Plant-Derived Antigens as Mucosal Vaccines

H. S. Mason and M. M. Herbst-Kralovetz

Abstract During the last two decades, researchers have developed robust systems for recombinant subunit vaccine production in plants. Stably and transiently transformed plants have particular advantages that enable immunization of humans and animals via mucosal delivery. The initial goal to immunize orally by ingestion of plant-derived antigens has proven difficult to attain, although many studies have demonstrated antibody production in both humans and animals, and in a few cases, protection against pathogen challenge. Substantial hurdles for this strategy are low-antigen content in crudely processed plant material and limited antigen stability in the gut. An alternative is intranasal delivery of purified plant-derived antigens expressed with robust viral vectors, especially virus-like particles. The use of pattern recognition receptor agonists as adjuvants for mucosal delivery of plant-derived antigens can substantially enhance serum and mucosal antibody responses. In this chapter, we briefly review the methods for recombinant protein expression in plants, and describe progress with human and animal vaccines that use mucosal delivery routes. We do not attempt to compile a comprehensive list, but focus on studies that progressed to clinical trials or those that showed strong indications of efficacy in animals. Finally, we discuss some regulatory concerns regarding plant-based vaccines.

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Contents

1 Introduction

Advances in plant biotechnology in the last two decades have facilitated production of recombinant proteins in plants, including subunit vaccine antigens. Several good review articles (Rybicki 2009; Streatfield 2006; Thanavala et al. 2006; Yusibov and Rabindran 2008) have summarized much of this work and have focused attention on the utility of plants for subunit vaccine expression. During the twenty years since the seminal work that demonstrated the potential of plant-derived vaccines (Curtiss and Cardineau 1990; Mason et al. 1992), a large body of literature has demonstrated expression of antigens in many plants, including edible plants like corn, potato, and tomato, as well as tobacco and its relatives. And yet, commercial development of plant-derived vaccine products has been very slow, for a variety of reasons, as discussed previously (Rybicki 2009). One of the biggest obstacles is the reluctance of the pharmaceutical industry to invest in a new technology when the existing seems adequate. However, a steady accumulation of promising data has shown that plant-based expression has great potential, especially if a few hurdles can be overcome.

The original and oft-touted goal for plant-derived vaccines was to provide cheap and conveniently delivered oral recombinant subunit vaccines to underdeveloped countries where poor economic conditions limit the application of modern medical science. The hypothesis was that ingestion of edible plant material, either

raw or minimally processed, could induce protective immune responses via activation of immune effectors in gut-associated lymphoid tissue (GALT) (Streatfield 2006; Thanavala et al. 2006). Oral delivery of bacterial antigens has generated protective immunity against enteric pathogens. However, there is scarce evidence that protection against respiratory or urogenital tract infectious disease can be achieved using the oral vaccine delivery route.

Digestive acids and proteases that degrade proteins for nutrient absorption are a substantial problem for oral vaccine development. Antigens must be relatively resistant to gut proteases, and typically very large antigen doses be administered. Encapsulation might better protect immunogens and their delivery into GALT (Streatfield 2006). However, little work in this area has been done with plant-derived antigens.

Another problem for edible vaccines is that accumulation of antigens in transgenic plant tissues is usually rather low ($\leq$1% of total soluble protein, TSP), creating a requirement for processing of plant material to concentrate the antigen. Moreover, the inherent plant-to-plant variability in antigen content requires processing of plant material to produce a well-characterized batch of antigen that can be administered in controlled dosages. Thus, plant-derived oral vaccine immunogens will require substantial purification and concentration, and probable delivery in capsules that protect them from proteolytic degradation in the small intestine.

Recently, plant-based recombinant protein production has been revolutionized by transient expression of antigens in plants using viral vectors (Gleba et al. 2007; Yusibov et al. 2006). The plant host is usually the tobacco relative *Nicotiana benthamiana* (Goodin et al. 2008), which is remarkably permissive to many plant viruses. Expression levels obtained with viral vectors in *N. benthamiana* leaves are variable depending on the protein, but are generally ten-fold greater than in stable transformants of the nuclear genome, and frequently in the range of 1–2 mg/kg of leaf mass. Thus, the prospect of plant-based expression and purification of vaccine antigens, especially virus-like particles (VLP) (Huang et al. 2009), for mucosal delivery is substantially brighter than that obtained using stably integrated transgenes. Moreover, the high yields of recombinant antigens that can be obtained using viral vectors may facilitate intranasal delivery, which requires smaller volumes and hence, more concentrated vaccine solutions than the oral delivery route.

An ideal mucosal vaccine would induce both antibody- and cell-mediated protection, not only at the relevant mucosal site, but also throughout the body. The most convenient means to achieve mucosal immunity in global health programs is oral delivery. Oral vaccination eliminates the possibility of transmission of other infectious diseases by contaminated needles, as well as elimination of pain associated with injections and the need for trained personnel to deliver the vaccines (Holmgren and Czerkinsky 2005; Lavelle 2005). However, nasal vaccines are not hampered by the physical and chemical barriers of the gut. Nasal vaccination has demonstrated particular potential with regard to induction of broadly disseminated immunity (Neutra and Kozlowski 2006; Staats et al. 1997). In humans, monkeys, and mice, nasal immunization induced antigen-specific mucosal IgA responses in

