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*Clin. Vaccine Immunol.* 2013, 20(1):1. DOI:  
10.1128/CVI.00280-12.  
Published Ahead of Print 24 October 2012.

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# An Adenovirus-Vectored Nasal Vaccine Confers Rapid and Sustained Protection against Anthrax in a Single-Dose Regimen

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***Bacillus anthracis* is the causative agent of anthrax, and its spores have been developed into lethal bioweapons. To mitigate an onslaught from airborne anthrax spores that are maliciously disseminated, it is of paramount importance to develop a rapid-response anthrax vaccine that can be mass administered by nonmedical personnel during a crisis. We report here that intranasal instillation of a nonreplicating adenovirus vector encoding *B. anthracis* protective antigen could confer rapid and sustained protection against inhalation anthrax in mice in a single-dose regimen in the presence of preexisting adenovirus immunity. The potency of the vaccine was greatly enhanced when codons of the antigen gene were optimized to match the tRNA pool found in human cells. In addition, an adenovirus vector encoding lethal factor can confer partial protection against inhalation anthrax and might be coadministered with a protective antigen-based vaccine.**

Anthrax is a naturally occurring bacterial disease caused by *Bacillus anthracis* infection, manifested in cutaneous, gastrointestinal, or inhalational form (1). Malicious dissemination of anthrax spores as an airborne bioweapon is a major biodefense concern since inhalation of anthrax spores is highly lethal (2). The harmful impact of anthrax on health is attributed to the production of a poly- $\gamma$ -D-glutamic acid (PGA) capsule, which confers resistance to phagocytosis postinfection (3), and the production of a tripartite exotoxin consisting of protective antigen (PA), lethal factor (LF), and edema factor (EF) (4). Although antibiotic therapy is effective against anthrax, latent spores may germinate many weeks postexposure, requiring prolonged administration of the antibiotic for full therapeutic effect (5, 6). Vaccination is the most effective medical intervention against anthrax, either as a preventative or in conjunction with antibiotic treatment for conferring postexposure prophylaxis (1).

The currently licensed human anthrax vaccine in the United States is anthrax vaccine adsorbed (AVA; BioThrax), which is produced by adsorbing cell-free filtrates of microaerophilic cultures of the *B. anthracis* V770-NP1-R strain onto aluminum hydroxide (1). The principal antigenic component of AVA is PA with undefined amounts of LF and EF (7). AVA requires five intramuscular injections over 18 months plus annual boosters to maintain the level of protection (8). The administration of AVA is associated with ~1% local, as well as systemic, adverse reactions (8). Although vaccination by injection of AVA confers complete protection against inhalation anthrax in animal models (9), it is unclear how well AVA can protect humans against inhalation anthrax (2). A variety of next-generation anthrax vaccines designed to address these issues are currently in development. Ideally, a new anthrax vaccine should be suitable to rapid mass manufacturing, mass administration by nonmedical personnel with minimal side effects, and preferably effective in a single-dose regimen.

Nasal administration of nonreplicating human adenovirus serotype 5 (Ad5)-vectored vaccines mimic a route of natural infection and stimulate both humoral and cellular immunity systemically and on mucosal surfaces (10–12). These features make them

ideal for the purpose of intranasal (i.n.) vaccination against pathogens.

We have shown previously that mice are protected against tetanus by i.n. immunizations with an Ad5 vector encoding tetanus toxin C fragment (13). We have shown that in the presence of strong preexisting anti-Ad5 immunity humans can be safely and effectively immunized with an Ad5-vectored nasal influenza vaccine (14). We provide in the present study experimental data that demonstrate that A/J mice with preexisting immunity to Ad5 can be immunized and protected by a single i.n. administration with an Ad5-vectored anthrax vaccine (AdVAV) encoding a codon-optimized PA gene. In addition, i.n. administration of an Ad5 vector expressing LF induces limited protection against spore infection and might be used in conjunction with a PA-based vaccine.

## MATERIALS AND METHODS

**Cell culture, medium, and reagents.** PER.C6 cells were provided by CruCell (Netherlands) and cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 10 mM MgCl<sub>2</sub>. Contaminant-free human embryonic kidney 293 (HEK293) cells (Medicine BioServices, Inc., La Jolla, CA) were maintained in RPMI 1640 supplemented with 10% FBS. RAW 264.7 mouse macrophage cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA) and cultured in DMEM supplemented with 10% FBS. The cells were incubated at 37°C in 10% CO<sub>2</sub>. Cell culture media and reagents were purchased from Mediatech, Inc. (Herndon, VA), and FBS was purchased from Serologicals Corp. (Norcross, GA).

