Development of a cAdVax-Based Bivalent Ebola Virus Vaccine That Induces Immune Responses against both the Sudan and Zaire Species of Ebola Virus

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Ebola virus (EBOV) causes a severe hemorrhagic fever for which there are currently no vaccines or effective treatments. While lethal human outbreaks have so far been restricted to sub-Saharan Africa, the potential exploitation of EBOV as a biological weapon cannot be ignored. Two species of EBOV, Sudan ebolavirus (SEBOV) and Zaire ebolavirus (ZEBOV), have been responsible for all of the deadly human outbreaks resulting from this virus. Therefore, it is important to develop a vaccine that can prevent infection by both lethal species. Here, we describe the bivalent cAdVaxE(GPs/z) vaccine, which includes the SEBOV glycoprotein (GP) and ZEBOV GP genes together in a single complex adenovirus-based vaccine (cAdVax) vector. Vaccination of mice with the bivalent cAdVaxE(GPs/z) vaccine led to efficient induction of EBOV-specific antibody and cell-mediated immune responses to both species of EBOV. In addition, the cAdVax technology demonstrated induction of a 100% protective immune response in mice, as all vaccinated C57BL/6 and BALB/c mice survived challenge with a lethal dose of ZEBOV (30,000 times the 50% lethal dose). This study demonstrates the potential efficacy of a bivalent EBOV vaccine based on a cAdVax vaccine vector design.

Ebola viruses (EBOV) are members of the filovirus family of viruses and cause a severe viral hemorrhagic fever with high mortality in humans and nonhuman primates, killing up to 90% of those infected. The disease is characterized by widespread petechial hemorrhages, focal necrosis of the liver, kidney, and spleen, shock, and ultimately, death. Despite considerable effort, no animal or arthropod reservoir capable of sustaining the virus between outbreaks has been identified (7, 9, 24). Moreover, the pathogenesis of Ebola hemorrhagic fever is not fully understood, and no vaccines or effective therapies are currently available.

Four distinct Ebola virus species have been identified to date: Sudan ebolavirus (SEBOV), Zaire ebolavirus (ZEBOV), Reston ebolavirus (REBOV), and Ivory Coast ebolavirus (ICEBOV). All human outbreaks and fatalities, however, have been attributed to ZEBOV and SEBOV, which together have resulted in over 1,000 cases of Ebola hemorrhagic fever since 1994 with a 50 to 81% mortality rate per outbreak (2). The best comprehensive, long-term solution for preventing EBOV infection would be the development of a safe and effective vaccine that could elicit protection against the deadliest EBOV species, ZEBOV and SEBOV. If this vaccine is to be effective for the people of Central Africa, it must be easy to mobilize and administer, and it must elicit protective immune responses with a minimal number of doses. Additionally, the current bioterrorist threat reinforces the need for the development of a vaccine whose immune induction is both swift and effective.

In order to design an effective vaccine against a fatal pathogen such as EBOV, it is important to induce effective immune responses that confer on the individual a protective immunity. Several studies have evaluated vaccine approaches incorporating components of the EBOV genome. In particular, protection in animals has been demonstrated with vaccine candidates expressing EBOV glycoprotein (GP) (4, 6, 10, 12, 22, 25, 33) or nucleoprotein (NP) (10, 22, 25, 31, 33). Protective immune responses following GP and NP vaccination may be attributed to induction of both humoral (4, 10, 12, 22, 25, 31, 33) and cell-mediated immune (CMI) responses (22, 25, 31, 33). However, most of these previous strategies were directed only at a single EBOV species, ZEBOV. In this study, we address the need for immunity against the two deadliest EBOV species, Zaire and Sudan, by developing and characterizing a bivalent EBOV vaccine that incorporates both virus species in the vaccine design.

Our vaccine strategy combines a benign infection caused by a replication-defective, complex adenovirus vaccine (cAdVax) vector with the antigenic potential conferred by highly induced expression of EBOV GP genes. It is our hypothesis that de novo synthesis and expression of EBOV antigens will mimic the antigen presentation that would occur from a natural EBOV infection, but without the pathogenicity and hemorrhagic fever associated with an actual EBOV infection. By mimicking EBOV infection, the presentation of EBOV antigen to the immune system should elicit an immune response.
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**ABSTRACT**

Ebola virus (EBOV) causes a severe hemorrhagic fever for which there are currently no vaccines or effective treatments. While human outbreaks have so far been restricted to sub-Saharan Africa, the potential exploitation of EBOV as a biological weapon cannot be ignored. Two species of EBOV, Sudan ebolavirus and Zaire ebolavirus (ZEBOV) have been responsible for all of the deadly human outbreaks resulting from this virus. Therefore, it is important to develop a vaccine that can prevent infection by both lethal species. Here, we describe the bivalent cAdVaxE(GPs/z) vaccine, which includes the SEBOV glycoprotein (GP) and ZEBOV GP genes together in a single complex adenovirus-based vaccine(cAdVAX) vector. Vaccination of mice with the bivalent cAdVaxE(GPs/z) vaccine led to efficient induction of EBOV-specific antibody and cellp-mediated immune responses to both species of EBOV. In addition, the cAdVax technology demonstrated induction of a 100% protective immune response in mice, as all vaccinated C57Bl/6 and BALB/c mice survived challenge with a lethal dose of ZEBOV (30,000 times the 50% lethal dose). This study demonstrates the potential efficacy of a bivalent EBOV vaccine based on a cAdVax vaccine vector design.

