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MOLECULAR STRATEGIES FOR THERAPY OF CYSTIC FIBROSIS

John A. Wagner, Anthony C. Chao, and Phyllis Gardner
Departments of Molecular Pharmacology and Medicine, Stanford University Medical Center, 300 Pasteur Drive, Stanford, California 94305

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ABSTRACT

Cystic fibrosis (CF), a lethal disease common to Caucasians, is characterized by a defect in the CF transmembrane conductance regulator and the resulting defective cAMP-regulated Cl^- secretion by epithelial cells. Clinical manifestations include both pancreatic and pulmonary insufficiency. Traditional therapeutic modalities address these problems with pancreatic enzyme replacement, vitamins and nutritional supplementation, antibiotics, and respiratory therapy. However, newer therapies directed at the specific underlying defects have emerged. In this review, we discuss agents that increase Cl^- secretion via preserved Cl^- secretory pathways, such as uridine triphosphate, or that enhance Na^+ resorption, such as amiloride, thereby correcting altered airway secretions. We also discuss agents, including deoxyribonuclease (DNase), that directly reduce sputum viscosity. CF is an early target for in vivo gene therapy, since it is a monogenic autosomal recessive disease in which restoration of normal cAMP-regulated Cl^- conductance can be achieved by complementation with a normal gene. The early clinical gene therapy work, with gene introduction by both viral and nonviral vectors, is discussed.

INTRODUCTION

Cystic fibrosis (CF), the most common lethal genetic disease in Caucasians, is caused by mutations in the CF transmembrane regulator (CFTR) and result-

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ing defective cAMP-regulated Cl^- secretion by epithelial cells (1–3). CFTR is a Cl^- channel regulated by cAMP-dependent protein kinase (PKA) and adenosine triphosphate (ATP). All of the characterized mutations in CF result in aberrant Cl^- secretion because of defective CFTR protein production, defective protein processing, defective regulation, or defective Cl^- conduction (4–6). Defective Cl^- secretion alters the volume and composition of epithelial surface liquids and leads to the pathophysiologic complications of CF. These complications include chronic inflammation, especially in the lung, the site of the most severe morbidity (1, 7–9). Substantive progress has occurred in identifying the molecular and cellular pathophysiology of CF. These developments have led to new prospects for molecular therapy of CF. This review details some of these new prospects for treatment of CF, including amiloride, uridine triphosphate, deoxyribonuclease, and gene therapy.

PROSPECTS FOR NEW THERAPIES

Many new prospects for therapy of CF have recently been described. These new therapies and potential therapies are based on greater understanding of the molecular and cellular pathophysiology of CF (reviewed in 2, 3, 6). Although CF involves epithelial tissues throughout the body—including respiratory, pancreas, liver, intestine, sweat gland, and reproductive epithelial cells—this review concentrates on respiratory epithelium, the site of the most severe pathophysiology and morbidity (1, 7–9). Normally, PKA and ATP open CFTR Cl^- channels. Net epithelial secretion of Cl^-, along with Na^+, controls the volume and hydration of airway mucus. In CF, mutations in CFTR prevent the normal secretion of Cl^- and result in altered composition and volume of airway mucus (reviewed in 6).

Decreased Cl^- secretion in CF initiates a pathophysiologic cascade. Increased viscosity and decreased volume of airway mucus lead to decreased clearance and bacterial infection, initially with *Haemophilus influenzae* and *Staphylococcus aureus,* and later with *Pseudomonas* species. Inflammation accompanies bacterial infection and leads to tissue damage, including bronchiolitis, chronic bronchitis, and bronchiectasis. Ninety-five percent of deaths in CF are secondary to pulmonary complications (1).

New therapies and potential therapies for CF are focused on particular components of this pathophysiological cascade. This review concentrates on therapies directed at correcting altered airway secretions, controlling inflammation, and correcting the basic defect in CFTR. Table 1 displays the pathophysiological schema and proposed therapies for CF. This review does not describe recent developments in existing therapies for CF such as antibiotics and chest physiotherapy.

Table 1 Pathophysiology and therapy for pulmonary manifestations of cystic fibrosis

Pathophysiology	Solution	Therapy
Mutant CFTR	Replace mutant with wild-type CFTR	Lung transplantation Protein replacement Gene therapy
Decreased Cl^- secretion	Increase Cl^- secretion through alternate pathway	UTP
Decreased volume of airway fluid	Block Na^+ absorption	Amiloride
Increased viscosity of airway fluid	Decrease viscosity	DNase
Decreased mucociliary clearance	Increase clearance	Chest physiotherapy
Bacterial infection	Decrease bacteria	Antibiotic
Inflammation	Reduce inflammation	Antiprotease NSAIDS Corticosteroid
Tissue damage	Replace damaged tissue	Lung transplantation and heart-lung transplantation

Alteration of Airway Secretions

Chloride secretion and Na^+ absorption control the volume and hydration of airway mucus. In CF, Cl^- secretion is severely restricted and Na^+ absorption is mildly increased; both processes tend to reduce hydration and increase viscosity of airway mucus. Thus, both Na^+ absorption and Cl^- secretion are targets for CF drug therapy.