Received 8 May 2012 Returned for modification 2 July 2012

Accepted 9 October 2012

Published ahead of print 24 October 2012

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doi:10.1128/CVI.00280-12

**Construction and production of adenoviral vectors.** (i) **Construction of adenoviral vectors encoding non-codon-optimized gene sequences: AdPAD4, AdPA63, AdPA83, AdLFn, and AdmLF7.** The full-length non-codon-optimized PA gene encoding PA83 and its signal peptide sequence was PCR amplified with template DNA prepared from a Sterne strain (34F2) of *B. anthracis* by using the specific primers 5'-GGA TTCGGTACCACCATGAAAAACGAAAAGTGTTAATACCA-3' and 5'-G AATTCTCTAGACCTTATCCTATCTCATAGCCTTTTTAGAA-3' and cloned into a pAdApt shuttle vector to generate a pAdApt/PA83 construct. pAdApt/PAD4 (encoding domain 4 of PA which includes C-terminal last 139 amino acids of the PA) was provided by Charles Turnbough (University of Alabama, Birmingham, AL). pAdApt/PA63 (encoding a 63-kDa active form of PA that includes amino acids 175 to 764 of the PA), pAdApt/LFn (encoding amino acids 10 to 254 of LF, a truncated version of LF that retained the N-terminal PA-binding domain without the catalytic domain of LF [15]), and pAdApt/mLF7 (encoding the full-length but atoxic *B. anthracis* LF, which has the E687C substitution [16]) were provided by Darrell Galloway (Ohio State University, Columbus, OH). The AdPAD4, AdPA63, AdPA83, AdLFn, and AdmLF7 viral vector seeds were created by cotransfection of the pAdApt/PAD4, pAdApt/PA63, pAdApt/PA83, pAdApt/LFn, and pAdApt/mLF7 with an Ad5 backbone plasmid pAdEasy-1 into the PER.C6 packaging cells (17).

(ii) **Construction of adenoviral vector encoding codon-optimized gene sequences, AdtPA83hu, AdtLFnhu, and AdPA83hu.** The tPA83hu fragment, an amino-terminal human tissue plasminogen activator (tPA) leader sequence, followed with a codon-optimized *B. anthracis* PA83 gene (without the PA signal peptide sequence), and the tLFnhu fragment, the tPA leader sequence followed by a codon-optimized fusion gene of the LFn fragment (encoding amino acids 1 to 254) and *Yersinia* V fragment were amplified by using a forward primer 5'-TAAGGGAAGCTTGCTAG CCACCATGGATGCAATGAAGAGA-3' and a reverse primer 5'-ATGG CTGTAACTAGAAGGCACAGCAGATCTTCA-3', which incorporate the HindIII, NheI, and HpaI cleavage sites, with VR1020/humanizedPA83 and VR1020/humanizedLFCrVLFn plasmids as DNA templates, respectively. The PCR generated tPA83hu fragment was digested with HindIII and HpaI and cloned into the HindIII and HpaI sites of a pAd shuttle vector, and the PCR-generated tLFnhu fragment was digested with NheI and HpaI and cloned into the NheI and HpaI sites of a pAd shuttle vector to construct pAdtPA83hu and pAdtLFnhu, respectively. A codon-optimized PA83 gene with its signal peptide sequence (but not the tPA leader sequence) was synthesized and cloned into HindIII and XbaI sites of the pAd shuttle vector to generate a pAdPA83hu. The pAdtPA83hu, pAdtLFnhu, and pAdPA83hu plasmids were used for generation of AdtPA83hu, AdtLFnhu, and AdPA83hu vectors as described previously (18).

(iii) **Production of adenoviral vectors.** All Ad5 vectors were propagated on PER.C6 cells and purified by ultracentrifugation over a cesium chloride gradient as described previously (13). The purified Ad5 vectors were sterilized by 0.22- $\mu$ m-pore-size filtration and stored at 4°C or -20°C in A195 adenoviral storage buffer (19). Ad5 vector titers were determined by using an Adeno-X rapid titer kit (Clontech, Mountain View, CA) on HEK293 cells. All of the insertions of above Ad5 vectors were checked by partial DNA sequencing (The Genomics Core Facility of the Heflin Center for Human Genetics, University of Alabama at Birmingham).

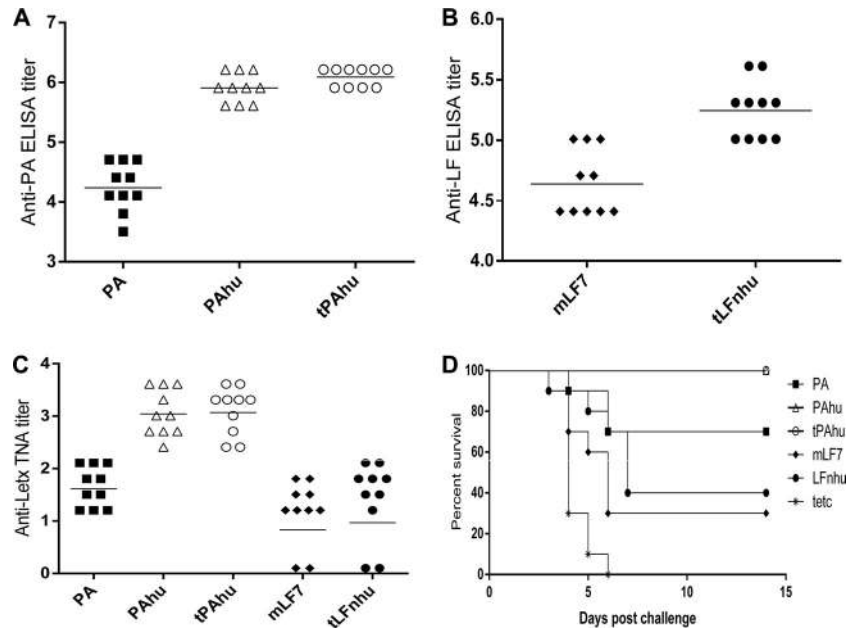
**Animal experiments.** The *B. anthracis* Sterne 34F2 strain (Colorado Serum Co., Denver, CO) is nonencapsulated (pXO2-) but toxigenic (pXO1+) with PA and LF expressed from the pXO1 plasmid. Live *B. anthracis* Sterne spores were mass produced and purified through gradients of Renografin-60 (Bracco Diagnostic, Princeton, NJ) as described previously (20). A/J mouse vaccinations with adenoviral constructs, followed by *B. anthracis* Sterne spore challenge, were performed at the Battelle Memorial Institute (Columbus, OH; studies 613-G005625, 649-G005625, and 675-G005625) and at the University of Alabama at Birmingham (Birmingham, AL), respectively. Briefly, young (2- to

