Induction of Protective Immunity to Anthrax Lethal Toxin with a Nonhuman Primate Adenovirus-Based Vaccine in the Presence of Preexisting Anti-Human Adenovirus Immunity

Masahiko Hashimoto,¹ Julie L. Boyer,¹ Neil R. Hackett,² James M. Wilson,³
and Ronald G. Crystal^{1,2*}

Department of Genetic Medicine¹ and Belfer Gene Therapy Core Facility,² Weill Medical College of Cornell University, New York, New York, and Gene Therapy Program, Division of Medical Genetics, Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania³

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Prevention or therapy for bioterrorism-associated anthrax infections requires rapidly acting effective vaccines. We recently demonstrated (Y. Tan, N. R. Hackett, J. L. Boyer, and R. G. Crystal, *Hum. Gene Ther.* 14:1673–1682, 2003) that a single administration of a recombinant serotype 5 adenovirus (Ad) vector expressing anthrax protective antigen (PA) provides rapid protection against anthrax lethal toxin challenge. However, approximately 35 to 50% of humans have preexisting neutralizing antibodies against Ad5. This study assesses the hypothesis that a recombinant adenovirus vaccine based on the nonhuman primate-derived serotype AdC7, against which humans do not have immunity, expressing PA (AdC7PA) will protect against anthrax lethal toxin even in the presence of preexisting anti-Ad5 immunity. Naive and Ad5-immunized BALB/c mice received (intramuscularly) 10⁸ to 10¹¹ particle units (PU) of AdC7PA, Ad5PA (a human serotype Ad5-based vector expressing a secreted form of PA), or AdNull (an Ad5 vector with no transgene). Robust anti-PA immunoglobulin G and neutralizing antibodies were detected by 2 to 4 weeks following administration of AdC7PA to naive or Ad5 preimmunized mice, whereas low anti-PA titers were detected in Ad5-preimmunized mice following administration of Ad5PA. To assess protection in vivo, naive or mice previously immunized against Ad5 were immunized with AdC7PA or Ad5PA and then challenged with a lethal intravenous dose of *Bacillus anthracis* lethal toxin. Whereas Ad5PA protected naive mice against challenge with *B. anthracis* lethal toxin, Ad5PA was ineffective in mice that were previously immunized against Ad5. In contrast, AdC7PA functioned effectively not only to protect naive mice but also to protect Ad5-preimmunized mice, with 100% survival after lethal toxin challenge. These data suggest the nonhuman-based vector AdC7PA is an effective vaccine for the development of protective immunity against *B. anthracis* and importantly functions as a “sero-switch” base for an adenovirus vaccine to function in the context of preexisting anti-Ad immunity.

Anthrax, the disease caused by *Bacillus anthracis*, is a threat as an agent of bioterrorism (27). In combination with the high mortality rate of anthrax, the capacity of *B. anthracis* to form stable spores that can be easily disseminated contributes to its potential use as an aerosolized biological weapon (27). Following inhalation, the spores are phagocytosed by macrophages and then transported to the regional lymph nodes; germination inside macrophages initiates a systemic infection (20). Although antibiotic therapy is recommended for diagnosed anthrax cases, these drugs do not neutralize the bacterial exotoxins produced by the bacteria or their consequent deleterious effects (12). The two exotoxins produced by *B. anthracis*, lethal toxin (LT) and edema toxin, are binary combinations of three proteins, protective antigen (PA), lethal factor (LF), and edema factor (2, 12, 21, 37). PA is a component of each toxin and is critical for toxin function by binding to the host cell receptor and facilitating translocation of the enzymatically active LF or edema factor proteins into the cytoplasm (7, 15, 19). Although LT is an isolated virulence factor from naturally

occurring anthrax infections, in a variety of model systems the effects of LT (PA plus LF) replicate the fatal pathophysiology of the disease (2, 6, 12, 16, 21, 34).

In the context that PA is a necessary component of *B. anthracis* lethality, it is the obvious target for anti-anthrax vaccines. PA is the major antigenic component of the anthrax vaccine available in the United States that is based on an aluminum hydroxide-adsorbed cell-free filtrate of an attenuated, nonencapsulated strain of *B. anthracis* (47). However, in addition to concerns regarding adverse effects of this vaccine (29), the administration schedule requires six doses over 18 months (<http://www.anthrax.mil/vaccine/schedule.asp>). In addition to this vaccine, a vaccine based on recombinant PA protein (rPA) has been developed by the United States military, but this vaccine also requires multiple administrations over several months for efficacy (32).