REDUCTION OF SODIUM ABSORPTION Na^+ absorption is increased in CF airway epithelium, leading to further decreases in the volume of airway surface liquids. Thus, reduction of Na^+ absorption is a potential therapeutic target in CF.

Amiloride is a Na^+ channel blocker used as a K^+ sparing diuretic. It is an effective blocker of Na^+ transport in human airway epithelia in vitro and in vivo (10, 11). Amiloride is only effective in blocking Na^+ transport when applied on the apical face of the epithelium (12). Because of the exclusively apical action of amiloride, aerosol administration is necessary for the airway. Aerosol administration of amiloride can achieve concentrations effective in reducing Na^+ permeability.

A single nebulized dose of 1 mm amiloride was shown to increase mucociliary clearance and cough clearance in 14 CF patients (13). No alterations were observed in spirometry or blood pressure. This study was later extended to a three-week trial of nebulized amiloride twice daily. Again, both mucociliary clearance and cough clearance were improved after 3 weeks of treatment (13, 14). Amiloride only improved these parameters in those CF patients less than

15 years old and in patients without previous pneumonia. Amiloride increased sputum Na^+ levels and decreased sputum viscosity. No adverse effects were observed, but sputum bacteria levels rose in the amiloride-treated group.

A double-blind, placebo-controlled, crossover pilot study further evaluated the safety and efficacy of amiloride versus placebo treatment in CF patients with preexisting lung disease (15). Eighteen adult CF patients ages 18 to 37 were recruited, and 14 patients finished the study. All patients were chronically colonized with *Pseudomonas aeruginosa.* Loss of pulmonary function indicative of worsening pulmonary disease and measured by forced vital capacity (FVC) was slowed by 60% during the 6-month course of this study in the amiloride treatment group. Amiloride also normalized the electrolyte composition of airway secretions, decreased viscosity, and improved mucociliary clearance and cough clearance. No pulmonary or systemic adverse side effects were noted. Bacterial levels were equal between the two groups. Necessary medical interventions with oral antibiotics, bronchodilators, and steroid treatments were not different between the two groups.

Recently, however, another randomized, double-blind, placebo-controlled, crossover trial of nebulized amiloride conducted in the presence of regular bronchodilator and/or antibiotic therapy revealed no additional therapeutic benefit (16). No significant changes in FVC, forced expiratory volume at 1 s (FEV_1), oxygen saturation, biorheological properties of the sputum, and other parameters were found between the amiloride treatment and the control group. Also, the frequency of infective exacerbation was the same between the amiloride and the control group.

Thus, the favorable effects of amiloride in CF treatment seem to require further substantiation. A multicenter, parallel-design, double-blind, placebo-controlled phase III trial is currently under way, which attempts to provide definitive data on this therapy. Furthermore, a possible interpretation of the above studies is that greater benefit is derived from amiloride treatment at earlier ages. A randomized, open-label trial of aerosolized amiloride in children between the ages of 4 and 11 is currently in progress (17). Preliminary results suggest that therapeutic concentrations of amiloride can be reached in the lungs of younger children with low systemic exposure. The safety of chronic amiloride treatment in younger children is also being evaluated in this study (17).

The biorheologic data (18) suggest that amiloride acts on epithelial Na^+ channels to reduce Na^+ absorption and thus inhibits water reabsorption and dehydration of airway fluid. Increased airway fluid hydration, in turn, allows improved mucociliary clearance and cough clearance. While this is the most likely mechanism for the therapeutic activity of amiloride in CF, the drug does have a number of other effects besides inhibition of Na^+ channels. These include inhibition of Na-H exchange and T-type Ca^{2+} channels at low doses of amiloride (19, 20) and block of Na-Ca exchange, Na-glucose cotransport,

and Na–K-ATPase at higher, millimolar concentrations of amiloride (19). Amiloride has also been shown to have antimicrobial activity against *Pseudomonas cepacia* and hemolytic streptococci, and antiinflammatory activity (21), as well as the ability to block production of a toxin produced by *P. aeruginosa.* (22). In addition, amiloride has been shown to attenuate β-adrenergic stimulation of cAMP synthesis and Cl^- secretion in human tracheal epithelial cells (23). Whether any of these other effects contributes to the beneficial action of amiloride in CF patients is unknown.

