Successful Treatment of Ebola Virus–Infected Cynomolgus Macaques with Monoclonal Antibodies

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Ebola virus (EBOV) is considered one of the most aggressive infectious agents and is capable of causing death in humans and nonhuman primates (NHPs) within days of exposure. Recent strategies have succeeded in preventing acquisition of infection in NHPs after treatment; however, these strategies are only successful when administered before or minutes after infection. The present work shows that a combination of three neutralizing monoclonal antibodies (mAbs) directed against the Ebola envelope glycoprotein (GP) resulted in complete survival (four of four cynomolgus macaques) with no apparent side effects when three doses were administered 3 days apart beginning at 24 hours after a lethal challenge with EBOV. The same treatment initiated 48 hours after lethal challenge with EBOV resulted in two of four cynomolgus macaques fully recovering. The survivors demonstrated an EBOV-GP–specific humoral and cell-mediated immune response. These data highlight the important role of antibodies to control EBOV replication in vivo, and support the use of mAbs against a severe filovirus infection.

INTRODUCTION

Ebola virus (EBOV) can cause a fulminant infection and rapid progression to death in up to 90% of infected humans. EBOV infection in humans or macaques results in the development of clinical sequelae with a high degree of similarity, justifying the use of macaques as the most relevant model of human infection (1, 2). Pigs are the only other animal lethally infected by a non-adapted EBOV; however, the disease in pigs is more respiratory in nature, and therefore different from humans (3). African green monkeys, hamadryad baboons, cynomolgus macaques, and rhesus macaques have all been used as nonhuman primate (NHP) filovirus infection models (2). Because African green monkeys and hamadryad baboons are somewhat resistant to some filoviruses, both cynomolgus and rhesus macaques have been routinely used for the study of EBOV, and both show the same disease profile for EBOV infections.

Although of relatively low consequence to public health worldwide, EBOV infections are a public health concern because of the high mortality rate and lack of prophylactic/therapeutic interventions. Recent advances in vaccine development against EBOV resulted in the identification of several successful immunization strategies to mount protective immune responses to macaques (4). The vesicular stomatitis virus (VSV)–based Ebola vaccine resulted in 50% protection in rhesus macaques when administered 20 to 30 min after a lethal challenge with EBOV (5). This finding demonstrated that fast-acting therapeutic interventions could be developed to protect macaques against a lethal EBOV exposure.

Indeed, other treatment protocols that provided partial survival in macaques have been identified. Continuous intravenous infusion for 7 days beginning 30 to 60 min after EBOV infection with recombinant human activated protein C (rhAPC), which inhibits blood coagulation and inflammation while promoting fibrinolysis, resulted in an 18% survival (6–9). Another inhibitor of coagulation, recombinant nectomide anticoagulant protein c2 (rNAPc2), resulted in 33% survival if given subcutaneously 10 minutes or 24 hours after EBOV challenge and treated daily for 14 or 8 days, respectively (10). Another type of therapy, antisense therapy for RNA viruses such as EBOV, was tested using a positively charged phosphorodiamidate morpholino oligomers (PMOplus), which is a synthetic antisense oligonucleotide analog that interferes with translation by forming base-pair duplexes with specific RNA sequences. PMOplus delivered by a combination of intraperitoneal and subcutaneous doses initiated 30 to 60 min after infection for 10 to 14 days resulted in 62.5% surviving the EBOV challenge (11). More recently, daily intravenous administration of small interfering RNAs (siRNAs) targeting the EBOV genome showed a 66% survival rate with four intravenous doses and 100% with seven doses (12). Notably, all these strategies required initiation within 20 to 60 min of virus challenge. All of the above studies for studying treatment protocols implemented after infection were conducted in rhesus macaques over cynomolgus macaques, largely because the average time to death in rhesus macaques is about 2 days longer (5, 12, 13), thereby offering more time for successful clinical intervention. Passive antibody transfer has also been investigated for EBOV treatment, with inconsistent results (14). Initially, whole blood from convalescing survivors of the 1995 Kikwit outbreak was given to EBOV–positive patients displaying symptoms. A marked improvement was seen: Seven of eight patients survived. However, in later studies, cynomolgus macaques were not protected when treated with purified equine immunoglobulin G (IgG) from EBOV-hypervaccinated horses (15, 16). The neutralizing monoclonal antibody (mAb) KZ52 provided complete protection in guinea pigs when given 1 hour before or after infection but failed to protect NHPs (17–19). Despite these inconsistent results, there is evidence that a strong early humoral immune response correlates with survival (20–22).