A major focus of our laboratory has been to use replication-defective recombinant adenovirus (Ad) vectors coding for PA as a strategy for an anti-anthrax vaccine that will be effective following a single administration (57). Relative to other genetic delivery methods, recombinant adenovirus vectors induce robust immune responses, likely because the Ad vector delivers the gene encoding the antigen directly to antigen-presenting cells (30, 56, 63). A plasmid DNA-based anti-PA vaccine is

* Corresponding author. Mailing address: Department of Genetic Medicine, Weill Medical College of Cornell University, 515 East 71st St., S-1000, New York, NY 10021. Phone: (212) 746-2258. Fax: (212) 746-8383. E-mail: geneticmedicine@med.cornell.edu.

effective against anthrax, but multiple administrations given over time are required to elicit protective immunity against PA (24a). By contrast, an anthrax vaccine based on a human serotype 5 adenovirus vector (Ad5) expressing PA only requires a single administration to be effective in protecting mice against a challenge with anthrax LT (57).

Although the human Ad5 has been effectively used as a base for vaccines in a variety of animal models, effective use of Ad5-based vaccines is limited because of the widespread preexisting immunity in humans against Ad5 (5, 9, 10, 13, 17, 22, 26, 41, 42, 49). Wild-type Ad5 is a ubiquitous pathogen; neutralizing titers found in up to 50% of the adult United States population may interfere with the efficacy of systemically delivered Ad vaccines based on the homologous serotype (5, 9, 10, 13, 17, 22, 26, 41, 42, 49). In rodents, it is possible to overcome preexisting anti-Ad immunity by increasing the dose of the vaccine carrier or by priming with naked DNA encoding the desired antigen and boosting with a recombinant Ad (62). However, humans have been repeatedly exposed to Ad and have immunological memory that may not be as readily overcome as the more moderate response in rodents to a single administration of a virus that does not replicate in this species.

The focus of the present study is to demonstrate that it is feasible to develop an anti-anthrax vaccine that will be effective even in the context of preexisting anti-Ad5 immunity by using AdC7, a novel nonhuman primate-based Ad serotype (5, 49, 51). Since AdC7 does not circulate in the human population, humans do not have neutralizing anti-AdC7 antibodies (5, 49, 51). To examine the impact of anti-Ad5 immunity on immunization with a heterologous Ad serotype, an Ad serotype C7 vector expressing a secreted form of *B. anthracis* PA (AdC7PA) was evaluated for the ability to protect immunized mice against anthrax toxin in the presence of preexisting anti-Ad5 immunity. The data demonstrate that in mice with preexisting anti-Ad5 immunity, administration of AdC7PA resulted in high anti-PA neutralizing antibody titers and protected the mice from LT challenge. By contrast, mice with preexisting anti-Ad5 immunity that were immunized with a similar Ad5-based vector (Ad5PA) had low anti-PA neutralizing titers and did not survive an LT challenge.

MATERIALS AND METHODS

Adenovirus vectors. Ad5PA, based on an E1[−]E3[−] serotype 5 Ad, includes an expression cassette with the cytomegalovirus immediate early promoter-enhancer, followed by the secretion signal peptide sequence from the mouse lysosome-associated membrane protein 1 gene, the full-length PA gene with codons optimized for mammalian cell expression, and then the simian virus 40 stop-polyadenylation signal (57). AdNull is a control vector with identical backbone but no transgene (25). AdC7PA is based on an E1[−]E3[−] serotype 7 chimpanzee Ad with an expression cassette identical to that of Ad5PA (49, 57). All vectors were produced in human embryonic kidney 293 cells (CRL-1573; ATCC, Manassas, VA) and purified with double CsCl gradient centrifugation (50). Dosing was based on particle units (PU), the physical number of particles of Ad as measured by spectrophotometry (39).

In vitro assessment of AdC7PA. Expression of PA protein from AdC7PA was determined by Western analysis. The AdC7PA and AdNull vectors were used to infect A549 human lung carcinoma cells (CCL-185; ATCC); at 48 h postinfection, supernatants were evaluated for the presence of PA protein by Western analysis with a PA-specific monoclonal antibody (Abcam, Cambridge, United Kingdom) and a goat anti-mouse immunoglobulin G (IgG) antibody-peroxidase conjugate (Sigma-Aldrich, St. Louis, MO).

