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Plant-based Mucosal Immunization

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Introduction

The annual appearance of new infectious diseases with no cure, and the increased incidence of re-emerging antibiotic-resistant infectious diseases have forced clinicians and biomedical scientists to devote greater efforts to the problem of disease prevention. Jennerian vaccine construction has been regarded for centuries as the strongest method for elimination of widespread infectious diseases such as cholera, smallpox, typhoid fever, polio, and hepatitis. Dependent on Jenner's original tenets, most of today's vaccines are still derived from killed or attenuated viruses and bacterial pathogens that can cause infection and contribute to the onset of autoimmune disease symptoms or allergic reactions in the patients they are attempting to protect. During the past two decades molecular biology and immunology have made significant contributions to the development of subunit mucosal vaccines as an alternative approach to parenteral immunization. An increasing body of experimental data is beginning to define the mechanisms underlying mucosal immunity and the critical role it plays in protecting the body's mucosal tissues from viral and bacterial infection (Kuby, 2000). As the importance of mucosal immunity for protection against infection continues to emerge, edible plants have gained increasing popularity as vehicles for delivery of vaccine antigens to the mucosal immune system.

Plant-synthesized recombinant antigen proteins are introduced to the mucosal immune system generally through consumption of fruit, tubers, roots, or leaves of the

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Abbreviations: APC, antigen-presenting cell; BADH, betaine aldehyde dehydrogenase; CPMV, cowpea mosaic virus; CTB, cholera toxin B subunit; ER, endoplasmic reticulum; FMDV, foot-and-mouth disease virus; GAD65, glutamic acid decarboxylase; HBsAg, hepatitis B surface antigen; Hg, mercury; IgG, immunoglobulin G; INS, proinsulin; LT-B, heat labile enterotoxin B subunit; M cells, microfold cells; MAb, monoclonal antibody; MHC, major histocompatibility complex; NVCP, Norwalk virus coat protein; ORSV, oddontoglossum ringspot virus; sIgA, secretory immunoglobulin A; Th1, T helper 1 cells; Th2, T helper 2 cells; TMV, tobacco mosaic virus; TSP, total soluble protein; VLP, virus-like protein; VP1, virus protein 1.

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transformed plants, and more recently, by ingestion of capsules of freeze-dried ground transformed plant tissues that can accurately define vaccine dosage. Among the advantages of plant-based mucosal vaccines are elicitation of secretory IgA antibodies, elimination of animal pathogens, and animal proteins that co-purify during vaccine protein isolation. Edible vaccines avoid needle-stick injury, decrease the spread of infectious disease by contaminated needles, and eliminate exposure to toxic preservatives (Hg) included in parenteral vaccines. In addition to reducing the need for qualified medical professionals for vaccine administration, an important economic advantage of plant-based vaccines is the elimination of refrigeration for vaccine storage, a costly requirement that significantly hinders the distribution of parenteral vaccines worldwide. The ease of distribution of seeds and edible plants that synthesize protective vaccine antigens permits propagation of vaccines against endemic pathogens at rural sites, with minimal expense, employing indigenous agricultural methods and machinery.

Most pathogens enter the body through mucosal surfaces exposed to the environment, such as the eyes, ears, nose, mouth, and intestinal, urinary, and respiratory tracts. This first line of defence is ignored by injected vaccines that elicit IgG production that contacts pathogens only after they have entered the body and have initiated multiplication and infection. In contrast, mucosal vaccines have the advantage of generating both IgG and especially secretory IgA antibodies, which can intercept the pathogen at or near the mucosal surface before it enters the circulation. The slow release of vaccine antigens from plant cells delays digestion of the antigen in the highly acidic environment of the stomach and protease-rich small intestine. This slow release of antigen can provide longer stimulation of the mucosal immune response (Mason *et al.*, 2002).

Plants are considered to be ideal systems for biosynthesis of pharmacologically active compounds. Based on their eukaryotic nature, plants can correctly fold complex proteins and subsequently glycosylate and phosphorylate specific sites necessary for recognition as functional proteins in mammals. Examples include post-translationally modified recombinant immunoglobulin proteins, and the complex fruit-specific type-2 ribosome-inactivating protein from transgenic tobacco plants (McGarvey *et al.*, 1995; Chen *et al.*, 2002).

