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Vesicular stomatitis virus-based vaccines protect nonhuman primates against aerosol challenge with Ebola and Marburg viruses

Thomas W. Geisbert^{a,b,c,d,e,*}, Kathleen M. Daddario-DiCaprio^{d,e}, Joan B. Geisbert^{a,e}, Douglas S. Reed^f, Friederike Feldmann^g, Allen Grolla^g, Ute Ströher^{g,h}, Elizabeth A. Fritz^e, Lisa E. Hensley^e, Steven M. Jones^{g,h,i}, and Heinz Feldmann^{g,h,1}

^aNational Emerging Infectious Diseases Laboratories Institute, Boston University School of Medicine, 715 Albany Street, Boston, MA, USA

^bDepartment of Microbiology, Boston University School of Medicine, 715 Albany Street, Boston, MA, USA

^cDepartment of Medicine, Boston University School of Medicine, 715 Albany Street, Boston, MA, USA

^dDepartment of Pathology, Uniformed Services University of the Health Sciences, Bethesda, MD, USA

^eVirology Division, U.S. Army Medical Research Institute for Infectious Diseases, Fort Detrick, Frederick, MD, USA

^fCenter for Vaccine Research, University of Pittsburgh, Pittsburgh, PA, USA

^gSpecial Pathogens Program, National Microbiology Laboratory, Public Health Agency of Canada, 1015 Arlington Street, Winnipeg, Manitoba, Canada

^hDepartment of Medical Microbiology, University of Manitoba, 730 William Avenue, Winnipeg, Manitoba, Canada

ⁱDepartment of Immunology, University of Manitoba, 730 William Avenue, Winnipeg, Manitoba, Canada

Abstract

Considerable progress has been made over the last decade in developing candidate preventive vaccines that can protect nonhuman primates against Ebola and Marburg viruses. A vaccine based on recombinant vesicular stomatitis virus (VSV) seems to be particularly robust as it can also confer protection when administered as a postexposure treatment. While filoviruses are not thought to be transmitted by aerosol in nature the inhalation route is among the most likely portals of entry in the setting of a bioterrorist event. At present, all candidate filoviral vaccines have been evaluated against parenteral challenges but none have been tested against an aerosol exposure. Here, we evaluated our recombinant VSV-based *Zaire ebolavirus* (ZEBOV) and Marburg virus (MARV) vaccines against aerosol challenge in cynomolgus macaques. All monkeys vaccinated with a VSV vector expressing the glycoprotein of ZEBOV were completely protected against an aerosol exposure of ZEBOV. Likewise, all monkeys vaccinated with a VSV vector expressing the glycoprotein of MARV were completely protected against an aerosol exposure of MARV. All

*Corresponding author at: Department of Microbiology, Boston University School of Medicine, 715 Albany Street, R514, Boston, MA 02118, USA Tel.: +1 617 638 4274; fax: +1 617 638 4286. geisbert@bu.edu (T.W. Geisbert).

¹Current address: Laboratory of Virology, Division of Intramural Research, National Institutes of Allergy and Infectious Diseases, Rocky Mountain Laboratories, 903 South 4th Street, Hamilton, MT 59840.

control animals challenged by the aerosol route with either ZEBOV or MARV succumbed. Interestingly, disease in control animals appeared to progress slower than previously seen in macaques exposed to comparable doses by intramuscular injection.

Keywords

Ebola virus; Marburg virus; Filovirus; Nonhuman primates; Aerosol; Vaccines

1. Introduction

Members of the family Filoviridae, Ebola virus (EBOV) and Marburg virus (MARV), cause severe hemorrhagic fever (HF) in humans and nonhuman primates. The *Marburgvirus* genus contains a single species: *Lake Victoria marburgvirus* (LVMARV). The *Ebolavirus* genus is subdivided into four distinct species: *Ivory Coast ebolavirus* (ICEBOV) (also known as *Cote d'Ivoire ebolavirus*, CIEBOV), *Reston ebolavirus* (REBOV), *Sudan ebolavirus* (SEBOV), and *Zaire ebolavirus* (ZEBOV) [1,2]. MARV, ZEBOV, and SEBOV are important human pathogens with case fatality rates frequently ranging between 70% and nearly 90% for ZEBOV, around 50% for SEBOV, and up to 90% for MARV outbreaks depending on the strain of MARV (reviewed in Ref. [2]). Currently, there are no vaccines or postexposure treatment modalities available for preventing or managing filoviral infections. However, remarkable progress has been made over the last few years in developing candidate preventive vaccines that can completely protect nonhuman primates against EBOV and MARV [3–11]. Among the most promising vaccines is a system based on recombinant vesicular stomatitis virus (VSV) which not only can protect nonhuman primates against EBOV and MARV when used as a single injection preventive vaccine but astonishingly showed 50–100% efficacy when employed as a postexposure treatment [12–14].

