# the Breath of Life

ATTEMPTS TO REPLACE OR CIRCUMVENT A DEFECTIVE GENE, OR COAX A DEFECTIVE PROTEIN BACK TO LIFE, ARE BRINGING HOPE TO PATIENTS WITH CYSTIC FIBROSIS.

by William A. Wells

Photographs by David Bartolomi

"bigger, bigger, moremoremore." The cries come every few minutes when Clinic E is open for business. The nurse in the pulmonary clinic at Lucile Packard Children's Hospital is directing her encouragement at a child blowing her way through a lung-function test. But the same sentiment carries over to the staff members, who are part of a revolution in cystic fibrosis (CF) treatment. That revolution, which has seen the life expectancy of CF patients double from 15 to 30 years in the last two decades, could make CF a lifelong, not life-threatening, illness.

But the reality for the patients in Clinic E today is lung mucus, and lots of it. Bacteria thrive in such sticky lungs, and these infections and the resultant lung destruction are the cause of death for 90 percent of those with CF. Antibiotics do bring patients some relief. But truly effective treatments for the roughly 30,000 sufferers nationwide will need to attack the basic defect underlying this inherited disease — a defect that was until recently a mystery.

A more direct approach suddenly became a possibility in 1989, with the discovery of the gene that is defective in CF sufferers. The gene provides the blueprint for a protein dubbed the cystic fibrosis transmembrane-conductance regulator, or CFTR. Normal CFTR proteins are found at the cell surface, where they snake in and out of the cell membrane, the outer lining of the cell, each forming a narrow tunnel. The cell relies on these tunnels to regulate the passage of electrically charged chloride ions through its membrane, which would otherwise be impermeable to a charged atom.

By regulating chloride ions, the cell controls its water content. Wherever chloride goes, its positively charged counterpart, sodium, will follow, and the resulting salt will draw water with it.

Originally, researchers believed that the lack of fully functional CFTR meant that cells in the lungs were unable to export chloride, and thus water. They reasoned this would make the mucus in the lungs too dry and sticky to do its job. Slippery mucus is needed to carry dust and invading microbes up and out of the lungs, powered by an escalator of tiny beating cilia on the lung cells.

New evidence suggests more complicated repercussions from faulty or absent

Hayley Wester, a CF patient and Stanford undergrad, takes a lung-function test.

CFTR channels. "Chloride can be either 'exported' or 'imported' depending on other molecular machinery in the cell," says Jeffrey Wine, PhD, of the departments of psychology and of molecular and cellular physiology. "We now know that both of these processes are compromised in CF. As a result, both decreased secretion of water, resulting in dry secretions, and decreased absorption of salt, resulting in salty secretions, can occur in various organs. In the lung, both probably contribute to CF symptoms," he says.

The salty secretions may explain the origin of an old German proverb: If you kiss the brow of an infant and it is salty, that child will die.

But for the most intellectually simple therapeutic approach, gene therapy, the details of the CF defect are irrelevant. The defective gene is simply replaced by delivering a normal copy.

CF gene therapy has attracted much interest as a test case for several reasons. The cause of CF is simple — defects in both copies of a single gene. The lungs, the most clinically critical tissue affected by CF, are accessible to drugs using aerosols. And only one cell in any given lung circuit needs a functional CFTR, suggesting that only a fraction of the cells will need to be corrected for the patient to benefit.

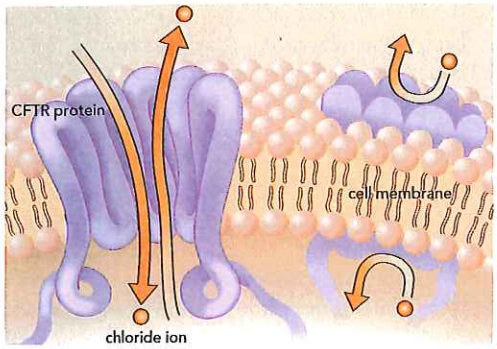
Delivering genes means delivering DNA. Some researchers have packaged the DNA in a cloak of greasy molecules, called a liposome, that can stick to cells and allow the DNA to be absorbed. But this method is very inefficient. John Wagner, MD, PhD, who directs CF gene therapy trials at Stanford, believes a different approach is needed. "Viruses have evolved to get their DNA into cells over millions of years," he says, "but we've only been doing this for about 30 years. We should take advantage of the molecular wisdom of viruses."