salivary glands, upper and lower respiratory tracts, small and large intestines, and most notably male and female reproductive tracts (Harandi et al. 2003; Imaoka et al. 1998; Kozlowski et al. 2002; Rudin et al. 1999; Staats et al. 1997). In addition, the nasal route of immunization can induce cytotoxic T lymphocytes (CTL) in distant mucosal tissues including the female reproductive tract (Gallichan and Rosenthal 1998). In both humans and mice, nasal immunization has produced greater systemic antibody responses than other mucosal immunization routes (Kozlowski et al. 1997, 2002; Staats et al. 1997). Kunkel and Butcher (2002) provided evidence from naïve human vaccine recipients that mucosal immunization can prime the immune system for both mucosal and systemic responses by inducing the expression of both mucosal and systemic homing receptors in responding lymphocytes. Thus, delivery of subunit antigens or VLP via the nasal route has excellent prospects as a vaccine strategy. For further reading on VLP vaccines, readers are directed to "Recent Advances in Microparticle and Nanoparticle Delivery Vehicles for Mucosal Vaccination" of this volume.

2 Plant Expression Systems

The strategies used for recombinant protein expression in plants are conceptually similar to those used for mammalian, yeast, or other eukaryotic hosts (Rybicki 2009; Thanavala et al. 2006; Yusibov and Rabindran 2008). They include stably integrated transgenes in the nuclear or chloroplast genomes, and transient expression using vectors that are either non-replicating or that utilize plant virus replication elements to amplify the mRNA for the target gene. Nuclear genes behave in a Mendelian fashion, and utilize the typical eukaryotic pathways of protein translation, processing, and subcellular localization. Organ- and development stage-specific promoters can be utilized, such that foreign proteins can be directed to accumulate in seeds (Nochi et al. 2007; Streatfield et al. 2003; Wu et al. 2007). Expression of antigens in seeds has a particular advantage in regard to protein stability due to drying of the storage tissue during seed development. Thus, seeds can be stored at ambient temperatures for months to years with little loss of protein activity.

Although site-specific recombination of plant nuclear genomes has been studied intensively (Hanin and Paszkowski 2003), it is currently not routine and usually rather inefficient. In most cases, the recombinant construct is delivered as DNA using *Agrobacterium tumefaciens*, a plant pathogen that has the ability to transfer genes into the plant cell nucleus (Gelvin 2003). The foreign DNA may be integrated stably into the host nuclear chromosomal DNA by non-homologous recombination at random sites, although some work suggests that transcriptionally active sites may be preferentially targeted. The transferred DNA may also reside in the nucleus without integration for several days, and can thus act as a template for transcription to effect transient expression. Unless the foreign DNA is constructed as a viral replicon, it will remain in low-copy numbers, but can be used to evaluate expression of foreign antigens (Huang and Mason 2004).

Agrobacterium-mediated DNA transfer can also be used to deliver viral replicons. DNA viruses such as geminiviruses have been used to develop gene amplification systems (Huang et al. 2009). Genomes of RNA viruses such as tobacco mosaic virus (TMV) can be constructed as cDNA fused to a plant promoter, which is transcribed in the nucleus to produce viral RNA that then moves to the cytoplasm to establish replication (Gleba et al. 2007). A method called "Magnifection" was developed to allow whole-plant inoculation of *N. benthamiana* by vacuum infiltration with *Agrobacterium* lines containing recombinant TMV replicons that lack the coat protein, resulting in foreign gene expression in all leaves (Gleba et al. 2005). Recombinant viral genomes can also be delivered directly as RNA after in vitro transcription from a plasmid by simply abrading the leaf surface and applying a solution of RNA (Gleba et al. 2007; Yusibov et al. 2006). In this case, the replicon needs to contain viral elements that mediate long-distance transport in the plant vasculature so that expression is not restricted only to the site of inoculation.

Chloroplast transformation is routine in only a few laboratories, but can yield very high-expression levels of some antigens, due to the high gene copy numbers from many chloroplasts per cell and genome molecules per chloroplast (Arlen et al. 2008; Singh et al. 2009). The DNA construct is delivered by micro projectile bombardment (gene gun), and site-specific recombination allows targeted gene insertion. One great advantage of chloroplast transgenes is that they are not usually subject to gene silencing mediated by RNA interference, which is a common problem with nuclear transgenes. Another advantage is that the chloroplast genome is exclusively maternally inherited, making gene containment much easier because pollen grains are devoid of plastids. However, the chloroplast genetic system is a prokaryotic one; thus, some protein processing events, notably glycosylation, will not occur.

3 Plant-Derived Human Vaccines

3.1 Norovirus

Noroviruses are non-enveloped positive-sense RNA members of Caliciviridae, and are enteric pathogens that cause gastroenteritis. The capsid protein of Norwalk virus (NVCP) can be expressed in insect cells (Jiang et al. 1992) or plants (Mason et al. 1996), and self-assembles into VLP (rNV) that are orally immunogenic. A clinical study evaluated the antibody responses after ingestion of 2 or 3 doses of 150 g raw potato tubers expressing rNV (Tacket et al. 2000). Although 19 of 20 subjects who ingested the rNV expressing potato developed measurable increases in circulating antibody-secreting cells, the overall response was rather weak. The variable rNV content of the tubers (215–750 μg per dose) and relatively poor VLP assembly in the potato (effective rNV dose of $\leq$325 μg per dose) were likely

limiting factors. However, a detrimental effect of the potato vehicle, perhaps due to poor release of VLP in the gut lumen, cannot be discounted. A clinical trial using orally delivered purified insect cell-derived rNV (i-rNV) produced stronger antibody responses at 250 μg per dose than were obtained with potato (Tacket et al. 2003), suggesting that purified rNV is a more potent oral immunogen.

