JOURNAL OF VIROLOGY, Mar. 2006, p. 2738–2746 Vol. 80, No. 6
0022-538X/06/$08.00+0 doi:10.1128/JVI.80.6.2738–2746.2006

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

Danher Wang,[1] Nicholas U. Raja,[1] Charles M. Trubey,[1] Laure Y. Juompan,[1] Min Luo,[1] Jan Woraratanadharm,[1] Stephen B. Deitz,[1] Hong Yu,[3] Benjamin M. Swain,[1] Kevin M. Moore,[1] William D. Pratt,[2] Mary Kate Hart,[2] and John Y. Dong[1,3]*

Division of Biodefense Vaccines, GenPhar, Inc., Mount Pleasant, South Carolina 29464-3066[1]; Virology Division, United States Army Medical Research Institute of Infectious Diseases (USAMRIID), Fort Detrick, Frederick, Maryland 21702-5011[2]; and Department of Microbiology and Immunology, Medical University of South Carolina, Charleston, South Carolina 29403[3]

Received 26 October 2005/Accepted 27 December 2005

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

* Corresponding author. Mailing address: GenPhar, Inc., 871 Lowcountry Blvd., Mount Pleasant, SC 29464. Phone: (843) 884-0120. Fax: (843) 884-0601. E-mail: dongj@genphar.com.

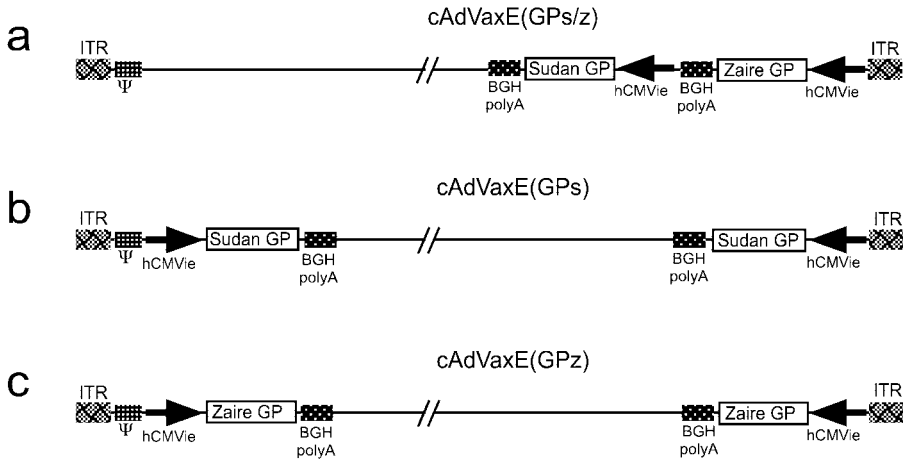

FIG. 1. Structures of the cAdVax vectors expressing multiple EBOV antigens. (a) Bivalent cAdVaxE(GPs/z) vaccine expresses single copies each of the SEBOV and ZEBOV GPs; (b) cAdVaxE(GPs) vaccine expresses two copies of the SEBOV GP; and (c) cAdVaxE(GPz) vaccine expresses two copies of the ZEBOV GP. cAdVax-based EBOV vaccine vectors were developed to express multiple EBOV genes for the purpose of developing an effective and safe Ebola virus vaccine. These vectors were based on a replication-defective cAdVax 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 deletions allow us to accommodate the insertion of gene sequences up to 7 kb in length. cAdVax vectors were constructed as described previously (14–16). ITR, inverted terminal repeat; hCMVie, human cytomegalovirus intermediate/early promoter; BGH polyA, bovine growth hormone polyadenylation site; Ψ, adenovirus packaging signal.

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(GPs/z). This vaccine efficiently expresses both the SEBOV GP 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% cosmic calf serum (HyClone, Logan, UT), while MC57G cells were maintained in Eagle's minimal essential medium supplemented with 10% cosmic calf serum, 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), 10^{-5} M β-mercaptoethanol, 10 mM HEPES, 1% penicillin-streptomycin, and 0.1 mM nonessential amino acids.

