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SUPPLEMENT ARTICLE

Efficacy of Ebola Glycoprotein-Specific Equine Polyclonal Antibody Product Against Lethal Ebola Virus Infection in Guinea Pigs

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Background. Filoviruses including Ebola, Sudan, and other species are emerging zoonotic pathogens representing a significant public health concern with high outbreak potential, and they remain a potential bioterrorism-related threat. We have developed a despeciated equine Ebola polyclonal antibody (E-EIG) postexposure treatment against Ebola virus (EBOV) and evaluated its efficacy in the guinea pig model of EBOV infection.

Methods. Guinea pigs were infected with guinea pig-adapted EBOV (Mayinga strain) and treated with various dose levels of E-EIG (20–100 mg/kg) twice daily for 6 days starting at 24 h postinfection. The E-EIG was also assessed for neutralization activity against related filoviruses including EBOV strains Mayinga, Kikwit, and Makona and the Bundibugyo and Taï Forest ebolavirus species.

Results. Treatment with E-EIG conferred 83% to 100% protection in guinea pigs. The results demonstrated a comparable neutralization activity (range, 1:512–1:896) of E-EIG against all tested strains, suggesting the potential for cross-protection with the polyclonal antibody therapeutic.

Conclusions. This study showed that equine-derived polyclonal antibodies are efficacious against lethal EBOV disease in a relevant animal model. Furthermore, the studies support the utility of the equine antibody platform for the rapid production of a therapeutic product in the event of an outbreak by a filovirus or other zoonotic pathogen.

Keywords. Ebola virus; EVD treatment; polyclonal antibody.

The majority of filoviruses are important human pathogens and can result in case fatality rates up to 90% depending on the species [1, 2]. Until recently, Ebola viruses (EBOVs) and related filoviruses, such as Marburg virus (MARV), have been responsible for sporadic and limited outbreaks, very often in isolated areas in central African countries. However, in March 2014, an outbreak was identified in West Africa involving countries that had never experienced an outbreak of EBOV disease (EVD). The outbreak swelled rapidly to unprecedented proportions, primarily involving Liberia, Guinea, and Sierra Leone. This outbreak resulted in a total of 28 616 cases and 11 310 deaths [3], demonstrating the potential for this virus to become disseminated, particularly if it reaches highly populated areas.

Ebola virus glycoprotein (GP) is the primary target of protective immunity with antibodies playing a critical role in the control of infection; therefore, antibody-derived therapies have emerged as a promising strategy for treating EVD. These include both monoclonal antibody (mAb)-based "cocktail" therapies [4–6] and polyclonal hyperimmune sera [7–9]. Recent studies have revealed that a mAb cocktail (Zmapp) provided full protection in nonhuman primates (NHPs) when given as late as 5 days postinfection (dpi) [10, 11]. Based on the successes demonstrated in NHPs, Zmapp was used to treat EBOV patients in West Africa, and 5 of the 7 treated patients survived infection [12]. The Zmapp cocktail has been tested in a randomized and controlled clinical trial in combination with standard of care in patients diagnosed with EVD in West Africa [13]. Although the trial failed to show statistical significance, results suggested higher survival in the Zmapp group (28 of 36) compared with standard of care alone (22 of 35).

Despite the promising data, mAbs have several limitations including development of EBOV escape mutants and high production costs. Studies have shown emergence of such escape mutants against individual mAbs as well as cocktails of mAbs resulting in reduced efficacy in animal models [10, 14]. However, with the rapid advance of several EBOV/MARV GP-based vaccine candidates such as virus-like particles (VLPs), there is an opportunity to use vaccines to develop

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a broad multivalent polyclonal therapeutic for future outbreaks of EVD. Polyclonal immune globulin therapeutics derived from horses is an attractive approach that can offer rapid scale-up in response to outbreaks while countering selection of antibody escape mutants by hitting multiple vulnerable epitopes of the ebolavirus. Clinical safety of equine immune globulin products is well established due to their lengthy use in the clinic to treat several human diseases including botulism [15], rabies [16], and diphtheria [17].

The immunogenicity and protective efficacy of VLPs expressing EBOV GP has been demonstrated against lethal EBOV challenge in numerous studies in rodents and NHPs [18–21]. The EBOV GP protein contains a heavily glycosylated region called the mucin-like domain, which is important for masking EBOV GP epitopes from cellular surface proteins including major histocompatibility complex I and β_1-integrin recognition, facilitating viral escape from immune detection [22–24]. Vaccination of mice with VLPs containing EBOV GP lacking its mucin-like domain (GPΔmuc) induced slightly higher levels of neutralizing antibodies compared with VLPs expressing wild-type EBOV GP, and both vaccines protected mice from EBOV infection [22]. Furthermore, our unpublished observations indicate that immunization of guinea pigs with EBOV GPΔmuc induces higher cross-neutralizing antibody titers against Sudan virus (SUDV). In this study, we describe the generation of EBOV-specific equine immunoglobulin G (IgG) using a prime-boost immunization strategy consisting of priming with a VLP vaccine encoding EBOV GP, nucleoprotein (NP), and viral protein 40 (VP40) followed by a boost with a GPΔmuc protein. We further demonstrate that postexposure treatment with a despeciated derivative of this equine IgG protects guinea pigs from lethal EBOV infection.

