellular adhesion and entry, and no single cell surface factor, and thereby no single site on the intact filovirus GP surface, is known to be necessary and sufficient for cell attachment.

Once in the endosome, however, the entry factor NPC1 appears essential for infection [33–36], and a theoretical antibody that blocks binding of this factor could be highly effective. However, the full-length, viral surface form of the GP, against which antibodies might be naturally elicited, does not bind the NPC1 receptor. The GP must first be cleaved by host proteases in the endosome to strip much of its mass prior to NPC1 engagement. If the NPC1 binding site is indeed masked on the viral surface, it could be difficult to elicit many high-affinity antibodies to that site in natural infection. One solution is to try to elicit such antibodies using immunogens engineered to have the NPC1 binding site well-exposed. Another solution is to use synthetic antibody technology [37], for which structure-based design and diversification could assist targeting of nonimmunogenic or shielded critical epitopes. Certainly, any antibodies that block NPC1 binding and confer neutralization would be promising candidates for protection studies in nonhuman primates. By contrast, KZ52 does not bind the expected receptor-binding site, but instead recognizes the base of the GP oligomer, likely conferring neutralization by blocking conformational rearrangements required for fusion of virus and host membranes [38].

Currently, KZ52 is the only mAb that has been evaluated individually for postexposure protection in nonhuman primates, and so it is unclear if a different single mAb could provide sufficient protection, or if no single mAb at all could provide sufficient protection in primate models. Also, it is not yet known if KZ52 could be highly effective as a component of a multi-antibody (and therefore multiepitope and multifunction) cocktail, even if it is insufficient alone. In electron micrographs, the filoviruses appear to be thickly coated with the GP; they are not largely ‘bald’ as are many HIV particles. Finally, the length of the viral

particle and the vast number of GP spikes that may need to be occupied by antibody in order for protection to occur, coupled with significant shedding of large sections of GP during entry, suggests that the most effective therapy would involve a cocktail of antibodies against different sites on the GP.

## Successful treatment with cocktails of antibodies

In the last year, four independent studies have demonstrated that cocktails of antibodies can confer protection to nonhuman primates when passively administered after otherwise lethal viral challenge [39–42]. In all of these studies, groups of antibodies were given – either polyclonal IgG or a cocktail of two to three mAbs – instead of a single mAb.

### Polyclonal IgG

In the first study, Dye *et al.* aimed to establish the precedent that nonhuman primates could be protected by antibody passively administered after challenge [39]. Differences between this and prior work were that the antibodies were species-matched to the animal receiving them, the antibodies were administered multiple times following challenge rather than just one dose after challenge, and that a polyclonal IgG was given, rather than a single mAb.

In an initial experiment, five naive rhesus macaques were challenged by intramuscular injection with 1000 plaque-forming units of MARV. In three of them, MARV-specific IgG was given within 15–30 min of challenge, with additional doses given 4 and 8 days after challenge. The remaining two macaques received irrelevant IgG or phosphate-buffered saline on the same dosing schedule as the MARV-specific IgG-treated animals. All animals that received MARV-specific IgG survived with no signs of disease or detectable viremia. All control animals died by day 8 with symptoms of hemorrhagic fever. Treated animals displayed and maintained MARV-specific serum IgG, but also developed MARV-specific IgM by days 4 and 6 after challenge. The IgM could only have resulted from an innate immune response against the challenging virus, as it was not delivered in the therapeutic. The animals' own immune response to the challenge seems to provide protection against future exposure, as all IgG-treated animals survived a rechallenge with MARV 77 days later, with no additional exogenous IgG administered, and no signs of illness or viremia occurring after challenge.

In a follow-up experiment, Dye *et al.* extended the treatment schedule, administering IgG on days 2, 4 and 8 after challenge, as opposed to initiating treatment 15–30 min after challenge [39]. Two of three treated animals survived with no detectable disease or viremia. A third displayed delayed onset of viremia, was given an additional dose of IgG on day 12, and survived with no detectable viremia by day 16. These results suggest that antibody treatment could be initiated 48 h after exposure and that repeated administration of antibody (rather than one single bolus after challenge) can help to clear remaining virus and provide complete protection when necessary. The 48-h window between exposure and initiation of treatment provides a time frame by which an exposed human could be evacuated to an appropriate facility for treatment. In a final experiment, the authors demonstrated that EBOV-challenged macaques can be protected by administration of MARV/EBOV-bispecific polyclonal IgG at 2, 4 and 8 days after challenge [39]. In summary, this landmark

study demonstrated that nonhuman primates could be protected from filovirus exposure when antibodies are passively administered 48 h after otherwise lethal viral challenge, and these studies provided tremendous optimism for further analysis.