**SUBJECT TERMS**

filovirus, Ebola, Sudan, Zaire, vaccine, bivalent, cAdVax-based, immune responses, laboratory animals, mice
against EBOV from both the humoral and cell-mediated arms of the immune system.

In this study, we develop and characterize a cAdVax-based bivalent EBOV vaccine candidate, known as cAdVaxE(GPz). This vaccine efficiently expresses both the SEBOV and ZEBOV GP genes from a single vaccine construct, demonstrating effective induction of both anti-EBOV GP serum antibody as well as EBOV-specific CMI responses. In addition, the coexpression of SEBOV GP and ZEBOV GP together by a single vaccine appeared to have a synergistic effect on the induction of bivalent humoral immune responses. Significantly, vaccination of mice with cAdVaxE(GPz) led to 100% protection of mice from lethal challenge with a mouse-adapted ZEBOV. This induction of a protective immune response with 100% efficiency indicates the potential for developing an effective bivalent EBOV vaccine based on the cAdVax technology.

MATERIALS AND METHODS

Cell lines. HEK293 (human embryonic kidney), Vero E6 (African green monkey kidney), BS-C-1 (African green monkey kidney), and MC57G cell lines were obtained from American Type Culture Collection (Manassas, VA). HEK293, Vero E6, and BS-C-1 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum (HyClone, Logan, UT), while MC57G cells were maintained in Eagle’s minimal essential medium supplemented with 10% calf serum (BioWhittaker), 2 mM-L-glutamine (BioWhittaker), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (BioWhittaker). Mouse splenocytes were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM-L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (BioWhittaker). Mouse splenocytes were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM-L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (BioWhittaker).

Construction of the cAdVax-based EBOV vaccine. The EBOV gene sequences included in the cAdVax vaccines were derived from the Sudan species (Bolivian strain) and the Zaire species (Zaire-95 strain). The EBOV GP genes were amplified by PCR, with each primer including specific restriction sites at the 5′ ends for subsequent cloning of the PCR fragments into pLaAd and pRAd plasmid shuttle vectors. In order to characterize immune responses to EBOV GP s, we subcloned each EBOV antigen into our pLaAd and pRAd shuttle vectors to create a series of cAdVax-based EBOV vaccines (Fig. 1). The cAdVax-based EBOV vaccine vectors were constructed as described previously (14–16). All adenovirus (Ad) vector genomes were based on a modified AdSub360, which contains deletions of E1, E3, and almost all E4 open reading frames (ORFs) with the exception of ORF6. The final Ad vector genomes were evaluated by sequencing analyses.

Studies of the EBOV GP gene have indicated that the EBOV GP is encoded in two reading frames, resulting in the expression of a secreted, nonstructural glycoprotein (SGP) in preference to the structural GP (19). The SEBOV GP gene sequence had been modified previously to delete the ATG start codon responsible for initiating SGP transcription. As a result, cAdVax vectors that included the SEBOV GP gene [cAdVaxE(GPs)] and cAdVaxE(GPz) vaccines] expressed predominantly the structural GP of SEBOV, while cAdVax vectors expressing the native ZEBOV GP sequences [cAdVaxE(GPs) and cAdVaxE(GPz) vaccines] expressed predominantly the SGP of ZEBOV (Fig. 2).

Complex adenovirus vector propagation, confirmation by sequencing analysis, and determination of titers. All vectors were propagated in HEK293 cells, using standard procedures (14–16). Briefly, HEK293 cells, which provide Ad E1a and E1b functions in trans, were transfected with the recombinant Ad-based EBOV vector genomic DNA using Lipofectamine reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. Transfected cells were maintained until adenovirus-related cytotoxic effects were observed (typically 7 to 14 days posttransfection), at which point the cells were harvested. After several rounds of single-plaque selection, candidate vaccine clones were confirmed by restriction map digestion as well as sequencing analysis of the virus DNA isolated from positive Ad vector plaques to assure that the vaccine preparation had no deletions or rearrangements. Entire viral transgene cassettes were completely sequenced, including promoter regions.

The final positive Ad vector clones were reamplified in HEK293 cells and purified by ultracentrifugation in cesium chloride gradients. Briefly, adenoviral lysates from 30 150-mm plates were banded twice on CsCl gradients, 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 Na2HPO4 · 2H2O, and 0.1% [vol/vol] dextrose), adjusted with NaOH to pH 7.5 and filter sterilized containing 10% glycerol, and stored in liquid N2. All vectors were titrated on HEK293 cells infected in serial dilution on triplicate columns of 12-well plates for PFU. The resulting titers were scored as PFU/ml. The final vaccine was confirmed again with restriction map digestion.