Little is known regarding how filoviruses are maintained in nature. All human outbreaks to date have been traced to Central Africa [2]. EBOV has decimated populations of wild apes in this same region [15]; however, apes and other nonhuman primates that have been associated with filoviral outbreaks are reservoir-incompetent species and like humans are accidental hosts [16]. Recent work has shown that bats may serve as a reservoir species for filoviruses [17,18] but it remains unclear whether other species are involved or exactly how transmission to humans and/or apes occurs. Once an individual is exposed to EBOV or MARV person-to-person transmission occurs by direct contact with blood or bodily fluids (saliva, sweat, stool, urine, tears, and breast milk) of an infected patient during the acute phase of illness [2,19]. Care-givers both at home and in hospitals are among populations at greatest risk. While studies have shown that EBOV and MARV can be spread through airborne particles/aerosols under controlled laboratory conditions [20–23], this type of spread rarely occurs among humans in a hospital or household setting during outbreaks.

Despite the relatively low and localized global occurrence of cases and the fact that transmission in nature is primarily by contact exposure, the filoviruses may be exploitable as agents of bioterrorism since they are highly infectious by the aerosol route, produce high morbidity and mortality in primates, and can be readily propagated *in vitro*. Indeed, the filoviruses have been classified as Category A bioterrorism agents by the Centers for Disease Control and Prevention [24]. When planning defenses against biological warfare agents such as filoviruses, it is important to consider that the inhalation route is the most likely portal of entry for agents disseminated as aerosols [25]. Moreover, it is known that the former Soviet Union experimented with aerosolized EBOV and MARV [26,27]. However, all previous vaccine candidates have been evaluated against peripheral or intraperitoneal filovirus injections while no vaccine has been tested against an aerosol challenge in non-

human primates. Here, we used cynomolgus macaque models of filoviral hemorrhagic HF to test the ability of our recombinant VSV-based ZEBOV and MARV vaccines to protect against homologous ZEBOV and MARV aerosol exposures, respectively.

2. Materials and methods

2.1. Vaccine vectors and viruses

The recombinant VSVs expressing either the glycoprotein (GP) of ZEBOV (VSV Δ G/ZEBOVGP) or MARV (Musoke strain) (VSV Δ G/MARVGP) were generated as described recently using the infectious clone for the VSV, Indiana serotype [28]. ZEBOV (strain Kikwit) was isolated from a patient of the ZEBOV outbreak in Kikwit in Ref. [29] while the Musoke strain of MARV was isolated from a human case in 1980 in Kenya [30].

2.2. Animal studies

Twelve healthy, filovirus-seronegative male cynomolgus macaques (*Macaca fascicularis*) (5–9 kg) were used for these studies. For the ZEBOV portion of this study, three animals were vaccinated by intramuscular (i.m.) injection with $\sim 2 \times 10^7$ pfu of VSV Δ G/ZEBOVGP (Subject 1, Subject 2, and Subject 3) and three animals were vaccinated i.m. with $\sim 2 \times 10^7$ pfu of VSV Δ G/MARVGP (Control 1, Control 2, and Control 3). The six cynomolgus macaques were challenged 28 days after the single dose vaccination by aerosol exposure to a target dose of 1000 pfu of ZEBOV. Before aerosol exposures a body plethysmograph was taken of each animal for 3 min to determine the animal's respiratory capacity. Each macaque was then inserted into a Class III biological safety cabinet and exposed in a head-only aerosol chamber to a ZEBOV aerosol created by a Collison nebulizer controlled by an automated bioaerosol exposure system for 10 min as previously described [31,32]. For the MARV portion of this study, four animals were immunized i.m. with $\sim 2 \times 10^7$ pfu of VSV Δ G/MARVGP (Subject 4, Subject 5, Subject 6, and Subject 7) and two animals with $\sim 2 \times 10^7$ pfu of VSV Δ G/ZEBOVGP (Control 4 and Control 5). These six macaques were challenged 28 days after the single dose vaccination by aerosol exposure to 1000 pfu of MARV (Musoke strain) in the same manner as described above for ZEBOV.

