Published in final edited form as:

Oral Dis. 2017 April ; 23(3): 337–346. doi:10.1111/odi.12614.

Immune reactivity after adenoviral-mediated aquaporin-1 cDNA transfer to human parotid glands

Ilias Alevizos¹, Changyu Zheng¹, Ana P. Cotrim¹, Corinne M. Goldsmith¹, Linda McCullagh^{1,+}, Theodore Berkowitz², Susan L. Strobl³, Anatoli Malyguine³, William C. Kopp⁴, John A. Chiorini¹, Nikolay P. Nikolov^{1,5}, Megan Neely², Gabor G. Illei^{1,6}, and Bruce J. Baum¹

¹Molecular Physiology and Therapeutics Branch, NIDCR, NIH, Bethesda, Maryland, USA

²Dept of Biostatistics & Bioinformatics, Duke University Medical Center, Durham, NC, USA

³Laboratory of Cell-Mediated Immunity, SAIC-Frederick, Maryland, USA

⁴Clinical Services Program, SAIC-Frederick, Maryland, USA

⁵Center for Drug Evaluation and Research, FDA, Silver Spring, MD, USA

⁶Clinical Development, MedImmune, Gaithersburg, MD, USA

Abstract

OBJECTIVES—The purpose of this study was to examine the humoral and cellular immune reactivity to adenoviral vector (AdhAQP1) administration in the human parotid gland over the first 42 days of a clinical gene therapy trial.

METHODS—Of eleven treated subjects, five were considered as positive responders (Baum et al, 2012). Herein, we measured serum neutralizing antibody titers, circulating cytotoxic lymphocytes, and lymphocyte proliferation in peripheral blood mononuclear cells. Additionally, after adenoviral vector stimulation of lymphocyte proliferation, we quantified secreted cytokine levels.

For correspondence: Dr. Ilias Alevizos, MPTB, NIDCR, NIH, Building 10, Room 1N110, MSC-1190, Bethesda, MD 20892 USA, Tel: +1-301-594-1193, alevizosi@mail.nih.gov.

⁺Deceased

Authors' Contributions

IA: participated in clinical evaluation of the patients and sample collection, participated in data organization, management and analyses, co-wrote the manuscript

CZ: participated in study design, performed experiments, approved manuscript

APC: participated in data organization management and analyses, participated in clinical evaluation of the patients and sample collection, approved manuscript

CMG, SS and AM: performed experiments, approved manuscript

LM: participated in clinical evaluation of the patients and sample collection

WCK: designed experiments, approved manuscript

JAC: designed experiments and interpreted data, approved manuscript

NPN: participated in clinical evaluation of the subjects, interpreted data, approved manuscript

TB and MN: analyzed data, approved manuscript

GGI: participated in study design, participated in clinical evaluation of the subjects, contributed to clinical assessment of the patients, approved manuscript

BB: designed the clinical trial, designed research experiments, interpreted data, co-wrote the manuscript

Competing financial interests

The authors declare no competing financial interests.

RESULTS—Responders showed little to modest immune reactivity during the first 42 days following gene transfer. Additionally, baseline serum neutralizing antibody titers to serotype 5-adenovirus generally were not predictive of a subject's response to parotid gland administration of AdhAQP1. Cytokine profiling from activated peripheral blood mononuclear cells could not distinguish responders and non-responders.

CONCLUSIONS—The data are the first to describe immune responses after adenoviral vector administration in a human parotid gland. Importantly, we found that modest (2–3 fold) changes in systemic cell-mediated immune reactivity did not preclude positive subject responses to gene transfer. However, changes beyond that level likely impeded the efficacy of gene transfer.

Keywords

Adenoviral; gene transfer; parotid; immune reactivity

Introduction

Adenoviral (Ad) vectors have been particularly useful in transferring genes to non-dividing cells, such as the epithelial cells found in salivary glands (Samuni & Baum, 2011). However, both adenovirus and Ad vectors typically elicit strong immunoreactivity (Jooss & Chirmule, 2003). Innate immune responses are generally considered to be the single greatest immune barrier limiting Ad vector gene transfer applications, though cell-mediated immunity certainly limits the extent of transgene expression observed (Gregory et al., 2011, Nayak & Herzog, 2010). Additionally, since most people have been naturally infected with adenovirus, neutralizing antibodies (NABs) are often present in their serum, and at high titers (Pine et al., 2011). However, there does not appear to be a strong relationship between the pre-existing NAb titer and the extent of gene transfer achieved, at least in humans (Campochiaro et al., 2006, Harvey et al., 2002).