Later, rNV was expressed at higher levels in transgenic tomato fruit using a plant codon-optimized NVCP gene (Zhang et al. 2006). Oral immunogenicity in mice was excellent: 4 doses of 0.4 g freeze–dried tomato fruit containing 64 μg NVCP (40 μg rNV) induced anti-rNV serum IgG and fecal IgA in ≥80% of mice, and 0.8 g doses generated systemic and mucosal antibodies in all mice. It was also shown that air-dried tomato fruit was at least as active in mice as freeze–dried tomato, indicating that sophisticated drying technology is not required for rNV in tomato. Interestingly, rNV delivered in freeze–dried potato tuber was less orally immunogenic in mice, due to oxidation by phenolic compounds and polyphenol oxidase in potato (Zhang et al. 2006). Tomato is much better in this respect, having low-phenolic content, as well as a high level of ascorbic acid that can act as antioxidant. Thus, dried rNV tomato fruit would be worthy of study in humans.

In recent years, our group has been experimenting with viral vectors for transient expression of rNV in plants because of the potential for high-level expression and the rapidity of protein production (Gleba et al. 2005; Marillonnet et al. 2004). We used the Magnifection system to express NVCP in the tobacco relative *N. benthamiana* at levels of 800 mg/kg leaf tissue (Santi et al. 2008). Further, we developed a VLP purification process that did not require ultracentrifugation but instead utilized pH 5.3 precipitation of the major leaf proteins, followed by filtration through a 100 kDa membrane to remove unassembled NVCP subunits. In the absence of adjuvant, the tobacco-derived rNV (t-rNV) was orally immunogenic in mice at a 100 μg dose, generating systemic and mucosal anti-rNV antibodies (Santi et al. 2008). Inclusion of cholera toxin adjuvant with t-rNV substantially increased anti-rNV responses.

In Velasquez et al. 2010 studies with mice, we have found that nasal co-delivery of t-rNV and a Toll-like receptor (TLR) agonist can produce robust systemic and mucosal antigen-specific antibody responses. We undertook these experiments to evaluate the potential of these VLP to induce distal mucosal IgA responses with or without adjuvant. In unpublished studies we examined the TLR9 agonist, CpG-containing oligodeoxynucleotides (CpG-ODN) as adjuvant because it is known to trigger an immunostimulatory cascade that culminates in the maturation, differentiation, and proliferation of multiple cell types, including B cells, and CpG-ODN had been used previously in mice by the nasal route (Abe et al. 2006; Balmelli et al. 1998; McCluskie et al. 2000). We nasally immunized conscious female BALB/c mice with 25 μg purified t-rNV alone or t-rNV with 10 μg type B CpG-ODN. Nasal immunization of conscious mice with VLP has been shown to target antigen to the nasopharyngeal-associated lymphoid tissue (NALT) but not deeper respiratory tract tissues (BALT); whereas nasal immunization of sedated mice is more analogous to intratracheal administration

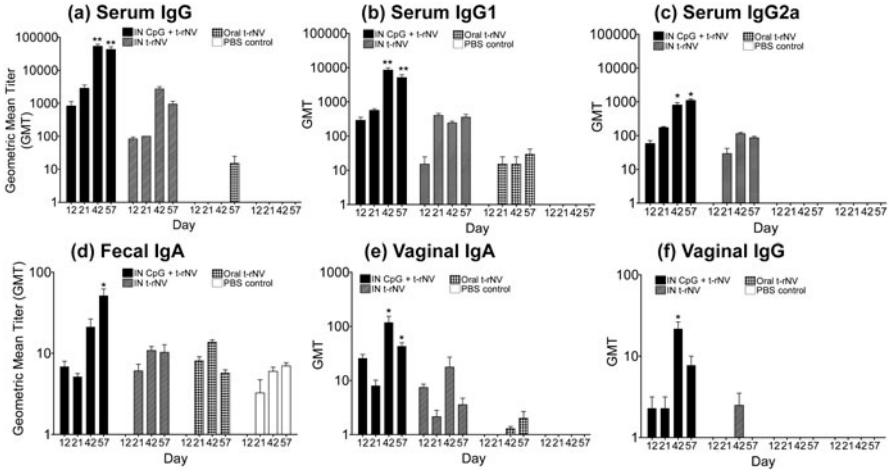

Fig. 1 TLR agonist significantly increases rNV-specific antibody production in mice immunized with tobacco-derived Norwalk virus-like particles (t-rNV). Conscious female BALB/c mice were immunized intranasally (IN) on days 0 and 21 with 25 μg t-rNV with or without 10 μg CpG ODN (10 μg), or orally (ORAL) on days, 0, 21, and 42 with 100 μg t-rNV or PBS vehicle alone. Serum (**a–c**), feces (**d**), and vaginal lavages (**e–f**) were collected at days 0, 12, 21, 42, and 56 and analyzed by ELISA for rNV-specific IgG or IgA. Data are geometric mean titers (GMT). Significantly higher levels of rNV-specific antibody production were observed throughout the time course in mice immunized IN with t-rNV and CpG ODN compared to t-rNV alone or vehicle alone-immunized mice. Prism software from GraphPad was used to conduct one-way ANOVA with Bonferroni post test ($p < 0.05$ was considered significant)

(Balmelli et al. 1998). We also immunized mice orally with 100 μg t-rNV alone. The rNV-specific IgG1 and IgG2a antibodies induced in serum were analyzed to determine if immunization had produced a Th1, Th2, or mixed Th1/Th2 immune response. Overall, rNV-specific serum IgG levels were higher in nasally versus orally immunized mice, with greatest levels achieved by nasal co-delivery of CpG-ODN with rNV (Fig. 1a–c). Within the nasal groups, both anti-rNV IgG1 and IgG2a in serum were significantly increased in the CpG-ODN immunization group relative to controls. This suggests that CpG-ODN drives a mixed Th1/Th2 response with respect to the production of anti-rNV antibodies.

