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Vaccine To Confer to Nonhuman Primates Complete Protection against Multistrain Ebola and Marburg Virus Infections▽

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Filoviruses (Ebola and Marburg viruses) are among the deadliest viruses known to mankind, with mortality rates nearing 90%. These pathogens are highly infectious through contact with infected body fluids and can be easily aerosolized. Additionally, there are currently no licensed vaccines available to prevent filovirus outbreaks. Their high mortality rates and infectious capabilities when aerosolized and the lack of licensed vaccines available to prevent such infectious make Ebola and Marburg viruses serious bioterrorism threats, placing them both on the category A list of bioterrorism agents. Here we describe a panfilovirus vaccine based on a complex adenovirus (CAdVax) technology that expresses multiple antigens from five different filoviruses de novo. Vaccination of nonhuman primates demonstrated 100% protection against infection by two species of Ebola virus and three Marburg virus subtypes, each administered at 1,000 times the lethal dose. This study indicates the feasibility of vaccination against all current filovirus threats in the event of natural hemorrhagic fever outbreak or biological attack.

Ebola virus (EBOV) and Marburg virus (MARV) are members of the virus family *Filoviridae* and are both classified as category A bioterrorism threats for several reasons. First, the filoviruses are highly lethal, causing severe hemorrhagic fever disease in humans and apes with high mortality rates (up to 90%). The recent description of massive gorilla and chimpanzee die-offs in the Democratic Republic of the Congo due to an EBOV outbreak serves as an unfortunate testament to the deadly nature of filovirus infections in primates (3). Second, in addition to being extremely pathogenic, filoviruses are highly infectious as aerosol droplets (23). Both EBOV and MARV aerosol infections are possible and are proven to be lethal in nonhuman primates (NHP). Jaax et al. first reported on the unintentional aerosol transmission of EBOV infection to a control NHP in a biocontainment facility (17), which was later demonstrated experimentally (18). Additionally, there is anecdotal evidence that the former Soviet Union explored the use of aerosolized MARV as a potential biowarfare agent in an offensive weapons program (1). Although filoviruses have been widely studied in recent years, there are no licensed filovirus vaccines currently available to prevent the spread of an outbreak or reduce its severity. Collectively, these facts make filoviruses a high priority on the U.S. government's list of biological threats.

Recently, a great amount of effort has been placed on developing safe and effective filovirus vaccines. However, despite these great efforts, there is still no licensed vaccine to counter filovirus outbreaks. Some of the difficulties imposed on developing such a vaccine are highlighted in the taxonomy of this family of viruses (9a). The family *Flaviviridae* is divided into two genera: *Ebolavirus* and *Marburgvirus*. The genus *Ebolavirus* is further divided into four species, *Zaire ebolavirus* (ZEBOV), *Ivory Coast ebolavirus*, *Sudan ebolavirus* (SEBOV), and *Reston ebolavirus*. The *Marburgvirus* genus, however, is considered to be represented by a single species (*Lake Victoria marburgvirus*). This taxonomic classification is partially based on sequence and serological differences in the glycoprotein (GP) molecule. Filovirus GP is the only surface protein of these viruses and is thus the most probable target of protective immune responses and vaccine development. Vaccine development difficulties stem from the divergence between the species (the amino acid sequence of GPs from ZEBOV and SEBOV share only about 50% sequence homology). However, even for MARV, where all strains and isolates are considered a single species, there are substantial antigenic differences between some of them on the basis of evaluations with polyclonal and monoclonal antibodies. For example, the Musoke and Ravn strains differ by 22% in overall amino acid sequences of GPs and by over 50% in what is thought to be the antibody-binding region (13). These antigenic differences account for a lack of cross-protective immunity between filovirus species. In short, immunity against EBOV will not cross-protect against MARV, and vice versa. The same can even be said for immunity between ZEBOV and SEBOV species (20).

Neither *Reston ebolavirus* nor *Ivory Coast ebolavirus* has any documented mortality in humans, and they are not generally considered significant biological weapon threats. Therefore, the number of filoviruses that a multiagent filovirus vaccine would have to protect against is thought to be at least three, ZEBOV, SEBOV, and MARV, and it is possible that antigens from multiple strains of MARV may be needed to fully cover

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the virus family. The newly emerged fifth EBOV species in Uganda, which is yet to be named, will also likely be pursued as a vaccine target in the near future (http://www.who.int/csr/don/2007_12_07/en/index.html).

Traditional vaccine platforms such as live-attenuated and killed-virus vaccines are unlikely to be used in humans due to safety risks of underattenuation or incomplete inactivation. For example, vaccination of guinea pigs with an inactivated whole-virus MARV vaccine was lethal in 20% of vaccinated animals (15). Therefore, much progress has been made using alternative vaccine platforms, such as recombinant viral vectors. For example, alphavirus replicons expressing MARV GP are very effective for protection of both rodents and NHP from lethal MARV challenge (14, 15). However, the same approach for an EBOV vaccine was protective in rodents but did not protect NHP from lethal ZEBOV challenge (11). More-consistent results have been produced using a live-attenuated recombinant vesicular stomatitis virus (VSV) vector. NHP vaccinated with the VSVΔG vector expressing either ZEBOV GP or MARV GP were completely protected against homologous virus challenge (20), and the vector has also shown 50% and 100% postexposure efficacy in protection against ZEBOV and MARV challenge, respectively (8, 9). Adenovirus (Ad) vectors, the most widely studied viral vectors, have also shown promising results against filoviruses. First-generation Ad vectors expressing ZEBOV GP have demonstrated 100% protection in NHP against homologous virus challenge (35, 36). Additionally, we previously demonstrated that second-generation complex Ad vectors expressing multiple filovirus GP antigens can provide complete protection of rodents against ZEBOV (39) or MARV challenge (40).

In the event of a filovirus outbreak or biological attack, the identity of the species or strain of filovirus will not be immediately known. Therefore, the ideal filovirus vaccine would be able to protect against all relevant subtypes in such a situation. We have initiated efforts to develop a panfilovirus vaccine using our complex Ad-based vaccine (CAdVax) vector, which allows the incorporation of multiple genes into a single vaccine component (40). Genome size restrictions of other vaccine vectors, such as the successful VSVΔG and first-generation Ad vectors mentioned above, allow the vectors to accommodate only a single filovirus GP gene. Therefore, a vaccine against multiple filovirus strains utilizing either of the aforementioned platforms would require a pool of individual vectors, one for each filovirus antigen. In contrast, the CAdVax platform offers the advantage of multiantigen expression from each vector, reducing the overall number of components required for a panfilovirus vaccine.

