ADVANCES IN THE LAB

To date, five gene transfer clinical trials for hemophilia have enrolled approximately 40 subjects. (For detailed information on these trials log onto www.hemophilia.org.) These trials were designed to evaluate safety; they were not intended to cure the disease. In general, all the trials have received high marks on the safety card. Nonetheless, scientists have been frustrated as only minimal increases in factor production have been observed in study participants. And so, before additional studies are conducted in the clinic, researchers in the lab continue to explore creative ways of tweaking the system, developing new approaches to raising factor levels and building a stronger foundation of basic knowledge. Three areas in particular received attention at NHF's gene therapy workshop in La Jolla, California, held from April 25 to 26, 2003. One theme focused on properties of the well-studied adeno-associated virus (AAV), the virus used in two of the clinical trials. The second pursues an alternative to gene transfer by attempting to improve existing recombinant proteins. The third investigates the destination of the gene following successful transfer.

BY JUDY BERLFEIN

GETTY IMAGES

Fiddling with Adeno-associated Virus

The uses of AAV as a vector for gene transmission has been discussed at previous gene therapy meetings. Scientists have shown that the shell enveloping the virus influences the efficiency of gene transfer. Scientists are familiar with six human AAV variants, named simply by number as serotype 1 through 6. Studies have shown that AAV2 is expressed for a long time, once transferred via vector into humans, however its expression is very low. Part of the reason AAV2 has not been successful is that antibodies are able to recognize it, as it comes from humans. The goal of James Wilson of the University of Pennsylvania was to find an AAV vector that is both efficient at gene transfer and has high expression. By using AAVs from primates, he solved the problem. The primate AAVs are not neutralized by antibodies generated to naturally acquired adenovirus infections in humans. Wilson's work has produced nearly 40 different AAV serotypes in monkeys and the newly found variants are proving more effective as gene transfer agents. One serotype in particular, AAV8, was highlighted at this year's meeting. Serum from humans demonstrated very little neutralizing ability to AAV8 vectors and AAV8 produced higher levels of factor production when used as a vector in mice and dog experimentation. Of course, scientists continue to experiment with AAV2 and the other human serotypes in an effort to increase their expression levels.

Improving Recombinant Factor

During the final hour of the two-day workshop, when participants joined in discussion, one overriding issue arose. Should the research community pursue gene transfer exclusively or should scientists and pharmaceutical companies put resources into other options? John Griffin of The Scripps Research Institute argued that more attention should be devoted to developing second and third generation factor products. Why can't we make recombinant factor work better and last longer in the circulation? This could translate to fewer needle sticks and lower costs.

That is precisely what Andrew Gale of The Scripps Research Institute hopes to accomplish with his mutant factor VIII (FVIII). By carefully studying the properties of existing FVIII, he has developed a mutant variation that will stay in the circulation longer. The normal sequence of events leading up to coagulation is fairly complex. Before FVIII can be effective, it must be activated by the protein thrombin. Activation involves excising the central chunk of the protein, leaving two small pieces loosely fastened together. The resulting molecule, FVIIIa facilitates clotting of the blood, but it is unstable. Though FVIIIa is required for clotting to occur, it falls apart quickly, rendering it inactive. In a normal system, this rapid inactivation serves to maintain a finely-tuned balance. FVIII acts effectively while preventing the hazards of the reverse scenario—excessive clotting.

Gale isn't thinking about a normal system though, he's focused on the hemophilic condition, where FVIII is an expensive commodity that must be infused on a regular basis. If he can lengthen the lifespan of the protein, perhaps infusions can be administered less frequently.

To achieve this goal, Gale has synthesized a mutant version of normal FVIII. Using strong chemical bonds to tether the fragile FVIIIa together, the protein remains intact and normal breakdown does not occur. Rather than losing its activity immediately, FVIII keeps on working. Gale stresses that the work is preliminary. "While the results so far are very encouraging," he says, "it still doesn't tell us a lot about what's happening *in vivo*—in living systems." The next step will be to test the mutant in animals.

Getting a Grasp on the Science—Where do the Vectors Go?

The gene therapy workshop has always emphasized both the fundamentals and the clinical aspects of gene transfer. This year, with knowledge of recent events in the French gene transfer trial on people's minds, in which two of nine children successfully treated with gene transfer for severe combined immunodeficiency disease (SCID) were later diagnosed with a leukemia-like cancer, presentations on basic science received special attention.