In these immunized animals, IgG levels had increased by 12 days following a single immunization and continued to rise following booster immunizations at days 21 (intranasal and oral) and 42 (oral only). Antibody levels began to plateau by day 57 (Fig. 1a–c). The orally delivered t-rNV at 100 μg was not very immunogenic; however, nasal immunization with 25 μg t-rNV produced robust immune responses. Fecal IgA was produced only in the CpG-ODN immunization groups (Fig. 1d). In addition, the levels of rNV-specific IgA and IgG in vaginal lavages were significantly elevated ($p < 0.001$) in nasal groups given rNV with CpG-ODN (Fig. 1e and f). To further characterize distal induction of antigen-specific IgA, we

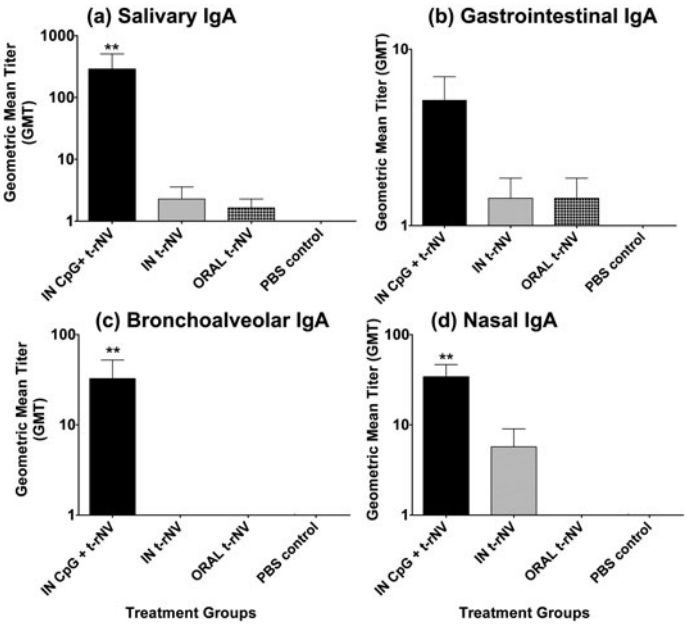

Fig. 2 Induction of antigen-specific IgA at distal mucosal sites following intranasal, but not oral administration of tobacco-derived Norwalk virus-like particles (t-rNV). Female BALB/c mice were immunized as described in the Fig. 1. Salivary samples (**a**), gastrointestinal lavages (**b**), bronchoalvelolar lavages (**c**), and nasal lavages (**d**) were collected from mice on day 56 and analyzed for rNV-specific IgA production by ELISA (presented as GMT). Distal mucosal sites contained significantly higher ($p< 0.001$) levels of antigen-specific IgA in CpG ODN + t-rNV IN vaccinated groups relative to t-rNV alone delivered via IN or ORAL routes

evaluated IgA production at additional mucosal sites, including salivary samples, gastrointestinal lavages, nasal washes, and bronchoalveolar lavages. At each of these distal mucosal sites, IgA production was significantly increased relative to controls in the rNV/CpG-ODN-immunized mice (Fig. 2a–d). These results demonstrate that t-rNV co-delivered with TLR agonist by the nasal route can induce robust antigen-specific IgA production at distal mucosal sites that would be important for mucosal protection against not only enteric organisms, but also respiratory and sexually–transmitted pathogens. Overall, the data demonstrate that immunization with t-rNV by the nasal route is more effective than the oral route for inducing robust rNV-specific systemic IgG and mucosal IgA antibody responses in upper and lower respiratory (nasal and bronchoalveolar), gastrointestinal (salivary, intestine and fecal), and female reproductive (vaginal) tracts. We are now planning a Phase I clinical trial to evaluate systemic and mucosal antibody responses to t-rNV after nasal delivery with a selected adjuvant.

3.2 Hepatitis B Virus

Chronic infection with hepatitis B virus (HBV) can result in liver cancer, accounting for up to one million deaths per year among approximately 350 million chronic carriers worldwide. HBV is transmitted mainly by blood or through sexual contact, but vertical transmission occurs frequently when HBV–infected mothers give birth. In this regard, a mucosal HBV vaccine may be an improvement over the current intramuscular hepatitis B surface antigen (HBsAg) vaccine made in recombinant yeast (McAleer et al. 1984). Moreover, an orally delivered plant-derived vaccine could improve patient compliance for multiple doses due to the convenience, and it would obviate the need for needles. However, we must take care to avoid interference with current efforts to expand HBV vaccination in the developing world, and move forward with a plant-derived vaccine only if distinct advantages are demonstrated.

