Salvation in a Snippet of DNA?

Hopes are running high for vaccines made from nothing more than a bit of pathogen DNA. But how they work is still mysterious, and none has yet succeeded in a clinical trial

By the early 1990s, Steve Hoffman of the Naval Medical Research Institute in Bethesda, Maryland, had run into a roadblock in his quest for a malaria vaccine. Hoffman and his collaborators had identified the portions of *Plasmodium falciparum*, a malaria-causing protozoan carried by mosquitoes, that they believed an effective vaccine should contain. They had gone on to try turning them into a vaccine by several of the most up-to-date means. One strategy spliced protozoan genes into vaccinia, a virus that, when injected into animals, would infect cells, producing the *P. falciparum* proteins. Another used genetically engineered *P. falciparum* proteins as the vaccine. Still another relied on small portions of the proteins, called peptides. But all these avenues deadended when the researchers tested their concoctions in mice. The animals did not develop robust immune responses that would protect them against malaria.

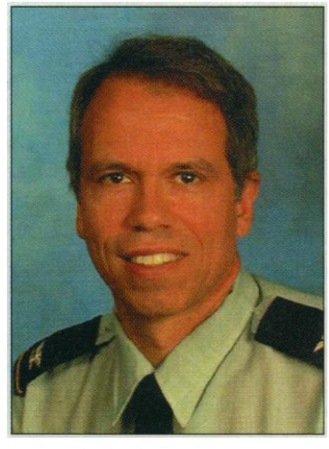
Naked truth 1. Steve Hoffman had success with a DNA malaria vaccine where other strategies failed.

Then Hoffman heard about DNA vaccines. Often known as gene vaccines or "naked" DNA, DNA vaccines turned many heads in 1993, when researchers published the first reports about their ability to stimulate an immune response in mice. Instead of peptides, proteins, or viral vectors, DNA vaccines consist of nothing more than a gene from the pathogen, stitched into a circular stretch of bacterial DNA called a plasmid. In theory, these vaccines are simpler to make and can stimulate a broader immune response because they closely follow the cellular pathway traveled by the real pathogen.

Martha Sedegah, a co-worker with Hoffman, says that when they put a DNA malaria vaccine into mice they saw a "tremendous" immune response. When they then "challenged" the mice with the most infectious strain of rodent malaria, the mice showed no signs of disease. After getting similarly impressive results in monkeys, the researchers this summer began safety trials on 25 human volunteers, hoping that this new approach will finally end the string of failures that has marked malaria vaccine research.

Yet even one of Hoffman's key collaborators on the human study offers a strong dose of caution about the approach. "I'm concerned about this general perception that DNA vaccines are going to save the world from all the failures of other vaccines," says Rip Ballou, a malaria researcher at the Walter Reed Army Institute of Research in Rockville, Maryland. "If our goal was to protect mice," says Ballou, who is pursuing a variety of other malaria vaccine strategies as well, "we'd have all the mice protected and we'd be out of business."

Welcome to the exploding and controversial field of DNA vaccines. Since researchers at Vical, a San Diego biotechnology company, first announced in the 23 March 1990 *Science* that naked DNA injected into animal cells would prompt the production of foreign proteins, the advances have been enormous. More than a half-dozen vaccines are already in clinical trials against such diverse diseases as AIDS, influenza, and cancer, and work is in progress on everything from rabies and genital herpes to measles, autoimmune diseases, and allergies. Meanwhile, ideas about entirely new ways to use DNA vaccines are proliferating (see sidebar). "I used to have a slide that listed all the pathogens for which DNA vaccines were under development," says immunologist Dennis Klinman of the Food and Drug Administration (FDA). "I can't use it anymore because the printing is too small."



Naked truth 2. Rip Ballou says high hurdles remain.

But many experienced vaccine developers like Ballou urge their colleagues to remember that the technique, however promising, is still in its infancy. Indeed, investigators are only beginning to understand how DNA vaccines actually work and how their efficacy might be boosted. Moreover, no DNA vaccine has yet proven its worth in a human study—a long, arduous journey even for vaccines made with techniques that have been used for centuries.

