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Antibody-Mediated Virus Neutralization Is Not a Universal Mechanism of Marburg, Ebola, or Sosuga Virus Clearance in Egyptian Rousette Bats

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Abstract

Although bats are increasingly being recognized as natural reservoir hosts of emerging zoonotic viruses, little is known about how they control and clear virus infection in the absence of clinical disease. Here, we test >50 convalescent sera from Egyptian rousette bats (ERBs) experimentally primed or prime-boosted with Marburg virus, Ebola virus, or Sosuga virus for the presence of virus-specific neutralizing antibodies, using infectious reporter viruses. After serum neutralization testing, we conclude that antibody-mediated virus neutralization does not contribute significantly to the control and clearance of Marburg virus, Ebola virus, or Sosuga virus infection in ERBs.

Keywords

Neutralization; Marburg virus; Ebola virus; Sosuga virus; bats

The order *Mononegavirales* is the taxonomic home of numerous zoonotic viruses, including filoviruses and paramyxoviruses. The Egyptian rousette bat (ERB; *Rousettus aegyptiacus*) is a natural reservoir host of Marburg virus (MARV; family *Filoviridae*, genus *Marburgvirus*) [1], a putative natural reservoir host of Sosuga virus (SOSV; family *Paramyxoviridae*, unclassified rubulavirus-like virus) [2], and a dead-end host of Ebola virus (EBOV; family *Filoviridae*, genus *Ebolavirus*) [3, 4]. Although ERBs infected with any one of these viruses exhibit no signs of clinical illness, humans often develop severe disease after infection. Shortly following experimental inoculation of ERBs with MARV [5–7] or SOSV (authors' unpublished data), the bats develop viremia and exhibit high viral loads in multiple tissue

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specimens, oral swab specimens, rectal swab specimens, and urine specimens. In contrast, ERBs experimentally infected with EBOV exhibit low viral loads in a limited number of tissue specimens and fail to develop viremia or detectable viral shedding [3, 4]. Despite these differences in viral infection dynamics, bats infected with any one of these viruses develop virus-specific immunoglobulin G (IgG) antibody levels that begin to rise around 10 days after infection and peak between 14 and 28 days after infection [3, 5–7]. For MARV [5–7] and SOSV, the end of viral shedding typically coincides with peak virus-specific IgG antibody levels.

The long-term dynamics of the virus-specific IgG antibody response and the outcomes of reexposure to the virus have been investigated in ERBs infected with MARV only. Schuh et al [7] demonstrated that, once bats attain peak virus-specific IgG antibody levels following experimental or “natural” infection with MARV, the antibody levels rapidly decline and fall below the threshold of seropositivity by 3 months after infection. After both groups of these seronegative bats were experimentally challenged with homologous virus 17–24 months later, they developed robust secondary immune responses by 7 days after infection, in the absence of detectable virus replication or shedding, suggesting that primary infection induces long-term protective immunity against reinfection with a homologous virus [8]. Storm et al [9] showed MARV-specific IgG antibodies were still detectable in the majority of experimentally infected ERBs 4 months after infection and in naturally infected bats 11 months after initial capture. Following experimental challenge of the latter group of bats with a heterologous strain of MARV, some of the bats had detectable viral RNA in the blood, and 1 bat had a MARV-positive nasal swab specimen. However, the bats developed secondary MARV-specific immune responses by 5 days after infection, and virus was not detected in any tissues that might lead to shedding and transmission, such as the salivary glands, intestine, reproductive tract, and bladder. This suggests that, although MARV IgG antibodies were not completely protective against reinfection with a heterologous MARV strain, they may have prevented widespread viral dissemination to tissues and shedding.

