# EDIBLE VACCINES

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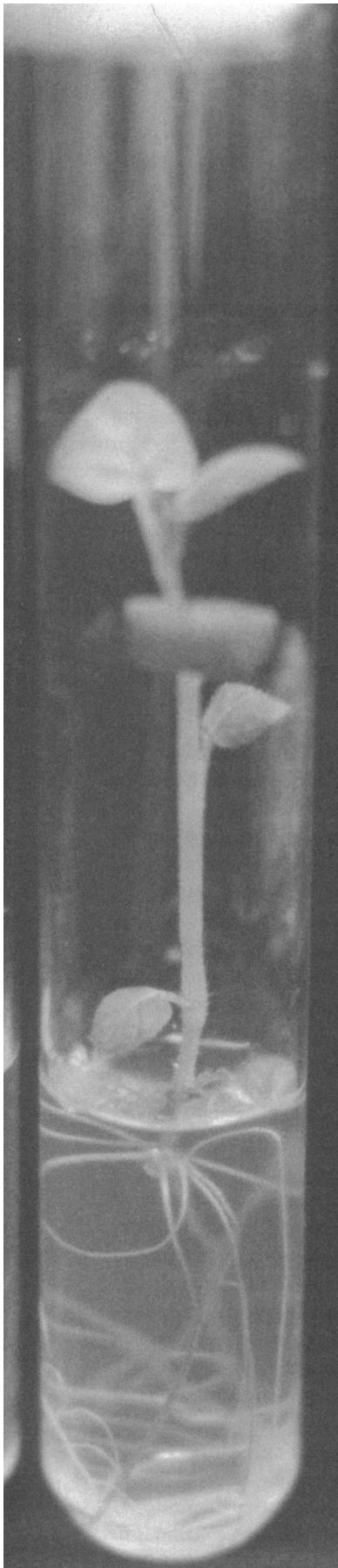

*SYNOPSIS*

VACCINES WERE THE RESULT of trial and error research until molecular biology and genetic engineering made possible the creation of many new and improved vaccines. New vaccines need to be inexpensive, easily administered, and capable of being stored and transported without refrigeration; without these characteristics, developing countries find it difficult to adopt vaccination as the central strategy for preventing their most devastating diseases. The authors describe a promising approach to inexpensive and effective vaccines: producing them in plants we commonly consume.

A one-year-old sits on her mother's lap eating a banana handed to her by a health worker. When she is done, the health worker checks off the box on the child's medical record indicating that she has been protected against three vaccine-preventable diseases. Pull back from this intimate scene and imagine a dusty courtyard in a developing country crowded with mothers feeding vaccine-containing bananas to their children. Science fiction? Maybe not for much longer.

Interdisciplinary research linking immunology and plant molecular biology has led to the creation of genetically engineered plants whose edible parts contain proteins that can function as oral vaccines. A new paradigm of vaccine production is emerging: very safe and low-cost vaccines available in a convenient delivery system. Scientists may even be able to engineer plants to produce multiple proteins in a single tissue to deliver multicomponent vaccines. Plant-based vaccines for oral delivery could simplify vaccination and increase compliance. The new technology is especially appropriate for developing countries where safe, inexpensive, and readily available vaccines are urgently needed for universal childhood immunization programs.

## The Children's Vaccine Initiative

In 1990, translation of scientific advancements into the new vaccines needed by developing countries was lagging. At the World Summit for Children in New York that year, a consortium of philanthropic, health, and intergovernmental organizations urged the world to harness new technologies to advance the immunization of children. The resulting Children's Vaccine Initiative (CVI) recognized the role that new technologies might play in improving current vaccines and developing new ones.

In 1992, WHO estimated that three to five million children's lives could be saved each year if new vaccines were available to control or prevent commonly occurring infectious diseases. Diarrheal and respiratory infections are major causes of infant mortality in the developing world.

