Transgenic Plants for Mucosal Vaccines

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Modern biotechnology provides a huge potential for human health improvement by the development of new therapies and vaccines against infectious diseases. In the best cases, vaccines can provide great economic value with cost-benefit ratios of 1:10 (Centers for Disease Control and Prevention, 1998). However, the development and implementation of new vaccines often encounter substantial cost and logistical barriers that are difficult to overcome in many countries that are economically depressed. The use of transgenic plant-derived recombinant proteins is a promising strategy that combines the innovations in medical science and plant biology to create affordable vaccines. The potential benefits are low cost, heat stability, oral administration, and convenience of production in developing countries. A growing number of laboratories are investing in plant-derived protein pharmaceuticals, expanding on the seminal works that first proposed this idea (Curtis and Cardineau, 1990; Mason *et al.*, 1992). The subject of plant-derived vaccines has been reviewed several times in recent years (Koprowski and Yusibov, 2001; Mason *et al.*, 2002), and a nearly comprehensive list of vaccine antigens produced in plants has been provided (Daniell *et al.*, 2001). While much research is yet needed to optimize plant production of vaccines and to validate them in large-scale clinical trials, the results to date show a very promising technology that is on the brink of commercial development. Ingestion of transgenic plants expressing vaccine antigens can, via the gut lymphoid system, result in specific mucosal secretory IgA (S-IgA) and serum IgG antibody responses. Although protective efficacy of a plant-derived vaccine has yet to be determined in humans, some challenge studies in animals have shown promising results and are discussed later in the text. The previous chapter in this series (Palmer *et al.*, 1999) described plant expression systems and their use in vaccine studies in some detail. In this chapter, we examine the advances in the field of transgenic plant-derived mucosal vaccines, focusing on the results of human clinical trials and on orally delivered animal vaccines. Another chapter in this volume (Lomonossoff, G.P.; see Chapter 59) describes work involving plant viruses to produce vaccines.

PLANT-DERIVED VACCINES FOR HUMANS: CLINICAL TRIALS

Bacterial diarrhea: enterotoxigenic *Escherichia coli* (ETEC) and *Vibrio cholerae*

Diarrhea caused by bacterial and viral infections can cause severe dehydration, which is particularly dangerous in children and the elderly. Especially in developing countries, infectious diarrheal diseases are major causes of morbidity and mortality. It is estimated that diarrhea causes up to 2.5 million deaths yearly, mostly in the age group of less than 1 year. Several different bacteria can cause acute gastroenteritis by colonizing the gastrointestinal (GI) tract via contaminated water or food. Some of these are capable of systemic infection by crossing the mucosal epithelium (septicemic forms); others colonize the intestinal tract and secrete toxins that are absorbed by the enterocytes and cause diarrhea (enterotoxigenic forms). Two of the most widely spread and well studied of the latter are enterotoxigenic *E. coli* (ETEC) and *Vibrio cholerae*. The toxins they produce, labile toxins (LTs) and cholera toxins (CTs), respectively, are very similar in primary sequence, structure, and mechanism of action (Sixma *et al.*, 1991). The ring-shaped pentamer formed by the five identical noncovalently linked B subunits is responsible for binding to the G_{M1} ganglioside (both CTs and LTs) and to other gangliosides (LTs only) on the mucosal epithelial cells, while the toxic A subunit is translocated into the epithelial cells of the GI tract, where its ADP-ribosyl transferase activity promotes the efflux of water and electrolytes. Both LT-B and CT-B are among the most potent oral immunogens known, with oral delivery efficiently causing accumulation of specific serum (IgG, IgA) and mucosal S-IgA antibodies (Holmgren *et al.*, 1993). Both LT and CT also function as mucosal adjuvants, stimulating antibody production

against codelivered antigens. The ganglioside-binding activity of the LT-B pentamer is required both for its mucosal immunogenicity and for the adjuvanticity of the holotoxin (Guidry *et al.,* 1997).