The missing link

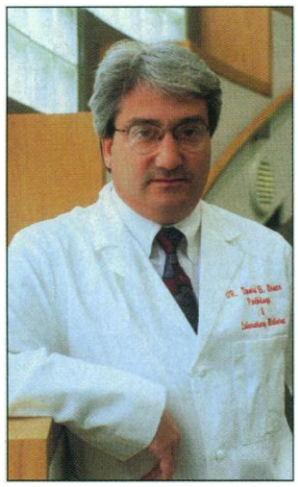
The potential advantages of DNA vaccines first made headlines in 1993 when Margaret Liu and colleagues at Merck Research Laboratories in West Point, Pennsylvania, showed that shots of naked flu genes could protect mice from lethal doses of influenza A. The novelty of the technique wasn't the only thing that caught the eye of other researchers; they were also intrigued by signs that the vaccine worked against a strain of flu that was thoroughly distinct from the strain used in the vaccine. Human flu vaccines, in contrast, only work against strains that match the vaccine.

The explanation Liu and colleagues proposed for this immunologic feat was that the DNA vaccine behaved like the many vaccines made from live, but weakened, pathogens. Ordinary flu vaccines are made from inactivated pathogens, which teach the immune system to make antibodies that target proteins on the invader's surface. But many pathogens mutate their surface proteins frequently, meaning antibodies against one strain of the invader may be ineffective against another, as is the case with influenza. By this logic, inactivated vaccines, along with "subunit" vaccines that contain parts of a pathogen, often only offer limited protection.

Dogma holds that live, attenuated vaccines can sidestep this dilemma because they both stimulate production of antibodies, and they excel at marshaling troops of so-called killer cells, or cytotoxic T lymphocytes (CTLs). These CTLs can not only identify and kill already infected cells but also home in on portions of pathogens that change little from strain to strain. "We've always known live, attenuated vaccines

were probably the most effective," says immunologist Frederick Vogel, who works in the AIDS vaccine branch of the National Institute of Allergy and Infectious Diseases (NIAID).

Live vaccines are so potent because they do their jobs from "the inside out," infecting a cell and then co-opting its genetic machinery to reproduce themselves. Infected cells display fragments of the invader on their cell surface together with molecules known as class I major histocompatibility complexes (MHC I). The MHC I display acts as a distress call for the so-called cell-mediated arm of the immune system, which dispatches CTLs that destroy the infected cells. But live vaccines also pose some risk, as they cause a mild form of the disease they're trying to prevent—and can sometimes lead to full-blown disease.



Mix 'n' match. Weiner's vaccines contain many ingredients.

Vogel sees DNA vaccines as "the missing link" between the two kinds of vaccines because they, too, "infect" a cell and prompt it to produce a broad-based immune response—yet, theoretically at least, they carry next to no risk. There are other, "safer" alternatives to attenuated vaccines: for example, stitching genes from a pathogen into a harmless virus, like vaccinia (which itself is the smallpox vaccine), which should also generate a CTL response. But this approach suffers from the immune system's ability to target and eliminate the viral vector before it can infect cells. Again, DNA vaccines offer an advantage. "We don't have to carry that baggage of vector or virus with it," says Vogel. "We don't have a response to anything but the [proteins] we want to be produced in the cell."

On top of DNA vaccines' impressive ability to rev up the immune system's CTL machinery, they are relatively simple to make. Denise Doolan, for instance, an immunologist in Hoffman's group, says it took her only 3 months, her first time out, to isolate the gene of interest from *P. falciparum*, insert it into a plasmid, mass-produce the plasmid in bacteria, and begin testing the vaccine in mice. Liu, who recently left Merck to become a vice president of vaccine research at Chiron in Emeryville, California, adds that unlike traditional protein or live virus vaccines, DNA vaccines for many different pathogens can all be made using roughly the same technology. "It's pretty much the same vector, just the gene is changed. It's like learning to make ice cream and then making different flavors." The ability to swap genes easily allows researchers to tailor-make vaccines for, say, different strains of a virus that are prevalent in specific locales. As a result, says Hoffman, "DNA vaccines could be revolutionary for fighting diseases of the developing world."