Swab samples (oral, nasal, rectal) and/or blood were taken before vaccination (day –28) and at days 2 (day –26), 14 (day –14), and 27 (day –1) after vaccination and at days 3, 6, 10, 14, and 28 after the ZEBOV or MARV challenges. Animal studies were performed in BSL-4 biocontainment at USAMRIID and were approved by the USAMRIID Laboratory Animal Care and Use Committee. Animal research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to the principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facility used is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.3. Hematology and serum biochemistry

Total white blood cell counts, white blood cell differentials, red blood cell counts, platelet counts, hematocrit values, total hemoglobin, mean cell volume, mean corpuscular volume, and mean corpuscular hemoglobin concentration were determined from blood samples collected in tubes containing EDTA, by using a laser-based hematologic Analyzer (Coulter Electronics, Hialeah, FL, USA). The white blood cell differentials were performed manually on Wright-stained blood smears. Serum samples were tested for concentrations of albumin (ALB), amylase (AMY), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), glucose (GLU), cholesterol (CHOL), total protein (TP), total bilirubin (TBIL), blood urea nitrogen (BUN),

and creatinine (CRE) by using a Piccolo Point-Of-Care Blood Analyzer (Abaxis, Sunnyvale, CA, USA).

2.4. Virus detection

RNA was isolated from blood and swabs using appropriate RNA isolation kits (QIAGEN, Mississauga, ON, Canada). For the detection of VSV we used a RT-PCR assay targeting the matrix gene (nt position 2355–2661, NC 001560). ZEBOV and MARV RNA were detected using primer pairs targeting the L genes (ZEBOV (AF 272001): RT-PCR: nt position 13344–13622; nested PCR-nt position 13397–13590; MARV (X 68494): RT-PCR-nt position 1966–2243; nested PCR-nt position 2017–2213). The low detection limit for these ZEBOV and MARV assays is 0.1 pfu/ml of plasma. Virus titration was performed by plaque assay on Vero E6 cells from all blood and selected organ (adrenal, testis, lymph nodes, liver, spleen, kidney, pancreas, lung, brain, and bone marrow) and swab samples. Briefly, increasing 10-fold dilutions of the samples were adsorbed to Vero E6 monolayers in duplicate wells (0.2 ml per well); thus, the limit for detection was 25 pfu/ml.

2.5. Humoral and cellular immune responses

IgG antibodies against ZEBOV and MARV were detected with an enzyme-linked immunosorbent assay (ELISA) using purified virus particles as an antigen source [5,6]. CD4 and CD8 T-cell responses to ZEBOV and MARV were evaluated by assessing the intracellular production of IFN- γ and TNF- α by flow cytometry as previously described [5,6].

3. Results

3.1. Clinical observations

We employed twelve cynomolgus macaques, of which five animals were immunized by i.m. injection with a single dose of VSV Δ G/ZEBOVGP (Subjects #1–3 and Controls #4–5) and the remaining seven with a single dose of VSV Δ G/MARVGP (Subjects # 4–7 and Controls #1–3). The animals were monitored closely for clinical symptoms and shedding of recombinant VSVs. Following vaccination none of the animals showed any signs of clinical symptoms indicating that the recombinant VSVs are apathogenic for these animals.

Animals were challenged 28 days after the single dose vaccine by aerosol exposure with either ZEBOV (Subjects #1–3 and Controls #1–3) or MARV (Subjects #4–7 and Controls #4–5). The three VSV Δ G/MARVGP-immunized animals (Controls #1–3), which served as controls in the ZEBOV challenge, started to show clinical signs of disease on day 6 after challenge including lymphopenia, thrombocytopenia, elevated levels of serum enzymes associated with liver function, depression, anorexia, and the appearance of macular rashes. The development of the changes in behavior and appearance occurred about one day later than previously reported for cynomolgous macaques challenged with the same ZEBOV seed and dose by i.m. injection [33]. Nonetheless, the animals succumbed to the ZEBOV infection on days 6, 7, and 8, respectively (Fig. 1A). In contrast, none of the three VSV Δ G/ZEBOVGP-immunized macaques became sick from the ZEBOV challenge and all three animals were fully protected.