While ERBs are able to effectively control and clear MARV, SOSV, or EBOV infection, it is unclear whether these events are mediated by the neutralizing ability of virus-specific IgG antibodies. Paweska et al [5], showed that 33% of ERBs inoculated with MARV possessed low levels of neutralizing antibodies (titers of 1:4 to 1:8) 21 days after infection; however; it is possible that viral neutralization activity increases later during convalescence or after a secondary MARV exposure. Although Storm et al [9] demonstrated that pooled sera from wild-caught, MARV-seropositive ERBs was able to minimally reduce the titer of a locally circulating strain of MARV, from 7.2 to 6.2 $\log_{10}$ 50% tissue culture infective doses/mL, pooling of the sera precluded examination of individual heterogeneities in virus neutralization ability and the ability to determine whether virus neutralization is a prominent feature of virus clearance at the population level. In this study, we tested >50 convalescent sera collected from ERBs that had been experimentally primed or prime-boosted with MARV, EBOV, or SOSV for the presence of virus-specific neutralizing antibodies, using homologous infectious reporter viruses.

METHODS

Biosafety

All neutralization assays were performed in a biosafety level 4 laboratory at the Centers for Disease Control and Prevention, and all filovirus work was performed in compliance with Select Agent Regulations (Animal and Plant Health Inspection Service and Centers for Disease Control 2014).

Informed Consent

Informed consent and approval by the institutional review board were obtained prior to using the SOSV convalescent human sera.

Sera

We prescreened convalescent and naive plasma/sera specimens, as well as the MR201 MARV monoclonal antibody [10], to identify appropriate positive and negative controls for subsequent virus-specific neutralizing assays (Table 1). Convalescent ERB plasma/sera specimens (the majority of which had high IgG antibody levels) from previous experimental virus infection studies were used as test samples (Table 1). Briefly, MARV convalescent bat plasma/sera specimens were generated in 3 ways. Bats in group 1 were experimentally primed with MARV and experimentally boosted with homologous virus 22 months later, and convalescent plasma specimens were collected 3 weeks later [7, 8]. ERBs in group 2 were “naturally” infected with MARV in a laboratory environment through contact with infectious bats and experimentally boosted with homologous virus 15 months later, and convalescent plasma specimens were collected 3 weeks later [7, 8]. Bats in group 3 were experimentally primed with MARV and experimentally boosted with homologous virus 2 months later, and convalescent sera were collected 3 weeks later. EBOV convalescent bat sera were generated by experimentally priming ERBs with EBOV, boosting them with homologous virus 2 months later, and collecting antisera 3 weeks later. SOSV convalescent bat sera were generated by experimentally inoculating ERBs with SOSV and collecting antisera 3 weeks later. The group 3 MARV convalescent bat sera and the EBOV convalescent bat sera were generated as part of an unpublished study evaluating the level of serological cross-reactivity between filovirus-specific bat antisera and heterologous filovirus antigen, while the SOSV convalescent sera originated from an unpublished experimental infection study designed to assess if the ERB is a competent natural reservoir host for SOSV.

Viruses

The following replication-competent, infectious recombinant (r) viruses that express the fluorescent ZsGreen1 (ZsG) protein in infected cells were used in the experiments: rMARV-ZsG (GenBank accession number MK271062; Huh7+2), rEBOV-ZsG (GenBank accession number KR781609; Huh7+2), and rSOSV-ZsG (GenBank accession number MG880225; BHK+3, Vero E6+3).

100% Focus Reduction Neutralization Test ($FRNT_{100}$)