Western blot. Vero cells were infected with EBOV vaccine constructs at a multiplicity of infection (MOI) of 100 for 48 h. Cell pellets were washed twice with phosphate-buffered saline (PBS) and lysed with lysis buffer (200 mM Tris-HCl [pH 7] with 8% Triton X-100, 2% NP-40, 20 mM NaCl, and 2 mM EDTA) on ice. Cell lysates were mixed with sample buffer (50 mM Tris [pH 6.8], 2% sodium dodecyl sulfate, 1% β-mercaptoethanol, 0.1% bromophenol blue, 10% sodium dodecyl sulfate, and 2% β-mercaptoethanol).
FIG. 2. EBOV vaccine candidates cAdVaxE(GPs/z), cAdVaxE(GPs), and cAdVaxE(GPz) demonstrate efficient GP expression. Vero cells were infected with EBOV vaccines cAdVaxE(GPs/z), cAdVaxE(GPs), and cAdVaxE(GPz). Cell lysates were resolved on a 4 to 15% gradient polyacrylamide gel under denaturing and reducing conditions. Membranes were probed with (a) anti-SEBOV GP mouse serum derived from mice vaccinated with cAdVaxE(GPs) or (b) anti-ZEBOV GP mouse monoclonal antibody (clone M-DAlI5-AAO5.ABII). Vero cells infected with a cAdVax vector expressing HCV antigens (mock Ad) served as a negative control for EBOV GP expression. Number scale indicates size in kilodaltons.

glycerol), heated at 100°C for 10 min, and separated on a 4 to 15% gradient sodium dodecyl sulfate-polyacrylamide gel (Bio-Rad, Hercules, CA). Separated proteins were then transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore Corporation, Bedford, MA). The membrane was stained with Poncau S for 15 min and washed with distilled water, and nonspecific antibody-binding sites were blocked with 5% nonfat dry milk in blocking buffer (0.05 M Tris [pH 7.5], 0.15 M NaCl, 0.01% NP-40, 0.3 mM NaN3) for 30 min. The membrane was later incubated for 1 h with anti-ZEBOV GP antibody (M-DA01-5A05.ABII, 5/22/96 mouse monoclonal antibody) or anti-SEBOV GP antibody [serum from mice vaccinated with cAdVaxE(GPs)] diluted 1:1,000 in blocking buffer containing 5% nonfat dry milk. After three washes with PBS containing 0.05% Tween 20, the blot was treated with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) antibody (KPL, Gaithersburg, MD) and diluted 1:10,000 for 1 h, and subsequent bands were visualized using the ECL Plus detection system (Amersham Biosciences, Piscataway, NJ).

Immunofluorescence assay to detect EBOV-GP expression. HeLa cells were seeded at 1.25 × 10⁶ cells/well in a 24-well plate. The next day, cells were infected at an MOI of 100 with cAdVaxE(GPs), cAdVaxE(GPz), cAdVaxE(GPz), or HC4 (a control cAdVax vaccine containing hepatitis C virus [HCV] envelope protein). Two days postinfection, the cells were washed once with PBS and then fixed with acetone for 10 min at −20°C. The cells were then probed with serum diluted 1:100 in PBS-2% FBS. Sera were pooled from five mice vaccinated with either cAdVaxE(GPs) or cAdVaxE(GPz) at 8 weeks postvaccination. For HeLa cells infected with cAdVaxE(GPs), cells were probed with a mixture of cAdVaxE(GPs) and cAdVaxE(GPz) sera, each diluted 1:100. Following two washes with PBS, cells were probed with anti-mouse IgG-fluorescein isothiocyanate (FITC) (Sigma) diluted 1:100 in PBS-2% FBS. Following three washes with PBS, cells were visualized with an Axiosvert-25 microscope (Carl Zeiss, Germany) and an FITC excitation/emission filter set (Chroma Technology Corp., Rockingham, VT).

Immunization of mice with cAdVax-based EBOV vaccines. Six- to 8-week-old C57BL/6 mice (Charles River, Wilmington, MA) were divided into four groups of 65 mice each and then immunized intramuscularly (i.m.) with 1 × 10⁷ PFU of cAdVaxE(GPs/z), cAdVaxE(GPs), cAdVaxE(GPz), or HC4 control. Immunizations were performed at 0, 16, and 24 weeks. All mice were maintained in accordance with Institutional Animal Care and Use Committee approved protocols. Each animal was analyzed independently. Vaccinated mice were visually monitored for any adverse effects resulting from immunization. Particular attention was paid to food and water intake, coat texture (ruffled coats are often a sign of illness), and excessive weight loss or gain.

Four mice from each group were euthanized every 2 weeks for weeks 0 to 30, as well as for week 38. At each time point, blood (via cardiac puncture) and spleens were harvested for immunogenecity analyses. At sacrifice, sera were prepared to determine the antibody titers and splenocytes were prepared to evaluate cellular immune responses.