In seeking the highest level of immune protection against all lethal filoviruses, we developed a panfilovirus vaccine that expresses the GP antigens of five different filoviruses covering all three significant species: ZEBOV, SEBOV, and MARV (Ci67, Ravn, and Musoke strains). The filovirus nucleoprotein (NP) is highly conserved among species, has been shown to induce effective cellular immune responses (44), and can enhance the efficacy of a GP-based vaccine (35). Therefore, since the CAdVax vaccine platform offers the advantage of multiantigen expression, we also included the NP genes of ZEBOV and MARV Musoke to maximize the breadth of immunity against the filoviruses. CAdVax vectors are able to accommodate large transgene inserts of up to 7 kilobases or six different genes, depending on the size of each respective gene. In order to ensure a balanced, high level of expression of each transgene, we included two filovirus genes per CAdVax vector. The final CAdVax-Panfilo vaccine formulation consisted of four vectors that cumulatively express five filovirus GP antigens and two filovirus NP antigens.

Vaccination of NHP with CAdVax-Panfilo was 100% protective against challenge with multiple filovirus species, including ZEBOV, SEBOV, MARV Musoke, and MARV Ci67. Additionally, all vaccinated animals survived rechallenge with a completely different species of filovirus. This study provides a strong proof of concept for a single vaccine against multiple filoviruses using the CAdVax platform.

MATERIALS AND METHODS

Viruses and vaccine components. The filoviruses used for challenge were from the U.S. Army Medical Research Institute of Infectious Diseases. MARV Musoke challenge stock (14) is a derivative (six passages in Vero 76 cells) of the virus originally isolated from a human case in Kenya in 1980 (34). The MARV Ci67 challenge stock is a variant obtained from Werner Slenczka and Stephan Becker, which was isolated in Marburg, Germany, during the first MARV outbreak (33) and has the same GP amino acid sequence as the more familiar Popp strain obtained in Frankfurt during the same outbreak. The ZEBOV challenge stock is the Kikwit strain and was provided by Peter Jahrling (10). The SEBOV challenge stock is the Boniface strain from the 1976 outbreak (45) and was provided by Thomas Geisbert. The Ravn strain of MARV was isolated from a fatal human case in 1987 in Kenya (19) and was used to evaluate immune responses.

The four CAdVax vaccine components used were generated as described recently (40, 41). The four vectors and the transgenes they expressed were as follows: EBO2, two copies of the ZEBOV NP gene; EBO7, SEBOV GP and ZEBOV GP genes; M8, Ci67 GP and Ravn GP genes; M11, Musoke GP and Musoke NP genes. The NP gene sequences for EBO2 were derived from the Kikwit strain of ZEBOV (GenBank accession number AF054908). The GP gene sequences for EBO7 were derived from the Boniface strain of SEBOV and the Kikwit strain of ZEBOV (GenBank accession numbers U28134 and U28077, respectively). These genes were modified to delete the RNA editing signal responsible for initiating a secreted, nonstructural form of GP (41). The GP gene sequences for M8 were derived from the Ci67 and Ravn strains of MARV (GenBank accession numbers AF005735 and AF005734, respectively). The NP and GP gene sequences for M11 were derived from the Musoke strain of MARV (GenBank accession number Z12132). These genes were amplified by PCR, and the PCR fragments were subcloned into pLAd or pRAd plasmid shuttle vectors. Each vaccine component was constructed using these shuttle vectors as previously described (29–31, 40, 41) and is based on a modified Ad5sub360 genome, which contains deletions of the E1 and E3 open reading frames (ORF) and all of the E4 ORF with the exception of ORF6. The genomic DNA from the final vaccine components was confirmed by sequence analyses. The control vaccine vector, HC4, was a CAdVax-based hepatitis C vaccine vector.

Vaccine component propagation, confirmation, and titer determination. All vaccine components were propagated in HEK293 cells obtained from the American Type Culture Collection (Manassas, VA) using standard procedures (29–31). Briefly, HEK293 cells were transfected with CAdVax vector genomic DNA using Lipofectamine reagent (Invitrogen, Carlsbad, CA) by following the manufacturer's instructions. Transfected cells were maintained until Ad-related cytopathic effects were observed, at which point the cells were harvested. After several rounds of single-plaque selection, candidate clones were confirmed by restriction map digestion and complete sequencing analysis of the virus DNA isolated from plaques to assure that no deletions or rearrangements in the vaccine components had occurred. The final vaccine components and the control Ad vaccine vector, HC4, were reamplified in HEK293 cells and purified by ultracentrifugation in cesium chloride gradients as previously described (29). Briefly, adenoviral lysates from 30 150-mm plates were banded twice on CsCl gradients and desalted twice with PD-10 size exclusion columns (Amersham Scientific, Piscataway, NJ) into HEPES-buffered saline (21 mM HEPES, 140 mM NaCl, 5 mM KCl, 0.75 mM $Na_2HPO_4 \cdot 2H_2O$, and 0.1% [wt/vol] dextrose adjusted to pH 7.5 with NaOH and filter sterilized) containing 10% glycerol and stored in liquid N_2.

All vaccine components were titrated on HEK293 cells infected in serial

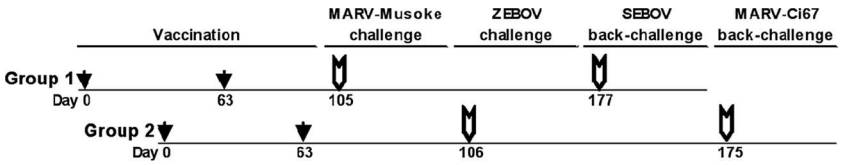

FIG. 1. Experimental design for vaccination and filovirus challenge. Cynomolgus macaques were divided into two groups of five per group, and each group was vaccinated on days 0 and 63 with CAdVax-Panfilo or a control CAdVax vector. Group 1 was challenged with 1,000 PFU of MARV Musoke on day 105, and group 2 was challenged with 1,000 PFU of ZEBOV on day 106. Group 1 was subsequently back-challenged with 1,000 PFU of SEBOV on day 177, and group 2 was back-challenged with 1,000 PFU of MARV Ci67 on day 175. Filled arrows, vaccination; open arrows, virus challenge.

dilution on triplicate columns of 12-well plates for PFU. The resulting titers were scored as PFU/ml. The respective transgene sequences of each final CAdVax vaccine vector (EBO2, EBO7, M8, and M11) were confirmed again with restriction map digestion. Protein expression from each vaccine component was confirmed by Western blotting, immunofluorescence assay, and determination of immunogenicity in mice as previously described (40, 41).

Animal studies. To evaluate the efficacy of our CAdVax-Panfilo vaccine, we designed a challenge experiment using two identical vaccine groups receiving either a MARV challenge followed by an EBOV challenge (group 1) or an EBOV challenge followed by a MARV challenge (group 2) (Fig. 1). Since multiple studies in the literature have indicated that EBOV immunity will not cross-protect against a MARV infection and vice versa (20, 37, 42, 43), the immune responses induced by the first challenge will have no bearing on an animal's survival after the second challenge. Thus, this experimental design allowed us to effectively analyze the vaccine's protective efficacy using a minimal number of NHP that received filovirus challenge.