These previous studies were performed using polyclonal IgG from vaccinated or convalescent animals. Quantities of such sera are limited, and for eventual treatment of exposed humans, human convalescent sera would be desired, but is even more limited in quantity. Hence, a major goal is to determine if mAb preparations, which can be produced recombinantly and on a large scale, can provide effective protection. Three separate studies performed in 2012 suggest that they can [40–42].

### Two-mAb cocktail

In this study, Marzi *et al.* treated rhesus macaques with a cocktail of two human–mouse chimeric mAbs, termed ch133 and ch226, that each neutralize EBOV *in vitro* [42]. Three macaques were each administered three intravenous doses of 50 mg of mAb in the cocktail at 24 h before, as well as 24 and 72 h after challenge. One of the three animals survived challenge with no clinical symptoms and reduced viremia levels. Two animals did not survive. In these animals, circulating mAb levels were observed to decrease significantly with a concomitant increase in viral load [42]. It was concluded that antibody-mediated protection could be improved if serum half-life of the mAbs were optimized, if they were used in combination with other mAbs or therapies or if antibodies were delivered that offered greater neutralization potency.

### Three-mAb cocktail

**ZMAb**—In a concurrent study, Qiu *et al.* achieved complete protection using a cocktail of three different mAbs [41]. The cocktail is termed ZMAb and contains mAbs 1H3, 2G4 and 4G7, each identified in mice vaccinated with EBOV GP borne on a vesicular stomatitis virus backbone [43], and each of the mAbs in this cocktail are known to be neutralizing [44]. Three doses of ZMAb at 25 mg/kg were administered to cynomolgus macaques at 24 h, 4 days and 7 days after challenge with EBOV; 100% of ZMAb-treated animals survived. When treatment was initiated 48 h after exposure, 50% of the animals survived [41], suggesting that earlier administration of antibody can be more effective. Hence, complete protection of nonhuman primates can be achieved by mAbs, when animals are treated 24 h after exposure. The ability of polyclonal IgG to protect at 48 h in the Dye *et al.* study suggests that the identity, quantity and potency of antibodies delivered is probably important for maximum clinical benefit [39]. Identification of the most effective antibodies and the most synergistic cocktails are essential subjects for future study.

**MB-003**—Olinger *et al.* administered a different cocktail of mAbs (termed MB-003, and containing the mAbs c13F6, h-13C6 and c6D8) at 50 mg/kg [40]. The mAbs were raised in mice immunized with Venezuelan equine encephalitis virus replicons encoding EBOV GP [45] and were subsequently deimmunized or chimerized for use in primates [46]. 100% protection was achieved when treatment with the MB-003 cocktail was initiated 1 h after challenge, and 67% protection was achieved when initiated at 24 or 48 h after challenge

[40]. Additional doses were administered every 2–3 days after the initial doses. Hence, protection is possible but, again, earlier intervention is preferable.

An important aspect of this study is that the antibodies were produced recombinantly in *Nicotiana benthamiana* (tobacco plant) cells, a production strategy that provides two potential benefits. Antibodies produced in this cell line are lacking a core fucose on the glycan attached to the Fc region. The nonfucosylated IgGs appear to have improved potency as a result of better binding to the FcγRIII [46]. Indeed, *N. benthamiana*-derived MB-003 confers better protection than Chinese hamster ovary cell-expressed MB-003 in mice, and comparable protection in nonhuman primates, at a third of the mammalian-derived dose [40]. The second essential aspect is that these plant cells can be produced inexpensively, on an industrial scale. This method of production is an option that could be pursued for generation of large quantities of material necessary for a biodefense or treatment stockpile.