Plant-based passive immunization

In 1989, the first functional antibody variable region was synthesized *in planta* (Hiatt *et al.*, 1989). Single gamma or kappa light chain cDNAs were transferred into separate tobacco plants (*Nicotiana tabacum*). The two plant-synthesized immunoglobulin chains were combined by reciprocal crossing of the transformed plants yielding F1 progeny that displayed functional antibody variable regions at approximately 1.3% of the total soluble transformed plant protein (TSP) (Hiatt *et al.*, 1989). These promising results stimulated further interest in using plants as production vehicles to replace the extraction of antibodies from the serum of immunized animals. Biosynthesis of antibodies from transgenic plants eliminates significant risks to the patient, including infection with co-injected mammalian pathogens, as well as allergic and autoimmune responses to pathogen and serum proteins.

In 1994, antibodies consisting of both light and heavy immunoglobulin chains

conferring bivalent antigen binding were expressed in transgenic tobacco plants (Ma *et al.*, 1994). Murine monoclonal antibody (MAb) capable of binding a surface protein from *Streptococcus mutans*, Guy's 13 MAb, was synthesized as an IgG with four domains. By altering the antibody's heavy chain, through replacement of its Cγ3 domain with Cα2 and Cα3 domains, Ma and his colleagues successfully assembled in transformed plants a chimeric immunoglobulin containing both gamma and alpha heavy chain domains (Ma *et al.*, 1994). In 1995, Ma and his colleagues successfully crossed these plants to generate progeny containing all four antibody chains in one plant. The transformed plants synthesized biologically active secretory antibody (sIgA) complexes at 5% to 8% of TSP (Ma *et al.*, 1995). Noteworthy was the finding that the secretory component protected the sIgA immunoglobulin from proteolysis, making it sufficiently durable for use *in vivo* (Underdown and Dorrington, 1974). Also of interest, the tobacco plant was able to assemble the antibody proteins in a single cell, in contrast to the two different cell types previously required for secretory antibody synthesis in mammalian cells (Frigerio *et al.*, 2000).

Additional studies on plant synthesized Guy's 13 antibody revealed a variation in the glycosylation pattern between the plant product and its murine counterpart IgG1 (Cabanes-Macheteau *et al.*, 1999). This study found that Guy's 13 MAb contained greater structural diversity in its N-linked glycans than its murine counterpart. However, glycosylation occurred at the same location on the Guy's 13 MAb as on the mouse Ab heavy chains, and the plant-synthesized immunoglobulin was more soluble and retained its bioreactive qualities (Cabanes-Macheteau *et al.*, 1999). The concern that the variation in glycosylation among plants and mammals might stimulate an unpredictable antigenic response was reduced when plant-synthesized Guy's 13 MAb was tested in mice. No detectable antibodies against the injected MAb product were found (Chargelegue *et al.*, 2000). When the Guy's 13 secretory monoclonal antibody was tested in humans, it was found to survive up to 3 days in the oral cavity, while mouse IgG1 survived only 1 day. The plant-synthesized secretory antibody was able to prevent oral colonization of streptococcal infections for up to four months, illustrating the efficacy of plant vaccination for oral caries disease prevention (Ma *et al.*, 1998).

A significant constraint in the engineering of functional antibodies in plants is the large size of the DNA molecules encoding the immunoglobulin protein. Until 1998, several plants were required for transfection with DNA molecules encoding different portions of the antibody. Thus, subsequent sexual crossing of these transformed plants was required to generate a single plant containing all the antibody components. More recently, using tobacco mosaic virus (TMV) as the vector, virus constructs containing both heavy and light immunoglobulin chains were co-transfected into a single plant. Using this method, the first plant to successfully assemble a full-size antibody molecule was created (Verch *et al.*, 1998). Roggero and his colleagues obtained successful biosynthesis of single-chain Fv antibody fragments in *Nicotiana benthamiana* using potato virus X (2001). This finding confirmed that plants were feasible production systems for the synthesis of antibodies.