Construction of the cAdVax-based EBOV vaccine. The EBOV gene sequences included in the cAdVax vaccines were derived from the Sudan species (Boniface 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 pLAd or pRAd plasmid shuttle vectors. In order to characterize immune responses to EBOV GPs, we subcloned each EBOV antigen into our pLAd and pRAd shuttle vectors to create a series of cAdVax-based EBOV vaccines (Fig. 1). The cAdVax-based EBOV vaccine vector genomes were constructed as described previously (14–16). All adenovirus (Ad) vector genomes were based on a modified Ad5sub360, 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(GPs/z) vaccines] expressed predominantly the structural GP of SEBOV, while cAdVax vectors expressing the native ZEBOV GP sequences [cAdVaxE(GPz) and cAdVaxE(GPs/z) 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 Ad5 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 cytopathic 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 $Na_2HPO_4 \cdot 2H_2O$, and 0.1% [wt/vol] dextrose; adjusted with NaOH to pH 7.5 and filter sterilized) containing 10% glycerol, and stored in liquid N_2. 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%

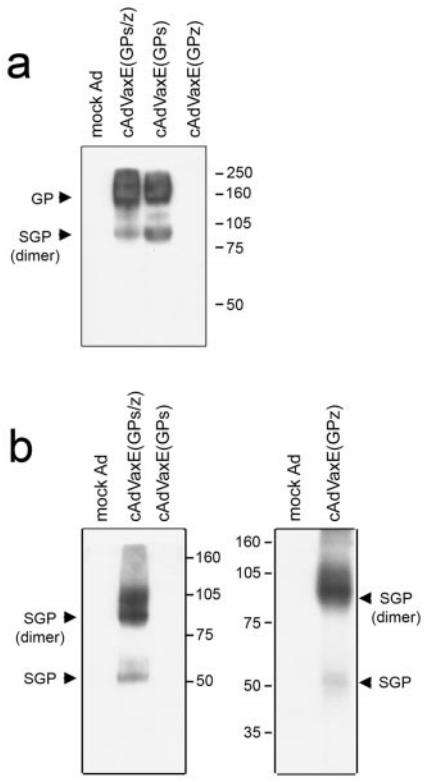

FIG. 2. EBOV vaccine candidates cAdVaxE(GPs/z), cAdVaxE(GPs), and cAdVaxE(GPz) demonstrate efficient GP expression. Vero cells were infected with EBOV vaccines cAdVaxE(GPs/z), cAdVaxE(GPs), and cAdVaxE(GPz). Cell lysates were resolved on a 4 to 15% gradient polyacrylamide gel under denaturing and reducing 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-DA01-AA05.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 Ponceau 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 NaN_3) for 30 min. The membrane was later incubated for 1 h with anti-ZEBOV GP antibody (M-DA01-AA05.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 1.25×10^4 cells/well in a 24-well plate. The next day, cells were infected at an MOI of 100 with cAdVaxE(GPs), cAdVaxE(GPz), cAdVaxE(GPs/z), 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 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/z), 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 Axiovert-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 68 mice each and then immunized intraperitoneally (i.p.) with 1×10^8 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 immunogenicity 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 $2,000 \times g$ 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 1×10^7 PFU/ml. Three days postinfection, cells were harvested using a cell scraper, washed once in PBS, and then lysed for 45 min at 4°C in RSB–NP-40–PMSF buffer (10 mM Tris-HCl [pH 7.5], 10 mM NaCl, 1.5 mM $MgCl_2$, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride). Lysed cells were centrifuged at $15,000 \times g$ 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 reagent; 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 TBS-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-S substrate (Research Diagnostics, Inc., Flanders, NJ) was added. The TMB-S reaction was stopped with 0.5 M HCl. Readings of optical density at 595 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 2×10^6 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 Zaire (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 2×10^5 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 Assessment and Accreditation of Laboratory Animal Care International.

Groups of 10 mice per group were vaccinated on days 0 and 35 by subcutaneous injection with 1×10^8 PFU of cAdVaxE(GPz) or PBS. Mice were bled on day 28 and day 63. Then, mice were transferred to a biosafety level 4 containment area and challenged on day 65 with i.p. inoculation of 1,000 PFU of mouse-adapted ZEBOV (approximately 30,000 times the dose lethal for 50% of adult mice [LD_{50}]). The mice were observed daily for at least 28 days, and morbidity and mortality were recorded. Both C57BL/6 and BALB/c mice were challenged.