CVI focused attention on the need to exploit technologies that would make vaccines—both their production and use—less expensive and more reliable, especially for the developing world. The Initiative emphasized the importance of oral vaccines because they eliminate the need for needles and syringes with their accompanying costs and risks. The pain of injections and the reaction of children is often a reason why subsequent vaccination visits are missed. (Oral vaccines may also induce mucosal immunity, creating a barrier in the gut, lungs, and urogenital tract that can block infection before the body must rely on a systemic response.)

CVI also promoted development of multicomponent vaccines in which antigens against several infectious agents could be delivered simultaneously, thus simplifying administration. Heat-stable vaccines, also advocated by CVI, would not need refrigeration—the traditional cold chain—which currently limits success and coverage in developing countries. Each of these desired features—low cost, improved reliability, elimination of injections, mucosal immunity, multicomponent vaccines, and heat-stability—can potentially be found in plant-based vaccines.

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## Innovations of the Past Decade

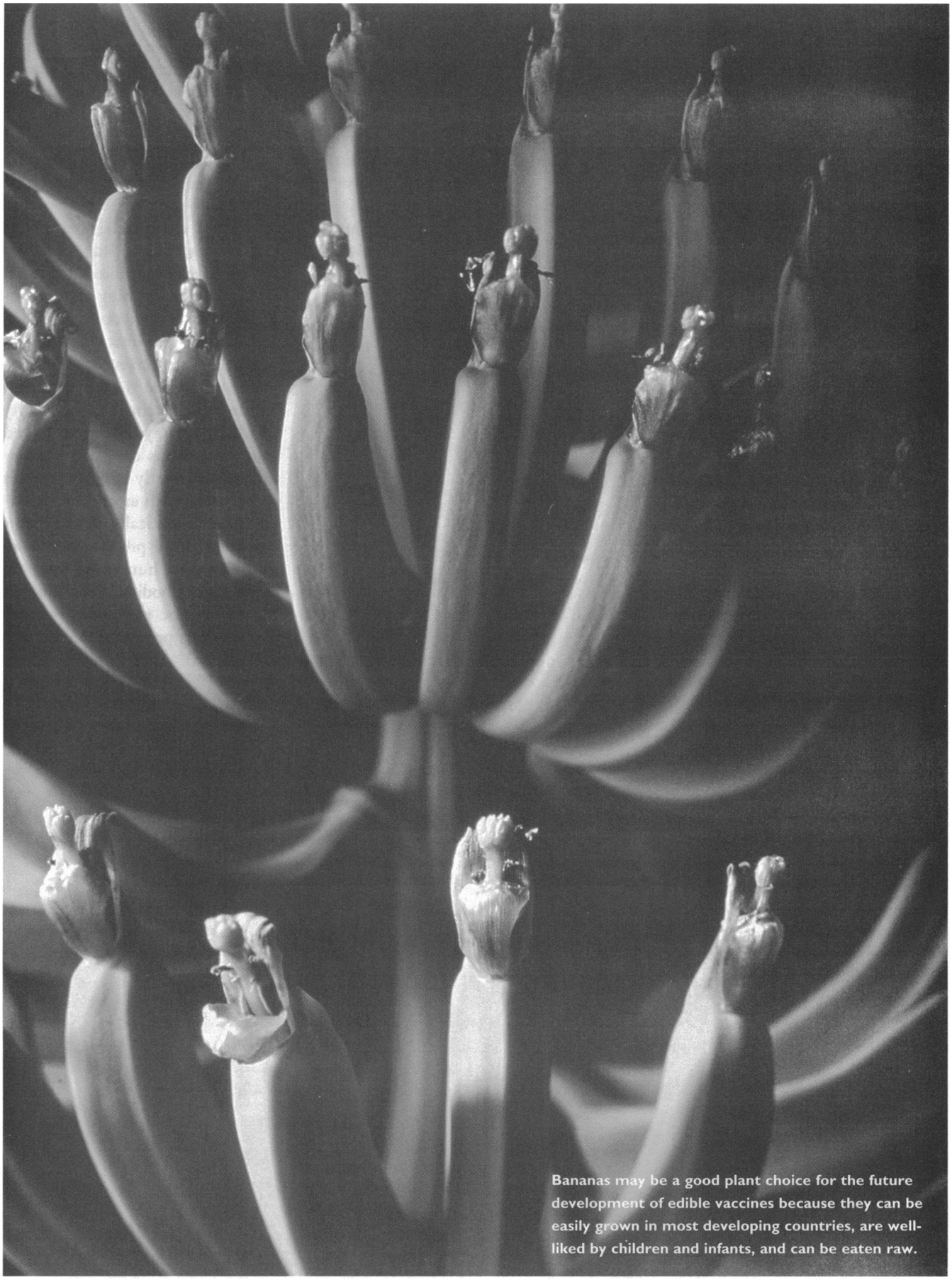
In 20 years since the introduction of genetic engineering, vaccine science has opened many new frontiers.