METHODS

Viruses and Cells

Strains of EBOV used in this study include 1976 Mayinga, 1995 Kikwit, and 2014 Makona (CO7) and other species from the ebolavirus genus such as Bundibugyo (BDBV), Taï Forest (TAFV), Reston (RESTV), and Sudan (SUDV) strain Boniface. Other filovirus genera tested include the Marburgviruses (MARV), strains Musoke and Ravn. The Mayinga strain of guinea pig-adapted EBOV (GA-EBOV) was used for guinea pig infections [25, 26]. These viruses were all handled in biosafety level 4 facilities (BSL-4) at the National Microbiology Laboratory of the Public Health Agency of Canada.

Vero E6 cells (African Green Monkey kidney epithelial cells) (American Type Culture Collection [ATCC]) were used to culture EBOV. Vero E6 cells were grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 5% bovine growth serum ([BGS] HyClone) and 1 mM L-glutamine. For SUDV, the virus was cultured in CV1 cells (African Green monkey kidney fibroblast cells) from ATCC, using Eagle's minimum essential medium + 5% BGS.

Ethics Statement

All experiments using guinea pigs were approved by the Animal Care Committee at the Canadian Science Centre for Human and Animal Health in protocol H-15-026. This protocol was carried out in accordance to the guidelines set by the Canadian Council on Animal Care. Horse immunization and plasmapheresis were approved by the Auburn University Institutional Animal Care and Use Committee (PRN 2014-2562).

Animals

For the in vivo studies, 250-gram male or female Hartley Guinea pigs were ordered from Charles River Laboratories (Montreal, Quebec). Guinea pigs were housed in negative pressured, HEPA-filtered caging units within BSL-4 and acclimatized for 1 week before infection. Eight horses (3 mares and 5 geldings; 6 Percherons, 2 Belgians) that were between 11 and 17 years of age with a mean body weight of 895 kg (range, 755–963 kg) were sourced from the Auburn University equine herd. All horses were maintained in mixed-grass, biosecure pastures and had ad libitum access to water and alfalfa hay.

Equine Immunization, Plasmapheresis, Hyperimmune Product Manufacturing

Virus-like particles containing EBOV GP, VP40, and NP were produced from Sf9 cell infection [27] and formulated with Titermax Gold (Sigma-Aldrich) adjuvant for immunization. Horses (n = 8) were immunized (primed) twice with 1 mg of VLPs. First immunization was given as intramuscular injection on day 0, and the subsequent immunization was given subcutaneously on day 21. Horses were boosted with 250 μg of EBOV GPΔmuc on days 42 and 63. Blood samples were obtained on days 0, 21, 42, 56, and 70 for evaluation of antibody response by enzyme-linked immunosorbent assay (ELISA) using EBOV GPΔTM (GP ectodomain lacking transmembrane domain). The maximal median effective concentration (EC_{50}) for each plasma sample was determined, and 1 horse was selected for plasmapheresis on day 90. Equine $F(ab')_2$ hyperimmune was produced from 20 liters of plasma using a validated manufacturing process that removes the Fc to reduce the risk of immunogenicity as described previously [15]. The control (placebo) $F(ab')_2$ was produced from plasma from naive horses using the same manufacturing process. Two lots of purified F(ab')2 (equine Ebola polyclonal antibody [E-EIG]) were produced, lot PD_740_VHF_15_001_001 (lot 1) and lot PD_740_VHF_15_001_002 (lot 2).

Microneutralization Assays

Cross-reactivity of E-EIG to other EBOV species was measured by microneutralization assays. In general, E-EIG was serially diluted (1:2) in DMEM + 1% BGS to a final dilution of 1:16 384 and incubated with 100 median tissue culture infectious dose ($TCID_{50}$) of each virus for 1 hour at 37°C. Ebola virus and serum mixture (50 μL) were then added to Vero E6 cells, with the exception of SUDV, which was added onto CV1 cells, and

allowed to adsorb for 1 hour at 37°C. After 1 hour, 100 µL fresh DMEM + 1%BGS + 1× L-glutamine were added to each well. Cytopathic effect (CPE) was observed after 14 days at 37°C.

Guinea Pig Experiments

Guinea pigs were infected by intraperitoneal (IP) injection with 1000 plaque-forming units (PFUs) of GA-EBOV. This study was divided into 2 equal cohorts. Groups of guinea pigs (n = 6) were treated via IP injection twice a day (b.i.d.) starting 1 dpi with E-EIG at different dose levels (20, 50, and 100 mg/kg) or 100 mg/kg placebo until 6 dpi. One group received 20 mg/kg dose b.i.d. following an abbreviated schedule of 3 days (1 to 3 dpi). As an additional control, a group of guinea pigs were left untreated to monitor adverse events of dosing. The primary endpoint was survival, defined as the percentage of exposed guinea pigs that survived to day 18. Survival and weight loss were monitored for 21 dpi, and animals were sacrificed when >25% weight loss or when predetermined clinical signs of disease were reached. In addition, on 3 and 5 dpi, guinea pigs were anesthetized with isoflurane, and blood, oral, and rectal swabs were collected to assess viral loads by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and $TCID_{50}$ assay.

Guinea pigs that survived GA-EBOV infection after E-EIG treatment were assessed for their protective immune response against subsequent GA-EBOV challenge. Animals for this portion of the study were anesthetized 28 dpi with isoflurane, and blood was collected to measure the EBOV-specific antibody response. Animals were then rechallenged by IP injection with 1000 PFU GP-EBOV and monitored for survival and weight loss for 18 days, and oral and rectal swabs were collected at 7 dpi to assess viral loads.