### Delayed treatment

Given these findings, it is likely an EBOV mAb or mAb cocktail therapy may be used either prophylactically or as an emergency post-exposure treatment for filovirus infection. Such a therapy could reduce deaths associated with outbreaks and mitigate risk for workers at risk of exposure. The fact that effective protection can still be achieved when treatment is initiated 24 h after exposure suggests that this is a viable treatment option for known accidental infection. It is likely that a laboratory or medical worker with a needlestick or scalpel injury could get to an appropriate treatment facility and have the antibody cocktail delivered within 24 h. However, for people living, working or traveling in endemic regions, it is possible that virus exposure could be unknown. Indeed, most people infected with filoviruses have been unknowingly exposed, including travelers, an ecologist, animal handlers, people receiving clinical treatment for other reasons, and workers in mines and factories that were later found to be inhabited by virus-infected bats. In these individuals, infection may only become noticed several days after exposure, once symptoms of hemorrhagic fever begin to develop. A more recent study by the same group demonstrates that MB-003 provides up to 45% protection when antibodies are administered only upon onset of both documented fever and a positive reverse transcription-PCR test for EBOV [27], which, in nonhuman primates, can occur 2–5 days after exposure.

### Combination therapy

Another recent paper indicates that a complementary, nonantibody therapy can be used in combination with these antibody cocktails to extend the treatment window. Qiu *et al.* demonstrate that administration of the ZMAb cocktail (antibodies 1H3, 2G4 and 4G7) to guinea pigs 3 days postexposure confers 33% survival [41]. Administration of an adenovirus-vectored interferon therapy (termed DEF-201 [47]) alone confers no survival, but does extend time to death. However, the combination of the antibody cocktail and DEF-201 provides 100% protection [48]. Hence, the addition of DEF-201 extends the window by which 100% protection can be achieved from 1 to 3 days. The advantage offered by addition of DEF-201 to the antibody therapy suggests that the balance between viral

replication and immune control can be tipped favorably. Analysis of this combination therapy in nonhuman primates is ongoing.

## Initial conclusions & going forward

These four studies in 2011 and 2012 together provide landmark demonstrations that antibody-based therapeutics are a viable avenue for filovirus infection. Polyclonal IgG can confer 100% protection when given 48 h after exposure. The first three mAb cocktails tested can offer up to 100% protection when given within 24 h, and 50–67% within 48 h. It seems that the longer the window between infection and treatment, the more and better antibodies one must provide. Hence, the initial sets of mAbs explored do not yet match the protection level of polyclonal convalescent sera at extended treatment windows. However, convalescent sera is limited in quantity, may not be species matched and can be variable. For treatment of human patients, and in production of multiple doses for stockpiling, we must provide human or humanized/chimerized mAbs that are well-characterized and consistent in composition and efficacy. MB-003 and ZMAb are certainly effective and provide immediate hope for persons exposed to EBOV.

Ongoing research will determine if these particular antibody combinations are the most effective possible against EBOV, or if a different formulation, or a different antibody, could improve efficacy or extend the treatment window. Most importantly, these antibodies are specific for EBOV and do not crossreact with the other filoviruses that also circulate and cause lethal disease, such as Sudan virus, Bundibugyo virus and MARV. Critical research questions to be answered going forward are:

- How do we design an antibody cocktail that is as good as or better than survivor/vaccinee sera?
- Which antibodies should be included in the cocktail? Which epitopes lead to the best protection?
- Are any of them synergistic?
- What combination of antibodies confers the greatest breadth and potency?
- Should the antibody cocktail be given with a supportive complementary therapy such as DEF201?

Understanding which antibody epitopes are recognized, and which lead to the most effective protection may be explored by understanding the changing structure of the filovirus surface GP to which these antibodies are directed. In order to understand which epitopes are recognized and which are most effective, it is imperative that we understand the structure of the viral surface GP and how that structure changes during infection.

## Filovirus GPs

The filoviruses express only one protein on the surface of the virion: the GP. GP drives attachment to, fusion with and entry of new host cells, and is the predominant target of antibodies that neutralize the viruses. GP is a 676-amino acid, heavily glycosylated protein that forms trimeric peplomers, or spikes, on the viral surface. Each monomeric GP precursor



is cleaved in producer cells by host furin to yield two subunits GP1 and GP2 that remain linked by a disulfide bond. The mature spike on the viral surface is formed by a trimer of GP1–GP2 heterodimers. Of the two subunits, GP1 mediates binding of host attachment factors/receptor, while GP2 contains the transmembrane domain that anchors the GP spike in the viral membrane. After receptor binding by GP1, an as-yet-unknown trigger causes GP2 to undergo a large and irreversible conformational rearrangement that drives fusion of virus and host membranes and allows the viral genetic material to enter the cytoplasm.