INCREASE OF CHLORIDE SECRETION Cl^- flux in epithelial cells is controlled by a cAMP-regulated CFTR pathway, which is defective in CF (reviewed in 6, 24). An alternate, Ca^{2+}-regulated pathway (reviewed in 25) is preserved in CF (26–28). One strategy for therapy of CF is to increase Cl^- secretion from CF airway epithelial cells by stimulating the alternate pathway. The challenge is to activate the alternate pathway and increase Cl^- secretion activation without generating adverse side effects. Mere activation of Cl^- channels in CF epithelia does not result in net Cl^- secretion, because of the increased Na^+ absorption (29). Net secretion is only affected if Cl^- channel activation is combined with amiloride to decrease the sodium permeability in CF epithelia. Thus, combined treatment with amiloride and Cl^- channel activators may be desirable for therapy of CF. Several compounds have been identified that stimulate Cl^- secretion with favorable side-effect profiles.

Nucleotide triphosphates have been shown to activate Cl^- channels in normal and CF epithelial cells. Both the exact receptor through which the nucleotide triphosphates act and the mechanism of action are controversial (30, 31). In the presence of amiloride, nucleotide triphosphates (NTP), uridine triphosphate (UTP), and adenosine triphosphate (ATP) increase Cl^- secretion in normal and CF airway epithelia in vitro and in vivo (29, 30). These nucleotide triphosphates likely raise intracellular Ca^{2+} through their interaction with extracellular 5′-nucleotide receptors. The nucleotide triphosphate–activated Cl^- secretion is likely mediated by the Ca^{2+}-activated alternate pathway for Cl^- channel activation (30), although other mechanisms have been proposed (32). In addition to activating Cl^- channels, nucleotide triphosphates increase ciliary beat frequency and induce degranulation of goblet cells in airway epithelia (33, 34).

UTP is likely to be a safer agent than ATP for the purpose of prolonged aerosolized therapy. Ectonucleotidases are present in airway secretions. Although adenosine, the breakdown product of ATP, does not induce bronchoconstriction in normal subjects, it enhances bronchoconstriction in asthmatic patients (35). Aeorsolized UTP has been shown to have no systemic toxicity in hamsters (36). Acute aerosolized UTP in normal humans produces a small statistically significant decrease in pO_2 that is reversible and not accompanied

by other changes in spirometry or arterial blood gases. Acute administration of UTP in CF patients produced a small, reversible decrease in FEV_1, as well as a small decrease in pO_2. UTP increased mucocilliary clearance in normal humans. Preliminary evidence indicates that acute aerosolized administration of UTP is safe in humans. Studies of chronic administration are ongoing. If chronic administration of UTP is safe, then aerosolized UTP may prove to be a useful adjunctive treatment of CF in combination with amiloride for a combined therapy of Cl^- and Na^+ transport defects.

Other types of investigational Ca^{2+} agonists in epithelial cells that may have favorable side-effect profiles are alkylmaltosides, di-*tert*-butylhydroquinones (DBHQ), a specific microsomal Ca^{2+}-ATPase inhibitor, and duramycin. Alkylmaltosides are mild nonionic surfactants developed originally as possible emulsifier agents for foods, and are nontoxic. Preliminary evidence suggests that alkylmaltosides increase Cl^- transport in normal and CF epithelial cells (37). Elevation of cytosolic [Ca^{2+}] can also be induced by microsomal Ca^{2+}-ATPase inhibitors such as thapsigargin, cyclopiazonic acid, and DBHQ by a non-IP_3-dependent mechanism (38–40). DBHQ, which is also referred to in the literature as 2,5-di-(*tert*-butyl)-1,4-hydroquinone, is a commercially available synthetic compound of potentially low toxicity. Although some similar compounds, such as 2-phenyl-1,4-benzoquinone, a metabolite of the citrus fruit fungicide O-phenylphenol (OPP), have been shown to have a weak DNA-damaging activity in rats, such effects are not detected in other DBHQ analogues like DBHQ itself and phenylhydroquinone (41). DBHQ was recently found to cause a sustained rise in intracellular [Ca^{2+}] and to enhance Cl^- secretion in a dose-dependent manner in the CF pancreatic epithelial cell line CFPAC-1 (AC Chao, K Kouyama, Y-J Dong & P Gardner, personal communication). Duramycin, a peptide antibiotic, was shown to stimulate Cl^- secretion in airway epithelial cells (43), apparently via a Ca^{2+}-dependent mechanism (44). However, because duramycin induces stimulation only within a narrow concentration range (43) and exerts nonspecific effects on membrane ion conductances, its therapeutic usefulness is in dispute (45).