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(GPs), 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(GPs) 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(GPs), 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

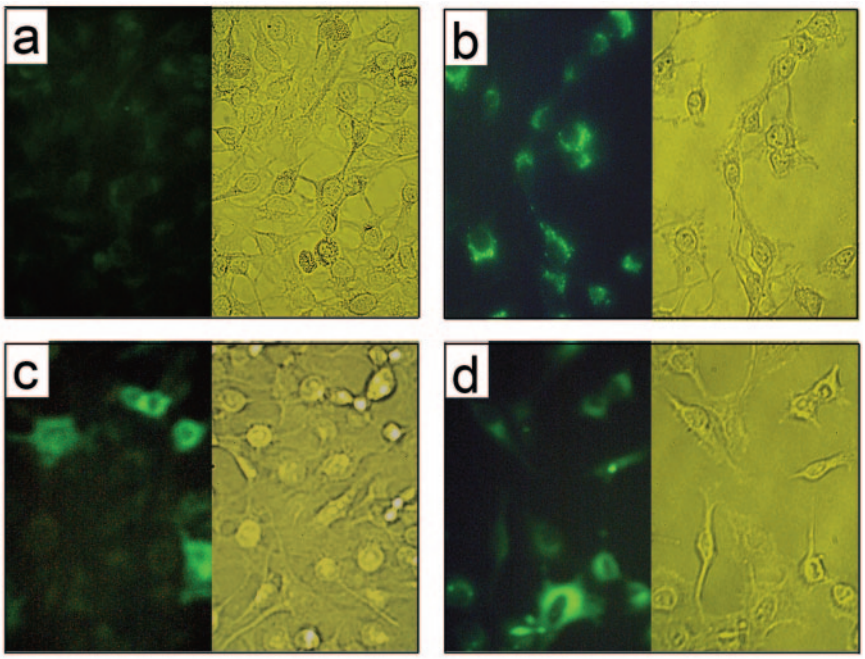

FIG. 3. Adenovirus-based EBOV vaccines efficiently overexpress two serotype EBOV glycoproteins. HeLa cells (1.25×10^5 cells/well) were seeded in 24-well tissue culture plates. The next day, cells were infected at an MOI of 100 with (a) HC4, a control adenovirus vaccine containing HCV envelope protein, (b) cAdVaxE(GPs/z) bivalent vaccine, (c) cAdVaxE(GPs), or (d) cAdVaxE(GPz). Two days postinfection, cells were washed, fixed, and then probed with sera derived from mice vaccinated with either cAdVaxE(GPs) (serum used to probe in panels b and c) or cAdVaxE(GPz) (serum used to probe in panels a, b, and d). Cells were then probed with anti-mouse IgG-FITC secondary antibody and visualized with an Axiovert-25 microscope with an FITC excitation/emission filter set.

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.

