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# Rapid/Sustained Anti-anthrax Passive Immunity Mediated by Co-administration of Ad/AAV

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Achieving both immediate and sustained protection against diseases caused by bacterial toxins and extracellular pathogens is a challenge in developing bio-defense therapeutics. We hypothesized that a single co-administration of an adenovirus (Ad) vector and an adeno-associated virus (AAV) vector, both expressing a pathogen-specific monoclonal antibody, would provide rapid, persistent passive immunotherapy against the pathogen. In order to test this strategy, we used the lethal toxin of *Bacillus anthracis* as a target of a monoclonal antibody directed against the protective antigen (PA) component of the toxin, using co-administration of an Ad vector encoding an anti-PA monoclonal antibody (Ad $\alpha$ PA) and an AAV vector encoding an anti-PA monoclonal antibody (AAVrh.10 $\alpha$ PA). As early as 1 day after co-administration of Ad $\alpha$ PA and AAVrh.10 $\alpha$ PA to mice, serum anti-PA antibody levels were detectable, and were sustained through 6 months. Importantly, animals that received both vectors were protected against toxin challenge as early as 1 day after administration and throughout the 6 month duration of the experiment. These data provide a new paradigm of genetic passive immunotherapy by co-administration of Ad and AAV vectors, each encoding a pathogen-specific monoclonal antibody, as an effective approach for both rapid and sustained protection against a bio-terror attack.

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## INTRODUCTION

Biological terrorism, the intentional use of biological agents to produce illness in a target population, poses a significant challenge relating to the development of strategies to protect the public.<sup>1</sup> Since it is impractical to vaccinate all of the population against a broad range of possible bio-terror agents, it is likely that public policy will be to administer protection to the at-risk population against a specific pathogen in response to a bio-terror attack.<sup>2</sup> In that context, the ideal bio-defense therapeutic should provide protection with an easily administered single administration that is rapid-acting and also long-acting (in case of a follow-up attack).

Against this background, we have developed a strategy that provides rapid (1 day) and sustained (at least 6 months) humoral

immunity with a single administration of a vaccine. Taking advantage of the different time-dependent expression profiles of adenovirus (Ad) (rapid, but short; 1–21 days) and adeno-associated virus (AAV) (slower, but persistent; 1 week to years) gene transfer vectors,<sup>3,4</sup> we have developed a dual-component vaccine strategy to deliver the genes coding for the heavy chains (HCs) and light chains (LCs) of monoclonal antibodies directed against extracellular pathogens and toxins. With a single administration, this dual-component vaccine is designed to provide protection from 1 day to at least 6 months.

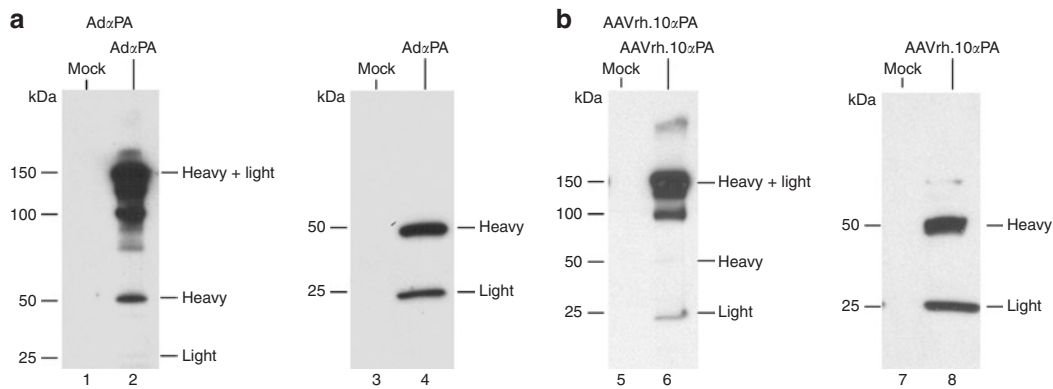
As an example to demonstrate the effectiveness of this approach, a group C serotype 5 Ad and a rhesus macaque serotype rh.10 AAV were designed to deliver the coding sequences for the HC and LC of a monoclonal antibody directed against the protective antigen (PA) component of the lethal toxin of *Bacillus anthracis*, the pathogen responsible for anthrax, a significant bio-terror threat.<sup>5</sup> The data demonstrates that a single administration of the Ad (Ad $\alpha$ PA) and AAV (AAVrh.10 $\alpha$ PA) vectors neutralizes the toxin and evokes systemic anti-toxin immunity. This immunity is effective in protecting mice against a lethal dose of anthrax toxin from day 1 up to at least 6 months. This combined Ad/AAV dual vaccine fulfills the criteria for an effective vaccine platform that could be used for providing rapid yet sustained protection against a variety of bio-terror pathogens and toxins against which humoral immunity is effective.

## RESULTS

### *In vitro* characterization of Ad- or AAV-expressed full length anti-PA antibody

In order to examine the expression of the anti-PA antibody by using Ad $\alpha$ PA, A549 cells were either infected with Ad $\alpha$ PA or mock-infected. At 48 hours after infection cell supernatants were collected, and the antibody expression was examined under non-reducing conditions so as to assess assembly of a full-size monoclonal antibody, and under reducing conditions so as to assess expression of the individual HC and LC (Figure 1a). Under non-reducing conditions, a complex of 150 kd (corresponding to the size of a completely assembled monoclonal antibody) was detected in Ad $\alpha$ PA-infected cells (lane 2) but not in mock-infected cells (lane 1). When the supernatants were assayed for antibody production under reducing conditions, the individual HC (50 kd) and LC (25 kd) were detected in Ad $\alpha$ PA-infected cells (lane 4) but not in mock-infected cells (lane 3).

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**Figure 1** Expression of a full-size anti-protective antigen (PA) mouse monoclonal antibody in cells infected with Ad $\alpha$ PA and AAVrh.10 $\alpha$ PA. A549 cells were infected with 5,000 particle units (pu) per cell of Ad $\alpha$ PA and 293orf6 cells were infected with  $10^4$  genome copies (gc) per cell of AAVrh.10 $\alpha$ PA. Infected cell supernatants were assessed for anti-PA antibody expression by Western analysis with a horseradish peroxidase–conjugated rabbit anti-mouse IgG antibody. **(a)** Expression of anti-PA antibody in supernatants from Ad $\alpha$ PA-infected A549 cells under nonreducing (native) conditions (lanes 1 and 2) and reducing conditions (lanes 3 and 4). Lanes 1 and 3, supernatants from mock-infected cells. Lanes 2 and 4, supernatants from Ad $\alpha$ PA-infected cells. **(b)** Expression of anti-PA antibody in supernatants from AAVrh.10 $\alpha$ PA-infected 293orf6 cells under nonreducing (native) conditions (lanes 5 and 6) and reducing conditions (lanes 7 and 8). Lanes 5 and 7, supernatants from mock-infected cells. Lanes 6 and 8, supernatants from AAVrh.10 $\alpha$ PA-infected cells. In all the panels, the heavy and light chains are identified.

