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***INHERITED DISEASE*** ***RESEARCH ARTICLE***

# *Inhibition of recombinant adeno-associated virus (rAAV) transduction by bronchial secretions from cystic fibrosis patients*

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*The conducting airways are the primary target for gene transfer in cystic fibrosis (CF), yet the inflammation associated with CF lung disease could potentially pose a significant barrier to gene transfer vectors, such as recombinant adeno-associated virus (rAAV). In order to investigate this possibility, aliquots of bronchoalveolar lavage (BAL) fluid from eight individuals with CF were tested for their in vitro inhibitory effects on rAAV transduction, along with BAL from non-CF individuals. While the non-CF BAL fluid was not inhibitory, seven of eight CF BAL samples had significant inhibitory activity, resulting in a five- to 20-fold reduction in transduction events. Inhibition of rAAV transduction by CF BAL could be reversed by alpha-1-antitrypsin (AAT), but not by DNase. When neutrophil elastase and neutrophil alpha defensins (human neutrophil peptides, HNP) were measured in these samples, they were elevated by 500- and 10 000-fold, respectively. The levels of HNP correlated inversely with the amount of rAAV transduction. Furthermore, rAAV transduction could be blocked by purified HNP in an AAT-reversible manner at HNP concentrations within the range measured in these fluids. We conclude that products of inflammation in CF BAL fluid are inhibitory to rAAV transduction, and that these effects may be reversible by AAT.* Gene Therapy (2000) **7,** 1783–1789.

**Keywords:** *gene therapy; adeno-associated virus; cystic fibrosis; alpha-1 antitrypsin; inflammation*

## *Introduction*

Recombinant adeno-associated virus (rAAV) vectors have been shown to safely mediate efficient DNA transfer in the lower airways of rabbits,[2,3] mice[4], and rhesus macaques,[5,6] although the efficiency of integration[5] and expression[6] has not consistently matched the efficiency of DNA transfer. Furthermore, in a phase I clinical trial, efficient gene transfer has been demonstrated in the maxillary sinuses of cystic fibrosis (CF) patients.[7] However, preliminary results from a phase I clinical trial of AAV-CFTR gene transfer to the nasal and bronchial epithelium[8] indicates that the DNA transfer efficiency has been low. Since all of the subjects in these studies have CF, it is unclear whether this inefficiency of vector entry is specifically related to CF airway disease or whether it represents a more general limitation to rAAV gene transfer in the human bronchial epithelium.

There are several potential obstacles to gene transfer in the bronchial epithelium.[1] These include physical blockade of apical surface by mucus glycoproteins, which due to their excessive sulfation in CF might competitively bind AAV capsids.[9] There are also potential inhibitory effects from bacteria, neutrophils, or other host defenses. Neutrophils represent the predominant cell type in the CF airway. The excessive neutrophil burden in CF leads to the release of excessive DNA, actin, free neutrophil elastase, alpha defensins and other substances that might inhibit rAAV-mediated gene transfer. Other processes, which could inhibit rAAV uptake, include the exposure of virus to Pseudomonas-produced proteases or to cognate or innate immune responses. In addition, it is not known whether the AAV attachment receptor (heparan sulfate proteoglycan) or its co-receptors are present on the apical surface of differentiated bronchial epithelial cells. We sought to address the issue of these potential inhibitory effects in a series of *in vitro* studies using bronchoalveolar lavage (BAL) fluid from CF patients. The results of these studies indicated that CF BAL fluid was inhibitory to rAAV transduction and that neutrophil alpha defensins accounted for a significant proportion of the observed inhibitory effect, while neutrophil elastase (NE) did not.

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## *Results*

### *Study population*

BAL fluids from eight subjects with CF were analyzed. This population included six males and two females and ranged in age from 18 to 40 years. Six of the eight were ΔF508 homozygotes. Pulmonary function varied widely from an FEV1 of 24% predicted in the subject with the most severe lung involvement to an FEV1 of 83% predicted in the least severely affected. The Shwachman glo-

bal severity scores[10] were somewhat less variable, ranging from 70 to 89.