Serum preparation. Serum was prepared from each blood sample by incubating the blood at room temperature for approximately 4 h to allow for clotting, followed by an overnight incubation at 4°C. The following day, clots were removed and blood was centrifuged at 2,000 × g for 10 min. Supernatants were transferred to sterile tubes, and the serum was stored at −80°C. Sodium azide was added to a final concentration of 0.05% as a preservative to these samples.

Enzyme-linked immunosorbent assay (ELISA). BS-C-1 cells were seeded in 100-mm plates. When cells reached 80 to 90% confluence, they were infected with cAdVaxE(GPs) or cAdVaxE(GPz) at 1 × 10⁶ PFU/ml. Three days postinfected, cells were harvested using a cell scraper, washed once in PBS, and then lysed for 45 min at 4°C in RSB–NP–PMSF buffer (10 mM Tris-HCl [pH 7.5], 10 mM NaCl, 1.5 mM MgCl₂, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride). Lysed cells were centrifuged at 15,000 × g for 5 min at 4°C to remove solid cellular debris. Protein concentrations were quantified by a modified Bradford protein assay according to the manufacturer’s instructions (advanced protein assay, Cytoskeleton, Inc., Denver, CO). Flat-bottomed microtiter plates were coated with cell lysates at 5 μg/ml for cAdVaxE(GPs) lysates and 28 μg/ml for cAdVaxE(GPz) [only medium supernatants were collected for cAdVaxE(GPz)-infected cells, since this vector readily produces SGP that is secreted into the medium]. Coated plates were blocked with 1% bovine serum albumin in Tris-buffered saline (TBS)-Tween.

The sera from vaccinated and control mice were diluted serially in PBS-2.5% nonfat dry milk-0.5% FBS-0.025% Tween 20. Duplicate samples of each serum dilution were added to the prepared ELISA plates. Following incubation of sera, wells were washed with PBS-Tween and then incubated with horseradish peroxidase-conjugated anti-mouse IgG antibody (KPL, Gaithersburg, MD) to detect positive binding of anti-Ebola antibodies. Following aspiration of secondary antibody and TBS-Tween washes, TMB-substrate [Research Diagnostics, Inc., Flanders, NJ] was added. The TMB-S reaction was stopped with 0.5 M HCl. Readings of optical density at 495 nm of each well were measured using the μQuant microtiter plate reader (Bio-tek Instruments, Inc., Winooski, VT). Antibody titers were determined by calculating the dilution of serum that corresponded to a signal of 3 times the background for that particular test. Mouse monoclonal anti-Ebola GP antibodies (provided by USAMRIID) were used as positive controls.

Mouse splenocyte preparations. Splenocytes were isolated from mouse spleens using 70-μm cell strainers (BD Falcon, Franklin Lakes, NJ). Red blood cells were removed using ammonium-chloride-potassium lysing solution (BioSource International, Camarillo, CA). Each individual animal was analyzed independently, with an assay of duplicate samples of 2 × 10⁷ splenocytes for each time point.

Antigenic peptide design and preparation. Cellular immune responses to Ebola GP proteins were detected by incubating splenocytes with overlapping peptide pools (15-mer peptides with a 10-amino-acid overlap) corresponding to both the conserved and heterologous regions of SEBOV and ZEBOV GP (GP targets). Conserved GP peptide pools were generated using the Sudan GP sequence and include the homologous amino-terminal region of Ebola GP1 (Sudan Maleo GP amino acids [aa] 40 to 187, yielding one pool of 20 peptides) and the homologous carboxy terminus of GP2 (Sudan Maleo GP aa 510 to 657, yielding one pool of 20 peptides). Heterologous GP peptide pools were generated for both the Sudan (Maleo) and Zaïre (Zaire-95) GP subtypes within the highly variable central region of GP (aa 313 to 509, yielding a pool of 27 peptides for each subtype). A pool of similarly constructed 15-mer peptides from dengue virus (serotype 2) E protein was used as a negative control and was subtracted as
background. Concanavalin A served as a positive control. Peptides were used at 5 μg/ml final concentration, keeping dimethyl sulfoxide concentration below 0.5% (vol/vol) in all final assay mixtures. All peptides were synthesized by Mimotopes (Victoria, Australia).

**Murine IFN-γ ELISPOT assay.** Peptides (at a final concentration of 5 μg/ml of each peptide per pool) were added to 96-well enzyme-linked immunospot (ELISPOT) plates (Millipore, Bedford, MA) coated with anti-mouse gamma interferon (IFN-γ) antibody (BD Biosciences, San Diego, CA). Splenocytes were then added to corresponding wells at a final concentration of 2 × 10^6 cells/well, in duplicate. After an 18-h incubation at 37°C, the cells were hypotonically lysed, and plates were washed extensively to remove cellular debris. A biotinylated, anti-mouse IFN-γ secondary antibody (BD Biosciences) was added and allowed to incubate at room temperature for 1 h. Following an additional wash step, alkaline phosphatase-labeled avidin (Sigma, St. Louis, MO) was added for 1 h and then the plates were washed again. BCIP-NBT (5-bromo-4-chloro-3-indolyl phosphate–nitroblue tetrazolium) solution (Pierce, Woburn, MA) was added, and spots were quantified using an AID ELISPOT reader (Autoimmun Diagnostika GmbH, Strassberg, Germany).