Plant systems have been used to express both LT-B and CT-B. For example, others reported the expression of ganglioside-binding pentameric LT-B in transgenic potato and tobacco plants (Haq *et al.,* 1995). Mice fed transgenic tubers showed production of serum and mucosal antibodies against LT-B. Later, a plant-optimized LT-B gene was synthesized with preferred codons and avoidance of spurious mRNA processing signals, ultimately resulting in increased expression in potatoes (Mason *et al.,* 1998). Mice were fed 5 g of these transgenic tubers, containing 50 µg of LT-B, at 4-day intervals over 3 weeks, and the titers of anti-LT-B serum IgG and fecal IgA antibodies were improved over those in the earlier experiment. These mice were partially protected from a 25-µg dose of LT, indicating the potential for efficacious use of the potato vaccine in humans. In a similar study, others found that LT-B expressed in corn and eaten as raw corn meal stimulated antibody responses in mice that were partially protective against toxin challenge (Streatfield *et al.,* 2001).

The preclinical studies with potatoes expressing LT-B led to the first trial of a transgenic plant-derived vaccine administered to humans (Tacket *et al.,* 1998). Fourteen volunteers ingested either 100 g of transgenic potato, 50 g of transgenic potato, or 50 g of nontransgenic potato. The LT-B content of the tubers varied between 3.7 and 15.7 µg per gram of tuber weight, and the doses were given on days 0, 7, and 21. Volunteers reported only a few instances of minor side effects (nausea, cramps, or diarrhea), and the raw potato was well-tolerated overall. Ten of 11 volunteers who ate the potatoes expressing LT-B (and none who ate placebo potatoes) developed at least fourfold increases in levels of toxin-neutralizing serum IgG antibodies against LT-B. Five of 10 volunteers showed at least fourfold rises in anti-LT-B IgA detected in stool samples. These data compared favorably with those from an earlier study in which volunteers were challenged with 10^9 ETEC cells (Tacket *et al.,* unpublished). This study was significant as the first ever to examine an edible plant vaccine in humans and showed great potential for this new strategy.

The CT-B molecule has also been expressed in transgenic potato tubers at up to 0.3% of TSP (Arakawa *et al.,*1997). The plant-produced CT-B assembled into the pentameric structure was shown to bind G_{M1} ganglioside and to react with specific anti-CT-B IgG antibody. Mice immunized by being fed raw potato tubers expressing CT-B developed specific serum and mucosal antibodies (Arakawa *et al.,* 1998). It is interesting that in previously immunized mice, the edible CT-B was able to trigger a significant boosting response. Moreover, the CT-B produced in plants was heat-resistant: transgenic potato tubers cooked until soft retained 50% of the ganglioside-binding activity.

Viral diarrhea: Norwalk virus

The Norwalk virus and related Norwalk-like viruses are responsible for 42% of outbreaks of acute epidemic gas-

troenteritis in the United States. The Norwalk virus capsid protein (NVCP) was the antigen chosen to develop an oral edible vaccine, since when expressed in insect cells it assembled into 38-nm Norwalk virus–like particles (VLPs) and reacted with serum of infected humans (Jiang *et al.,* 1992). Tobacco and potato plants were transformed with constructs harboring the NVCP sequence; the plant recombinant protein assembled into VLPs identical to the insect cell–derived antigen (Mason *et al.,* 1996). Mice that were gavaged with partially purified VLPs from tobacco leaf or fed with transgenic tubers developed serum IgG and fecal IgA antibodies specific for NVCP.

A clinical trial was performed with the same potatoes used for the preclinical study (Tacket *et al.,* 2000). Of 20 adult volunteers, 10 received two doses (days 0 and 7) and 10 received three doses (days 0, 7, and 21) of 150 g of raw transgenic potato tubers containing NVCP at 215 to 750 µg/dose. It is important to note that tuber expression was quite variable, and at most only half of NVCP in these potatoes was assembled as VLP; thus, the effective dose of potato vaccine was ~325 µg/dose. Unassembled subunits are likely to be much less stable in the GI tract and thus less immunogenic. However, 19 of 20 subjects in the experimental group showed significant increases in the numbers of IgA antibody–forming cells (AFCs), ranging from 6 to 280 per 10^6 peripheral blood mononuclear cells (PBMCs), and 6 of 20 subjects in this group developed increases in IgG AFCs. Four volunteers showed increases in serum IgG anti-NVCP antibody titers, 4 had increased serum IgM, and 6 showed increased IgA in their stool samples (17-fold mean increase). Although the antibody responses were less impressive than those obtained with LT-B, the study showed that a plant-derived protein other than LT-B and CT-B can stimulate human immune responses after oral delivery. Insect cell–derived 250-µg doses of purified Norwalk VLP provided more effective seroconversion (Ball *et al.,* 1999); thus it is likely that part of the potato-delivered NVCP was unavailable for uptake in the GI tract. More recent studies in transgenic tomato fruits with a plant-optimized NVCP gene resulted in higher expression and more potent immune responses in mice fed freeze-dried tomatoes (X. Zhang and H.S. Mason, unpublished results). A clinical trial is planned in which dried tomato powder formulated in gelatin capsules will be used to evaluate safety and immunogenicity (D. Kirk, H.S. Mason, and C.J. Arntzen, trial investigators).