### *Inhibition of AAV transduction by BAL fluid from CF patients*

As an initial test of the concept that CF airway secretions may be inhibitory to AAV vector transduction we performed the following experiment. Packaged AAV-CMV-GFP virions (aliquots containing $10^7$ IU = $2 \times 10^9$ DNase-resistant particles per well) were pre-incubated (37°C, 4 h) with a 50% (v/v) dilution of bronchoalveolar lavage (BAL) fluid from subjects either with or without CF. These aliquots were then used to transduce cultures of $5 \times 10^5$ IB3–1 cells (a CF bronchial epithelial cell line) which had been pre-infected with Ad5 at a multiplicity of infection (MOI) of 5 p.f.u. per cell 4 h before vector administration. The addition of Ad was used to hasten the endpoint of the experiment. Pre-infection with Ad was performed before exposure to the BAL fluid to eliminate the possibility that substances in the BAL were affecting Ad infection rather than AAV vector transduction itself. The experiments were performed in triplicate. The absolute transduction efficiency in the presence of non-CF BAL fluid at the 50% concentration was 48.4%, which was no different from the 50% observed without BAL fluid. For clarity, the results with the CF BAL fluids are shown as a percentage of the non-CF baseline. In seven of the eight CF BAL fluids, there was statistically significant inhibition of transduction efficiency in the presence of a 50% concentration of CF BAL fluid (Figure 1). In the one patient with a missense mutation the transduction efficiency of rAAV-GFP is not effected by incubation with BAL. We speculate that this is due to milder airway disease characterized by less inflammation as is typical for those patients having missense *versus* ΔF508 mutations.

### *Inhibition of rAAV transduction is reversible by alpha-1-antitrypsin but not by DNase*

There are a number of factors present in CF BAL, which could be inhibitory to AAV transduction, including free DNA (which has been shown to be inhibitory to Ad transduction), neutrophil-derived elastase and/or defensins, immunoglobulins, Pseudomonas exotoxins, and free sulfated proteoglycans. In order to test for the contributions of the first two of these factors, the above experiments were repeated with the addition of two specific agents (alpha-1-antitrypsin (AAT), and DNase) designed to counteract them. Three different concentrations of BAL were used in this experiment (0.5%, 5% and 50%) and in the group with the highest dose of the CF BAL, two different concentrations of AAT (20 μg/ml and 200 μg/ml) and two different concentrations of DNase (0.18 and 1.8 units/μl) were used. The addition of AAT dramatically restored the efficiency of AAV-GFP vector transduction, while DNase did not (Figure 2). In fact, the addition of DNase further abrogated AAV-GFP transduction. IB-3 cells transduced with rAAV-CMV-GFP and incubated with AAT alone demonstrated a transduction efficiency of 63.2 ± 10.22 *versus* 100.0 ± 28.8 in the non-CF baseline (data not shown). These data indicate that an inhibitory activity in CF bronchial secretions can have a deleterious

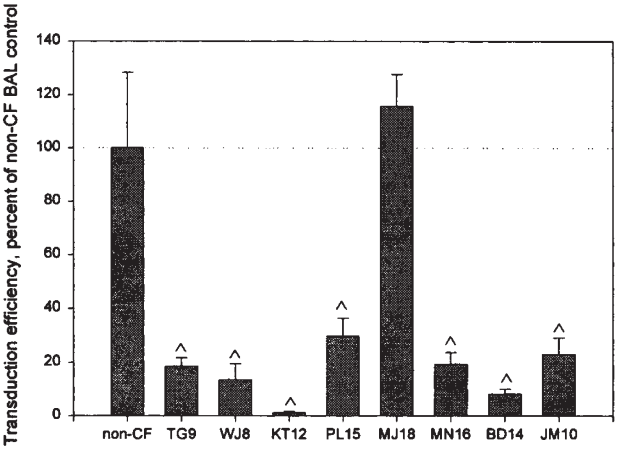


***Figure 1*** *Inhibition of rAAV transduction by CF BAL. The transduction efficiency of UF5 after exposure to BAL fluid from a non-CF individual or from each of eight CF subjects are shown. There was no significant difference between the non-CF BAL fluid sample and the saline-incubated control. For clarity, transduction efficiencies have been normalized to a percentage of the non-CF BAL control value. ^ Indicates a statistically significant decrease in transduction efficiency as compared with the control.*