**Mucosal immunology.** For two centuries, medicine has manipulated the human immune response with vaccines. [See "Edward Jenner's Vaccine" March/April 1997 *PHR*, p. 116–21.] Disease-causing agents enter the human body by many routes. An immune response is triggered by these invasions: specialized cells of the immune system (macrophages, lymphocytes, B-and T-cells) migrate to the point of invasion to block the spread of the infectious agent. The next time the same invader appears, the body remembers and the response is faster and stronger. Vaccines attempt to mimic the foreign organisms, thereby stimulating this protective immunity.

In the last decade, we have learned that mucous surfaces of the gastrointestinal, respiratory, and urogenital tracts and of the conjunctiva function as a distinct part of the immune system, providing a first defense against entry of foreign organisms. The mucosal immune response is initiated by lymphoid cells that are responding to antigens located at the mucous surfaces. Breakthroughs in mucosal immunology have given us a new way of thinking about vaccine delivery. We can now use oral vaccines to stimulate mucosal immunity, our first line of defense.

**Biotechnology.** The field of biotechnology has provided new strategies for vaccine design based on techniques that make it possible to isolate genes from pathogenic viruses or bacteria. When certain pathogen genes are moved into other organisms—from a virus to yeast, for example—the receiving organism begins to mimic aspects of the original pathogen, including the production of antigens that are the "signature" of the original pathogen. These are called *subunit* vaccines because they are only a small part of the pathogen. The new combined, or recombinant, organism is incapable of causing the disease itself because it only contains one subunit of the pathogen. This technology was applied successfully to produce a vaccine for hepatitis B. Yeast cells grown in fermentation vats now make hepatitis B antigen on a commercial scale. The "signature" hepatitis B antigens are extracted from the yeast cells and purified for injection into humans.

This method produced the first commercial example of a recombinant subunit vaccine. This vaccine is extremely safe and effective but relatively expensive. Although the price has dropped from $35.00 per adult dose to about $1.00 for large international purchases, the Institute of Medicine of the National Academy of Sciences continues to list the development of a lower-cost recombinant hepatitis B vaccine as a top priority. (In contrast, vaccines developed early in this century now cost as little as ten cents per dose when purchased by UNICEF.)



Bananas may be a good plant choice for the future development of edible vaccines because they can be easily grown in most developing countries, are well-liked by children and infants, and can be eaten raw.

As our understanding grows of the how the immune system recognizes individual proteins to which it has been exposed before, other recombinant subunit vaccines will be developed offering exciting disease prevention opportunities. But recombinant vaccine production is likely to remain dependent on costly fermentation and protein purification technology. Moreover, vaccines produced this way often need to be refrigerated and injected.

