



A fluorescence video-endoscopy technique for detection of gene transfer and expression

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The green fluorescent protein (GFP) has previously been adapted as a reporter for gene transfer and expression in mammalian cells in culture and in tissue sections. Herein is described a new method for detecting GFP *in situ* within epithelia accessible to fiberoptic endoscopy by incorporating fluorescent filters for detection of GFP into an existing fiberoptic endoscopy system. This device was used to detect expression of GFP from adeno-associated virus (AAV; dose of 3×10^7 IU) and adenovirus (Ad; dose of 1×10^9 to 1×10^{10} p.f.u.) vectors within the bronchial epithelium of New Zealand white rabbits. GFP expression was confirmed by fluorescence-activated cell sorting (FACS),

direct fluorescence microscopy of cytospin preparations of brushed cells, and by fluorescence microscopy of fixed tissue sections. This reporter gene/detection system was then used to track the time course of expression of the AAV vector in the bronchial epithelium over the first 30 days after administration. The transduction frequency in the treated region of the epithelium peaked at around 50% at 21 days after transduction. Vector expression was still present at around 20% efficiency at 30 days after administration. This example indicates how this method could be used to reliably track gene transfer in living animals or patients.

Keywords: adeno-associated virus (AAV); gene therapy; adenovirus (Ad); green fluorescent protein (GFP); endoscopy; bronchoscopy

Introduction

In order to achieve therapeutic gene transfer *in vivo* a vector must distribute to the appropriate cells or tissues, bind to cell-surface receptors, internalize, migrate to the nucleus and be converted into a form capable of transcription activity. As gene transfer strategies are tested, it is essential to have sensitive and specific assays for these processes. One approach to this problem has been to use reporter genes whose products can be assayed reproducibly in a quantitative or semi-quantitative fashion. These reporters have varied in their sensitivity, however, and most have been detected in large blocks of tissue at the time of necropsy. In the living organism, repeated biopsies or cytologic brush sampling has been required to obtain cellular material for assay. This sampling process has the potential to induce cellular proliferation or inflammation which can alter the nature of vector expression or remove the very cells of interest.

The *Aequorea victoria* green fluorescent protein (GFP) has recently been modified, or 'humanized', to enhance its sensitivity as a reporter which can be detected by fluorescence microscopy or flow cytometry.^{1,2} One unique property of GFP is that the native protein is fluorescent within living cells, potentially allowing for *in situ* detection within the living animal, thereby obviating the sam-

pling issues described above. The goal of the current study was to develop a technique for *in situ* GFP detection based on a conventional fiberoptic endoscopy system, frequently used for bright field visualization of the endobronchial surface of the airway, a site important for cystic fibrosis (CF) gene therapy. In this report, we describe a newly devised fluorescence video fiberoptic endoscopy (FVFE) system which could potentially be used to track the distribution of gene transfer within the airway, as well as the level and duration of expression from candidate viral vectors such as adeno-associated virus (AAV) or adenovirus (Ad).

Results

Design and testing of the FVFE device

The basic device consists of a modification of an Olympus (Roswell, GA, USA) pediatric fiberoptic bronchoscope (3.5 mm external diameter) attached to an Olympus CLV-U40 light source and the Olympus EVIS video imaging system (Figure 1). This device was modified to allow for fluorescent detection by (1) the insertion of the appropriate excitation filter for GFP (Chroma (Burlington, VT, USA) HQ480/40, peak transmission at 480 nm) within the primary filter wheel of the CLV-U40 light source and (2) the insertion of the appropriate detection/emission filter for GFP (Chroma HQ510LP, peak transmission at 510 nm) between the eyepiece of the bronchoscope itself and the camera head attachment.