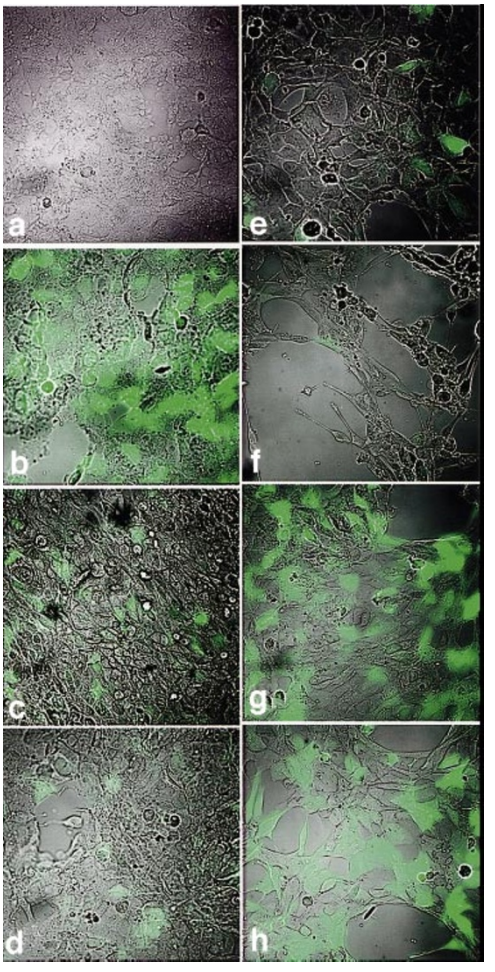


***Figure 2*** *Alpha-1-antitrypsin antagonizes the inhibitory effects of CF BAL, but DNase does not. Aliquots of $10^7$ IU of AAV-CMV-GFP vector were pre-incubated with PBS containing either 0.5% (b), 5% (c), or 50% (d) CF BAL fluid and used to inoculate Ad5-pre-infected IB3–1 cells. Additional mixtures of 50% CF BAL fluid were co-incubated with either 0.18 U/μl (e) or 1.8 U/μl (f) concentrations of DNase I or with 20 μg/ml[23] or 200 μg/ml (h) of human AAT. Ad5-pre-infected IB3–1 cells that were not incubated with AAV-CMV-GFP served as a negative control (a).*

effect on AAV particles, and perhaps may render them more DNase sensitive.

This experiment was repeated with parallel samples containing CF BAL alone, or BAL plus either DNase (to eliminate free DNA), AAT (to inactivate neutrophil elastase or defensin), or DNase/AAT in combination. Additional samples were pre-adsorbed with either protein G (to eliminate immunoglobulins), anti-Pseudomonas exotoxin antibodies (to eliminate Pseudomonas exotoxins), or protamine-agarose (to bind up and eliminate free heparan sulfate proteoglycan, which is known to be an important AAV binding receptor.[9] The results indicate that only AAT treatment rescued the transduction efficiency to levels above the BAL-only (inhibited) baseline. AAT had a four-fold induction and resulted in an absolute transduction efficiency at or near the non-inhibited control (Figure 3a).