Viral Assays

Viral loads were measured by qRT-PCR using primers and probes detecting the EBOV L gene. Viral ribonucleic acid (vRNA) was extracted using the QIAamp Viral RNA Mini kit (QIAGEN). Levels of EBOV L were quantified using Light Cycler 480 RNA Master Hydrolysis kit (Roche) along with the following primer and probe set: EBOV L forward primer CAGCCAGCAATTTCTTCCAT, EBOV L reverse primer TTTCGGTTGCTGTTTCTGTG, EBOV L probe1 FAM-ATCATTGGCGTACTGGAGGAGCAG, and EBOV L probe 2 FAM-TCATTGGCGTACTGGAGGAGCAGG. Thermocycling conditions were as follows: 61°C for 3 minutes, 95°C for 30 seconds, followed by 40 cycles of 95°C for 15 seconds and 60°C for 40 seconds. Genome equivalents were calculated based on a standard curve generated from a plasmid containing EBOV L.

Viral titers in samples were measured by $TCID_{50}$ assay on Vero E6 cells. Samples were serially diluted (1:10), 100 µL of each dilution was added to Vero E6 cells in triplicate, and plates were incubated at 37°C with 5% CO_2. The presence of CPE was determined after 14 days, and titers, reported as $TCID_{50}$/mL of sample, were calculated using the Reed and Muench [28] method.

Antibody Assays

Reactivity of E-EIG (Lot 1) against EBOV VP40 and NP was assessed by ELISA. Plates were coated with 100 ng/well of either purified EBOV VP40 (IBT) or EBOV NP (Sino Biological) in phosphate-buffered saline (PBS) overnight at 4°C. Plates were blocked with StartingBlock T20 (Thermo Fisher Scientific), washed 3 times with PBS/0.05% Tween 20, incubated with E-EIG serial dilutions (1:3) for 2 hours at room temperature, washed, incubated with secondary peroxidase conjugated antihorse IgG (1:5000; Sigma-Aldrich) for 1 hour at room temperature, washed, and developed with TMB solution (Life Technologies) for 30 minutes at room temperature. Absorbance values at 650 nm were obtained, and data were transformed into a 4-parameter nonlinear regression curve to determine EC_{50} values.

The EBOV-specific IgG titers were determined in guinea pig serum by ELISA. The procedure was as described above, with the exception that plates were coated with whole EBOV lysate (1:1000). As a negative control, wells were coated with whole MARV lysate (1:1000). Serum serial dilutions (1:4) were incubated on the plates for 1 hour at 37°C, followed by a secondary peroxidase-conjugated antiguinea pig IgG (KPL) for 1 hour at 37°C. Sample wells were considered positive when the net optical denisty (OD) (sample OD minus negative control OD at the same dilution) was greater than 1.0.

Statistical Analysis

Fisher's exact test and log-rank (Mantel-Cox) test were used to compare survival rates and median time to death, respectively, between groups. Results were considered significant when $P < .05$.

RESULTS

Immunization of Horses and Manufacturing of Equine $F(ab')_2$ Product

Horses were immunized with VLPs containing EBOV GP, VP40, and NP and boosted with EBOV GPΔmuc protein as shown (Figure 1A). Blood samples were collected from each horse to evaluate the antibody response against EBOV GPΔTM. The EC_{50} titers, expressed as the reciprocal dilution, gradually increased until day 70 and ranged from 5×10^3 to 10^5 (Figure 1B). Based on the EC_{50} titer results, plasma was collected from the horse with the highest titer by plasmapheresis on day 90 for further manufacturing. The purified $F(ab')_2$ (E-EIG) was further evaluated by in vitro assays and in the guinea pig model of infection.

In Vitro Characterization of Equine Ebola Polyclonal Antibody

The 2 lots used in these studies contained a total protein concentration of ~52 mg/mL (lot 1) or 58 mg/mL (lot 2). Gel electrophoresis and protein staining showed greater than 96% purity for lot 1, consistent with purity for both lots (Figure 2A). The neutralization potency of E-EIG was tested in an assay using vesicular stomatitis virus (VSV) pseudotyped with GP of EBOV (EBOV-VSV-Luc) and containing a luciferase reporter