Immunization, serum collection, and LT challenge. Female BALB/c mice, 4 to 6 weeks old, were purchased from Jackson Laboratories (Bar Harbor, ME) or

Taconic, Inc. (Germantown, NY) and housed under pathogen-free conditions. All vaccinations used intramuscular injection with 50 μ l of the vaccine preparations in the quadriceps on each side. Ad vectors were diluted with saline to the specified dose. At the indicated times postimmunization, mice were bled from the tail vein (each, 100 μ l of blood), samples were centrifuged (3,000 $\times$ g; 20 min), and sera were stored at -20°C until assayed for anti-PA antibodies by enzyme-linked immunosorbent assay (ELISA) or macrophage protection assay as described below.

The challenge with LT was carried out by intravenous injection of mixed recombinant PA and recombinant LF. The lethal dose of LT was determined empirically for every batch of purified PA and LF as described below. Following challenge, survival was monitored daily for 14 days.

Antibodies against PA. Anti-PA antibodies were quantified by ELISA and neutralizing anti-PA antibodies were quantified by a macrophage protection assay (15, 57). For ELISA, flat-bottomed 96-well plates were coated with 100 μ l of PA antigen at 1 $\mu\text{g}/\text{ml}$ overnight at 4°C . The plates were washed and blocked with 5% dry milk in phosphate-buffered saline (PBS), pH 7.4, for 30 min at 23°C and washed three times with PBS. Serial dilutions of serum (each, 100 μ l; 1:2 dilutions) were added to each well, starting with a 1:10 dilution and incubated for 1 h at 23°C . The plates were washed three times with PBS with 0.05% Tween 20; 100 μ l/well of anti-mouse antibody-peroxidase conjugate was added and the plates were incubated for 1 h at 23°C . The plates were washed four times with PBS-Tween 20 and once with PBS. Peroxidase substrate (100 μ l/well, catalogue no. 172-1064, Bio-Rad, Hercules, CA) was added and incubated for 15 min at 23°C , followed by the addition of a stop solution of 2% oxalic acid (100 μ l/well). Absorbance at 415 nm was read with a microplate reader (Bio-Rad). Secondary antibodies to IgG were obtained from Sigma (at 1:2,000; anti-IgG [A6782] was used at 1:10,000). Antibody titers were calculated using a log optical density – log dilution interpolation model and a cutoff value equal to twofold the absorbance of the background (45, 46, 57).

Anti-PA neutralizing activity was quantified using a mouse macrophage protection assay as previously described using 264.7 murine macrophage-like cells (ATCC, TIB-71) (15, 57). Cells were plated in flat-bottom 96-well culture plates at a concentration of 3×10^4 cells/well in Dulbecco's modified Eagle's Medium with 10% fetal bovine serum, 4.5 g/liter glucose, and 2 mM L-glutamine and incubated for 24 h at 37°C . Pooled sera from a group of immunized mice were twofold serially diluted with culture medium and incubated with rPA protein (List Biologicals) at 0.2 $\mu\text{g}/\text{ml}$ for 1 h and 37°C to allow neutralization to occur. LF protein (List Biologicals) was added to this mixture to achieve a final concentration of 0.1 $\mu\text{g}/\text{ml}$ for both PA and LF. Medium from the cells in 96-well plates was aspirated and replaced by the serum-PA-LF mixture at 100 μ l/well. Cells in control wells were incubated with 100 μ l of medium only, PA only, LF only, PA plus LF without sera, or 0.1% Triton X-100 (for total lysis). After 4 h of incubation at 37°C , 10 μ l of Alamar blue solution (Biosource International, Camarillo, CA) was added to each well; this dye is reduced by live cells, and the reduced form has a strongly fluorescent red color. Cells were incubated for 4 h. The plates were then evaluated in a fluorescent multiplate reader at an excitation setting of 530 nm and emission setting of 580 nm. The fluorescent values in the PA-plus-LF wells were used as baseline (0% protection) and the readings in medium-only wells were used as maximal response (100% protection). Neutralizing titers were calculated by a log-log linear fit model as the dilution that gave 50% protection (45, 57).