Healthy feral 6- to 11-kg cynomolgus macaques (*Macaca fascicularis*) of Mauritius origin were obtained from Primate Products, Inc. (Miami, FL). For the first experiment, the macaques were divided into two vaccine groups and controls and were vaccinated and challenged in parallel with different viruses at the indicated times (see Fig. 1). This approach, although requiring significantly more biosafety level 4 laboratory space, is necessary to avoid the rechallenge of the NHP with viruses of the same species. The macaques in the vaccine groups (five per group) were anesthetized by intramuscular injection of ketamine HCl (10 mg/kg of body weight), followed by intramuscular vaccination with an equal mixture of 1×10^{10} PFU of each vaccine component: EBO2, EBO7, M8, and M11 (resulting in 4×10^{10} total PFU per animal). Control animals received 4×10^{10} PFU of the HC4 vaccine vector, also via the intramuscular route. Animals were vaccinated on day 0 and given a booster immunization of the same vaccine formulation on day 63. Fifteen weeks after vaccination (day 105/106), viral challenges were performed with the two groups of monkeys. Animals were anesthetized again as above, and group 1 was inoculated subcutaneously with MARV Musoke, while group 2 was inoculated intramuscularly with ZEBOV, using approximately 1,000 PFU of each filovirus. EBOV and MARV each have different established routes of administration (intramuscular and subcutaneous, respectively) in the NHP model of infection; this is the reason for the two different challenge routes of infection used in this study. Macaques were closely monitored for 28 days for signs of clinical disease. Ten weeks after the initial challenge (day 175/177), the animals in groups 1 and 2 were rechallenged with 1,000 PFU of a different filovirus (SEBOV and MARV Ci67, respectively) by the same respective route of administration.

All filovirus-infected animals were handled under maximum containment in an animal biosafety level 4 facility at the U.S. Army Medical Research Institute of Infectious Diseases. The research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the National Research Council's *Guide for the Care and Use of Laboratory Animals* (25). The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Hematology, blood biochemistry, and humoral immune responses. Phlebotomy was performed on the femoral vein using a venous blood collection system (Becton Dickinson, Franklin, NJ). Viremia was assayed by traditional plaque assay (24). Hematological values were determined from blood samples collected in tubes containing EDTA using a hematologic analyzer (Coulter Electronics, Hialeah, FL). Liver-associated enzymes were measured using a Piccolo point-of-care blood analyzer (Abaxis, Sunnyvale, CA). Levels of filovirus-specific antibodies were determined from serum or plasma samples by enzyme-linked immunosorbent assay (ELISA) using inactivated filoviruses as immune targets, as previously described (42). Briefly, filovirus preparations were inactivated by irradiation and used to coat polyvinyl chloride ELISA plates (Dynatech Laboratories, Chantilly, VA). The plates were coated with 50 μl per well of each respective filovirus preparation diluted in phosphate-buffered saline to an approximate concentration of 1 mg/ml total protein. After the plates were incubated overnight at 4°C, the assays were carried out as previously described (42). Antibody titers were defined as the reciprocals of the highest dilutions giving a net optical density values ≥0.2.

RESULTS

CAdVax-Panfilo-vaccinated animals survive lethal challenge with two different filoviruses. Cynomolgus macaques were separated into two groups, and macaques in each group were vaccinated with 4×10^{10} PFU of CAdVax-Panfilo on days 0 and 63, followed by the initial filovirus challenge on day 105 (group 1) or 106 (group 2). Group 1 was challenged with 1,000 PFU MARV Musoke, while group 2 was challenged with the same dose of ZEBOV (Fig. 1). After challenge, the two groups of vaccinated animals showed no signs of fever, as measured by rectal temperature changes, whereas control animals developed fever within 5 days of challenge by either filovirus species and died within 8 days of challenge (Table 1; Fig. 2A and C). Additionally, vaccinated animals showed no changes in the serum levels of liver enzymes after challenge with either MARV or EBOV, whereas control animals showed dramatic increases in alkaline phosphatase and aspartate transaminase enzyme levels 5 to 8 days after virus challenge (Table 1; Fig. 3A and C). These changes are consistent with a massive hepatic insult that is typical of filovirus hemorrhagic fever. Finally, all vaccinated animals showed no detectable viremia or hematology abnormalities (data not shown). There was a single exception to these findings in an animal from group 1, which showed a slight increase in liver enzymes on day 5 after MARV Musoke challenge (Fig. 3A). However, this animal did not show any other signs of infection or disease, including fever (Fig. 2A). From these data, we can conclude that the multivalent filovirus vaccine was 100% protective against lethal MARV Musoke and ZEBOV challenges.

CAdVax-Panfilo-vaccinated animals survive back-challenges with additional filovirus subtypes. Since 100% of the vaccinated animals in both challenge groups survived with no signs of disease, we explored the effects of rechallenging the NHP with a different species of filovirus approximately 10 weeks after the first challenge. This time, group 1 was rechallenged with the Boniface strain of SEBOV while group 2 was rechallenged with MARV Ci67 (Fig. 1). As before, the vaccinated animals showed no signs of fever (Table 1; Fig. 2B and D) or elevations

TABLE 1. Panfilo vaccine protects NHP against multistrain EBOV and MARV challenges[a]

Group	Initial challenge		Back-challenge		Clinical findings	Day of death
	Virus	S/T (%)	Virus	S/T (%)		
1	Musoke	5/5 (100)	SEBOV	5/5 (100)	All NHP had no symptoms	
2	ZEBOV	5/5 (100)	Ci67	5/5 (100)	Small transient ALT increase; small increase in AST in one NHP; all others had no symptoms	
Control 1A	Musoke	0/1 (0)			Fever; ALP ↑ ↑ ↑; AST ↑ ↑ ↑; ALT ↑; GGT ↑	8
Control 2A	ZEBOV	0/1 (0)			Fever; ALP ↑ ↑; AST ↑ ↑	6
Control 1B			SEBOV	0/2 (0)	Fever; ALP ↑ ↑ ↑; AST ↑ ↑ ↑; ALT ↑; GGT ↑	6, 8
Control 2B			Ci67	0/1 (0)	Fever; ALP ↑ ↑ ↑; AST ↑ ↑ ↑; ALT ↑ ↑; GGT ↑ ↑	7