Other agents capable of activating Cl^- channels include P_1 purinoceptor agonists (46, 47), A_1 adenosine receptor antagonists (48), and ketoconazole, an epoxygenase inhibitor (49).

REDUCTION OF MUCUS VISCOSITY WITH DEOXYRIBONUCLEASE (DNase) Initial increases in airway secretion viscosity in CF are due to reduced Cl^- flux. This alteration in secretion viscosity interferes with mucociliary clearance, which eventually results in chronic infection and chronic inflammation. The viscous purulent sputum in chronic inflammation results from the breakdown of bacteria and granulocytes, and obstructs airways. A major component of the sputum is granulocyte DNA. In fact, DNA constitutes 10% of sputum by dry

weight (50). The finding that granulocyte DNA is a major contributor to sputum viscosity suggested that DNase could play a role in reducing the viscosity of CF mucus, an observation confirmed experimentally (51).

Originally, bovine DNase was used to reduce mucus viscosity, with mixed results (52, 53). More recently, recombinant human DNase I (rhDNase I; dornase alfa) has been used. rhDNase I at 50 μg/ml increases sputum pourability assay (51). A phase I trial using rhDNase I in CF patients showed that rhDNase I was well treated (54). A phase II trial showed improvement by 13.3% in both FVC and FEV_1, with 0.6, 2.5, and 10 mg twice-daily doses of rhDNase I (55). A phase III, parallel-design, randomized placebo-controlled, double-blind, 51-center study confirmed the efficacy of rhDNase I (56). Over the 24-week study period, rhDNase I in once- or twice-daily dosing reduced the number of infections requiring parenteral antibiotics and increased lung function. In addition, rhDNase I decreased the subjective sense of dyspnea and increased the overall perception of well-being. Patients receiving twice-daily rhDNase I spent a statistically significant 1.2 fewer days in the hospital. Self-limited alteration in voice was the main adverse effect. No anaphylaxis or change in mortality was observed among the groups during the study. In addition, two phase II trials have found favorable effects and observed no significant safety issues in more seriously ill hospitalized CF patients (55, 57). rhDNase I was approved by the FDA in 1994 and appears to be a promising new therapy for CF patients with a wide spectrum of disease.

REDUCTION OF MUCOS VISCOSITY WITH GELSOLIN Purulent CF sputum also contains filamentous actin. Gelsolin is a human plasma protein that severs actin filaments. Recently, gelsolin was shown to decrease CF sputum viscosity in vitro better than DNase I (58). Gelsolin deserves further investigation as a potential therapeutic mucolytic agent in CF.

Control of Inflammation

Inflammation secondary to bacterial infection in CF lung disease ultimately leads to tissue damage, including bronchiolitis, chronic bronchitis, and bronchiectasis. Control of inflammation in CF lung disease may significantly delay morbidity. Several of the agents used to control inflammation are discussed below.

α_1 ANTITRYPSIN AND OTHER ANTIPROTEASES Inflammation in the chronically infected CF lung and resulting excess of neutrophil elastase can overwhelm the activity of α_1 antitrypsin, even though the absolute amount of α_1 antitrypsin activity present is normal (59). Two weeks of intravenous antibiotics in CF patients reduces serum and lung fluid elastase concentrations, which

demonstrates that the extent of the imbalance between elastase and α_1 antitrypsin activities is directly related to the burden of infection.

Because endogenous antiprotease levels are overwhelmed by excess elastase levels, a potential therapy for CF is treatment with exogenous antiprotease. Aerosol treatment of CF patients with purified human α_1 antitrypsin reduces elastase activity in lung fluids and restores neutrophil bacterial killing activity (60). Research on aerosolized α_1 antitrypsin treatment of CF lung disease is currently limited because of the great cost of the purified human enzyme. Availability of recombinant human α_1 antitrypsin should make this therapy more economically feasible.

Other promising antiproteases include α_1-proteinase inhibitor and secretory leukocyte protease inhibitors (61–63). Antioxidants are under consideration as potential antiinflammatory agents in CF (64, 65).

CROMOLYN This agent stabilizes mast cell membranes and thus prevents mediator release and inhibits early- and late-phase allergic response. A short-term study revealed encouraging results on CF patients (66), but a longer-term study did not confirm these results (67). Therefore, cromolyn is not currently recommended for therapy in CF except in CF patients with concurrent asthma.

CORTICOSTEROID The corticosteroids are powerful antiinflammatory agents as well as immunosuppressants. They inhibit chemotaxis, reduce adhesion and activation of inflammatory cells, lower cytokine expression, and decrease arachidonic acid metabolism, which results in reduced synthesis of leukotrienes and prostaglandins.