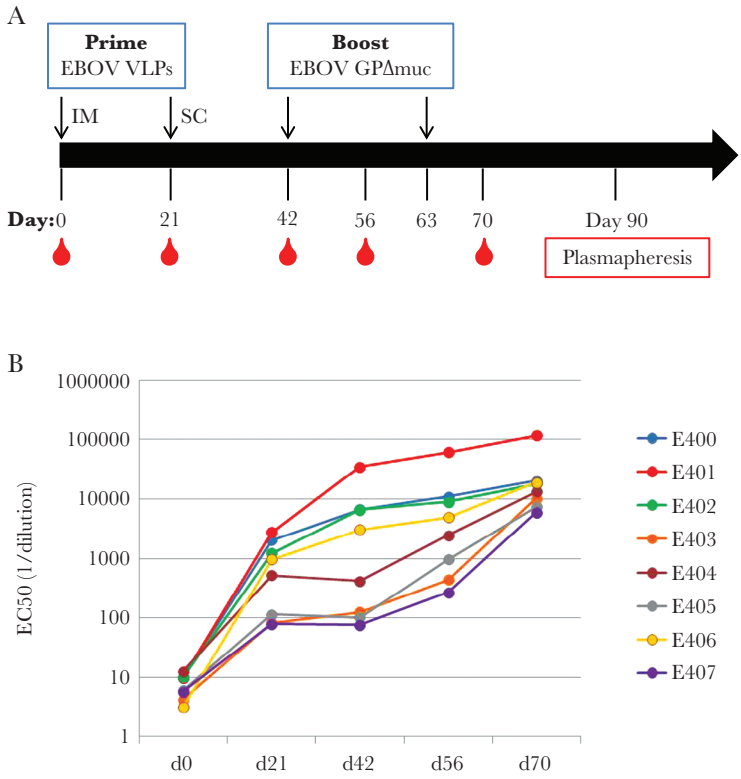

Figure 1. Production of Ebola virus (EBOV)-specific equine F(ab')$_2$ antibody product. (A) Immunization and plasmapheresis schedule. Horses (n = 8) were immunized with 1 mg EBOV virus-like particles (VLPs) via intramuscular (IM) injection or subcutaneous (SC) injection, followed by 2 boosts with 250 µg of EBOV GPΔmuc. Blood samples were collected on days (d) 0, 21, 42, 56, and 70. (B) Anti-EBOV glycoprotein (GP) antibody response in plasma of immunized horses measured by enzyme-linked immunosorbent assay against EBOV GPΔTM. The median maximum effective concentration (EC_{50}) for each plasma sample is shown.

gene as previously described [29]. The neutralization ability of both lots was comparable with EC_{50} values of 1.68 and 2.75 µg/mL, respectively (Figure 2B). The antibody response of lot 1 against EBOV VP40 and NP was also assessed. The EC_{50} value for EBOV VP40 was determined to be 4.51 µg/mL, whereas the EC_{50} value for EBOV NP was not determined due to low reactivity of lot 1 towards NP.

Cross-Reactivity Against Related Filoviruses

The cross-reactivity of E-EIG (lot 1) was assessed against various strains of EBOV (Mayinga, Kikwit, Makona) and the other recognized virus species from ebolavirus genus including SUDV, TAFV, RESTV, and BDBV. The results demonstrated a comparable and strong neutralization activity (range, 1:512–1:896) of E-EIG against strains of EBOV, TAFV, and BDBV (Table 1). Strong cross-reactivity against most viruses from the ebolavirus genus indicates the potential for use of E-EIG as a cross-protective polyclonal antibody therapeutic.

Protective In Vivo Efficacy of Equine Ebola Polyclonal Antibody

The in vivo efficacy of E-EIG (lot 1) was evaluated in groups of guinea pigs (n = 6) that were infected with GA-EBOV on day 0. Starting on 1 dpi, animals were treated twice daily with various doses of E-EIG or placebo as described under Methods. All animals from the high-dose groups (50 and 100 mg/kg) and 5 of 6 (83%) animals from 20 mg/kg-dose group survived to day 18. Survival was significant compared with the placebo or untreated group (P = .0022 for 50 and 100 mg/kg-dose group and P = .015 for 20 mg/kg-dose group; Figure 3A). The group treated with 20 mg/kg at an abbreviated schedule had significantly lower survival (33%, P = .45) compared with placebo. Weight loss correlated with survival rates, where animals in the untreated and placebo groups had significant weight loss, followed by animals in the low-dose group treated for 3 days with minor weight loss, and no weight loss in the animals treated with higher doses (Figure 3B). Median survival time was significantly longer for E-EIG at 20 mg/kg with the abbreviated dosing schedule (14 days) compared with 7 days for placebo group (P < .0001). The median survival time was not calculable for the rest of the groups.

Animals were also monitored for viral loads on 3 and 5 dpi by measuring vRNA and infectious EBOV in blood and swab samples by qRT-PCR and $TCID_{50}$ assay, respectively. These time points were selected because the onset of viremia in guinea pigs is known to occur on day 3, and the peak is expected on day 5. As expected, most control animals (11 of 12) showed

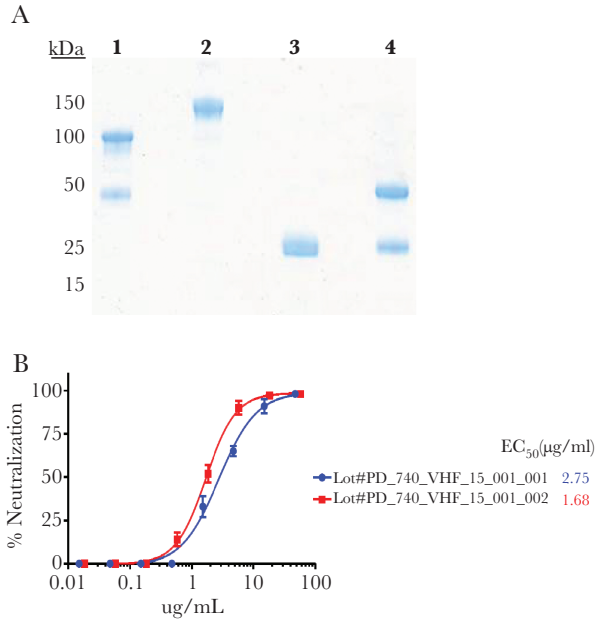

Figure 2. Characterization of equine Ebola polyclonal antibody (E-EIG) in vitro. (A) Purity analysis of E-EIG by sodium dodecyl sulfate gel electrophoresis (Lot 1). Sterile-filtered Fab (nonreduced) in lane 1, sterile-filtered whole immunoglobulin G ([IgG] nonreduced) in lane 2, sterile-filtered Fab (reduced) in lane 3, and sterile-filtered whole IgG (reduced) in lane 4. (B) Neutralizationof Ebola virus vesicular stomatitis virus-Luc by E-EIG. Abbreviation: EC_{50}, median maximum effective concentration