Therefore, we optimized a treatment protocol with three of eight EBOV glycoprotein (GP)–specific mAbs (1H3, 2G4, and 4G7) that were previously generated from mice vaccinated with the VSVΔG/EBOV-GP vaccine (23, 24). In enzyme-linked immunosorbent assays (ELISAs), mAb 2G4 bound to GP2, and 4G7 bound to epitopes in the C-terminal
portion of GP1 of the EBOV-GP, whereas 1H3 bound to the soluble GP (sGP) portion (amino acids 1 to 295) (23). None of these EBOV-GP–specific mAbs are cross-reactive with Marburg virus or other EBOV species but did react with other EBOV strains. Individually, these antibodies protected mice and were more efficacious when administered 24 or 48 hours after the infection than at 1 to 4 days before infection. Although individually the antibodies were less protective in guinea pigs, a combination of the three neutralizing antibodies (1H3 + 2G4 + 4G7: 15 mg/kg combined total) was fully protective when administered on day 2 after infection (24).

On the basis of the success of the combination therapy in guinea pigs, the next step was to test the mAb cocktail in the NHP model. The current study builds upon the optimized treatment protocol in rodent experiments (24), presents a combination of three neutralizing mAbs (named ZMAb) to treat NHPs that have been infected with EBOV, and examines their survival, clinical manifestations, and immune response to the EBOV infection.

RESULTS

Clinical observations
Cynomolgus macaques were used as the NHP model to test whether and how effectively the ZMAb treatment could improve survival when administered after a high-dose EBOV infection. Eight cynomolgus macaques were randomized into two groups of four animals (two of each sex) and infected intramuscularly with 1000 plaque-forming units (PFU) of EBOV (Fig. 1A). Each group was then treated intravenously with three doses of a mAb mix (ZMAb) (25 mg/kg), which is composed of three EBOV-GP–specific neutralizing mAbs (1H3, 2G4, and 4G7). Although the dose at 15 mg/kg was 100% effective in the guinea pig study (24), the dose at 25 mg/kg was chosen because the accepted human dose is between 25 and 50 mg/kg. Each dose was given 3 days apart, with one group starting ZMAb treatment at 24 hours and the other group at 48 hours after challenge. A 3-day dosing protocol was chosen because the subjects are sedated each time, and sedation every second day would not allow much time for recovery and would have been overly

Fig. 1. ZMAb improves survival of EBOV-challenged NHPs. (A) Nine cynomolgus macaques were challenged with 1000 PFU of EBOV (red arrow). Two groups (n = 4) were treated with ZMAb beginning either at 24 hours (days 1, 4, and 7; blue arrows) or at 48 hours (days 2, 5, and 8; pink arrows). A control animal (n = 1) received PBS (days 1, 4, and 7). The animals were sampled on treatment days plus days 14 and 21 (black arrows) and examined for (B) serum murine mAb levels by an Ebola virus–like particle (eVLP) ELISA, (C) survival, and (E) viremia measured by real-time RT-PCR (GEQ, genome equivalents) or (F) TCID50. Only PCR-positive samples were assayed for TCID50. (D) Historical cynomolgus macaque controls over the last 2 years, using the same challenge virus stock and dose, are presented with their time to death after challenge. All macaques have consistent symbols across panels.
taxing. As a positive control for EBOV infection, one NHP was challenged with 1000 PFU of EBOV and then treated with phosphate-buffered saline (PBS) on days 1, 4, and 7.

To confirm delivery of ZMAb, we determined the serum levels of mouse IgG by ELISA at 4, 7, and 14 days post-infection (dpi) and 5, 8, and 14 dpi for the 24- and 48-hour group, respectively (Fig. 1B). The serum samples were taken immediately before the animals received the ZMAb bolus. There were variable levels of mAbs in the blood ranging from 80 to more than 1000 μg/ml before the second and third treatment at 4 to 5 and 7 to 8 dpi before decreasing in all but one animal (B3) at later time points. Overall, in all cases, there were detectable levels of mAbs.

The survival of the subjects was monitored daily, and a significant difference was found in the survival of the 24- and 48-hour treatment groups (P = 0.0062) in comparison to the control group with historical controls included. The PBS-treated control animal was euthanized on day 5 when the clinical score reached the euthanization criteria (Fig. 1C). The control animal confirmed productive infection after EBOV challenge with a time to death that was similar to those of 12 previous historical control animals (average time to death = 6.5 days) from other challenge studies conducted within the previous 16 months using the same virus stock and conditions (dose, route, etc.) (Fig. 1D).

Two animals from the 48-hour group (B3 and B2) were euthanized at 8 and 11 dpi, respectively, when they reached the humane endpoint score. The remaining six animals survived and fully recovered despite evidence of virus replication, albeit with lower viral titers than seen in the nonsurvivors.