Interest in steroid therapy for CF was sparked in 1985 by the publication of a double-blind trial in which CF patients with relatively mild lung disease were treated systemically with 2 mg/kg prednisone or placebo for 4 years (68). Prednisone treatment resulted in fewer CF-patient hospital admissions, better lung function, reduced immune activity as evidenced by decreased IgG levels, and relatively few side effects.

A larger, multicenter, double-blind trial employing alternate-day 1 mg/kg prednisone, 2 mg/kg prednisone, and placebo attempted to replicate these favorable results (69). FVC was increased throughout the two years of the study in the low-dose group compared to placebo. The high-dose group also showed improvement, but it was less marked than the low-dose group. FEV_1 was statistically improved only at the 12-month time point in the low-dose group compared to control. If the population was subdivided into *P. aeruginosa* carriers and noncarriers, then those colonized showed statistically significant improvement at all time points in the 1 mg/kg group compared to placebo. No significant differences were observed in hospitalization rates. Unfortunately, the high-dose arm of the study required early termination secondary to adverse

effects, largely growth retardation. The low-dose group also had significantly more growth retardation than control and no catch-up growth observed in the one year following termination of the study. Despite the beneficial effects noted in the low-dose group, prednisone therapy is not recommended for CF patients because of the high frequency of adverse side effects.

The inhaled route of steroid administration is associated with fewer side effects in patients with asthma and chronic obstructive pulmonary disease. One study of local steroids revealed no beneficial effects in the lungs of CF patients (70).

NONSTEROIDAL ANTIINFLAMMATORY DRUGS Ibuprofen, prototypical of nonsteroidal antiinflammatory drugs (NSAIDS), decreases synthesis and release of leukotrienes and prostaglandins by inhibiting arachidonic acid metabolism, and decreases chemotaxis adhesion, activation, and degranulation of neutrophils. Concentrations of ibuprofen between 25 and 100 μg/ml lower leukotriene B_4. In preliminary studies in animals Konstan and coworkers reported evidence for protection of the lungs from chronic inflammatory damage (71). This group of investigators is currently conducting a clinical trial involving 84 CF patients between the ages of 5 and 39 years. Results from this study are expected in 1994.

Although NSAIDS show protective effects against airway inflammatory damage, a number of NSAIDS, particularly niflumic and flufenamic acids, also decrease airway Cl^- secretion (72, 73). The inhibitory effect of NSAIDS on Cl^- secretion does not seem to be due to the inhibition of cell cyclooxygenase (72). Whether or not other NSAIDS affect epithelial Cl^- transport is unknown.

Correction of the Defect

The ideal treatment for CF is correction of the defect in CFTR. This might be accomplished by several different methods. The best-tested method for correcting the basic defect in CF is lung or heart-lung transplantation with organs from non-CF donors. Excellent reviews are available on the state of the art in organ transplantation in CF patients (74, 75). Experimental methods for correcting the basic defect in CF include protein-replacement therapy involving application and incorporation of wild-type CFTR into CF epithelial cells, and gene therapy using the expression of wild-type CFTR in affected CF epithelial cells. These methods for correction of the basic defect in CF are discussed below.

PROTEIN REPLACEMENT If CF results from the lack of functional CFTR in epithelial cells, then delivery and incorporation of normal, wild-type CFTR should correct CF pathophysiology. Protein replacement therapy of CF with

wild-type CFTR would require the production of large amounts of recombinant wild-type CFTR, as well as delivery of this large membrane-spanning protein to affected airway epithelial cells in amounts that correct the defect in Cl^- permeability but do not prove toxic to the cell. Production of large amounts of recombinant protein is certainly possible; however, purification of this membrane-bound protein for delivery to humans may prove challenging. Delivery of recombinant proteins has been accomplished by aerosol in the case of α_1 antitrypsin (76), but because CFTR is a membrane-spanning protein, its delivery will require carrier lipid—possibly in the form of liposomes. The technical challenges to protein therapy for CF are large, but are currently being examined.

GENE THERAPY Gene therapy, which involves expression of the wild-type CFTR into affected CF epithelial cells in a process termed gene augmentation (77), is currently under active investigation as a treatment for CF. The feasibility of gene therapy for CF was demonstrated by transfection of the wild-type CFTR cDNA by means of vaccinia or retroviral vectors into CF epithelial cells in vitro, with resulting correction of the Cl^- transport defect (78, 79). CF is an attractive disease for gene therapy because it is relatively common (79a) and lethal (median survival approximately 29 years), with limited therapeutic options. In addition, it is a monogenic disease, which can be corrected by CFTR gene transfer in a small percentage of affected cells [estimated to be approximately 5–10%; (80–82)]. Furthermore, the initial target organ for gene transfer is the lung, since the major morbidity and mortality are secondary to disease of the airways. The lung also presents the advantage of accessibility by aerosolized administration.