viremia (10^4 to 10^7 genome copies/mL) on 3 dpi, whereas none of the E-EIG treated animals were positive by qRT-PCR on day 3 (Figure 4A). The viral load increased sharply (10^8 to 10^{10} genome copies/mL) in all animals from both control groups by 5 dpi, suggesting widespread systemic disease, whereas treated animals by comparison remained negative by qRT-PCR, except 1 sample in the 20 mg/kg-abbreviated treatment group. Samples that were positive by qRT-PCR with cycle threshold (Ct) values <31 were further titrated by $TCID_{50}$ assay. The titration confirmed high levels of infectious virus in the blood on days 3 and 5 in control animals (Figure 4B), which correlated well with viral RNA copies by qRT-PCR assay (Figure 4A).

Although 50% of animals from each control and E-EIG-treated group were positive for vRNA in the oral and rectal swabs at 3 dpi (Figure 5A), they were all negative for infectious virus by $TCID_{50}$ assay (Figure 5B). Although 60% (15 of 24) of E-EIG-treated animals were positive for vRNA at 5 dpi, none had infectious EBOV in the swab samples. On the other hand, vRNA-positive animals at 5 dpi in placebo control and untreated groups increased to 80% and 100%, respectively. As with the blood samples, only swab samples with Ct values <31 were tested for infectious virus. Infectious virus was detectable in the rectal swab for both control groups and in the oral swab for 1 animal in the placebo-treated group. These results suggest that treatment with E-EIG reduced the virus shedding via the oral and rectal routes.

Overall, there was a significant decrease in the proportion of guinea pigs with viremia in the treated groups compared with the placebo group. Only 1 animal from the 20 mg/kg treatment group with the abbreviated schedule was positive for vRNA in the blood among all treated animals, although it was negative

Table 1. E-EIG Neutralization Activity Against Selected Ebolaviruses

Genus	Species	Virus	Strain	Neutralization Titer[a]
Ebolavirus	Zaire ebolavirus	EBOV	Mayinga	512
			Kikwit	512
			Makona C07	640
	Bundibugyo ebolavirus	BDBV		896
	Taï Forest ebolavirus	TAFV		512
	Sudan ebolavirus	SUDV		16
	Reston ebolavirus	RESTV		-
Marburgvirus	Marburg marburgvirus	MARV		-
		RAVV		-

Abbreviations: E-EIG, equine Ebola polyclonal antibody; RAVV, Ravn virus.

[a]Neutralization activities towards EBOV strains Mayinga, Kikwit, and Makona C07, and other ebolavirus genus species SUDV, TAFV, RESTV, and BDBV were determined by microneutralization assay. Titers are reported as the reciprocal value of the lowest dilution with complete virus neutralization.

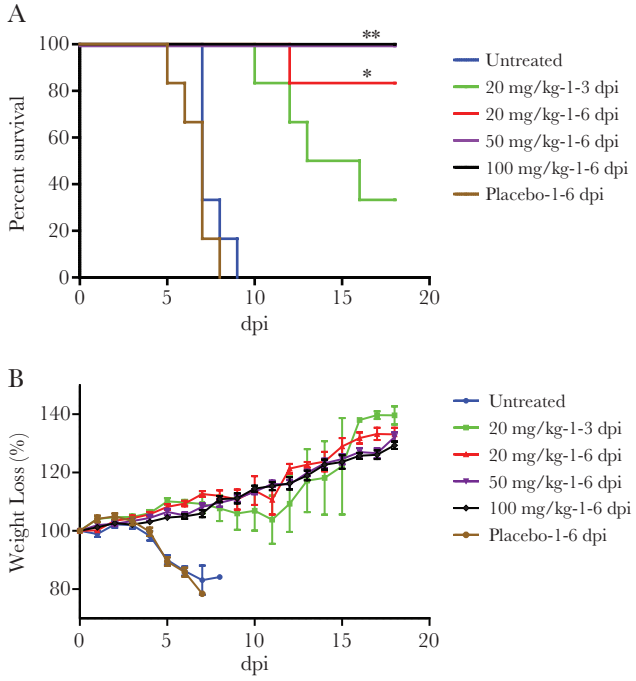

Figure 3. In vivo efficacy of equine Ebola polyclonal antibody (E-EIG) in guinea pig model. Groups of guinea pigs (n = 6) were infected with 1000 plaque-forming units of guinea pig-adapted Ebola virus by intraperitoneal injection and treated with E-EIG (20, 50, 100 mg/kg) for either 3 days (1–3 days postinfection [dpi]) or 6 days (1–6 dpi), or with 100 mg/kg placebo (1–6 dpi), or left untreated. (A) Percentage of survival and (B) weight loss are shown. Statistical significance is denoted by **, $P < .01$ and *, $P < .05$.