3-month-old) female A/J mice (Jackson Laboratory, Bar Harbor, ME) were immunized by i.n. inoculation of 30 to 50  $\mu$ l of Ad5 vectors into one of the nostrils of an anesthetized mouse. Blood samples were collected pre- and postvaccination and stored at -20°C until analyzed. Bronchoalveolar lavage (BAL) was taken by flushing the lungs with 1.0 ml of phosphate-buffered saline (PBS) and stored at -20°C until analyzed. Immunized A/J mice were challenged by i.n. instillation of  $5 \times 10^5$  CFU of *B. anthracis* Sterne spores ( $\sim 50 \times$  the 50% lethal dose [LD<sub>50</sub>]) in a volume of 10  $\mu$ l as previously described (20). Animals were maintained in the animal facility at the Battelle Memorial Institute and University of Alabama at Birmingham. Daily care was provided by trained technicians at Battelle Memorial Institute and University of Alabama at Birmingham Animal Resource Center. Challenge studies were performed in designed challenge rooms. All experiments in mice were performed according to institutional guidelines.

**Anti-PA and anti-LF IgG and IgA antibody titers.** Anti-PA and anti-LF immunoglobulin G (IgG) titers in mouse serum samples were determined by enzyme-linked immunosorbent assay (ELISA) using purified PA or LF protein (List Biological Laboratories, Campbell, CA) as the capture antigen. Briefly, 96-well plates were coated with 100 ng of purified PA or LF protein/well dissolved in PBS at 4°C overnight. Plates were washed with Tris-buffered saline (TBS; 50 mM Tris-HCl, 0.15 M NaCl [pH 7.3]) and blocked with 1% bovine serum albumin in TBS. The serum samples and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Promega Corp., Madison, WI) were incubated sequentially on the plates with extensive washing between incubations. All incubations were carried out at 37°C for 1 h. The presence of bound antibody was detected after 10 min of incubation in the presence of  $\alpha$ -phenylenediamine substrate (Sigma), and the absorbance was read at 490 nm using a Molecular Devices model Precision microplate reader. The endpoint was determined as the dilution of serum producing the same optical density at 490 nm as a 1/100 dilution of preimmune serum. Sera negative at the lowest dilution tested were assigned endpoint titers of 1. Titers of anti-PA and anti-LF IgA in mouse BAL samples were measured by ELISA as described above. The second antibody used was HRP-conjugated goat anti-mouse IgA (Southern Biotech, Birmingham, AL).

**Anti-Letx neutralizing antibody titers.** Serum samples were analyzed for anti-lethal toxin (anti-Letx) neutralizing antibodies as described for a toxin neutralization assay (21) with modifications. RAW 264.7 mouse macrophage cells were placed in flat-bottom 96-well microtiter plates at a concentration of  $5 \times 10^4$  cells/well in culture medium incubated for 24 h at 37°C. Serum samples from each mouse were serially diluted and incubated with an equal volume of Letx (200 ng of PA/ml plus 200 ng of LF/ml in cell culture medium) for 1 h at room temperature to allow neutralization to occur. Media from the cells in 96-well plates were aspirated and replaced with the serum-Letx mixture at 100  $\mu$ l/well. Cells in control wells were incubated with 100  $\mu$ l of medium only or Letx without mouse serum. After  $\sim 20$  h of incubation at 37°C, the cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT) assay as described previously (20). Samples in duplicate were read on a Precision microplate reader (Molecular Devices) at 490 nm. The survival of control cells without exposure to Letx was arbitrarily defined as 100% protection, and the reading of the Letx-only wells was defined as 0% protection. The neutralization titer was determined as the reciprocal of the highest serum dilution resulting in 50% protection of the cells.