Hepatitis B virus

The World Health Organization (WHO) has estimated that there are approximately 350 million chronic carriers of hepatitis B virus (HBV). Infection with HBV leads to liver cancer resulting in as many as 1 million deaths annually. Transmission of HBV is primarily through blood and/or sexual contact, but there is also a high incidence of mother-child transmission during childbirth. Thus a vaccine that provides mucosal immune protection in addition to serum antibodies could be an improvement over the currently used intramuscularly injected hepatitis B surface antigen (HBsAg), pro-

duced in transgenic yeast cells (McAleer *et al.*, 1984). Two types of hepatitis B vaccine are licensed by the U.S. Food and Drug Administration (FDA) for human use, both purified for intramuscular injection. The first is based on HBsAg derived from human plasma; the second is a recombinant yeast-derived HBsAg. Beyond the potential for mucosal immunity, an orally administered plant-derived vaccine would be useful for several reasons: it could improve the success rate of full immunization because the vaccine could be taken without the requirement of returning to a facility that can provide injections; it would be heat stable and could thus reach children in more remote areas; and it would not require the use of needles that pose the risk of infection themselves. However, in light of the increasingly global use of yeast-derived hepatitis B vaccine in both developed and developing countries, any plant-derived vaccine would have to offer distinct advantages and not interfere with efforts to expand vaccination.

The report of expression of HBsAg in tobacco leaf was the first description of plant expression of vaccine immunogen (Mason *et al.*, 1992). The recombinant tobacco HBsAg was recovered as spherical particles of an average diameter of 22 nm, similar to the native subviral particle from blood of infected humans and similar to the yeast-derived vaccine (McAleer *et al.*, 1984). The tobacco-derived HBsAg stimulated immune responses in mice vaccinated intraperitoneally that were similar to those produced by the commercial vaccine, including production of all IgG subclasses and IgM (Thanavala *et al.*, 1995). In addition, T cells from the mice immunized with the plant-derived HBsAg proliferated in response to a yeast-derived HBsAg, indicating the fidelity of the T-cell epitopes on the plant-produced protein. Studies that followed aimed to improve the amount of antigen produced by the plant system by optimizing different constructs (Richter *et al.*, 2000; Sojikul *et al.*, 2003), as well as proving that orally delivered plant material could elicit an immune response (Kong *et al.*, 2001). A construct containing the unmodified HBsAg driven by the constitutive CaMV 35S promoter and terminated by the potato PinII polyadenylation signal yielded the highest expression of HBsAg in potato tubers (Richter *et al.*, 2000). Immuno-electron microscopy of sectioned potato leaf showed that spherical and filamentous subviral particles assembled *in vivo* and accumulated in endoplasmic reticulum (ER)-derived vesicles. Mice fed once a week for 3 weeks with 5 g of raw tubers (containing an average of 42 μg of HBsAg/dose) were compared with mice that were gavaged with the purified yeast recombinant HBsAg (Kong *et al.*, 2001). In either case, 10 μg CT per dose was given as a mucosal adjuvant. The potato-delivered HBsAg was a more potent immunogen when delivered orally: a primary serum IgG antibody response began after two feedings of the transgenic potatoes (containing a total of 84 μg of HBsAg), whereas no primary immune response was detected after two doses of yeast-derived HBsAg (containing a total of 300 μg of HBsAg). The authors suggested that the difference was due to enhanced stability of the antigen when it is retained in the plant cells. Serum IgG antibody levels in the potato-primed mice declined after a few weeks but rebounded dramatically with high and lasting titers after a single intraperitoneal injection with a subimmunogenic dose of yeast recombinant vaccine. Furthermore, mice primed with a subimmunogenic intraperitoneal dose of the commercial vaccine and fed with HBsAg potatoes responded with a long-lasting secondary antibody response. The antibody titer decreased with time but remained above the estimated protection level (10 IU/L) up to 5 months after the challenge, when the experiment was terminated (Kong *et al.*, 2001). This result suggests that priming with a single injection followed by additional doses of orally delivered plant-derived vaccine could be used in humans. A change in immunization strategies would be especially useful in developing countries, where the injection delivery of multiple doses of vaccine is a significant problem for massive immunization.