**Vaccination and challenge of mice.** Mouse-adapted Ebola virus was obtained from Mike Bray (1). Challenge studies were conducted at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID), 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 Guide for the Care and Use of Laboratory Animals (8a). The facility where this research was conducted is fully accredited by the Association for the Care of Laboratory Animals (ACLAP). The facility is also fully accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC). The facility is approved for research involving vertebrate animals by the U.S. Department of Agriculture (USDA) and the U.S. Department of Health and Human Services (DHHS).

**RESULTS**

The ZEBOV, SEBOV, REBOV, and ICEBOV species of EBOV are genetically distinct members of the filovirus family of viruses. Of the four EBOV species, ZEBOV and SEBOV are the two deadliest, as they have each given rise to multiple outbreaks with up to 90% mortality (2). For this reason, it is imperative that an EBOV vaccine be designed to prevent disease caused by both the Zaire and Sudan EBOV species (i.e., a bivalent EBOV vaccine). We have developed three cAdVax-based EBOV vaccines, described below. These vaccines have been genetically designed to express the EBOV antigens from either the Sudan or Zaire virus species or both.

**Construction of a bivalent EBOV vaccine vector—cAdVax vectors expressing GP from two EBOV species.** In order to develop a safe and effective bivalent EBOV vaccine, we have constructed and characterized a bivalent cAdVax-based EBOV vaccine vector, known as cAdVaxE(GPs/z), to express the EBOV GP from both the Sudan and Zaire species of EBOV (Fig. 1a). As additional vaccine candidates and controls, monovalent vaccines expressing single-species GP genes were also developed (Fig. 1b and c). The cAdVax vectors were based on a replication-defective adenovirus vaccine vector platform that differs from other Ad-based vectors in that it contains multiple deletions within the Ad E1, E3, and E4 (except ORF6) genes and multiple insertion sites in the Ad genome (14–16). These modifications enable the vector to accommodate relatively large amounts of exogenous DNA (up to 7 kb) and render the vector deficient for replication.

Each vaccine construct expresses GP from SEBOV, ZEBOV, or both species: cAdVaxE(GPs/z) contains one copy each of the SEBOV GP and ZEBOV GP genes; cAdVaxE(GPs) contains two copies of the SEBOV GP gene; and cAdVaxE(GPz) contains two copies of the ZEBOV GP gene (Fig. 1). Several rounds of single-plaque isolation and purification were completed to ensure that the vaccine preparations were homogeneous and free from contaminants. Vectors were purified using density gradient centrifugation according to previously established protocols (14–16). Purified preparations were titrated on HEK293 cells to determine their infectious activity and scored as PFU per ml. By design, each of these vaccines, upon transduction, should be able to induce cellular expression of their respective EBOV antigens, without the consequence of expressing vector components (i.e., Ad proteins).

**Efficient de novo EBOV GP expression mediated by cAdVax vectors.** After completing construction of the vaccine vectors, it was important to verify that they induce EBOV GP expression upon infection. The SEBOV GP DNA, generously provided by Kevin Anderson, USAMRIID, was modified previously by deletion of the ATG start codon responsible for initiating transcription of the nonstructural secreted form (SGP; ~50 kDa). Therefore, the SEBOV GP was expected to predominantly express the structural GP (~160 kDa), while ZEBOV GP (which retained the native, unmodified sequence) was expected to predominantly express the nonstructural SGP. To confirm that the expected EBOV GP products were being induced by the cAdVax vaccines, Vero cells were infected with the EBOV vaccines cAdVaxE(GPs/z), cAdVaxE(GPz), and cAdVaxE(GPz) at MOIs of 100 and assayed for GP expression by Western blotting (Fig. 2).

The EBOV vaccines were found to mediate high levels of GP expression upon in vitro infection of Vero cells (Fig. 2). As expected with the modified SEBOV GP gene, the SEBOV vectors predominantly favored expression of structural GP over the secreted form. Specifically, cAdVaxE(GPs/z) and cAdVaxE(GPz) efficiently induced 160- and 100-kDa SEBOV GP bands, which correspond well to the sizes of the Golgi-specific GP precursor (160 kDa) (26) and a homodimer of SGP (50 kDa) (19), respectively (Fig. 2a). A faint 110-kDa band may indicate the presence of the endoplasmic reticulum form GP precursor (26).

In contrast, ZEBOV GP was predominantly expressed as SGP (Fig. 2b). This was expected, as the ZEBOV GP gene was unmodified (19). Two distinct bands were visualized at 50 and 100 kDa (Fig. 2b). These two bands correspond well to SGP being expressed as both a monomer and a homodimer. The predominance of the homodimer form of SGP over the monomeric form is also consistent with the literature (20). An additional faint 160-kDa band may also be present in these blots, which may indicate the presence of the Golgi-specific GP precursor as well (Fig. 2b).