In order to examine expression and secretion of the anti-PA antibody by AAVrh.10 $\alpha$ PA, 293orf6 cells were either infected with AAVrh.10 $\alpha$ PA or mock-infected. At 72 hours after infection cell supernatants were collected, and the antibody expression was examined under both nonreducing and reducing conditions (**Figure 1b**). Under nonreducing conditions, a 150 kD full-size monoclonal antibody was detected in AAVrh.10 $\alpha$ PA-infected cells (lane 6) but not in mock-infected cells (lane 5). The individual HC and LC were detected under reducing conditions in AAVrh.10 $\alpha$ PA-infected cells (lane 8) but not in mock-infected cells (lane 7).

When antibody production from either vector was analyzed under nonreducing conditions, the presence of additional bands that could correspond to free HC and LC or partially assembled antibodies were detected. These protein products could be a limitation of the expression system or, alternatively, could be an artifact of *in vitro* expression. In order to determine the production of antibodies in response to the vectors *in vivo*, sera from mice that received  $10^{11}$  particle units (pu) of Ad $\alpha$ PA plus  $10^{11}$  genome copies (gc) of AAVrh.10 $\alpha$ PA were analyzed for antibody production over a 2 week period. The analysis was carried out using Western blot under nonreducing conditions with an anti-*c-myc* antibody specific for the *c-myc* epitope tag at the carboxy terminus of the antibody HC. The results indicated that only fully assembled antibodies were detectable *in vivo*, thereby suggesting that the presence of antibody fragments *in vitro* was an artifact of the *in vitro* system.

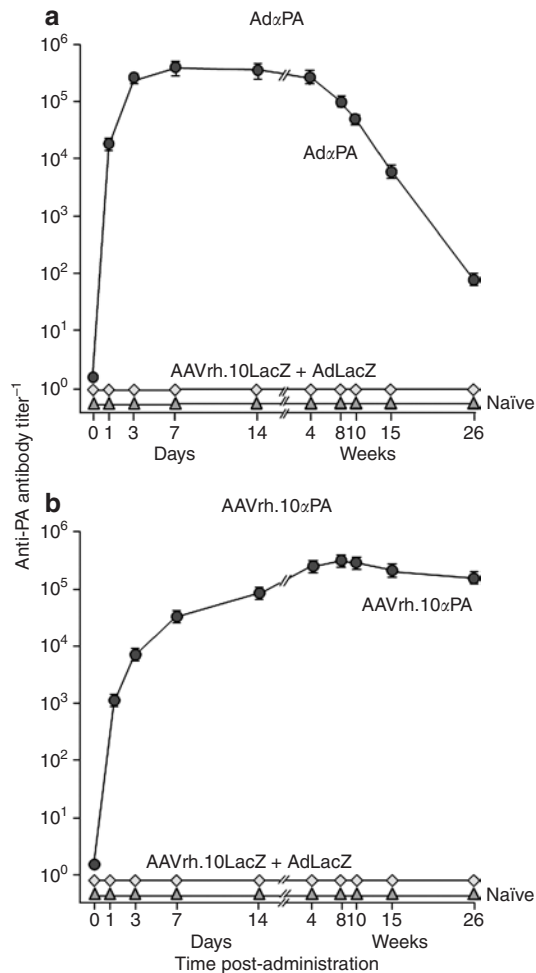
### **In vivo** expression of anti-PA monoclonal antibody from Ad $\alpha$ PA or AAVrh.10 $\alpha$ PA

The *in vivo* expression profile of the anti-PA antibody was determined in mice injected with either Ad $\alpha$ PA or AAVrh.10 $\alpha$ PA. Serum anti-PA antibody levels were determined by a PA-specific enzyme-linked immunosorbent assay over a 6 month time course. Mice that received Ad $\alpha$ PA had high serum anti-PA antibody levels as early as 1 day after administration (**Figure 2a**). These antibody titers peaked at day 7 after administration and then decreased, reaching a minimal level at the end of the 26 week experiment. In contrast, mice injected with a control vector expressing an

unrelated transgene, or naïve mice, did not have any measurable anti-PA antibody titers at any time point. When AAVrh.10 $\alpha$ PA was administered to mice, anti-PA antibody levels gradually increased through day 14 and reached a maximum value at 8 weeks after administration (**Figure 2b**). These levels were sustained throughout the 26 week experiment. No anti-PA antibodies were detected in the sera of mice injected with a control vector expressing an unrelated transgene, or in the sera of naïve mice. These results indicate that full-size anti-PA antibodies can be expressed *in vivo* from either Ad or AAV gene transfer vectors but, importantly, with different time-dependent expression profiles as expected from the two vectors.

### **Protection against *in vivo* lethal toxin challenge by anti-PA antibodies expressed from either Ad $\alpha$ PA or AAVrh.10 $\alpha$ PA**

In order to determine the efficacy of viral vector–expressed anti-PA antibodies against the effects of lethal toxin, mice were injected with Ad $\alpha$ PA or AAVrh.10 $\alpha$ PA and were then challenged at various time points from day 0 up to 26 weeks after vector administration (**Figure 3**). Administration of Ad $\alpha$ PA to mice resulted in protection from lethal toxin challenge as early as 1 day after administration (**Figure 3a**; 100%). This protective effect continued through 8 weeks after administration (100%), but declined by 6 months (0%), demonstrating rapid, but not sustained, efficacy of Ad-delivered anti-PA antibody. In contrast, naïve mice or mice injected with a control vector expressing an unrelated transgene were not protected against lethal toxin challenge at any time point. The administration of AAVrh.10 $\alpha$ PA to mice also resulted in protection against toxin challenge, but with different kinetics (**Figure 3b**). This protective effect was not seen prior to 2 weeks after administration (100%), but the effect persisted thereafter throughout 26 weeks after administration (100%), demonstrating delayed but sustained efficacy of AAV-delivered anti-PA antibody against lethal toxin challenge. None of the naïve mice, or mice that received a control vector, survived the challenge. This shows that, as expected from the serum anti-PA antibody levels, the kinetics of protection by Ad $\alpha$ PA or