Crystal structures are available for oligomeric prefusion GPs of EBOV (strain Mayinga) [38], Sudan virus (strain Gulu) [49] and Sudan virus (strain Boniface) [50]. The prefusion GP trimer is shaped like a chalice, in which the three GP1 subunits form a bowl, from which each monomer angles outward from a central concavity (Figure 1). The three GP2 subunits encircle the GP1s and anchor them together at the base. The oligomeric assembly is stabilized mainly by trimerization of the GP2s and by interactions between GP1 and GP2 as the GPs wrap around the outside of the GP1 subunits in the trimer. In response to an as-yet-unidentified trigger within host cell endosomes, GP2 unwinds from around GP1 and undergoes a series of large-scale conformational changes collapsing into a six-helix bundle conformation that concomitantly drives fusion of viral and cellular membranes. Crystal structures are available for the postfusion six-helix bundle conformation of the GP2 subunit of EBOV [51–53] and MARV [54], and indicate that GP2 adopts a very different 3D structure postfusion than prefusion. Conformational anti-GP2 antibodies are expected to be specific for one of the pre- or post-fusion structures.

Based on the prefusion crystal structures, the GP1 subunit can be divided into subdomains termed the base, the head, the glycan cap and the mucin-like domain (Figure 2). The base of GP1 is the region encircled by GP2. The head sits atop the base and contains the putative receptor-binding site. The glycan cap is on top of the head and contains four N-linked glycosylation sites. Outward from the glycan cap is the unusual, heavily glycosylated, mucin-like domain. The mucin-like domains are each approximately 150 amino acids in length with little predicted secondary or tertiary structure and seven N-linked and approximately 15–20 O-linked glycans attached. These domains are approximately 75 kDa in size each, and hence, are approximately 50% of the mass of the GP monomer.

Curiously, these domains are not required for attachment or entry of pseudoviruses in cell culture. GPs engineered with deletions of these domains mediate viral entry as well as, or perhaps a bit better than particles pseudo-typed with wild-type GP [55,56]. The function of these large mucin-like domains and their requirement in authentic filoviruses are both somewhat unclear, although they have been found to sterically shield antibody epitopes on the rest of GP [57], downregulate expression and recognition of  $\beta$ -integrin and MHC type I on the surface of infected/transfected cells [58] and to confer possible cytotoxicity and damage to vascular integrity [56]. Immunization studies suggest that these mucin-like domains could be immunodominant [45]. They are certainly expected to be quite large in size and may be a dominant structural feature of the viral surface GP.

How do these GP-studded filamentous viruses enter cells? First, the filoviruses do not bind any single and essential receptor on the cell surface. Multiple attachment factors on the cell

surface have been proposed, but no individual molecule on the cell surface is always required for entry [59,60]. Instead, the filoviruses are likely captured by any one of several possible lectins and may interact with a protein termed TIM-1 [61]. The virus then appears to enter cells through macro-pinocytosis [62–66]. Once in the endosome and subsequently, the lysosome, the GP undergoes a radical structural transformation. There, the GPs are cleaved by host cathepsin proteases [67–69], which delete approximately 80% of the mass of the GP1 receptor-binding subunit, including most of its carbohydrate and all antibody epitopes in the mucin-like domains or glycan cap. After much of GP1 is removed in the endosome, the receptor binding sites are thought to become exposed, and the remaining GP core capable of binding to its likely receptor [68,70–72], the NPC1 protein [33–35,73]. Hence, GP only binds NPC1 after entry into the host cell endosome, typically after cleavage and removal of much of the bulk of GP and a large number of antibody epitopes. This structural remodeling has significant consequences for the antibody response.

### Where are antibodies known to bind on the filovirus GP?

Two GP-specific neutralizing antibodies, termed KZ52 and 16F6, have been crystallized in complex with Ebola and Sudan virus GP, respectively (Figure 1). KZ52 was identified in a human survivor of the 1995 Kikwit, Zaire outbreak and is specific for EBOV [30,74]. 16F6 was raised in mice immunized with irradiated Sudan virions, and is specific for Sudan virus [49]. Neither antibody recognizes the expected receptor-binding site. Instead, both antibodies recognize the base of their cognate GP oligomer, where GP2 meets GP1 [38,49,50,75]. KZ52 and 16F6 remain bound at endosomal pH and after enzymatic stripping of the mucin-like domains and glycan caps. Both antibodies appear to anchor the trimer together and may neutralize entry by preventing the conformational changes associated with fusion.