As a preliminary test of the specificity of the filter set,

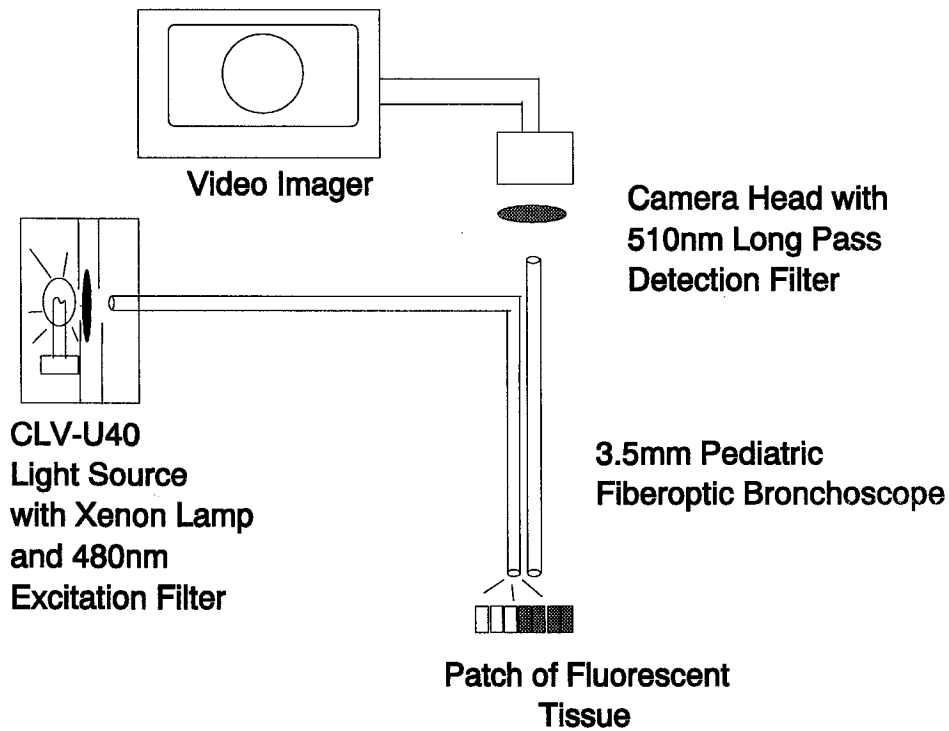


Figure 1 Diagram of fluorescence video-endoscopy (FVFE) detection system. Key elements of the FVFE device are depicted (see text for details).

a 1.5 ml polypropylene microfuge tube containing approximately 0.2 ml of a crude GFP protein preparation was placed against a white background and visualized with and without the barrier detection filter in place (Figure 2). In the absence of the barrier filter the intensity of light reflected from the GFP was similar to that reflected by the white background. When the barrier filter was inserted, the background signal decreased dramatically, enabling selective visualization of the GFP signal.

In vivo assessment of GFP expression in the rabbit airway

In order to determine whether the FVFE system would allow for visualization of GFP fluorescence within bronchial epithelial cells *in situ* on the airway surface, doses of 1×10^9 p.f.u. ($n = 2$) or 1×10^{10} p.f.u. ($n = 2$) of Ad-CMV-GFP were administered to the endobronchial surface of the right lower lobe (RLL) of New Zealand white rabbits, while three saline-instilled rabbits served as controls. The

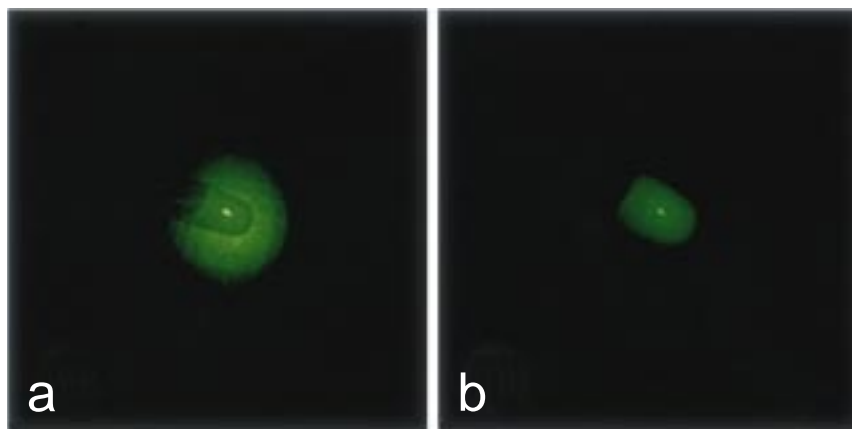


Figure 2 Demonstration of specificity of the FVFE system for green fluorescence versus reflected light. (a) 0.2 ml of a crude GFP extract in a 1.5 ml microfuge tube held against a white background and shown under conditions where the excitation filter was in place but the barrier detection filter was removed. Note the similarity in signal intensity between the GFP itself and the white background (reflected signal). (b) The same tube of GFP against the same background shown with both the excitation and detection filters in place. Note the elimination of signal attributable to light reflected off the white background.

bronchial surface was examined by FVFE at 3 days after vector administration, which has been found to be the time of peak of vector expression in previous studies. Diffuse, intense fluorescence was visualized over the entire mucosal surface of the right lower lobe and the adjacent right lower lobe bronchus of rabbits treated with Ad-CMV-GFP (Figure 3a), while no specific signal was observed in the saline-instilled rabbits (Figure 3c). The thin rim of signal seen at the upper left edge of field in Figure 3c was no longer visualized when the scope was flexed perpendicular to that area, probably indicating that it was due to tangentially reflected light.