Some gene therapy trials have bundled CFTR into the DNA of adenovirus, one of the causes of the common cold. Adenovirus is, however, a relatively complex virus. When it infects cells, its genes direct the production of certain proteins that are recognized by the immune system. Thus, even when these genes are first removed from the adenovirus, infected cells are met by a strong immune response that destroys them, and the newly inserted CFTR gene dies with them. Subsequent doses of the adenovirus are met by an even stronger response.

What was needed was a DNA vehicle with the simplicity of the liposomes, but the efficiency of a virus. "We want something that is stealthier and that the body can't recognize. We want a better Trojan horse," says Wagner, a clinical and molecular pharmacology fellow.

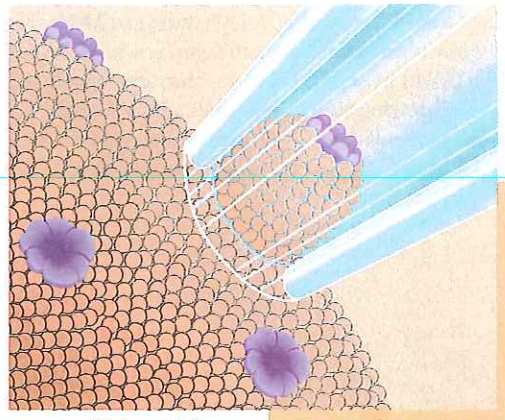
So Stanford researchers, including Wagner; Richard Moss, MD, professor of pediatrics and director of the Cystic Fibrosis Center; and Phyllis Gardner, MD, associate professor of molecular pharmacology and principal investigator for the gene therapy trial, collaborated with Targeted Genetics Corp. of Seattle, Wash., and settled on a simpler virus, called adeno-associated virus (AAV).

This tiny virus has only two genes, and even those can be chopped out to make room for CFTR. With none of its own genes there is no chance that the virus will make its own proteins and provoke an immune response.




FAULTY TUNNELS LEAD TO CF SYMPTOMS

Above: A fully functional CFTR protein (left) serves as a tunnel, allowing chloride ions to pass in and out of a cell. Cystic fibrosis patients have a genetic defect that results in faulty CFTR proteins. Some patients have proteins that fail to embed themselves in cell membranes; others have proteins that bind to the membranes but don't allow choride to pass through (right). Below: The patch clamp technique allows researchers to study a single molecule within a cell's membrane.



Diagrams by Ian Worpole

AAV had a test run last year in a Phase 1 trial at Stanford's NIH-funded General Clinical Research Center. Instead of applying the virus to the lungs, the obvious CF study site, the Stanford researchers applied it to the si nuses, a set of branched tissue-lined cavities within the face. Sinus tissue resembles lung tissue but is more convenient to study, said Wine, who pioneered this strategy.

At the highest dose, Wagner says, they saw no detectable immune response or adverse effects and the gene was transferred into 10 to 100 percent of the cells. Although CFTR carried by adenovirus had been eliminated within two weeks, the AAV version was only beginning to decline at 10 weeks.

A recently begun Phase II trial will address whether the treatment is actually beneficial. The sinuses are again the testing ground.

But a positive result in the sinuses is not a guarantee of success in the lungs. They have a much higher density of glands that are plugged up with mucus, so reaching the right cells with an aerosolized virus preparation may be difficult.

The Stanford team, which is coordinating with researchers doing similar studies at Johns Hopkins University, is extremely optimistic about the Phase II study. If the second trial is positive, the Stanford and Johns Hopkins teams will together move into another Phase II trial, this time targeting the lungs. But they are wary of promising too much.

"Some of the excitement in the field gets translated into overly high expectations," says Wagner. "Gene therapy will be more like a drug that is used to treat the disease, not a cure for the disease."

This is all the more reason to follow up other lines of treatment. The CF center is testing a new antibiotic to combat the bacteria that

## How do you study single channels?

Much of the basic science that drives CF treatments relies on a sensitive device called a patch clamp. This device allows the researchers to monitor the opening and closing of single ion channels. "It's quite astonishing," says Wine, "to be able to tell what a single molecule is doing."