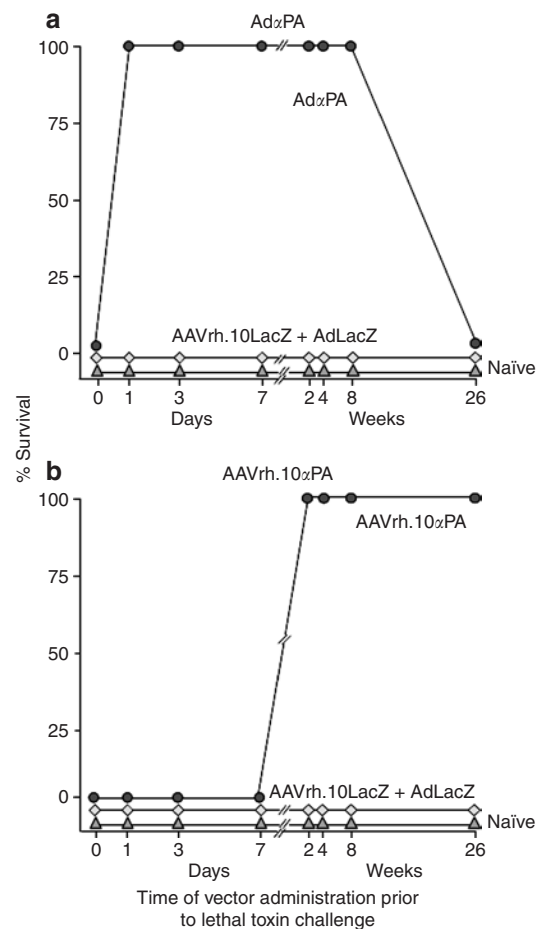


**Figure 2** Time course of serum anti-protective antigen (PA) antibody levels after administration of Ad $\alpha$ PA or AAVrh.10 $\alpha$ PA to C57BL/6 mice. Mice ( $n = 5$ /group) were injected with Ad $\alpha$ PA [ $10^{11}$  particle units (pu)] intravenously or with AAVrh.10 $\alpha$ PA [ $10^{11}$  genome copies (gc)] intrapleurally. Naïve mice, and mice injected through similar routes with AdLacZ ( $10^{11}$  pu) and AAVrh.10LacZ ( $10^{11}$  gc) were included as negative controls. Serum antibody levels were measured using PA-specific enzyme-linked immunosorbent assay from day 0 through to 26 weeks after administration. **(a)** Serum anti-PA antibody levels after administration of Ad $\alpha$ PA. **(b)** Serum anti-PA antibody levels after administration of AAVrh.10 $\alpha$ PA. Values shown are mean values  $\pm$  SEM.

AAVrh.10 $\alpha$ PA are different, with both vectors effectively directing the expression of protective anti-PA antibodies, but at different time points.

### Antibody expression and protection from lethal toxin challenge when Ad $\alpha$ PA and AAVrh.10 $\alpha$ PA are co-administered to mice

Serum anti-PA antibody levels were assessed in mice injected with both Ad $\alpha$ PA and AAVrh.10 $\alpha$ PA from day 0 through 26 weeks after vector administration, using a PA-specific enzyme-linked immunosorbent assay (Figure 4a). Anti-PA antibody titers were high as early as 1 day after injection, continued to rise to a maximal level at day 14 and remained at high levels through 26 weeks. No serum anti-PA antibody titers were detectable in naïve animals or in animals injected with a control vector expressing an unrelated transgene. When animals that received both Ad $\alpha$ PA and

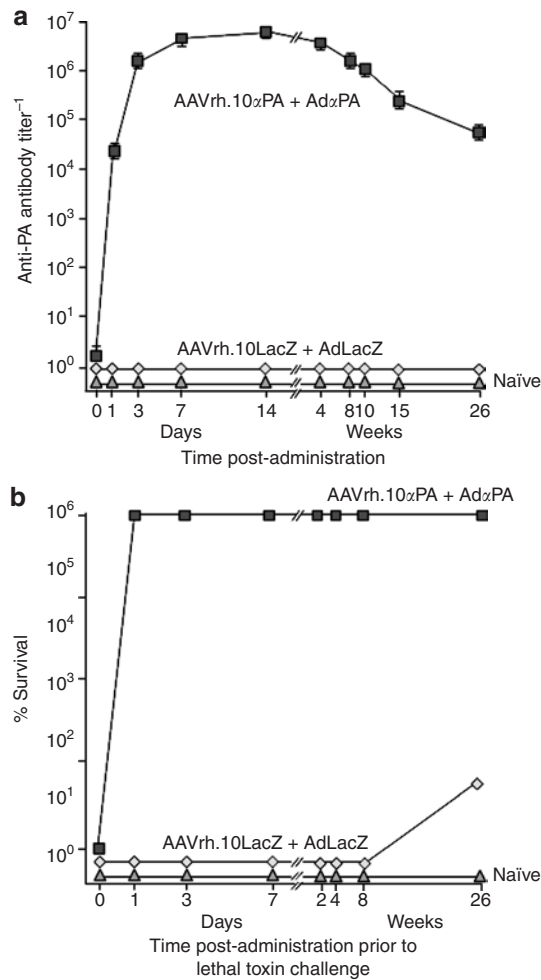


**Figure 3** Survival of Ad $\alpha$ PA- or AAVrh.10 $\alpha$ PA-treated C57BL/6 mice following challenge with *Bacillus anthracis* lethal toxin at various time points relative to receiving the vectors. Mice ( $n = 5$ /group) were injected with Ad $\alpha$ PA [ $10^{11}$  particle units (pu)] intravenously or with AAVrh.10 $\alpha$ PA [ $10^{11}$  genome copies (gc)] intrapleurally. From day 0 through to 26 weeks after administration, the animals were challenged with an intravenous bolus of lethal toxin. Naïve mice and mice injected through similar routes with AdLacZ ( $10^{11}$  pu) or with AAVrh.10LacZ ( $10^{11}$  gc) were included as negative controls. **(a)** Survival of mice challenged with lethal toxin at various time points after Ad $\alpha$ PA administration. **(b)** Survival of mice challenged with lethal toxin at various time points after AAVrh.10 $\alpha$ PA administration. After toxin challenge at each time point, the survival of the animal was monitored for 14 days. PA, protective antigen

AAVrh.10 $\alpha$ PA were challenged with lethal toxin at various time points between day 0 and 26 weeks after administration, the protective effects were evident as early as 1 day after injection (100% survival) and lasted through 26 week (100% survival; Figure 4b). In contrast, the control mice were not protected. This shows that a single administration of Ad $\alpha$ PA and AAVrh.10 $\alpha$ PA is sufficient to generate both rapid and long-term protection against the action of anthrax lethal toxin.