Cells were obtained from this region by passing a cytologic brush through the suction channel of the bronchoscope. Approximately 50% of these cells showed GFP expression when analyzed by standard fluorescence microscopy using a filter set with the same excitation and emission spectra (Figure 4). These animals were then killed and formalin-fixed, paraffin-embedded 5- μ m sections were examined for epifluorescence using the same filter set, and once again large patches of epithelium were found to be fluorescent in this region of the bronchial epithelium, while saline controls showed very little or no autofluorescence (Figure 4). These data confirmed the correlation between microscopy and FVFE imaging.

Adeno-associated virus (AAV)-vector mediated GFP-expression and confirmation of expression by fluorescence-activated cell sorting (FACS)

In an analogous experiment, doses of approximately 3×10^7 IU ($n = 8$) of the AAV-CMV-GFP vector, TR-UF5, were administered to the right lower lobe of rabbits. In this instance the mucosal fluorescence as judged by FVFE was visualized at 13, 21 and 30 days, which should include the

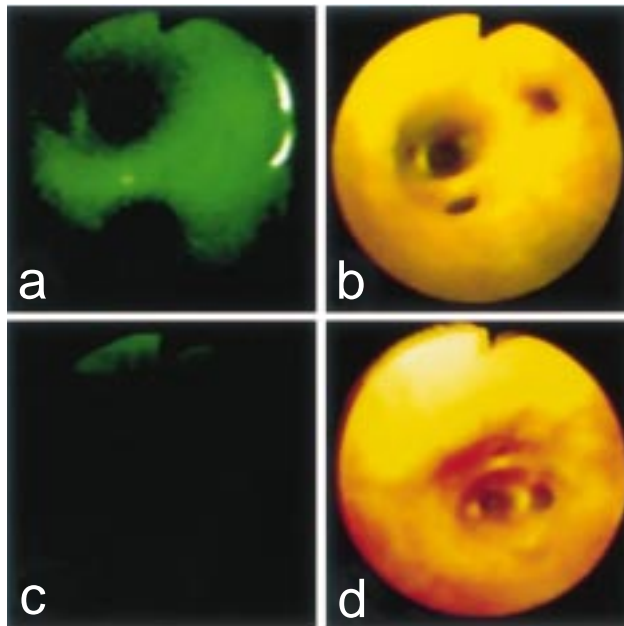


Figure 3 *In vivo* detection of GFP in the bronchial epithelium of New Zealand white rabbits. panels a and b show the fluorescence and bright field images, respectively, of the endobronchial surface of the right lower lobe of a New Zealand white rabbit 3 days after instillation of 1×10^9 p.f.u. of the Ad-CMV-GFP vector, UF7. Note the bright green fluorescence in panel a. Panels c and d show the corresponding fluorescence and bright field images from a saline-instilled control rabbit.

peak of expression based on earlier experiments.^{2,3} The fluorescence signal was somewhat less intense and appeared more discretely localized in patches or strips of the mucosa (Figure 5). This particular image corresponded with GFP fluorescence of approximately 20% as judged by fluorescence microscopy in cells brushed from the right lower lobe of the same animal. In contrast, the tracheas of these animals showed a minimal amount of reflected signal, comparable to that seen in the saline control animals (Figure 5). As was the case with the Ad-treated rabbits, fixed tissue sections confirmed the presence of patches of fluorescent epithelium. Also of note, histologic examination of lung sections from some vector-treated animals showed mild mucosal inflammation in both the Ad and AAV treatment groups (Figure 6). Similar changes were seen to a lesser degree in some vehicle control animals and there were no significant alveolar changes. Furthermore, there was no early mortality among vector-treated rabbits and no observable effect on respiratory rate, respiratory distress or weight gain.

In order to confirm independently the discrete population of GFP-expressing bronchial epithelial cells being visualized, fluorescence-activated cell sorting (FACS) was performed on cells brushed from the area of vector delivery. The forward scatter/side scatter profile of the cells obtained by brushing was correlated with morphology of cells obtained by cyto centrifugation of the same samples in order to include only bronchial epithelial cells in the fluorescence analysis and exclude red blood cells. Under these conditions, a discrete population of fluorescent bronchial epithelial cells was detected (Figure 7a) in the AAV-CMV-GFP-treated lobes, which represented as much as 55% of the bronchial epithelial cell population. In contrast, low levels of autofluorescence were observed in brushed bronchial epithelial populations from saline-treated animals ($3.07 \pm 2.75\%$) or from the untreated contralateral lobes ($5.78 \pm 5.11\%$) of treated rabbits.