[a] Group 1 and 2 NHP were immunized on days 0 and 63 with 4×10^{10} PFU of vaccine (1×10^{10} PFU each of EBO7, EBO2, M8, and M11). EBO7 expresses GPs of SEBOV and ZEBOV, EBO2 expresses two copies of ZEBOV NP, M8 expresses GPs of Ci67 and Ravn, and M11 expresses Musoke GP and NP. Fifteen weeks after immunization, vaccinated animals were divided into two groups, which were challenged with MARV (group 1; Musoke) or EBOV (group 2; ZEBOV). Ten weeks after the primary challenge, group 1 animals were back-challenged with a different EBOV species (SEBOV) and group 2 animals were back-challenged with a different MARV strain (Ci67). Individual control groups were also included for each of the four filovirus challenges. NHP were administered 1,000 PFU of filovirus at each challenge. Rectal temperature and liver enzyme levels were measured pre- and postchallenge, and any changes from baseline were noted as clinical findings. S/T, number of survivors/total challenged; ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; ↑, 50 to 150 enzyme U/liter of blood; ↑ ↑, 151 to 400 enzyme U/liter of blood; ↑ ↑ ↑, >400 enzyme U/liter of blood.

in liver enzymes (Table 1; Fig. 3B and D), whereas the control animals all developed viral hemorrhagic fever and died. As there is no cross-protective immunity between filovirus species (20, 37, 42, 43), we can conclude that the results from the secondary challenge can be attributed to the vaccine's protection and not to immune responses generated from the primary challenge.

CAdVax-Panfilo induces balanced humoral immune responses against multiple filoviruses after vaccination. We also closely analyzed vaccinated NHP antibody responses to EBOV and MARV by ELISA. Using inactivated filoviruses as immune targets, we found that all vaccinated animals from groups 1 and 2 mounted strong antibody titers against all five filoviruses with similar kinetics (Fig. 4). By having two groups

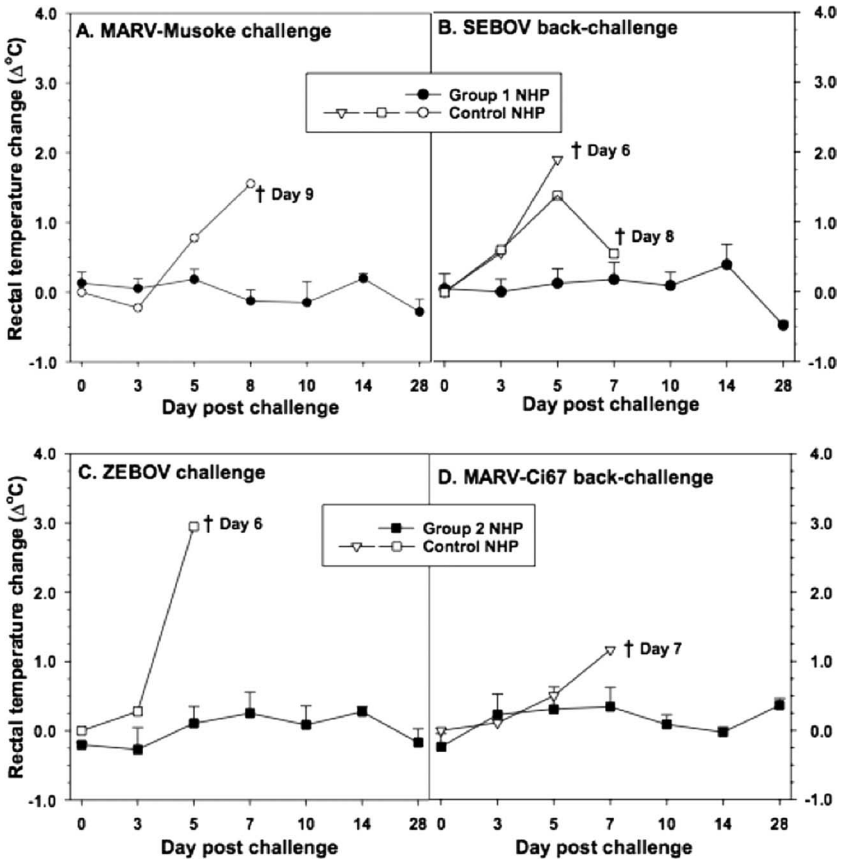

FIG. 2. Relative rectal temperature changes before and after filovirus challenge. Changes from baseline rectal temperature values were measured in NHP challenged with 1,000 PFU of (A) MARV Musoke, (B) SEBOV, (C) ZEBOV, and (D) MARV Ci67. Group 1 NHP were challenged with MARV Musoke and back-challenged with SEBOV. Group 2 NHP were challenged with ZEBOV and back-challenged with MARV Ci67.

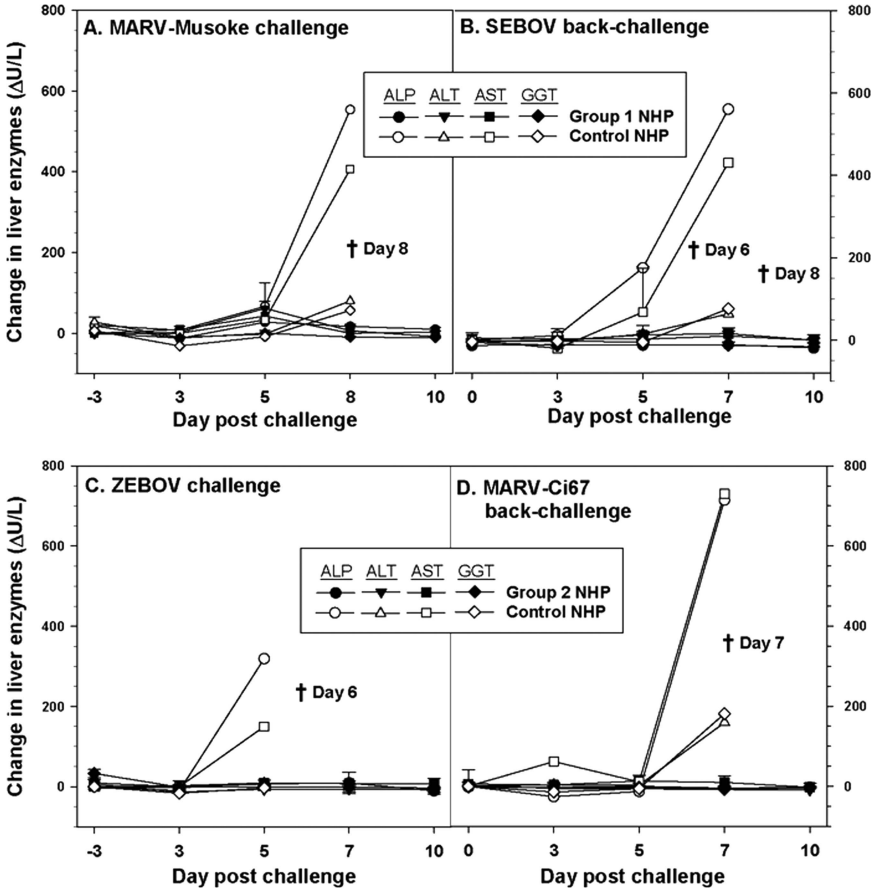

FIG. 3. Relative serum liver enzyme levels before and after filovirus challenge. Changes from baseline liver enzyme values were measured in NHP challenged with 1,000 PFU of (A) MARV Musoke, (B) SEBOV, (C) ZEBOV, and (D) MARV Ci67. Group 1 NHP were challenged with MARV Musoke and back-challenged with SEBOV. Group 2 NHP were challenged with ZEBOV and back-challenged with MARV Ci67.