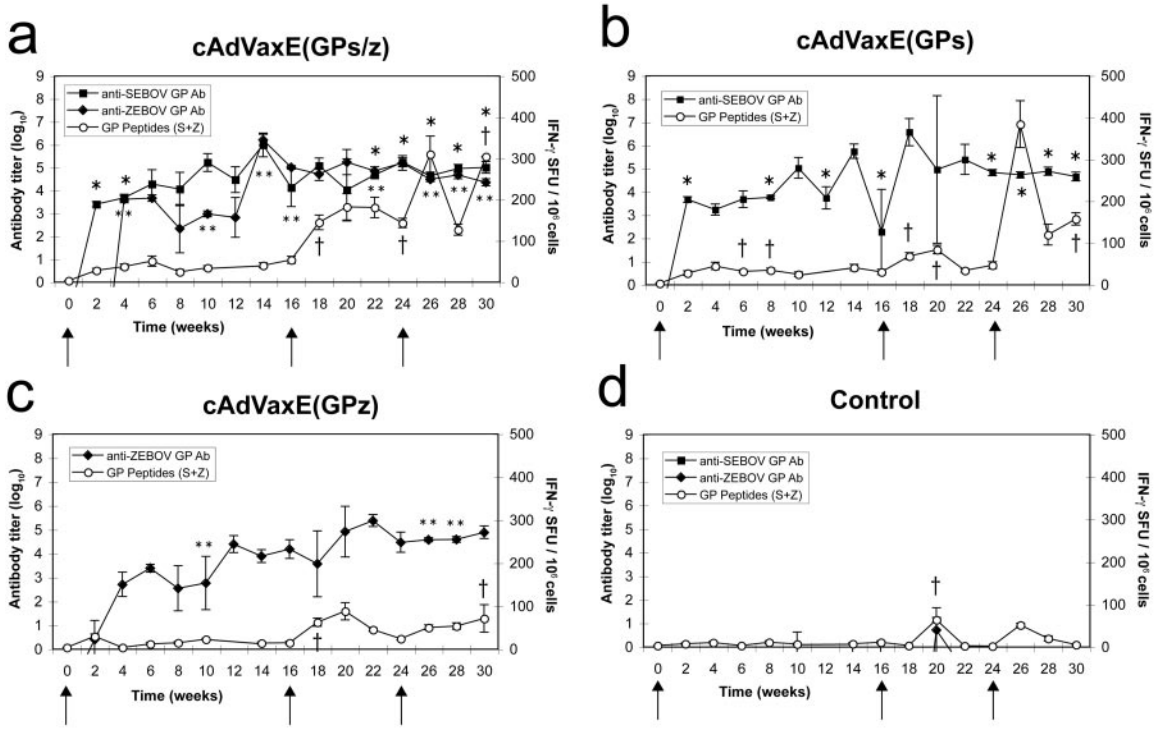

FIG. 4. Immunization of mice with cAdVax-based EBOV vaccines induces EBOV-specific antibody and CMI responses. C57BL/6 mice were vaccinated on weeks 0, 16, and 24 by i.p. injection with 1×10^8 PFU of (a) cAdVaxE(GPs/z), (b) cAdVaxE(GPs), (c) cAdVaxE(GPz), or (d) HC4 control. Arrows indicate vaccination time points. Four mice from each group were euthanized every 2 weeks to assay both antibody and CMI responses. Each animal was analyzed individually for anti-EBOV-specific antibodies by ELISA (filled symbols; see left *y* axis) as well as for EBOV-specific CMI activity by IFN-γ ELISPOT (open symbols; see right *y* axis). Lysates from BS-C-1 cells transduced with cAdVaxE vaccines served as targets for the ELISAs. Peptides derived from EBOV GP sequences (see Materials and Methods) served as immune targets for the ELISPOT assays. IFN-γ spot-forming units induced by control peptides (derived from dengue virus E protein) were subtracted as background. Statistically significant differences ($P < 0.05$) were determined by using a one-tailed, paired *t* test. Ab, antibody; S, Sudan ebolavirus; Z, Zaire ebolavirus; SFU, spot-forming units; *, statistically significant difference in anti-SEBOV GP Ab titers, compared to HC4 control; **, statistically significant difference in anti-ZEBOV GP Ab titers, compared to HC4 control; †, statistically significant difference in CMI responses, compared to dengue virus E peptide controls.

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(GPs) 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