In order to define the time course of AAV vector expression in the rabbit bronchus more clearly, this analysis was repeated serially on a subgroup ($n = 3$) of the eight AAV-CMV-GFP-treated rabbits (Figure 7b). In performing this analysis, the FVFE method was used to direct the cytologic brush to the GFP-expressing region of the bronchus. In each case, the rank order of fluorescence intensity as judged by FVFE matched that observed by FACS on the brushed cells. The time course data indicate that AAV vector expression peaks at 21 days, which is much later than that seen in previous studies with Ad vectors^{3,4} and nonviral systems,⁵ and is consistent with that noted in other AAV vector studies.⁶

Immune response to GFP-expressing cells

The apparently steep decline at 30 days is in contrast to our earlier findings with AAV-CFTR vectors in the rabbit⁶ and rhesus airway,⁷ as is the finding of mucosal inflammation, mentioned above. In order to assess whether these differences might be due to an immune response to the GFP protein, we determined the antibody titers to GFP and to the AAV capsid protein, VP-3 by means of a standard enzyme-linked immunosorbent assay (ELISA). Levels of antibody to VP-3 did not rise in any of the vector-treated animals between the pretreatment blood sampling and the 30-day post-treatment sampling, and all remained at or below the upper limit of the range in vehicle controls (Figure 8b). In contrast,

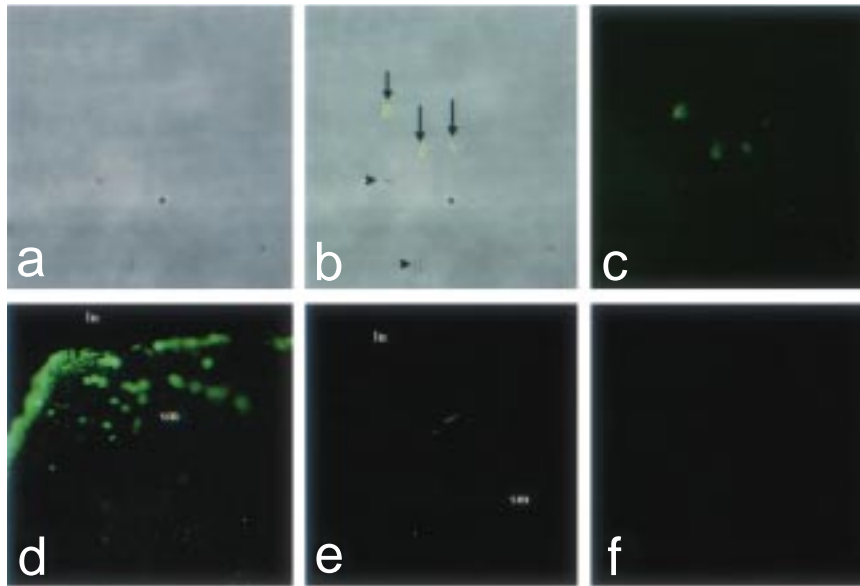


Figure 4 Direct fluorescence microscopy confirms GFP expression in brushed bronchial epithelial cells and tissue sections. (a, b and c) Bright field, overlay, and fluorescence images are shown respectively, of bronchial epithelial cells brushed from a rabbit 30 days after receiving approximately 3×10^7 IU of the AAV-CMV-GFP vector, UF5 (original magnification $\times 400$). Cells were mounted on a glass microscope slide by cytocentrifugation and examined by fluorescence microscopy using a GFP-specific filter set. Fluorescent epithelial cells are indicated by downward arrows, non-fluorescent cells by the right-pointing arrowheads. (d and e) 5- μ m paraffin-embedded sections of formalin-fixed lung samples from an Ad-CMV-GFP-treated and a saline control rabbit, respectively. Note patches of brightly fluorescent epithelium in d (lu, bronchial lumen, sm, submucosa).

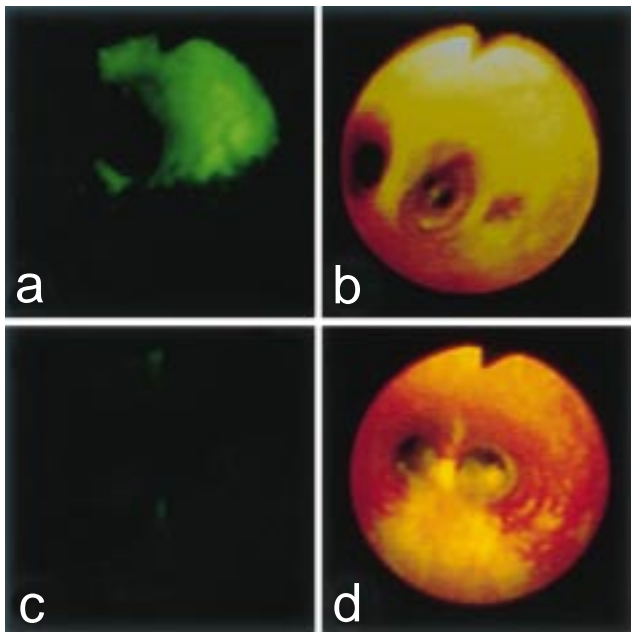


Figure 5 FVFE detection of AAV-CMV-GFP vector expression in the rabbit bronchial epithelium. (a and b) The fluorescence and bright field images, are shown respectively of the endobronchial surface of the right lower lobe of an AAV-CMV-GFP-treated rabbit examined 30 days after vector delivery. Note the sharp line of demarcation in a between fluorescent and non-fluorescent areas. For comparison, c and d show the fluorescence and bright field images from an untreated area of airway (trachea), which again demonstrates the low level of autofluorescence and the lack of significant spread of vector from the right lower lobe back up into the trachea.

the anti-GFP antibody titers rose in all of the vector-treated animals, in most cases to levels above the saline-control reference range (Figure 8a).