of macaques immunized with the same vaccine components but challenged with different filoviruses, we were also able to compare the humoral immune responses before and after individual challenges. Interestingly, across all comparisons, we found no noticeable differences in antibody titers in animals before and after challenge with either filovirus species. A possible explanation may be that the humoral responses elicited by the multivalent vaccine had already reached maximal threshold levels for each filovirus prior to the challenges. Therefore, antibody titers could not be elevated any further, even after challenge with 1,000 PFU of filovirus. This suggests the possibility that a single vaccination with CAdVax vaccine could be sufficient to induce maximal levels of immune responses and protect from lethal filovirus infection. This hypothesis is mutually supported in that immune responses reached a plateau at day 63 after the first vaccination and did not increase further after the booster vaccination or after each challenge. However, a separate experiment must be performed comparing vaccination schedules of prime only versus prime plus boost in order to confirm this hypothesis.

DISCUSSION

This study demonstrates the efficacy of a multivalent filovirus vaccine and represents a significant step forward in addressing the public health threat from these deadly viruses. In this study, cynomolgus macaques vaccinated with a two-dose regimen of the CAdVax-Panfilo vaccine were protected against the three filovirus species of major concern to human health: ZEBOV, SEBOV, and MARV.

The Panfilo vaccine approach is relevant both for biodefense applications and for the early response and containment of natural filovirus outbreaks, which have continued to emerge in central Africa since the mid-1990s with steady increases in frequency in recent years (http://www.who.int/mediacentre/factsheets/fs103/en/index1.html), including the recent EBOV outbreak in the Democratic Republic of the Congo (46), the MARV outbreak in Angola in 2005 (26), and the current EBOV outbreak in Uganda, which is due to a new, unnamed fifth species of EBOV (http://www.who.int/csr/don/2007_12_07/en/index.html). Because the identification of the causative agent during the early phase of acute filovirus outbreaks is usually unknown, first responders and caregivers could use a multivalent filovirus vaccine to both protect themselves and curtail the spread of disease through herd immunity. Sullivan et al. first suggested the possibility of using an Ad-based vaccine for containment during acute filovirus outbreaks (35). However, the vaccine vector described in that report carried only a single gene (GP or NP gene) for a single filovirus species

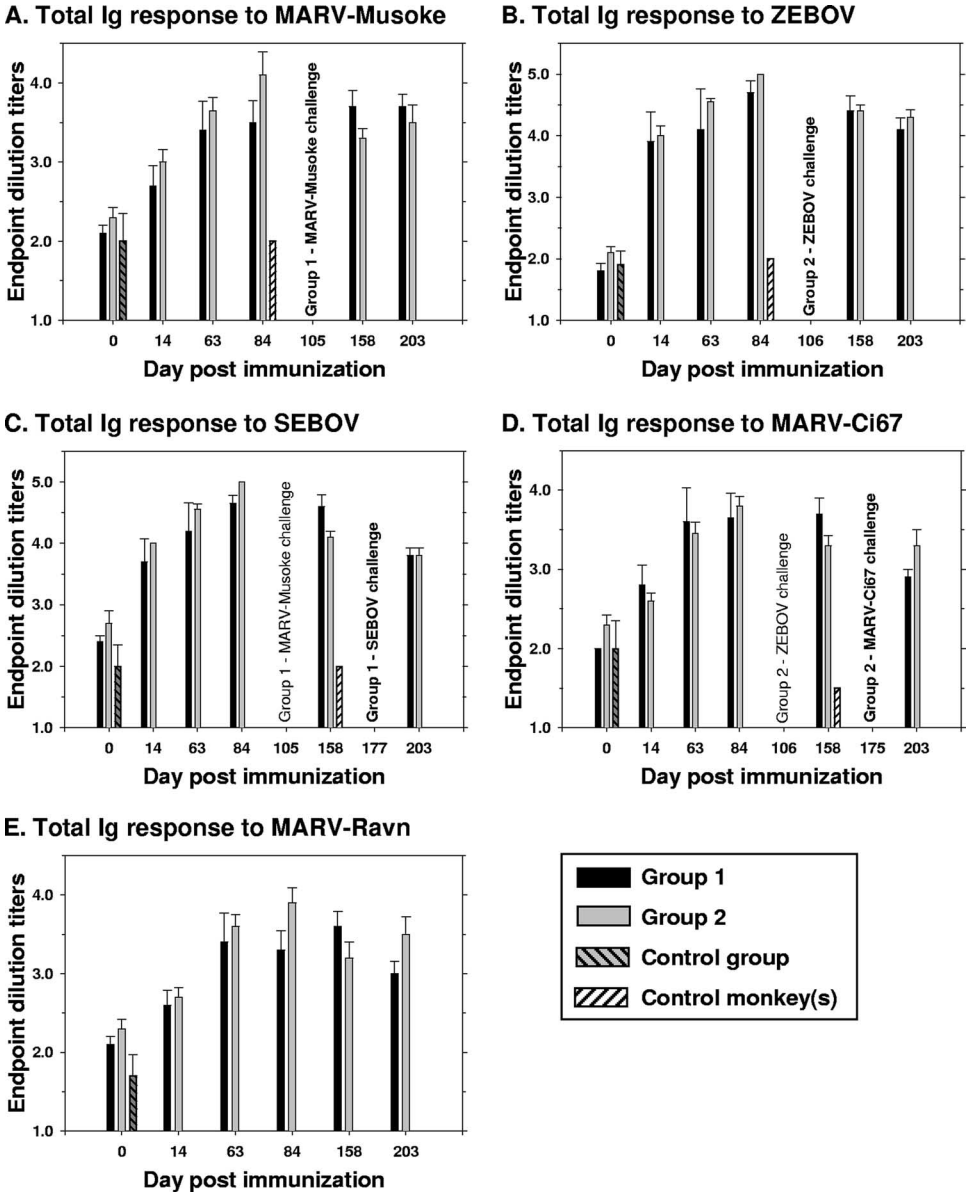

FIG. 4. Humoral immune responses to filoviruses before and after challenges. Geometric mean titers (plus standard errors of the means) of total immunoglobin (Ig) in response to (A) MARV Musoke, (B) ZEBOV, (C) SEBOV, (D) MARV Ci67, and (E) MARV Ravn were measured by ELISA using inactivated filovirus preparations as immune targets. Group 1 NHP were challenged with MARV Musoke and back-challenged with SEBOV. Group 2 NHP were challenged with ZEBOV and back-challenged with MARV Ci67. The control group of NHP ($n = 5$) were evaluated on day 0 prior to challenge.