Unfortunately, while research on which proteins make effective vaccines and on the genes that control their production proceeds in many laboratories, much less research has been devoted to the organisms in which to express these genes—microbes or plants that will produce the immunogenic protein in large quantities. The recombinant HBV vaccine produced by yeast cells grown in large fermenters provides a benchmark of efficacy and safety for vaccines produced in recombinant organisms. Transgenic plants, that is plants in which genes from other species have been introduced, may prove to be an equally useful way to produce proteins encoded by specific genes.

**Plant biotechnology.** A revolution in our basic understanding of plants, fueled by genetic engineering techniques, has occurred over the last 15 years. Today we can introduce foreign genes into almost all major food crops, and these genes are faithfully passed on to future generations. Studying the heredity of plants by inserting foreign genes has led to a detailed understanding of how gene expression is regulated in different tissues and at different times in the plant life cycle. We now know how to cause foreign genes to be expressed only in selected tissues—such as fruits or grains. Plant biotechnology has yielded commercial agricultural products: plants resistant to insects, viruses, fungi, and herbicides have been created, and foods with modified ripening characteristics are now commercially available.

Within the last few years, research laboratories have experimented with the use of plants for "biomanufacturing" of specialty products. They have exploited transgenic plants designed to accumulate particularly valuable proteins or enzymes, such as ones of potential pharmaceutical value.[1] Plants can be made to produce molecules as diverse as human serum and secretory antibodies. Formation of these complex molecules requires many cellular processes; the fact that

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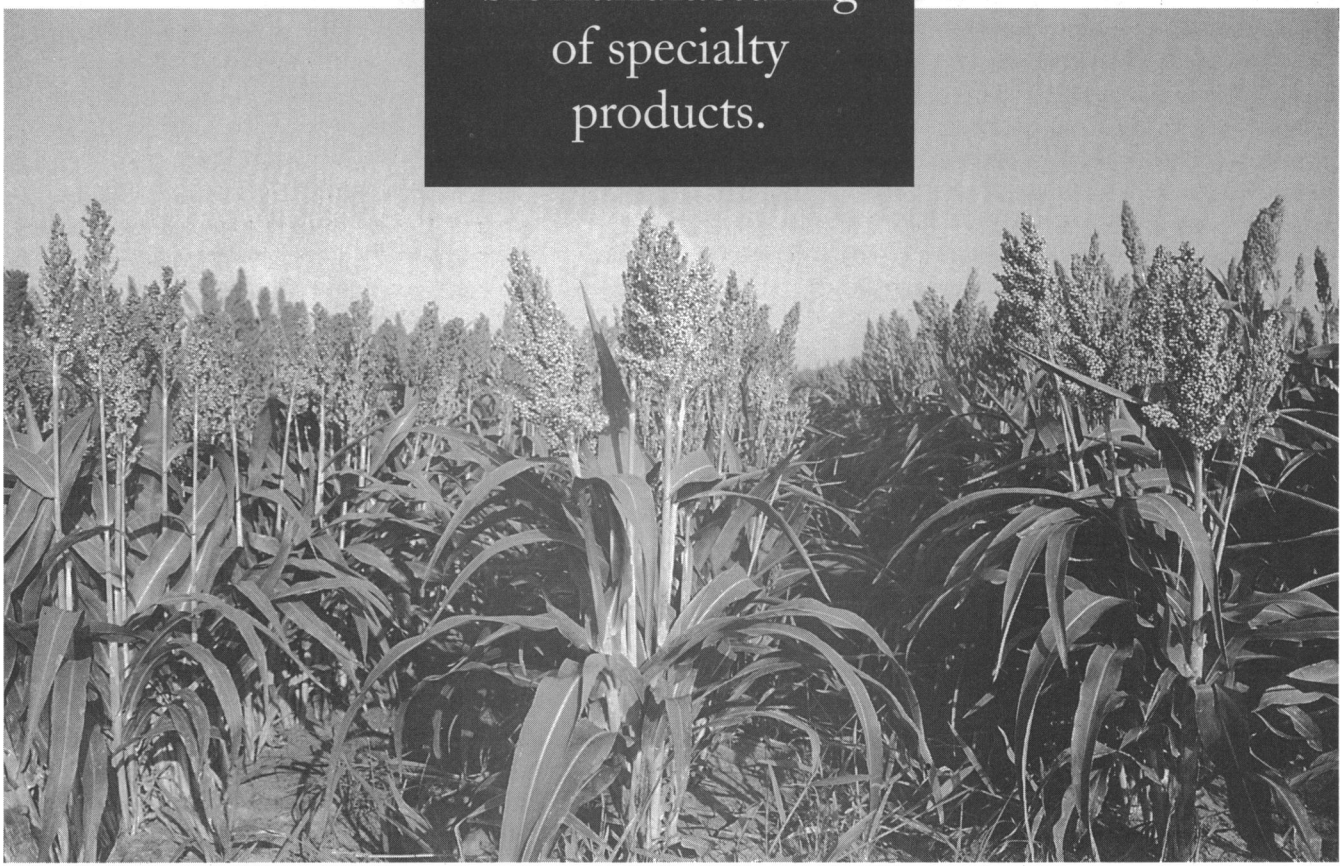