Anti-adenovirus neutralizing antibodies. Anti-adenovirus neutralizing antibody titers were evaluated as previously described, as the ability of the serum to prevent induction of cytopathic changes, following infection with wild-type human Ad5 (22). A549 cells were seeded at a density of 3×10^4 cells/well in 96-well plates 4 h before infection. Mouse sera were inactivated by being heated to 55°C for 45 min and then serially twofold diluted in Iscove's modified Eagle's medium (IMEM) containing 2% fetal bovine serum (FBS). Ad5 was diluted in IMEM containing 2% FBS to a concentration of 3×10^3 PFU/ μ l. Diluted virus (10 μ l) was combined with 50 μ l of each diluted serum sample and incubated at 37°C for 1 h to allow neutralization to occur. Following this incubation, the virus-serum mixture was added to the A549 cells and incubated for 90 min at 37°C . This medium was then replaced by 150 μ l of IMEM containing 10% FBS/well. After 7 days of incubation at 37°C , the plates were stained with methylene blue (Fisher LC169601). As a positive control, human serum known to have anti-Ad5 neutralizing antibodies was used; as a negative control, human serum known to have no anti-Ad5 neutralizing antibodies was used. The anti-Ad5 neutralizing antibody titer was determined to be the serum dilution that protected >90% of the cells from cytopathic effect.

Production and purification of LT. LT was generated by combining PA and LF proteins produced and purified from bacteria. The PA or the LF genes were cloned downstream of the T7 promoter in the pRSET prokaryotic expression

FIG. 1. Expression of *B. anthracis* PA in cells infected with AdC7PA. A549 cells were infected with AdC7PA or AdNull (an Ad5 vector with no transgene) at 5,000 PU/cell or left uninfected. At 48 h postinfection, supernatants were collected and analyzed for PA protein expression by Western analysis with an anti-PA monoclonal antibody. Lane 1, supernatant from uninfected cells; lane 2, AdNull-infected cells; lane 3, AdC7PA-infected cells; lane 4, purified rPA.

plasmid (Invitrogen, Carlsbad, CA) for expression as six-His fusion proteins. The resulting plasmids were transformed into the BL21.DE3 strain of *Escherichia coli*. Bacteria were grown under antibiotic selection to an optical density at 600 nm of 0.6 to 0.8; expression of the PA or LF proteins was induced with isopropyl-beta-D-thiogalactopyranoside (52). The recombinant proteins were purified with a Ni^{2+} column (ProBond kit; Invitrogen) under native conditions (resuspension of bacteria in 50 mM NaPO_4 and 2.5 M NaCl, pH 8.0; wash column with 50 mM NaPO_4 and 35 mM imidazole, pH 8.0; and elution with 50 mM NaPO_4 and 250 mM imidazole, pH 8.0). The purity of the proteins was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the identity was confirmed by Western analysis with anti-PA or anti-LF antibodies (Abcam). The biological activity of the LT and dose for challenge experiments were determined by in vivo dose-dependent survival experiments with mice (60 μg of PA and 25 μg of LF via the tail vein). Individual challenge experiments were done with the same preparation of recombinant proteins.

RESULTS

In vitro characterization of AdC7PA. AdC7PA, a nonhuman, primate-based, $\text{E1}^-\text{E3}^-$ Ad gene transfer vector, contains a gene encoding PA from *B. anthracis*. For optimal expression of PA in mammalian cells, the coding sequence was modified to contain mammalian-preferred codons, as previously described (57). To examine the expression and secretion of PA by the vector, A549 cells were infected with AdC7PA; as controls, cells were infected with AdNull or left uninfected. At 48 h postinfection, supernatants from infected cells were evaluated for PA expression by Western analysis (Fig. 1). AdC7PA-infected cell supernatants contained detectable amounts of PA protein (Fig. 1, lane 3). In contrast, supernatants from AdNull-infected cells (Fig. 1, lane 2) or uninfected cells (Fig. 1, lane 1) did not contain PA protein.

In vivo characterization of immune responses elicited by AdC7PA. The immune response following a single intramuscular administration of AdC7PA was evaluated with BALB/c mice. At 6 weeks postadministration, serum from immunized