To increase the amount of antibody accumulation in transformed plants, an endoplasmic reticulum retention signal amino acid sequence (KDEL) was inserted immediately downstream of the antibody transgene to be incorporated into the plant genome. Addition of the KDEL sequence substantially increased the amount of

recombinant protein synthesized in transformed leaf tissues (Haq *et al.*, 1995; Fiedler *et al.*, 1997). Addition of the KDEL sequence to the 3' end of the transferred gene resulted in increased accumulation of functional secretory antibody protein scFv to 4–6.8% of TSP in transformed tobacco leaves, and to 3–4% in seeds from transformed plants (Fiedler *et al.*, 1997). These encouraging results suggested that accumulation of the recombinant protein in the ER enhanced folding of the protein into biologically active antibody complexes.

Plant-generated active mucosal immunity

Arranged in domes along the lumen of the small intestine, specialized gut epithelial microfold cells (M cells) sample proteins and other macromolecules travelling through the intestine. The M cells, located in the intestine immediately above large lymphoid follicles (Peyer's patches), deliver soluble and particulate antigens from the lumen of the intestine to underlying dendritic cells and macrophage antigen-presenting cells (APCs) located within the Peyer's patches. The APCs take up antigen proteins into pinocytotic or endocytic vesicles, process them with endogenous proteases, and display immunogenic peptides (epitopes) on major histocompatibility complex (MHC) receptors on the cell surface. Cognate B and T cells capable of responding to the specific antigens bind the antigen to their T cell receptors. With the help of co-stimulatory factors synthesized by both T cells and APCs, specific cytokines are released from the activated T cells that more precisely determine the nature of the immune response, e.g. activation of Th2 lymphocytes results in the release of cytokine types which stimulate B lymphocytes, resulting in specific antibody synthesis in activated B cells (plasma cells). Th1 helper cell activation of cytotoxic lymphocytes induces apoptosis in somatic cells infected by intracellular pathogens. Thus, while eliminating the costs of antigen protein purification, plant-synthesized antigens deliver specific antigenic proteins to the APCs of the mucosal immune system that generate a variety of specific immune responses related to the nature of the delivered antigen.

Engineering plants to synthesize specific immunodominant antigen proteins (subunit vaccines), strong mucosal immune responses can be directed against the pathogen in the lumen of the gut, avoiding the possibility of pathogen transfer into the circulatory system. However, since subunit vaccines contain individual pathogen antigens, they frequently convey significantly less protection against infection than vaccines containing killed or live attenuated viral or bacterial pathogens that supply a large number and variety of epitopes. However, killed or live attenuated vaccines harbour potential risks not observed in subunit vaccines, such as reactogenicity and possible infection from pathogenic microbes contaminating the vaccine. Since whole organism-based vaccines are frequently generated in mammalian cell systems, undesirable allergic or autoimmune reactions from co-purified mammalian proteins can be generated following vaccination, especially when multiple booster inoculations are required to enhance the humoral immune response. The substantial drawbacks associated with parenteral vaccination are a strong driving force for finding methods to improve the immunogenicity of mucosal vaccines. To achieve this goal, a variety of methods have been constructed to synthesize pathogen antigens in plant tissues, including stable genetic transformation of plant cells, the expression of antigenic

Table 8.1. Plant virus vaccines against protozoan, bacterial, and viral pathogens

Host	Antigen protein	Virus†	Yield*	Reference
Tobacco	Foot-and-mouth disease virus (FMDV) structural protein (VP1)	TMV	0.15**	Wigdorovitz *et al.*, 1999b
	Malarial epitopes	TMV	1.2	Turpen *et al.*, 1995
	Influenza virus haemagglutinin (HA) and HIV glycoprotein (gp120) epitopes	TMV	2.0	Sugiyama *et al.*, 1995
	HIV V3 loop of glycoprotein gp120	TBSV	0.9	Joelson *et al.*, 1997
	Rabies virus nucleoprotein–glycoprotein chimera (Drg24)	AMV	0.025**	Modelska *et al.*, 1998
	B cell lymphoma single-chain Fv antibody	TMV	0.03**	McCormick *et al.*, 1999
	Staphylococcus aureus fibronectin-binding protein (FnBP) D2 peptide	PVX	0.2	Brennan *et al.*, 1999b
	Hepatitis C virus (HCV) hypervariable region 1 (HVR1)-CTB fusion	TMV	0.08**	Nemchinov *et al.*, 2000
	Respiratory syncytial virus (RSV) G protein	AMV	0.8	Belanger *et al.*, 2000
	Rabbit haemorrhagic disease virus (RHDV) structural protein (VP60)	PPV	0.63	Fernandez-Fernandez *et al.*, 2001
	Human papillomavirus 16 E7	PVX	0.004	Franconi *et al.*, 2002
Cowpea	Human rhinovirus 14 and human immunodeficiency virus (HIV) epitopes	CPMV	1.5	Porta *et al.*, 1994
	Mink enteritis virus (MEV) nucleoprotein–glycoprotein chimera (Drg24)	CPMV	1.2	Dalsgaard *et al.*, 1997
	Pseudomonas aeruginosa OMP F	CPMV	1.0	Brennan *et al.*, 1999a
	Staphylococcus aureus fibronectin-binding protein (FnBP) D2 peptide	CPMV	1.2	Brennan *et al.*, 1999b