Black boxes

If all this seems a little too pat, well, it might be. Perhaps most sobering is that researchers still only have vague ideas about how DNA vaccines even produce an immune response. "It's a black box," says Carl Dieffenbach, associate director of the basic science program in the division of AIDS at NIAID.

As Dieffenbach explains, when researchers at Vical and subsequently at Merck first published their DNA vaccine work, they proposed that muscle cells where the vaccine is injected take up the DNA. The cells then express the proteins encoded by the DNA—which are called "antigens" because they're meant to stimulate an immune response—on their surfaces, in conjunction with MHC I molecules. "But muscle cells really are lousy antigen-presenting cells," notes Dieffenbach. In particular, the surfaces of muscle cells are devoid of the crucial "costimulatory" molecules, which are present on most, if not all, antigen-presenting cells.

Researchers like immunologist Eyal Raz of the University of California, San Diego (UCSD), bring up another puzzle: Mice immunized with a DNA vaccine produce only a tiny amount of the protein encoded in the pathogen gene, raising the question of how such a vaccine can trigger any immune response, let alone robust CTL responses. "We measured picogram quantities of gene product being produced in vivo," says Raz. "If I take this quantity of antigen and directly immunize mice with that, it's almost guaranteed that you will get no immune response. So why do gene vaccines work?"

Researchers are finally beginning to get inside the black box to answer these puzzles. Stephen Johnston of the University of Texas Southwestern Medical Center in Dallas says there have been hints from the outset that something other than muscle cells was doing the antigen presentation. One hint came from what he and his colleagues call "the Van Gogh experiment." The group shot a mouse in the ear with a gene vaccine and then quickly cut off the ear. The mice still produced a long-lasting immune response, indicating that the cells in the ear could not have been producing the proteins. "Cells that move picked up the DNA and expressed things," concludes Johnston.

In a series of papers published this past spring and summer, Liu's group at Merck, as well as a collaboration between Brian Barber's lab at the University of Toronto and Harriet Robinson's at the University of Massachusetts (she has since moved to Emory University in Atlanta), has shown definitively that the cells that do the presentation are dendritic cells, rather than muscle cells. Dendritic cells, which can be found everywhere in the body except for the brain, are known as "professional" antigen-presenting cells because their main function is to patrol for foreigners and then deliver whatever trespassers they find to the immune system.

Researchers have also made headway recently in understanding why DNA vaccines pack such a relatively potent immunogenic punch in spite of the tiny amount of gene product measured in vaccinated animals. Several groups over the past 3 years, including Arthur Krieg's at the University of Iowa in Iowa City and Raz's at UCSD, have proposed that the secret to this disproportionate response is the bacterial DNA surrounding the pathogen gene, which also stimulates the immune system. Bacterial DNA, they note, includes "sequence motifs" that our immune systems view as foreign. That finding raises the possibility that adding more bacterial sequences can increase the wallop of a DNA vaccine.

Researchers are also exploring other strategies for boosting the immune response triggered by DNA vaccines. While the immune response these vaccines milk out of a tiny quantity of antigen is startling, it is still underwhelming by the standards of other vaccines. "It's not a knockout vaccine that really gives you an extremely potent immune response," says Hildegund Ertl, a viral immunologist at the Wistar Institute in Philadelphia. "If you compare the immune response you get to a DNA vaccine to one that you get using a regular attenuated or inactivated virus, it's often lower."

HUMAN TRIALS OF DNA VACCINES

Institution	Disease
Johns Hopkins University	Influenza
Naval Medical Research Institute	Malaria
University of Alabama, Birmingham	Colon cancer
University of Cincinnati	Hepatitis B
University of Pennsylvania	AIDS, cutaneous T cell lymphoma
University of Washington	Herpes
University of Wisconsin, Aurigin	Hepatitis B

Ways to Vary the Gene Vaccine Theme

When researchers first reported in *Science* more than 7 years ago that directly injecting DNA into a muscle could lead to the production of proteins (see main text), they concluded that the finding "may provide alternative approaches to vaccine development." That's turned out to be a classic scientific understatement: So many researchers are now developing so-called DNA vaccines that entire scientific meetings are devoted to the topic, it has its own Internet site,* and biotech companies are tripping over each other to get into the business. And, as is true in any booming scientific field, researchers now have many creative variations on the original theme.