human albumin folds correctly to produce the authentic molecule or that the multiple subunits of the antibody assemble properly is proof that human and plant cells share fundamental biosynthetic processes. We can therefore conclude that plants have the capacity to "manufacture" valuable human pharmaceutical proteins.

## Putting It All Together

About five years ago, my colleagues and I were strongly influenced by the CVI objectives calling for new technology that would make inexpensive, multicomponent vaccines available for worldwide childhood immunization. In particular, we were impressed by the call for technology leading to oral vaccines. We have evaluated the possibility that immunogenic proteins produced in transgenic plants might induce immunity when the plant material is eaten.

In our evaluation of transgenic plants as a new means of vaccine production, we pursued three separate courses.[1] First, we experimented to determine the capacity of plants to produce foreign proteins that retain antigenic characteristics necessary to induce immunity. Second, we evaluated the immunogenicity of ingested plant-derived proteins (was immunity still provided after the food had been eaten?). Third, we sought an appropriate food crop that could be used for both production and distribution of vaccines, with attention to the needs of the developing world.

**Hepatitis B surface antigen expression in plants.** We began our studies of vaccine production in transgenic plants using the gene that encoded the hepatitis B surface antigen ($HB_sAg$). This surface antigen was chosen because (*a*) the commercially available vaccine for hepatitis B and the associated human immune response had been very well characterized; (*b*) the structure of the immunogenic form of the surface antigen protein was known; and (*c*) an inexpensive recombinant hepatitis B vaccine was deemed highly desirable for both developed and developing countries.

In our initial studies, the gene for $HB_sAg$ was introduced into cells of tobacco plants, and transgenic plants were regenerated.[2] We chose tobacco for its ease of genetic manipulation and because of the abundant literature on how gene expression is controlled in this "model laboratory plant." When we extracted leaf material from the transgenic plants, virus-like particles were recovered, characterized, and found to be very similar in structural properties to the recombinant $HB_sAg$ in commercial vaccine produced in yeast cells.

To evaluate the immunogenicity of plant-derived $HB_sAg$, we collaborated with Dr. Yasmin Thanavala of the Roswell Park Cancer Institute. We used plant-derived $HB_sAg$ to vaccinate mice by injection and recovered anti-$HB_sAg$ antibodies that reacted with authentic $HB_sAg$ from human serum. This was our first indication that the antigenic properties of the protein were indeed maintained in recombinant plants. Subsequently, we isolated T-cells from mice immunized with the tobacco-derived $HB_sAg$. When grown in culture, these T-cells could be activated using the commercial hepatitis B vaccine or by a synthetic peptide that mimics a portion of the antigen, the "a" epitope determinant of $HB_sAg$. This showed that the T-cells had a memory of earlier exposure to a similar antigen—the tobacco derived surface antigen. In summary, these immunology studies showed that recombinant $HB_sAg$ recovered from plant cells retains the characteristics or regions (epitopes) that stimulate both B- and T-cells. We demonstrated that plant cells have the capacity not only to synthesize this protein but to allow it to assemble in an immunologically active form.[3]