†AMV = alfalfa mosaic virus, CPMV = cowpea mosaic virus, PPV = plum pox virus, PVX = potato virus X, TMV = tobacco mosaic virus, TBSV = tomato bushy-stunt virus.
*mg of chimeric virus particles per g fresh weight of tissue.
**mg of protein isolated from virus per g fresh weight of tissue.

proteins in or on the surface of plant viruses, and more recently, the transformation of plant chloroplasts and other plastids.

Plant viruses as antigen production and delivery systems

Infection with genetically modified plant viruses has been used effectively to introduce foreign genes into plant cells, and has been shown to yield high quantities of recombinant vaccine proteins (Ma and Vine, 1999) (*Table 8.1*). Foreign genes encoding antigens carried within the virus genome can express the antigen protein in plant tissues during virus infection. In some cases, the antigen protein can be assembled in the infected cell into empty virus-like particles (VLPs) containing the foreign antigen inserted into the virus capsid protein (Mason *et al.*, 1996).

Cowpea mosaic virus (CPMV) is a frequent candidate for antigen biosynthesis as it contains sites in the virus genome where foreign protein DNA sequences can be inserted without interrupting virus-specific sequences essential for virus multiplication (Porta *et al.*, 1994). In one example, an epitope from virus protein 1 (VP1) of foot-and-mouth disease (FMDV) was successfully inserted into the small protein subunit of CPMV. Upon infection of cowpea plants with the modified virus, FMDV-specific RNAs were able to replicate within the cowpea protoplast, and were later

identified by binding FMDV-specific antibodies to the genetically modified protein (Carrillo *et al.*, 1998).

Another RNA virus, tobacco mosaic virus (TMV), showed that recoverable recombinant viral particles could be extracted from virus-infected tobacco plants in amounts up to 50% of plant dry weight (Ma and Vine, 1999). Large-size foreign genes integrated into viral RNA were shown to interfere with assembly of recombinant viral particles. This size limitation has been partially overcome by creating hybrid viral vectors containing genes from TMV and odontoglossum ringspot virus (ORSV), which can assist in virus capsid protein folding, resulting in more efficient viral particle assembly (Donson *et al.*, 1991).

Infection with genetically modified RNA viruses, such as CPMV and TMV, provides an effective mechanism for synthesis of large quantities of vaccine antigen protein in plants (*Table 8.1*). Unfortunately, the size of the antigen epitope that can be successfully integrated into the virus genome is limited to short peptides that can significantly decrease the immunogenicity of the vaccine. Peptides as large as 25 amino acids have been introduced successfully into TMV, while peptides of up to 30 amino acids were successfully introduced into CPMV (Ma and Vine, 1999). In addition to TMV and CPMV, a variety of plant viruses has been successfully used as vectors for the production of vaccine antigens in crop plants (*Table 8.1*). To obtain virus amplification, the altered virus must remain pathogenic to the plant. Thus, propagation of genetically modified virus is labour intensive, and is not suited for production of large-scale virus-infected crops. Due to the need for pathogen containment, recombinant virus infection as a method for generation of plant-based vaccines is suited mainly for greenhouse propagation conditions.