Recently, we reported using a first generation, serotype 5 Ad (Ad5) vector, AdhAQP1, to transfer the aquaporin-1 cDNA to single parotid glands in subjects with salivary hypofunction resulting from radiation therapy during their treatment for a head and neck cancer (Baum et al., 2012). Five of the eleven individuals enrolled and treated in this study exhibited both objective (increased parotid saliva flow rate) and subjective (e.g., less oral dryness) outcomes during the first 42 days of the study; these five individuals are termed responders in the current report. Interestingly, all four of the subjects who received vector at doses $>1 \times 10^6$ vector particles/ul parotid infusate showed clinical, radiographic and/or laboratory indicators of inflammation (Baum et al., 2012). Three of these individuals experienced no benefits from the gene transfer maneuver, i.e., were non-responders (see Baum et al, 2012; Table 1 this report), including both individuals administered the highest dose of vector delivered (5.8×10^8 vector particles).

Herein, we report humoral and cellular immune responses observed during the first 42-day study period, in all treated subjects, and examine analytes (biomarkers) that could predict response to treatment. Since most of the immune related responses to Ad5 vectors occur within the first few weeks after delivery, we limited the analysis to data derived for each

subject during the first 6 weeks after AdhAQP1 delivery to the single parotid gland, i.e., a timeframe identical to that described in Baum et al (2012).

Importantly, we found that four of the five subjects who responded positively to vector administration exhibited some systemic indicators consistent with a modest vector-associated cell-mediated immune response, i.e., increased cytotoxic lymphocytes (CTLs) and/or increased lymphocyte proliferation. However, there was no statistically significant relationship between a subject's response to gene transfer and the pattern and expression of cytokines released, as measured by cytokine multiplex assays. Additionally, serum neutralizing antibodies (NAbs) in general showed modest change following vector administration over the 42-day study period in all subjects independent of pre-existing titers, and there was no simple relationship between subjects' responses to treatment with their baseline NAb titers.

Materials and Methods

Patients and study design

A Phase I/II clinical trial to test the safety and biologic efficacy of AdhAQP1 administration to a single previously irradiated parotid gland in eleven subjects using an open label, single dose, dose-escalation design was conducted (four dose tiers from 4.8×10^7 to 5.8×10^9 vector particles [vp]/gland, see Table 1; <http://www.clinicaltrials.gov/ct/show/NCT00372320?order=>, registered on September 2, 2006). This study was approved by the National Institute of Dental and Craniofacial Research Institutional Review Board, the United States Recombinant DNA Advisory Committee, the NIH Biosafety Committee, and the United States Food and Drug Administration (IND BB-13,102). Additionally, an independent Data and Safety Monitoring Board approved and monitored the study. Informed consent was obtained from all subjects prior to the initiation of any procedures. All methods were carried out in accordance with the approved guidelines (Baum et al., 2012). Treated subjects were followed at scheduled intervals. Multiple safety parameters were evaluated and the efficacy of the intervention was assessed by measurements of citrate-stimulated parotid salivary flow rate. Visual analogue scales (Pai et al., 2001) were used for symptom assessment.

Neutralizing antibody assay

For this report all serum NAb assays were performed at SAIC-Frederick in batches done during June-July 2011 (for subjects 19, 25, 40, 50, 73, 99, 103, 105, 118) or December 2011 (for subjects 4 and 116). To measure anti-Ad5 NAbs in the subjects' serum samples we used a previously described assay (Sprangers et al., 2003, Zheng et al., 2010). Briefly, this assay measured the ability of serum dilutions to prevent a recombinant Ad5 vector, AdCMVLuc encoding luciferase, from transducing 293 cells (Zheng et al., 2010). Titers are reported as the dilution of serum that resulted in 50% inhibition of the transduction of 293 cells with AdCMVLuc.

Elispot assay (cytotoxic lymphocytes)

Peripheral blood mononuclear cells (PBMCs) were thawed and rested for 2 hours in the presence of 30U/mL of DNase (10,000U/mL, Roche Cat# 04716728001) in 5% HuAB

ELISPOT media containing RPMI 1640 (Invitrogen, Cat #21807), 25mM Hepes (Invitrogen, Cat# 15630-080), 1X Pen/Strep-L-glutamine (100X Gibco BRL, Cat#10378-016), and 5% human AB serum (Gemini Cat#100-512). Cells were washed 2 times, counted on Coulter Z1, viability determined by trypan blue exclusion and adjusted to a viable cell count of 3×10^6 /ml in 5% HuAB ELISPOT media. ELISPOT opaque plates (Millipore, MSIPS40W10) were precoated with 1 μ g/well of capture antibody (anti-human IFN- γ mAb 1-D1K; Mabtech, Inc., Cincinnati, OH Cat #3420-3-1000) the previous day. The day of the assay the plates were washed 4 times and blocked with assay media for 2 hours. The blocking media was discarded and PBMCs were plated at 3×10^5 cells/well in triplicate. PBMCs were stimulated with 1 mg/ml 293 cell lysate prepared from cells transduced with the AdhAQP1 vector. A lysate from non-transduced 293 cells was used as a non-specific control and stimulation with phytohemagglutinin (PHA) was a positive control for T-cell responsiveness. The 293 cell lysates ($\pm$ AdhAQP1 transduction) were prepared as follows. Confluent 293 cells, grown in 15 cm culture dishes, were scraped into 20 ml culture media/dish. Cells were centrifuged at 2000 rpm and the media removed. The cell pellet was washed 3-times with phosphate buffered saline (PBS) via re-suspension and re-centrifugation. Thereafter, 2.5 ml 1mM NaHCO₃ was added to pellets from each 15 cm dish, the pellets re-suspended, and the re-suspension subjected to three rounds of freezing and thawing, followed by vortexing. This crude lysate was then centrifuged at 6000xg for 10 min at 4°C, the supernatant removed and PBS added to the original volume (equivalent to 2.5 ml/pellet) and this extract was used as the lysate for ELISPOT assays. For the AdhAQP1-transduced 293 cells we used 50 $\times$ 15 cm dishes and this procedure led to a yield of 612 mg protein (5.1 μ g/ μ l), while for the non-transduced 293 cells this procedure yielded 775 mg protein (6.2 μ g/ μ l) from 70 $\times$ 15 cm dishes.

