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Blood-clotting factors produced by these lettuce plants could eliminate the problem of immune rejection.

IMMUNOLOGY

Oral solutions

Pills made from lettuce leaves could help to prevent one of the most serious complications of haemophilia treatment.

BY ELIE DOLGIN

The food in Anita's bowl is not your average dog chow. Although the dish contains pellets and wet food, there is also a sprinkling of green powder — the product of a trailblazing experiment to address a potentially lethal complication of haemophilia treatment. Anita, so named because her red coat reminded breeders of the character from the animated film *One Hundred and One Dalmatians*, is a keagle (a mix of a beagle and a Cairn terrier) with haemophilia B.

Like people with this rare genetic disorder, Anita is naturally deficient in factor IX, a protein that helps the blood to form clots. When treated with replacement coagulation proteins, the dog naturally develops antibodies, or inhibitors, against the therapy — a problem that is also seen in some 5% of humans with haemophilia B. In these people, the immune system identifies the therapeutic protein as dangerous, causing the body to stop accepting the protein as a normal part of the blood, and destroys it before it can stop the bleeding. Continuing to take factor-replacement therapies can result in life-threatening allergic reactions, such as anaphylaxis.

The problem is even worse with haemophilia A, a disease that is four times more common than haemophilia B and in which the missing link in the coagulation chain is a protein called factor VIII. Around 30% of people with haemophilia A develop antibodies against replacement factor VIII.

Therapies are available to eliminate these antibodies. Some people, for example, undergo an intensive treatment called immune tolerance induction therapy, which involves regular intravenous administration of coagulation factors. But this is time consuming and costly (around US$1 million for an average five-year-old patient), and the treatment works in only about three-quarters of patients. "The challenges of treating haemophilia with inhibitors are just staggering," says Timothy Nichols, director of the Francis Owen Blood Research Laboratory at the University of North Carolina at Chapel Hill, which maintains the colony of haemophiliac dogs to which Anita belongs (see page S18).

Inducing immune tolerance in people who have developed inhibitors is one approach. But avoiding the problem altogether would be even better. "If you can prevent antibody formation in the first place, by finding some way of producing immunological tolerance that gets around that type of protocol, that would be a major advantage," says David Lillicrap, a clinician and researcher who specializes in bleeding disorders at Queen's University in Kingston, Ontario, Canada.

The green powder in Anita's dish might do just that. The oral treatment is a concentrate of freeze-dried lettuce-leaf cells, each containing around 10,000 chloroplasts — the organelles responsible for photosynthesis — that have been genetically engineered to produce factor IX. These proteins cannot themselves be used to prevent bleeding episodes, because the cellular machinery found in plants cannot package the human clotting factors into the biologically active form. What they can do, however, is prevent the immune system from mounting an attack against subsequent therapy.

The researchers behind the bioengineered lettuce have shown that inhibitor formation and severe allergic reactions can be prevented in mice by feeding the animals with a product based on these plants[1,2]. If the strategy also works in Anita and her kennel mates — and ultimately in humans — it could form the basis of the first product to protect against the immune responses associated with haemophilia treatment.

Anita is one of only two dogs to have received the bioengineered lettuce. "So far, it's going very well," says lead researcher Henry Daniell, director of translational research at the University of Pennsylvania School of Dental Medicine in Philadelphia.

AN ACT OF TOLERANCE

In 2006, Lillicrap demonstrated that a simple oral treatment could train the immune system not to produce inhibitors. Working with a mouse model of haemophilia A, he and his colleagues gave the mice a purified fragment of the human factor VIII protein, through the nose or mouth. The researchers found that the treatment afforded some protection against antibody development after factor VIII replacement therapy[3]. But the approach did not deliver sufficient amounts of the factor to immune cells in the gut or nasal passage to fully quash inhibitor formation.

Daniell came up with an improved delivery system. He focused first on haemophilia B. Adapting a technique[4] that he had previously developed to delay the onset of type 1 diabetes, Daniell and his group genetically modified tobacco plants to express human factor IX in their chloroplasts. (Daniell has since switched to using lettuce.)

Chloroplast DNA is separate from the genome DNA in the plant nucleus, and the large numbers of these tiny organelles in the

cell allow huge volumes of the coagulation protein to accumulate in each tobacco leaf. Once ingested, the plant cell wall protects the coagulation protein from being destroyed by stomach acid. Gut microorganisms farther down the digestive tract then chew away at the cell wall, releasing the clotting-factor protein.

To target the proteins to the immune system, Daniell then attached a second protein that has high binding affinity for a receptor found on the inside of the human gut. With this fused construct tethered to the intestinal wall, the coagulation protein could be absorbed into the body and processed by the specialized cells in the immune system that induce tolerance.

Working with Roland Herzog, a molecular biologist at the University of Florida in Gainesville, Daniell then tested the plant-based product in animal models. In 2010, they showed that oral delivery of factor IX expressed in chloroplasts in this way led to almost undetectable inhibitor levels in mice, and no sign of anaphylactic shock[1]. "The mice are healthy, they show no allergic responses and they don't form the inhibitors," Herzog says. "That's pretty exciting."