Formation of genetically modified viral coat proteins can serve as an alternative method for antigen biosynthesis in plants. Norwalk virus, known to induce acute gastroenteritis in humans, has been used successfully as a vaccine delivery vehicle. Foreign peptides can be inserted into the Norwalk virus capsid protein while maintaining its ability to generate virus-like particles (VLPs) (Mason *et al.*, 1996). Norwalk virus coat protein (NVCP) expressed in transgenic potatoes was shown to properly assemble into VLPs, and to initiate antiviral IgA and IgG production following oral administration in mice. When NVCP transgenic potatoes were fed to 20 adult volunteers in a clinical trial conducted by the Centre for Vaccine Development, University of Maryland, 19 of the 20 showed significant increases in anti-NVCP IgA antibody secretion (Tacket *et al.*, 2000). Six out of 20 volunteers produced detectable increases in IgG antibody-secreting cells. Clearly, modified NVCP can be an effective stimulator of mucosal immunity. However, limitations occur in terms of size of the foreign protein that can be inserted into the capsid protein for successful VLP formation (Mason *et al.*, 1996).

Transgenic plants as vaccine production and delivery systems

In 1990, the first mucosal vaccine candidate was synthesized in plants, when a gene encoding a surface antigen of *Streptococcus mutans* (spa A) was introduced to tobacco cells via *Agrobacterium*-mediated transformation (Curtiss and Cardineau, 1990). This achievement was remarkable as the spa A gene encodes a very large protein of more than 1500 amino acids in length, and was expressed at 0.2% of TSP.

Another early transgenic plant vaccine was directed against hepatitis B, a major cause of chronic hepatitis in humans. The hepatitis B surface antigen (HBsAg) was assembled into virus-like particles in transgenic tobacco, yielding up to 0.01% of total soluble protein (Mason *et al.*, 1992). Although an effective and safe yeast-derived vaccine targeting hepatitis B was licensed in 1986, comparison of the oral immunogenicity of HBsAg derived from yeast and transgenic potatoes indicated that the plant-derived HbsAg stimulated primary immune responses after only two feedings, while a primary humoral immune response was not detected after two doses of HBsAg derived from yeast (Kong *et al.*, 2001). This finding in favour of plant vaccination is partially accredited to the fact that HBsAg was not subjected to degradation in the gut, based on bioencapsulation of the antigen within the cellulose plant cell wall.

Clinical trials using plant-based vaccines against HBsAg have generated promising results. One human trial tested responses following ingestion of transgenic lettuce containing HBsg (Kapusta *et al.*, 1999). Levels of anti-HBsAg IgG greater than 10 International Units per litre, were found in two out of three volunteers following two doses of approximately 1 μg of the antigen.

The heat-labile enterotoxin (LT) of enterotoxigenic *Escherichia coli* (ETEC), 80% similar in molecular structure and amino acid sequence to cholera toxin (Gill and Meren, 1978; Rappuoli *et al.*, 1999), colonizes the small intestine (Sack, 1980). The LT toxin is made up of a single A subunit and five B subunits, arranged in a pentameric configuration into which the single A subunit is inserted. Pathogenic effects of ETEC infections are due specifically to the toxic A subunit, which catalyses ADP ribosylation of adenylate cyclase, a key enzyme involved in signal transduction and protein phosphorylation. In the pentameric configuration, the B subunit has the ability to bind G_{M1} ganglioside sugar receptors on the intestinal epithelial cell membrane, which assists in penetration of the toxic A subunit into the cell cytoplasm (Rappuoli *et al.*, 1999). The B subunit of LT (LT-B) was the first bacterial enterotoxin fragment synthesized in transgenic plants. Oral immunization of mice with tobacco tissues synthesizing LT-B showed that the plant-produced toxin antigen could stimulate a significant mucosal immune response (Haq *et al.*, 1995).

Transgenic potatoes containing LT-B provided the first edible-plant vaccine to be tested in human clinical trials (Tacket *et al.*, 1998). In a double-blind study, conducted by the Centre for Vaccine Development, University of Maryland School of Medicine, USA, adult volunteers were fed 50 g or 100 g of raw LT-B potatoes or non-transgenic potatoes, once a week over a period of 3 weeks. Ten out of 11 subjects fed the LT-B potatoes showed at least a fourfold increase in serum LT-neutralizing anti-LT-B IgG levels (Tacket *et al.*, 1998). Five out of 10 volunteers showed increased anti-LT-B IgA in stool samples. The results of this early clinical study demonstrated that ingestion of transgenic plants was not toxic, and that plant-delivered antigens could effectively stimulate a mucosal immune response in humans.