After overnight incubation at 37°C in 5% CO₂, the plates were washed 6x with 0.05% Tween in Dulbecco's PBS to wash the cells and remove the cellular debris. The plates were incubated 2 hours at room temperature with biotinylated IFN- γ antibody (Mabtech #3420-6-100, diluted 1:2000 in PBS/1% bovine serum albumin/0.05% Tween). Thereafter, the plates were washed 4x with Dulbecco's PBS to remove excess antibody. Streptavidin-alkaline phosphatase (Mabtech #3310-10, diluted 1:3000 in PBS/1% bovine serum albumin) was added and incubated for 1 hour at room temperature. Spots were visualized by adding 100 μ l/well of BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium) (KPL, Cat# 50-81-07 Gaithersburg, MD) for 10 min. for maximal spot development. The reaction was stopped with washing the plates, 3 times, with distilled water and the membranes dried. Spots were enumerated on a CTL-ImmunoSpot[®] S5 micro analyzer (Cellular Technology Limited, Shaker Heights, OH). Two frozen normal donor controls with known responsive values were run with each assay to assure quality control of the results. For all assays, at least one of the two controls was within 2 SDs of the lab-generated mean for a pool of T cell epitopes from the human cytomegalovirus, Epstein-Barr virus, and flu viruses used for positive control antigens to elicit responses from antigen-specific memory T cells.

Lymphocyte proliferation

PBMCs were treated as above and adjusted to a viable cell count of 1×10^6 /ml in 10% HuAB Proliferation media containing RPMI 1640, but with the addition of 0.1mM non-essential amino acids (Gibco BRL, Cat#11140-050), 1mM sodium pyruvate (Gibco BRL, Cat#11360-070), 50uM 2-mercaptoethanol (Sigma, Cat#M7522) to that described above, with 10%, versus 5%, human AB serum. PBMCs were then plated into 96-well round bottom polystyrene plates at 10^5 cells/well in triplicate. The plated PBMCs were stimulated as described by Harvey et al (Harvey et al., 1999a, Harvey et al., 2001) with 2.5µg protein/ml Ad5 vector (AdMLPα.1AT; (Mastrangeli et al., 1994, Rosenfeld et al., 1991)) for 6 days or with 2.5µg/ml phytohemagglutinin (PHA-P, Sigma, Cat#L0917) for 3 days at 37°C in 5% CO₂. Cells cultured under similar conditions without any stimulation served as the negative control. After the indicated incubation period, the plates were centrifuged and 75µl of supernatant fluid were harvested from individual triplicate wells, combined and aliquoted into 2 vials. Then, 0.5µCi of [³H]-thymidine (Perkin Elmer, Cat#NET-027) was added to each well and incubated for 4 hours at 37°C in 5% CO₂ and thereafter plates were frozen at -20°C overnight. The plates were thawed, the cells harvested onto filtermats (Perkin-Elmer, Cat#1450-421) and allowed to dry several hours. The filters were then placed into sample bags (Perkin Elmer, Cat#1450-432) containing scintillation fluid (Perkin Elmer, Cat#1205-440) and counted with a Beta Scintillation Counter (MicroBeta Trilux, Perkin-Elmer). Proliferation responses were calculated as a stimulation index (SI), as determined by the mean ratio of antigen-stimulated counts per minute (cpm) over background cpm. Two frozen normal donor controls with known responsive values to PHA at day 3 incubation and pokeweed mitogen at day 6 incubation were run with each assay to assure quality control of the assay results. For all assays, at least one of the two controls was within 2 SDs of the lab-generated means for PHA and pokeweed mitogen.

Cytokine multiplex assay

Cytokines were tested in supernatant fluids collected from the above described lymphocyte proliferation plates using a multiplex Th1/Th2 panel from Meso Scale Discovery (Cat.# K15010B) following the manufacturers instructions. The multiplex is an electrochemiluminescence assay and plates were read on the MSD Sector Imager 6000. Cytokines included in the panel were IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p70, IL-13, and TNF-α. Each proliferation-derived supernatant sample was tested in duplicate and all samples from a single individual were tested in the same assay along with three internal control samples. When the number of samples exceeded the number that could be tested on a single plate one sample, usually the baseline, was tested on both plates. The standard curve range for the assay was 2.4 pg/ml to 10,000 pg/ml.