The first sign of disease in the VSV Δ G/ZEBOVGP-immunized animals (Controls #4–5), which served as controls for the MARV challenge, occurred on day 6 after MARV challenge when one animal (Control #4) presented with a small macular rash on one arm. Both of these animals showed reduced activity by day 9 and both were depressed and anorexic. By day 10, both animals had macular rashes and other evidence of clinical illness including lymphopenia, thrombocytopenia, and elevated levels of serum enzymes associated with liver

function. As was noted with the ZEBOV control animals, the development of the changes in behavior and appearance occurred about 1–2 days later than previously observed for cynomolgous macaques challenged with the same MARV seed and dose by i.m. injection (6, TW Geisbert, unpublished observation). Nevertheless, these animals succumbed to the MARV infection on days 10 and 13, respectively (Fig. 1B). In contrast, none of the four VSVΔG/MARVGP-immunized macaques became sick and all four animals were fully protected against the MARV aerosol challenge. None of the protected animals in either challenge experiment displayed any clinical signs or visual symptoms of EBOV or MARV disease.

3.2. Viremia

To determine whether viremia or shedding of the recombinant VSVs occurred after immunization whole blood and swab samples from all twelve of the vaccinated animals were analyzed by RT-PCR and virus isolation. A transient and low level ($3.3 \log_{10}$ pfu/ml) recombinant VSV viremia was detected by virus isolation only at day 2 after vaccination in plasma from four of the VSVΔG/ZEBOVGP-vaccinated animals (Subjects #1–3 and Control #1). We did not detect recombinant VSV in swab samples of any animal from this study.

Blood samples were also analyzed after ZEBOV or MARV challenge for evidence of filoviral replication by plaque assay and RT-PCR. By day 6, all three ZEBOV control animals (Control #1–3) developed high ZEBOV titers in plasma as detected by plaque assay ($>10^6 \log$ pfu/ml) (Fig. 2A). In comparison, one of the MARV control animals (Control #5) was viremic at day 6 by plaque assay ($\sim 10^3 \log$ pfu/ml) (Fig. 2B) and high titers of MARV were detected by plaque assay in both animals at day 10 ($>10^7 \log$ pfu/ml). RT-PCR was more sensitive and showed evidence of MARV in plasma of these two control animals by day 3 postinfection. In contrast, no ZEBOV or MARV was detected in plasma or swabs by virus isolation or RT-PCR in any of the animals vaccinated with either VSVΔG/ZEBOVGP or with VSVΔG/MARVGP and challenged with ZEBOV or MARV, respectively.

Organ infectivity titration of tissues from the control animals showed ZEBOV or MARV in nearly all tissues examined (Table 1). Interestingly, the tissue tropism in these aerosol-exposed animals appeared to be consistent with results obtained in animals exposed by i.m. injection (33, TW Geisbert, unpublished observation). Notably, viral loads obtained from lungs of aerosol-exposed macaques were no greater than viral loads obtained from lungs of macaques exposed by i.m. injection whereas liver (MARV) and spleen and adrenal gland (ZEBOV and MARV) were primary target organs regardless of route of exposure.

3.3. Evaluation of antibody and cellular immune responses

The antibody responses of the cynomolgus macaques vaccinated with VSVΔG/ZEBOVGP or VSVΔG/MARVGP were evaluated after vaccination (days –14 and 0) and after ZEBOV or MARV challenge (day 28) by IgG ELISA. By the day of ZEBOV challenge (day 0) all VSVΔG/ZEBOVGP-immunized animals had developed modest IgG antibody titers against ZEBOV GP (Fig. 3A). These titers increased after the ZEBOV challenge. By the day of MARV challenge (day 0) all VSVΔG/MARVGP-immunized animals had developed modest IgG antibody titers against MARV GP (Fig. 3B). These titers increased slightly in two of the four animals after the MARV challenge.

To better understand the cellular responses of T-cell populations found in peripheral blood mononuclear cell fractions of specifically and nonspecifically vaccinated animals in mediating protection against ZEBOV or MARV challenge, flow cytometry was employed during the course of study. There was no evidence of either IFN- γ or TNF- α production in

CD4 or CD8 T-cell populations either before or after the ZEBOV or MARV challenges in any of the animals employed in this study (data not shown).