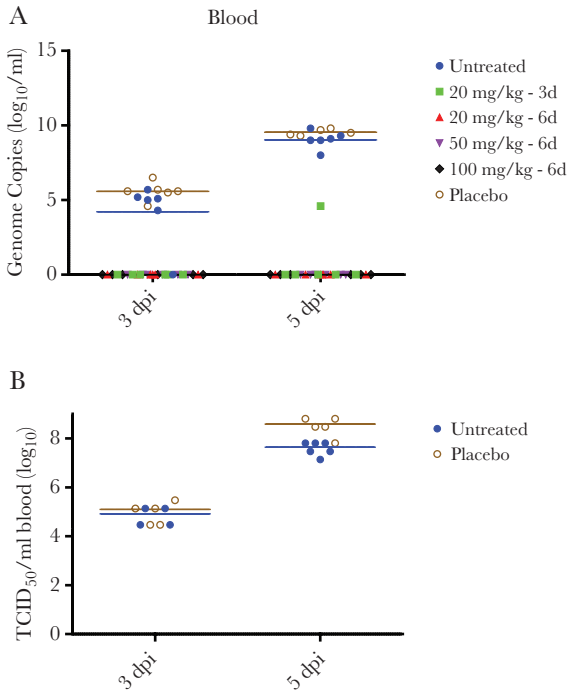

Figure 4. Detection of Ebola virus (EBOV) ribonucleic acid (RNA) and replicating virus in the blood. Groups of guinea pigs (n = 6) were infected with guinea pig-adapted Ebola virus and treated with equine Ebola polyclonal antibody (20, 50, 100 mg/kg) for either 3 days (3d) or 6 days (6d), or with 100 mg/kg placebo (6d), or untreated. (A) Viral RNA in blood was determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) targeting the EBOV L gene. Data are shown as genome copies (log_{10}) per milliliter of blood and was estimated with the use of a L-gene plasmid-based standard curve. (B) The EBOV titers in blood as determined by median tissue culture infectious dose ($TCID_{50}$) assay. Only samples that were positive by qRT-PCR (cycle threshold value <31) were titered.

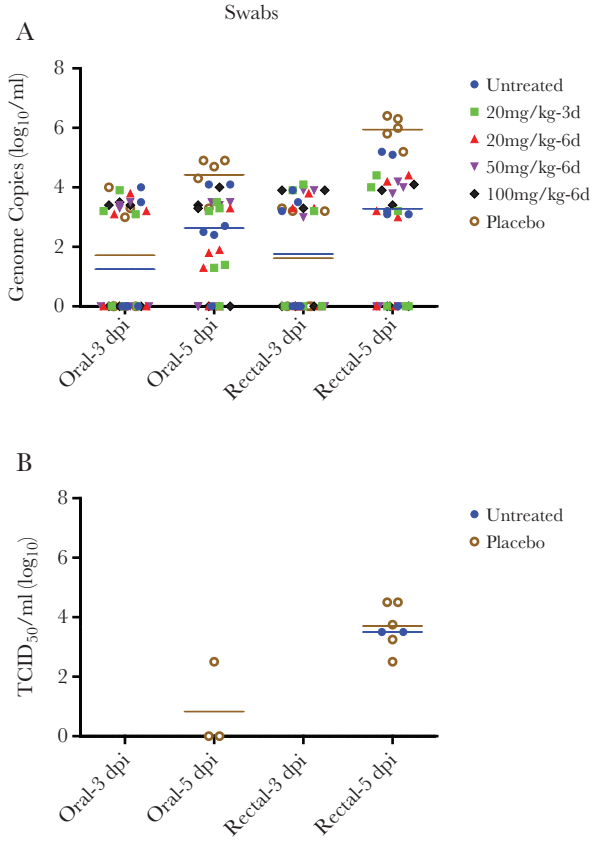

Figure 5. Ebola virus (EBOV) shedding in guinea pigs infected with guinea pig-adapted (GA)-EBOV and treated with equine Ebola polyclonal antibody (E-EIG) or placebo. Groups of guinea pigs (n = 6) were infected with GA-EBOV and treated with E-EIG (20, 50, 100 mg/kg) for either 3 days ([d] 1–3 d postinfection [dpi]) or 6d (1–6 dpi), 100 mg/kg placebo (1–6 dpi), or untreated. (A) Viral ribonucleic acid in oral and rectal swabs obtained on day 3 and 5 postinfection were determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) targeting EBOV L gene. Data are shown as genome copies (log_{10}) per milliliter and was estimated with the use of a L-gene plasmid-based standard curve. (B) The EBOV titers in oral and rectal swabs as determined by median tissue culture infectious dose ($TCID_{50}$) assay. Only samples that were positive by qRT-PCR (cycle threshold value <31) were titered.

for infectious virus. Many treated animals were positive for vRNA in oral and rectal swabs; however, infectious virus was not recovered from these animals. Taken together, survival and viremia data suggest that E-EIG treatment significantly increases survival when administered before the onset of viremia and, importantly, may block shedding of infectious virus, thereby potentially interfering with transmission.

Rechallenge

All E-EIG-treated animals that survived the infection were rechallenged on either day 33 (cohort 2) or 35 (cohort 1) after the initial challenge, to evaluate whether the immune response developed during the first challenge can provide lasting immunity without further intervention. Administration of E-EIG in the initial protection experiment was initiated 24 hours postinfection, but because no viremia was detected in the E-EIG-treated animals by 3 dpi, it is possible that the inoculum was neutralized in these animals before any viral replication occurred. In this case, a lack of replicating virus could hinder the induction of a protective primary immune response towards EBOV. Alternatively, daily dosing of E-EIG may have limited significant viral replication but still allowed priming of the immune system with low levels of replicating virus, resulting in production of neutralizing antibody responses in survivors.