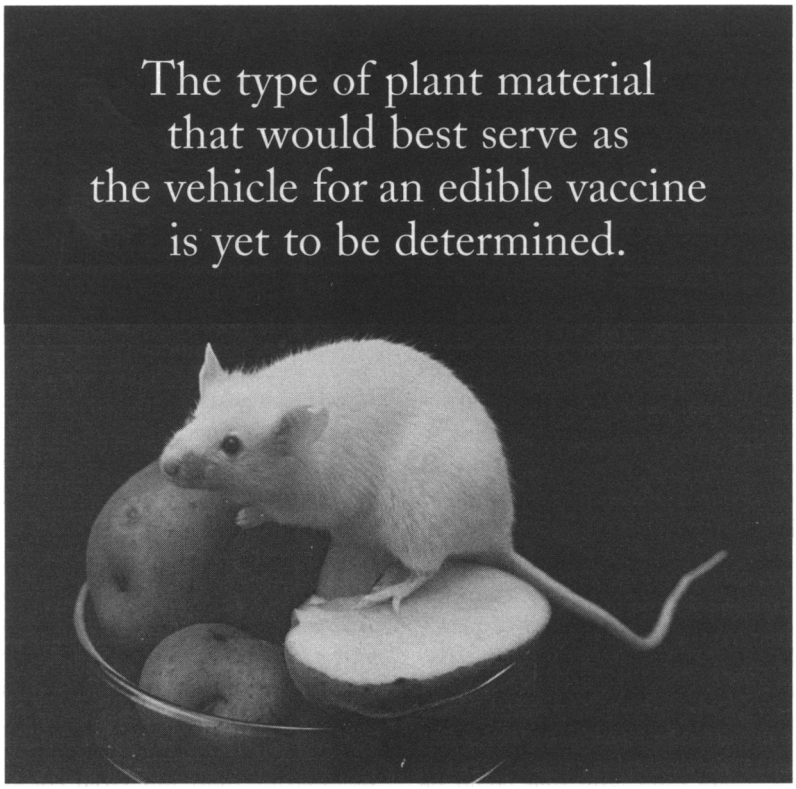
**Vaccines against diarrhea.** In the developing world, diarrhea is a principal cause of infant mortality. Despite advances in the use of oral rehydration therapy, one-third of the deaths from diarrhea are in children under 5 years of age and another one-quarter are in children ages 5 to 14. There are no immediate prospects for a solution to this problem in the countries where disease prevention is needed most. Affluent countries conduct little research on diarrheal disease vaccines because in those countries good sanitation and drinking water have removed the life-threatening aspects of the problem. Moreover, vaccines produced by the vaccine industry using "good manufacturing practices" that are required by most industrial countries are likely to be very expensive when they are first marketed. Perhaps our research on plant expression systems will lead to a new technology that can be transferred to the developing world to produce vaccines where they are needed.

Because my colleagues and I wanted to test the immunogenicity of ingested recombinant antigens produced in plants, we made an early choice to focus on diseases of the gut. We collaborated with enteric disease researchers—particularly with the laboratory teams of John Clements at Tulane Medical School, which studies bacterial diseases, and Mary Estes at the Baylor College of Medicine, which studies viral diarrheas.

