



Better days: Alain Fischer and Marina Cavazzana-Calvo announce the successful results of their gene-therapy trial on immune-deficient children in April 2000.

A tragic setback

With one French gene-therapy patient having developed a form of cancer, a frantic detective effort is under way to determine what went wrong — and to assess the risks faced by others. Erika Check reports.

Until a few weeks ago, a three-year-old boy whose identity remains confidential was a beacon of hope for gene therapists. He is one of 11 children with severe combined immune deficiency (SCID) to receive a pioneering treatment from a team led by Alain Fischer and Marina Cavazzana-Calvo of the Necker Hospital for Sick Children in Paris. It worked, providing the first proof that gene therapy can cure a life-threatening disease¹.

Now, however, the boy has developed a leukaemia-like condition that most experts believe was caused by the very treatment that cured his SCID. The field of gene therapy is in turmoil, as scientists and medical authorities try to figure out how to proceed with trials in SCID and other diseases². For Fischer, the past few weeks have been a blur of meetings, e-mails and telephone calls, as he has kept colleagues informed of developments. But already his work has begun to reveal what

went wrong in this tragic case. And future findings may allow an assessment of the risks faced by other patients.

The 11 children in Fischer and Cavazzana-Calvo's trial, 9 of whom have been cured, suffered from a form of SCID caused by a defect in a gene on the X chromosome. The gene encodes a subunit of a cell-surface receptor that allows developing immune-system cells to respond to growth signals called cytokines. Without this subunit, known as the γ -c chain, children fail to develop the mature T cells, B cells and natural killer cells needed to fight off infections. The condition can be cured by a perfectly matched bone-marrow transplant, but few children have perfect donors. Of those who receive imperfectly matched transplants, up to 30% die.

The French trial offered new hope to children with the disease by replacing the defective gene. Doctors at Great Ormond Street

Children's Hospital in London have since treated four patients. Gene therapists in the United States and Italy³ have treated another form of SCID, caused by a defect in the gene for the enzyme adenosine deaminase (ADA), which is needed for immune-cell development. And another US group has treated one patient whose SCID results from a deficiency in a gene called *JAK3*, which produces a protein that is involved in transmitting the signals received by cytokine receptors.

In each of these trials, the corrective genes have been packaged into modified retroviruses, which can incorporate themselves into a host cell's DNA. These retroviruses have been stripped of most of their viral genes to prevent them from causing dangerous infections, but gene therapists cannot control where retroviral vectors insert themselves. So one long-standing worry is that a vector could disrupt important genes through 'insertional mutagenesis' — and if

the gene involved is one that normally regulates cell growth and division, cancer could result.

This seems to be what happened in the case of the French boy, who has developed a leukaemia-like condition in which one particular type of T cell began to proliferate uncontrollably. As a result, SCID gene-therapy trials in France and the United States have been suspended, Italian authorities have suspended the enrolment of new patients, and planned trials in Japan have been put on hold. Germany, meanwhile, had already suspended a range of gene-therapy trials involving retroviral vectors in the wake of a report⁴ that a such a vector has caused leukaemia in mice.

But given that many patients have been treated in gene-therapy trials involving retroviral vectors, and there is no other known instance of vector-triggered cancer, most experts believe that the benefits still outweigh the dangers. Britain's authorities, for instance, did not suspend trials in light of the French setback. And an advisory committee to the US Food and Drug Administration (FDA) has now advised that trials under the agency's jurisdiction should resume. "None of these types of adverse events has ever been seen before," says Savio Woo, a gene therapist at Mount Sinai School of Medicine in New York. "The frequency is really very low."

But other experts argue that it's too early to know how great the risk is. The patients exposed to retroviral gene-therapy vectors were all treated in the past few years. So it is possible that some will develop cancer at some time in the future. This is why Fischer is now working to determine what, exactly, went wrong in his unfortunate patient — and whether any other children face a similar fate.

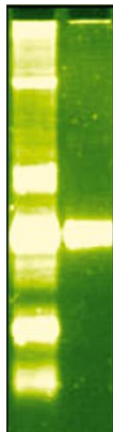
To do so, Fischer has enlisted the help of molecular biologist Christof von Kalle of the Cincinnati Children's Hospital. While at the University of Freiburg in Germany, von Kalle and his colleague Manfred Schmidt developed a technique called linear amplification-mediated PCR (LAM-PCR) to detect where a retroviral vector inserts into human DNA⁵.