There is one published report on the immunogenicity in humans of orally delivered HBsAg expressed in plants (Kapusta *et al.*, 1999). Two of three volunteers who ate two 150-g doses of transgenic lettuce (containing ~1 to 2 μg HBsAg per dose) developed a modest but protective (>10 IU/L) serum antibody titer after the second dose. The serum antibody titers declined rapidly after 4 weeks, probably because of the very low dosage; however, the study showed that presumably naïve subjects could be seroconverted by oral delivery of plant-expressed HBsAg. In the United States, others directed a clinical trial at the Roswell Park Cancer Institute (Buffalo, NY) to test the oral immunogenicity of recombinant potatoes expressing HBsAg (Kong *et al.*, 2001). Since chronic HBV infection can lead to liver cancer, the FDA was concerned that oral delivery of HBsAg could cause oral tolerance. Thus, the study was limited to volunteers who had previously been vaccinated and seroconverted with the standard injectable HBsAg. The experiment involved 33 volunteers who ate either two (days 0 and 28) or three (days 0, 14, and 28) 100-g doses of HBsAg potato tubers containing ~1 mg HBsAg per dose, as measured by the Auszyme monoclonal immunoassay (Abbott Laboratories, Abbott Park, IL). A group of 10 volunteers ate nontransgenic potatoes only. The potato HBsAg vaccine stimulated boosting of serum IgG antibody titers in more than half of the volunteers (Thanavala *et al.*, unpublished results, 2003), which suggests that oral ingestion of plant-produced HBsAg could be a viable delivery system for an HBV vaccine.

Rotavirus vaccines currently under study

Rotavirus is a common form of diarrhea and is estimated to cause approximately 600,000 deaths annually, primarily among children in developing countries. It is the most frequent cause of childhood diarrhea-related hospitalizations in the United States. Group A rotavirus (GAR) is one of the leading causes of acute infectious viral diarrhea in humans and livestock. The rotavirus major inner capsid protein (VP6) has been defined as a protective antigen (Choi *et al.*, 1999), and VP6 constitutes about 51% by weight of the virion protein and is well conserved in each virus serogroup (Kapikian *et al.*, 1990). In addition to the capsid proteins, a

nonstructural protein, NSP4, and its 22–amino acid peptide, which functions as a viral enterotoxin, were also identified (Ball *et al.*, 1996). Induction of antibodies against the NSP4 22 amino acid peptide alone might confer protection from clinical disease, without the need for induction of antibodies against the structural proteins (O'Neal *et al.*, 1997). Others have shown that VP6 elicits protection against rotavirus shedding in a mouse model, and this protection was attributed to a CD4+ T cell epitope in the protein. These attributes indicate that the VP6 protein has potential as a candidate subunit vaccine against rotavirus (Choi *et al.*, 1999).

Others have reported that expression of VP6 from bovine group A rotavirus in transgenic potato plants occurs at levels in leaf and tuber tissues of 0.006% and 0.002% of TSP, respectively (Matsumura *et al.*, 2002). Mice were vaccinated intraperitoneally with concentrated potato tuber extracts estimated to contain 750 ng of VP6, which resulted in anti-GAR antibodies detected by Western blotting. Expression of VP6 in potato tubers was boosted by using two tandem repeats of the 5′-upstream sequence of the CaMV 35S promoter and the 5′ untranslated region of tobacco mosaic virus, which yielded 0.1% VP6 of TSP in tubers, a 17-fold increase. Subsequent experiments will test oral immunization with tuber-produced VP6.

Adopting a different approach, others used a fusion protein approach with the NSP4 22–amino acid peptide and subunits of the cholera toxin (Yu and Langridge, 2001). This group reported the expression in potato tuber of a recombinant holotoxin in which the 22–amino acid immunodominant murine rotavirus enterotoxin NSP4 to the C-terminus of CT-B and an ETEC bacterial fimbrial protein (CF1) fused to the N-terminus of the A subunit. The recombinant A and B particles assemble into a holotoxin-like molecule. CD-1 mice orally immunized with five doses of tuber tissues containing 10 μg of fusion protein per dose showed serum and mucosal antibodies to NSP4, as well as high levels of IL-2 and IFN-γ, indicating a Th1-type immune response. Offspring born to dams orally immunized with CT-B-NSP4 fusion proteins were partially protected from challenge from live murine rotavirus. The success of this multiple fusion protein strategy shows the possibility of a plant-based multi-component vaccine against several diarrheal pathogens.