Two other antibodies have been crystallized in complex with their GP-derived linear epitopes (Figure 3). These are 13F6, a component of the MB-003 cocktail, which binds residues 405–413 in the EBOV mucin-like domain [45,76] and 14G7, which binds residues 477–492, also in the mucin-like domain [45,77]. 13F6 contains a rare V $\lambda$ x light chain [45], and the crystal structure of 13F6 revealed that the rare light chain confers noncanonical structures to the light chain complementarity determining regions [76]. The structure also revealed that 13F6 recognizes its peptide epitope in a shallow groove that runs diagonally across the antigen-binding site, rather than between light and heavy chains as would be typical of an antipeptide antibody. Although the mucin-like domain is highly glycosylated, 13F6 appears to recognize polypeptide, between the glycans, and the epitope adopts a flat, straight structure bound to 13F6. 14G7 also recognizes mucin-like domain polypeptide between glycans, but unlike that of 13F6, the peptide epitope of 14G7 forms a structured double  $\beta$ -turn that binds into a groove between light and heavy chains [77].

KZ52, 16F6, 13F6 and 14G7 are the only four anti-filovirus antibodies that have been crystallized thus far. Numerous other ones exist however, and some epitope information is known from either escape studies or peptide mapping (Figure 4). For example, the two mAbs in the cocktail of Marzi, *et al.* are 226/81 and 133/3.16 [42]. Escape from 226/81 is mediated by mutations at amino acids 134, 194 and 199 in the GP1 base [78]. Escape from

mAb 133/3.16 is mediated by a mutation at amino acid 549 in GP2 [78]. The ZMAb cocktail contains mAbs 1H3, 2G4, and 4G7 [41]. Escape from mAb 1H3 is mediated by a change in amino acid 274, which maps to the glycan cap [43]. 1H3 probably binds at the top of the GP trimer. Escape from mAb 2G4 is mediated by mutation a mutation at amino acid 508, which maps to the GP2 fusion loop [43]. 2G4 could thus map to GP2 or else its epitope could be affected by a structural change in the GP trimer. mAb 4G7 likely binds an epitope involving the C-terminal region of GP1 and GP2 [43]. In the MB-003 cocktail are mAbs 13C6, 13F6 and 6D8 [40]. 13C6 binds a conformational epitope in GP1 that is shared with the secreted GP version of the viral GP [45]. 13F6 and 6D8 bind to different linear peptides in the mucin-like domain.

The crystallized antibodies 13F6 and 14G7 represent competition groups I and III, respectively, of a number of different antibodies against these same epitopes [45]. Another competition group, group II, recognizes amino acids 389–405 of EBOV GP, and is represented by the mAb 6D8 in the MB-003 cocktail [45]. The large number of antibodies against the mucin-like domain suggests that this domain could be immunodominant or at least a statistically large portion of the intact GP surface.

## Protection in the absence of neutralization

Although antimucin antibodies bind GP with nanomolar affinity, they generally do not neutralize. Some however, like 14G7, neutralize in the presence of complement [40,45]. The inability of these antibodies to confer neutralization on their own is likely because they, and the mucin-like domain epitopes to which they are bound, are removed from GP by cathepsin cleavage in the endosome. Neutralization is typically an assay of the ability of an antibody to prevent entry. Viruses complexed with antimucin antibodies can still enter into the endosome, where the antibodies are cleaved off. Hence, these antibodies do not neutralize.

They are, however, protective in mouse, guinea pig and nonhuman primate models. How can it be that these antibodies do not neutralize, but are nonetheless protective? The answer is that these antibodies prevent budding of nascent virions from infected cells [79] and/or may confer antiviral protection *in vivo* through their Fc functions, including complement-dependent viral lysis, complement-dependent cytotoxicity, and antibody-dependent cell-mediated cytotoxicity [40]. Hence, the unusual features of the filovirus GP mean that neutralization assays are not the only possible predictor of antibody success *in vitro*. Other types of assays (budding, antibody-dependent cell-mediated cytotoxicity and plaque reduction) are also required in order to identify mAbs that may be protective *in vivo*. As a result, multiple different types of assays must be used for *in vitro* characterization and downselection of the array of possible mAbs that could be used in therapeutic cocktails.