All GP isoforms expressed following cAdVaxE(GPs/z), cAdVaxE(GPz), and cAdVaxE(GPz) infections in vitro displayed the typical appearance of a fully glycosylated protein, as the glycoprotein bands were found to be diffuse rather than sharp bands on the Western blot. Additionally, in support of the antigenic differences between SEBOV GP and ZEBOV GP and the need to develop a bivalent vaccine against both EBOV species, the anti-SEBOV GP mouse serum did not cross-react with ZEBOV SGP (Fig. 2a), nor did a monoclonal antibody for ZEBOV GP cross-react with the SEBOV GP (Fig. 2b). Finally, cAdVaxE(GPs/z) efficiently coexpressed
both the SEBOV and ZEBOV GPs, confirming an effective bivalent design.

cAdVaxE vaccines efficiently overexpress two EBOV species glycoproteins both intracellularly and on the cell surfaces of vector-transduced cells. Cell surface expression of GP and intracellular expression of SGP would indicate proper expression, folding, and localization of the vaccine-induced GP isoforms. Western blot analyses indicated that the cAdVaxE(GPs) vaccine induced expression of both GP and SGP (Fig. 2a), while cAdVaxE(GPz) predominantly expressed SGP with little transmembrane GP expression (Fig. 2b). These findings were supported by immunofluorescence assay staining of transduced HeLa cells (Fig. 3). cAdVaxE(GPs) demonstrated mostly cell surface staining (GP) with some cytoplasmic staining (SGP) (Fig. 3c), while cAdVaxE(GPz) staining was more indicative of cytoplasmic staining (SGP) (Fig. 3d). The bivalent vector, cAdVaxE(GPs/z), demonstrated an almost punctate cell surface staining of GP (Fig. 3b).

Vaccination with Ad-based EBOV vaccines induces efficient EBOV-specific antibody responses. It is our belief that cell surface expression of GP and secretion of SGP will mimic EBOV-infected cells and therefore induce neutralizing responses against the naturally formed viral membrane proteins that are identical to those produced in a virus infection. When the envelope proteins of both the Zaire and Sudan EBOV species are included, this should initiate a bivalent neutralizing response (i.e., immunity against two species). In addition, we hypothesize that presentation of the GP and SGP to circulating T cells will induce broadly reactive CMI responses to further strengthen immune protection in the presence of neutralizing responses. In order to test for immune response induction by our vaccine vectors, we injected C57BL/6 mice i.p. with 1 × 10^8 PFU per vaccine of cAdVaxE(GPs/z), cAdVaxE(GPs), or cAdVaxE(GPz). As a negative control, one group was immunized with 1 × 10^8 PFU of a cAdVax-based hepatitis C vaccine, known as HC4, which induces expression of HCV and not EBOV antigens. Mice were boosted with 1 × 10^8 PFU of their respective vaccines at week 16 and week 24 from the primary immunization. Sera and splenocytes from vaccinated mice were harvested biweekly for assay of antibody and CMI activities, respectively.

With the repeated dosing schedule used for vaccination, we would expect an initial primary antibody response after the first injection, followed by a considerable increase in antibody titer after either the first or second boost, indicating induction of a secondary immune response. In order to assay for induction of EBOV-specific antibody, we conducted ELISAs for each vaccination group and the negative control group. As shown in Fig. 4 (filled symbols), each of the EBOV vaccines induced strong antibody responses against their respective species antigens. Secondary immune responses were evident for the cAdVaxE(GPs) and cAdVaxE(GPz) vaccines, particularly after the first boost at week 16, peaking at weeks 18 to 22 postvaccination. Antibody responses from the bivalent cAdVaxE(GPs/z) vaccine seemed to reach maximal levels (6 log titers) following the primary vaccination and were not further induced following the booster injections. In contrast, antibody induction by the control vaccine remained low to undetectable.
Antibody responses to EBOV are species specific, indicating the need for a bivalent vaccine. Both the SEBOV and ZEBOV species are known to cause lethal EBOV outbreaks with significant morbidity and mortality (2). Therefore, the development of a bivalent vaccine with the capability of protecting against both viral species is essential. As SEBOV and ZEBOV are two antigenically distinct species (17–20), we predicted that the monovalent GP vaccines, cAdVaxE(GPs) and cAdVaxE(GPz), would induce antibodies specific to their respective GPs but not to heterologous GP species. This is shown in Fig. 5, where cAdVaxE(GPs) induced strong antibody responses against SEBOV GP, with less potent response to ZEBOV GP. Similarly, cAdVaxE(GPz) induced definitive anti-ZEBOV GP antibody responses with diminished response to SEBOV GP. Both of these differences were found to be statistically significant ($P < 0.05$).

On the other hand, the bivalent vaccine, cAdVaxE(GPs/z), was able to induce antibody responses specific to both EBOV species (Fig. 4a and 5). Even 14 weeks after the final immunization (week 38), both SEBOV- and ZEBOV-specific antibody titers remained high (between 5 log and 6 log titers) (Fig. 5) in these vaccinated mice. Furthermore, the antibody titers induced by the bivalent vaccine appeared to be higher than those induced by the individual monovalent vaccines to their respective species GPs (Fig. 5).