PLANT-DERIVED VACCINES FOR ANIMALS

Swine transmissible gastroenteritis virus

Swine transmissible gastroenteritis (TGE) is an economically important herd health problem globally. It is a highly contagious viral diarrheal disease in neonatal pigs that can result in up to 100% mortality among severe cases (Saif and Wesley, 1992; see Chapter 61). Because of the rapid course of disease and the immaturity of their immune systems, newborn piglets are unable to develop their own protective immunity and depend upon the presence of S-IgA in mother's milk for protection against the virus. To date,

commercially available vaccines, either attenuated or inactivated, are unable to adequately protect piglets (Tuboly *et al.*, 2000). Swine TGE is caused by the transmissible gastroenteritis virus (TGEV), a coronavirus with an immunodominant spike (S) protein. The S protein can induce neutralizing antibodies against the virus, and protective immunity in the host is directed toward this protein, which contains four major antigenic sites in its N-terminal region (Jiminez *et al.*, 1986). The S protein has been expressed in many systems, including baculovirus (Tuboly *et al.*, 1994) and human adenovirus (Torres *et al.*, 1996).

Others have reported the expression of the TGEV S protein in transgenic *Arabidopsis thaliana*, a convenient plant model system (Gomez *et al.*, 1998). The level of S protein obtained in leaves was between 0.03% and 0.06% of TSP. Immunization of mice intramuscularly with crude plant extracts induced TGEV-specific antibodies, with a virus-neutralizing index of 2.2 to 3.5, in comparison with an index of 5 for rabbit sera, with anti-TGEV antibodies used as a positive control. The S protein has been expressed in tobacco leaves at 0.1% to 0.2% of TSP (Tuboly *et al.*, 2000). Transgenic leaf extracts given intraperitoneally to pigs stimulated TGEV-specific serum antibodies with low levels of virus-neutralizing activity. In this study, the N-terminal domain of the S protein was codon-optimized for tobacco cells, which resulted in expression levels that were 10 times higher than had been obtained previously (Gomez *et al.*, 1998). No data on mucosal immunization were described in either study report; however, the results indicate that the plant-expressed S protein presents virus-neutralizing epitopes and is thus a competent immunogen.

Perhaps the most promising study showing protection with a mucosally delivered plant-based vaccine involved TGEV S protein expressed in corn (Streatfield *et al.*, 2001; Lamphear *et al.*, 2002). This group reported on the oral immunization of 10-day-old, specific-pathogen-free, TGEV-seronegative piglets with transgenic corn meal expressing the S protein. Test animals were fed nontransgenic corn mixed with 50 g of transgenic corn containing 2 mg of the S protein in a medicated milk replacer daily over a 10-day period, and control groups were orally immunized with either a commercial modified live vaccine or nontransgenic corn. All piglets were then orally challenged with a virulent form of the virus, and clinical symptoms were evaluated. While 50% of the pigs vaccinated with transgenic corn developed diarrhea, 78% in the group immunized by the commercially available vaccine and 100% of those that were fed with nontransgenic corn became ill. These observations suggest that the corn-derived S protein generated an immune response adequate to confer partial protection from the virus. No specific antibody assays were reported for this study; however, animals vaccinated with the commercial vaccine appeared to recover much faster, an observation suggesting that the commercial vaccine generated a stronger cytotoxic T-cell (CTL) response that was boosted by infection. In subsequent experiments (Lamphear *et al.*, 2002), piglets orally immunized with the corn-expressed S protein had a memory immune response

leading to rapid accumulation of virus-neutralizing antibodies when orally immunized piglets were exposed to enough TGEV to cause subclinical infection. A study of different dosage regimens (4, 8, or 16 consecutive days) with S protein corn or with the commercial vaccine showed animals on the S protein corn 4-day regimen had no morbidity, while 50% of animals fed nontransgenic corn displayed symptoms. The animals fed the transgenic corn on the 8- and 16-day dosage regimens showed 20% and 36% morbidity, respectively, indicating that the 4-day dosage regimen was more effective in protecting the pigs. The frequency of dosage is therefore critical for this vaccine. In the group immunized with the commercial vaccine the morbidity rate was 9%, in comparison with zero for the corn-derived-vaccine recipients on the 4-day dosage regimen, a result which suggests that the corn-derived S protein is marginally more effective. However, this work should be explored further with virus challenge titers that are relevant in the field, rather than just enough to cause subclinical infection.