FIG. 2. Induction of PA-specific immune responses following immunization with AdC7PA. (A) Anti-PA antibodies in mouse serum elicited by AdC7PA, following intramuscular immunization of mice. Groups of five BALB/c mice were immunized intramuscularly with AdC7PA (10^8 , 10^9 , 10^{10} , or 10^{11} PU) and sera were collected 0, 2, 4, and 6 weeks after immunization. Reciprocal anti-PA IgG titers were measured by ELISA and are presented as mean values $\pm$ standard error of the mean (SEM). A nonparametric statistical analysis (Mann-Whitney) of pairwise comparisons between all groups indicated that the differences in anti-PA IgG titers between groups are significant ($P < 0.04$ for all comparisons). (B) Anti-LT neutralizing antibodies in AdC7PA-immunized mice. Neutralizing antibody titers were measured by the ability of serial dilutions of immunized mouse sera (five mice/group) to protect murine macrophages from anthrax LT (PA and LF). Neutralizing activity was evaluated as a function of time (0, 2, 4, and 6 weeks) and AdC7PA dose (10^8 , 10^9 , 10^{10} , or 10^{11} PU). The data are presented as the average of titers ($\pm$ SEM) in individual mice from a representative experiment. A statistical analysis (Mann-Whitney nonparametric) indicates that all groups are statistically distinct from each other ($P < 0.02$ for pairwise comparisons between the following doses: 10^{11} and 10^{10} , 10^{11} and 10^9 , and 10^{10} and 10^9).

mice was analyzed for anti-PA IgG titers by ELISA, and a dose-response increase in anti-PA IgG titers was observed (Fig. 2A). A dose of 10^{10} PU elicited increasing titers over time, reaching a maximum of 2.5×10^3 at 6 weeks.

Serum from AdC7PA-immunized mice was also analyzed for the presence of anti-PA neutralizing antibodies. The development of LT neutralizing activity paralleled the induction of anti-PA IgG titers (Fig. 2B). At doses of 10^{10} and 10^{11} PU, detectable levels of anti-PA neutralizing antibodies were present at 2 weeks postimmunization and rose to a maximum titer at 6 weeks postadministration.

To evaluate the protective efficacy of the humoral immune responses elicited by AdC7PA, AdC7PA-immunized mice were challenged with an intravenous lethal dose of anthrax LT. The induction of anti-PA IgG and neutralizing antibody titers

FIG. 3. Survival of AdC7PA-immunized mice following intravenous LT challenge. Groups of five BALB/c mice were immunized intramuscularly with AdC7PA at 10^8 , 10^9 , 10^{10} , or 10^{11} PU. Six weeks after immunization, the mice were challenged with *B. anthracis* LT and survival was monitored for 14 days. A statistical survival analysis (Kaplan-Meier) indicated that the protection conferred by each immunizing dose of AdC7PA is statistically significant relative to naive animals ($P < 0.05$).

correlated with protection of immunized mice from an intravenous challenge with anthrax LT (Fig. 3). When AdC7PA was administered at a dose of 10^{10} PU or greater, 100% of mice were protected from a LT challenge. Control animals that were immunized with AdNull or were not immunized did not survive the challenge.

Effect of preexisting anti-Ad5 immunity on AdC7PA or Ad5PA immunization. The most commonly used vector for preclinical vaccination studies, human Ad5, is a ubiquitous pathogen; neutralizing titers found in up to 50% of the adult United States population may interfere with the efficacy of systemically delivered Ad vaccines based on the homologous serotype (5, 9, 10, 13, 17, 22, 26, 41, 42, 49). To determine the effect of preexisting anti-human Ad5 neutralizing antibody titers on immunization with Ad5PA or AdC7PA, mice were preimmunized with AdNull, an Ad5-based E1-E3⁻ vector with no transgene. This resulted in significant anti-human Ad5 neutralizing antibody titers (Table 1) that are comparable to those observed in the human population. When anti-PA IgG titers were measured in AdC7PA-immunized mice with preexisting anti-Ad5 immunity, there was a small reduction in the levels of anti-PA antibodies (Fig. 4A). However, in Ad5PA-immunized mice with preexisting anti-Ad5 immunity, there was a very large decrease in serum anti-PA antibody levels. The level of anti-human Ad5 neutralizing antibody titers elicited by preimmunization with AdNull significantly reduced anti-PA neutralizing antibody titers in mice subsequently immu-