**Plant-produced vaccines to prevent bacterial diarrhea.** Bacteria that cause diarrheal disease are transmitted by contaminated food or water. Once in the gastrointestinal tract, they colonize the surface of the gut and begin secreting enterotoxins that bind to the mucosal cells lining the gut. Binding and subsequent uptake of the toxins leads to the massive cellular damage and water loss that characterizes diarrheal disease. Cholera and "traveler's diarrhea" are caused by bacteria that produce toxins composed of two parts: a binding protein subunit (which is functionally inactive by itself) and an associated enzymatically active protein that causes the cellular damage.

The binding subunit of *E. coli*'s heat-labile enterotoxin (LT-B) was our obvious candidate to evaluate its production in plants because it has been extensively characterized in vaccine studies. It is very similar in structure and immunological properties to one of the parts of cholera toxin, the B subunit (CT-B) which has been used for oral immunization in field studies

in Bangladesh. These studies showed that CT-B when taken by mouth was moderately useful in preventing cholera and gave cross-protection against bacterial diarrhea caused by enterotoxic *E. coli* (often called ETEC or traveler's diarrhea).




We have conducted experiments to understand how high levels of an antigenic protein can be produced in edible, transgenic plant tissues. How much protein would have to be eaten to trigger an immune response in the vacinee? Our first success came when we designed a modified LT-B gene that caused the antigenic protein to accumulate in protein storage bodies in potato cells. This gave us potato tubers that could be fed to mice. (We had selected potato plants for these experiments not because people eat raw potatoes, but because we could rather quickly generate transgenic plants and because test animals such as mice will readily eat them.)

We found serum antibodies specific for LT-B in blood samples of animals fed the potatoes, and secretory antibodies in their intestinal fluids; both antibodies were effective in inactivating the *E. coli* toxin.[4] In addition, the mice challenged by mouth with purified, authentic bacterial toxin were protected from its effects, as measured by their resistance to gut injury—specifically intestinal swelling. Because mice are not susceptible to enterotoxic *E. coli* infection, the efficacy of our plant-based material could not be evaluated in challenge trials with the bacterium.

Our encouraging results have led us to begin planning human clinical trials, collaborating with Drs. Myron Levine and Carol Tackett of the Center for Vaccine Development at the University of Maryland. Human trials will demand transgenic plants that accumulate high concentrations of LT-B, to deliver an adequate dose in the small amounts that can be consumed raw.

Production of high levels of LT-B in plants has been more difficult to achieve than was originally anticipated, principally because LT-B was found to damage the plants when expressed at high levels in potatoes. We discovered this fact after creating an entirely synthetic gene for LT-B and expressing it constituitively (in all cells) in transgenic plants. The synthetic gene, which had been designed for optimized expression in plants, did indeed result in higher LT-B protein accumulation. This success was muted by the observation that the plants with high levels of the protein were stunted and grew slowly; we believe that the native form of LT-B causes cellular damage at high concentrations. Since these observations, we have refined our procedure for LT-B synthesis in plant cells and will develop plants that accumulate high levels of the desired protein only in mature tissues that can be collected before any phytotoxicity—cellular plant damage—sets in. These studies are ongoing. In the meantime, we are pursuing human clinical trials using plants that accumulate LT-B protein at a moderate, non-phytotoxic level to verify that when eaten they are immunogenic in humans.

**Plant-produced vaccines to prevent viral diarrhea.** Many viruses can cause diarrheal disease. Norwalk virus (NV), a member of the *Caliciviridae* family, is a causal agent of severe epidemic outbreaks of viral diarrhea. Dr. Mary Estes and her colleagues at the Houston Medical Center have identified the gene for the surface protein of Norwalk virus and have demonstrated the potential value of this purified protein as a vaccine. In cooperative studies, my colleagues and I have used the NV gene to create transgenic potato plants. We collected potatoes from these plants, peeled them, and fed 5-gm samples to mice on an immunization schedule analogous to Dr. Estes's previous experiments using purified NV protein. The mice produced serum and secretory antibodies against the recombinant protein.[5] We continue to work with Dr. Estes and her colleagues to move toward human clinical trials since our transgenic potatoes contain an amount of the antigenic protein that is anticipated to be effective.