4. Discussion

Few studies have evaluated the pathogenic potential of filoviruses in animals exposed by the aerosol route [20–23]. Infection of nonhuman primates after aerosol exposure to ZEBOV has been reported and was uniformly lethal in both studies [21,23]. With regard to MARV, aerosol exposure was also shown to be lethal [20,22]. However, in one study in African green monkeys there appeared to be a reduced lethality associated with aerosol exposure to MARV [20]. Despite an overall paucity of information on aerosol exposure in animals, the requirements for an EBOV or MARV vaccine suitable for military use include protection against aerosol exposure. In this study we show that our recombinant VSV-based ZEBOV and MARV vaccines completely protect cynomolgus macaques against lethal challenge with either homologous ZEBOV or MARV, respectively. This is the first demonstration of protection against an aerosol challenge with any filovirus vaccine candidate as all previous work in nonhuman primates has been done against either i.m. [3,5–11] or intraperitoneal challenge routes [4].

Consistent with the previous study suggesting that an aerosol exposure to MARV may be less pathogenic than a challenge by i.m. injection [20], our results showed that disease in cynomolgus monkeys appeared to be protracted for aerosol-exposed macaques with animals succumbing on days 10 and 13 after MARV challenge. Our previous studies using the same seed stock and dose of MARV (Musoke strain) administered to cynomolgus macaques by i.m. injection produces a uniformly lethal infection with animals succumbing primarily on day 9 (mean = 9.3, range = 9–10, TW Geis-bert, unpublished observation). While there were only two control animals exposed to MARV by the aerosol route in the current study the animal that died on day 13 was clearly delayed when compared with historical control cynomolgus macaques exposed by i.m. injection. We did not see any clear delay in time to death of the three cynomolgus monkeys exposed to ZEBOV by aerosol when compared to animals exposed by i.m. injection. Animals exposed to ZEBOV by aerosol in this study succumbed on days 6, 7, and 8, respectively compared to a mean time to death of 6.4 days (range 5–8) for animals exposed to the same virus and dose by i.m. injection [2]. Although the animal that succumbed to ZEBOV on day 8 is at the far end of the window for time to death and the disease did appear to progress slower in all three animals when looking at other clinical parameters such development of lymphopenia and thrombocytopenia, elevation in levels of enzymes associated with liver function, and the development of a macular rash.

Clearly, further studies are needed to characterize the pathogenesis of filoviruses in animals exposed by the aerosol route. However, based on our findings in the current study it appears that aerosol exposure to a filovirus may be no more difficult to protect against than a parenteral exposure. This is not surprising as the lung is not a primary target organ for filoviruses in primates [33]. Even in the current study where macaques were exposed to ZEBOV or MARV by aerosol, the lung appeared to be minimally involved compared with other organs such as liver and spleen. These results support the view that monocytes/macrophages and other antigen-presenting cells are initial sites of filovirus infection [33,34] and these cells migrate and rapidly disseminate the virus throughout the host where the virus replicates extensively and uncontrollably in preferred target organs. Thus, it appears that regardless of the portal of entry the overall pathogenesis is similar and the primary difference if any is in the length of the disease course. These results are also consistent with a study where rhesus macaques were exposed to ZEBOV by oral or conjunctival routes yet the disease pathology was indistinguishable from animals exposed by i.m. injection [35].

The fact that i.m. injection appears to produce a very rapid disease course is also consistent with human data obtained from outbreaks. Specifically, the mean incubation period for cases of ZEBOV from the 1976 outbreak known to be due to injection resulting from the reuse of needles was 6.3 days versus 9.5 days for contact exposures [36]. Moreover, the case fatality rate in this outbreak was 100% (85 of 85) in cases associated with injection compared to ~80% (119 of 149) in cases of known contact exposure [36].