The perception among plant vaccinologists that a plant-derived HBV vaccine is important is apparent from the large body of work demonstrating expression of HBsAg in a variety of plants including potato, lettuce, tomato, tomatillo, corn, and banana (Streatfield 2005). The first publication on plant-based vaccine antigen expression was with HBsAg in transgenic tobacco (Mason et al. 1992). After optimization of HBsAg expression in transgenic potato tubers (Richter et al. 2000), its oral immunogenicity in mice (albeit dependent upon inclusion of CT; Kong et al. 2001), encouraged a human trial. Due to FDA concerns that immunologically naïve subjects might experience antigenic tolerance after ingestion of HBsAg, the clinical study used HBsAg seropositive volunteers who had been previously vaccinated with the yeast recombinant HBsAg (Thanavala et al. 2005). Titers of anti-HBsAg in serum were increased in 9/17 and 10/16 volunteers who consumed 2 or 3 doses of transgenic potatoes, respectively, with each dose containing roughly 850 μg HBsAg. This finding suggests that oral boosting with plant-derived HBsAg could be incorporated as a viable component of immunization programs. Oral delivery of HBsAg can activate systemic memory cells generated by intramuscular immunization, However, the possibility of priming immunization by HBsAg potato ingestion cannot be excluded. An earlier study had shown that presumably naïve human subjects who ingested transgenic lettuce expressing low levels of HBsAg underwent seroconversion (Kapusta et al. 1999). It is interesting to note that neither of these clinical trials employed an adjuvant, which suggests that oral delivery can be substantially optimized in further work.

The fear of oral tolerance is one that must be taken seriously; however, very little work has addressed this issue with plant-derived vaccines. One recent paper examined regulatory T cells (Tregs) in humanized mice orally immunized with transgenic HBsAg tobacco (Kostrzak et al. 2009). T regulatory cells mediate immunological unresponsiveness to self-antigens and are implicated in oral tolerance (Sakaguchi et al. 2008). The humanized mice were immunized by gavage with different amounts of powdered tobacco leaves, and Foxp3 + CD3 +CD4 + Tregs were measured in spleen and peripheral lymph nodes (Kostrzak et al. 2009).

Serum IgG and fecal IgA were inversely correlated with antigen dose, while the frequency of Tregs positively correlated with tobacco dose, leading the authors to conclude that oral tolerance occurred at higher doses. While the data are not strongly conclusive due to the relatively poor antibody response rate among groups, they do suggest that, in the absence of adjuvant, oral plant vaccines may induce tolerance, and that higher-vaccine dosages are not necessarily better. Nonetheless, much more work is needed with different antigens, plant vehicles, and dosages in order to make firm conclusions regarding the potential of plant-derived antigens to induce oral tolerance.

3.3 Rabies Virus

Rabies is a zoonotic disease that is invariably fatal after development of clinical symptoms (Nagarajan et al. 2008). Because most human deaths from rabies occur in developing countries, new technologies like plant-derived recombinant antibodies and vaccines could make a significant impact. Yusibov and colleagues (Yusibov et al. 2002) have shown that oral delivery of rabies virus epitopes expressed in spinach is immunogenic in humans. A chimeric peptide containing determinants from rabies virus glycoprotein (G protein) and nucleoprotein (N protein) was fused to the N-terminus of the alfalfa mosaic virus coat protein, and expressed with viral vectors in tobacco and spinach. Virus particles were expressed at 0.4 mg per g leaf tissue, of which 70% was recovered in purified form. In mice, 3 intraperitoneal doses of recombinant virus particles (with 35 μg of rabies peptide) generated neutralizing antibodies and protected against lethal rabies virus challenge.

Thus, a human study was performed using 10 volunteers who had previously received the conventional rabies vaccine (as with the HBV potato vaccine, there were concerns about potential oral tolerance). Five subjects were fed 20 g raw recombinant spinach leaves (0.6 mg of recombinant virus with 84 μg chimeric rabies peptide per dose) a total of 3 times at biweekly intervals, while another five subjects ingested control vector-only spinach. Three of the antigen-treated volunteers, but none of the individuals in the control group, showed significant boosting of anti-rabies virus antibody in serum. Based on these results, naïve subjects were tested. Nine volunteers consumed 3 doses of raw rabies-recombinant spinach leaves (150 g per dose) and 5 were fed control spinach. All subjects then received a single dose of commercial rabies vaccine 7 days after the last oral dose. Six of the subjects who ate the rabies antigen-containing spinach showed significant increases in serum IgG or IgA specific for rabies virus (Yusibov et al. 2002). These results are similar to those obtained with HBsAg-expressing potatoes (Thanavala et al. 2005), and although a greater response rate would be desirable, the studies demonstrate the potential for successful oral delivery of plant-derived vaccines in humans.

Little further work on rabies vaccines has been reported, but in one study (Loza-Rubio et al. 2008) the rabies virus G protein was expressed in transgenic maize seeds at 1% TSP. Mice were fed a single dose of transgenic seeds containing 50 μg of G protein, and challenged 90 days later with a lethal dose of rabies virus (100 LD_{50}). The vaccine induced virus-neutralizing antibodies and impressively protected all mice against challenge. These results suggest that maize seeds may be an ideal vehicle for expression and delivery of rabies G protein.