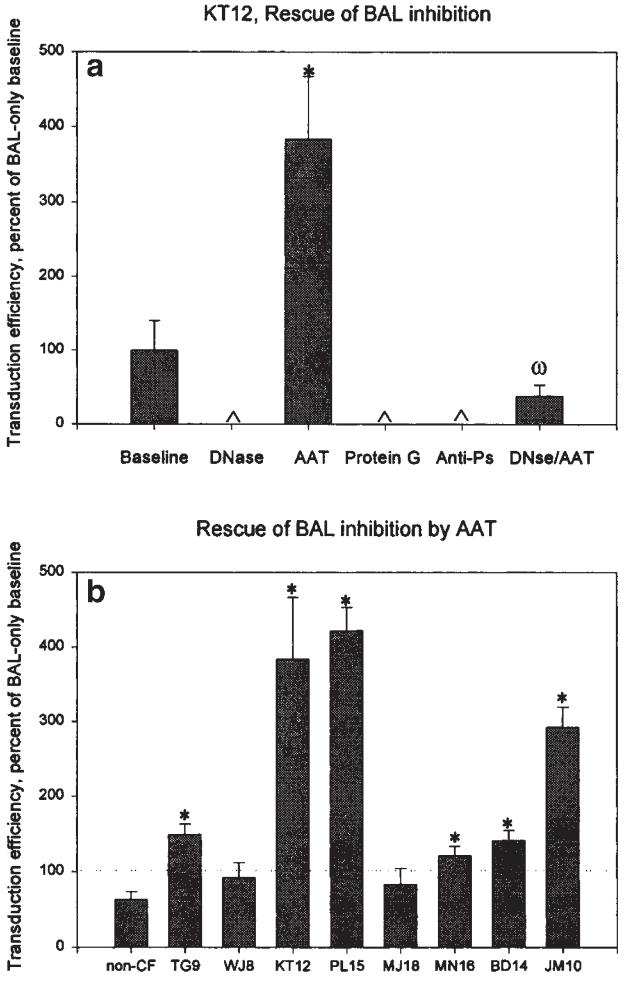


***Figure 3*** *Reversal of BAL inhibitory effects by AAT. In panel (a) is shown a comparison of the transduction efficiency after inhibition by BAL fluid from one of the CF subjects, KT12, marked as baseline. Comparisons to this baseline were made to co-incubation of KT12 BAL with DNase, AAT, a combination of DNase and AAT (Dn/AT), or by pre-adsorption of BAL with protein G-agarose (ProG), anti-Pseudomonas aeruginosa exotoxin antibody (anti-Pa) or protamine-agarose (Protam). Panel (b) shows the effect of AAT co-incubation on each of the eight CF BAL fluids. For comparison, each individual patient's BAL only transduction efficiency was used as the baseline. ∧ Indicates a statistically significant decrease in transduction efficiency, while * indicates a statistically significant increase. ω in panel a indicates a significant difference from both the baseline and the DNase only values.*

In panel b (Figure 3), the results of additional experiments are shown in which all eight CF BAL samples were tested for reversal of inhibitory effects by AAT. Not surprisingly, the one BAL (MRJ18), which did itself not cause any inhibition of transduction, also showed no change in the presence of AAT. In six of the seven cases where the BAL fluid did inhibit transduction, AAT was efficacious at reversing this effect. In contrast, DNase addition failed to augment transduction efficiency in any of these samples (Figure 4).

### *The inhibition of rAAV transduction by CF BAL is associated with elevations in BAL levels of neutrophil defensins and neutrophil elastase*

The beneficial effect of AAT on reversing CF BAL inhibition of AAV could be due to AAT's interaction with any of several substances in the CF airways. Two important candidates are neutrophil elastase and human neutrophil peptide (HNP, the primary neutrophil defensin). In order to address whether the samples which showed inhibition of AAV transduction also showed elevation of these substances, these molecules were assayed directly by ELISA (Table 1). The most striking findings in this analysis are that the ELF neutrophil elastase levels are 500-fold higher than normal, and the neutrophil defensin levels are 10000-fold higher than normal.

Furthermore, the one BAL fluid which did not show inhibition of AAT transduction (MRJ) actually had the lowest absolute BAL-HNP level. The three samples with the highest BAL-HNP levels (KKT12, WBJ18 and MAN16) represented three of the four BAL samples with the greatest level of inhibition. Uncorrected BAL levels are important in attempting to correlate levels with inhibition of transduction, since the original BAL inhibition studies were not normalized to ELF. In order to more formally test the potential association between HNP and rAAV inhibition, we plotted efficiency of rAAV transduction *versus* BAL HNP levels, and found an inverse correlation with $R^2 = 0.62$ (Figure 5).

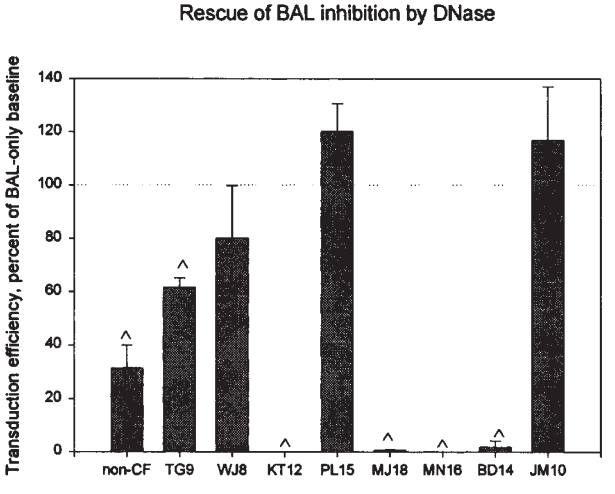


***Figure 4*** *BAL inhibition of rAAV is exacerbated by DNase addition. The UF5 transduction efficiency in the presence of each BAL co-incubated 1.8 U/μl of DNase (Pulmonzyme; Genetech) was compared with the transduction efficiency in the presence of the corresponding BAL fluid alone. Transduction efficiencies are expressed as a percentage of this BAL-only baseline. ∧ Indicates a statistically significant decrease as compared with the baseline.*

**Table 1** Epithelial lining fluid (elf) levels of neutrophil elastase (NE) and human neutrophil peptides (HNP), the primary neutrophil defensin

| *Patient* | $[NE]_{elf}$, nM | $[HNP]_{elf}$, nM | $[HNP]_{bal}$, nM |
|---|---|---|---|
| *Normal range* | *4 to 7* | *31 to 79* | |
| BRD | 431.8 | 112900 | 33200 |
| JDM | 476.0 | 57400 | 8200 |
| MAN | 4526.4 | 1391000 | 75600 |
| PJL | 7739.5 | 2469900 | 36700 |
| TGO | 2467.8 | 557500 | 12200 |
| WBJ | 4650.0 | 1590000 | 53000 |
| KKT | 163.8 | 70100 | 50100 |
| MRJ | 1382.0 | 63700 | 5900 |
| Mean | 2729.0 | 789100 | 34000 |

ELF values are derived by multiplying BAL fluid values by the lavage dilution factor. The dilution factor is the ratio of BAL urea nitrogen/blood urea nitrogen for each individual sampling.