Notable levels of virus were detected by real-time reverse transcription–polymerase chain reaction (RT-PCR) from the blood of all treated animals with the exception of one subject (A2) in the 24-hour group (Fig. 1E). The control and the two euthanized animals had genome equivalent (GEQ) copy numbers above 10^4 particles/ml of blood, whereas treated animals surviving challenge developed viremia of 10^2 to 10^4 GEQ/ml of blood that peaked around 7 dpi before coming down to undetectable levels by day 14 for all but one animal from the 48-hour group (B1) (Fig. 1E). Infectious virus was recovered by limiting dilution analysis from the blood samples in only two (A1 and B1) of six survivors at 7 and 8 dpi, respectively (Fig. 1F). These were 1.25 logs lower than that detected in the nonsurvivors. EBOV particles were detected by RT-PCR in the oral, nasal, and rectal mucosa of the nonsurvivors between 4 and 12 dpi but not in the mucosa of the survivors at any time points (Table 1). These areas were chosen as

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three representative shedding sites for where mucosal secretions could contain virus that could be transmitted to others. Together, these data suggest that the survivors had lower virus titers than the nonsurvivors.

The most prominent clinical signs observed in nonsurvivors were reminiscent of typical NHP EBOV infections with some or all of the following features: fever, reduced activity, lower food and water intake leading to weight loss, and occasional early signs of cutaneous hemorrhage (such as petechiae; Fig. 2, A and B, and Table 2). Two of the survivors (A4 and B4) demonstrated signs of disease including reduced activity, lower food/water consumption with weight loss, and decreased excretion of feces. Clinical scores remained relatively low for all other animals (Fig. 2C). The most noticeable changes in hematology and blood chemistry were observed between 5 and 15 dpi in the nonsurvivors showing leukocytopenia, lower platelet counts, and elevated liver enzyme [alanine aminotransferase (ALT)/alkaline phosphatase (ALP)] indicative of hematologic and liver dysfunction commonly associated with EBOV infection (25). In contrast, hematology and blood chemistry profiles remained largely unchanged in survivors with the exception of a transient thrombocytosis between 7 and 21 dpi for all but one animal (A1; Fig. 2, D to F).

**EBOV-GP–specific immune responses**

Immune responses were monitored to see whether ZMAb could provide a transient control on EBOV replication long enough for the subjects to mount a protective immune response leading to survival. To characterize the humoral response, we quantified neutralizing antibody levels and total levels of IgM and IgG antibody binding the EBOV-GP from serum samples by a neutralizing antibody assay or ELISA, respectively. Although neutralizing antibody activity may be attributed to both the ZMAb and the endogenous antibodies, monitoring of IgM and IgG was specifically targeted to endogenous NHP antibody responses using secondary IgM or IgG specific for NHP antibody in the ELISA assays.

Significant levels \((P \leq 0.05)\) of total NHP IgM specific to EBOV-GP were detected early from 5 to 7 dpi and remained relatively constant in all survivors up to 28 dpi (Fig. 3A). One nonsurvivor (B3) had IgM levels below the assay detection limit, whereas the other nonsurvivor (B2) had levels comparable to those of other animals surviving the lethal challenge at 5 and 8 dpi, at which time this animal reached the humane endpoint and was euthanized. Significant \((P \leq 0.05)\) but variable levels of NHP IgG anti–EBOV-GP were detected from 8 to 21 dpi that generally increased over time to 28 dpi in all treated animals with the exception of the nonsurvivor B2 in the 48-hour group (Fig. 3B).

Neutralizing antibody levels were generally low, with reciprocal titers of 1:10 to 1:100 (Fig. 3C), in comparison to previous EBOV studies where a higher neutralizing antibody titer was 1:128 to 1:512 (26–28). The neutralizing levels peaked around 7 to 8 dpi before diminishing to undetectable levels by 14 to 21 dpi. This assay cannot distinguish between the NHP neutralizing antibodies and the ZMAb. However, EBOV-specific neutralizing antibodies produced by NHPs

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**Fig. 2.** Clinical overview. During the EBOV experiment, various clinical parameters were assessed for the 24-hour, 48-hour, and control animals. (A) The percent change in weight was expressed as a change from baseline values taken on day 1 (control and 24 hours) or day 2 (48 hours). (B) Temperature for each animal on each exam date. (C) The animals were scored daily for signs of illness, and the daily clinical score reached over 19 days is presented. (D to F) Variations in blood biochemistry and hematological levels were noted for (D) alanine aminotransferase (ALT), (E) alkaline phosphatase (ALP), and (F) platelets (PLT). All macaques have consistent symbols across panels.
should be increasing at this time, yet the neutralizing antibody levels decrease by 14 to 21 dpi, therefore suggesting that neutralizing activity was mainly provided by ZMAb. Currently, we do not have the clearance to determine whether IFN-γ production was specifically due to CD4+ and CD8+ cells, with CD4+ cells expressing IFN-γ secreting cells in PBMCs stimulated in vitro in all but one animal (A4; Fig. 4A). Although the PBMCs responded to all four peptide pools in three of four of the 24-hour group, the predominant response overall was to pool 3 (amino acids 347 to 495) followed by pool 2 (amino acids 180 to 347). The 48-hour group responded predominantly to pools 2 and 4, although the response to pool 3 was slightly weaker. Flow cytometry analysis revealed differences between animals regarding the immune cell subpopulations responsible for the synthesis of IFN-γ (Fig. 4B). Two animals (A1 and A2) had predominantly CD8+ IFN-γ cells, whereas survivors A3 and B1 had a greater percentage of CD4+ IFN-γ cells. Subject B4 had both CD4+ and CD8+ IFN-γ cells, whereas A4 had essentially no IFN-γ response. Examining the overall CD8+ IFN-γ response demonstrated that the PBMCs responded to all four peptide pools in all NHPs except A4, with pools 3 (amino acids 347 to 495) and 4 (amino acids 496 to 679) eliciting the greatest percentage of CD8+ IFN-γ cells (Fig. 4C). The CD4+ IFN-γ response was also broad, because most subjects responded to all four pools (Fig. 4D). However, a single pool was not predominant overall.