In fact, the difference in anti-ZEBOV titers between the cAdVaxE(GPs/z) vaccine and cAdVaxE(GPz) was found to be statistically significant ($P < 0.05$); however, the anti-SEBOV titers induced by the cAdVaxE(GPs/z) and cAdVaxE(GPz) vaccines were not found to be statistically different. These data appear to suggest a synergistic response when both species GPs are expressed together in a single vaccine construct, further supporting the single-construct bivalent vaccine design.

Antigen synthesis de novo induced CMI responses to EBOV GP antigens. Important players in the cell-mediated immune arm of the adaptive immune system include the cytotoxic T lymphocytes. The role of this subset of T cells is to destroy virus-infected cells and thereby prevent the production of nascent viruses by infected host cells. Therefore, induction of a productive cellular immune response against a viral pathogen is desirable for the development of a protective vaccine. In order to analyze the CMI responses induced by our cAdVax-based EBOV vaccines, we developed an IFN-γ ELISPOT assay using overlapping EBOV GP peptide pools (15-mer peptides with a 10-amino-acid overlap) as cytotoxic-T-lymphocyte targets. A pool of 15-mer peptides derived from dengue virus E protein served as a negative control. Background IFN-γ levels induced by the dengue virus E peptides were subtracted...
from each EBOV GP peptide ELISPOT value to give a background-subtracted value which is represented in Fig. 4 (open symbols). Splenocytes were harvested concurrently with the serum samples assayed in the antibody studies.

Both EBOV-specific CMI and antibody responses appeared to follow a similar time course, as both responses seemed to peak and plateau within a few weeks of each other for each corresponding vaccine (Fig. 4). These data suggest that the cAdVaxE vaccines efficiently induce both humoral and CMI responses to EBOV infection in mice. CMI responses appeared to be particularly high for the bivalent cAdVaxE(GPs/z) vaccine, rising to as high as 300 IFN-γ spot-forming units/10⁶ cells by week 30 (Fig. 4a).

**Vaccinated mice survived lethal Ebola virus challenge.** It is our hypothesis that induction of potent antibody and CMI responses is essential for the induction of protective immunity and that the levels of humoral and cellular immune responses induced by the cAdVaxE-based vaccines would be sufficient for such protection. For challenge studies in mice, a mouse-adapted EBOV strain has been developed (1). Mice challenged with this mouse-adapted EBOV strain consistently develop viremia and die 7 to 8 days after challenge (1, 30, 31). Currently, only a ZEBOV-derived mouse-adapted EBOV strain has been developed (1). Mice challenged with this mouse-adapted EBOV strain consistently develop viremia and die 7 to 8 days after challenge (1, 30, 31).

**TABLE 1. Two mouse strains are protected from lethal challenge with mouse-adapted ZEBOV**

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Vaccine</th>
<th>ELISA GMT</th>
<th>S/T</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>cAdVaxE(GPs)</td>
<td>3.0</td>
<td>10/10</td>
</tr>
<tr>
<td></td>
<td>Control (PBS)</td>
<td>&lt;2.0</td>
<td>0/10</td>
</tr>
<tr>
<td>BALB/c</td>
<td>cAdVaxE(GPs)</td>
<td>2.5</td>
<td>10/10</td>
</tr>
<tr>
<td></td>
<td>Control (PBS)</td>
<td>&lt;2.0</td>
<td>0/10</td>
</tr>
</tbody>
</table>

*a Mice were vaccinated by subcutaneous injection with 1 × 10⁸ PFU of cAdVaxE(GPs) or PBS on days 0 and 35.

* b GMT, geometric mean titer calculated from endpoint titers of prechallenge sera in ELISA using irradiated, sucrose-purified mouse-adapted ZEBOV virions as antigen.

* S/T, survivors/total challenged with 1,000 PFU of mouse-adapted ZEBOV (30,000 LD₅₀) given i.p. on day 65. The mice were observed daily for at least 28 days, and morbidity and mortality were recorded.
guinea pigs with up to four plasmid vectors, each expressing a single-species EBOV GP, in a single injection (23). While these animals survived challenge by ZEBOV, no data were shown to demonstrate that these animals developed either a humoral or a CMI response to any EBOV species other than Zaire. In addition, while the vesicular stomatitis virus-based vaccine was protective against ZEBOV challenge, it was unable to protect cynomolgus macaques from challenge with SEBOV (6). Other current vaccine strategies such as Ebola virus-like particles have demonstrated protective efficacy (27); however, these particles are inefficient to produce and would require cotransfection of several plasmids simultaneously to develop a bivalent vaccine approach. In addition, our vaccine demonstrated 100% protection of two mouse strains against viral challenge, while the alphavirus replicon (10, 30, 31), baculovirus (8), vaccinia virus (4), and DNA plasmid (25, 33) approaches were all found to be only partially protective in small-animal models.