(ZEBOV). If an outbreak were caused by a different species of filovirus, such as SEBOV or any of the MARV subtypes, then such a filovirus vaccine will likely be ineffective.

While a similar Panfilo vaccine could be formulated using a mixture of first-generation Ad vectors (each expressing a single filovirus GP or NP gene), it would require seven different vectors to express the same number of antigens as CAdVax-Panfilo. The same could be said for the VSVΔG filovirus vaccine vectors, which also are capable of expressing only a single GP or NP antigen. The CAdVax platform has the capability to express multiple antigens from a single vaccine component, thus requiring fewer components to express a large number of antigens. While we included only two filovirus genes per vector in this study, CAdVax vectors are capable of accommodating more than two transgenes (16, 28, 32), depending on the size of the gene of interest. This capability can greatly simplify production processes and quality control measures for large-scale manufacturing, especially in the event of a biological attack scenario, when large amounts of vaccine would be needed in a short amount of time.

Many experimental filovirus vaccines have shown protective efficacy against ZEBOV and MARV. However, to date, no published data are available demonstrating clear protection against SEBOV, nor are there data describing polyvalent

vaccine formulations against multiple filovirus species. The VSVΔG/ZEBOV GP vector described by Jones et al. was 100% protective in NHP against the homologous ZEBOV challenge. However, when the same vaccinated animals were subsequently back-challenged with SEBOV, only one out of four macaques survived (20). In contrast, 100% of NHP receiving CAdVax-Panfilo survived challenge with ZEBOV and SEBOV. Jones et al. also demonstrate 100% efficacy of the VSVΔG/MARV GP vector against two different subtypes of MARV (Musoke and Popp) (20). Unfortunately, interpretation of those challenge results is complicated by the fact that the NHP were first challenged with (and thus immunized against) MARV Musoke prior to back-challenge with the antigenically related Popp strain. In the present report, NHP were back-challenged with a filovirus of completely different genus to eliminate any possibility for cross-protection among EBOV species or among MARV strains.

It is estimated that 35% to 55% of the world's population is seropositive for neutralizing antibodies against Ad (6, 27), in particular Ad subtype 5 (Ad5), the subtype on which the CAdVax vectors are based. This has led to frequent suggestions that these circulating Ad-neutralizing antibodies might limit any Ad-based vaccine vector's efficacy by neutralization of the vector prior to efficient transgene expression, which has been demonstrated in animal models (4, 12, 22, 47). However, other experiments with NHP have suggested that preexisting Ad5 immunity can be overcome by increasing the dose of the Ad-based vaccine (5). Human clinical trials have also produced data supporting this notion. For example, a phase I/II trial studying an Ad5-based human immunodeficiency virus vaccine found that preexisting Ad5 immunity significantly impacted the vaccine's performance but that this inhibition could be overcome by increasing the dose of vaccine (7). Additionally, it is suggested that vaccination by alternate routes of administration (such as oral or intranasal) rather than injection can overcome preexisting vector immunity (2, 47). This suggestion has also been supported by data generated from a human clinical trial studying the intranasal delivery of an Ad5-based influenza vaccine, which found no correlation between vaccine immunogenicity and the levels of Ad5-neutralizing antibodies in vaccine recipients as long as the vaccine was delivered intranasally (38). So far, much of the experimental data supporting the negative aspects of Ad5 preexisting immunity have all been generated using mouse models (4, 12, 22, 47), which are not even capable of supporting a wild-type Ad5 infection. The true significance of Ad5 preexisting immunity for the performance of Ad-based vaccines remains a frequent topic of debate.

A major concern with developing components for a multivalent format is the possibility of vaccine interference between gene products within a component and between different components. Such interference has been observed, for example, in clinical trials of a tetravalent mixture of four live-attenuated dengue vaccines, in which immune response induction favored dengue virus serotype 3 over the other three serotypes (21). In our comparison of the different ELISA titers to the various filoviruses, there appears to be no evidence of this occurring. These data suggest a lack of dominance of any one antigen over the other, and the challenge results confirm the panfilovirus vaccine's capability to induce a balanced protection against all filovirus species/subtypes tested.

In summary, we have demonstrated the feasibility and efficacy of the CAdVax-Panfilo vaccine to protect NHP against superlethal challenges and rechallenges with multiple species and subtypes of filoviruses. This vaccine platform is highly efficient, economical to produce, and amenable to multivalent formats. Additionally, Ad vectors have been studied in hundreds of clinical trials worldwide and have a favorable safety profile for human use. These results support a more advanced development and study of CAdVax-Panfilo to address both natural and manmade threats from these deadly viruses.