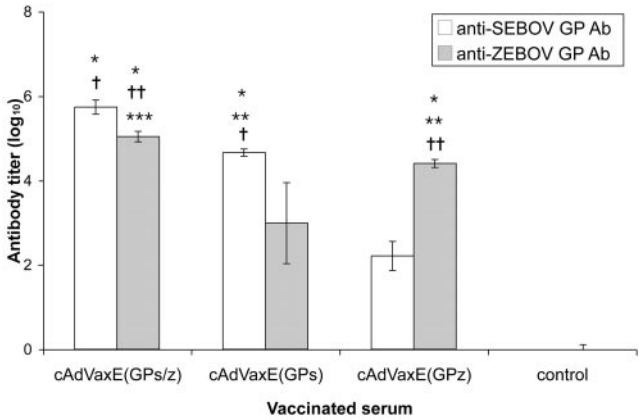

FIG. 5. Demonstration of the bivalency of the cAdVaxE(GPs/z) vaccine, in comparison to the monovalent cAdVaxE(GPs) and cAdVaxE(GPz) vaccines. Mice were vaccinated as described in the legend to Fig. 4. Vaccinated mouse sera from mice immunized with cAdVaxE(GPs/z), cAdVaxE(GPs), cAdVaxE(GPz), or HC4 control were harvested on week 38 and assayed for anti-SEBOV GP and anti-ZEBOV GP antibodies by ELISA. Statistically significant differences ($P < 0.05$) were determined by using a one-tailed, paired *t* test. GP, glycoprotein; SEBOV, *Sudan ebolavirus*; ZEBOV, *Zaire ebolavirus*; Ab, antibody; *, statistically significant difference from HC4 control vaccinations; **, statistical difference between the anti-SEBOV GP and anti-ZEBOV GP antibody titers induced by an individual vaccine; ***, statistical difference between bivalent cAdVaxE(GPs/z) GP titers and cAdVaxE(GPz) anti-ZEBOV GP titers; †, statistically significant difference from cAdVaxE(GPs) anti-ZEBOV GP (heterologous GP) titers; ††, statistically significant difference from cAdVaxE(GPz) anti-SEBOV GP (heterologous GP) titers.

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 Ebola viruses 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/10^6 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 cAdVax-based vaccines would be sufficient for such protection. For challenge studies in mice, a mouse-adapted Ebola virus strain has been developed (1). Mice challenged with this mouse-adapted Ebola virus strain consistently develop viremia and die 7 to 8 days after challenge (1, 30, 31). Currently, only a ZEBOV-derived mouse-adapted Ebola virus is available (1), and therefore the bivalency of the cAdVaxE(GPs/z) vaccine in mice could not be assessed at this time. However, we were able to evaluate the protective efficacy of the cAdVax vaccine design against ZEBOV challenge.

C57BL/6 mice were vaccinated on day 0 and day 35 with 1×10^8 PFU of either cAdVaxE(GPz) or PBS and then challenged on day 65 with 1,000 PFU of mouse-adapted Ebola virus (Table 1). As indicated in Table 1, C57BL/6 mice vaccinated with cAdVaxE(GPz) were 100% protected from lethal Ebola virus challenge (challenge with 30,000 times the LD_{50}), while all control mice succumbed to ZEBOV infection and died. A second challenge experiment was conducted on BALB/c mice under the same experimental conditions (Table 1). The cAdVax vaccine also demonstrated 100% efficacy in this mouse strain, as all 10 out of 10 mice survived challenges, while all control mice again were susceptible to disease and died. The results from these two experiments indicate an efficient induction of a protective immunity in a small-animal model and serve as the first major milestone to the development of a successful vaccine. Therefore, these data indicate great promise for cAdVax-based EBOV vaccines for future studies.

DISCUSSION

Currently, there is no preventative treatment against the deadly hemorrhagic fever caused by EBOV infection. Due to the highly contagious and deadly nature of filoviruses, there is great concern that these lethal agents may be used as biological weapons or terrorism agents against civilized nations, in addition to the fear that these viruses may spread into populated urban areas as a result of increasing modern travel. Consequently, the development of effective EBOV vaccines to prevent the further evolution and spread of EBOV has become a great interest to many research groups.

In this study, we evaluate the first bivalent EBOV vaccine designed to prevent infection by both the Zaire and Sudan species. Our approach differs from other vaccine strategies, which are all monovalent in design and therefore express single antigens from only the Zaire species (3, 5, 6, 8, 10–13, 22, 25, 27–30). Among the many tested approaches in developing EBOV vaccines, vector-mediated antigen transfer, using either the first-generation adenoviral vector (21–23) or a replication-competent vesicular stomatitis virus vector (6), appears to be the most promising. Both studies demonstrated protection of nonhuman primates against ZEBOV challenges. However, one major difficulty that remains in the development of an effective EBOV vaccine is the requirement for a bivalent capability to induce protective immune responses against two EBOV species, Zaire and Sudan, which have been responsible for all human deaths due to EBOV infection thus far.