### DISCUSSION

The ideal vaccine for a population threatened with a bio-terror attack should be an easily administered vaccine requiring only a single administration; it should be rapid acting (to provide immediate protection) and should also provide sustained protection (in case of a follow-up attack). This study demonstrates that it is



**Figure 4** Serum anti-protective antigen (PA) antibody levels and survival from lethal toxin challenge after co-administration of AdαPA and AAVrh.10αPA to C57BL/6 mice. Mice ( $n = 5$ /group) were co-administered AdαPA [ $10^{11}$  particle units (pu)] intravenously and AAVrh.10αPA [ $10^{11}$  genome copies (gc)] intrapleurally. Naïve mice and mice co-administered AdLacZ ( $10^{11}$  pu) or AAVrh.10LacZ ( $10^{11}$  gc) were included as negative controls. **(a)** Serum anti-PA antibody levels were measured using PA-specific enzyme-linked immunosorbent assay from day 0 through to 26 weeks after administration. Values shown are mean values  $\pm$  SEM. **(b)** Mice co-administered AdαPA and AAVrh.10αPA were challenged with an intravenous bolus of *Bacillus anthracis* lethal toxin at various time points relative to receiving the vectors, from day 0 through to 26 weeks. After toxin challenge at each time point, the survival of the animal was monitored for 14 days.

feasible to design a vaccine to provide passive protection against pathogens and/or toxins, which can be neutralized with a monoclonal antibody. This antibody can be delivered genetically by co-administering two gene therapy vectors, an Ad (providing rapid protection from 1 to 8 weeks) and an AAV (providing delayed but sustained protection from 2 to at least 26 weeks). The current study was able to demonstrate that, when the *B. anthracis* lethal toxin was administered to C57BL/6 mice, a single co-administration of an Ad vector (AdαPA) and an AAV vector (AAVrh.10αPA), each expressing a neutralizing anti-PA monoclonal antibody, was sufficient to produce rapid, sustained protection against anthrax lethal toxin. For these experiments, the doses of each individual vector were selected on the basis of data from previous experiments

that demonstrated maximal levels of gene expression. Although the selected doses may appear to be high [ $10^{11}$  pu for AdαPA and  $10^{11}$  gc for AAVrh.10αPA], in comparison with doses used for correction of genetic disorders with gene transfer vectors, it is unclear whether a direct scaling up from mice to humans will be necessary for the vaccine to be effective. After administration of AdαPA alone, protective antibody titers were  $>10^4$  from day 1 through to at least 10 weeks, and the mice were protected from lethal toxin challenge from day 1 through to 8 weeks. With administration of AAVrh.10αPA alone, protective antibody titers were  $>10^4$  from 7 days through to at least 26 weeks, the duration of the experiment, and the mice were protected from 2 weeks after vaccine administration through 26 weeks. When the two vectors were co-administered, protective antibody titers were  $>10^4$  from day 1 through 26 weeks, and the mice were 100% protected from lethal toxin challenge throughout that period. This also fulfilled the criteria for the ideal vaccine strategy, with a single administration providing rapid and long-term protection. The duration of antibody expression in these experiments is striking. Although this has not been measured directly, we do not expect that anti-idiotypic antibodies were formed. If indeed they had been formed, there would have probably been a decrease in circulating specific antibody prior to the 26 week time point.

## Anthrax

The inhaled form of anthrax, a disease caused by *B. anthracis*, is highly lethal and constitutes a bio-terrorism threat.<sup>5-7</sup> Anthrax lethal toxin is the major causative agent for this disease.<sup>8</sup> PA is the cell surface binding component of the lethal toxin that binds to tumor endothelial marker-8 or human capillary morphogenesis protein-2 on the surface of macrophages and endothelial cells.<sup>9,10</sup> Membrane-bound PA is cleaved by a furin or furin-like protease that facilitates assembly into a heptameric oligomer.<sup>8,11,12</sup> The enzymatic component of the lethal toxin, namely, the lethal factor, binds to the PA heptamer and the complex is internalized with consequent immunosuppressive and toxic effects.<sup>8,13,14</sup>

The currently available therapies for anthrax include antibiotics and vaccines. Antibiotics can be effective, but even with treatment, a high degree of mortality is still associated with anthrax, and there are forms of anthrax that have been engineered to be resistant to antibiotic therapy.<sup>6,15,16</sup> The currently licensed vaccine, AVA Biothrax, and the next generation anthrax vaccine, rPA102, are effective in inducing long-term immunity against disease.<sup>17-19</sup> However, both require a multiple-dose immunization schedule and a minimum of 4 weeks to develop protective antibody titers. These regimens are obviously too protracted to be effective in response to a deliberate *B. anthracis* release<sup>19</sup> (<http://www.anthrax.osd.mil/vaccine/schedule.asp>).

Protection against anthrax is correlated with the induction of humoral immune responses against PA.<sup>20-22</sup> The efficacy of AVA Biothrax and rPA102 correlates with the development of PA-specific humoral immune responses, and the therapeutic value of passive immunization for anthrax has been demonstrated to be effective against both spore and toxin challenges in a variety of experimental systems.<sup>23-31</sup> It is possible to protect experimental animals against anthrax with monoclonal antibodies, and one monoclonal antibody has been assessed

in a Phase I clinical study.<sup>23–32</sup> Although these antibody-based therapies are effective, their main limitation is the half-life of the antibody molecule, with a single administration resulting in only short-term efficacy.<sup>32</sup>