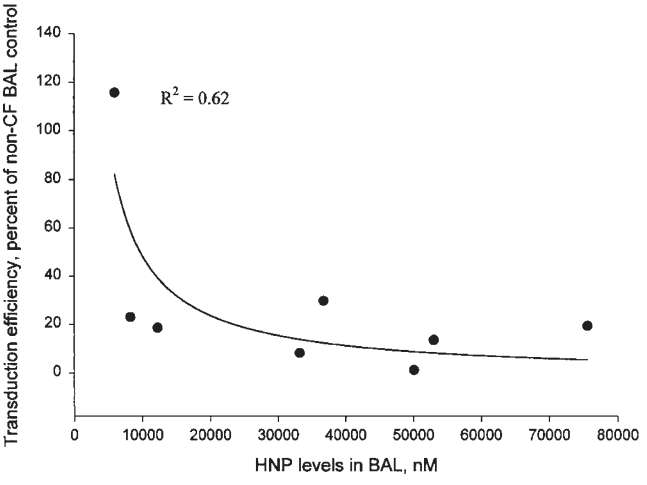


***Figure 5*** *Inverse correlation between HNP levels and transduction efficiency. The transduction efficiency with each of the CF BAL fluids tested was compared with the total BAL fluid level of HNP (uncorrected). A non-linear curve fit performed with Sigma plot 5.0 is shown.*

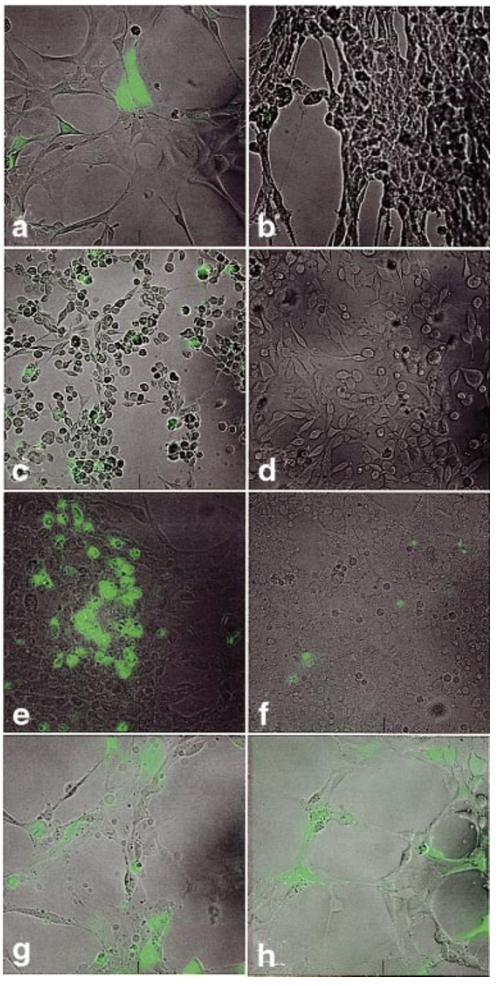


***Figure 6*** *Purified HNP is also inhibitory to rAAV transduction but NE is not. Superimposed Nomarski contrast and epifluorescence images of Ad-infected IB3–1 cells transduced with UF5 are shown. (a) UF5 alone; (b) no-UF5 control; (c) UF5 with CF BAL and AAT (200 μg/ml); (d) UF5 and CF BAL alone; (e) UF5 with HNP (100 μM) and AAT; (f) UF5 with HNP alone; (g) UF5 with NE (250 nM) and AAT; (h) UF5 with NE alone. Differences in morphology are due to the cytotoxicity of the HNP in the BAL samples.*

### *RAAV transduction is inhibited by purified HNP but not by purified NE*

The above-mentioned association of HNP levels with rAAV transduction was unexpected, since alpha defensins have previously been shown to inhibit only enveloped viruses. In order to determine directly whether HNP could be inhibiting rAAV, we exposed rAAV-GFP vector to a 1:1 mixture of HNP1 and HNP2 at a total final concentration of up to 100 μM, which is well within the range observed in the CF BAL fluids. RAAV transduction was again inhibited in an AAT-reversible manner (Figure 6). In contrast, no such inhibition was observed with NE, even at concentrations of up to 250 nM. This along with the inverse correlation mentioned above, tends to implicate HNP as an inhibitor of rAAV transduction in the CF airway.