In developing vaccines against any virus, there are always concerns about broad protection and the ability to protect against different species, strains, or isolates of the same virus. Here, we demonstrated that our VSV-based vaccines protected nonhuman primates against homologous aerosol challenges with either ZEBOV or the Musoke strain of MARV, respectively. We have previously shown that our VSVΔG/MARVGP preventive vaccine expressing the Musoke strain GP can completely protect nonhuman primates against heterologous peripheral challenge with either the seemingly more pathogenic Angola strain or the more distantly related Ravn strain of MARV [7]. We have also shown that a vaccine that can protect against different species of EBOV will require inclusion of antigens specific for each distinct species [6]. While most EBOV vaccine studies have focused on ZEBOV, we recently showed that a VSVΔG/SEBOVGP vaccine completely protected macaques against a homologous SEBOV peripheral challenge when administered as a postexposure treatment [14]. While aerosol challenge studies will be needed to confirm the results of these peripheral challenge studies, the results from the current aerosol studies suggest that the VSVΔG/MARVGP vaccine based on the Musoke strain of MARV will be able to protect against aerosol challenge with other MARV strains and that any EBOV vaccine will likely require inclusion of species-specific antigens.

Regarding the mechanism by which the VSVΔG/ZEBOVGP and VSVΔG/MARVGP vaccines confer protection, as in our previous studies both vaccines appeared to be associated with a strong humoral immune response [6,7]. However, for EBOV it is likely that a robust humoral immune response while possibly necessary is insufficient to confer protection. Specifically, passive antibody studies in nonhuman primates using a variety of anti-ZEBOV immune reagents including polyclonal equine immune globulin [29], a recombinant human monoclonal antibody [37], and convalescent monkey blood [38] have uniformly failed to provide protection and more importantly have failed to provide any beneficial effect. In this study and in previous studies [6,7] we failed to detect a cellular immune response against MARV. In a previous study we observed a cellular immune response against ZEBOV [6] whereas in this study we did not observe a cellular response against ZEBOV. However, as has been reported by others our findings illustrate the limitation of the evaluation of cellular immune responses using small numbers of functional measurements (such as interferon-gamma) [39]. It is evident that the T cell cytokine profile generated in response to an immunogen can be rather complex. In addition, even with successful cellular responses, the frequency of antigen-specific T cells can still be quite low (typically less than 0.1% of lymphocytes) [40,41]. It is likely that the intracellular cytokine assay that we have employed in the current study is not sensitive or thorough enough to detect a cellular immune response against ZEBOV or MARV. Future studies will need to develop a more sensitive and systematic evaluation of antigen-specific T cells which will require the coordinate measurement of several cytokines across different populations of T cells which is now possible using multicolor flow cytometry assays (e.g., 18-color flow cytometry).

The primary objective of the current study was to determine whether our VSV-based ZEBOV and MARV vaccines could completely protect nonhuman primates against an aerosol challenge. Our results unequivocally show that both of these VSV-based vaccines can confer complete protection against either i.m. [6,7] or aerosol challenges (this study) of

high doses of infectious filoviruses. It is likely that these results will have importance for other vaccine systems. However, because the host immune response elicited by different vaccine platforms could be different it is not possible to say with absolute certainty that the results shown here for the VSV-based filovirus vaccine system will hold true for all filovirus vaccine candidates and each will therefore need to be evaluated independently.

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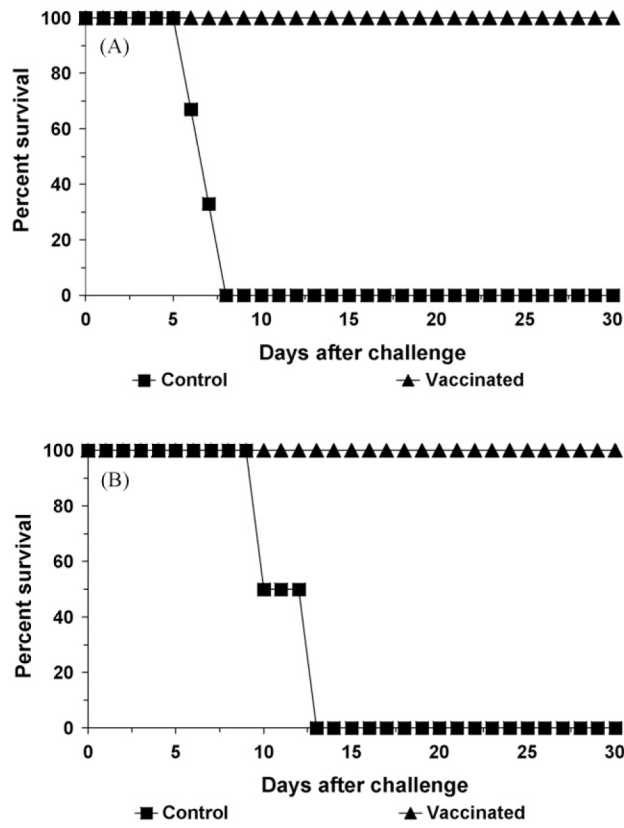


Fig. 1. Kaplan–Meier survival curves for cynomolgus macaques vaccinated against ZEBOV or MARV and challenged against ZEBOV (A) or MARV (B).