Test and control sera were heat inactivated at 56°C for 30 minutes prior to testing. All sera were screened for the presence of virus neutralizing antibodies, using a $FRNT_{100}$. Two-fold serial dilutions of the sera, ranging from 1:8 to ≥1:128 (from 325 to 20 μg/mL for MR201), were prepared in dilution medium (FlouroBrite Dulbecco's modified Eagle's medium [DMEM] containing penicillin/streptomycin and GlutaMAX). Each serum dilution series was mixed with an equal volume of dilution medium (100 μL) containing 150 $TCID_{50}$/20 μL of virus and then incubated on a plate rocker for 1 hour at 37°C. All serum dilution series included serum, virus, and reagent controls. Following incubation, 40 μL of each serum-virus mixture was inoculated in quadruplicate onto monolayers of 95% confluent Vero E6 cells in 96-well clear-bottomed black plates containing maintenance medium (FlouroBrite DMEM supplemented with 5% heat-inactivated fetal bovine serum [HI-FBS], penicillin/streptomycin, and GlutaMAX). Plates were then incubated at 37°C in 5% CO_2 (duration, 2 days for SOSV, 3 days for EBOV, and 4 days for MARV). Following incubation, plates were viewed under a fluorescence microscope, and each well was scored for the presence, reduction, or absence of virus, as indicated by cells containing green foci. The $FRNT_{100}$ was defined as the reciprocal of the highest serum dilution for which the average virus infectivity was reduced by 100% when compared to the corresponding virus control wells.

$FRNT_{50}$

Test sera and relevant controls that exhibited $FRNT_{100}$ values ≥8 or a dilution effect were tested by $FRNT_{50}$. One set of 2-fold serial dilutions of the serum samples, ranging from 1:8 to ≥1:2048 (from 325 to 1 μg/mL for MR201), was prepared in dilution medium, while the second set was prepared in dilution medium containing 5% guinea pig complement (GPC). Each serum dilution series was mixed with an equal volume of dilution medium (100 μL) containing 125–164 $TCID_{50}$/20 μL of virus and then incubated on a plate rocker for 1 hour at 37°C. Following incubation, 40 μL of each serum-virus mixture and controls were inoculated in quadruplicate onto monolayers of 95% confluent Vero E6 cells in 96-well black-bottomed plates. After the plates were incubated on a plate rocker for 1 hour at 37°C, the virussera mixtures were removed, the cell monolayers were washed once with PBS, and 250 μL of carboxymethyl cellulose agar (1:1 mixture of 3% carboxymethyl cellulose sodium salt to 2X MEM supplemented with 8% HI-FBS, penicillin/streptomycin, and sodium pyruvate) was added to the wells. Plates were then incubated at 37°C in 5% CO_2 (duration, 2 days for SOSV and 4 days each for EBOV and MARV). The BioTek Cytation 3 Cell Imaging Multi-Mode Reader imaged and counted the number of green foci in each well. The $FRNT_{50}$ was defined as the reciprocal of the highest serum dilution for which the average virus infectivity was reduced by 50% when compared to the corresponding virus control wells.

RESULTS

The MARV $FRNT_{100}$ value of MR201 was >325 μg/mL (>32.5 μg); however, it exhibited a visual reduction in the number of viral foci at higher antibody concentrations (dilution effect) and was selected as the positive control for subsequent MARV neutralization assays. Two naive ERB plasma specimens (726415 and 725904) exhibited MARV $FRNT_{100}$ values

<8 and were selected as negative controls for subsequent testing. All 30 MARV convalescent ERB plasma/sera specimens from groups 1–3 exhibited MARV $FRNT_{100}$ values <8; however, 1 plasma specimen from group 1 (685734) exhibited a dilution effect at lower plasma concentrations and was subjected to further testing. The MARV $FRNT_{50}$ values for 685734 and the negative controls (726415 and 725904) were <8 (with and without GPC). Consistent with previous results [10], the MARV $FRNT_{50}$ values of the MR201 positive control were 325 μg/mL (32.5 μg; with and without GPC).

The EBOV convalescent nonhuman primate (NHP) pooled sera (703547; EBOV $FRNT_{100}$ = 16) and the naive NHP pooled sera (703694; EBOV $FRNT_{100}$ <8) were selected as the positive and negative controls, respectively, for subsequent testing. All 20 EBOV convalescent ERB sera exhibited EBOV $FRNT_{100}$ values <8; however, 4 sera (214838, 546240, 691073, and 721253) exhibited dilution effects at lower serum dilutions and were selected for further testing. The EBOV $FRNT_{50}$ values with and without GPC for all 4 of these EBOV convalescent ERB sera, as well as the naive NHP pooled sera negative control (703694), were <8. As previously shown [11], the EBOV convalescent NHP pooled sera positive control (703547) was able to neutralize EBOV infectivity ($FRNT_{50}$ = 16, and $FRNT_{50}$ with GPC = 128).