Researchers felt that conclusions about gene transfer should not be drawn too quickly based on this specific trial and this sentiment was present throughout the conference. In fact, while lessons should be learned, the lessons may not apply across the board. The SCID trial used a retrovirus that cannot perform its function without integrating into the host's genome. AAV and adenovirus (AV), which have been investigated in hemophilia trials, function differently; adenovirus essentially never integrates into the genome and AAV only integrates in a small percentage of cases.

When asked if you can compare the SCID results with hemophilia trials using

AAV and AV, Mark Kay of Stanford University Medical Center replied, "These are two very, very different kinds of situations." He explains that at least three factors have been identified that could have contributed to the development of leukemia in the SCID children. First, the integrated gene had the potential to enhance cell growth. Second, the transfer was performed in stem cells from the bone marrow. The genetically corrected cells have a selective growth advantage and these cells divide both rapidly and often, a condition that increases the possibility of introducing damage or causing errors. And finally, the gene integrated into the genome in close proximity to an oncogene, a gene that can participate in the development of a malignancy or cancer.

Kay points out that in hemophilia the focus has been primarily on liver and muscle cells, typically slow-dividing cells. One of the earliest hemophilia trials used a retrovirus, but since then the studies have used AAV and AV. While Kay stresses the difference between the hemophilia work and the SCID trial, he states openly, "There are still a lot of unknowns." His lab is working to close some of these gaps.

While Kay believes the risks of AAV causing cancer are slight, he understands that mapping the precise behavior of AAV will help to shed light on all aspects of gene transfer. Kay and fellow researcher Hiroyuki Nakai have demonstrated that a small portion of AAV, about 5% to 10% of the AAV genome, does integrate into the cellular DNA. It seems to be inserting in locations where genes are actively making proteins. What does that mean in the long term? At this point, no one knows for sure. Kay says he was encouraged to hear in a presentation by David Russell of the University of Washington that while AAV integrates, it appears to do so at naturally occurring breaks. Retroviruses, in contrast, force their way into the chromosome at random locations. The assumption is that AAV might be less likely to cause collateral damage if it's quietly attaching itself to the end of a broken chromosome, whereas retroviruses may interrupt a normal function with potentially detrimental effects.

Because of the concerns raised by the SCID trial, integration remains a troubling issue. Some researchers attempt to minimize risk by avoiding viruses that integrate.

Michéle Calos of Stanford University Medical Center plans to tackle the challenge using reverse psychology. Rather than circumventing integration, her method requires specific integration, though she plans to know in advance the exact destination of the gene. Rather than packaging the gene in viral vectors, her group is sending "naked" DNA directly into the cells without any shuttle vehicle. The gene is not travelling alone however, as it is chaperoned by an additional snippet of DNA coding for an enzyme known as integrase. The sole job of integrase is to ensure that foreign DNA inserts into the chromosome at specific, rather than random, locations. Scientists have mapped these locations and are now busy determining if these are safe landing zones. Using integrase, scientists will know exactly where the gene has gone and avoid the unpredictable nature of integration. Hopefully, this will eliminate a degree of uncertainty. That's certainly a plus for gene transfer though, hurdles still remain. One of the challenges is to find an appropriate means of delivering the DNA. Historically, viruses have been used for gene transfer because they are specifically designed to enter cells and provide efficient transport of the desired genes. Calos' "naked" DNA, on the other hand, needs a push to cross the cell membrane. This has been achieved successfully in mice using high-pressure infusions through the tail vein. A pressure gradient forces the free-floating DNA out of the blood vessel into the liver cells. Translating this to humans, however, is not a straightforward proposition, as we obviously lack a tail vein. Calos is hopeful that a high-pressure infusion technique, being evaluated in dogs and rabbits, will ultimately succeed in humans.

Calos' work is still in the early stages. Her group has tested the approach in mice and induced production of adequate levels of factor. Calos recognizes, however, that though hundreds of mice have been cured of hemophilia with gene transfer, it won't be until they scale up to larger animals that they will know if the system has potential for humans.

Many of the same scientists, including Mark Kay and James Wilson, return each year to the workshop providing a sense of continuity. But an influx of new faces, such as those of Andrew Gale and Michéle Calos, bring fresh ideas and techniques, invigorating the process. It is this healthy mixture of old and new that keeps participants optimistic about the steady progress of treatment for hemophilia.