3.4 Measles Virus

Measles virus (MV) causes substantial morbidity and mortality worldwide, but developing countries carry the heaviest burden due to the instability of the attenuated virus vaccine and the requirement for inoculation needles (Webster et al. 2005). Moreover, evidence indicates that vaccinated individuals generate less robust and long-lasting antibody titers than individuals who recovered from a natural MV infection (Muller et al. 2003). Thus, boosting with a convenient mucosally delivered MV vaccine would be a great boon to health, especially in resource-poor countries. Two groups of researchers have approached this problem using plant-derived vaccines. Webster and colleagues expressed a soluble form of the MV hemagglutinin (MV-H) surface protein in stable transgenic tobacco and lettuce (Webster et al. 2006, 2005). The MV-H was stable in freeze–dried lettuce leaf stored at room temperature for 6 months, and showed only 30–40% loss after 13 months (Webster et al. 2006). Moreover, the dried MV-H was orally immunogenic in mice after resuspension and delivery by gavage with a crude saponin adjuvant. Since no reference standard was available, the amount of MV-H antigen in the lettuce could not be accurately determined. Best results were obtained when mice were systemically primed with a MV-H DNA vaccine, then orally boosted five times with lettuce expressing MV-H. The mean titers of MV-specific serum IgG were boosted tenfold by the oral doses, while control lettuce had no effect. The virus-neutralizing titers were roughly 3.5-fold higher in MV-H lettuce boosted mice than in control lettuce mice. These data indicate the potential for oral boosting with plant-derived MV-H for humans that have been intramuscularly vaccinated but experience waning of anti-MV antibody titer.

Another group has used a MV polyepitope strategy, expressing four copies of the loop–forming hemagglutinin protective B cell epitope fused to four repeats of the human promiscuous T cell epitope of tetanus toxoid in transgenic carrots (Bouche et al. 2005). Although the vaccine was delivered by the intraperitoneal route and not mucosally, the antigen was immunogenic and generated serum antibodies that neutralized ten different MV strains. This approach might be developed for oral or nasal delivery, with appropriate mucosal adjuvants, in order to produce a vaccine that is more convenient for populations in developing

countries. No plant-derived MV vaccines have been tested in humans yet, but the data above show very promising indications that oral boosting could provide substantial benefits.

3.5 Enteric Bacterial Infections

The first plant-derived vaccine immunogen to be tested in humans was the B subunit protein of the enterotoxigenic *Escherichia coli* heat–labile toxin (LT) expressed in transgenic potatoes (Mason et al. 1998; Tacket et al. 1998). The non-toxic LTB protein mediates binding of the LT holotoxin to epithelial cells, triggering intracellular delivery of the toxic A subunit. The rationale for a mucosal vaccine rationale presumes that oral delivery of LTB will induce local secretion of anti-LTB antibodies in the gut, which will prevent the binding of the holotoxin to enterocytes. Data from mouse studies supports this hypothesis, and mice that ingested LTB-containing potato tubers were partially protected against LT challenge, as indicated by reduced fluid secretion in the small intestine (Mason et al. 1998). The clinical trial showed that ingestion of LTB in potatoes stimulated anti-LT serum IgG in 9 of 11 subjects, serum IgA in six subjects, and fecal IgA in 5 of 10 subjects; and further showed that the antibodies could neutralize LT (Tacket et al. 1998). Another clinical trial using transgenic maize germ (embryo of seeds) expressing LTB yielded similar results (Tacket et al. 2004). The fact that LTB is one of the most orally immunogenic proteins known does not diminish the impact of these findings, which provide clear evidence that the ingestion of crude plant material, in the absence of adjuvant, could potentially stimulate protective antibody responses to enteric pathogens in humans.

Recently, transgenic rice seeds expressing the structurally and functionally similar B subunit of cholera toxin were developed (Nochi et al. 2007). Cholera toxin B subunit (CTB) was expressed in rice seeds using the *GluB*-1 seed storage protein promoter at up to 30 μg per seed (2.1% TSP). It was localized in protein storage bodies, which allowed high stability (immunogenicity after 1.5 years at room temperature), and resistance to pepsin treatment. Thus, rice and corn appear to be good vehicles for delivery of antigens in the gut.

A plant-derived vaccine against enterohemorrhagic *E. coli* (O157:H7) has also been created by expressing part of the bacterial adhesin intimin in cultured transgenic tobacco cells (Judge et al. 2004). The C-terminal domain of intimin (Int261) mediates binding to the translocated intimin receptor, as well as host–specific surface molecules, for bacterial colonization in the gut. Oral immunization of mice by ingestion of Int261 expressing tobacco cells after intraperitoneal priming with plant-derived Int261 stimulated specific fecal IgA in 7 of 10 mice, and reduced bacterial shedding after challenge with O157:H7 (Judge et al. 2004). However, low expression of Int261 in the transgenic tobacco cells limited the amount of antigen that could be produced. Using viral vectors and transient expression, our research team has substantially enhanced Int261 expression in leaves of *N. benthamiana* (E. Topal & H. Mason, manuscript in preparation).

Further testing in more appropriate hosts, such as cattle, will be needed to assess the efficacy of this and other intimin expressing plant-based vaccines for preventing infection by *E. coli* 0157:H7.

4 Plant-Derived Animal Vaccines

Plant-derived vaccines for animals are likely to be realized sooner than those for humans, due to more relaxed regulations (Rybicki 2009). In fact, the only licensed plant-derived vaccine is for prevention of Newcastle disease virus (NDV) in chickens, comprising recombinant hemagglutinin–neuraminidase protein expressed in cultured transgenic tobacco cells and prepared as an injectable vaccine for chickens (Dow AgroSciences, www.dowagro.com/animalhealth/resources/faq.htm#faq11). As discussed below, several other plant-derived mucosally delivered vaccines against animal diseases have shown potential.