### *Quantification of HNP-mediated inhibition of rAAV transduction using flow cytometry*

In order to verify HNP-mediated inhibition of rAAV-GFP transduction and compare the degree of inhibition with that observed with BAL, a number of flow cytometry experiments were performed. In these experiments, the HeLa-derived rAAV helper cell line, C12, was used for transduction, since these cells appeared to be more easily handled as single cell suspensions after transduction. As shown in the example (Figure 7), flow cytometric analysis indicated that under optimal conditions, rAAV-CMV-GFP transduction resulted in expression of GFP in approximately 50.1% of cells (Figure 7b), while in a control population of cells only 2.5% were scored as positive due to autofluorescence (Figure 7a). Exposure of rAAV to CF BAL (Figure 7c) caused a decrease in expression down to 14.3% (meaning that a 3.5-fold reduction of efficiency was noted), while exposure to purified HNP at a concentration of 250 nM, similar to that measured in the CF ELF (Figure 7d), caused a decrease in expression down to 33.9% (meaning that a 1.5-fold reduction was noted). Based on these estimates, it appeared that HNP accounted for roughly half of the noted inhibition. Meanwhile, exposure to purified elastase (Figure 7e) did not cause any decrease in GFP expression (49.0% *versus*

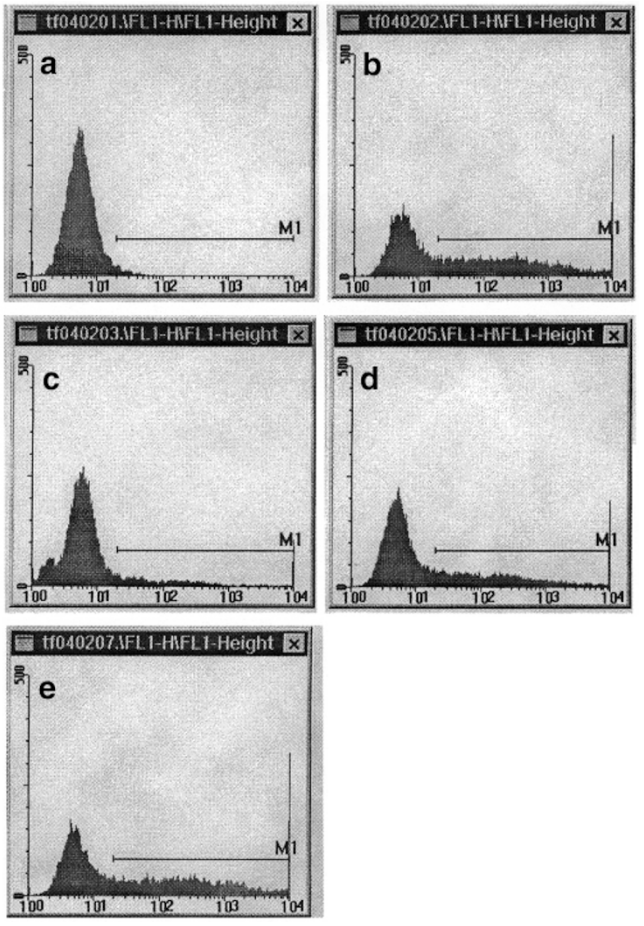


***Figure 7*** *Quantitation of HNP-mediated inhibition of rAAV transduction using flow cytometry. The HeLa-derived rAAV helper cell line, C12, was infected with Ad and transduced with rAAV-CMV-GFP. Transduction efficiency was measured by flow cytometry. Fluorescence intensity is shown on the x-axis in each histogram, while the number of cells counted in each window is shown on the y-axis. Cells within the M1 gate were scored as positive. (a) untreated negative control C12 cells; (b) Ad-infected, UF5 transduced positive control cells; (c) CF-BAL-mediated inhibition of UF5 expression; (d) partial inhibition by purified human neutrophil peptide (HNP), 250 nm; (e) lack of inhibition by purified neutrophil elastase.*

50.1%). Thus, the flow cytometry results in C12 cells confirmed the fluorescence microscopy results in IB3–1 cells, and indicated that about half of the CF BAL-mediated inhibition was due to neutrophil defensin, while NE did not appear to be contributing to this phenomenon.