## Gaps in knowledge

Although we know that these existing cocktails of anti-EBOV antibodies can confer efficacy, there remain critical gaps in our knowledge. We do not know where these antibodies bind on the 3D shape of GP, we do not know which epitopes would elicit antibodies with additive or synergistic effects and we do not know what the most efficacious cocktail is. Furthermore, we have yet to assemble cocktails of mAbs against the remaining

pathogenic filoviruses – Sudan, Bundibugyo and Marburg viruses – largely because not enough mAbs against these viruses yet exist. Furthermore, although the Reston species has not yet been shown to be pathogenic in humans, it is just as lethal to nonhuman primates as any other species of ebolavirus. It is not yet known how many mutations would cause Reston virus to become pathogenic for humans. Note that only one or two mutations are sufficient to adapt other filoviruses to pathogenicity in rodent models [80,81]. Certainly, panels of antibodies against Sudan, Bundibugyo, Marburg and Reston viruses are needed, in addition to those against EBOV.

## The Filovirus Immunotherapeutic Consortium

Recent discoveries described here provide a strong impetus to further develop and translate these mAbs for emergency use in humans. In order to reliably save the life of a fever patient, or a scientist or medical worker with an occupational exposure, we might develop the most potent cocktail possible. But, what antibodies should the cocktail contain? And how will we find them among the array of antibodies available in individual laboratories across the globe? How can we support the expensive nonhuman primate studies required? A large consortium of investigators has formed in order to gather as many antibodies as possible that are known against EBOV and perform a singular, field-wide analysis as efficiently and effectively as possible.

In this program, all antibodies will be expressed on an identical IgG framework, blinded and compared in a battery of standardized *in vitro* and *in vivo* assays. Epitopes will be mapped by x-ray crystallography and single particle electron microscopy. Through this collaborative analysis, the consortium will sort the pool of antibodies into groups by competition and function, and rank the antibodies in each group by efficacy. The most effective mAbs will be combined in different cocktails in order to determine which antibodies confer additive or synergistic effects and what the best antibody cocktail is from antibodies currently known against the virus. The resulting cocktail would be advanced for human use, and could certainly be used as a benchmark for comparison to additional antibodies and other types of therapies that will likely be developed in the future. This work will also form an essential knowledge base of why certain antibodies are the most effective, how they confer protection, and if filovirus crossreactive protection is possible.

As a result of this collaboration, the filovirus field now has the opportunity to identify the most effective antibodies from the array of antibodies now known. The consortium aims for these studies to be conducted in a public way, through dissemination and tracking of project progress on an open-access website, so that the research field and the population of scientists and doctors who may need this cocktail for their own occupational exposure may achieve consensus on cocktail selection. The effort will begin with antibodies against the EBOV, and will continue with antibodies against Sudan, Bundibugyo and Marburg viruses, when more antibodies against those filoviruses become available. All investigators wishing to contribute antibodies to the analysis are welcome and should contact the present author.

## Conclusion & future perspective

Over the last 30 years, multiple doctors, researchers and family members have died as a result of occupational exposure as they cared for filovirus-infected patients or performed filovirus research. Even today, occupational exposure is followed by days of great anxiety as signs of disease are watched for. At the moment, there are no specific treatments beyond supportive care that are available for human use. However, the studies described here strongly suggest that in the near future, medicine will have more to offer. The success of these pilot studies on immunotherapeutics against EBOV now opens the door to refinement of these antibody cocktails and translation of these therapies for human use, and it seems quite likely that an effective treatment will be in the hands of doctors in the next few years. Importantly, the proofs of principle provided by these pilot studies illuminates the likely return on investment in continued research in this area. The success of the work reviewed here opens the door to next-tier studies that will illuminate the mechanism of action of these antibodies and those elicited by natural infection. Continued research will identify new mAbs and different combinations of mAbs that may have greater potency or even crossreactivity, against Ebola and the other filoviruses that cause human disease. Hence, in the next 5–10 years, it is reasonable to expect that we will have amassed a knowledge base of how immune protection against a filovirus can be achieved and an armamentarium of effective virus-specific therapies. As a result, occupational and natural filovirus infection may no longer be a possible death sentence, but instead, a survivable occurrence.