4.1 Foot-and-Mouth Disease Virus

Foot-and-mouth disease virus (FMDV) is a very important veterinary pathogen because it is highly infectious in animals and has economically devastating effects on meat and milk production. The FMDV VP1 capsid protein contains virus-neutralizing determinants (Wigdorovitz et al. 1999). Several studies have shown expression of VP1 in various plant hosts (Yusibov and Rabindran 2008). Transgenic VP1 alfalfa leaves were immunogenic in mice by intraperitoneal or oral delivery (Wigdorovitz et al. 1999). For oral delivery, mice were fed 0.3 g of fresh leaves three times per week for 2 weeks. Ten days later serum was obtained. All mice in two separate experiments developed serum antibodies against FMDV particles, at titers ranging up to 320. Moreover, 14 of 17 mice were protected against intraperitoneal challenge with FMDV, as measured by the absence of viremia 36 h later, while none of the control mice were protected.

A different strategy fused a VP1 epitope (amino acids 135–160) to β-glucuronidase (GUS), a stable enzyme that is readily measured using a fluorometric assay (Dus Santos et al. 2002). Expression in transgenic alfalfa plants ranged from 0.05 to 0.1% of TSP, and crude extracts injected by the intraperitoneal route elicited completely protective immune responses in mice. Another fusion protein study used VP1 amino acids 128–164 replacing the N-terminal 35 residues of bamboo mosaic virus coat protein, and the recombinant virus used to infect leaves of *Chenopdium quinoa* (a spinach relative) (Yang et al. 2007). Infected leaves produced chimeric virions expressing VP1 epitopes in its coat protein. Although the yield of recombinant protein was not quantified, the data suggest accumulation to $\sim$5–10% TSP (Yang et al. 2007). Intramuscular immunization of pigs with 5 mg of VP1 virions resulted in the induction of anti-FMDV neutralizing antibodies, VP1-specific IFN-γ-producing cells, and complete protection against

FMDV challenge. The high antigen dose used in these studies indicates robust expression of this viral coat protein fusion.

In view of the promising results obtained through oral delivery of plant-derived VP1 (Wigdorovitz et al. 1999), it is perhaps surprising that these later fusion protein strategies that yielded improved expression were not tested by the oral route. Further investigation is needed in order to evaluate oral delivery with adjuvants, and in animal species that have more economic relevance.

4.2 Transmissible Gastroenteritis Virus

Transmissible gastroenteritis virus (TGEV) is a coronavirus that significantly affects swine production (Hammond and Nemchinov 2009). The disease is highly infectious, and the severe diarrhea and vomiting produce high mortality in young piglets. The envelope spike (S) protein is a target for neutralizing antibody, and thus has been expressed in plant systems for oral delivery (Howard 2004; Lamphear et al. 2004). Transgenic corn seed expressing TGEV-S protein fed to piglets stimulated antibody production and partially protected animals from viral challenge (Howard 2004). The truncated soluble S protein product was targeted to the cell wall instead of protein bodies, which allowed accumulation of antigen at 13 mg/kg seed, and a dose of 20–30 mg S protein in a single feeding. Further studies examined the potential to immunize gilts (young sows) and stimulate anti-S antibody secretion in the milk for protection of suckling piglets (Lamphear et al. 2004). Oral dosing by ingestion of TGEV-S corn by gilts on days −35 and −14 before farrowing (day 0), after having received the modified live TGEV vaccine orally at days −115 and −102, stimulated significantly higher TGEV-neutralizing activity in serum, colostrum (day 0), and milk (day 3) compared to a placebo group. Antibody isotypes were not determined, but the authors suggest that the milk antibodies later than day two are mostly IgA (Lamphear et al. 2004). Protective efficacy was not examined, but the neutralizing titers in milk suggest that suckling piglets would have been protected. However, the milk titers dropped precipitously at day 7–14, suggesting that continued boosting would be necessary to maintain protection.

An interesting chimeric antibody against TGEV was expressed in plants and tested for its ability to protect piglets after oral delivery (Monger et al. 2006). These investigators fused an anti-TGEV single-chain antibody (scFv) to the CH4 domain of IgE to mediate dimerization. The protein was expressed in cowpea leaves using a cowpea mosaic virus vector, achieving accumulation of ∼2%TSP. This plant-derived antibody (dubbed "ε-small immune protein" or εSIP) showed similar TGEV-neutralizing activity as the parent antibody 6A.C3 or the εSIP expressed in mammalian cells. Moreover, oral gavage in piglets reduced viral titers in lung and gut of piglets challenged with TGEV, though less effectively than antibody 6A.C3. These studies illustrate the potential for plant-derived antibodies to provide mucosal protection against enteric viral disease, and deserve further attention for application in veterinary and human medicine.

4.3 Infectious Bursal Disease Virus

Infectious bursal disease virus (IBDV) is highly contagious and deadly in young chickens. It thus has a major economic impact on the poultry industry. IBDV is a member of the *Birnaviridae* with two double–stranded RNA genome segments (Wu et al. 2007). The VP2 viral capsid protein is the immunodominant antigen that generates antiviral neutralizing antibodies. The currently used vaccines are either attenuated or killed viruses, but suffer problems regarding the potential for the live virus vaccine to recombine and generate variants, or poor efficacy in the case of the whole-killed vaccine.