Initial screening of the SOSV convalescent human serum (SOSV $FRNT_{100}$ = 800) and 2 naive ERB plasma specimens (726415 and 725904; SOSV $FRNT_{100}$ <8) confirmed their selection as the positive and negative controls, respectively. All 3 SOSV convalescent ERB plasma specimens exhibited SOSV $FRNT_{100}$ values <8; however, 2 plasma specimens (289953 and 290040) exhibited dilution effects at lower plasma dilutions. Further testing of these 2 samples revealed they were only able to neutralize SOSV in the presence of GPC (289953: $FRNT_{50}$ <8, and $FRNT_{50}$ with GPC = 16; 290040: $FRNT_{50}$ <8, and $FRNT_{50}$ with GPC = 32). The SOSV convalescent human serum positive control ($FRNT_{50}$ = 8192, and $FRNT_{50}$ with GPC = 32 768) and the 2 naive ERB plasma negative controls (726415 and 725904; $FRNT_{50}$ <8 with and without GPC) performed as expected.

DISCUSSION

In this study, we systematically tested the ability of >50 MARV, EBOV, and SOSV convalescent ERB sera to neutralize live homologous virus. Our results demonstrate that the effective control and clearance of these viruses by ERBs does not appear to be mediated by virus-specific neutralizing antibodies. However, testing of additional SOSV convalescent sera from ERBs, including samples collected later in convalescence and after secondary viral exposure, is needed to firmly rule out antibody-mediated virus neutralization as a mechanism for SOSV control. Nevertheless, the results of this study suggest that ERBs may rely on other antibody-mediated functions (ie, phagocytosis or antibody-dependent cellular cytotoxicity) and/or the innate immune response to modulate infection with these viruses that are otherwise pathogenic to humans. A recent analysis of the ERB genome uncovered expanded and diversified natural killer cell receptor families, major histocompatibility complex class I genes, and type I interferons, and a theoretical functional analysis of these genes suggested that they might allow bats to tolerate viral infection in the absence of clinically apparent disease [12].

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Disclaimer. The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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Table 1.

Details of the Control and Test Samples Used in this Study

Identifier	Origin	Sample Type	Time of Boost; Time of Collection[a]	Gamma-Irradiated	IgG Indirect ELISA Result (% Above Cutoff[b])	$FRNT_{100}$	$FRNT_{50}$	$FRNT_{50}$ + GPC
MR201	Monoclonal antibody	MARV antibody	NA	No	ND	>325 μg/mL; DE	325 μg/mL	325 μg/mL
726415	ERB	Naive sera	NA	No	Negative[b,c] (−100)	<8	<8	<8
725904	ERB	Naive sera	NA	No	Negative[b,c] (−101)	<8	<8	<8
550417	ERB, group 1	MARV antiplasma	22 mo; 3 wk	No	Positive[b] (28)	<8	ND	ND
685891	ERB, group 1	MARV antiplasma	22 mo; 3 wk	No	Positive[b] (148)	<8	ND	ND
214605	ERB, group 1	MARV antiplasma	22 mo; 3 wk	No	Positive[b] (49)	<8	ND	ND
685734	ERB, group 1	MARV antiplasma	22 mo; 3 wk	No	Positive[b] (253)	<8; DE	<8	<8
686146	ERB, group 1	MARV antiplasma	22 mo; 3 wk	No	Positive[b] (60)	<8	ND	ND
684822	ERB, group 2	MARV antiplasma	15 mo; 3 wk	No	Positive[b] (165)	<8	ND	ND
721442	ERB, group 2	MARV antiplasma	15 mo; 3 wk	No	Positive[b] (261)	<8	ND	ND
726397	ERB, group 2	MARV antiplasma	15 mo; 3 wk	No	Positive[b] (193)	<8	ND	ND
684904	ERB, group 2	MARV antiplasma	15 mo; 3 wk	No	Positive[b] (227)	<8	ND	ND
720802	ERB, group 2	MARV antiplasma	15 mo; 3 wk	No	Positive[b] (69)	<8	ND	ND
215330	ERB, group 3	MARV antisera	2 mo; 3 wk	Yes	Positive[c] (459)	<8	ND	ND
219792	ERB, group 3	MARV antisera	2 mo; 3 wk	Yes	Positive[c] (331)	<8	ND	ND
438185	ERB, group 3	MARV antisera	2 mo; 3 wk	Yes	Positive[c] (397)	<8	ND	ND
438440	ERB, group 3	MARV antisera	2 mo; 3 wk	Yes	Negative[c] (−43)	<8	ND	ND
440976	ERB, group 3	MARV antisera	2 mo; 3 wk	Yes	Positive[c] (82)	<8	ND	ND
441384	ERB, group 3	MARV antisera	2 mo; 3 wk	Yes	Positive[c] (104)	<8	ND	ND
441951	ERB, group 3	MARV antisera	2 mo; 3 wk	Yes	Positive[c] (454)	<8	ND	ND
442331	ERB, group 3	MARV antisera	2 mo; 3 wk	Yes	Positive[c] (97)	<8	ND	ND