Virus-specific antibody responses were monitored by measuring serum levels of EBOV-specific IgG by ELISA before rechallenge. Most survivors from the first challenge had significant antibody titers against EBOV, suggesting that there was sufficient viral replication to prime the immune system (Table 2). The EBOV-specific IgG titers varied from 50 to 3200 on day 0 of rechallenge. Out of 24 treated guinea pigs from 4 different E-EIG treatment groups, 19 survived after the first challenge study, and, of these, only 3 were negative for EBOV-specific IgG. Survival after rechallenge and antibody levels before challenge is shown in Table 2. Only the 3 animals that did not have detectable IgG titers before challenge did not survive reinfection. Oral and rectal swabs taken on day 7 after rechallenge demonstrated that these 3 animals were positive for vRNA, and high levels

Table 2. Disposition of Survivors From Treatment Experiment After Rechallenge With GA-EBOV[a]

E-EIG Treatment Groups	Survivors From 1st Challenge	EBOV IgG Response Before 2nd Challenge	Outcome of 2nd Challenge	Genome Copies/mL (Oral/Rectal)	$TCID_{50}$/mL (Oral/Rectal)
Untreated control (n = 6)	-	NA	NA	NA	NA
Placebo control (n = 6)	-	NA	NA	NA	NA
20 mg/kg 1, 2, 3 Days (n = 6, 2 Survivors)					
Guinea pig no. 2	+	1:800	Survived	–/–	NA
Guinea pig no. 4	+	1:800	Survived	–/–	NA
20 mg/kg 1, 2, 3, 4, 5, 6 Days (n = 6, 5 Survivors)					
Guinea pig no. 1	+	Negative	Died	$10^{7}/10^{6.3}$	$10^{6.5}/10^{4.8}$
Guinea pig no. 2	+	1:800	Survived	$10^{3.1}/10^{3.1}$	NA
Guinea pig no. 3	+	1:800	Survived	–/–	NA
Guinea pig no. 5	+	1:800	Survived	–/–	NA
Guinea pig no. 6	+	1:800	Survived	–/–	NA
50 mg/kg 1, 2, 3, 4, 5, 6 Days (n = 6, All Survivors)					
Guinea pig no. 1	+	1:200	Survived	–/–	NA
Guinea pig no. 2	+	Negative	Died	$10^{7.1}/10^{5.5}$	$10^{6.8}/10^{3.8}$
Guinea pig no. 3	+	1:50	Survived	–/–	NA
Guinea pig no. 4	+	1:800	Survived	–/–	NA
Guinea pig no. 5	+	1:3200	Survived	–/–	NA
Guinea pig no. 6	+	1:3200	Survived	–/–	NA
100 mg/kg 1, 2, 3, 4, 5, 6 Days (n = 6, All Survivors)					
Guinea pig no. 1	+	1:800	Survived	–/–	NA
Guinea pig no. 2	+	1:50	Survived	–/–	NA
Guinea pig no. 3	+	1:200	Survived	–/–	NA
Guinea pig no. 4	+	1:3200	Survived	–/–	NA
Guinea pig no. 5	+	1:800	Survived	–/–	NA
Guinea pig no. 6	+	Negative	Died	$10^{3.4}/10^{6.6}$	$NA/10^{5.3}$

Abbreviations: Ct, cycle threshold; E-EIG, equine Ebola polyclonal antibody; EBOV, Ebola virus; GA-EBOV, guinea pig-adapted EBOV; IgG, immunoglobulin G; NA, values were not determined; $TCID_{50}$, median tissue culture infectious dose; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; vRNA, viral ribonucleic acid.

[a]Levels of EBOV-specific IgG were determined in survivors before rechallenge. Genome copies/mL were determined in oral and rectal swab samples at day 7 after rechallenge. (–/–) denotes the absence of vRNA in both samples. Virus titers ($TCID_{50}$/mL) were only determined for samples with Ct <31 based on EBOV L qRT-PCR results of the swab samples.

of infectious virus were recovered from the swabs (Table 2). It is interesting to note that 1 animal that had an IgG titer to EBOV of 1:800 showed detectable virus by qRT-PCR in swabs after rechallenge. This guinea pig was from the group originally treated with 20 mg/kg for 6 days. Despite evidence of viral replication in this animal, live virus could not be detected by $TCID_{50}$ assay, and it survived rechallenge. All other animals that survived rechallenge, including those that exhibited no viremia or virus shedding in the initial treatment experiment, showed no detectable evidence of viral replication after rechallenge.

DISCUSSION

In this study, we describe the use of an EBOV GP-specific equine $F(ab')_2$ product (E-EIG) as an effective postexposure treatment against lethal EVD in guinea pigs. The ability of E-EIG to cross-neutralize other EBOV strains (Mayinga, Kikwit, and Makona) and genus species (TAFV, RESTV, and BDBV) highlights the potential of this product to be used broadly against numerous ebolaviruses. Although the E-EIG response is strongest towards EBOV GP, we showed that E-EIG also recognizes EBOV VP40 but not NP, suggesting a possible minor role for EBOV VP40-specific antibodies in protection.