Airway epithelial cells include tracheal, bronchial, bronchiolar, alveolar, and submucosal cells. The appropriate cell type for CFTR gene transfer is currently unknown. In the human proximal airway, CFTR expression is highest in submucosal gland epithelial cells; however, CFTR is also expressed at a lower level in surface epithelial cells (83, 84). The human distal airway contains a subpopulation of surface epithelial cells with high expression, and there is also some expression in alveolar cells (85). Proximal airway surface epithelial cells are much more accessible to aerosol delivery of vector (86); thus the first phase of gene therapy will target the surface epithelium. Ideally, CFTR gene transfer could be directed at airway stem cells, but the identity of the airway stem cell remains an elusive cell biology question.

Other important questions with regard to gene therapy in CF include the choice of vector system, safety of gene transfer and expression, regulation and amount of expression, the results of over-expression, and the immunogenicity conferred by the procedure. These questions are discussed individually with each gene-transfer system below. Methods for CFTR gene transfer that are

currently under investigation include viral-mediated gene transfer, liposomes, and receptor-mediated gene transfer by means of DNA-ligand complexes. Gene transfer by means of traditional plasmid constructs versus autonomously replicating episomes is also being studied.

Several recombinant viruses are being developed for gene transfer. Various viral-mediated gene transfer systems are chosen for their relatively high efficiency over inert transfer mechanisms and can be differentiated on several properties, including suitability for ex vivo versus in vivo administration, ability to confer stable versus transient expression of transgene, and pathogenic potential for both the recipient and the general population. Two major viral vectors under consideration for CFTR gene transfer are recombinant adenovirus-based vectors (87–89) and recombinant adeno-associated virus (90).

Adenovirus is a double-stranded DNA virus that naturally infects the respiratory and gastrointestinal tracts of humans. Adenoviral-mediated gene transfer is felt to have promise for CFTR gene transfer and is under investigation at numerous centers. This method has many advantages and disadvantages. Advantages include (*a*) ability to produce high-viral titers, (*b*) tropism for respiratory epithelium, (*c*) the lack of requirement for host cell division, (*d*) adequate capacity for DNA insert size, (*e*) history of use in safe and effective vaccine development, (*f*) relatively low pathogenicity of the wild-type virus and (*g*) lack of reported associated malignancies. Disadvantages are significant, however, including (*a*) production of cytopathic effects in transfected cells; (*b*) conferral of only transient gene correction due to the episomal, nonintegrating location of the transducing genome in the nucleus of the host cell; (*c*) expression of viral proteins with induction of host immune response, which limits the potential for repetitive administration; (*d*) possible pathogenicity, specifically adenoviral pneumonia and generalized viremia, in immunocompromised hosts; and (*e*) the possibility of recombination with wild-type viruses and replication, with release into the general population.

First-generation adenoviral vectors, rendered replication incompetent by deletion of the E1 region genes, are currently under evaluation by a number of groups (82, 91–93). By use of a cell line that provides in transit the essential regulatory genes E1a and E1b, it is possible to produce recombinants containing a transgene in which the virus is rendered replication defective, theoretically obviating some of the major disadvantages of this vector, including cytopathic effects and elicitation of a host immune response. Replication may still occur under conditions where the E1 region is provided by coinfection with or recombination with an E1-containing adenovirus (87, 88). Initial studies have been performed in vitro that have demonstrated transfer of the CFTR cDNA and correction of the CF epithelial cell Cl^- secretory defect (82, 91). CFTR gene transfer has also been evaluated in a number of animal model

systems, including the cotton rat (a good model because the respiratory epithelium is permissive for human adenoviruses, and infection produces a similar histopathologic response) (94, 95) and nonhuman primates (95–97) in order to define the therapeutic window between biological efficacy and toxicity. In general, the studies were supportive of a dose-dependent efficacy. Reports of toxicity were mixed, varying from virtually no evidence of a local or systemic inflammatory response after initial or repeat administration in cotton rats and rhesus monkeys (95) to diffuse alveolar wall damage with intraalveolar edema between 4 and 21 days after administration at higher doses (10^9 to 10^{10} cfu/ml) in baboons (97).