LAM-PCR is a variant of the standard polymerase chain reaction, used to pull out a specific DNA sequence from a sample of DNA. PCR requires a short 'primer' sequence that binds tightly to part of the sequence being sought. Using a primer for the retroviral vector, therefore, it is possible to use PCR to selectively amplify the vector sequence, if it is present, and so to detect whether the vector has incorporated into a particular cell or tissue. But that still leaves the question of where in the genome it is integrated.

Von Kalle and Schmidt's technique uses a



Christof von Kalle (above, left) has devised a way to track the insertion sites of gene vectors. Successive analyses can highlight the potentially cancerous transition from a healthy T-cell population with a range of insertion sites (left, left column) to one dominated by a single site.



retroviral vector primer tagged with a molecule called biotin, which binds tightly to another molecule called avidin. Performing PCR and washing the DNA sample over a column of avidin beads pulls out amplified DNA that contains the retroviral vector. The amplified sequences are then cut into pieces using a restriction enzyme and small 'linker' pieces of DNA with a known sequence are attached to the ends of the resulting fragments. Using these linker sequences as primers for further PCR, it is then possible to amplify the DNA that flanks the inserted vectors, so that it can be analysed to determine where the vectors have landed.

When Fischer and von Kalle used LAM-PCR to analyse the French patient's cancerous T cells, they found that the vector had inserted itself into a gene called *LMO-2*, mutations in which are known to be involved in childhood cancers. What's more, von Kalle found that the child's T cells were expressing *LMO-2*, which is abnormal. This has convinced most experts that the powerful promoter sequence in the vector, included to boost expression of the corrective gene, acti-

vated *LMO-2*, triggering the child's cancer. "Altogether, I think insertional mutagenesis is the most likely explanation," Fischer says.

Now, Fischer wants to use LAM-PCR to determine whether such problems are likely to occur in future, by making a catalogue of vector-insertion sites in SCID gene-therapy patients. So far, he and von Kalle have examined two other patients from the French trial. In total, they have now identified more than 100 insertion sites. None of those examined so far are in genes that scientists think are involved in cancer.

As von Kalle and Fischer fill in their insertion map, they may also find more information about where retroviruses prefer to insert themselves. In August, Frederic Bushman, a molecular virologist at the Salk Institute for Biological Studies in La Jolla, California, reported that HIV-1 inserts more often into genes than into non-coding regions of DNA⁶. The SCID trials involve a different virus, called the Moloney retrovirus. But if it, too, shows a penchant for inserting into genes, it will mean that the risks associated with SCID gene therapy are greater than was thought.

Identifying every single insertion site in a child's cells is an arduous process. Even this may not yield a complete picture of the risks that the children face, because promoter sequences can influence the expression of genes at remote sites in the genome. And in any case, we don't yet know the identity of all of the genes that can contribute to cancer.

Carrying out successive LAM-PCR analyses over time on individual patients could, however, tip gene therapists off to a developing leukaemia-like condition. Leukaemia occurs when an individual immune cell starts proliferating wildly so that, over time, it dominates the population

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of cells circulating through the body. In LAM-PCR, this would show up by a patient initially carrying T cells with a range of different insertion sites. But over time, T cells with one particular insertion type would become more and more common until they were completely dominant.

Looking back at blood samples taken from the French boy, von Kalle has found that this is exactly what happened. Early on, the patient's T cells carried about 50 different insertion sites. But eventually, all of his T cells were clones of one cell with the LMO-2 insertion.

Gene therapists would like to include LAM-PCR analysis as a monitoring tool in SCID trials. But they note that not all children with a single insertion site have leukaemia. Paediatrician Donald Kohn of the Children's Hospital in Los Angeles, who is running a trial on ADA SCID, treated one patient nine years ago whose T cells all carry the same insertion site. Yet this patient appears completely healthy. Kohn believes that, in this case, the vector integrated into a bone-marrow stem cell that has divided normally to derive the child's entire T-cell population, rather than there being a cancerous proliferation of a particular T cell. For this reason, Kohn says that analyses of insertion sites must be combined with detailed clinical information.