### Viral vector–based transfer of therapeutic antibodies

Genetic transfer of therapeutic antibodies is an attractive strategy as both short-term and long-term therapy. It has been possible to express antibody molecules by using a variety of viral vectors including baculovirus, rhabdovirus, vaccinia virus, Ad, and AAV.<sup>33–40</sup> Previous studies in our laboratory had demonstrated that genetic delivery of a PA-specific single chain antibody with an Ad gene transfer vector is effective in rapidly protecting experimental animals from lethal toxin within 1 day of administration, but the effect lasted only for 10 days, because of the short half-life of the antibody.<sup>41</sup> In contrast to single-chain antibodies, *in vivo* delivery of full-length monoclonal antibodies with viral vectors results in more sustained serum antibody levels. Expression of an anti-HER-2 monoclonal antibody from an Ad vector resulted in detectable serum antibody levels in nude mice from day 3 through at least 4 weeks after administration, and these levels were high enough to significantly reduce the growth of HER-2-positive tumor xenografts in these animals.<sup>39</sup> The administration of an Ad vector expressing an anti-thyroglobulin antibody to mice resulted in serum anti-thyroglobulin levels that were detectable for at least 3 months after administration.<sup>37</sup> It has also been possible to achieve sustained therapeutic levels of a monoclonal antibody with AAV gene transfer vectors *in vivo*. Significant levels of a human immunodeficiency virus–specific monoclonal antibody were detectable in the sera of mice following intramuscular administration of an AAV2 vector encoding an anti-gp160 antibody for 6 months after administration.<sup>36</sup> Stable antibody expression was observed for 4 months in nude mice that had received an AAV8 vector encoding a monoclonal antibody specific for the vascular endothelial growth factor receptor-2, and this was associated with a reduction of vascular endothelial growth factor receptor-2–positive tumor growth when tumor cells were introduced into the animals.<sup>38</sup> In subsequent experiments, *in vivo* serum levels of the anti-vascular endothelial growth factor receptor-2 antibody were detectable for 6 months after administration to C57BL/6 mice.<sup>40</sup>

In this study, continuous expression of an anti-PA monoclonal antibody following delivery with an AAVrh.10 gene transfer vector resulted in detectable levels of serum antibodies that were protective against lethal toxin challenge for the 6 month duration of the study. Administration of Ad $\alpha$ PA was effective in protecting animals against lethal toxin challenge as early as 1 day after administration. This demonstrates that co-administration of Ad and AAV vectors expressing neutralizing anti-PA antibodies may be a strategy to provide rapid, sustained, and effective protection against anthrax. It is likely that the effect of the genetic delivery of anti-PA antibody by Ad $\alpha$ PA may be enhanced in conjunction with antibiotic therapy, and that viral vector–based antibody delivery could be used as an adjunct to antibiotic therapy to shorten the course of antibiotic treatment significantly.

## MATERIALS AND METHODS

**Ad and AAV vectors.** Ad $\alpha$ PA and AAVrh.10 $\alpha$ PA vectors were constructed so as to direct the expression of a full-size (HC and LC) murine monoclonal antibody against anthrax toxin PA. Constructs containing the HC and LC variable domain complementary DNA (cDNA) sequence of a high-affinity, lethal toxin-neutralizing, anti-PA antibody (14B7-1H) were provided by J. Maynard (Department of Chemical Engineering and Institute for Cellular and Molecular Biology, University of Texas, Austin, TX).<sup>42</sup> Full-size HC and LC were generated by incorporating the murine IgG1 constant domain and the murine  $\kappa$  constant domain onto the variable regions by overlap polymerase chain reaction. In designing the Ad $\alpha$ PA and AAVrh.10 $\alpha$ PA vectors, one major challenge was to balance the expression of the HC and LC cDNAs. We found, by trial and error, that the use of a poliovirus internal ribosome entry site was most effective for the Ad vector, whereas the use of a furin cleavage site and the self-cleaving 2A peptide from the foot-and-mouth disease virus was the most effective for the AAV vector.<sup>38,43</sup>

Ad $\alpha$ PA is a serotype 5, E1–E3-Ad gene transfer vector encoding the anti-PA LC and HC cDNAs separated by a poliovirus internal ribosome entry site to facilitate expression of both protein subunits from a single CMV promoter. The expression cassette in the Ad $\alpha$ PA vector contains (5' to 3') the CMV promoter/enhancer followed by cDNAs encoding the anti-PA LC, poliovirus internal ribosome entry site, the anti-PA HC, and the simian virus 40 polyadenylation signal. AdLacZ, an identical vector except that it contains  $\beta$ -galactosidase in the expression cassette, was used as a negative control in this study. The AdLacZ vector contains (5' to 3') the CMV promoter/enhancer followed by cDNA encoding  $\beta$ -galactosidase, and the simian virus 40 polyadenylation signal. Ad $\alpha$ PA and AdLacZ were produced in 293 cells and purified by centrifugation twice through cesium chloride gradient as previously described.<sup>44</sup> The particle units of each recombinant Ad preparation were determined spectrophotometrically.<sup>45</sup>

AAVrh.10 $\alpha$ PA is a nonhuman primate-derived AAV serotype rh.10 gene transfer vector encoding the anti-PA HC and LC, with the subunits separated by both furin and self-cleaving 2A protease sites for expression from a single CMV promoter. The AAVrh.10 $\alpha$ PA expression cassette contains (5' to 3') the CMV promoter/enhancer followed by cDNAs encoding the anti-PA HC, a 13 amino acid *c-myc* tag, a 4 amino acid furin cleavage site, the 24 amino acid self-cleaving 2A peptide from foot-and-mouth disease virus, the anti-PA LC, and the human growth hormone polyadenylation signal.

AAVrh.10 $\alpha$ PA was produced using three plasmids: (i) pAAV $\alpha$ PA, an expression plasmid containing (5' to 3') the AAV2 5'-inverted terminal repeat including packaging signal ( $\Psi$ ), the anti-PA antibody expression cassette, and the AAV2 3'-inverted terminal repeat; (ii) pAAV44.2, a plasmid that provides rep proteins derived from AAV2 and cap proteins derived from AAVrh.10; and (iii) pAdDeltaF6, an Ad helper plasmid that provides Ad helper functions of E2, E4, and VA RNA.<sup>46–48</sup> pAAV $\alpha$ PA (600  $\mu$ g), pAAV44.2 (800  $\mu$ g), and pAdDeltaF6 (1.2 mg) were co-transfected into human embryonic kidney 293 cells (American Type Culture Collection, Manassas, VA), which contain an integrated copy of the Ad E1 region, using Polyfect (Qiagen, Valencia, CA). At 72 hours after transfection, the cells were harvested, a crude viral lysate was prepared using three cycles of freeze/thaw, and clarified by centrifugation. AAVrh.10 $\alpha$ PA was purified by iodixanol gradient and QHP anion exchange chromatography. The purified AAVrh.10 $\alpha$ PA was concentrated using a BioMax 100 membrane concentrator (Millipore, Billerica, MA) and stored in phosphate-buffered saline, pH 7.4 (PBS) at  $-80^{\circ}\text{C}$ . AAVrh.10LacZ was used as a negative control vector. The genomic structure is identical to AAVrh.10 $\alpha$ PA, except that the transgene in the expression cassette is  $\beta$ -galactosidase. Vector genome titers were determined by TaqMan real-time-polymerase chain reaction using a CMV promoter–specific primer–probe set (Applied Biosystems, Foster City, CA). An AAV $\alpha$ 1AT plasmid DNA standard of known copy number was used for generating a standard curve, from which the genome copy number of each AAV preparation was determined.