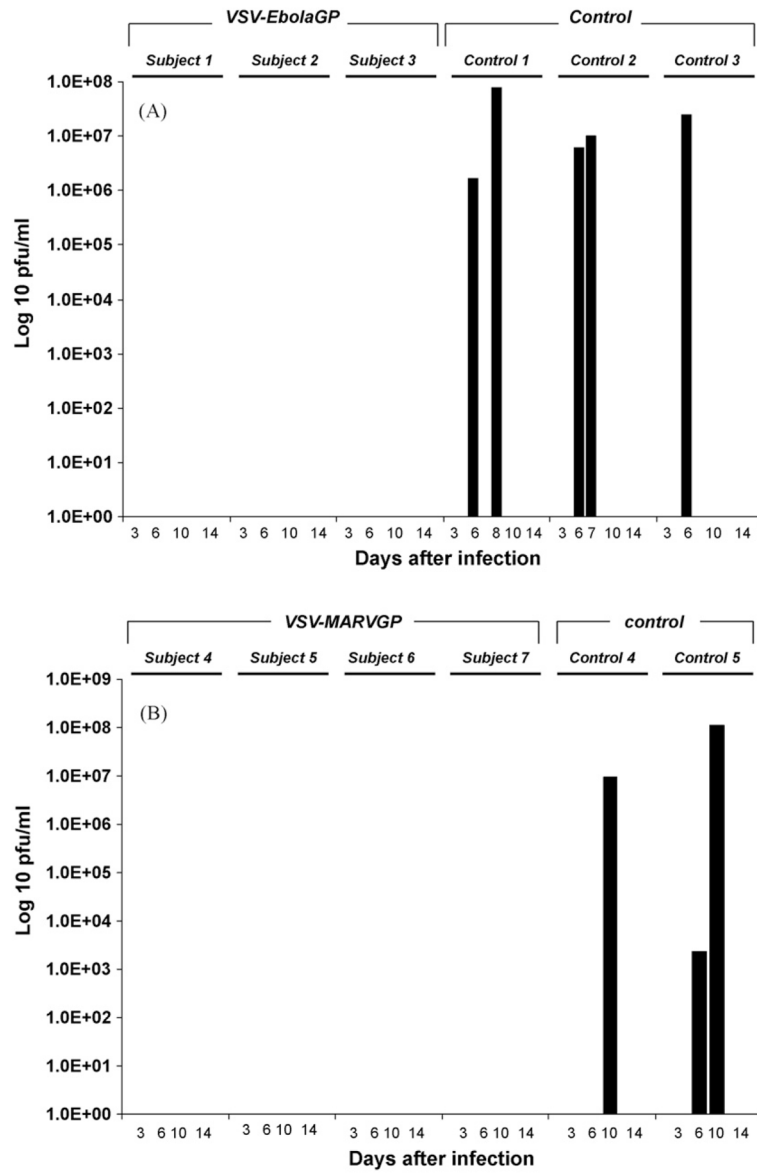


Fig. 2. (A) Plasma levels of ZEBOV (pfu/ml) from cynomolgus macaques challenged with ZEBOV. (B) Plasma levels of MARV (pfu/ml) from cynomolgus macaques challenged with MARV.