Interleukin-2 (IL-2), which is another important cytokine in the cell-mediated immune response during the amplification phase, was found more equally in both CD4+ and CD8+ cells, with CD4+ cells expressing IL-2, IFN-γ, or both were investigated by ICS (Fig. 5). Most of the IL-2+ cells were overwhelmingly CD4+, although a small portion of CD8+ cells were IL-2+ (Fig. 5B). In contrast, IFN-γ expression was found more equally in both CD4+ and CD8+ cells, with CD4+ cells covering 30% of the skin, moderate rash as areas of petechiae covering 10% of the skin, and severe rash as areas of petechiae and/or ecchymosis covering >40% of the skin. Leukocytopenia and thrombocytopenia were defined as a ≥30% decrease in numbers of white blood cells (WBCs) and platelets, respectively. Leukocytopenia and thrombocytopenia were defined as a twofold or greater increase in numbers of platelets and WBCs over baseline and also a WBC count >11,000, ↑ twofold- threefold increase; ↑↑, four- to fivefold increase; ↑↑↑, greater than fivefold increase. ALP, alkaline phosphatase; ALT, alanine aminotransferase; BUN, blood urea nitrogen; GLOB, globulin; GLU, glucose; TBIL, total bilirubin; ND, not done; —, no change.

Table 2. Clinical findings. Fever was defined as a temperature ≥1.0°C higher than baseline. Mild rash was defined as focal areas of petechiae covering <10% of the skin, moderate rash as areas of petechiae covering 10 to 40% of the skin, and severe rash as areas of petechiae and/or ecchymosis covering >40% of the skin. Leukocytopenia and thrombocytopenia were defined as a ≥30% decrease in numbers of white blood cells (WBCs) and platelets, respectively. Leukocytopenia and thrombocytopenia were defined as a twofold or greater increase in numbers of platelets and WBCs over baseline and also a WBC count >11,000, ↑ twofold- threefold increase; ↑↑, four- to fivefold increase; ↑↑↑, greater than fivefold increase. ALP, alkaline phosphatase; ALT, alanine aminotransferase; BUN, blood urea nitrogen; GLOB, globulin; GLU, glucose; TBIL, total bilirubin; ND, not done; —, no change.
sometimes more dominant than CD8⁺ cells, and vice versa (Fig. 5C). The percentage of CD8⁺ IFN-γ⁺ and CD4⁺ IFN-γ⁺ cells also producing IL-2 was generally ≤30% for CD8 and ≥73% for CD4. Overall, there appeared to be a strong EBOV-GP–specific cell-mediated immune response at 21 dpi in the survivors.

**Escape mutations in ZMAb-treated nonsurvivors**

To identify whether the two 48-hour–treated nonsurvivors were not protected by the ZMAb treatment because of escape mutations, we extracted the EBOV virus RNA from blood on day 8 for B2 and day 11 for B3 and subjected it to sequence analysis. The sequences were compared with the challenge virus sequence. Two mutations were found in EBOV isolated from B2 and were located at amino acids 275 and 508 of EBOV-GP (Fig. 6). These mutations were not found in any other natural EBOV isolates when searching GenBank. On the other hand, B3 did not have any escape mutants. B3 was euthanized on day 11 for humane reasons because of hindlimb paralysis. However, the clinical score for B3 was improving at this time because the subject was beginning to eat more and the fever was declining (Fig. 2, B and C).

**DISCUSSION**

EBOV infections result in mortality rates as high as 90%. With no vaccine approved for human use, there is an urgent need for a therapeutic protocol that would improve survival in humans after exposure. Ideally, an effective treatment could be delivered once symptoms appear, but EBOV kills rapidly, providing limited time to intervene. Current therapies have only been 100% effective when given within 1 hour; however, most people present at clinic when symptoms appear several days after infection. Therefore, the goal of this study was to find a safe and viable treatment that would significantly improve survival at later time points after infection.