Identifier	Origin	Sample Type	Time of Boost; Time of Collection[a]	Gamma-Irradiated	IgG Indirect ELISA Result (% Above Cutoff[b])	$FRNT_{100}$	$FRNT_{50}$	$FRNT_{50}$ + GPC
443629	ERB, group 3	MARV antisera	2 mo; 3 wk	Yes	Positive[c] (75)	<8	ND	ND
546951	ERB, group 3	MARV antisera	2 mo; 3 wk	Yes	Positive[c] (628)	<8	ND	ND
547158	ERB, group 3	MARV antisera	2 mo; 3 wk	Yes	Negative[c] (−33)	<8	ND	ND
642904	ERB, group 3	MARV antisera	2 mo; 3 wk	Yes	Positive[c] (228)	<8	ND	ND
656933	ERB, group 3	MARV antisera	2 mo; 3 wk	Yes	Positive[c] (320)	<8	ND	ND
684800	ERB, group 3	MARV antisera	2 mo; 3 wk	Yes	Positive[c] (372)	<8	ND	ND
685742	ERB, group 3	MARV antisera	2 mo; 3 wk	Yes	Positive[c] (151)	<8	ND	ND
691089	ERB, group 3	MARV antisera	2 mo; 3 wk	Yes	Positive[c] (281)	<8	ND	ND
691313	ERB, group 3	MARV antisera	2 mo; 3 wk	Yes	Positive[c] (269)	<8	ND	ND
725971	ERB, group 3	MARV antisera	2 mo; 3 wk	Yes	Positive[c] (318)	<8	ND	ND
726247	ERB, group 3	MARV antisera	2 mo; 3 wk	Yes	Positive[c] (618)	<8	ND	ND
726539	ERB, group 3	MARV antisera	2 mo; 3 wk	Yes	Positive[c] (285)	<8	ND	ND
703547	NHP	EBOV antisera (pooled)	Unknown	Yes	Positive[d] (high IgM/IgG)	16	16	128
703694	NHP	Naive sera (pooled)	NA	Yes	Negative[d]	<8	<8	<8
214703	ERB	EBOV antisera	2 mo; 3 wk	Yes	Positive[d] (742)	<8	ND	ND
214755	ERB	EBOV antisera	2 mo; 3 wk	Yes	Positive[d] (708)	<8	ND	ND
214838	ERB	EBOV antisera	2 mo; 3 wk	Yes	Positive[d] (677)	<8; DE	<8	<8
220406	ERB	EBOV antisera	2 mo; 3 wk	Yes	Positive[d] (624)	<8	ND	ND
369437	ERB	EBOV antisera	2 mo; 3 wk	Yes	Positive[d] (772)	<8	ND	ND
421936	ERB	EBOV antisera	2 mo; 3 wk	Yes	Positive[d] (422)	<8	ND	ND
441204	ERB	EBOV antisera	2 mo; 3 wk	Yes	Positive[d] (372)	<8	ND	ND
442221	ERB	EBOV antisera	2 mo; 3 wk	Yes	Positive[d] (180)	<8	ND	ND
444308	ERB	EBOV antisera	2 mo; 3 wk	Yes	Positive[d] (24)	<8	ND	ND