FIG. 4. Induction of PA-specific immune responses following immunization with AdC7PA in the presence of preexisting anti-Ad5 immunity. (A) Effect of preexisting anti-Ad5 immunity on anti-PA IgG titer in AdC7PA-immunized mice. Ad5Null (10^{10} PU) or saline was administered intramuscularly three times to BALB/c mice at 0, 4, and 6 weeks. At 8 weeks after initiation of the experiment, AdC7PA was administered intramuscularly (10^{10} PU; $n = 5$ per group) or Ad5PA (10^{10} PU; $n = 5$ per group). Anti-PA IgG titers were measured by ELISA 2 and 4 weeks following immunization with the PA expression vectors. All data are presented as the geometric means ($\pm$ SEM) of titers from individual mice. The difference in anti-PA IgG titers between animals that received Ad5Null/AdC7PA or Ad5Null/Ad5PA is significant ($P < 0.05$; Mann-Whitney nonparametric test). (B) Effect of preexisting anti-Ad5 immunity on the time course of anti-PA neutralizing antibody induction in AdC7PA-immunized mice. BALB/c mice received Ad5Null (10^{10} PU) three times intramuscularly or saline, as described above. At 8 weeks after the initiation of the experiment, Ad5PA (10^{10} PU) or AdC7PA (10^{10} PU) was administered by an intramuscular route. At 0, 2, and 4 weeks following the second immunization, anti-PA neutralizing antibody titer was measured by the ability of serial dilutions of immunized mouse sera (pooled samples; five samples/group) to protect murine macrophages from anthrax LT. All data are presented as the average ($\pm$ SEM) of three replicate measurements from a representative experiment.

TABLE 1. Development of anti-Ad5 neutralizing antibodies in Ad5Null-immunized mice^a

Mouse line	Anti-Ad neutralizing antibody titer ⁻¹
Naive	0
Ad5Null	176 $\pm$ 39

^a Ad5Null (10^{10}) or saline was administered intramuscularly to BALB/c mice (10 mice per group) at 0, 4, and 6 weeks. At 8 weeks after the initiation of the experiment, anti-Ad neutralizing antibody titers were measured by the ability of serial dilutions of immunized mouse sera to protect A549 cells from wild-type Ad5 infection. The values represent the titers for all mice in the group $\pm$ SEM.

nized with Ad5PA (Fig. 4B). By contrast, anti-human Ad5 neutralizing antibody titers had a minimal impact on the development of anti-PA neutralizing antibody titers in mice immunized with AdC7PA. The effect of preexisting anti-human Ad5 neutralizing immunity on the development of anti-PA humoral immunity directly correlated with protection from an intravenous LT challenge (Fig. 5). Survival was reduced from 100% in mice immunized with Ad5PA to 0% in similarly immunized mice that had preexisting anti-Ad5 immunity. In contrast, survival of mice immunized with AdC7PA (100%) or mice immunized

FIG. 5. Effect of preexisting Ad immunity on survival of AdC7-immunized mice following intravenous LT challenge. Ad5Null (10^{10} PU) or saline was administered intramuscularly to BALB/c mice (five mice/group) three times as described in the legend to Fig. 4. Eight weeks after initiation of the experiment, AdC7PA (10^{10} PU) or Ad5PA (10^{10} PU) was administered intramuscularly. Four weeks after the second immunization, the mice were challenged with *B. anthracis* LT, and survival was monitored for 14 days. A statistical survival analysis (Kaplan-Meier) indicates significant differences between the Ad5PA-Ad5PA versus Ad5PA-AdC7PA groups ($P = 0.0143$) and between the naive versus Ad5Null-AdC7PA groups ($P = 0.0143$).

with AdC7PA in the presence of anti-Ad5 immunity (80%) was similar.

DISCUSSION

Rapidly acting, effective vaccines against anthrax are necessary to protect the population in the event of a deliberate release of *B. anthracis*. The present study demonstrates that this can be achieved using AdC7PA, a vaccine based on an E1⁻E3⁻ AdC7 nonhuman primate adenovirus to deliver PA (codon modified for optimal human expression) derived from *B. anthracis*. Administration of AdC7PA to mice elicits neutralizing antibodies against *B. anthracis* LT in naive mice, as well as mice immunized against Ad5, a common adenovirus for which up to 50% of humans have protective immunity. Importantly, a single administration of AdC7PA protected mice from challenge with LT even in the context of anti-Ad5 immunity, whereas an Ad5-based anthrax vaccine was ineffective in the presence of anti-Ad5 immunity. Together, these data support the concept that AdC7PA may be effective in a broad spectrum of the human population, independent of prior human Ad exposures.