The present study demonstrates that administration of a combination of three neutralizing mAbs directed against the EBOV-GP can provide 100% or 50% protection when given 24 or 48 hours after exposure, respectively. The treatment resulted in lower virus loads, mild symptoms, and no shedding. The fact that the survivors demonstrated mainly normal blood biochemistry and hematology points out the effectiveness of the ZMAb treatment in preventing morbidity, which is also a desired feature of any therapeutic intervention. Additionally, the subjects were able to mount a noticeable EBOV-GP–specific humoral and cell-mediated immune response. The CD8⁺ response was generally broad, but predominantly against peptide pool 3, which spans amino acids 348 to 495 of EBOV-GP. The CD4⁺ response was also broad yet somewhat equivalent for all of the peptide pools. This is not surprising because the NHPs are outbred. Indeed, it is possible that the ZMAb treatment reduced EBOV replication long enough for an immune response to develop.

Overall, a 100% survival rate when starting treatment as late as 24 hours is 24-fold better than the current therapies that initiate treatment within 1 hour. The ability to achieve 50% protection when treatment starts at 48 hours after infection suggests that ZMAb delivered in this time period may also be a viable treatment option. Aside from an accidental laboratory infection, the administration of a life-saving treatment is likely to take place several hours if not days rather than minutes after infection. Rhesus macaques, which have a delayed time

![Fig. 3. EBOV-GP–specific antibody response. (A and B) During the EBOV challenge experiment, the NHP serum was quantified by eVLP ELISA to determine the levels of EBOV-GP–specific NHP (A) IgM and (B) IgG. (C) A fluorescence neutralization assay using EBOV-eGFP was used to determine the EBOV-GP–specific NHP neutralizing antibody levels. All macaques have consistent symbols across panels.](http://www.sciencemag.org/img/doi/10.1126/scitranslmed.3003574)
to death more in line with human infections, may further improve the percent survival in the 48-hour treatment group. Therefore, the capacity to improve survival by initiating treatment as late as 48 hours is a substantial advance over currently published protocols.

Previous attempts at developing treatment protocols with mAb KZ52 (19) or polyclonal antibodies (14–16) against EBOV-GP have failed to save lethally infected macaques. When comparing the previous mAb treatment protocol to the ZMAb treatment, the human KZ52 could have failed to protect because of several factors (19). First, the KZ52 study used rhesus macaques, whereas this ZMAb study used cynomolgus macaques, leaving open the possibility of some interspecies differences. However, when comparing ZMAb and KZ52 in the same guinea pig model, the ZMAb is more effective, in general, than KZ52: The ZMAb provides 100% protection when given 48 hours after challenge compared with 1 hour for KZ52. Additionally, EBOV-infected cynomolgus macaques die 2 days earlier than rhesus macaques, thereby making a successful intervention more difficult in the cynomolgus model. Together, these studies suggest that the ZMAbs may be more efficacious than KZ52.

Second, the ZMAbs and KZ52 are different isotypes: ZMAbs are mouse IgG2a,κ and IgG2b,κ isotypes, whereas KZ52 is human IgG1. Although the mouse isotypes are known to bind to the human Fc receptors, there may be a difference in the half-life between the human and the mouse mAb in the NHP. The half-life of KZ52 was 7 days (18), but the ZMAb half-life is currently unknown; therefore, further studies are needed to tease apart any functional differences.

Finally, there are intrinsic differences in the treatment protocols between these studies. The ZMAb treatment used three antibodies recognizing three different areas of GP, which may make ZMAb more effective than KZ52, which only recognizes one epitope. Notably, the KZ52 study used a dose of 50 mg/kg in comparison to our dose of ZMAb of 25 mg/kg with 8.3 mg/kg of each mAb. The present regimen used 25 mg/kg total of the ZMAb, a clinically relevant dose for the administration to humans (30).

In the other published NHPs studies, polyclonal antibodies were used, by passive transfer of either EBOV-immune NHP whole blood (14) or equine IgG (15, 16) into naïve NHPs. Studies using polyclonal antibodies have failed to provide complete protection against EBOV in NHPs. Until recently (31), the current study demonstrates successful antibody therapy against EBOV infection in NHPs and therefore strongly suggests that protective regimens using other EBOV monoclonal or polyclonal antibodies could be developed.