Discussion

The ability for gene transfer and expression to be detected non-invasively in the airway epithelium is of great interest since recent studies of viral and nonviral vector-mediated CFTR gene transfer in the airway have been somewhat limited by sampling issues⁸ and by changes in the mucosa which may have related to both the delivery process and the sampling process itself.⁹ We present here a relatively non-invasive method for assessment of gene transfer which was made possible by the recent development of the highly sensitive ‘humanized’ version of the GFP reporter gene.¹ The major limitation on the sensitivity of this technique is that a population of autofluorescent cells comprised $3.07 \pm 2.75\%$ of cells brushed from vehicle controls and $5.78 \pm 5.11\%$ of cells brushed from the contralateral lungs of treated animals. It is unclear whether the trend to a higher level of fluorescence in the contralateral lungs of treated animals was due to spill-over of vector, as was seen in one earlier study,⁶ or to random variability in the levels of autofluorescence. In either case the technique may be unreliable at expression efficiencies below 5%.

The assessment of gene transfer in the context of AAV-mediated transduction of the bronchial epithelium is also of interest, since a number of potentially conflicting observations regarding AAV-mediated gene transfer and expression have recently been reported. While some level of AAV gene transfer was readily detected within the lungs of rabbits in an earlier study,⁶ the ability of AAV vectors to express in primary cells was found to be

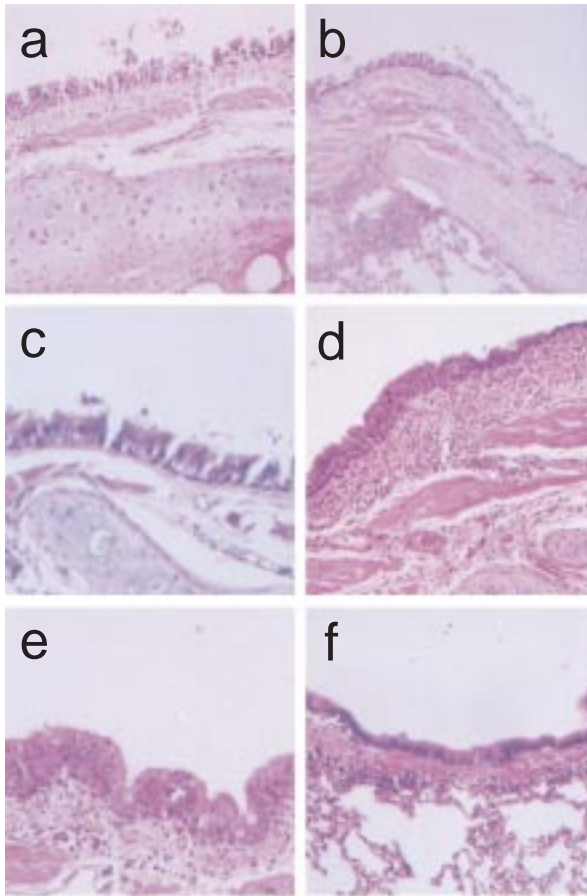


Figure 6 Mild mucosal inflammation was observed in GFP-expressing animals. A transverse 5- μ m section was made through the right lower lobe bronchus at the point of segmental branching of each rabbit. Sections were stained with hematoxylin and eosin and examined on a Zeiss (Thornwood, NY, USA) Axioskop upright microscope for evidence of inflammatory cell infiltration, goblet cell hyperplasia and basement membrane thickening. (a) Vehicle control ($\times 200$ original magnification); (b) Ad-GFP ($\times 200$); (c) Ad-GFP ($\times 400$); (d) AAV-GFP ($\times 400$); (e) AAV-GFP ($\times 400$); (f) AAV-GFP ($\times 200$). Mild inflammatory cell inflammation is seen in the submucosa in panels b and d.

greatly reduced as compared with immortalized cells.¹⁰ Furthermore, some have reported that irradiation, DNA-damaging agents, or adenoviral sequences^{11–13} were required for AAV-mediated expression to occur, presumably by increasing DNA polymerase activity and triggering a conversion of the AAV genome from single-stranded DNA to double-stranded DNA.