**Anti-Ad5 neutralizing antibody assay.** Anti-Ad5 neutralizing antibody titers were measured by incubating diluted serum samples with wild-type (wt) Ad5 as described previously (13). Briefly, 293 cells were placed in flat-bottom 96-well microtiter plates at a concentration of  $2.5 \times 10^4$  cells/well in culture medium incubated for 24 h at 37°C. Serum samples from each mouse were serially diluted in serum-free culture medium. Then, 60  $\mu$ l of each dilution was mixed with  $1.7 \times 10^4$  infectious units (IFU) of wt Ad5 in 60  $\mu$ l of medium containing 2% FBS with antibiotic for 1 h at room temperature to allow neutralization to occur. Media from the cells in 96-well plates were aspirated and replaced with 100  $\mu$ l of serum-



**FIG 1** Comparison of immune responses in A/J mice induced by Ad5 vectors encode codon-optimized and non-codon-optimized PA or LF genes. (A to C) Anti-PA IgG ELISA titer (A), anti-LF IgG ELISA titer (B), and anti-Letx neutralizing antibody titer (C) in mouse serum were compared 4 weeks after a single i.n. immunization with AdPA83 (PA), AdPA83hu (PAhu), AdtPA83hu (tPAhu), AdmLF7 (mLF7), and AdtLFnhu (tLFnhu) at a dose of  $10^7$  IFU per vector. All mice immunized with Adtetc control vectors and all preimmune sera had anti-PA or anti-LF IgG ELISA titers of  $\leq 100$  and had anti-Letx neutralizing antibody titers of  $< 8$ . (D) Survival of vaccinated A/J mice against a  $50 \times \text{LD}_{50}$  *B. anthracis* Sterne spore challenge. The results of anti-PA and anti-LF IgG ELISAs and anti-Letx neutralizing assays are plotted as the  $\log_{10}$  titers. Bars are geometric mean of the anti-PA and anti-LF ELISA titers or the anti-Letx neutralizing antibody titers.

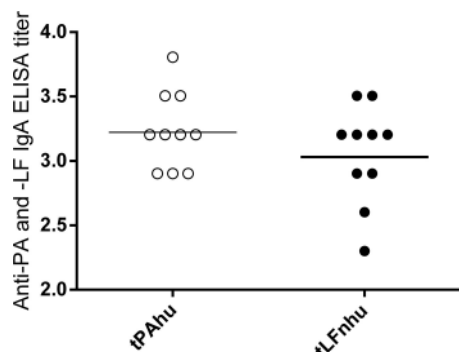
Ad5 mixture/well. The cells in control wells were incubated with  $100 \mu\text{l}$  of medium only or wt Ad5 without mouse serum. After 2 h of incubation at  $37^\circ\text{C}$ , the serum-Ad5 mixture was replaced with cell culture medium. Cell viability at 4 days postinfection was determined by MTT assay. The survival of control cells without exposure to Ad5 was arbitrarily defined as 100% protection, and the readings of the Ad5-only wells were defined as 0% protection. Neutralization titer was determined as the reciprocal of the highest serum dilution resulting in 50% protection of the cells.

**Statistical analysis.** All statistical analysis was performed using GraphPad Prism version 5.04 (GraphPad Software, San Diego, CA). Log-rank (Mantel-Cox) tests were performed for comparing survival curves and the mean times to death (MTD) in challenged animals. One-way

analysis of variance with Tukey's multiple-comparison post tests were performed to compare anti-Letx neutralizing antibody titers and anti-PA and anti-LF IgG ELISA titers. Statistical significance was set at  $P < 0.05$ .