Importantly, there is a distinct difference between our cAdVax-based vaccines and those vaccines based on the first-generation Ad. The major advantage of the cAdVax system over the first-generation Ad vector is the ability to express multiple (up to six) antigens in a single construct. Upon vaccination, all of the antigens carried by the vector will be produced at high levels within the cells transduced at the site of vaccination. We hypothesize that vector-based vaccine gene transfer induces a de novo antigen synthesis, which results in a natural antigen expression and presentation on cell surfaces. This mimics a natural infection by the pathogenic viruses and induces potent immune responses without causing the disease. Vaccines based on antigen synthesis de novo create a major advantage over protein-based subunit vaccines that are only capable of presenting linear epitopes. They also have an advantage over recombinant protein antigen synthesis in eukaryotic cells in which the correct conformation of the glycoproteins that contain the receptor-binding site may be destroyed in the extensive purification processes. In contrast, GP antigens synthesized de novo would theoretically retain the natural conformations and posttranslational modifications of the native GPs and therefore would include intact viral receptor-binding sites, where virus-neutralizing epitopes would be located.

Because we constructed cAdVax vaccines that expressed the GP of the Sudan [cAdVaxE(GPs)] or Zaire [cAdVaxE(GPz)] species of EBOV, as well as the bivalent cAdVaxE(GPs/z) vaccine that expresses the GP of both species of EBOV, we were able to study type-specific and cross-reactive immune responses and the true bivalent immunity against both species of virus. We have clearly shown that immune responses are specific to each species of EBOV, although some level of cross-reactivity between the two different species was observed in ELISA. The significance of the cross-immune responses detected by ELISA in broad immune protection is questionable. It is not known, in natural infections, whether patients who have recovered from one species of EBOV infection would develop protective immune responses against other species, due to the high mortality of the infection and the rareness of these diseases. However, in the nonhuman primate study conducted by Jones et al., monkeys challenged with ZEBOV were not protected against challenge by SEBOV (6), thereby indicating the need for a bivalent vaccine designed to prevent infections by both species. Studies of cross protection have been further complicated by the lack of an effective neutralizing assay (5). It is generally believed that neutralizing assays are not predictive of protection (5), as protective immunity has been observed despite negative results from plaque reduction assays (32).

In addition to antibody responses, the cell-mediated arm of the immune system is critically important in defense against virus infections. Activated T lymphocytes play an essential role in destroying infected cells, preventing viral replication, reducing viral load, and eventually eliminating the infection. In the case of filovirus infections, mortalities often occur before sufficient time is allowed for the activation of CMI responses. We hypothesize that activation of an EBOV-specific CMI response prior to exposure to EBOV would give the cellular arm of the immune system a chance to establish itself and proliferate quickly in the event of an infection.

In our study, we have shown that antigen synthesis de novo can effectively induce CMI responses against the EBOV GPs, based on ELISPOT analyses. We attribute this to the persistent stimulation of the immune system by exogenously induced EBOV antigen production and presentation. In combination with the humoral responses, we believe that this EBOV-specific CMI response will play important roles in protective immunity against EBOV.

Ultimately, induction of protective immune responses against EBOV infections is the main goal for any vaccine strategy. In this study, we were able to demonstrate 100% protection of two genetically distinct strains of mice (C57BL/6 and BALB/c) against a mouse-adapted ZEBOV challenge given at 30,000 times the LD50. Currently, a mouse model for SEBOV has not yet been established, and a nonhuman primate model for SEBOV has also been unavailable until recently (6). In future studies, we plan to include additional challenge tests with SEBOV in nonhuman primates to fully evaluate the bivalent capability of the vaccine. However, because our cAdVaxE(GPs/z) vaccine is capable of inducing immune responses against both SEBOV and ZEBOV that are similar to those the cAdVaxE(GPz) vaccine makes against ZEBOV, we believe that vaccination with cAdVaxE(GPs/z) has the potential to protect animal models from EBOV infections by both species. Further tests will be necessary to determine whether this holds to be true.

To our knowledge, this is the first demonstration of a bivalent EBOV vaccine to coexpress multiple serotype proteins in a single vaccine construct, eliciting efficient humoral and cellular immune responses to both SEBOV and ZEBOV antigens. Among the many advantages of the cAdVax vaccine platform is its ability to express multiple antigens in a single vaccine construct, thereby simplifying the production and approval processes that would be necessary to bring a final Ebola virus vaccine to the public. In establishing a vaccine comprised of a single vaccine vector, this decreases production costs and FDA approval costs as well as ensuring that each transduced cell expresses all incorporated antigens at a 1:1 ratio. Importantly, the cAdVax vaccine demonstrated efficient induction of a protective immune response, demonstrating 100% protection of two strains of mice against lethal EBOV challenge. Taken together, our data suggest that a cAdVax-based multiple antigen vaccine, such as cAdVaxE(GPs/z), represents a promising candi-
date for the development of an effective bivalent vaccine against EBOV infections.

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REFERENCES