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REFERENCES

1. **Alibek, K., and H. Handelman.** 1999. Biohazard. Random House, New York, NY.
2. **Appaiahgari, M. B., M. Saini, M. Rauthan, Jyoti, and S. Vrati.** 2006. Immunization with recombinant adenovirus synthesizing the secretory form of Japanese encephalitis virus envelope protein protects adenovirus-exposed mice against lethal encephalitis. Microbes Infect. **8:**92–104.
3. **Bermejo, M., J. D. Rodriguez-Teijeiro, G. Illera, A. Barroso, C. Vila, and P. D. Walsh.** 2006. Ebola outbreak killed 5000 gorillas. Science **314:**1564.
4. **Capone, S., A. Meola, B. B. Ercole, A. Vitelli, M. Pezzanera, L. Ruggeri, M. E. Davies, R. Tafi, C. Santini, A. Luzzago, T. M. Fu, A. Bett, S. Colloca, R. Cortese, A. Nicosia, and A. Folgori.** 2006. A novel adenovirus type 6 (Ad6)-based hepatitis C virus vector that overcomes preexisting anti-Ad5 immunity and induces potent and broad cellular immune responses in rhesus macaques. J. Virol. **80:**1688–1699.
5. **Casimiro, D. R., L. Chen, T. M. Fu, R. K. Evans, M. J. Caulfield, M. E. Davies, A. Tang, M. Chen, L. Huang, V. Harris, D. C. Freed, K. A. Wilson, S. Dubey, D. M. Zhu, D. Nawrocki, H. Mach, R. Troutman, L. Isopi, D. Williams, W. Hurni, Z. Xu, J. G. Smith, S. Wang, X. Liu, L. Guan, R. Long, W. Trigona, G. J. Heidecker, H. C. Perry, N. Persaud, T. J. Toner, Q. Su, X. Liang, R. Youil, M. Chastain, A. J. Bett, D. B. Volkin, E. A. Emini, and J. W. Shiver.** 2003. Comparative immunogenicity in rhesus monkeys of DNA plasmid, recombinant vaccinia virus, and replication-defective adenovirus vectors expressing a human immunodeficiency virus type 1 *gag* gene. J. Virol. **77:**6305–6313.
6. **Chirmule, N., K. Propert, S. Magosin, Y. Qian, R. Qian, and J. Wilson.** 1999. Immune responses to adenovirus and adeno-associated virus in humans. Gene Ther. **6:**1574–1583.
7. **Cohen, P.** 2006. Immunity's yin and yang. A successful vaccine must first avoid being eliminated by pre-existing immunity before it can promote a protective immune response. IAVI Rep. **10**(1)**:**1–5.
8. **Daddario-DiCaprio, K. M., T. W. Geisbert, U. Stroher, J. B. Geisbert, A. Grolla, E. A. Fritz, L. Fernando, E. Kagan, P. B. Jahrling, L. E. Hensley, S. M. Jones, and H. Feldmann.** 2006. Postexposure protection against Marburg haemorrhagic fever with recombinant vesicular stomatitis virus vectors in non-human primates: an efficacy assessment. Lancet **367:**1399–1404.
9. **Feldmann, H., S. M. Jones, K. M. Daddario-DiCaprio, J. B. Geisbert, U. Stroher, A. Grolla, M. Bray, E. A. Fritz, L. Fernando, F. Feldmann, L. E. Hensley, and T. W. Geisbert.** 2007. Effective post-exposure treatment of Ebola infection. PLoS Pathog. **3:**e2.

9a. **Feldmann, H., T. W. Geisbert, P. B. Jahrling, H.-D. Klenk, S. V. Netesov, C. J. Peters, A. Sanchez, R. Swanepoel, and V. E. Volchkov.** 2005. Family *Filoviridae*, p. 645–653. *In* C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger, and L. A. Ball (ed.), Virus taxonomy: classification and nomenclature. Eighth report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, San Diego, CA.

10. **Geisbert, T. W., L. E. Hensley, T. Larsen, H. A. Young, D. S. Reed, J. B. Geisbert, D. P. Scott, E. Kagan, P. B. Jahrling, and K. J. Davis.** 2003. Pathogenesis of Ebola hemorrhagic fever in cynomolgus macaques: evidence that dendritic cells are early and sustained targets of infection. Am. J. Pathol. **163:**2347–2370.
11. **Geisbert, T. W., P. Pushko, K. Anderson, J. Smith, K. J. Davis, and P. B. Jahrling.** 2002. Evaluation in nonhuman primates of vaccines against Ebola virus. Emerg. Infect. Dis. **8:**503–507.
12. **Hashimoto, M., J. L. Boyer, N. R. Hackett, J. M. Wilson, and R. G. Crystal.** 2005. Induction of protective immunity to anthrax lethal toxin with a nonhuman primate adenovirus-based vaccine in the presence of preexisting anti-human adenovirus immunity. Infect. Immun. **73:**6885–6891.