Sullivan et al. only partially addressed this issue by vaccinating

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

Mouse strain	Vaccine[a]	ELISA GMT[b] (log_{10})	S/T[c]
C57BL/6	cAdVaxE(GPz)	3.0	10/10
	Control (PBS)	<2.0	0/10
BALB/c	cAdVaxE(GPz)	2.5	10/10
	Control (PBS)	<2.0	0/10

[a] Mice were vaccinated by subcutaneous injection with 1×10^8 PFU of cAdVaxE(GPz) 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.

[c] S/T, survivors/total challenged with 1,000 PFU of mouse-adapted ZEBOV (30,000 LD_{50}) 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 LD_{50}. 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.

ACKNOWLEDGMENTS

We acknowledge Russell Bakken, Michael Bailey, and Ana Kuehne for their technical assistance in performing the mouse challenge studies.

This work was supported in part by a grant from the Department of Defense, award no. DAMD17-02-2-0035.

REFERENCES

1. **Bray, M., K. Davis, T. Geisbert, C. Schmaljohn, and J. Huggins.** 1999. A mouse model for evaluation of prophylaxis and therapy of Ebola hemorrhagic fever. J. Infect. Dis. **179**(Suppl. 1)**:**S248–S258.
2. **Centers for Disease Control and Prevention.** 6 May 2005, posting date. Ebola cases and outbreaks. Centers for Disease Control and Prevention, Atlanta, Ga. [Online.] http://www.cdc.gov/ncidod/dvrd/spb/mnpages/dispages/ebotabl.html.
3. **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.
4. **Gilligan, K. J., J. B. Geisbert, P. B. Jahrling, and K. Anderson.** 1997. Assessment of protective immunity conferred by recombinant vaccinia viruses to guinea pigs challenged with Ebola virus, p. 87–92. *In* F. Brown, D. Burton, P. Doherty, J. Mekalanos, and E. Norrby (ed.), Vaccines 97. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
5. **Hart, M. K.** 2003. Vaccine research efforts for filoviruses. Int. J. Parasitol. **33:**583–595.
6. **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.
7. **Leirs, H., J. N. Mills, J. W. Krebs, J. E. Childs, D. Akaibe, N. Woollen, G. Ludwig, C. J. Peters, and T. G. Ksiazek.** 1999. Search for the Ebola virus reservoir in Kikwit, Democratic Republic of the Congo: reflections on a vertebrate collection. J. Infect. Dis. **179**(Suppl. 1)**:**S155–S163.
8. **Mellquist-Riemenschneider, J. L., A. R. Garrison, J. B. Geisbert, K. U. Saikh, K. D. Heidebrink, P. B. Jahrling, R. G. Ulrich, and C. S. Schmaljohn.** 2003. Comparison of the protective efficacy of DNA and baculovirus-derived protein vaccines for EBOLA virus in guinea pigs. Virus Res. **92:**187–193.

8a. National Research Council. 1996. Guide for the care and use of laboratory animals. National Academy Press, Washington, D.C.