One of the more compelling plant-derived vaccine successes to date is recombinant VP2 delivered orally. The VP2 was expressed in leaves of *Arabidopsis thaliana* at levels up to 4.8% TSP (Wu et al. 2004a), which is among the highest obtained for a recombinant subunit vaccine in stably transformed plants. Leaves of transgenic plants were dried, powdered, and resuspended in water for oral delivery (Wu et al. 2004b). One-week old birds received five oral doses at 3 days intervals (~5 μg VP2 per dose), or the live intermediate vaccine Bursine-2, or priming with Bursine-2 followed by boosting with oral VP2. Although the Bursine-2 primed birds developed serum anti-VP2 antibodies earlier, the titers were similar in all three groups after 5 weeks. Furthermore, all groups exhibited similar protection (80–90%) after challenge with virulent IBDV (Wu et al. 2004b).

Later, VP2 was expressed in stable transgenic rice seeds at up to 4.5% TSP, or 40 μg per seed (Wu et al. 2007). Two week old chickens were fed 4 weekly doses of 1, 3, or 5 g rice seed, or they received the B87 live attenuated nasal virus vaccine. Anti-VP2 serum antibodies increased during the 2 weeks following immunization, with the 5 g oral group attaining levels similar to those in the B87 nasal vaccine group. Among the different VP2-rice oral groups, antibody levels were positively correlated with the dosage. Interestingly, the 5 g oral VP2 group was better protected against virulent IBDV challenge than the B87 vaccine group (5/6 vs. 2/6, respectively). Although the bursal lesion score used to calculate efficacy could be considered somewhat subjective, efficacy was correlated with oral antigen dose (Wu et al. 2007). We have estimated that the 5 g rice seed contained roughly a 10 mg dose VP2 protein, which is rather high. In this regard, seeds have a distinct advantage over leaves, by enabling a high dose as well as excellent protein stability on long-term storage.

4.4 Actinobacillus Pleuropneumoniae

Actinobacillus pleuropneumoniae is an agent of porcine pleuropneumonia, which is an important swine disease. Although the pathogenic mechanism is not fully understood, Apx toxins are associated with bacterial virulence (Shin et al. 2005). The Apx I, II, and III toxins mediate virulence by inserting into host cell

membranes and creating pores; thus they may be good targets for immune intervention in this disease. One group has demonstrated the protective potential of ApxIA and ApxIIA proteins as vaccine antigens after oral delivery in mice (Lee et al. 2006; Shin et al. 2005, 2007). Their first study used the yeast *Saccharomyces cerevisiae* for expression of ApxIIA and oral delivery in mice, showing ApxIIA-specific IgA production in lung and intestine, as well as partial (≤50%) and dose-dependent protection against challenge with *A. pleuropneumoniae*.

In a second study, stable transgenic tobacco was used to express ApxIIA at relatively low levels (≤0.1% TSP) (Lee et al. 2006). Mice were gavaged with 4 weekly doses of dried tobacco leaf (1 mg or 5 mg dry mass, which we estimate contained 30 or 150 ng ApxIIA) suspended in PBS, which evoked modest serum IgG specific for ApxIIA in the higher dose group. Challenge of the mice by intraperitoneal injection of *A. pleuropneumoniae* killed all mice in the control non-transgenic tobacco group within 72 h, while 50% of the higher–dose ApxIIA tobacco group survived. By comparison, 90% of mice immunized subcutaneously with recombinant *E. coli*-expressed ApxIIA survived (Lee et al. 2006). Although the ultimate fate of the mice beyond 72 h was not reported, it appears that oral delivery of ApxIIA tobacco afforded some protection. A later oral immunization study using both ApxIA and ApxIIA expressed in yeast showed enhanced protective efficacy over either toxin delivered alone, suggesting that multiple toxins should be included in a swine vaccine (Shin et al. 2007). Certainly, the levels of antigen expression in plants must be increased in order to facilitate more robust protection by oral delivery. Moreover, testing in swine must be performed in order to examine the potential for protection by oral delivery of the Apx antigens.

5 Conclusion

Plant-based expression of vaccine antigen and oral delivery of crude or minimally processed plant tissues is most promising for antigens expressed in seeds, such as rice or corn. The seed vehicle provides a stable environment for accumulated proteins, thus the need for refrigeration could be obviated or minimized. Nonetheless, only a few antigens have been expressed in seeds at sufficient levels to afford protection in animals upon ingestion. Due to the variable nature of protein structure and biochemical characteristics, it is difficult to predict which proteins will accumulate well and be correctly folded to display protective epitopes. The generation and screening for optimal expression in stable transgenic plant lines is a time- and labor-intensive process, but in some cases may yield excellent results.

Regulatory agencies have a substantial concern regarding the use of plants, especially plants used as food for humans, for the production of pharmaceutical proteins and vaccines. This issue was discussed in a recent review (Rybicki 2009). Although the World Health Organization, United States Department of Agriculture, and the Food and Drug Administration agree that applicable regulatory and good manufacturing practice requirements are in place for plant-derived vaccines,

enthusiasm is dimmed by fears that noncompliance could compromise food security. Thus, investment of funds for commercial development of vaccines in food crops is likely to suffer.

Meanwhile, much recent work has focused on the use of non-food plants (tobacco and relatives) for production of vaccine antigens using robust transient expression via plant virus replicons (Gleba et al. 2007; Huang et al. 2009; Santi et al. 2006). These systems provide expression levels high enough to facilitate purification of antigens in good yield and concentrated enough to use for intranasal delivery, or to incorporate into oral delivery formulations. Ongoing work to develop mucosal adjuvants will synergize with these efficient plant expression systems to provide more alternatives for vaccination and, ultimately, vaccines tailored for particular pathogens and their hosts. Agonists for pattern recognition receptors (e.g. Toll-like receptors) show great promise as mucosal adjuvants, and deserve further research and development.

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