There are a number of limitations to consider in this study. Using three neutralizing mAbs rather than one to improve survival is an effective strategy. However, of the two animals that died in the 48-hour group, NHP B3 did not have any escape mutants, B2 carried an escape mutant with two mutations in EBOV-GP (amino acids 275 and 508). Amino acid 275 corresponds to the 1H3 binding site in sGP, whereas amino acid 508 corresponds to the 2G4 binding site in GP2, and possibly the 4G7 binding site in GP1 (23). These data suggest that the mAb combination therapy could result in the selection of escape mutants. Another limitation is that the C1 control was treated with PBS and not an irrelevant mouse IgG. Therefore, we cannot formally rule out possible non-specific therapeutic effects of mouse IgG. However, the two treated animals that...
did not survive, B2 and B3, provide additional internal controls of lethal infection as well as for the nonspecific effects of mouse antibodies. Another potential limitation is our use of cynomolgus macaques, which could account for some differences in survival times from previously published studies. Although most of the studies for treatment of EBOV after infection have used rhesus macaques, both cynomolgus and rhesus macaques are the “gold standard” for EBOV infections. However, the capacity to achieve 100% survival in the more stringent cynomolgus macaque model suggests that treatment may be more potent than presently depicted, and that 100% protection might be achieved in rhesus macaques when delivered as late as 48 hours after infection.

This study highlights the ability of antibodies to control EBOV replication in macaques. Despite sustained efforts dedicated to identification of immune parameters correlating with survival of EBOV-infected animals or humans, no clear consensus biomarker has been identified. A recent report showed that CD8+ T cell depletion reduced the survival of monkeys vaccinated with an adenovirus-based Ebola vaccine (32). However, the animals that did not survive in the CD8+-depleted group also had poor CD4+ T cell activation levels before the depletion. The current study also failed to correlate activated CD8+ T cells with clinical outcome. Instead, the only common feature observed in the nonsurvivors is the absence of production of at least one antibody isotype (IgM or IgG). According to Plotkin (33) and Jones et al. (34), the success of this passive transfer of antibody should mean that antibodies can be used as a marker of protection. Moreover, in the current study, subject A4 did not have a cellular response yet survived the infection. A4 did, however, have a strong antibody response, thereby providing more support for the hypothesis that antibodies could be used as a marker of protection.

These findings have implications for developing future mAbs against highly pathogenic viruses, even those whose replication is suspected of being controlled by the cellular immune response. Consistently, successful strategies using a mAb after infection were recently reported against...
Nipah and Hendra viruses (35). This fresh wave of treatment options against some of the most feared viral hemorrhagic fever viruses may provide concrete solutions to both the public health and the biosafety/biosecurity sectors. Although questions remain regarding sustained protection against a second exposure to EBOV after ZMAb treatment, combining ZMAb with other experimental treatment strategies such as siRNA, oligomers, adenovirus-based vaccines, and the comparative effectiveness of the murine-based ZMABS examined here with humanized or chimeric ZMABS (36), this study supports the testing of ZMAb in preclinical trials. Moreover, dose studies and incorporation of additional mAbs may further improve treatment efficacy. Together, these studies reinforce the potential use of mAbs as a promising treatment option for severe filoviral infections.

MATERIALS AND METHODS

Viruses and peptides

EBOV strain Kikwit 95 was produced on Vero E6 cells in complete minimal essential medium (cMEM), 2% fetal bovine serum (FBS), and 0.5% penicillin/streptomycin. EBOV-eGFP (enhanced green fluorescent protein) is EBOV strain Mayinga encoding the GFP reporter gene between NP and VP35 (37). Peptides (spanning EBOV, strain Kikwit 1995 GP) consisted of 15 mers with 11 amino acid overlaps. There were four peptide pools containing 42 peptides each as follows: pool 1 (amino acids 1 to 179), pool 2 (amino acids 180 to 347), pool 3 (amino acids 348 to 495), and pool 4 (amino acids 496 to 676).

Animal experiments

Nine healthy male or female cynomolgus macaques (Macaca fascicularis; 2.5 to 4.9 kg) from a certified Canadian NHP colony (Health Canada Animal Resources Division, Ontario) received commercial monkey chow, treats, vegetables, and fruits. Husbandry enrichment consisted of commercial toys and visual stimulation. After a 10-day acclimatization, macaques were randomized into two groups of four and one NHP received PBS only as a positive control for infection. Each subject received 1000 PFU (1 ml each into two sites intramuscularly) of EBOV in Dulbecco’s modified Eagle’s medium (DMEM). At the indicated times after challenge, the subjects were treated intravenously with mAb mix (ZMAb) (25 mg/kg) containing three EBOV-GP-neutralizing mAbs (1H3, 2G4, and 4G7) as a 5-ml slow bolus in the saphenous vein. The subjects were monitored daily and scored for disease progression with an internal filovirus scoring protocol approved by the Canadian Science Centre for Human and Animal Health (CSCHAH) Animal Care Committee. The scoring rated changes from normal in the subject’s posture/activity, attitude, activity level, feces/urine output, food/water intake, weight, temperature, respiration, and scored disease manifestations such as a visible rash, hemorrhage, cyanosis, or flushed skin. Tests on examination dates taken immediately before the animals received the ZMAb bolus included weight, temperature (rectal), blood, and oropharyngeal, nasal, and rectal swabs at 1, 4, 7, 14, 21, and 28 dpi for the 24-hour group and at 2, 5, 8, 14, 21, and 28 dpi for the 48-hour group. Examinations included hematological analysis (Ani-Blood Counter, scil Vet abc) and blood biochemistry for albumin, ALP, ALT, amylase, blood urea nitrogen, carbohydrate antigen, creatinine, globulin, glucose, K+, Na+, phosphate, total bilirubin, and...
protein (VetScan vs2, Abaxis). Surviving animals were kept until day 28. Animal studies were performed under CL4 conditions and approved by the CSCHAH Animal Care Committee following the guidelines of the Canadian Council on Animal Care.