However, several groups have reported efficient AAV-mediated gene transfer and expression in slowly dividing cells such as skeletal myocytes,^{14–16} neurons,¹⁷ and retinal cells^{2,18} in the absence of irradiation or adenovirus. Our study, while not designed as a comprehensive assessment of *in vivo* AAV vector expression, suggests that AAV vector expression occurs without Ad but is delayed for several weeks. This reinforces earlier findings from our group,^{6,7,19,20} as well as recent data from Snyder *et al*,²¹ using AAV in the liver. Although there is no direct evidence for this in the current study, one may speculate that the enhancement by Ad which others have observed is essentially a kinetic effect, and that given sufficient time DNA repair enzymes will serve to complete leading

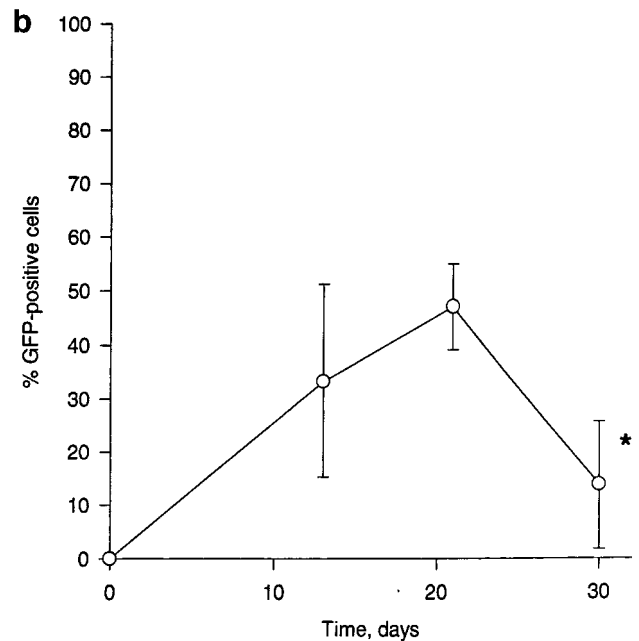
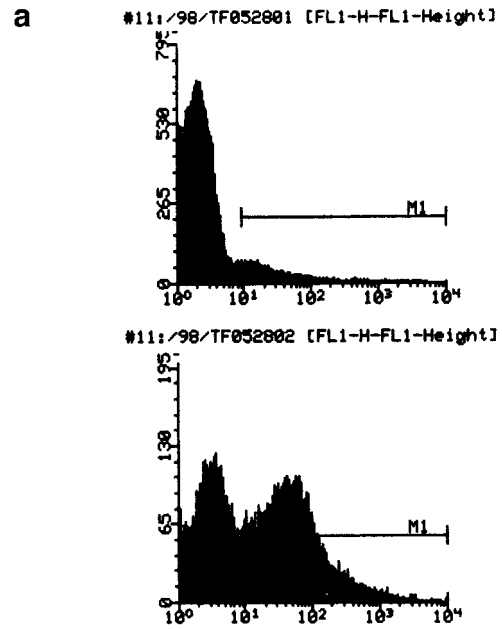


Figure 7 Fluorescence-activated cell sorting (FACS) confirms AAV-CMV-GFP expression after *in vivo* delivery. (a) Bronchial epithelial cells brushed at 13 days from the AAV-CMV-GFP-treated (RLL, lower histogram) and untreated (LLL, upper histogram) portions of the airway of a rabbit, were analyzed by FACS. Cells with a light-scatter profile characteristic of epithelial cells only were included, while red blood cells were excluded from the analysis. A discrete population of fluorescent bronchial epithelial cells representing 55% of the total epithelial cell pool is shown in the lower histogram, while the background level of cells scored as positive in the upper histogram is less than 10%. (b) Time course of AAV-CMV-GFP expression in the rabbit bronchus. Analysis similar to that depicted in a was performed on each of three AAV vector treated rabbits at 13, 21 and 30 days after vector instillation. The mean and standard deviation of each set of values is shown. (*, statistically significant decrease at $P < 0.05$).