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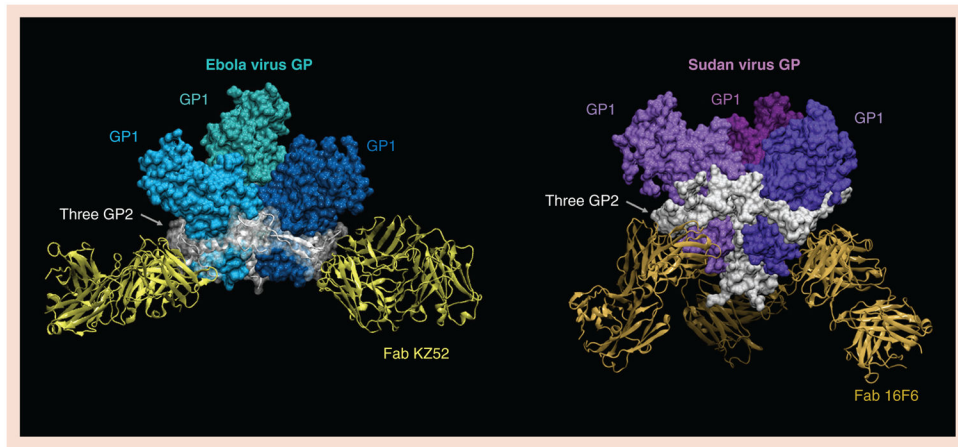
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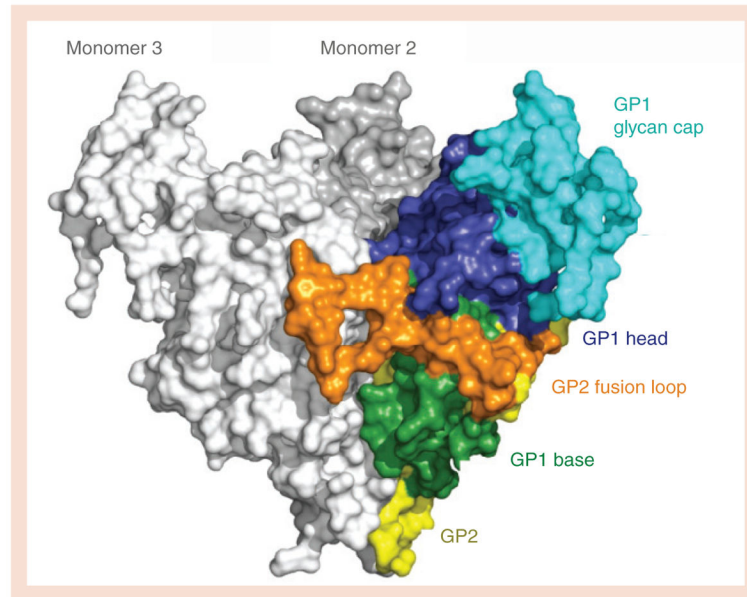
### Executive summary

- The filoviruses (Ebola and Marburg viruses) can cause highly lethal hemorrhagic fever. There is a continued high likelihood of natural or occupational exposure to the viruses, but no specific treatments are approved for human use.
- Antibody therapy is appealing for its likely safety, bioavailability and efficacy, as well as the ability to offer such treatment in a time frame in which most exposed persons could travel to an appropriate facility for treatment.
- Recent studies show that passively administered antibodies can provide effective postexposure protection.
- Different cocktails of two to three specific monoclonal antibodies have been identified that protect nonhuman primates against Ebola virus exposure.
- Polyclonal antibody has been shown to provide effective protection against Marburg virus exposure, suggesting that cocktails of monoclonal antibodies, when available, may be similarly effective against Marburg virus exposure.
- Ongoing research aims to elucidate which epitopes lead to the most effective protection, which antibodies can be combined for greatest synergy, and whether any single monoclonal antibody can achieve the protection of a cocktail.