Statistical Analysis

To describe changes over time between responders and non-responders, as defined above, subject-specific trajectories were plotted for each analyte for each time-point and coded by responder-status. The mean biomarker levels and 95% confidence intervals were plotted by responder-status at each time point and were compared between responders and non-responders at each time point separately using a two-sample t-test to determine if (1) there is

ever a time point prior to 42 days when the analyte levels are statistically different between the groups and (2) there is an analyte that exhibits statistical difference before the other biomarkers under consideration. A biomarker that satisfies both conditions could potentially be used to predict whether a subject will eventually respond to AdhAQP1 gene therapy. As a sensitivity analysis, median biomarker levels, as opposed to mean levels, were compared because of the presence of outliers among the subjects at various time points. The Wilcoxon test was used to compare median biomarker level between responders and non-responders.

Given the small sample size, the analysis did not account for the varying dose tiers of AdhAQP1 vector assigned to the 11 subjects (the results presented are averages over the 4 different dosing tiers) and ignored the correlation in biomarker measures within subjects over time. The presented results were a split-time analysis rather than modeling longitudinal trajectories over time. Additionally, the results were not adjusted for the multiple tests performed across the measured biomarkers and across time points.

Results and Discussion

Analyses of a Phase I/II clinical trial examining immune responses offer several challenges. First, and most important, the sample size is always small and the confounders multiple. Secondly, human biologic variability is usually large. In general, we used a two-prong approach to analyze the results. Initially, we looked at the humoral and cellular immune responses for differences between all responders and all non-responders, searching for potential “universal” changes that could help us understand or could predict responsiveness. Thereafter, we looked at the individual level searching for patterns that could lead to clinically relevant information and associations.

Neutralizing antibody assay

The Ad5-NAb titers did not change substantially (from zero to ~8-fold) over the first 42 days of the study, irrespective of the vector dose concentrations and responder status (Fig. 1; Table 1). For example, three subjects with a baseline serum NAb titer of <1:2 were non responders (40, 73, 116), while two subjects who had quite high serum NAb titers (103, 118): <1:1024 and <1:8192, respectively, were responders. Of the two individuals showing the greatest elevation in serum NAb titer over the 42-day study period (50 and 105; both 8-fold increase, 50 increasing from <1:2 to <1:16 and 105 increasing from <1:32768 to <1:262144), the former was a responder while the latter was not. The significance of these findings is twofold. First, for the local delivery of an Ad5 gene transfer vector in the parotid glands, even the highest dose used herein (5.8×10^9 vp/gland) appeared to not dramatically provoke a systemic humoral immune response, at least in the two subjects treated with that dose. Secondly, the initial serum anti-Ad5 NAb titers observed were not simple predictors of a subject's ability to respond positively to the gene transfer maneuver, something that the inclusion/exclusion criteria for future studies can take into consideration. However, it is important to note that subject 105 did have the highest pre-treatment serum NAb titer (<1:32768) and also experienced the most significant spectrum of inflammatory responses to AdhAQP1 vector delivery (Table 1).

Elispot assay (cytotoxic lymphocytes)

CTLs can be key effector cells in the immune-mediated destruction of virally-infected cells. Therefore, we measured both the frequency and function of antigen specific CTLs with an IFN- γ ELISPOT assay by stimulating PBMCs with 1mg/ml 293 cell lysate prepared from cells transduced with the AdhAQP1 vector (Malyguine et al., 2012). There were no statistically significant differences in the mean number of activated PBMCs between grouped responders and non-responders in any of the time points measured (Figure 2; supplemental Figure 1). However, differences between responders and non-responders were highest on Day 14 and, even if not statistically significant for all the subjects, it may prove to be clinically notable. This timing of the presence of the largest number of CTLs corresponds with data shown in previously published reports in other tissue targets (McKelvey et al., 2004, Mendell et al., 2015). Looking at the individual plots (Figure 2; supplemental Figure 1), three of the non-responders (4, 105, 116) exhibited a quite high elevation (8–10 fold) in the number of activated PBMCs on day 14.

Lymphocyte proliferation

As noted earlier, proliferation responses of PBMCs to the Ad5 antigen were calculated as a SI. Grouping responders and non-responders, a difference in the mean values at Day 14 was observed, although it was not statistically significant (Figure 3; supplemental Figure 2). However, along with the IFN- γ Elispot assay differences between responders and non-responders occurring at the same time point, it appears this result warrants increased attention and, perhaps, suggests the possible benefit of using systemic blockers of inflammation over this period. Looking at the individual plots (Figure 3; supplemental Figure 2), two of the non-responders (4, 116) exhibited an increase in the SI 10-fold, while no responder exhibited showed an increase >3-fold in their SI over this study period. Subject 105's SI index did not increase until later and it was ~4-fold, which was similar to its day 42 value.