**EBOV-GP–specific neutralizing mAb production and purification**

The creation of the EBOV-GP–specific neutralizing mAbs (1H3, 2G4, and 4G7) has been described previously (23). For mAb purification, initial hybridoma cell cultures were grown in RPMI 1640 (Invitrogen), 1 mM L-glutamine, 10% FBS, and 1× penicillin/streptomycin (Invitrogen) and then expanded in Hybridoma-SFM (Invitrogen), 1 mM L-glutamine, and 1× antibiotic-antimycotic (Invitrogen) in roller bottles at 37°C and 5% CO2 for 7 to 14 days. Supernatant was clarified by centrifugation and concentrated 10-fold with an Amicon Stirred Cell system [30-kD molecular weight cutoff (MWCO) filter, Millipore]. The antibodies were purified on a HiTrap Protein G HP column (GE Healthcare) with Protein A Binding Buffer and IgG Elution Buffer (Thermo Scientific) according to the manufacturers’ instructions. Positive fractions were pooled and buffer was exchanged into PBS with Centriprep units (30-kD MWCO filter, Millipore). Antibody purity was assessed at >98% by SDS–polyacrylamide gel electrophoresis.

**Enzyme-linked immunosorbent assay**

ELISAs for monkey IgG, IgM, and mouse IgG were performed on NHP sera as described previously (13). Assays were performed once with each sample assayed in triplicate. For monkey IgG and IgM, a sample was considered positive when the absorbance was higher than the mean plus 2 SDs of the before treatment negative control from each monkey. For quantification of the mouse IgG levels, a pool of the three treatment antibodies (2 mg/ml each, 6 mg/ml total) was serially diluted and used as the standard. The average values from day 0 were subtracted from the average exam date values.

**EBOV titration**

The EBOV median tissue culture infectious dose (TCID50) virus titers were determined by adding 10-fold serial dilutions of whole-blood samples from NHPs to Vero E6 cells. The assay was performed once with four replicates per dilution. Plates were scored for cytopathic effect on 10 dpi, and titers were calculated with the Reed and Muench method.

For the GEQ titers, total RNA was extracted from blood with the QIAamp Viral RNA Mini Kit (Qiagen). EBOV was detected by quantitative RT-PCR with the LightCycler 480 RNA Master Hydrolysis Probes (Roche) assay targeting the RNA polymerase (nucleotides 16472 to 16538, AF086833). Reaction conditions were as follows: 63°C for 3 min, 95°C for 30 s, and cycling of 95°C for 15 s, 60°C for 30 s for 45 cycles with StepOne Plus (Applied Biosystems). The lower detection limit for this assay is 1 PFU/ml. The primer sequences are as follows: EBOVLF2 (CAGCCAGCAATTTCTTCCAT), EBOVLR2 (TTTCCGTTGCTGTITCTGTG), and EBOV LP2 FAM (FAM-ATCATTTGGCGTACTGGAGGACGAG).

**EBOV-neutralizing antibody titration**

NHP sera were inactivated at 56°C for 45 min and then serially diluted twofold with DMEM in triplicate before incubating at 37°C for 60 min with an equal volume of EBOV-eGFP (100 PFU per well). The virus-serum mixture was added to Vero E6 cells and incubated at 37°C for 48 hours. The plates were fixed in 10% phosphate-buffered formalin, and GFP levels were quantified by fluorescence plate reader (Advanced Imaging Devices). The highest serum dilution scoring greater than 50% reduction in eGFP expression was considered positive for neutralizing antibodies, and the neutralization titer was reported as the reciprocal of this dilution.

**Cytokine assays**

PBMCs were isolated by diluting whole blood 1:1 with PBS before overlaying onto Ficoll. After centrifugation at 750 g for 45 min, the buffy coat was washed twice and then resuspended in (cRPMI) RPMI 1640, 0.1 mM MEM nonessential amino acid, 1 mM sodium pyruvate, 10 mM Heps, 2 mM L-glutamine, 55 μM β-mercaptoethanol, 10% FBS, and 1% penicillin/streptomycin. INF-γ ELISpot assays (BD Biosciences) were performed according to the manufacturer’s protocol (4), with 5 × 106 PBMCs in cRPMI plus EBOV peptide pool (2.5 μg/ml), and incubated for 18 hours. Spots were visualized with AEC substrate (BD Biosciences) and quantified with the ELISpot Plate Reader (AID Cell Technology).