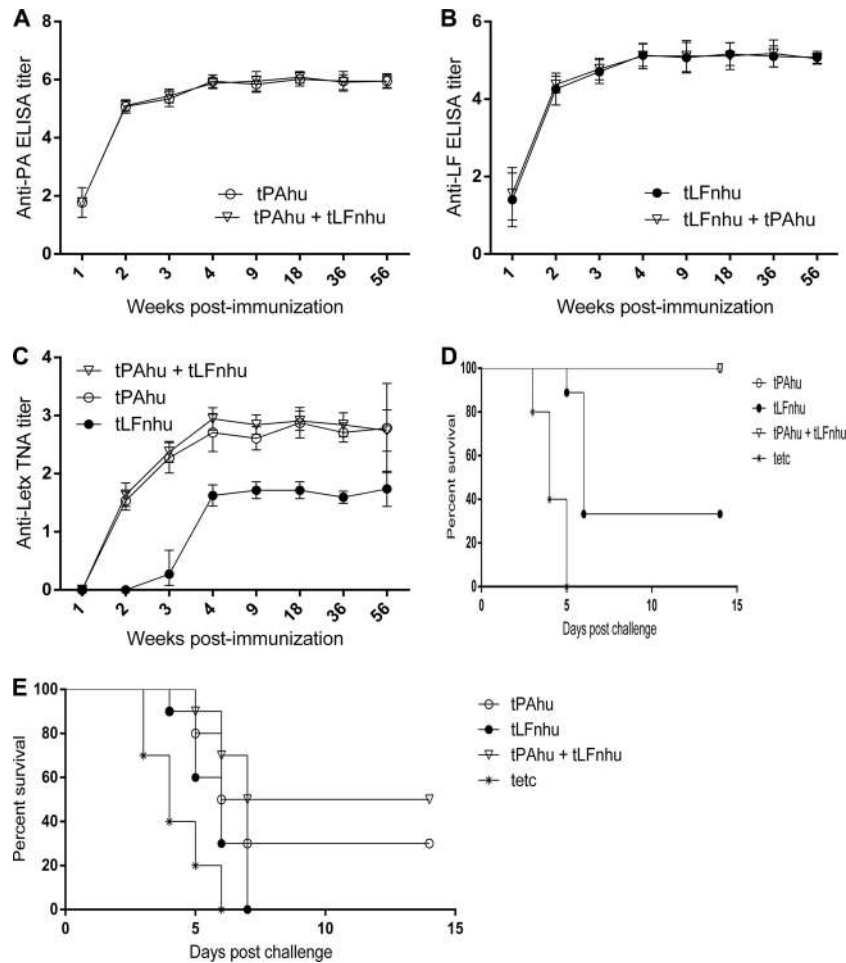
## RESULTS

**Codon-optimized Ad5-vectored anthrax vaccines offer better protection against anthrax spore challenges than their cognate counterparts.** In developing the Ad5-vectored anthrax vaccines, five Ad5 vectors—AdPAD4, AdPA63, AdPA83, AdLFn, and AdmLF7—encoding non-codon-optimized DNA sequence for subdomains and full length of PA or LF proteins were constructed. Animal experiments indicated that the Ad5 vectors encoding full-length PA or LF were able to achieve higher levels of serum antigen-specific IgG antibodies, stronger anti-Letx neutralizing antibody responses, and better protection against lethal anthrax spore challenges than their truncated counterparts. However, only partial protection was afforded against a  $5 \times 10^5$  CFU *B. anthracis* Sterne spore ( $\sim 50 \times \text{LD}_{50}$ ) challenge after a single i.n. immunization with  $10^7$  IFU of AdPA83, the most immunogenic vaccine construct, per mouse (Vaxin, Inc., unpublished data). To improve the potency of Ad5-vectored anthrax vaccines, codon optimization of PA and LF genes and fusion of the tPA leader sequence approaches were applied to generate AdtPA83hu, AdtLFnhu, and AdPA83hu. The relative immunogenicities among these Ad5 vectors encoding codon-optimized and non-codon-optimized PA and LF were then assessed by i.n. vaccination of groups A/J mice (10 mice per group) with  $10^7$  IFU of AdPA83, AdPA83hu, AdtPA83hu, AdmLF7, and AdtLFnhu, respectively, per mouse. A recombinant Ad5 vector (Adtetc encoding tetanus C fragment) served as an Ad5 vector control. Analysis of the sera by ELISA showed that the geometric mean titers of anti-PA IgG antibody



**FIG 2** Anti-PA and anti-LF IgA ELISA titers in the BAL fluid were measured 4 weeks after a single i.n. immunization with AdtPA83hu (tPAhu) or AdtLFnhu (tLFnhu) at a dose of  $10^7$  IFU per vector. All mice immunized with Adtetc vector controls and all preimmune sera had anti-PA and anti-LF IgA ELISA titers of  $\leq 100$ . The results of anti-PA and anti-LF IgA ELISAs are plotted as the  $\log_{10}$  titers. Bars are geometric means of the anti-PA or anti-LF ELISA titers.



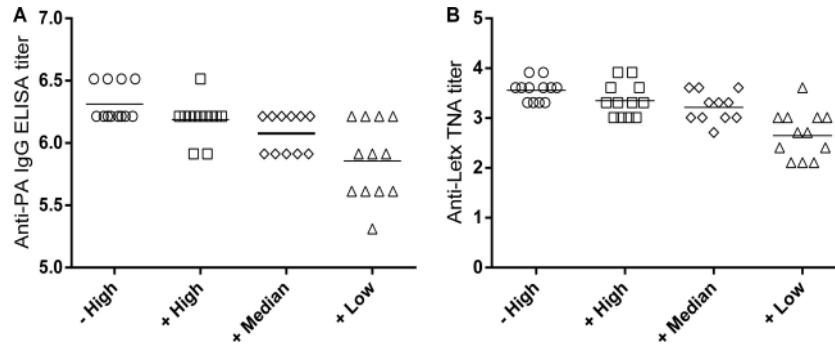


**FIG 3** Kinetics of immune responses in A/J mice. (A to C) The anti-PA IgG ELISA titer (A), anti-LF IgG ELISA titer (B), and anti-Letx neutralizing antibody titer (C) in mouse serum were measured at the indicated time points after a single i.n. immunization with  $10^7$  IFU of AdtPA83hu (tPAhu),  $10^7$  IFU of AdtLFnhu (tLFnhu), or  $10^7$  IFU of AdtPA83hu plus  $10^7$  IFU of AdtLF4hu (tPAhu+tLFnhu). All mice immunized with  $10^7$  IFU of Adtetc vectors and all preimmune sera had anti-PA and anti-LF IgG ELISA titers of  $\leq 100$  and had anti-Letx neutralizing antibody titers of  $< 8$ . The results of anti-PA and anti-LF IgG ELISAs and anti-Letx neutralizing assays are plotted as the  $\log_{10}$  titers. All of the data are shown as geometric means with 95% confidence intervals of 8 to 10 mice per group. (D and E) Protection of A/J mice against a  $50 \times LD_{50}$  *B. anthracis* Sterne spore challenge at 2 weeks (E) and 56 weeks (D) postimmunization.