Cytokine multiplex assay

We also looked for trends or specific pathways that could be identified with the multiplex Th1/Th2 cytokine panel, measuring the levels of IFN- γ , IL-1 β , IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p70, IL-13, and TNF- α for the same time points as the other immunoassays. In Figure 4 and supplemental Figure 3 we report the individual changes for each cytokine over time and the median differences between responders and non-responders across the time points.

We used the Wilcoxon test and confidence interval, which are based on the pseudo-median and not the raw median. The descriptive and inferential summaries may appear to be discrepant but in reality they are not. That is, the difference in pseudo-medians may not be the same as the difference in the raw medians, which are usually reported in descriptive summaries. Nevertheless, this is not true for an inferential analysis based on means – the estimate from the two-sample t-test is equal to the difference in raw means based on the groups being compared. In supplemental Figure 3 we show that the pattern of inferential results does not change materially between the median-based (left hand side of the figure) and the mean-based (right side of the figure). By grouping the subjects as responders and non-responders, as described above, we found no evidence of statistical difference between

groups for any of the measured cytokines at any time point (all confidence intervals contained zero). However, for some there was evidence of a trend that failed to reach the level of statistical significance (likely due to the small sample size). This may warrant follow-up studies with larger numbers of subjects.

Even though the inherent limitation of a small sample size in a Phase I/II clinical trial precludes the generation of definitive conclusions, there are three key findings from the present study. First, at the doses employed baseline serum anti-Ad5 NAb titers generally were not predictive of a subject's response to parotid gland administration of AdhAQP1. Some subjects with high pre-existing NAb titers (e.g., 103, 118; 1:1024 and 1:8192, respectively) responded positively to treatment, i.e., vector delivery to glands clearly was not blocked by the high, serum anti-Ad5 NAb titer present, while others with low NAb titers (e.g., 40, 73, 116; 1:2 for all) were non-responders (Table 1). Furthermore, changes in serum NAb titers following vector administration were relatively small (zero to 8-fold) and generally similar in both responders and non-responders (Figure 1). Additionally, comparable results were found for anti-Ad5 NAb titers measured in saliva from the targeted parotid gland, although full sample analyses were only available for subjects 50, 99 and 103 (responders) and 73 and 105 (non-responders) and at much lower titers than the serum values (data not shown). All five of these subjects had baseline salivary Ad5-NAb titers of <1:16. Indeed, the very low levels of NABs present in parotid saliva provide the most likely reason for noninterference of salivary epithelial cell transduction via intraductal cannulation. For four of the five subjects for whom baseline and longitudinal salivary Ad5-NAb analyses were performed, two showed no change through day 42 (50 and 73), while two showed a maximum of a 2-fold increase in titer, i.e., to <1:32 (99 and 103). However, subject #105, who had the highest baseline serum Ad5-NAb titer (<1:32,768) showed a 512-fold increase in salivary anti-Ad5 NAb titer in the targeted gland on day 14, which remained constant through day 42. As noted in Table 1, this subject also exhibited frank gland inflammation, i.e., a flocculent swelling and high ⁶⁷Ga uptake (see also (Baum et al., 2012), suggesting that the elevated salivary anti-Ad5 NAb titer present likely was due to leakage from serum into the forming saliva.

Doubtless many factors influenced an individual subject's responsiveness to AdhAQP1 delivery in this Phase I/II clinical trial. However, from the present results, albeit with a small number of treated subjects, baseline serum Ad5-NAb titers alone appeared not to be simple predictors of parotid gland responsiveness. Overall, the findings herein regarding serum NAb titers and response to treatment are generally similar to those reported by Harvey et al following Ad5 vector administration clinically to several non-malignant tissues and quite different from results following Ad5 vector administration to naïve laboratory animals(Harvey et al., 1999a,b).

The next key finding herein was that four of the five subjects who responded positively to vector administration nonetheless exhibited some systemic indicators consistent with a modest vector-associated cell-mediated immune response. In three of five responders (50, 99, 118) there were increases (at peak, 2–3 fold; Figure 2 and supplemental Figure 1) in detection of CTLs, assessed by an IFN- γ Elispot assay. Three of six non-responders (4, 105, 116) also exhibited increased reactivity in this assay (Figure 2 and supplemental Figure 1),

but the fold increases were much greater than found with the responders. These same three non-responders also showed the greatest changes in their parotid gland ^{67}Ga citrate scans at 24-hours post-vector administration (Table 1; (Baum et al., 2012); see below). Similarly, in three of five responders (19, 99, 118) there were modest increases in Ad5 antigen-induced PBMC proliferation (at peak, 3 fold; Figure 3 and supplementary. Figure 2), while the same three non-responders with an elevated CTL response (4, 105, 116) again showed much greater lymphocyte proliferation levels. For all non-responder subjects, the Elispot and proliferation assay results were consistent with clinical findings ((Baum et al., 2012); Table 1). Thus, the present data suggest that modest (2–3 fold changes) systemic cell-mediated immune reactivity does not preclude positive subject responses to gene transfer following Ad5 vector administration to parotid glands. However, at least among the subjects treated herein, changes beyond that level likely impede the efficacy of gene transfer.