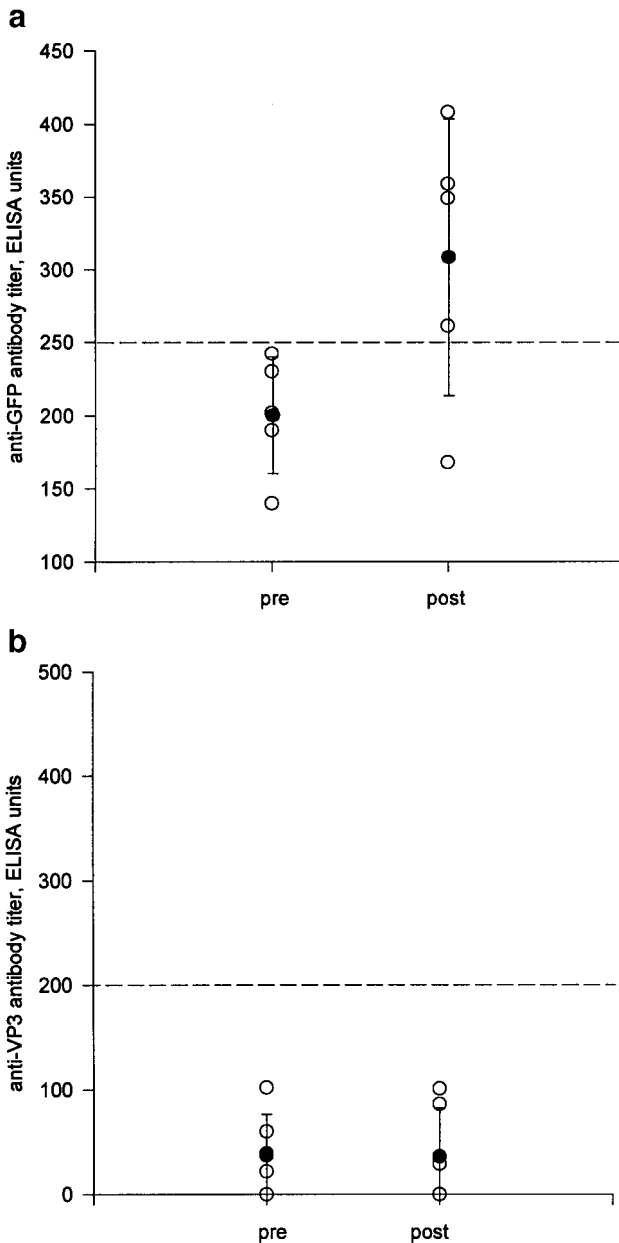


Figure 8 AAV-CMV-GFP vector-treated animals mounted a humoral immune response to GFP but not to AAV capsid protein. The levels of anti-GFP (a) and anti-AAV-VP3 (b) antibodies present in the sera of rabbits before (pre-) and 30 days after (post-) AAV-CMV-GFP vector delivery were determined by ELISA. Open circles show the values for each individual AAV-CMV-GFP vector-treated animal ($n = 5$). Closed circles and error bars show the mean and standard deviations of each group of measurements. The upper limits of the range of values measured in negative control sera in each assay are shown by the horizontal dashed lines.

strand synthesis of the AAV genome and allow for the expression observed here.

The immune response to GFP expressed from an AAV vector in this study is interesting. Several previous studies have assessed the immune responses to AAV vectors and their transgene products with varying results. In two of our own studies, the humoral immune response to AAV capsid proteins after single-dose administration of AAV-CFTR to rhesus monkeys and to humans was examined.⁷ In neither case was there a significant increase

in anti-VP3 antibody titers after single-dose administration. There are several other AAV gene transfer studies that have addressed this issue in greater detail. Of the several studies of AAV vector gene transfer in muscle, both Fisher *et al*¹⁶ and Kessler *et al*¹⁴ reported a low-grade immune response to AAV capsid after a single dose but no detectable response to a variety of prokaryotic and human foreign transgene products expressed from AAV in immunocompetent mice. The results of Xiao *et al*¹⁵ were similar in that anti-AAV neutralizing titers were low but detectable after a first dose (range 1:250 to 1:1250), and there was no detectable response to the transgene product. However, they found that after a second dose, the anti-AAV titers increased by 25-fold to greater than 1:30 000. Two other studies have had different results. A study by Snyder *et al*²¹ found neither an immune response to AAV nor to the transgene product after liver delivery, while Herzog *et al*²² found a vigorous response to human coagulation factor IX (hFIX) expressed from an AAV vector in murine muscle. The last of these studies differs most from the others, and the authors pointed out that the binding of the hFIX to type IV collagen may have altered the response to the transgene product. Finally, a study by Halbert *et al*²³ found that anti-AAV neutralizing antibody titers increased greatly after vector administration to the rabbit airway. In the latter study, vector preparations contained large amounts of wild-type AAV which may have affected the results. If there is a pattern to these results, it suggests that single-dose administration of AAV generally results in a low-level (or occasionally absent) response to the AAV capsid protein, which is the only protein component of the recombinant virion, but which is not subsequently expressed in transduced cells (at least in wild-type-free preparations). Furthermore, the response to the transgene product is curiously absent in many, but not all, instances. The latter finding may be due to a relative inability of AAV vectors to transduce antigen-presenting cells.¹⁶

The observed ability of endoscopic detection of GFP to serve as an *in vivo* marker of gene transfer and expression is of broad potential application in gene therapy, as well. Reporter genes have been used in clinical trials of gene transfer for cell marking from its earliest days. The potential use of GFP as a tracer or biological indicator has potential usefulness for diseases such as cystic fibrosis where broad distribution across an epithelial surface is presumably required, or for cancer applications where injection into all sites of tumor may be of critical importance. While immune reactions to GFP will be a concern in clinical settings, it is possible that the benefit of having more accurate assessment of the distribution of gene transfer may outweigh that potential risk in certain circumstances.