In addition to cellular inflammation, adenoviral gene transfer is associated with elicitation of a host immune response and production of antibodies. The immune response to both wild-type and replication-deficient E1- and E3-deleted adenoviral vectors was evaluated for efficiency of gene delivery and expression after single or repeated in vivo administration (98). Nasal administration of wild-type adenoviral vector resulted in a strong humoral immunologic response with development of human adenovirus neutralizing antibody (HANA) in serum, independent of the dose administered, which was consistent with in vivo replication of the wild-type virus. By contrast, the replication-deficient vector resulted in a graded HANA titer that correlated strongly with the administered dose, consistent with a lack of replication of the mutant virus. The study further tested the hypothesis that efficiency of repeat adenoviral vector administration is related to the level of blocking antibody (HANA) developed in response to a prior adenoviral vector administration. On repeat administration, the reporter activity of the repeat wild-type adenoviral vector administration was found to be inversely proportional to the serum HANA titer and correlated less well with the actual dose of the administered vector. These two observations suggested a causal relationship between the reduced efficiency of the second vector administration and the development of a humoral immune response resulting from the first vector administration. Overall, this study strongly supports the contention that long-term efficacy of viral-mediated CFTR gene transfection is critically dependent on avoidance of the development of host blocking antibodies, and supports the use of second- and higher-generation vectors that minimize viral protein production.

Along these lines, second-generation recombinant adenoviruses in which deletions in addition to E1 (E2A or E3) further cripple the ability of the virus to express viral proteins and decrease the extent of CTL-mediated rejection of virally infected cells are under development. One such variant, harboring the β-galactosidase reporter transgene—in which a temperature-sensitive mutation is introduced into the transgene such that, at nonpermissive temperature, this virus fails to express late gene products even when E1 is expressed in trans.

This variant allows both a blunting and delaying of the inflammatory response and an enhanced persistence of transgene expression by fivefold (99).

The efficacy and safety of adenoviral-mediated gene transfer ultimately requires evaluation in human clinical trials. Five separate clinical trials in humans have been approved by the National Institutes of Health Recombinant DNA Activities Committee (RG Crystal, December, 1992; M Welsh & A Smith, 1992; J Wilson, December, 1992; RW Wilmott, JA Whitsett & BC Trapnell, March 1993; R Boucher, March 1993), with protocols published for at least two of these (100, 101). Preliminary results from the gene-therapy protocol initiated by the Welsh and Smith group have been published (102). The adenovirus used by this group is called Ad2/CFTR-1 and consists of E1-deleted adenovirus type 2 that encodes CFTR and retains the E1a promoter and the E3 region. Ad2/CFTR-1 expresses CFTR in CF cells and corrects Cl^- and fluid transport in CF cells (82). It has also been expressed in cotton rats and rhesus monkeys and has an encouraging safety profile (95). Initial published results (102) on human gene therapy trials included 3 CF patients, in whom the vector was applied to 0.5 cm^2 of nasal mucosa on each side and 3 doses tested between MOI 1 and 25. The safety profile from this study was encouraging. Ad2/CFTR-1 could not be cultured from these patients. No changes were observed in histories and physical exams, serum chemistries, chest films, pulmonary function tests, or arterial blood gases. Clinical syndromes ranging from mild nasal inflammation to bronchitis were seen in each patient in the weeks following vector application, but the relationship to vector administration was not definite. An RNA-specific template PCR confirmed the presence of CFTR RNA in patient biopsies up to 15 days after the procedure (the longest time period tested). Nasal voltage measurements reveal a partial return to the normal ion-transport phenotype within 7 days, with complete return to baseline by 35 days after treatment with Ad2/CFTR-1.

Recombinant adeno-associated virus (rAAV) is also under investigation as a potential vector for CFTR gene transfer. AAV is a small (4.7 kb), single-stranded parvo virus, which is naturally defective for replication so that it requires coinfection with a helper virus—either adenovirus or herpesvirus—for replication in a productive life cycle. In the absence of a helper virus, AAV undergoes high-frequency stable DNA integration into a specific target site in chromosome 19 (103). The ability to integrate into the host genome suggests that stable, long-term expression is achievable. Furthermore, site-specific integration reduces the likelihood of random integration into a tumor-suppressor gene with resultant oncogenesis. Like adenovirus, AAV has a natural tropism for respiratory and gastrointestinal epithelial cells. It is, however, nonpathogenic; 85% of normal individuals have antibodies to AAV with no history of associated disease. There is no expression of viral proteins; therefore there is little inflammatory or immune response by the host. In addition, it confers high

efficiency and reproducibility of gene transfer in a stable particle, allowing for easy purification and storage for long periods.