To determine whether increased doses of E-EIG are able to afford better postexposure protection against GA-EBOV, a dose-dependent study was performed. Because the half-life of equine-derived $F(ab')_2$ antibodies is described to be between 8 and 12 hours in guinea pigs [30], animals were treated twice daily to maintain levels of E-EIG in circulation and improve the protective response. Complete protection was observed for the 2 highest dose groups (50 and 100 mg/kg), with no weight loss or signs of disease noted. When the E-EIG dose was reduced to 20 mg/kg, a reduction in survival (83%) was observed, with further reduction to 33% when the treatment regimen was reduced from 6 to 3 days b.i.d. Reduced survival in the 20 mg/kg groups also correlated with increased weight loss and viral loads.

Based on the survival and viremia data, E-EIG treatment significantly increases survival when administered before the onset of viremia and, importantly, may block shedding of infectious virus, thereby potentially interferring with transmission. Although vRNA was detected in a number of swab samples from treated animals, as well as in the blood for 1 animal in the 20 mg/kg 3-day b.i.d. group, infectious virus was not detected. Detection of vRNA without recovery of infectious virus particles has been observed with filoviruses and for other virus families, including

Nipah viruses [31]. Furthermore, human patients treated with the mAb cocktail ZMapp had detectable EBOV RNA 4 weeks after the onset of illness [32]. This observation may suggest that virus remains as immune complexes, but it is unclear whether these complexes remain in circulation for extended periods of time or whether they are regenerated from ongoing viral replication. Therefore, future studies to determine whether the virus continues to replicate in target organs despite lack of viremia and shedding as well as how long vRNA may persist even after the clearance of clinical symptoms would be interesting to pursue.

To determine whether successful treatment with E-EIG interferes with the induction of adaptive immunity, a rechallenge experiment was performed. Of 19 survivors from the treatment experiment, 3 had high levels of infectious virus recovered from their swab samples and did not survive rechallenge. It is interesting to note that these animals were also the only ones negative for EBOV-specific IgG. All animals that survived rechallenge were positive for EBOV-specific IgG before rechallenge. They had titers ranging from 1:50 to 1:3200, indicating seroconversion even though viremia and virus shedding was suppressed by E-EIG treatment to undetectable levels in practically every animal. These results suggest that the treatment did not interfere with development of humoral immunity in most animals. Furthermore, oral/rectal swabs from the rechallenge survivors were negative for vRNA with the exception of 1 animal, although it remained negative for infectious virus. The observation that viral replication did occur in 4 of the rechallenged animals and that 3 succumbed to the disease suggests that there needs to be a balance between the immune response to infection and viral neutralization from E-EIG treatment. It is interesting to note that animals that failed to develop their own humoral responses to EBOV were distributed among the treatment groups, suggesting that individual responses to infection and treatment may play an important factor in immunity in addition to the dose of E-EIG provided. Our results emphasize the importance of testing survivors treated with immunotherapy for the presence of EBOV-specific antibodies to ascertain their susceptibility to reinfection.

Although complete viral neutralization by passive antibody therapy is important to control virus replication, this may also prevent induction of a sufficient natural immune response to the incoming virus. Without proper stimulation of the immune response, in particular the humoral arm of immunity, an individual may be susceptible to future infections by the same virus. A recent case report of a patient treated with mAb cocktail therapy that recovered from EBOV infection relapsed 9 months later with meningoenchephalitis [33]. It is interesting to note that EBOV-specific antibodies were detected in this individual after the initial illness, suggesting that other factors may play a role in the susceptibility of a host to reinfection. As the 2014 West African outbreak subsided, there have been reports of EBOV persistance for months in survivors from the outbreak, particularly in immune-privileged sites [33–35].

The devastation caused by the 2014 West African outbreak highlights the deficiencies in available treatment options against EVD. The outbreak fast tracked several potential candidates into clinical trials, including the monoclonal cocktail Zmapp [10, 11, 36]; however, some barriers to treatment to consider are supply, cost, and potential for emergence of EBOV escape mutants with monoclonal treatment [10, 14, 37–39].

CONCLUSIONS

In this study, we describe the production of an equine-derived EBOV-GP polyclonal hyperimmune with $F(ab')_2$ antibody that (1) shows strong cross-neutralizing capabilities to other EBOV species, (2) is relatively easy and cost-effective to mass produce, and (3) is highly efficacious at providing postexposure protection in the guinea pig model of EBOV disease. Although studies have shown that equine-derived IgG products that are protective in guinea pigs failed to protect cynomolgous macaques from EBOV infection [40, 41], evidence suggests that protection may improve with increased dosing. In a recent study, cynomolgous macaques treated with equine IgG daily for 5 consecutive days starting 24 hours after lethal EBOV exposure were completely protected [42]. Based on our results, we show that the timing and dose of E-EIG administration are important factors to consider for successful treatment. Further testing in NHP models to determine the optimal dosing regimen and window for postinfection treatment for further development of this product as an EVD therapeutic is warranted.

Notes

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Potential conflicts of interest. S. K. is an employee of Emergent BioSolutions Canada, which manufactured the equine Ebola polyclonal antibody (E-EIG) used in these studies. F. W. H., H. V., K. A. H., and M. J. A. are employees of Integrated BioTherapeutics, Inc. who developed and manufactured virus-like particles for immunization. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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