induced by AdPA83hu and AdtPA83hu were 45.3 and 68.6 times that induced by AdPA83 ( $P < 0.001$  for both AdPA83hu versus AdPA83 and AdtPA83hu versus AdPA83;  $P > 0.05$  for AdtPA83hu versus AdPA83hu) (Fig. 1A). The geometric mean titers of anti-LF IgG antibody stimulated by AdtLFnhu were 4.0 times that induced by AdmLF7 after 4 weeks with a single i.n. administration (Fig. 1B). To determine the functionality of antibodies to PA and LF elicited by the immunization, the abilities of sera to protect mouse macrophage cells from Letx mediated lysis were measured. As shown in Fig. 1C, the highest anti-Letx antibody titers were detected in mice immunized with AdtPA83hu. Both AdtPA83hu and AdPA83hu could induce significantly higher anti-Letx antibody titers than did AdPA83 ( $P < 0.001$  for both AdPA83hu versus AdPA83 and AdtPA83hu versus AdPA83;  $P > 0.05$  AdtPA83hu versus AdPA83hu). Furthermore, the anti-LF antibodies elicited by both AdtLFnhu and AdmLF7 were able to inhibit the cytotoxic effects of Letx (Fig. 1C). To test the effectiveness of Ad5-vectored anthrax vaccines with regard to protection against anthrax infection, immunized mice were challenged with  $50 \times LD_{50}$  *B. anthracis* Sterne spores after 4

weeks vaccination (Fig. 1D). All mice receiving the control Adtetc died within 6 days, and 100% of the mice vaccinated with AdPA83hu and AdtPA83hu survived the spore challenge. Protection was also achieved in 70, 30, and 40% of the mice vaccinated with AdPA83, AdmLF7, and AdtLFnhu, respectively ( $P > 0.05$  for both AdPA83hu versus AdPA83 and AdtPA83hu versus AdPA83;  $P < 0.001$  for both AdPA83 versus Adtetc and AdtLFnhu versus Adtetc;  $P < 0.05$  AdmLF7 versus Adtetc). These results indicate that optimization of codon usage improved the protection potency of an AdVAV and fusion with the tPA leader sequence may further enhance its immunogenicity.

**Intranasal vaccination with Ad5-vectored anthrax vaccines induces secretory IgA antibodies.** Intranasal vaccination is an effective approach for optimal protection of mucosal surfaces by inducing mucosal and systemic immunity. To test the feasibility of AdVAVs for induction of mucosal immunity, groups of A/J mice (10 mice per group) were immunized with  $10^7$  IFU of AdtPA83hu, AdtLFnhu, or Adtetc/mouse. Mouse BAL samples from immunized mice were collected at 4 weeks postvaccination and assessed



**FIG 4** Immunogenicity of Ad-vectored nasal anthrax vaccines in the presence of preexisting anti-Ad5 vector immunity. The A/J mice were first i.n. inoculated with  $10^7$  IFU of wt Ad5 per mouse and then after 2 weeks were i.n. immunized with  $10^8$  IFU of AdtPA83hu plus  $5.4 \times 10^6$  IFU of AdmInhAhu (+High),  $10^7$  IFU of AdtPA83hu plus  $5.4 \times 10^5$  IFU of AdmInhAhu (+Median), or  $10^6$  IFU of AdtPA83hu plus  $5.4 \times 10^4$  IFU of AdmInhAhu (+Low). One group of mice without Ad5 priming were immunized with  $10^8$  IFU of AdtPA83hu plus  $5.4 \times 10^6$  IFU of AdmInhAhu (-High) as controls. Anti-PA IgG ELISA titers (A) and anti-Letx neutralizing antibody titers (B) were determined at 4 weeks postimmunization. The results of the anti-PA IgG ELISAs and anti-Letx neutralizing assays are plotted as  $\log_{10}$  titers. Bars indicate geometric means of the anti-PA ELISA titers or anti-Letx neutralizing antibody titers.

by ELISA. As shown in Fig. 2, strong anti-PA and anti-LF secretory IgA antibodies were induced by AdtPA83hu and AdtLFnhu, respectively (Fig. 2).