Anthrax vaccines. The original anti-anthrax vaccine approved for human use in the United States is anthrax vaccine adsorbed (AVA), based on a filtrate of a nonlethal strain of *B. anthracis*, which is available only for the military and for civilians in occupations at risk (www.anthrax.mil) (29). Even if this vaccine were readily available for the general population, its utility in the event of a biological attack is restricted because a multimonth administration regimen is required to confer protective immunity (www.anthrax.mil/vaccine/schedule.asp). A vaccine based on recombinant PA is being evaluated by the United States military; although protective against anthrax in experimental animals, it is expected that multiple administra-

tions will be required for efficacy in humans (28). Similarly, a plasmid DNA-based anti-PA vaccine is effective at preventing *B. anthracis* infection, but multiple administrations given over time are required to elicit protective immunity (18). In contrast to the AVA vaccine and recombinant protein- or plasmid-based vaccines, AdC7PA functions to protect against *B. anthracis* LT following a single vaccine administration. Should this single-dose effectiveness hold up in future efficacy trials against challenge with *B. anthracis* spores with experimental animals, theoretically it should function in a similar fashion in humans.

Development of an Ad-based vaccine. There are 51 human Ad serotypes that are classified on the basis of biological, chemical, immunological, and structural properties into six subgroups and then into serotypes based on neutralization by antisera to other Ad serotypes (11, 54). Preexisting anti-Ad5 immunity has been demonstrated to reduce the effectiveness of Ad5-based vectors in studies in mice, rhesus monkeys, and humans in early phase I clinical trials (3, 8, 13, 23, 43, 44, 49, 55, 62). Therefore, the development of Ad vaccine vectors that elicit strong antigen-specific immune responses even in the presence of preexisting anti-Ad5 immunity is relevant. One strategy to circumvent preexisting anti-Ad neutralizing immunity is to base the vaccine on rare human Ad serotypes (53). When recombinant human serotype Ad35 vectors expressing a luciferase reporter gene are administered to mice in the presence of preexisting anti-Ad5 immunity, luciferase expression is readily detectable, whereas mice receiving an Ad5-based vector expressing a luciferase reporter gene in the presence of preexisting anti-Ad5 immunity demonstrate a 90% reduction in luciferase activity relative to control mice that do not have preexisting anti-Ad5 immunity (58). The immunogenicity of Ad35-based vaccines has been demonstrated for a simian immunodeficiency virus antigen (4). The seroprevalence of anti-Ad35 neutralizing antibodies is extremely low in the human population, and no cross-neutralization between Ad5 and Ad35 is evident. Human serotype Ad7-based vectors have also been developed and demonstrated to transduce a variety of murine tissues (1). In gene transfer studies, it has been possible to overcome preexisting anti-Ad immunity by switching the serotype of the Ad vector for sequential administration (31, 35, 36, 40). However, cross-reactive antivector immune responses between heterologous human serotypes have been reported and may be a major impediment to their use to circumvent preexisting anti-Ad neutralizing immunity in humans (24).

An alternative strategy to circumvent preexisting anti-Ad immunity is the use of nonhuman Ad serotypes (5, 13, 14, 26, 33, 38, 40, 43, 44, 48, 49, 51, 59–61). Nonhuman primate-derived Ad vaccine vectors were developed to overcome preexisting immunity to common human Ad serotypes and to broaden the repertoire of Ad vaccines for booster immunizations. Ad vaccine vectors based on nonhuman primate serotypes do not circulate in the human population and are therefore not affected by preexisting immunity (5, 13). Vaccines based on the serotype C68, C6, and C7 vectors are effective in generating potent transgene product-specific humoral and cellular immune responses against both human immunodeficiency virus and rabies antigens (13, 14, 43, 44, 59–61). These responses can also be boosted by the sequential use of heterologous vectors (43, 44, 49).

Preexisting immunity to common adenovirus serotypes found in a large fraction of the human population will likely impair the efficacy of recombinant Ad vaccine vectors based on these serotypes. In some reports, the adverse effects of preexisting Ad5 immunity can be overcome by increasing the dose of the vaccine or by using a DNA vaccine expressing the same transgene product for priming (62). In addition to increasing the cost of a vaccine, prime-boost regimens are not practical in the event of a bioweapons attack, where a rapid protective response is required. This study demonstrates the potential utility of Ad vaccine vectors based on nonhuman primate serotypes in the presence of preexisting anti-Ad5 immunity. This strategy may be an effective approach for developing Ad vaccine vectors for responses to bioterror attacks that avoid reduced effectiveness in the presence of preexisting anti-human Ad immunity.

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