Identifier	Origin	Sample Type	Time of Boost; Time of Collection[a]	Gamma-Irradiated	IgG Indirect ELISA Result (% Above Cutoff[b])	$FRNT_{100}$	$FRNT_{50}$	$FRNT_{50}$ + GPC
444345	ERB	EBOV antisera	2 mo; 3 wk	Yes	Positive[d] (516)	<8	ND	ND
546240	ERB	EBOV antisera	2 mo; 3 wk	Yes	Positive[d] (716)	<8; DE	<8	<8
547881	ERB	EBOV antisera	2 mo; 3 wk	Yes	Positive[d] (345)	<8	ND	ND
652967	ERB	EBOV antisera	2 mo; 3 wk	Yes	Positive[d] (328)	<8	ND	ND
656745	ERB	EBOV antisera	2 mo; 3 wk	Yes	Positive[d] (541)	<8	ND	ND
686263	ERB	EBOV antisera	2 mo; 3 wk	Yes	Positive[d] (987)	<8	ND	ND
691073	ERB	EBOV antisera	2 mo; 3 wk	Yes	Positive[d] (726)	<8; DE	<8	<8
720908	ERB	EBOV antisera	2 mo; 3 wk	Yes	Positive[d] (437)	<8	ND	ND
721106	ERB	EBOV antisera	2 mo; 3 wk	Yes	Positive[d] (428)	<8	ND	ND
721253	ERB	EBOV antisera	2 mo; 3 wk	Yes	Positive[d] (764)	<8; DE	<8	<8
726214	ERB	EBOV antisera	2 mo; 3 wk	Yes	Positive[d] (469)	<8	ND	ND
NA	Human	SOSV antisera	Unknown	No	Positive[e] (high IgM/IgG)	800	8192	32 768
726415	ERB	Naive plasma	NA	No	Negative[e] (−130)	<8	<8	<8
725904	ERB	Naive plasma	NA	No	Negative[e] (−110)	<8	<8	<8
289338	ERB	SOSV antiplasma	NA, 3 wk	No	Positive[e] (33)	<8	ND	ND
289953	ERB	SOSV antiplasma	NA, 3 wk	No	Positive[e] (503)	<8; DE	<8	16
290040	ERB	SOSV antiplasma	NA, 3 wk	No	Positive[e] (320)	<8; DE	<8	32

Abbreviations: DE, dilution effect; EBOV, Ebola virus; ERB, Egyptian rousette bat; ELISA, enzyme-linked immunosorbent assay; $FRNT_{100}$, 100% focus reduction neutralization test; $FRNT_{50}$, 50% focus reduction neutralization test; GPC, guinea pig complement; IgG, immunoglobulin G; IgM, immunoglobulin M; MARV, Marburg virus; NA, not applicable; ND, not done; NHP, nonhuman primate; SOSV, Sosuga virus.

[a] "Time of boost" denotes the time from initial virus exposure to virus boost; "time of collection" denotes the time from virus boost to plasma/sera specimen collection.

[b] MARV recombinant nucleoprotein.

[c] MARV infectious-based lysate (200704852 Uganda Bat).

[d] EBOV infectious-based lysate (Mayinga).

[e] SOSV infectious-based lysate.