Finally, we examined supernatants obtained following the above PBMC stimulation using a multiplex assay for a panel of cytokines. We found this assay was unhelpful in distinguishing responders and non-responders, with no consistent patterns seen in each group (Figure 4 and supplementary Figure 3). Recently, it has been reported that pre-existing natural immunity to Ad5 (Ad5 seronegative, 18 vs titers >200) in HIV-negative volunteers was associated with differences in T-cell reactivity, measured as cytokine profiles in PBMCs stimulated *ex vivo* with an empty Ad5 vector (Pine et al., 2011). Herein, six subjects exhibited NAb titers 18. All (25, 40, 73, 116, all non-responders; and 99, 50 both responders) generally showed few changes in their *ex vivo* PMBC cytokine profile (Figure 4). Conversely, four subjects with very high NAb titers (1024; 4 and 105, non-responders; 103 and 118, responders) displayed heterogeneous cytokine profiles, with no clear pattern (Figure 4). In retrospect, instead of determining cytokine profiles *ex vivo* with Ad5 antigen-stimulated PMBCs, it may have been more informative for us to examine saliva directly from the targeted gland for the presence of inflammatory cytokines, since in patients with Sjögren's syndrome, local (salivary) cytokines are elevated significantly when compared to serum levels (Chiappin et al., 2007, Grisius et al., 1997).

As noted above, while host defense against adenovirus involves both innate and adaptive immune responses, the predominant response is through innate immunity (Gregory et al., 2011, Nayak & Herzog, 2010). Indeed, the multifaceted innate immune response, not adaptive immunity, is generally considered to be the limiting factor in successful Ad vector-mediated gene transfer, accounting for as much as 90% of vector clearance within the first 24 hours following intravenous vector administration (Nayak & Herzog, 2010). Furthermore, macrophages and other phagocytic cells involved in innate responses can directly influence adaptive immune responses, through production of inflammatory cytokines, such as TNF α and IL-6, as well as IL-8, which acts as a major chemoattractant for neutrophils (Gregory et al., 2011).

In the present study, following vector administration three of the six non-responders (4, 105, 116) showed clear evidence of a significant innate immune response (marked increases above baseline levels of ^{67}Ga citrate uptake in the targeted parotid gland at 24 hours; 49%, 29%, 22%, respectively), while at this time point none of the responders showed levels of ^{67}Ga citrate uptake >11% above baseline (Baum et al., 2012). This is consistent with

findings following Ad5-mediated salivary gland gene transfer in rodents (Adesanya et al., 1996, Wang et al., 2000), and perhaps suggests the need for co-administration of anti-inflammatory glucocorticoids in some individuals to ablate this immune reactivity (Seregin et al., 2009). Indeed, an elevated ^{67}Ga scan at 24-hours may be the easiest and most cost-effective early predictor of a negative response to Ad5-mediated gene transfer in a salivary gland.

In summary, these data represent the first immune reactivity data obtained following Ad5 vector-mediated gene transfer to salivary glands in humans. However, the aggregate results are not surprising, based on previous human studies of both Ad infections and Ad vector-mediated gene transfer performed in other tissue targets, including skin and a related epithelial organ, lung (Harvey et al., 2001).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The Intramural Research Program of the National Institute of Dental and Craniofacial Research supported this study.