Daniell then modified the tobacco leaves to express factor VIII and shipped powders of the leaves to Herzog. Earlier this year, the two researchers and their teams documented[2] suppression of inhibitor formation and even reversal of pre-existing inhibitors in mouse models of haemophilia A.

INHIBITORY CONTROL

Other strategies being pursued to prevent the formation of inhibitors of clotting-factor therapy include immunosuppressants and drugs that deplete specific immune cells. However, these therapies have many side effects, including increased susceptibility to infection.

A potentially safer option comes from Selecta Biosciences, a company in Watertown, Massachusetts. Selecta has developed a nanoparticle delivery system in which an immune-modifying compound is contained in biodegradable plastic particles just 150 nanometres across. When injected together with factor VIII into mouse models of haemophilia A, the nanoparticles deliver their payload to cells in the lymphoid tissue that are responsible for initiating immune responses. These cells, in turn, instruct factor-VIII–specific immune cells to become tolerant to the coagulation protein, resulting in suppression of misdirected antibody responses to the replacement therapy — all without affecting the rest of the immune system.

David Scott and his colleagues at the Uniformed Services University of the Health Sciences in Bethesda, Maryland, teamed up with Selecta to show that inhibitors remained undetectable for at least six months after treatment with the nanoparticle formulation[5]. "This underscores the point that we're actually teaching the immune system to become tolerant to factor VIII," says Selecta's chief scientific officer, Takashi Kei Kishimoto.

The nanotechnology approach that is being tested for inhibitor control could also improve the haemophilia treatment that is now at the cutting edge of clinical research: gene therapy. Using the standard gene-therapy approach, researchers have shown that they can achieve long-term expression of factor IX in adults with haemophilia B at sufficiently high levels to convert the bleeding disorder into a mild disease (see page S6). There has so far been no reported evidence of inhibitor formation in the small number of human participants in clinical trials for this viral therapy[6].

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Green power: from leaf to powder to capsule.

Still, the standard form of liver-targeted gene therapy carries a range of potential complications, including the risk of harmful mutations and of the body mounting an immune response against the viral vectors used to carry the correct forms of the defective genes responsible for haemophilia. That is why several research groups are attempting to replace viral vectors with nanoparticles that can deliver gene therapies as 'DNA pills'.

PILL PROTECTION

DNA pills combine DNA plasmids — circular pieces of bacterial DNA containing the gene encoding either factor VIII or factor IX — with nanoparticles made of chitosan, a tough polymeric carbohydrate found in the exoskeleton of crustaceans. Chitosan protects the therapeutic gene product and chaperones it through the gut. "The oral route has significant appeal," says Gonzalo Hortelano, a gene-therapy researcher at McMaster University in Hamilton, Canada. "The key is to achieve a system of delivery that's persistent, effective and completely safe."

Independent studies by Hortelano's group and other research teams in Germany and the United States have shown that this oral gene therapy does not activate the immune system. Indeed, exposure of the protein produced by the nanoparticle-based gene therapy to the gut mucosa prevents inhibitor development and restores clotting-factor activity in mouse models of both haemophilia A[7,8] and B[9]. "This approach really could hold big benefit for patients," says Jörg Schüttrumpf, a transfusion-medicine specialist who led one of the studies performed at the German Red Cross Blood Donor Service in Frankfurt.

Kam Leong, a biomedical engineer at Columbia University in New York City whose team was the first to demonstrate success with this approach in mice[7], has even tried feeding the chitosan–DNA nanoparticles to dogs with haemophilia A. Leong found some evidence of gene transfer and a reduction in inhibitors in the animals. But bleeding times were not reduced, which would be expected if sufficient levels of factor VIII were being produced. "It is still a very inefficient process," Leong says, "so it requires continued optimization."

Although the ideal remains a gene therapy that both corrects the disease and offers immune tolerance, some scientists have focused on treating inhibitor formation, without worrying about fixing the disease. Under this strategy, people would still need to take factor-replacement therapies, but they could do so without fear of inhibitor development.

With this in mind, independent teams led by Scott and Herzog took the conventional viral-vector approach to inducing tolerance through gene therapy. But rather than delivering the entire gene for the clotting-factor proteins to cells, as most gene therapies do, the researchers used the viruses to engineer immune-regulating B cells to express a fragment of the clotting factor fused to an immune molecule called an immunoglobulin. This led to long-lived tolerance in mouse models of haemophilia A[10] and B[11].

Pursuing such gene-therapy approaches offers a degree of bet hedging, says Herzog. "Each strategy has potential advantages and disadvantages," he points out, "and we do not really know yet what will work or may work best in people." With so many therapeutic tactics moving through the preclinical pipeline, scientists and clinicians remain hopeful that at least one will ultimately succeed, eliminating the problem of inhibitor formation for people with haemophilia altogether. ■

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