**Intranasal immunization of Ad-vectored anthrax vaccines elicits partial protective immunity within 2 weeks, and the robust immunity lasts at least 1 year.** To determine the kinetics and durability of the immune response, groups of A/J mice (10 mice per group) with a single i.n. administration of  $10^7$  IFU of AdtPA83hu, AdtLFnhu, or both/mouse were evaluated. Significant levels of anti-PA IgG antibody (Fig. 3A), anti-LF IgG antibody (Fig. 3B), and anti-Letx antibody (Fig. 3C) were detected after 2 weeks immunization, and the antibody titers increased and peaked around week 4 and remained unchanged to the time of spore challenge at week 56 ( $P > 0.05$  for the anti-Letx antibody titers at 4 weeks postvaccination versus the anti-Letx antibody titers at 56 weeks postvaccination in animals immunized with AdtPA83hu, AdtLFnhu, or both). All animals vaccinated with AdtPA83hu and AdtPA83hu+AdtLFnhu and three of nine mice immunized only with AdtLFnhu survived the challenge (Fig. 3D). To determine the onset of protective immunity, groups of A/J mice were challenged 2 weeks after vaccination (Fig. 3E). Thirty percent in the AdtPA83hu-immunized group and 50% in the AdtPA83hu+AdtLFnhu-vaccinated group survived ( $P < 0.001$  for both AdtPA83hu versus Adtetc and AdtPA83hu+AdtLFnhu versus Adtetc). All mice receiving Adtetc or AdtLFnhu alone died after spore challenge. However, the mean time to death (MTD) was 4.8 days for mice immunized with AdtLFnhu, while the MTD for Adtetc-immunized control mice was 3.3 days ( $P < 0.05$ ). Our findings demonstrate that a single i.n. vaccination with an AdvAV can provide rapid and long-term protection against a lethal challenge of *B. anthracis* spores. The anti-LF immune responses induced by AdtLFnhu increase the anti-Letx antibody titers minimally and enhanced the protective immunity induced by AdtPA83hu (Fig. 3C and E), although the differences between AdtLFnhu+AdtPA83hu and AdtPA83hu alone were not statistically significant.

**Preexisting immunity to Ad5 did not impair the potency of an Ad-vectored nasal anthrax vaccine.** To determine the effect of preexisting anti-Ad5 immunity on i.n. vaccination of an AdvAV, A/J mice (12 animals per group) were primed by i.n. installation with  $10^7$  IFU of wt Ad5/mouse or control buffer on day 0. On day

14, serum anti-Ad5 neutralizing antibody titers ranging from 1:32 to 1:256 (with a geometric mean at 1:57) were detected in mice primed with wt Ad5 on day 0. These mice were i.n. immunized with  $10^8$  IFU of AdtPA83hu/mouse plus  $5.4 \times 10^6$  IFU of AdmInhAhu/mouse (an Ad5 vector encoding *B. anthracis* immune inhibitor A gene) (high dose),  $10^7$  IFU of AdtPA83hu/mouse plus  $5.4 \times 10^5$  IFU of AdmInhAhu/mouse (median dose),  $10^6$  IFU of AdtPA83hu/mouse plus  $5.4 \times 10^4$  IFU of AdmInhAhu/mouse (low dose) on day 14. One group of mice without wt Ad5 priming were immunized with  $10^8$  IFU of AdtPA83hu plus  $5.4 \times 10^6$  IFU of AdmInhAhu (high dose) as controls. Although vaccination of mice without wt Ad5 priming produced slightly higher anti-PA IgG and anti-Letx antibody titers, the differences between two groups (high dose) were not statistically significant ( $P > 0.05$  for both anti-PA IgG and anti-Letx antibody titers) (Fig. 4). All of the mice immunized with AdtPA83 plus AdmInhAhu with or without wt Ad5 priming were protected against a  $50 \times \text{LD}_{50}$  *B. anthracis* Sterne spore challenge. All animals in control groups succumbed to the anthrax spore challenge (Table 1). These results demonstrated that animals possessing high levels of antibodies against Ad5 can be effectively vaccinated against anthrax with an i.n. AdvAV.

## DISCUSSION

To develop the next generation of safe and effective anthrax vaccines, it is crucial that the vaccine can induce rapid and sustained protective immunity in a single-dose regimen with no adverse side effects. Manufacturing, distribution, and administration must be rapid, easy, and economical. To address these requirements, several AdvAVs expressing PA or LF proteins were tested. Certain attributes of Ad5 vectors make them attractive for use as nasal vaccine platforms. (i) Replication-competent adenovirus (RCA)-free Ad5-vectored vaccines can be manufactured in PER.C6 cells quickly using disposable manufacturing technologies (17, 18, 22). (ii) Novel formulations allow Ad5-vectored vaccines to be stored in liquid buffer (19) or as lyophilized dry powder (23) at  $4^\circ\text{C}$  for at least a year and newly developed thermostabilization techniques allow Ad5 vectors to be stored at room temperature up to  $45^\circ\text{C}$  for 6 months with minimal declining infectivity (24). (iii) Ad5-vectored vaccines may be self-administered with a spray in an emergency event.

TABLE 1 Protection of immunized mice with or without wt Ad5 priming against a lethal-dose anthrax spore challenge<sup>a</sup>

Group	No. of animals/group	Vector/buffer	Dose/animal	Ad5 priming	No. of survivors/ no. of animals tested
1	12	AdtPA83hu + AdmInhAhu	High dose	–	12/12
2	12	A195 buffer	None	–	0/12
3	12	AdtPA83hu + AdmInhAhu	High dose	+	12/12
4	11 <sup>b</sup>	AdtPA83hu + AdmInhAhu	Median dose	+	11/11
5	12	AdtPA83hu + AdmInhAhu	Low dose	+	12/12
6	12	AdmInhAhu	10 <sup>8</sup> IFU	–	0/12
7	12	A195 buffer	None	+	0/12
8	12	Naive control	None	–	0/12

<sup>a</sup> High dose, 10<sup>8</sup> IFU of AdtPA83hu/mouse plus 5.4 × 10<sup>6</sup> IFU of AdmInhAhu/mouse; median dose, 10<sup>7</sup> IFU of AdtPA83hu/mouse plus 5.4 × 10<sup>5</sup> IFU of AdmInhAhu/mouse; low dose, 10<sup>6</sup> IFU of AdtPA83hu/mouse plus 5.4 × 10<sup>5</sup> IFU of AdmInhAhu/mouse; none, no AdtPA83hu and AdmInhAhu treatment.