**In vitro assessment of  $\alpha$ PA expression.** Expression of the anti-PA antibody from Ad $\alpha$ PA and AAVrh.10 $\alpha$ PA following infection of cells *in vitro* was assessed by Western analysis. Ad $\alpha$ PA was used for infecting A549 cells, and the infected-cell supernatants were harvested at 48 hours after infection. AAVrh.10 $\alpha$ PA was used for infecting 293orf6 cells, and the infected-cell supernatants were harvested at 72 hours after infection. All supernatants were concentrated using Ultracel YM-10 centrifugal filter devices (Millipore, Billerica, MA) and evaluated for the expression of anti-PA antibody by Western analyses under nonreducing and reducing conditions, using a horseradish peroxidase–conjugated anti-mouse IgG1 secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and ECL reagent (Amersham, Piscataway, NJ).

**Administration of Ad and AAVrh.10 vectors to mice.** In order to avoid issues of the Ad and AAV vectors interacting in the same cells, the vectors were administered at different sites. Male C57BL/6 mice were administered the Ad $\alpha$ PA vector or the AdlacZ control vector through the intravenous route and the AAVrh.10 $\alpha$ PA vector or the AAVrh.10lacZ vector through the intrapleural route. In previous experiments with AAV gene transfer vectors of the same serotype, the intrapleural route of administration was found to result in optimal levels of gene expression. On the basis of this information, the AAV vectors were administered intrapleurally in these experiments.<sup>48</sup>

Recombinant AAVrh.10 vectors ( $10^{11}$  gc) were delivered into the pleural space of the left lungs of 6-week-old male C57BL/6 mice ( $n = 5$ /group). The mice were anesthetized using a combination of ketamine (100 mg/kg) and xylazine (10 mg/kg) by intraperitoneal injection. The trachea was cannulated with a 20-gauge angiocatheter (Becton Dickinson, Franklin Lakes, NJ), and mechanical ventilation was achieved using a small animal ventilator (Harvard Apparatus, Holliston, MA). Tidal volume and respiratory rate were set 0.7–1.0 ml  $\times$  70/minute. An anterolateral chest skin incision ~1 cm in length was made using scissors. The thoracic cage was exposed and a thoracotomy was done in the third intercostal space. The AAVrh.10 vectors were administered in 100  $\mu$ l of PBS through the exposed pleural space using a 1 ml insulin syringe. After placement of the angiocatheter through the pleural cavity, the thoracic cage was closed with 4–0 absorbable sutures (Polysorb, Auburn, NY). The angiocatheter was removed after air was evacuated using a syringe manually. The skin was closed using a second layer of the same sutures, the ventilator was stopped, and the tracheal tube was removed.

Recombinant Ad vectors ( $10^{11}$  pu) were delivered intravenously to mice. The mice were anesthetized using a combination of ketamine (100 mg/kg) and xylazine (10 mg/kg) by intraperitoneal injection; recombinant Ad vectors in diluted in PBS (100  $\mu$ l) were administered via the tail vein. Co-administration of recombinant AAV vectors ( $10^{11}$  gc) and recombinant Ad vectors ( $10^{11}$  pu) was accomplished by introduction of each vector through different routes as described earlier.

**In vivo assessment of  $\alpha$ PA levels.** Male C57BL/6 mice, 4–6 week of age, from The Jackson Laboratory (Bar Harbor, ME) or Taconic (Germantown, NY), were housed in a pathogen-free environment. The mice were administered Ad $\alpha$ PA, AAVrh.10 $\alpha$ PA, or a combination of Ad $\alpha$ PA and AAVrh.10 $\alpha$ PA. Naïve mice and mice injected with a combination of AdLacZ and AAVrh.10LacZ were used as negative controls. Following vector administration, serum was collected via the tail vein, centrifuged at 8,000g for 20 minutes, and stored at  $-20^{\circ}\text{C}$ . Anti-PA antibody levels in mouse sera were assessed by a PA-specific enzyme-linked immunosorbent assay using flat-bottomed 96-well EIA/RIA plates (Corning, New York, NY) coated with 0.1  $\mu$ g PA (List Biological Lab, Campbell, CA) per well in a total volume of 100  $\mu$ l of PBS, pH 7.4 overnight at  $4^{\circ}\text{C}$ . The plates were washed with PBS containing 0.05% Tween-20 (PBS–Tween) and blocked with 5% dry milk in PBS for 30 minutes at  $23^{\circ}\text{C}$ . Serial serum dilutions were added to each well and incubated for 90 minutes at  $23^{\circ}\text{C}$ . The plates were washed four times with PBS–Tween, 100  $\mu$ l of 1:1,000 diluted horseradish peroxidase–conjugated goat anti-mouse IgG1 (Santa Cruz Biotechnology, Santa Cruz, CA)

in PBS containing 1% dry milk was added and incubated for 90 minutes at  $23^{\circ}\text{C}$ . The plates were washed four times with PBS–Tween and once with PBS. Peroxidase substrate (100  $\mu$ l/well; Bio-Rad, Hercules, CA) was added, incubated for 15 minutes,  $23^{\circ}\text{C}$ , and this was followed by the addition of a stop solution of 2% oxalic acid (100  $\mu$ l/well). Absorbance at 415 nm was read with a microplate reader (Bio-Rad, Hercules, CA). Antibody titers were calculated with a log(OD)–log(dilution) interpolation model using a cut-off value equal to twofold of the absorbance of background.<sup>49</sup>

**Ad $\alpha$ PA plus AAVrh.10 $\alpha$ PA-mediated protection of mice from lethal toxin challenge.** In order to assess the ability of Ad $\alpha$ PA, AAVrh.10 $\alpha$ PA or Ad $\alpha$ PA plus AAVrh.10 $\alpha$ PA to protect mice against challenge with anthrax lethal toxin, male C57BL/6 mice ( $n = 5$ /group) were injected with Ad $\alpha$ PA, AAVrh.10 $\alpha$ PA, or a combination of Ad $\alpha$ PA and AAVrh.10 $\alpha$ PA. Naïve mice and mice injected with a combination of AdLacZ and AAVrh.10LacZ were used as negative controls. At various time points ranging from 1 to 180 days after vector administration, each mouse was challenged by intravenous administration of anthrax lethal toxin, consisting of a mixture of 30  $\mu$ g PA and 12.5  $\mu$ g lethal factor in a total volume of 100  $\mu$ l PBS.<sup>50</sup> Survival was monitored twice daily for 14 days after lethal toxin challenge.