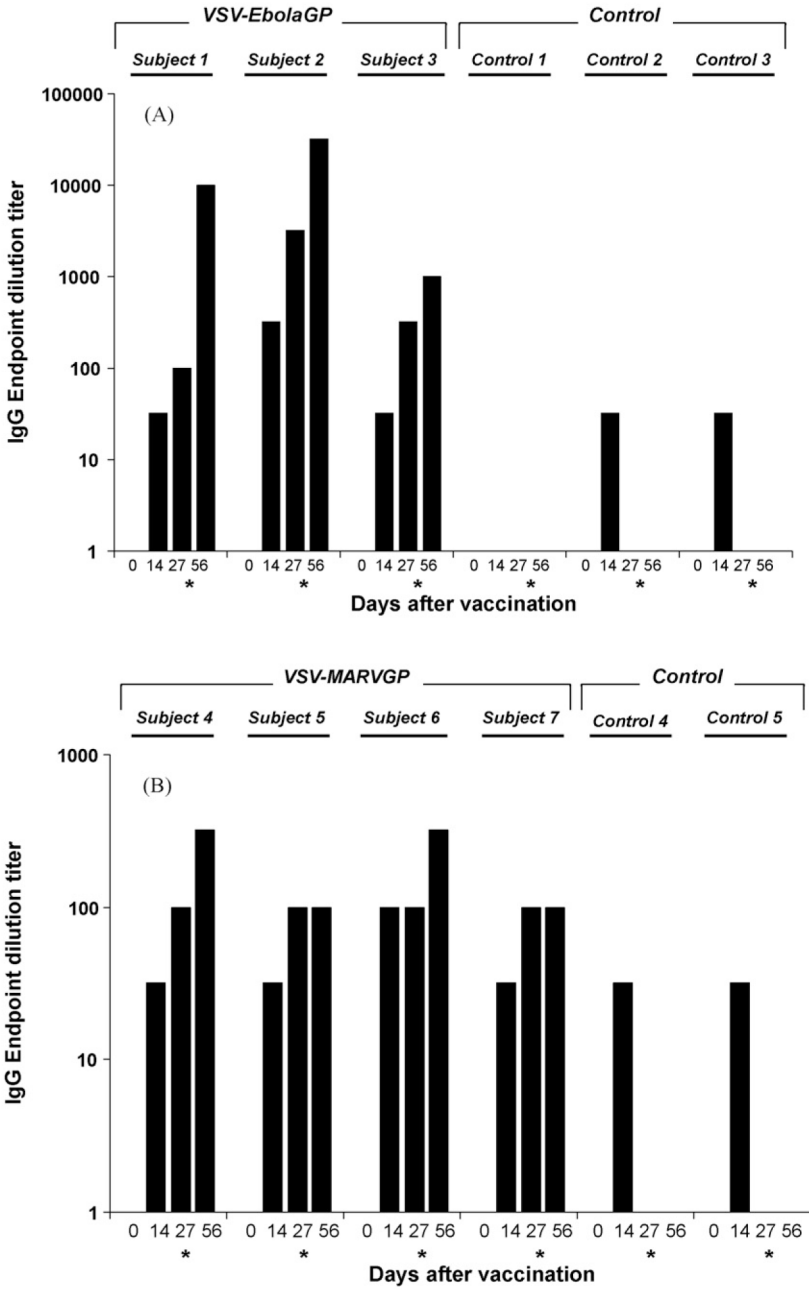


Fig. 3. Circulating levels of IgG against ZEBOV (A) or MARV (B) from cynomolgus macaques challenged with either ZEBOV (A) or MARV (B). (*) Day of filovirus challenge (28 days after single injection vaccination).

Table 1

Organ infectivity titers.

Animal no.	Virus	Day of death	LVR	SPL	LNG	KID	ADR	PAN	AxLN	InLN	MsLN	BRN	TST	BM
Cont 1	ZEBOV	8	6.31 ^a	7.41	6.84	6.47	7.95	4.08	6.84	6.68	6.97	4.68	6.22	7.03
Cont 2	ZEBOV	7	6.63	5.84	6.11	5.57	5.72	3.10	6.78	6.90	6.89	3.65	4.99	4.78
Cont 3	ZEBOV	6	6.24	7.23	6.35	4.87	6.09	0.00	5.94	6.18	6.70	4.78	5.51	5.44
Cont 4	MARV	13	8.27	7.14	6.08	5.83	5.92	7.40	7.38	7.65	7.26	6.76	6.60	3.90
Cont 5	MARV	10	9.01	7.99	6.63	6.17	7.44	5.72	7.76	7.35	7.35	5.03	6.06	7.54

LVR, Liver; SPL, Spleen; LNG, Lung; KID, Kidney; ADR, Adrenal gland; PAN, Pancreas; AxLN, Axillary lymph node; InLN, Inguinal lymph node; MsLN, Mesenteric lymph node; BRN, Brain; TST, Testis; BM, Bone marrow.

^aLog₁₀ pfu/g of tissue.