<sup>b</sup> Lost one animal without replacement.

In research for new anthrax vaccines, numerous DNA constructs and viral vectors encoding codon-optimized or non-codon-optimized anthrax immunogens have been tested in anthrax vaccine studies, but the protective efficacies as vaccine candidates have not been compared in a same system. In the present study, we compared the relative levels of serum antigen-specific antibody titers and anti-Letx neutralizing antibody titers, as well as the strength of protective immunity induced by Ad5 vectors encoding codon-optimized or non-codon-optimized PA and LF genes in the A/J mouse model. Our results suggest that codon optimization of anthrax immunogens can enhance the humoral immune response of an AdVAV (Fig. 1), although the levels of protection against a lethal spore challenge among the AdPA83, AdPA83hu, and AdtPA83hu groups were not statistically significant in this particular experiment (Fig. 1D).

We demonstrated here that 100% of A/J mice can be protected from a lethal anthrax spore challenge after a single-dose i.n. vaccination with AdtPA83hu vectors (Fig. 1D and 3D). The duration of immunity with AdtPA83hu and/or AdtLFnhu has been measured in mice with demonstrable high serum anti-Letx antibody titers and antigen specific IgG titers that persist for at least 1 year (Fig. 3A to C). Moreover, partial protective immune responses can be rapidly generated within 2 weeks (Fig. 3E). These results are consistent with previous reports that Ad5-vectored vaccines could provide rapid and sustained protective immunity (11, 12, 25–28).

There is compelling evidence that the upper respiratory tract and other mucosal tissues are early targets of inhalational anthrax spore infection (29–31). A nasal anthrax vaccine that potentially induces both systemic and respiratory mucosal immune responses may be a favorable candidate against inhalational anthrax (32–34). Nasal delivery of an Ad5-vectored human immunodeficiency virus vaccine induced stronger mucosal immunity (35), and a nasal Ad5-vectored vaccine provided better protection against airway *Mycobacterium tuberculosis* than intramuscularly injected Ad5-vectored vaccines (36). In contrast to vaccines requiring artificial adjuvants and multiple immunizations for inducing sufficient mucosal immunity, an AdVAV can provide self-adjuvanting activity (37) capable of eliciting high titers of secreted IgA in animals after single-dose administration (Fig. 2). In addition, i.n. Ad5-vectored vaccines can also induce strong cell-mediated immunity (38, 39). It would be interesting to test whether an AdVAV can elicit cellular immune responses that may be beneficial in combating early infection when spores are engulfed by pulmonary phagocytes.

Studies by us and others indicate that administration of Ad5-vectored vaccines via the i.n. route might overcome preexisting immunity against the Ad5 vector (14, 40–42). In the present study, we demonstrated that an AdVAV, administered i.n. 2 weeks after priming with wt Ad5, could effectively immunize A/J mice (Fig. 4). Additional nonclinical and clinical information is needed to clarify the influence of the preexisting Ad5 immunity and to confirm the suitability of an i.n. AdVAV for human use.

Anthrax vaccines that can induce immune responses against both PA and an additional component such as PGA (43), LF (21), or *Bacillus* collagen-like protein of *anthracis* (BclA) (44) may be more effective against anthrax than the vaccines based on PA alone. Consistent with previous reports (21, 45), we observed that immune responses to LF alone could provide partial protection against a lethal anthrax spore challenge (Fig. 1D and 3D). Our study also suggested that multivalent AdVAVs could be mixed and delivered simultaneously (Fig. 3C and E). Additional testing to determine whether Ad5-vectored anthrax vaccines encoding PA and additional components would provide better protection against anthrax by targeting the early stages of spore infection as well as the toxins produced by the vegetative bacilli is warranted.

In summary, the data presented here show that (i) i.n. AdVAV can induce both systemic and mucosa immunity responses, (ii) i.n. immunization with AdtPA83hu provides full protection in vaccinated A/J mice against a lethal anthrax spore challenge, (iii) a single dose of AdVAV can elicit rapid and sustained protective immunity, (iv) AdVAV can effectively immunize A/J mice in the presence of preexisting immunity to Ad5, and (v) AdVAV has merit for further studies in the search a new and improved vaccine against aerosolized anthrax spores.

#### ACKNOWLEDGMENTS

This study was supported in part by the National Institutes of Health (1-UC1-AI067198-01).

We thank Megan Lallier and Robert Davenport for their outstanding technical assistance, Darrell Galloway and Charles Turnbough for providing plasmids, and Bo Andersen and Vyjayanthi Krishnan for critical review of the manuscript. We also thank the Battelle Memorial Institute and University of Alabama at Birmingham for providing facilities for animal testing, as well as Crucell Holland BV for providing the PER.C6 cell line, and Medicine Bio-Services, Inc., for providing contaminant-free HEK293 cells.

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