Immunologist Maurizio Zanetti of the University of California, San Diego, and his colleagues recently developed an approach that is far afield from the early notion of injecting DNA vaccines into muscles. As he and his co-workers describe in the 15 September issue of *Nature Biotechnology*, the immune system itself can be the target for the DNA vaccine, serving double duty by having its cells both produce the proteins coded for by the DNA fragment and mount the appropriate immune response to those foreign "antigens." Specifically, Zanetti has recruited B lymphocytes, the cells that manufacture antibodies, to do the work of DNA vaccination.

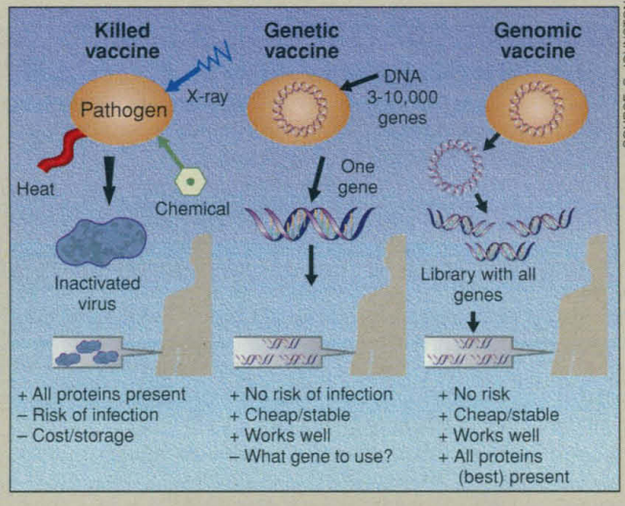
Zanetti decided to exploit B lymphocytes because they are "extremely powerful pieces of machinery" for making proteins. He cites estimates that the average B cell can spit out 1000 antibody molecules per second. "If this [antibody] happens to be your antigen, that's a lot of antigen being produced," says Zanetti.

At the heart of Zanetti's vaccine, designed to foil the malaria-causing *Plasmodium falciparum*, is a gene for an immunoglobulin—an antibody protein—mixed with a gene from the malaria parasite. Zanetti and his colleagues found that when they inject a plasmid, or a circlet of DNA, containing this hybrid "transgene" into the B cell–rich spleens of mice, B cells take it up and produce an antibody studded with a piece of *P. falciparum*. This, in turn, triggers a broad, long-lasting immune response to the so-called "antigenic antibody."

Rip Ballou of the Walter Reed Army Institute of Research in Rockville, Maryland, a veteran malaria vaccine developer and a Zanetti collaborator, calls Zanetti's technique "pretty remarkable" but says he's "not sure how practical the approach is." One problem is the need for an injection directly into the spleen. And because the new genes become integrated into the B cell genome rather than just borrowing the cells' protein-synthesizing machinery, as they do in muscle, the approach raises concerns about long-term safety. "Would it cause problems?" Ballou asks. "Who knows?"

Zanetti, however, is optimistic: "I hope we can develop methods that allow us to vaccinate without being invasive." One possibility, he says, is an aerosolized form of the vaccine, delivered through the nose, the inside of which Zanetti sees as "a lymphoid organ in direct contact with the air."

Equally ambitious is Stephen Johnston's unusual twist on the DNA vaccine technique: "expression library immunization" (ELI). Johnston, a biochemist at the University of Texas Southwestern Medical Center in Dallas, has been exploring ways to use the technique to shortcut the search for the parts of HIV, say, or malaria or tuberculosis needed to trigger the most effective immune response for vaccination. "If you have an immunologically complicated organism, you don't have to go through each and every protein to figure out which one is protective," explains Dennis Klinman, an immunologist with the Food and Drug Administration. ELI, he says, "allows the immune system to make that decision itself."