## Discussion

In these experiments, CF BAL fluid inhibited rAAV transduction *in vitro*. However, AAT reversed this effect in most cases. Furthermore, there was an association between elevated BAL levels of neutrophil alpha defensins and the inhibitory effect. These factors could provide part of the explanation for the low efficiency of gene transfer observed with rAAV in the inflamed lower airways of CF patients.

The findings presented here are similar to inhibitory effects of recombinant Ad vectors in previous studies.[11] In a study of Ad vector transduction by Stern *et al*,[1] an inhibitory effect of CF sputum was also prominent. However, in that case the effect was reversible by DNase. In contrast, the inhibitory effect observed here on rAAV was not alleviated by DNase pretreatment. In fact, the inhibition was often exacerbated by DNase. This might indicate an effect that degraded rAAV capsid integrity, thus making the vector genomes more accessible to DNase. An alternative explanation could relate to the increase in free NE after DNase therapy, that has previously been reported.[12] Other studies have suggested that mucus itself may interfere with gene transfer. In this vein, we investigated the possibility that free sulfated proteoglycans could be inhibitory to rAAV transduction.[9] While our results did not indicate a significant contribution from free heparan sulfate, our model would not reflect the potential barrier of cell-bound mucins on the apical surface of the bronchial epithelium. Anti-AAV antibodies could also create a barrier to rAAV in the airway. A significant proportion of the human population have serum neutralizing antibodies directed against AAV capsid proteins. However, previous studies in humans both with natural wt-AAV infection[13] and with rAAV administration to the maxillary sinus,[20] have shown that the presence of serum neutralizing antibodies does not preclude AAV infection. Similar studies in animals have yielded conflicting results.[4,14,15]

One novel observation in our studies was the potential role of alpha defensins (HNP) in innate immunity to AAV. HNP has been increasingly recognized as an important effector molecule in neutrophil-mediated inflammation. This class of peptides has been increasingly recognized to play an important role in host defense against an array of microorganisms, including bacteria, fungi and viruses.[16–18] Although we do not know the mechanism, it is interesting that HNP affects a non-enveloped virus. Preliminary electron microscopy does not indicate that any aggregation of HNP with the AAV is taking place. Other studies with BAL fluid, however, have demonstrated that BAL increases DNase sensitivity, which is a reasonable indicator of physical distortion.[19] It seems reasonable then that HNP may also function via distortion of the capsid. Since neutrophilic infiltration is prominent in the CF airway, excessive levels of HNP should have been expected. Nevertheless, the degree of elevation of defensin levels in the BAL was massive, and this could have other important effects on the propagation of the inflammatory response.

Overall, this study suggests that the inflamed airway may not be an ideal target for rAAV-mediated transduction. The reversal of rAAV inhibition by AAT suggests that pretreatment with AAT aerosolization may be a useful adjunct to rAAV-CFTR delivery. Another approach that could ultimately be considered is to initiate therapy early in infancy, before the onset of significant airway inflammation. Recent studies indicate, however, that inflammation is already present at some level at the time of diagnosis in many CF patients.[20] Even if patients could be identified by screening of the newborn, the long-term safety profile of rAAV in this population is not sufficiently established to warrant neonatal gene therapy in the near future. Given this, a short-term pretreatment strategy with AAT and/or other anti-inflammatory agents may be the most practical approach to circumventing the observed effects.

## Materials and methods

### Acquisition of BAL fluid

BAL fluid samples used in these studies were taken from the remaining unused BAL gathered in association with a phase I trial of AAV-CFTR gene transfer to the nasal and bronchial epithelium of adult CF patients that was reviewed and approved by the University of Florida Institutional Review Board. Informed consent had been obtained from each subject as part of that original protocol. BAL was performed using standard protocols on four separate occasions, was transported on ice to the laboratory, and then was frozen at –80°C for later analysis. Control (non-CF) BAL fluid was obtained from samples designated for discard from the University of Florida Cytopathology Laboratory. The BAL had been taken from individuals undergoing clinically indicated diagnostic bronchoscopy for reflux aspiration-related lung disease.

### RAAV packaging

The rAAV-CMV-green fluorescent protein (GFP) vector, UF5,[21] was used for studies of *in vitro* inhibition of rAAV by BAL fluid. The UF5 vector was packaged by cotransfection of human embryonic kidney 293 cells (which are Ad E1a and E1b expressors) with a helper plasmid, pDG that contains both the additional required Ad helper functions (E2a, E4, VA) and the AAV *rep* and *cap* genes. RAAV particles were then purified using a newly described combination of iodixanol-gradient ultracentrifugation and heparin agarose affinity column chromatography.[19] Physical titers were obtained by quantitative-competitive PCR and biological titers by the infectious center assay.