To use the patch clamp, Wine sucks a tiny piece of a cell's outer membrane into a thin glass tube so it makes a tight seal against the glass. He then applies a voltage across the membrane, so charged particles in the surrounding solution, such as chloride ions, are driven in one direction. These ions can cross the membrane only through special channels and only if the channels are open. The membrane patches are less than a hair's width across, so many of them contain only one or a few channels. When a channel opens, the researchers can detect the opening (and then closing) as a discrete flow of current across the membrane.

Wine has used this method to show that some cells have a mechanism for locking the CFTR channel open for long times. If he can mimic this phenomenon in patients, he may be able to boost the effectiveness of normal CFTR delivered by gene therapy.

Kopito has found that one part of the open/close switch is ATP, the energy currency used by cells. Splitting apart one molecule of ATP opens the channel, and splitting apart another closes it up again. Such details about the operation of CFTR can be discovered only in a controlled environment, such as the glass tube of a patch clamp.

CYSTIC FIBROSIS RESEARCHER AND PHYSICIAN JOHN WAGNER (OPPOSITE) EXAMINES AN AUTORADIOGRAM.

most often infect the lungs of CF patients; using the Web to conduct a nutrition study (pancreatic problems mean that many CF patients are malnourished); and testing a newer, faster CT scanner that could replace the lung-function test, especially for Moss' youngest patients, in whom lung-function testing is most difficult.

The center is also conducting a Phase I trial with a new drug that gums up elastase, a protein from white blood cells. The rationale for developing this drug, produced by Dupont Merck Pharmaceuticals Inc., comes from a new view of CF in which the immune system is the culprit.

Moss found his first clue in infants. "Inflammation is present in the lungs of infants as early as you can look for it, even though in some cases there is no evidence for infection," he says. Perhaps, Moss thought, the immune system is too eager. Indeed, he has found that CF patients produce abnormally low levels of an anti-inflammatory messenger, called IL-10. Why a defect in a chloride channel should affect the amount of IL-10 is not known.

Infections may be due, at least in part, to the high salt concentration outside CF patients' lung cells, which deactivates various "homemade" antibacterial substances. These infections lead to the recruitment of white blood cells called neutrophils, which flood into the lungs by the millions because too little IL-10 is present to stop them. As many as half of all the body's neutrophils can wind up in the lungs, and the short-lived cells begin dying all over the place. The garbage trucks of the immune system cannot keep up, and the contents of the neutrophils spill out into the lungs.

One of those contents is elastase, the target of the Dupont Merck drug. Elastase is normally kept within a compartment of the neutrophils where bacteria are sent to be destroyed. Now, however, the elastase attacks not bacteria but lung tissue. This destruction, and the other debris from the neutrophils including their DNA, constitutes a significant proportion of the lung sludge that plagues CF patients.

Whatever twists and turns are occurring in the immune system, the underlying problem is a lack of CFTR, and anything that can restore CFTR function should help patients. Ron Kopito of the Department of Biological Sciences has found, at least in the laboratory, that the most prevalent mutant version of CFTR can be brought back to life. According to Moss, this mutation, called deltaF508, is present in about 75 percent of all cases of CF.

The mutant gene still directs the cell to make a protein, but one that lacks a building block. As a result, the protein can't fold into the correct shape; the end product is like a tangled ball of wool. Kopito added a chemical and showed that this convinced the mutant protein to fold. "And if you can trick the protein to fold properly," says Kopito, "the mutant can actually function quite well."

The chemical that Kopito used cannot be used in humans, but Moss is working on another compound, CPX, a relative of caffeine, which has similar activity. This was recognized by NIH researchers who were studying whether CPX would stimulate other chloride channels. They found instead that CPX attached itself directly to deltaF508 CFTR. CPX may either help deltaF508 to fold, or rev up the small amount of deltaF508 protein that does get to the cell membrane.

Phyllis Gardner's work has spurred researchers to look for drugs that stimulate other chloride channels. Gardner helped show that other chloride channels can be opened in cells that lack CFTR. Drugs that open this second class of channels could effect a CFTR bypass; an aerosolized drug is being developed by the Inspire company of North Carolina.