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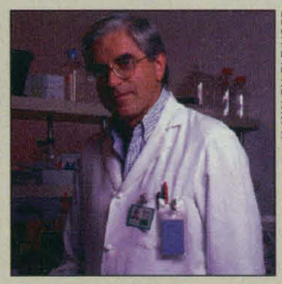


K. WALSH/OLR/UCSD

Moon shots. Both the genomic vaccine's library approach *(above)* pioneered by Stephen Johnston *(left)* and the "antigenic antibody" of Maurizio Zanetti *(right)* take DNA vaccines to new terrain.

Johnston and his colleagues take DNA from a pathogen and randomly break it into fragments. They then clone the fragments into groups of, say, 1000 plasmids. After injecting different plasmid groups into test animals, the researchers "challenge" them with the pathogen. Animals that don't develop disease must have been protected by one or more out of the 1000 plasmids they received. The researchers then take that 1000 and break it into groups of 100 and retest. "Now if the animal is protected, we know one out of that 100 did it, and we repeat that process until we get down to a single plasmid or a few plasmids we know are conferring protection," Johnston says.

For a small to medium-sized virus, Johnston says it should take no more than 6 months to run through the procedure, and 9 months to a year for a bacterium. "The nice thing is once you've done it," he says, "you've got everything." He and his colleagues are now using ELI to find every possible antigenic protein coded for by the 4000 genes of the tuberculosis pathogen, *Mycobacterium tuberculosis*.

Once this procedure is finished—and Johnston's group has done it so far in three bacterial systems including *Mycoplasma pulmonis* and *M. tuberculosis*—the proteins identified as immunogenic can be put to work in any delivery system. "There are as many as 20 different pathogen or microbial genome sequences that will be entered into databases this year alone," says Johnston. "We can then systematically sort through them for [relevant antigens to] the key pathogens in a very short time, rather than having to rely on the hunt-and-peck technique people have used for the past 30 years." –G.T.

* DNA Vaccine Web is at www.genweb.com/Dnavax/dnavax.html

DNA vaccines are hampered because a plasmid simply doesn't wreak havoc with the cells it co-opts. Ertl explains that a vaccinia-based vaccine, for example, infects cells, busily copies itself, then kills the host cell, which releases tons of virus. "That stuff gets picked up by antigen-presenting cells," says Ertl. "But by themselves, DNA vaccines will not kill a host cell." She adds that DNA vaccines are not very efficient at entering cells and commandeering their machinery to produce the desired antigens.

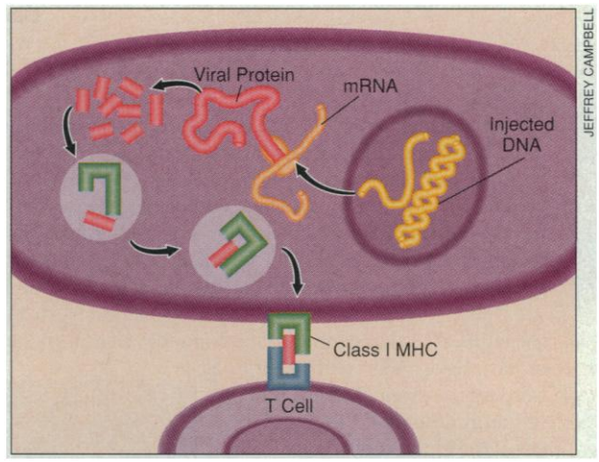
To overcome these kinds of "limiting steps," Ertl and others have been adding to the plasmids what they call genetic adjuvants. Ertl has focused on genes that code for immune system messengers called cytokines, which can summon specific sets of immune cells to cells carrying a pathogen or foreign DNA, amplifying the immune response. Ertl and her colleagues began with a cytokine called granulocyte-macrophage colony-stimulating factor, or GM-CSF. They took the gene for GM-CSF and packed it in the plasmid with a gene for a rabies antigen to create a souped-up vaccine. When given to mice, it triggered an immune response as much as 50-fold stronger than the vaccine without adjuvants. Johnston says his lab has tried the approach with a half-dozen different cytokines and seen them enhance immunogenicity of the vaccine by as much as 100-fold.