These efforts indicate the feasibility of producing a plant-based TGEV vaccine in transgenic plants. The work on the corn-derived S protein is especially interesting in that corn is a main constituent of livestock diets and does not require extensive heating during processing. Furthermore, the stability of antigen in the corn kernel matrix has been established, and a dosage size of 2 mg of S protein in 50 g of corn meal (Streatfield *et al.,* 2001) indicates reasonable expression levels were attained for this antigen in maize.

Foot-and-mouth disease
Foot-and-mouth disease virus (FMDV) is considered the most economically important veterinary pathogen because of its highly infectious nature, ability to cause persistent infections, and long-term effects on the condition and productivity of the many animal species it affects. Countries where FMDV infections are active face many trade restrictions (Knowles and Samuels, 2003). Once an outbreak of the disease occurs, formal quarantine approaches are generally employed to contain the disease. Vaccination of all susceptible hosts with inactivated virus is a viable strategy for control of the disease, but is not used in most countries because of the expense or the difficulty in distinguishing immunized from infected animals. FMD is caused by several strains of virus that belong to the genus *Aphthovirus*, of the Picornaviridae family. Critical epitopes resulting in the induction of neutralizing antibodies have been identified on the VP1 structural protein of FMDV (Brown, 1992).

One group reported the expression of the FMDV VP1 in transgenic *A. thaliana* plants (Carillo *et al.,* 1998). In this study, BALB/c mice were immunized intraperitoneally with plant extracts in incomplete Freund's adjuvant, which stimulated serum antibodies that reacted strongly with intact FMDV particles. The mice were challenged intraperitoneally with a virulent virus and were protected, as determined by absence of viremia 48 hours postinoculation, while the control groups were not protected. Others reported the expression of VP1 protein in transgenic alfalfa; in this study, mice

fed 0.3 g of transgenic leaves on a fortnightly basis in three doses of 15 to 20 mg of total soluble protein each developed virus-neutralizing antibodies, and 66% to 75% of animals were protected from intraperitoneal challenge (Wigdorovitz *et al.,* 1999).

A common problem in all FMDV studies has been the low level of expression of the VP1 protein, resulting in limited immunogenicity. For example, one group developed a methodology for rapidly screening transgene expression levels in large numbers of transgenic plants from independent transgenic events (Dus Santos *et al.,* 2002). This methodology involved the fusion of FMDV peptides to the readily assayable *gus* reporter gene and yielded a high correlation between expression of GUS and a VP135–160 epitope to which it was fused (between 0.05% and 0.1% of TSP). Mice immunized intraperitoneally with crude extracts from selected transgenic plants readily developed strong and completely protective immune responses. At the observed levels of expression, this peptide vaccine resulted in higher antibody titers than those previously reported when the full VP1 protein was expressed in alfalfa (Carillo *et al.,* 1998).

Rabies
Rabies is a viral disease of mammals and is most commonly transmitted through the bite of a rabid animal. This disease is unusual in that it affects humans, wildlife, and domestic animals and is readily transmitted between animals and humans. In the United States, most rabies cases reported to the Centers for Disease Control and Prevention each year occur in wild animals such as raccoons, skunks, bats, and foxes, and a few (less than 10%) have been reported to occur in domestic animals such as cats, cattle, and dogs. However, rabies remains a major threat to humans in the world today, with about 50,000 fatal cases reported by the WHO each year (Plotkin, 2000). Globally, an estimated 10 million people receive postexposure antiserum treatment each year, after being exposed to rabies-suspect animals (WHO Fact Sheet number 99, 2001). Modern vaccines for humans are expensive, especially for densely populated countries in Africa and Asia, where rabies is endemic and remains a major health problem. For example, it has been reported that in India, most individuals exposed to rabies receive a primitive brain tissue vaccine because the safer and more efficacious modern products are unaffordable (Koprowski and Yusibov, 2001).