9. **Peterson, A. T., D. S. Carroll, J. N. Mills, and K. M. Johnson.** 2004. Potential mammalian filovirus reservoirs. Emerg. Infect. Dis. **10:**2073–2081.
10. **Pushko, P., M. Bray, G. V. Ludwig, M. Parker, A. Schmaljohn, A. Sanchez, P. B. Jahrling, and J. F. Smith.** 2000. Recombinant RNA replicons derived from attenuated Venezuelan equine encephalitis virus protect guinea pigs and mice from Ebola hemorrhagic fever virus. Vaccine **19:**142–153.
11. **Pushko, P., J. Geisbert, M. Parker, P. Jahrling, and J. Smith.** 2001. Individual and bivalent vaccines based on alphavirus replicons protect guinea pigs against infection with Lassa and Ebola viruses. J. Virol. **75:**11677–11685.
12. **Pushko, P., M. Parker, J. Geisbert, D. Negley, A. Schmaljohn, A. Sanchez, P. B. Jahrling, and J. F. Smith.** 1997. Venezuelan equine encephalitis virus replicon vector: immunogenicity studies with Ebola NP and GP genes in guinea pigs. Vaccines **97:**253–258.
13. **Riemenschneider, J., A. Garrison, J. Geisbert, P. Jahrling, M. Hevey, D. Negley, A. Schmaljohn, J. Lee, M. K. Hart, L. Vanderzanden, D. Custer, M. Bray, A. Ruff, B. Ivins, A. Bassett, C. Rossi, and C. Schmaljohn.** 2003. Comparison of individual and combination DNA vaccines for B. anthracis, Ebola virus, Marburg virus and Venezuelan equine encephalitis virus. Vaccine **21:**4071–4080.
14. **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.
15. **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.
16. **Rubinchik, S., J. Woraratanadharm, J. Schepp, and J. Dong.** 2003. Improving the transcriptional regulation of genes delivered by adenovirus vectors. Methods Mol. Med. **76:**167–199.
17. **Sanchez, A., M. P. Kiley, B. P. Holloway, and D. D. Auperin.** 1993. Sequence analysis of the Ebola virus genome: organization, genetic elements, and comparison with the genome of Marburg virus. Virus Res. **29:**215–240.
18. **Sanchez, A., and P. E. Rollin.** 2005. Complete genome sequence of an Ebola virus (Sudan species) responsible for a 2000 outbreak of human disease in Uganda. Virus Res. **113:**16–25.
19. **Sanchez, A., S. G. Trappier, B. W. Mahy, C. J. Peters, and S. T. Nichol.** 1996. The virion glycoproteins of Ebola viruses are encoded in two reading frames and are expressed through transcriptional editing. Proc. Natl. Acad. Sci. USA **93:**3602–3607.
20. **Sanchez, A., Z. Y. Yang, L. Xu, G. J. Nabel, T. Crews, and C. J. Peters.** 1998. Biochemical analysis of the secreted and virion glycoproteins of Ebola virus. J. Virol. **72:**6442–6447.
21. **Sullivan, N., Z. Y. Yang, and G. J. Nabel.** 2003. Ebola virus pathogenesis: implications for vaccines and therapies. J. Virol. **77:**9733–9737.
22. **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.
23. **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.
24. **Turell, M. J., D. S. Bressler, and C. A. Rossi.** 1996. Short report: lack of virus replication in arthropods after intrathoracic inoculation of Ebola Reston virus. Am. J. Trop. Med. Hyg. **55:**89–90.
25. **Vanderzanden, L., M. Bray, D. Fuller, T. Roberts, D. Custer, K. Spik, P. Jahrling, J. Huggins, A. Schmaljohn, and C. Schmaljohn.** 1998. DNA vaccines expressing either the GP or NP genes of Ebola virus protect mice from lethal challenge. Virology **246:**134–144.
26. **Volchkov, V. E., H. Feldmann, V. A. Volchkova, and H. D. Klenk.** 1998. Processing of the Ebola virus glycoprotein by the proprotein convertase furin. Proc. Natl. Acad. Sci. USA **95:**5762–5767.
27. **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.
28. **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.
29. **Watanabe, S., T. Watanabe, T. Noda, A. Takada, H. Feldmann, L. D. Jasenosky, and Y. Kawaoka.** 2004. Production of novel Ebola virus-like particles from cDNAs: an alternative to Ebola virus generation by reverse genetics. J. Virol. **78:**999–1005.
30. **Wilson, J. A., M. Bray, R. Bakken, and M. K. Hart.** 2001. Vaccine potential of Ebola virus VP24, VP30, VP35, and VP40 proteins. Virology **286:**384–390.
31. **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.
32. **Wilson, J. A., M. Hevey, R. Bakken, S. Guest, M. Bray, A. L. Schmaljohn, and M. K. Hart.** 2000. Epitopes involved in antibody-mediated protection from Ebola virus. Science **287:**1664–1666.
33. **Xu, L., A. Sanchez, Z. Yang, S. R. Zaki, E. G. Nabel, S. T. Nichol, and G. J. Nabel.** 1998. Immunization for Ebola virus infection. Nat. Med. **4:**37–42.