### BAL inhibition experiments

All transduction experiments were performed using the CF bronchial epithelial cell line, IB3–1 (genotype ΔF508/W1282X).[22] Cells were pre-infected with Ad5 (MOI = 5 IU per cell) 1 to 4 h before rAAV infection. Aliquots of packaged UF5 vector were incubated with the BAL fluid to be tested before inoculation on to the IB3–1 monolayer. Cells were incubated for 24 to 48 h after vector transduction and then scored either by direct fluorescence microscopy (using a Zeiss (Göttingen, Germany) Axioskop upright fluorescence microscope) or by flow cytometry using a Becton Dickinson (Franklin Lakes, NJ, USA) FACSCalibur system.[23] Data from these counts were analyzed using standard two-tailed *t* test, accepting a type I error of 0.05 to define statistical significance.

### Determination of human neutrophil defensin concentration

The defensin concentration in BAL was determined by indirect sandwich ELISA. Immulon-2 plates (Dynatech, Chantilly, VA, USA) were coated overnight at room temperature with monoclonal mouse anti-human defensin antibody (Bachem, Torrance, CA, USA) suspended in 0.1 M carbonate buffer, pH 9.6. The plates were washed four times in PBS-Tween and blocked with 1% gelatin in 20 mM Tris-HCl, 500 mM NaCl, pH 7.5 (TBS) for 1 h at room temperature. A standard curve was prepared using synthezised defensins (Bachem) diluted in TBS+0.01% CETAB (hexadecyltrimethylammonium bromide) (Fluka Chemical Corporation, Milwaukee, WI, USA). The samples were applied directly and endpoint dilution of 1:2 was performed using TBS+CETAB diluent. After incubating for 2 h the plates were washed as above. Polyclonal rabbit anti-human defensin antiserum (Domcroft, Lovettsville, VA, USA) diluted 1/500 in TBS and CETAB was added to the wells and incubated for 1.5 h. After washing, the plates were incubated for 1 h in goat anti-rabbit IgG peroxidase conjugate antibody (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA) diluted 1/1000 in TBS plus 1% gelatin. The plates were developed by adding 100 μl of OPD (Sigma, St Louis, MO, USA), 0.2 mg/ml in 20 mM citrate buffer pH 4.7, containing 0.25 μg/ml 30% $H_2O_2$. The reaction was stopped by the addition of 2.5 N $H_2SO_4$. The plates were read using Molecular Devices (Sunnyvale, CA, USA) SPECTRAmax plate reader. Sample concentrations were calculated from best of fit curve of the standards and values corrected for BAL fluid dilution to give ELF concentration of HNP. This ELISA showed an interassay reproducibility of >95% and was sensitive to 0.78 nM of HNP.

### Determination of antigenic neutrophil elastase

Immulon 2 plates (Dynatech) were coated with sheep anti-human neutrophil elastase (ICN, Costa Mesa, CA, USA), diluted in Voller's buffer pH 9.6 at 4°C overnight. Standard human neutrophil elastase (Athens Research and Technology, Athens, GA, USA) was prepared by incubating 8 nM neutrophil elastase with 1 mM phenylmethylsulfonylfluoride (PMSF) (Sigma) for 30 min at room temperature. Plates were washed with PBS-Tween followed by the addition of samples or standard, pretreated in 1 mM PMSF (Sigma) and diluted 1:2 in PBS-tween+0.1% BSA. After incubation at 37°C for 1 h, plates were washed three times in PBS-Tween and rabbit anti-neutrophil elastase (Athens Research and Technology) diluted in PBS-Tween was added to the plates. After another 1 h incubation at 37°C the plates were washed and horseradish peroxidase-conjugated goat anti-rabbit IgG (Boehringer Mannheim), was added to the plates and incubated at 37°C for 1 h. The plates were developed by OPD addition. Reactions were terminated after 10 min by the addition of 8 N $H_2SO_4$, and the optical density at 490 nm was determined using a SPECTRAmax (Molecular Devices) plate reader. The amount of neutrophil elastase presented in the samples was interpolated from a standard curve of neutrophil elastase using the Molecular Devices SoftmaxPRO program and the values were corrected for BAL fluid dilution to give neutrophil elastase ELF concentration.

## Acknowledgements

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