As the approaches to CF treatment and the number of trials proliferate, the life expectancy of the patients increases. "We've made enormous strides," says Gardner, "but we need to make more." The first step for these doctors is to keep their patients breathing. "Anyone who has had difficulty breathing knows it's a frightening experience," says Moss, "and CF patients live with that." At least now CF patients survive to breathe with difficulty; the next step is to enable them to breathe with ease. SM

## The advantage of having a defective CFTR gene

The high incidence of mutant CFTR genes in humans is surprising. One explanation for the persistence of mutant genes is that the carriers (unaffected individuals with only one defective gene) have a selective advantage.

For CF, that advantage appears to be resistance to the effects of intestinal infections. The toxin produced by many of the bacteria that cause travelers' diarrhea can lock the CFTR channels open, causing loss of ions and water. With fewer channels, carriers stand a better chance of survival because they lose less water than those with two normal CFTR genes.

Now Gardner is trying to bring that advantage to everyone. She is testing compounds that keep CFTR closed, in the hope that they can be used to help those with either travelers' diarrhea or more serious intestinal diseases such as cholera.

## CF drives basic science

The inspiration for clinical treatments often comes from basic biological research. For CF, says Wine, the reverse is also true.

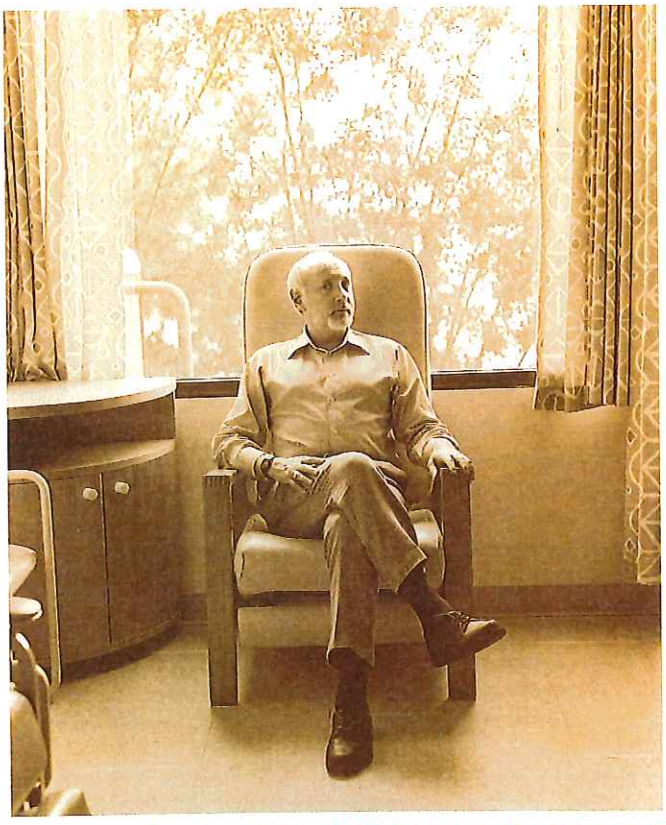
This is clearest in Kopito's work. "In trying to understand how CFTR works we discovered a new way in which the cell sorts its proteins," says Kopito.

The new sorting pathway is one of many that cells use to direct proteins to different locations. When proteins are to be inserted in the outside lining of the cell, for example, they are first sent to a membrane-enclosed compartment — part of an extensive network of such compartments, called the endoplasmic reticulum or ER.

While studying the folding of deltaF508 CFTR (see main text), Kopito found that the unfolded CFTR reached the ER but was then disposed of by an assembly of proteins called the proteasome. This cellular garbage disposal was known to chew up unwanted proteins that were floating around in the cell, but it was not thought that it could access proteins, such as CFTR, enclosed in membranes.

Researchers went on to show that the proteasome can dispose of membrane-enveloped proteins other than CFTR, suggesting that this is a general mechanism for the disposal of misfolded proteins.

This CFTR study may not lead to a treatment, but it has added one more road to the cellular map.



RICHARD MOSS EYES THE IMMUNE SYSTEM AS CULPRIT