Such in-depth monitoring will help scientists to understand more about the risks of insertional mutagenesis in SCID gene therapy. But there is still a host of unanswered questions about other factors that might have predisposed the French child to cancer. A phenomenon called 'selective advantage' could be at work. As SCID patients are born with defective immune systems, only cells that integrate the corrective gene will survive. And these cells must divide millions of times to create a functional immune system. This could boost the chance that a cell will develop new harmful mutations, and could also increase the risk that mutated cells will thrive.

Then there are factors that are particular to the French patient's case. Two of his relatives had childhood cancers, which may indicate an inherited susceptibility. And he suffered a bout of chicken-pox before his immune system had fully developed — stimulating his immune system in a way that may have triggered T-cell proliferation. Matters are further complicated by the fact that the boy has a form of cancer that has never been seen before. His T cells, for instance, do not produce typical leukaemia proteins. "It's not at all clear what this disease is," says Ted Friedmann of the University of California, San Diego, who chairs the US National Institutes of Health's Recombinant Advisory Committee (RAC), which reviews federally funded gene-therapy trials.

All of this makes it difficult to draw general conclusions from the case. "There are a lot



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of questions about the degree to which we can extrapolate to other trials," says Amy Patterson, head of the office that supports the RAC. But at the RAC's next meeting, on 4 December, it will have to begin wrestling with the issues.

One question that is likely to come up concerns the safety of 'gene-marking trials' in which doctors use retroviral vectors not to correct a genetic defect, but to introduce a marker gene to track a particular group of cells as they migrate through the body. For example, in patients given bone-marrow transplants, the introduced cells can be tagged with a genetic marker so that doctors can follow them and determine whether they develop normally. Patterson says that 41 marking trials are currently under way in the United States. But in the light of the French patient's cancer, some experts are wondering whether such trials should be discontinued.

Vector calculations

In the long term, gene therapists must consider whether the risks posed by insertional mutagenesis should cause them to move away from current retroviral vectors. They might, for instance, abandon retroviruses in favour of vectors that do not integrate into the genome. These aren't devoid of problems — as demonstrated by the 1999 death of teenager Jesse Gelsinger, killed by an inflammatory reaction to an adenovirus vector in a gene-therapy trial at the University of Pennsylvania in Philadelphia. But adenoviral vectors have since been refined to make them less likely to provoke dangerous immune reactions. Other non-

integrating vectors, such as modified Epstein-Barr and herpes viruses, are also being considered.

But it should be possible to engineer retroviruses to reduce the risks posed by insertional mutagenesis. One obvious possibility is to equip the vectors with 'suicide' genes. For instance, a vector could include a gene for a protein that renders any cell that produces it susceptible to the antiviral drug gancyclovir. If doctors noticed that cells that have integrated the vector were growing out of control, they could then administer the drug and nip the cancer in the bud.

Such approaches are already being used experimentally to attack difficult-to-treat cancers. Dan Salomon of the Scripps Research Institute in La Jolla, California, who heads the FDA advisory committee on gene therapy, says that scientists must investigate whether such methods are fully effective. But if they are shown to work well, suicide genes might become a standard safety measure in future gene-therapy trials. Observes Salomon: "I think Alain Fischer would be very happy right now if he had a suicide gene in his original vector."

Other researchers are investigating 'insulating' sequences to minimize the interaction between vector and host DNA. The initial idea was to prevent host genes from interfering with expression of the corrective gene. For instance, David Emery at the University of Washington in Seattle has put a genetic sequence from chickens into a retroviral vector, and found that the modification allowed cells that integrated the vector to express more of the therapeutic gene⁷. Hopefully, this buffering effect will also work in reverse, preventing the corrective gene from interfering with host genes.

While they work on new strategies, gene therapists will have to stick with some imperfect techniques. The French case is an individual tragedy, but experts point out that such events are, unfortunately, part and parcel of the business of developing new therapies. "It's no surprise that the more effective the gene-therapy transfer becomes, the more we're going to see the adverse consequences that are intrinsic to that technology," says Freidmann.

Rather than backing away from gene therapy, argue its practitioners, the important lessons to be learned from the French case lie in a deeper understanding of the risks that will help doctors to predict and respond to similar events in the future. ■

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