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## REFERENCES

- Lane, HC and Fauci AS (2005). Microbial bioterrorism. In: Kasper, DL *et al.* (eds). *Harrison's Principles of Internal Medicine*. McGraw-Hill Medical Publishing Division: New York, pp. 1279–1293.
- Rotz, LD, Dotson, DA, Damon, IK and Becher, JA (2001). Vaccinia (smallpox) vaccine: recommendations of the Advisory Committee on Immunization Practices (ACIP), 2001. *MMWR Recomm Rep* **50**: 1–25.
- Stilwell, JL and Samulski, RJ (2003). Adeno-associated virus vectors for therapeutic gene transfer. *Biotechniques* **34**: 148–150, 152, 154.
- Hackett, NR and Crystal RG (2004). Adenovirus vectors for gene therapy. In: Lasic, D and Templeton, NS (eds.). *Gene Therapy: Therapeutic Mechanisms and Strategies*. Marcel Dekker, Inc.: New York. pp.17–30.
- Friedlander, AM (2000). Anthrax: clinical features, pathogenesis, and potential biological warfare threat. *Curr Clin Top Infect Dis* **20**: 335–349.
- Inglesby, TV, O'Toole, T, Henderson, DA, Bartlett, JG, Ascher, MS, Eitzen, E *et al.* (2002). Anthrax as a biological weapon, 2002: updated recommendations for management. *JAMA* **287**: 2236–2252.
- Fennelly, KP, Davidow, AL, Miller, SL, Connell, N and Ellner, JJ (2004). Airborne infection with *Bacillus anthracis*—from mills to mail. *Emerg Infect Dis* **10**: 996–1002.
- Collier, RJ and Young, JA (2003). Anthrax toxin. *Annu Rev Cell Dev Biol* **19**: 45–70.
- Bradley, KA, Mogridge, J, Mourez, M, Collier, RJ and Young, JA (2001). Identification of the cellular receptor for anthrax toxin. *Nature* **414**: 225–229.
- Scobie, HM, Rainey, CJ, Bradley, KA and Young, JA (2003). Human capillary morphogenesis protein 2 functions as an anthrax toxin receptor. *Proc Natl Acad Sci USA* **100**: 5170–5174.
- Gordon, VM, Klimpel, KR, Arora, N, Henderson, MA and Leppla, SH (1995). Proteolytic activation of bacterial toxins by eukaryotic cells is performed by furin and by additional cellular proteases. *Infect Immun* **63**: 82–87.
- Milne, JC, Furlong, D, Hanna, PC, Wall, JS and Collier, RJ (1994). Anthrax protective antigen forms oligomers during intoxication of mammalian cells. *J Biol Chem* **269**: 20607–20612.
- Cunningham, K, Lacy, DB, Mogridge, J and Collier, RJ (2002). Mapping the lethal factor and edema factor binding sites on oligomeric anthrax protective antigen. *Proc Natl Acad Sci USA* **99**: 7049–7053.
- Turk, BE (2007). Manipulation of host signalling pathways by anthrax toxins. *Biochem J* **402**: 405–417.
- Stepanov, AV, Marinin, LI, Pomerantsev, AP and Staritsin, NA (1996). Development of novel vaccines against anthrax in man. *J Biotechnol* **44**: 155–160.
- Choe, CH, Bouhouala, SS, Brook, I, Elliot, TB and Knudson, GB (2000). *In vitro* development of resistance to ofloxacin and doxycycline in *Bacillus anthracis* Sterne. *Antimicrob Agents Chemother* **44**: 1766.
- Ivins, BE, Pitt, ML, Fellows, PF, Farchaus, JW, Benner, GE, Waag, DM *et al.* (1998). Comparative efficacy of experimental anthrax vaccine candidates against inhalation anthrax in rhesus macaques. *Vaccine* **16**: 1141–1148.
- Demicheli, V, Rivetti, D, Deeks, JJ, Jefferson, T and Pratt, M (1998). The effectiveness and safety of vaccines against human anthrax: a systematic review. *Vaccine* **16**: 880–884.