The frequency of CD4+ or CD8+ cells producing IFN-γ and IL-2 was assessed by flow cytometry with BD Biosciences reagents. PBMCs, at 106 cells per well in cRPMI with EBOV peptide pool (5 μg/ml) plus GolgiPlug (1 μl/ml). PBMCs stimulated overnight were stained with anti-human CD4–peridinin chlorophyll protein–Cy5.5 and CD8–allophycocyanin mouse antibodies (clones B27 and MQ1-17H12, respectively) followed by a 20-min incubation in Cytofix/Cytoperp. Intracellular cytokines were detected by staining with anti-human IFN-γ–fluorescein isothiocyanate and IL-2–phycoerythrin mouse antibodies (clones B27 and MQ1-17H12, respectively) diluted in Perm/Wash buffer. Then, 300,000 events were acquired (Accuri), and data were analyzed with FCS Express 4 (DeNovo Software).

**Sequencing of EBOV-GP**

Total RNA was extracted from 8-dpi blood samples of NHP B2, B3, and the challenge virus with the QIAamp Viral RNA Mini Kit (Qiagen). EBOV-GP was amplified with OneStep RT-PCR Kit (Qiagen) according to the manufacturer’s instructions. Reaction conditions were as follows: 50°C for 30 min, 95°C for 15 min, and then 39 cycles of 95°C for 15 s, 50°C for 30 s, and 72°C for 30 s. PCR product purification was performed with QIAquick PCR Purification kit (Qiagen) as per the manufacturer’s instructions and sequenced on ABI 3720XL DNA Sequencer with Applied Biosystems BigDye Terminator Version 3.1 chemistry. The sequences were assembled with DNASTAR Lasergene 9 SeqMan software.

The primer sequences are as follows: GP1,2 Xho I Fwd (1 to 18): GACCTCGAGATGCGCCTACAGGATATT; GP1,2 Nhe I Rev (2030 to 2040): GACGCTAGCTAAAAGCAAAATTGGC; GP1,2 Fwd01 (264 to 285): CAGGTCGGTGTCCTCCACCAAG; GP1,2 Fwd02 (528 to 546): CGCTGAAGGTTGTGTGG; GP1,2 Rev02 (546 to 528): GCAACGCACCTTACG; GP1,2 Fwd03 (757 to 776): CTGTCACCAGCTAAGGAC; GP1,2 Rev03 (746 to 721): GTGAACTTGTTACGG; GP1,2 Fwd04 (1051 to 1070): GTTCAAGTGCAAGTCAAG; GP1,2 Rev04 (1070 to 1051): CTTTGACTGTCACATT; GP1,2 Fwd05 (1311 to 1330): GACGGAGGTACCGACTTCC; GP1,2 Rev05 (1330 to 1311): GGAGTGGTACCTGTGTA; GP1,2 Fwd06 (1563 to 1582): GGAGTTGATCTG; GP1,2 Rev06 (1582 to 1563): CGATTGCAGCCTTCTAC; GP1,2 Rev07 (1835 to 1817): GGTTCGATACAGCGTCC.
negative control from each monkey; therefore, any positive results would mean $P \leq 0.05$.
Successful Treatment of Ebola Virus–Infected Cynomolgus Macaques with Monoclonal Antibodies

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A Race Against Time
Although rare, Ebola infection figures prominently in the public’s fear of an infectious disease outbreak because of its marked, rapid, and fatal manifestation. This fear is fueled by our complete helplessness when it comes to fighting Ebola—there’s no vaccine, and any treatment options we do have only work if administered within minutes—or at most hours—after infection. Qiu et al. address this impotence head-on by demonstrating that administration of a three-antibody cocktail to macaques within 24 hours of infection yields 100% survival.

The authors treated the macaques 24 or 48 hours after Ebola virus challenge with a virus-neutralizing antibody cocktail (ZMab). The three antibodies in the mix each bind to distinct regions of the Ebola envelope glycoprotein (GP) and show efficacy in small-animal models. When the cocktail was given at 24 hours after infection, 100% of the monkeys survived; if the same dose of the cocktail was administered 48 hours after infection, the survival rate was 50%. Surviving macaques developed both Ebola-specific antibodies and T cell responses, which suggests that the passive neutralizing antibody transfer may keep the virus in check long enough for endogenous immunity to take over. Timing, dose, and composition must be optimized before this therapy moves into humans, but the new findings add sand to the hourglass and provide hope for an expanded treatment window for Ebola virus infection.