References

- Adesanya MR, Redman RS, Baum BJ, O'Connell BC. Immediate inflammatory responses to adenovirus-mediated gene transfer in rat salivary glands. *Hum Gene Ther.* 1996; 7:1085–93. [PubMed: 8773510]
- Baum BJ, Alevizos I, Zheng C, Cotrim AP, Liu S, McCullagh L, Goldsmith CM, Burbelo PD, Citrin DE, Mitchell JB, Nottingham LK, Rudy SF, Van Waes C, Whatley MA, Brahim JS, Chiorini JA, Danielides S, Turner RJ, Patronas NJ, Chen CC, Nikolov NP, Illei GG. Early responses to adenoviral-mediated transfer of the aquaporin-1 cDNA for radiation-induced salivary hypofunction. *Proc Natl Acad Sci U S A.* 2012; 109:19403–7. [PubMed: 23129637]
- Campochiaro PA, Nguyen QD, Shah SM, Klein ML, Holz E, Frank RN, Saperstein DA, Gupta A, Stout JT, Macko J, DiBartolomeo R, Wei LL. Adenoviral vector-delivered pigment epithelium-derived factor for neovascular age-related macular degeneration: results of a phase I clinical trial. *Hum Gene Ther.* 2006; 17:167–76. [PubMed: 16454650]
- Chiappin S, Antonelli G, Gatti R, De Palo EF. Saliva specimen: a new laboratory tool for diagnostic and basic investigation. *Clin Chim Acta.* 2007; 383:30–40. [PubMed: 17512510]
- Gregory SM, Nazir SA, Metcalf JP. Implications of the innate immune response to adenovirus and adenoviral vectors. *Future Virol.* 2011; 6:357–374. [PubMed: 21738557]
- Grisius MM, Bermudez DK, Fox PC. Salivary and serum interleukin 6 in primary Sjogren's syndrome. *J Rheumatol.* 1997; 24:1089–91. [PubMed: 9195514]
- Harvey BG, Hackett NR, El-Sawy T, Rosengart TK, Hirschowitz EA, Lieberman MD, Lesser ML, Crystal RG. Variability of human systemic humoral immune responses to adenovirus gene transfer vectors administered to different organs. *J Virol.* 1999a; 73:6729–42. [PubMed: 10400771]
- Harvey BG, Hackett NR, Ely S, Crystal RG. Host responses and persistence of vector genome following intrabronchial administration of an E1(-)E3(-) adenovirus gene transfer vector to normal individuals. *Mol Ther.* 2001; 3:206–15. [PubMed: 11237677]
- Harvey BG, Maroni J, O'Donoghue KA, Chu KW, Muscat JC, Pippo AL, Wright CE, Hollmann C, Wisnivesky JP, Kessler PD, Rasmussen HS, Rosengart TK, Crystal RG. Safety of local delivery of low- and intermediate-dose adenovirus gene transfer vectors to individuals with a spectrum of morbid conditions. *Hum Gene Ther.* 2002; 13:15–63. [PubMed: 11779412]

- Harvey BG, Worgall S, Ely S, Leopold PL, Crystal RG. Cellular immune responses of healthy individuals to intradermal administration of an E1-E3-adenovirus gene transfer vector. *Hum Gene Ther.* 1999b; 10:2823–37. [PubMed: 10584928]
- Jooss K, Chirmule N. Immunity to adenovirus and adeno-associated viral vectors: implications for gene therapy. *Gene Ther.* 2003; 10:955–63. [PubMed: 12756416]
- Malyguine AM, Strobl S, Dunham K, Shurin MR, Sayers TJ. ELISPOT Assay for Monitoring Cytotoxic T Lymphocytes (CTL) Activity in Cancer Vaccine Clinical Trials. *Cells.* 2012; 1:111–26. [PubMed: 24710418]
- Mastrangeli A, O'Connell B, Aladib W, Fox PC, Baum BJ, Crystal RG. Direct in vivo adenovirus-mediated gene transfer to salivary glands. *The American journal of physiology.* 1994; 266:G1146–55. [PubMed: 8023944]
- McKelvey T, Tang A, Bett AJ, Casimiro DR, Chastain M. T-cell response to adenovirus hexon and DNA-binding protein in mice. *Gene Ther.* 2004; 11:791–6. [PubMed: 14961070]
- Mendell JR, Sahenk Z, Malik V, Gomez AM, Flanigan KM, Lowes LP, Alfano LN, Berry K, Meadows E, Lewis S, Braun L, Shontz K, Rouhana M, Clark KR, Rosales XQ, Al-Zaidy S, Govoni A, Rodino-Klapac LR, Hogan MJ, Kaspar BK. A phase 1/2a follistatin gene therapy trial for becker muscular dystrophy. *Mol Ther.* 2015; 23:192–201. [PubMed: 25322757]
- Nayak S, Herzog RW. Progress and prospects: immune responses to viral vectors. *Gene Ther.* 2010; 17:295–304. [PubMed: 19907498]
- Pai S, Ghezzi EM, Ship JA. Development of a Visual Analogue Scale questionnaire for subjective assessment of salivary dysfunction. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* 2001; 91:311–6. [PubMed: 11250628]
- Pine SO, Kublin JG, Hammer SM, Borgerding J, Huang Y, Casimiro DR, McElrath MJ. Pre-existing adenovirus immunity modifies a complex mixed Th1 and Th2 cytokine response to an Ad5/HIV-1 vaccine candidate in humans. *PLoS One.* 2011; 6:e18526. [PubMed: 21533229]
- Rosenfeld MA, Siegfried W, Yoshimura K, Yoneyama K, Fukayama M, Stier LE, Paakko PK, Gilardi P, Stratford-Perricaudet LD, Perricaudet M, et al. Adenovirus-mediated transfer of a recombinant alpha 1-antitrypsin gene to the lung epithelium in vivo. *Science.* 1991; 252:431–4. [PubMed: 2017680]
- Samuni Y, Baum BJ. Gene delivery in salivary glands: from the bench to the clinic. *Biochim Biophys Acta.* 2011; 1812:1515–21. [PubMed: 21763423]
- Seregin SS, Appledorn DM, McBride AJ, Schuldt NJ, Aldhamen YA, Voss T, Wei J, Bujold M, Nance W, Godbehere S, Amalfitano A. Transient pretreatment with glucocorticoid ablates innate toxicity of systemically delivered adenoviral vectors without reducing efficacy. *Mol Ther.* 2009; 17:685–96. [PubMed: 19174760]
- Sprangers MC, Lakhai W, Koudstaal W, Verhoeven M, Koel BF, Vogels R, Goudsmit J, Havenga MJ, Kostense S. Quantifying adenovirus-neutralizing antibodies by luciferase transgene detection: addressing preexisting immunity to vaccine and gene therapy vectors. *J Clin Microbiol.* 2003; 41:5046–52. [PubMed: 14605137]
- Wang S, Baum BJ, Yamano S, Mankani MH, Sun D, Jonsson M, Davis C, Graham FL, Gauldie J, Atkinson JC. Adenoviral-mediated gene transfer to mouse salivary glands. *J Dent Res.* 2000; 79:701–8. [PubMed: 10728970]
- Zheng C, Nikolov NP, Alevizos I, Cotrim AP, Liu S, McCullagh L, Chiorini JA, Illei GG, Baum BJ. Transient detection of E1-containing adenovirus in saliva after the delivery of a first-generation adenoviral vector to human parotid gland. *J Gene Med.* 2010; 12:3–10. [PubMed: 19941317]