Materials and methods

Construction of FVFE system

A standard Olympus EVIS bronchoscopy system was modified by the incorporation of an excitation and detection filter manufactured by Chroma Technologies. The Olympus CLV-U40 light source contains a xenon lamp and a primary filter wheel into which was inserted a Chroma HQ480 excitation filter (480 nm) which had been

custom fit to the size of an open slot in the filter wheel. In order to mount a detection barrier filter within the light path for captured light, a Chroma HQ510LP (510 nm) was custom fit to be inserted between the eyepiece of the pediatric bronchoscope and the camera head attachment. Once inserted the detection filter could not be easily moved in and out of the light path. However, the excitation filter having been mounted on the existing filter wheel, could be moved in and out of the light path using the standard control mechanism of the Olympus system.

In vivo detection of GFP in the bronchial epithelium of New Zealand white rabbits

The AAV-CMV-GFP vector, pTRUF5, expresses the 'humanized' version of the *A. victoria* GFP gene in which codon usage has been corrected to correspond with that commonly employed by higher eukaryotes and is driven by the cytomegalovirus (CMV) immediate-early promoter, as has been described elsewhere.² AAV vectors were produced using a standard co-transfection technique in adenovirus-infected 293 cells followed by CsCl gradient ultracentrifugation and heat-inactivation as previously described.^{2,20,24} Biological titers of infectious recombinant AAV and wild-type AAV were determined by the infectious center assay and physical titers were determined by quantitative-competitive PCR. Using these methods, the particle to infectious unit ratio was approximately 100:1. The batch of vector used in these experiments contained approximately 250 infectious units of wt-AAV per animal dose, and less than 100 infectious units of Ad per dose. The analogous Ad5 vector, UF7, contains the identical promoter and GFP coding sequence in an Ad type 5 backbone.

New Zealand white rabbits (approximately 3 kg) were acquired from Hazelton (Herndon, USA) Laboratories and maintained under conditions approved by the institutional animal care and use committee. Animals were sedated with intramuscular acepromazine (3 mg) and ketamine (150 mg) and given supplemental oxygen by face mask throughout the procedure. A pediatric 3.5 mm fiberoptic was used for both vector instillation and later for fluorescence imaging and cytologic brushing. Three groups of animals were studied, including saline-instilled ($n = 3$), Ad-vector-instilled ($n = 4$), and AAV-vector-instilled ($n = 8$). Assessment of expression by FVFE and cytologic brushing was performed at 3, 13 and 30 days after vector delivery. In a subgroup of the AAV-treatment group ($n = 3$), cells brushed from the area of maximal fluorescence were analyzed for GFP by fluorescence-activated cell sorting (FACS) using a Beckton Dickinson (Philadelphia, USA) flow cytometer. At 3 days for Ad vector animals and at 30 days for AAV-treated and saline control groups, animals were killed by pentobarbital injection and underwent necropsy following the last bronchoscopy. Lung samples were fixed in 10% formalin, paraffin-embedded and sectioned at 5 μ m. One section of each was stained with hematoxylin and eosin for standard histopathologic examination, while another unstained section was used for fluorescence microscopy to determine GFP expression.

Determination of anti-GFP and anti-AAV antibody titers by ELISA

Serum was obtained from rabbits immediately before (pre-) and 30 days after (post-) endobronchial adminis-

tration of 3×10^7 IU of AAV-CMV-GFP. Levels of antibody directed against GFP and AAV-VP3 were determined by means of an ELISA assay. Immulon 4 (Dynatech, Chantilly, USA) flat-bottom microtiter plates were coated with 100 ng of the appropriate protein, blocked with 0.5% bovine serum albumin, and then incubated with a 1:100 dilution of each test rabbit serum. After binding with a goat anti-rabbit IgG-peroxidase-conjugated secondary antibody (1:5000 dilution), an OPD (*o*-phenylenediamine dihydrochloride)-detection reaction (Sigma, St Louis, MO, USA) was performed to quantify antibody levels in each test serum.

Acknowledgements

This work was funded by grants from the National Institutes of Health (DK51809, HL51811 and RR00082), and the Cystic Fibrosis Foundation. Many thanks to Dr Nicholas Muzyczka for providing assistance with the vector preparations used in these experiments. Thanks also to Drs Tom Reynolds, Morrey Atkinson and Barry Byrne for supplying VP-3 for the ELISA assays. The authors may be entitled to patent royalties for technology described in this paper.

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