What makes the genetic adjuvants so intriguing, says David Weiner, a molecular immunologist at the University of Pennsylvania School of Medicine in Philadelphia, is that by adding them to a vaccine in particular combinations, investigators may ultimately be able to fine-tune the immune response, bringing out precisely the level of antibody or CTL response needed to attack a particular disease. Weiner and his colleagues, Richard Ciccarelli at Apollon in Malvern, Pennsylvania, and John Kim at Penn, for instance, have explored several AIDS vaccines with different combinations of genetic adjuvants and HIV genes.

DAVID POWERS

Tutti-frutti DNA? Margaret Liu says it's easy to make different flavors.

When they included a cytokine called interleukin-12, he says, they saw "enormous boosting, up to 10-fold enhancement" of CTLs in animal experiments, and a simultaneous suppression of the antibody response. Conversely, he says, GM-CSF turned antibody production on high. When they delivered the vaccines with the costimulatory molecule CD86 (a cell surface receptor formerly known as B7-2), they once again got dramatic enhancement of CTLs, but this time without suppressing antibody response. "You can see immediately what you can do with these combinations of genes," says Weiner. "There's really a lot to play with here, and a lot to learn about how the immune system works."



JEFFREY CAMPBELL

Double trouble. DNA vaccines trigger production of both antibody and killer cells.

Naked fears

If researchers at times seem giddy about the potential of DNA vaccines, there's still an undercurrent of concern about injecting naked DNA into humans. In particular, it's unknown whether the foreign DNA will integrate into the chromosomes, potentially leading to mutations or abnormalities. In short, says Klinman, "we're worried about whether these things could produce cancers." Klinman notes that vaccines, unlike drugs, primarily go into healthy children: "So if something had the potential to produce a tumor, it would have long time and large population base to work on."

The FDA is also worried that the vaccines might make mischief by generating anti-DNA antibodies, which play a role in autoimmune diseases such as lupus. So far, this doesn't seem to be the case, but Klinman and his colleagues have created animal models suggesting that young children with certain genetic predispositions could be at risk for certain rare forms of organ-specific autoimmune problems. "Having now seen that in an experimental mouse model," he says, "we know to be on the lookout for that when we go into human trials."

Despite these worries, the speed at which DNA vaccines are moving into clinical trials is accelerating. In the first few years of DNA vaccine development, the FDA and other regulatory bodies were hesitant to let researchers move into clinical trials with such a radical technology. The researchers, in turn, complained that their proposals were snarled in regulatory red tape and going nowhere. Early this year, however, the World Health Organization released its guidelines for DNA vaccine production, and the FDA released a "Points to Consider" document, covering the requisites of testing and manufacture from preclinical trials on up to human tests. Now, say researchers, the bottleneck has vanished and proposals to do clinical trials are whizzing through the FDA.

Weiner, for instance, has four small human trials of DNA vaccines in progress for HIV to see whether they are safe and can stimulate immune responses. "The DNA vaccine gives us a lot of targets to go after," he says, "just as multiple drug therapy works better than single drug therapy. If you let the immune system attack viruses at multiple points it should limit, at least conceptually, the ability of the virus to escape all attack."

Other researchers are also designing trials of DNA vaccines for cancer, where the goal is not to prevent the disease but to spur the immune system in people who already have it. The strong CTL response induced by DNA vaccines, says Weiner, might prove valuable for fighting tumors, where killer T cells seem to play a primary role, at least in animal models. What's more, says Klinman, the ease of production of DNA vaccines suggests it might be practical to create a unique vaccine for each patient at considerably less cost and effort than a tailor-made conventional vaccine.

At the rate DNA vaccines are currently moving into clinical trials, answers about their true worth should begin to surface during the next few years. "One can be cautiously optimistic, but one still has to be cautious," says Barry Rouse, a viral immunologist at the University of Tennessee, Knoxville, who works with herpes vaccines. "Just because they're there, doesn't mean they're the answer to the maiden's prayer. Expecting a DNA vaccine to work where other vaccines haven't worked still requires a leap of faith, a big leap of faith."

–Gary Taubes