Despite the numerous theoretical advantages of AAV vector systems, there are at least two potential disadvantages. Integrated AAV-DNA can be rescued, i.e. excised and replicated, if coinfection by a helper virus occurs at a later date. Since natural infection with adenovirus or herpesvirus is prevalent in the general population, there is the possibility of rescue of the recombinant AAV-CFTR vector with release into the general population. An additional disadvantage of AAV as a vector system is limitation of DNA insert size. The 4.7 kb AAV genome consists of two genes, *rep* and *cap*, flanked by inverted terminal repeats (ITRs), which serve as origins of DNA replication and packaging signals (104). AAV vectors are constructed by substitution of foreign DNA for either or both the *rep* and *cap* genes. Because of the large size of the CFTR coding sequence (4.5 kb) relative to AAV, the selection of an optimal small promoter is very important (90). The group of Flotte and Carter have addressed this latter problem in two ways. This group initially tested the AAV p5 promoter, which is derived from the *rep* gene and able to form a cassette of suitable size (263 bp) with the left-hand ITR, and found it to be efficient for expression of reporter genes in both stable and transient assays (105). They then made the serendipitous observation that ITR has transcription-promoter activity (90). By using an AAV-ITR-CFTR vector, the group demonstrated both in vitro (90) and in vivo (106) efficacy of gene transfer. After in vivo delivery of AAV-ITR-CFTR vector to one lobe of the rabbit lung through a fiberoptic bronchoscope, CFTR RNA and protein could be detected in the infected lobe up to 6 months after vector administration (106).

The largest experience with gene transfer to date has been by means of retroviral gene transfer, usually through an ex vivo approach (77, 107). More recently, retroviruses have been used to introduce genes directly into blood vessels and liver (108, 109). This method of gene transfer, which requires actively dividing cells, has not been felt to be generally applicable to CF because airway cells are largely quiescent and nondividing. One study, however, reported efficient (5–10%) gene transfer to regenerating, mitotic human bronchial epithelium of xenografts (110), which raises the possibility that retroviral-mediated CFTR gene transfer to the airway in vivo may be feasible if the proper regenerative state of airway epithelium can be induced.

Viral systems are not the only potential means for CFTR gene transfer. Liposomes are cationic lipid vesicles that bind negatively charged DNA and fuse to cell membranes for DNA transfer. Liposomal-mediated gene transfer has been demonstrated in a variety of cell systems (111, 112) and liposome-mediated gene delivery has been shown to correct the ion-transport defect in CF-mutant mice (81). Safety is an important advantage of liposomes over other gene-transfer methods. Liposomal aerosol treatment has been shown to

be safe in mice, sheep, and humans. A randomized, double-blind clinical trial investigating liposomal transfer of CFTR to nasal epithelial sheets in CF patients was started in 1993 in the UK (113); results are pending at the time of this review.

DNA-ligand complexes are made by combining negatively changed DNA with polycations like protamine or polylysine. These DNA-ligand complexes deliver DNA by incorporation of the complex with, for example, the transferrin receptor (114, 115). This delivery system has the advantage of targeting specific cell types by exploiting cell-specific receptors.

Mammalian artificial chromosomes is a technology in its infancy. There are a number of potential advantages, including stability, lack of nuclear integration, an episomal configuration, constant copy number, lack of other gene expression, and no viral pathogenicity. However, problems with this technology, including technical limits to manipulation of large construct and lack of definition of human centromeres, may limit its use. A promising system has been developed by the Calos group. By use of modules of specific human cDNA sequences, this group was able to demonstrate that autonomous replication efficiency increased with successive addition of human modules, to essentially 100% by six copies, suggesting that autonomous replication in human cells is stimulated by simple sequence features that occur frequently in human DNA (116).

Clearly, many questions remain with regard to gene therapy for CF. Which vector system is preferable is only the first question. The "magic bullet" of CF gene therapy would be efficient, tropic for the airway, targeted to nondividing cells, safe, and capable of prolonged expression and repeated administrations, if necessary.

CONCLUSION

Many components of the pathophysiological cascade in CF lung disease are targeted in current CF therapy research (Table 1). One of the most promising is rhDNase I, approved by the FDA in 1994, which is a safe and effective mucolytic in CF patients with a wide spectrum of disease. More research is required, but aerosolized UTP may be a useful adjunctive treatment of CF, in combination with amiloride for a combined therapy of Cl^- and Na^+ transport defects. Despite the theoretical benefits of antiinflammatory therapy, no antiinflammatory therapy is currently recommended for CF lung disease. The future of CF treatment is likely to be in the correction of the basic defect. Striking advances have been made in gene-transfer research, especially using recombinant adenovirus-mediated gene transfer. Gene therapy of CF is a growing technology and many new developments can be expected in the coming years.

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