19. Gorse, GJ, Keitel, W, Keyserling, H, Taylor, DN, Lock, M, Alves, K *et al.* (2006). Immunogenicity and tolerance of ascending doses of a recombinant protective antigen (rPA102) anthrax vaccine: a randomized, double-blinded, controlled, multicenter trial. *Vaccine* **24**: 5950–5959.
20. Pitt, ML, Little, SF, Ivins, BE, Fellows, P, Barth, J, Hewetson, J *et al.* (2001). *In vitro* correlate of immunity in a rabbit model of inhalational anthrax. *Vaccine* **19**: 4768–4773.
21. Little, SF, Ivins, BE, Fellows, PF, Pitt, ML, Norris, SL and Andrews, GP (2004). Defining a serological correlate of protection in rabbits for a recombinant anthrax vaccine. *Vaccine* **22**: 422–430.
22. Grunow, R, Porsch-Ozcuremez, M, Spletstoesser, W, Buckendahl, A, Hahn, U, Beyer, W *et al.* (2007). Monitoring of ELISA-reactive antibodies against anthrax protective antigen (PA), lethal factor (LF), and toxin-neutralising antibodies in serum of individuals vaccinated against anthrax with the PA-based UK anthrax vaccine. *Vaccine* **25**: 3679–3683.
23. Little, SF, Ivins, BE, Fellows, PF and Friedlander, AM (1997). Passive protection by polyclonal antibodies against *Bacillus anthracis* infection in guinea pigs. *Infect Immun* **65**: 5171–5175.
24. Beedham, RJ, Turnbull, PC and Williamson, ED (2001). Passive transfer of protection against *Bacillus anthracis* infection in a murine model. *Vaccine* **19**: 4409–4416.
25. Kobiler, D, Gozes, Y, Rosenberg, H, Marcus, D, Reuveny, S and Altboum, Z (2002). Efficiency of protection of guinea pigs against infection with *Bacillus anthracis* spores by passive immunization. *Infect Immun* **70**: 544–560.
26. Mohamed, N, Clagett, M, Li, J, Jones, S, Pincus, S, D'Alia, G *et al.* (2005). A high-affinity monoclonal antibody to anthrax protective antigen passively protects rabbits before and after aerosolized *Bacillus anthracis* spore challenge. *Infect Immun* **73**: 795–802.
27. Wild, MA, Xin, H, Maruyama, T, Nolan, MJ, Calveley, PM, Malone, JD *et al.* (2003). Human antibodies from immunized donors are protective against anthrax toxin *In vivo*. *Nat Biotechnol* **21**: 1305–1306.
28. Sawada-Hirai, R, Jiang, J, Wang, F, Sun, SM, Nedellec, R, Ruther, P *et al.* (2004). Human anti-anthrax protective antigen neutralizing monoclonal antibodies derived from donors vaccinated with anthrax vaccine adsorbed. *J Immune Based Ther Vaccines* **2**: 5.
29. Peterson, JW, Comer, JE, Noffsinger, DM, Wenglikowski, A, Walberg, KG, Chatuev, BM *et al.* (2006). Human monoclonal anti-protective antigen antibody completely protects rabbits and is synergistic with ciprofloxacin in protecting mice and guinea pigs against inhalation anthrax. *Infect Immun* **74**: 1016–1024.
30. Rivera, J, Nakouzi, A, Abboud, N, Revskaya, E, Goldman, D, Collier, RJ *et al.* (2006). A monoclonal antibody to *Bacillus anthracis* protective antigen defines a neutralizing epitope in domain 1. *Infect Immun* **74**: 4149–4156.
31. Vitale, L, Blanset, D, Lowy, I, O'Neill, T, Goldstein, J, Little, SF *et al.* (2006). Prophylaxis and therapy of inhalational anthrax by a novel monoclonal antibody to protective antigen that mimics vaccine-induced immunity. *Infect Immun* **74**: 5840–5847.
32. Subramanian, GM, Cronin, PW, Poley, G, Weinstein, A, Stoughton, SM, Zhong, J *et al.* (2005). A phase 1 study of PAmAb, a fully human monoclonal antibody against *Bacillus anthracis* protective antigen, in healthy volunteers. *Clin Infect Dis* **41**: 12–20.
33. Liang, M, Guttieri, M, Lundkvist, A and Schmaljohn, C (1997). Baculovirus expression of a human G<sub>2</sub>-specific, neutralizing IgG monoclonal antibody to Puumala virus. *Virology* **235**: 252–260.
34. BenAmmar-Ceccoli, S, Humblot, S, Crouzier, R, Acres, B, Kieny, MP, Herlyn, D *et al.* (2001). Recombinant vaccinia viruses expressing immunoglobulin variable regions efficiently and selectively protect mice against tumoral B-cell growth. *Cancer Gene Ther* **8**: 815–826.
35. Morimoto, K, Schnell, MJ, Pulmanusahakul, R, McGettigan, JP, Foley, HD, Faber, M *et al.* (2001). High level expression of a human rabies virus-neutralizing monoclonal antibody by a rhabdovirus-based vector. *J Immunol Methods* **252**: 199–206.
36. Lewis, AD, Chen, R, Montefiori, DC, Johnson, PR and Clark, KR (2002). Generation of neutralizing activity against human immunodeficiency virus type 1 in serum by antibody gene transfer. *J Virol* **76**: 8769–8775.
37. Noel, D, Pelegrin, M, Kramer, S, Jacquet, C, Skander, N and Piechaczyk, M (2002). High *In vivo* production of a model monoclonal antibody on adenoviral gene transfer. *Hum Gene Ther* **13**: 1483–1493.
38. Fang, J, Qian, JJ, Yi, S, Harding, TC, Tu, GH, VanRoey, M *et al.* (2005). Stable antibody expression at therapeutic levels using the 2A peptide. *Nat Biotechnol* **23**: 584–590.
39. Jiang, M, Shi, W, Zhang, Q, Wang, X, Guo, M, Cui, Z *et al.* (2006). Gene therapy using adenovirus-mediated full-length anti-HER-2 antibody for HER-2 overexpression cancers. *Clin Cancer Res* **12**: 6179–6185.
40. Fang, J, Yi, S, Simmons, A, Tu, GH, Nguyen, M, Harding, TC *et al.* (2007). An antibody delivery system for regulated expression of therapeutic levels of monoclonal antibodies *In vivo*. *Mol Ther* **15**: 1153–1159.
41. Kasuya, K, Boyer, JL, Tan, Y, Alipui, DO, Hackett, NR and Crystal, RG (2005). Passive immunotherapy for anthrax toxin mediated by an adenovirus expressing an anti-protective antigen single-chain antibody. *Mol Ther* **11**: 237–244.
42. Maynard, JA, Maassen, CB, Leppla, SH, Brasky, K, Patterson, JL, Iverson, BL *et al.* (2002). Protection against anthrax toxin by recombinant antibody fragments correlates with antigen affinity. *Nat Biotechnol* **20**: 597–601.
43. Pelletier, J, Kaplan, G, Racaniello, VR and Sonenberg, N (1988). Cap-independent translation of poliovirus mRNA is conferred by sequence elements within the 5' noncoding region. *Mol Cell Biol* **8**: 1103–1112.
44. Rosenfeld, MA, Yoshimura, K, Trapnell, BC, Yoneyama, K, Rosenthal, ER, Dalemans, W *et al.* (1992). *In vivo* transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. *Cell* **68**: 143–155.
45. Mittereder, N, March, KL and Trapnell, BC (1996). Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. *J Virol* **70**: 7498–7509.
46. Xiao, X, Li, J and Samulski, RJ (1998). Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J Virol* **72**: 2224–2232.
47. Rabinowitz, JE, Rolling, F, Li, C, Conrath, H, Xiao, W, Xiao, X *et al.* (2002). Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity. *J Virol* **76**: 791–801.
48. De, BP, Heguy, A, Hackett, NR, Ferris, B, Leopold, PL, Lee, J *et al.* (2006). High levels of persistent expression of alpha1-antitrypsin mediated by the nonhuman primate serotype rh.10 adeno-associated virus despite preexisting immunity to common human adeno-associated viruses. *Mol Ther* **13**: 67–76.
49. Plikaytis, BD, Turner, SH, Gheesling, LL and Carlone, GM (1991). Comparisons of standard curve-fitting methods to quantitate *Neisseria meningitidis* group A polysaccharide antibody levels by enzyme-linked immunosorbent assay. *J Clin Microbiol* **29**: 1439–1446.
50. Tan, Y, Hackett, NR, Boyer, JL and Crystal, RG (2003). Protective immunity evoked against anthrax lethal toxin after a single intramuscular administration of an adenovirus-based vaccine encoding humanized protective antigen. *Hum Gene Ther* **14**: 1673–1682.