13. **Hevey, M., D. Negley, J. Geisbert, P. Jahrling, and A. Schmaljohn.** 1997. Antigenicity and vaccine potential of Marburg virus glycoprotein expressed by baculovirus recombinants. Virology **239:**206–216.
14. **Hevey, M., D. Negley, P. Pushko, J. Smith, and A. Schmaljohn.** 1998. Marburg virus vaccines based upon alphavirus replicons protect guinea pigs and nonhuman primates. Virology **251:**28–37.
15. **Hevey, M., D. Negley, L. VanderZanden, R. F. Tammariello, J. Geisbert, C. Schmaljohn, J. F. Smith, P. B. Jahrling, and A. L. Schmaljohn.** 2001. Marburg virus vaccines: comparing classical and new approaches. Vaccine **20:** 586–593.
16. **Holman, D. H., D. Wang, K. Raviprakash, N. U. Raja, M. Luo, J. Zhang, K. R. Porter, and J. Y. Dong.** 2007. Two complex, adenovirus-based vaccines that together induce immune responses to all four dengue virus serotypes. Clin. Vaccine Immunol. **14:**182–189.
17. **Jaax, N., P. Jahrling, T. Geisbert, J. Geisbert, K. Steele, K. McKee, D. Nagley, E. Johnson, G. Jaax, and C. Peters.** 1995. Transmission of Ebola virus (Zaire strain) to uninfected control monkeys in a biocontainment laboratory. Lancet **346:**1669–1671.
18. **Johnson, E., N. Jaax, J. White, and P. Jahrling.** 1995. Lethal experimental infections of rhesus monkeys by aerosolized Ebola virus. Int. J. Exp. Pathol. **76:**227–236.
19. **Johnson, E. D., B. K. Johnson, D. Silverstein, P. Tukei, T. W. Geisbert, A. N. Sanchez, and P. B. Jahrling.** 1996. Characterization of a new Marburg virus isolated from a 1987 fatal case in Kenya. Arch. Virol. Suppl. **11:**101–114.
20. **Jones, S. M., H. Feldmann, U. Stroher, J. B. Geisbert, L. Fernando, A. Grolla, H. D. Klenk, N. J. Sullivan, V. E. Volchkov, E. A. Fritz, K. M. Daddario, L. E. Hensley, P. B. Jahrling, and T. W. Geisbert.** 2005. Live attenuated recombinant vaccine protects nonhuman primates against Ebola and Marburg viruses. Nat. Med. **11:**786–790.
21. **Kanesa-thasan, N., W. Sun, G. Kim-Ahn, S. Van Albert, J. R. Putnak, A. King, B. Raengsakulsrach, H. Christ-Schmidt, K. Gilson, J. M. Zahradnik, D. W. Vaughn, B. L. Innis, J. F. Saluzzo, and C. H. Hoke, Jr.** 2001. Safety and immunogenicity of attenuated dengue virus vaccines (Aventis Pasteur) in human volunteers. Vaccine **19:**3179–3188.
22. **Kobinger, G. P., H. Feldmann, Y. Zhi, G. Schumer, G. Gao, F. Feldmann, S. Jones, and J. M. Wilson.** 2006. Chimpanzee adenovirus vaccine protects against Zaire Ebola virus. Virology **346:**394–401.
23. **Leffel, E. K., and D. S. Reed.** 2004. Marburg and Ebola viruses as aerosol threats. Biosecur. Bioterror. **2:**186–191.
24. **Moe, J. B., R. D. Lambert, and H. W. Lupton.** 1981. Plaque assay for Ebola virus. J. Clin. Microbiol. **13:**791–793.
25. **National Research Council.** 1996. Guide for the care and use of laboratory animals. National Academy Press, Washington, DC.
26. **Ndayimirije, N., and M. K. Kindhauser.** 2005. Marburg hemorrhagic fever in Angola–fighting fear and a lethal pathogen. N. Engl. J. Med. **352:**2155–2157.
27. **Nwanegbo, E., E. Vardas, W. Gao, H. Whittle, H. Sun, D. Rowe, P. D. Robbins, and A. Gambotto.** 2004. Prevalence of neutralizing antibodies to adenoviral serotypes 5 and 35 in the adult populations of The Gambia, South Africa, and the United States. Clin. Diagn. Lab. Immunol. **11:**351–357.
28. **Raja, N. U., D. H. Holman, D. Wang, K. Raviprakash, L. Y. Juompan, S. B. Deitz, M. Luo, J. Zhang, K. R. Porter, and J. Y. Dong.** 2007. Induction of bivalent immune responses by expression of dengue virus type 1 and type 2 antigens from a single complex adenoviral vector. Am. J. Trop. Med. Hyg. **76:**743–751.
29. **Rubinchik, S., J. S. Norris, and J. Y. Dong.** 2002. Construction, purification and characterization of adenovirus vectors expressing apoptosis-inducing transgenes. Methods Enzymol. **346:**529–547.
30. **Rubinchik, S., D. Wang, H. Yu, F. Fan, M. Luo, J. S. Norris, and J. Y. Dong.** 2001. A complex adenovirus vector that delivers FASL-GFP with combined prostate-specific and tetracycline-regulated expression. Mol. Ther. **4:**416–426.
31. **Rubinchik, S., J. Woraratanadharm, J. Schepp, and J. Y. Dong.** 2003. Improving the transcriptional regulation of genes delivered by adenovirus vectors. Methods Mol. Med. **76:**167–199.
32. **Schepp-Berglind, J., M. Luo, D. Wang, J. A. Wicker, N. U. Raja, B. D. Hoel, D. H. Holman, A. D. Barrett, and J. Y. Dong.** 2007. Complex adenovirus-mediated expression of West Nile virus C, PreM, E, and NS1 proteins induces both humoral and cellular immune responses. Clin. Vaccine Immunol. **14:**1117–1126.
33. **Slenczka, W. G.** 1999. The Marburg virus outbreak of 1967 and subsequent episodes. Curr. Top. Microbiol. Immunol. **235:**49–75.
34. **Smith, D. H., B. K. Johnson, M. Isaacson, R. Swanapoel, K. M. Johnson, M. Killey, A. Bagshawe, T. Siongok, and W. K. Keruga.** 1982. Marburg-virus disease in Kenya. Lancet **i:**816–820.
35. **Sullivan, N. J., T. W. Geisbert, J. B. Geisbert, L. Xu, Z. Y. Yang, M. Roederer, R. A. Koup, P. B. Jahrling, and G. J. Nabel.** 2003. Accelerated vaccination for Ebola virus haemorrhagic fever in non-human primates. Nature **424:**681–684.
36. **Sullivan, N. J., A. Sanchez, P. E. Rollin, Z. Y. Yang, and G. J. Nabel.** 2000. Development of a preventive vaccine for Ebola virus infection in primates. Nature **408:**605–609.
37. **Swenson, D. L., K. L. Warfield, D. L. Negley, A. Schmaljohn, M. J. Aman, and S. Bavari.** 2005. Virus-like particles exhibit potential as a pan-filovirus vaccine for both Ebola and Marburg viral infections. Vaccine **23:**3033–3042.
38. **Van Kampen, K. R., Z. Shi, P. Gao, J. Zhang, K. W. Foster, D. T. Chen, D. Marks, C. A. Elmets, and D. C. Tang.** 2005. Safety and immunogenicity of adenovirus-vectored nasal and epicutaneous influenza vaccines in humans. Vaccine **23:**1029–1036.
39. **Wang, D., M. Hevey, L. Y. Juompan, C. M. Trubey, N. U. Raja, S. B. Deitz, J. Woraratanadharm, M. Luo, H. Yu, B. M. Swain, K. M. Moore, and J. Y. Dong.** 2006. Complex adenovirus-vectored vaccine protects guinea pigs from three strains of Marburg virus challenges. Virology **353:**324–332.
40. **Wang, D., N. U. Raja, C. M. Trubey, L. Y. Juompan, M. Luo, J. Woraratanadharm, S. B. Deitz, H. Yu, B. M. Swain, K. M. Moore, W. D. Pratt, M. K. Hart, and J. Y. Dong.** 2006. Development of a cAdVax-based bivalent ebola virus vaccine that induces immune responses against both the Sudan and Zaire species of Ebola virus. J. Virol. **80:**2738–2746.
41. **Wang, D., A. L. Schmaljohn, N. U. Raja, C. M. Trubey, L. Y. Juompan, M. Luo, S. B. Deitz, H. Yu, J. Woraratanadharm, D. H. Holman, K. M. Moore, B. M. Swain, W. D. Pratt, and J. Y. Dong.** 2006. De novo syntheses of Marburg virus antigens from adenovirus vectors induce potent humoral and cellular immune responses. Vaccine **24:**2975–2986.
42. **Warfield, K. L., C. M. Bosio, B. C. Welcher, E. M. Deal, M. Mohamadzadeh, A. Schmaljohn, M. J. Aman, and S. Bavari.** 2003. Ebola virus-like particles protect from lethal Ebola virus infection. Proc. Natl. Acad. Sci. USA **100:** 15889–15894.
43. **Warfield, K. L., D. L. Swenson, D. L. Negley, A. L. Schmaljohn, M. J. Aman, and S. Bavari.** 2004. Marburg virus-like particles protect guinea pigs from lethal Marburg virus infection. Vaccine **22:**3495–3502.
44. **Wilson, J. A., and M. K. Hart.** 2001. Protection from Ebola virus mediated by cytotoxic T lymphocytes specific for the viral nucleoprotein. J. Virol. **75:**2660–2664.
45. **World Health Organization.** 1978. Ebola haemorrhagic fever in Sudan, 1976: report of a WHO/international study team. Bull. W. H. O. **56:**247–270.
46. **World Health Organization.** 2007. Outbreak news. Ebola virus haemorrhagic fever, Democratic Republic of the Congo–update. Wkly. Epidemiol. Rec. **82:**345–346.
47. **Xiang, Z. Q., G. P. Gao, A. Reyes-Sandoval, Y. Li, J. M. Wilson, and H. C. Ertl.** 2003. Oral vaccination of mice with adenoviral vectors is not impaired by preexisting immunity to the vaccine carrier. J. Virol. **77:**10780–10789.