The rabies virus infects the central nervous system, causing encephalopathy and inevitable death. The rabies virus glycoprotein (G protein) is the major viral protein responsible for the induction of a protective immune response, while the nucleoprotein (N protein) triggers T cell responses, facilitating the production of neutralizing antibodies and other immune functions (Tollis *et al.,* 1991). Oral vaccination with the baculovirus-expressed G protein was shown to protect raccoons from lethal challenge with the virus, and boosting with the N protein enhanced the protective immune response (Fu *et al.,* 1993). A plant-based edible rabies vaccine that is very low in cost and orally administered would be very useful in preexposure vaccination of children and older

persons in developing countries where rabies is highly endemic.

It has been reported that the production in transgenic tomato fruit of the rabies virus G protein can be estimated at 0.001% of TSP (McGarvey *et al.*, 1995). However no immunization studies were described with this protein. This group observed that the tomato-synthesized G protein was smaller than the mammalian protein and yet larger than the predicted size for the unglycosylated protein, indicating that the plant glycosylation occurred, but perhaps the absence of sialic acid in plant glycans results in the observed smaller molecule size. Chimeric plant viruses were used to express immunogenic rabies virus G protein B-cell epitope and a T-cell epitope from the N protein (Yusibov *et al.,* 1997). This work suggests that optimized transgenic plant expression could produce a viable rabies vaccine.

Poultry vaccines

The poultry industry is a significant component of global agriculture, and its success depends on the ability to maintain healthy birds. Vaccination is one of the key strategies for disease management, and in many cases, birds are immunized with attenuated live vaccines. The use of edible vaccines for the poultry industry should result in significant cost savings by greatly simplifying the current vaccination strategies and avoiding the practice of immunization via live vaccines. Infectious bursal disease virus (IBDV) is a globally important poultry pathogen. The major antigen in this virus is the structural protein VP2.

One group is currently working on a USDA-funded project for the production of a recombinant VP2 protein in transgenic plants (Scissum-Gunn [Alabama State University] and colleagues). In this work, oral immunization and boosting of 3-week-old unvaccinated chicks, challenge with IBDV, and serum and bursal tissue examinations were planned. Preliminary results following oral vaccination with recombinant VP2 expressed in transgenic *A. thaliana* were encouraging, and future expression in alfalfa is planned (K. Scissum-Gunn, personal communication, 2003).

SUMMARY AND CONCLUSIONS

Based upon the results presented in this chapter, it is clear that plant-based vaccines hold great promise and will likely form an integral part of the future arsenal of antigen production systems for vaccination. Transgenic plant-derived vaccine antigens have been shown to be orally immunogenic in humans and several animals species, and partial protection from pathogen challenge was shown for TGEV and FMDV in mice. Several issues remain to be addressed, including potential risk factors associated with the production of these pharmaceuticals in transgenic plants. First, progress needs to be made in improving the yield of antigens, in ensuring uniformity of yield, in enhancing immunogenicity of orally administered vaccines, and in assessing the potential for development of immune tolerance. Second, there are several

regulatory issues. For materials produced in the field, interaction with biotic and abiotic factors in the environment could affect expression stability and potentially compromise the integrity of the product. Thus, it is important to ensure sufficient analysis of the plant-derived product to validate its uniformity and functionality. Potential risk to the environment and the associated public perception of risk must be carefully considered. To that end, the USDA recently proposed strict measures to minimize environmental risks, including physical containment and rigorous regulation of transportation, processing, and disposal of plant-made pharmaceuticals. Biological containment practices to minimize unintentional release include the use of male sterile lines to minimize transgene escape via pollen and tissue or organ-specific expression of recombinant protein. The choice of crop for production of vaccines has a direct bearing on the ability to contain transgenes, with pollen-shedding crops presenting a greater challenge than those like tomatoes or other self-pollinating species. Strategies for monitoring genes within the environment should be employed, such as use of dominant marker genes, high-resolution detection systems, and efficient production, processing, and monitoring systems.

Finally, active participation of the plant biotechnology industry is an important step in the development of plant-based vaccines. Dow AgroSciences, LLC (DAS; Indianapolis, IN) has invested substantial effort toward the development of plant-derived vaccines for animals (Butch Mercer, DAS, personal communication). Results of DAS-supported studies are encouraging and may lead to commercialization of this exciting new technology.

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