Figure 1.

Ad5 Neutralizing antibodies titers for all subjects until Day 42 are represented by blue squares for responders and by orange circles for non-responders. Subjects receiving the first, lowest vector dose (Dose 1) are traced over shown with solid lines; subjects receiving Dose 2 are depicted with dash-gap lines; Dose 3 subjects are shown with square dotted lines and Dose 4 subjects are shown with square dot-gap-dash lines. Two subjects had identical titers and their traces on the figure are superimposed (subjects #40 and # 73 had titers of <1:2 throughout all reported time points). There are no patterns in the change of titers between responders and non-responders for any of the different doses. See Table on for additional information on baseline titers and vector doses.

Figure 2.

Cytotoxic lymphocyte responses in all subjects over the 42-day study period. Data for individual subjects are indicated by symbols and, in the lower panel, also divided as to responders and non-responders. See Materials and Methods for experimental details.

Figure 3.

Lymphocyte proliferation responses in all subjects over the 42-day study period. Data for individual subjects are indicated by symbols and, in the lower panel, divided as to responders and non-responders. See Materials and Methods for experimental details.

Figure 4.

Ex vivo secreted cytokine levels in supernatants from the lymphocyte proliferation experiments shown in Figure 3. Data for individual subjects are indicated by symbols and are separated as to responders (left side) and non-responders (right side). See Materials and Methods for experimental details.

Table 1

Summary of subjects' characteristics in AdhAQP1 clinical trial¹

Dose Group	Subject #	Age (yrs)	Gender	Responder ²	Baseline Ad5 NAb ³	Gland target	Peak flow (ml/min)	Day of peak flow	Fold flow increase above baseline	4 Clinical evidence of inflammation
4.8×10 ⁷	40	68	M	No	<1:2	Left	N/A	N/A	N/A	None
	25	60	M	No	<1:4	Right	N/A	N/A	N/A	None
	19	58	M	Yes	<1:64	Right	0.403	7	5.52	None
2.9×10 ⁸	50	58	M	Yes	<1:2	Left	0.291	42	2.01	None
	73	71	M	No	<1:2	Right	N/A	N/A	N/A	None
	99	53	M	Yes	<1:16	Left	0.497	14	5.40	⁶⁷ Ga +
1.3×10 ⁹	105	57	F	No	<1:32768	Left	N/A	N/A	N/A	P, L, ⁶⁷ Ga ++
	118*	56	M	Yes	<1:8192	Left	0.107	28	2.43	L
	103	62	M	Yes	<1:1024	Right	0.221	28	1.63	None
5.8×10 ⁹	4	53	M	No	<1:2048	Right	N/A	N/A	N/A	L, ⁶⁷ Ga +++
	116	62	M	No	<1:2	Left	N/A	N/A	N/A	⁶⁷ Ga ++

¹ Some of this information (dose, age, gender, gland target) was previously published in Table 1 of Baum et al (2012)² Responders, data highlighted in gray, were individuals who exhibited both (i) at least a 50% increase in saliva flow rates after administration of AdhAQP1 to the targeted parotid gland and (ii) an improvement in xerostomic symptoms as measured using a Visual Analogue Scale.³ All serum NAb titers reported herein were performed in two large batches over a short time interval. Note that there are some differences in the baseline values presented previously in Baum et al (2012).⁴ P = physical signs (visible); L = laboratory chemistry and/or hematology results; ⁶⁷Ga = based on a ratio of ⁶⁷Ga citrate scan results between the targeted and contralateral parotid gland at 24 hours post-vector delivery. + ratio > 10% higher; ++ ratio > 20% higher; +++ ratio > 30% higher. Details for these were reported in Baum et al (2012).

* This subject received a dose 71.5 % higher than intended due to a pharmacy dilution error (Baum et al, 2012).