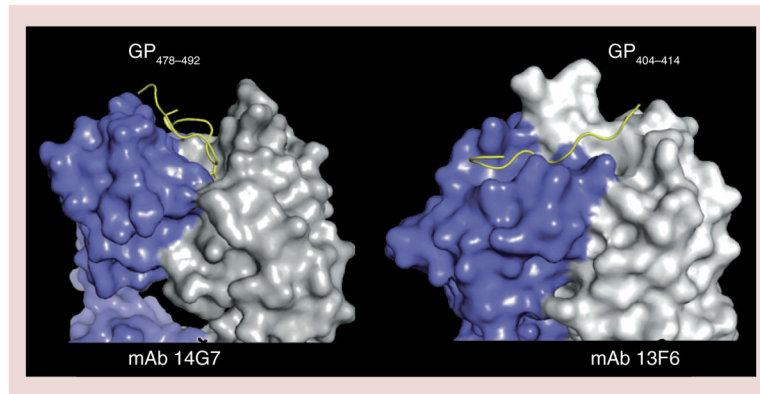


**Figure 1. Crystal structures of ebolaviruses**

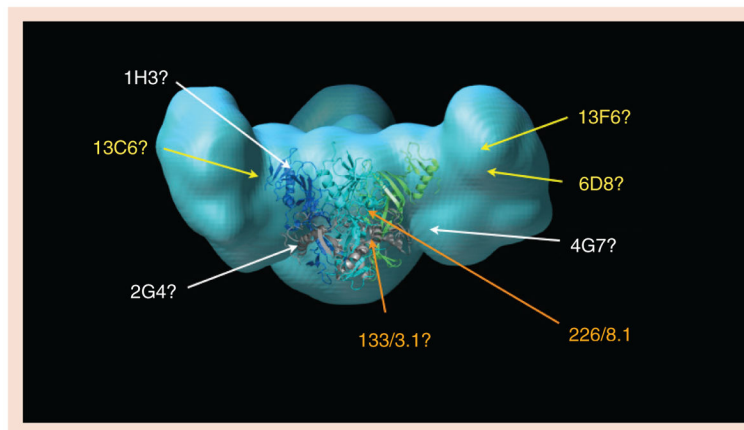
Ebola virus GP in (A) complex with Fab KZ52 and (B) Sudan virus (Gulu) GP in complex with Fab 16F6. GP1 subunits are colored blue for Ebola virus and purple for Sudan virus. In both oligomers, all three GP2 subunits are colored white, and wrap around the outside of trimer tethering the GP1 subunits together. Although raised in different species against antigenically distinct viruses, both Fab fragments recognize a site at the bottom of their cognate GP oligomer, where GP2 meets GP1.  
GP: Glycoprotein.



**Figure 2. Crystal structure of Sudan (Gulu) glycoprotein, illustrating the molecular surface** One monomer is colored according to subdomain with the GP1 base in green, head in blue and glycan cap in cyan. The GP1 mucin-like domain was deleted from the construct for crystallization. The GP2 fusion loop (orange) wrapped around the outside of the trimer, packing into GP1 of the neighboring monomer (monomer 3). The rest of the visible portion of GP2 (yellow) forms a helix broken into four segments, that wraps around the back of the GP1 subunit and descends downward toward the viral membrane (at the bottom of the image).  
GP: Glycoprotein.



**Figure 3. Crystal structures of Fabs 13F6 and 14G7 in complex with synthetic peptides representing their Ebola virus GP mucin-like domain epitopes (yellow)**  
Light chains are colored purple and heavy chains are colored white.  
GP: Glycoprotein; mAb: Monoclonal antibody.



**Figure 4. Possible binding sites of the different monoclonal antibodies in recent anti-Ebola virus cocktails**

In light blue is a model of the fully glycosylated mucin-containing Ebola virus glycoprotein (GP), with a crystal structure of the mucin-deleted core drawn at center in ribbon representation. In the GP core ribbon model, GP1 subunits are blue and green while the GP2 subunits are colored gray. Antibody epitopes are roughly located by the site of amino acid mutations that confer escape. Two mAbs in the Marzi *et al.* cocktail, 226/8.1 and 133/3.16, are listed in orange [42]. mAbs in the MB-003 cocktail of Olinger *et al.* are listed in yellow [40]. mAbs in the Qiu *et al.* ZMAb cocktail are in white [41]. Although the identities of the amino acids in the mucin-like domain to which 13F6 and 6D8 bind are known, the location of these linear epitopes in the 3D space of the mucin-like domain is not yet known. Experimental mapping of antibody epitopes is a major goal of the Filovirus Immunotherapeutic Consortium.