## Questions and Opportunities

Our research has demonstrated that transgenic plants have the capacity to synthesize and accumulate antigenic proteins that retain the immunological properties of their native counterparts in infectious viruses or bacteria. In the

## Vaccines for Animal Diseases

Oral vaccines can also provide efficient and humane strategies for disease prevention in agricultural animals and pets as well as in feral populations. Our studies of edible vaccines suggest that the design of analogous vaccines in feed crops is a viable strategy. Seed-specific protein production technology for crops such as corn or soybeans is available, and the stability of foreign proteins in dried seeds has been demonstrated.

Recently another research group has shown that transgenic plants can be created with the gene encoding the glycoprotein (G-protein) that coats the outer surface of the rabies virus. Although the immunogenicity of these materials has yet to be reported, it is encouraging to note that bait containing the same G-protein produced in a more traditional fermentation system was effective in immunizing raccoons orally, providing protection against naturally occuring rabies virus.

case of HBsAg and the NV-surface protein, virus-like particles accumulate in plant cells. These particles are comprised of many individual protein subunits. They are likely to induce strong immune responses because particles seem to have greater immunogenicity when eaten than do soluble (individual) proteins.

Questions remain. Still to be conducted are studies that can evaluate dosage requirements for plant-delivered vaccines. Will proteins that are not normally eaten and not native to plants be effective in inducing an immune response after having gone through the digestive system? We believe that the structure of plant cells may be an influencing factor: plant cells constitute a natural bioencapsulation system. The surrounding layers of cell wall, cell membrane, and (in some cases) internal membrane compartments encapsulate and may protect an antigenic protein from digestive degradation as the plant cell traverses the stomach. But does gradual release of the desired protein in the gut as the plant cells are degraded in the normal digestive process limit the amount of antigen available at any one time? If so, dosage levels may have to be adjusted accordingly.

Most food proteins do not trigger an immune response. Rather, ingestion is more likely to induce of a state of immune tolerance (the inability of the immune system to detect the protein). Now we must learn if food-based vaccines when eaten would eventually induce tolerance rather than immunity against the desired antigen. If so, controlled use and dosages will be required for "edible vaccines." This should not be an insurmountable difficulty, however, since only a small amount of plant material would need to be propagated for wide-scale vaccine delivery and its distribution could remain under the control of public health authorities.

Multiple vaccines produced in a single plant would be a major advantage of vaccine production using recombinant plants. No theoretical limit exists on the number of different genes that could be introduced into a single plant species but research is needed to evaluate the effects of multiple new proteins on plant cell growth and development. However, we anticipate the plant tissue could meet some of the objectives expressed in The Children's Vaccine Initiative with respect to delivering multiple antigens in one delivery system. Moreover, plant-produced vaccines might obviate the need for a "cold chain" if produced and used in developing countries.

The type of plant material that would best serve as the vehicle for an edible vaccine is yet to be determined. Our own research team has focused on the use of bananas. This crop has three desirable attributes: bananas are grown in almost all tropical and subtropical developing countries throughout the world; they can be eaten uncooked (which avoids denaturation of subunit proteins during cooking); and they are a food that is widely consumed by infants and children. We have developed a methodology for the genetic transformation of bananas and are currently cloning genetic regulatory elements that we believe will cause the production of the vaccine proteins in the developing fruit. Bananas have a downside that is a technical limitation for research. The time from genetic transformation until harvest and evaluation of the fruit is long—at least two years. Thus our first transgenic banana plants containing genes encoding candidate vaccines are still in the laboratory stage and have not borne fruit, literally or figuratively.

Plant-based vaccines will not be available tomorrow, but the efforts of researchers from fields as diverse as agronomy and medicine make it possible to believe that the image of the child being vaccinated as she eats a banana is not so far-fetched.

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