Although somewhat different in structure and receptor-binding specificity from LT-B, the cholera toxin B subunit (CTB), synthesized in *Vibrio cholerae*, was also found to be a potent stimulator of mucosal immunity. Oral inoculation of mice with CTB was shown to stimulate mucosal and systemic anti-CTB antibody synthesis, making it an ideal candidate for generating edible vaccines in plants (Elson and Ealding, 1984). *Vibrio cholerae* causes a devastating diarrhoeal disease that continues

to generate epidemics throughout African, Asian, South American, and Latin American countries.

Cholera toxin (CT), like LT, is composed of five identical B subunits and one toxic A subunit made up of two peptides, A1 and A2 (Gill, 1976) (*Figure 8.1*). Five CTB subunits assemble to form a pentameric molecular structure into which the A1 and A2 peptides, linked together by a single disulphide bond, are inserted (*Figure 8.1*). The A1 subunit is an ADP ribosyl transferase, responsible for the toxin's pathogenic activity (Gill, 1976). Interestingly, the pentameric ring, composed of five identical B subunits, binds specifically to G_{M1} ganglioside receptors on the gut epithelial cell (enterocyte) and M cell membranes (Holmgren *et al.*, 1975). The ability of CTB to target G_{M1} ganglioside receptors on intestinal epidermis suggested that it could be an effective carrier for delivery of antigens from transgenic plants to the mammalian mucosal immune system. Unlike LT-B, CTB is significantly resistant to heat denaturation, a distinct advantage for generating vaccines in plants that require cooking for digestibility. In preliminary transformation experiments, CTB protein was synthesized in transformed potato plants (Arakawa *et al.*, 1997). Immunoblot and sandwich ELISA experiments indicated that the CTB monomers assembled into G_{M1} ganglioside, binding oligomers of a size corresponding to pentameric CTB (Arakawa *et al.*, 1997). In mouse immunization experiments, the plant-based vaccine was shown to protect immunized mice against cholera toxin-induced diarrhoea (Arakawa *et al.*, 1998a).

Genetic fusion of a rotavirus non-structural protein immunodominant epitope (NSP4) to the C-terminus of the CTB subunit resulted in significant antibody production against NSP4 in orally immunized mice (Yu *et al.*, 2000; Arakawa *et al.*, 2001). This result was considered to be related to the ability of the CTB carrier ligand to target enterocyte and M cell membrane G_{M1} ganglioside receptors, enhancing uptake of the antigen into gut epidermal and M cells (Arakawa *et al.*, 1998a). To enhance CTB oligomerization, an endoplasmic reticulum (ER) retention signal, SEKDEL, was linked to the 3' end of the CTB gene (Arakawa *et al.*, 1997). The SEKDEL sequence was first shown to facilitate oligomerization of the LT enterotoxin B subunit in the plant (Haq *et al.*, 1995). Further ELISA experiments showed that addition of the ER retention signal to the 3' end of the NSP4 22 amino acid epitope significantly increased CTB–NSP4 fusion protein amounts in transformed plants, from 0.01% to 0.3% of TSP (Arakawa *et al.*, 1998a). Both serum and intestinal CTB-specific antibody titers were generated in response to immunization with 3.0 g of transgenic potato tubers delivered orally to mice (Arakawa *et al.*, 1998a). In more recent experiments, the CTB-coding sequence was integrated into the tobacco chloroplast genome (Daniell *et al.*, 2001a). The CTB protein accumulated in the transformed cells as oligomers up to 4.1% of TSP, a 410-fold increase in expression from unmodified LTB gene expression in the nuclear genome (Daniell *et al.*, 2001a).

The structure of cholera toxin was found to permit integration of antigen peptides into both A and B subunits. Replacement of the toxic A1 with a streptococcal adhesion protein generated an antibody response against the adhesion protein in mice immunized with the substituted holotoxin (Hajishengallis *et al.*, 1995). Immunoblot studies in our laboratory showed that the CTB and CTA subunits could assemble into oligomers of heterohexameric size in transformed potato plants (Chong and Langridge, 2001). Recognizing that the CTA2 and CTB components of CT were able to assemble

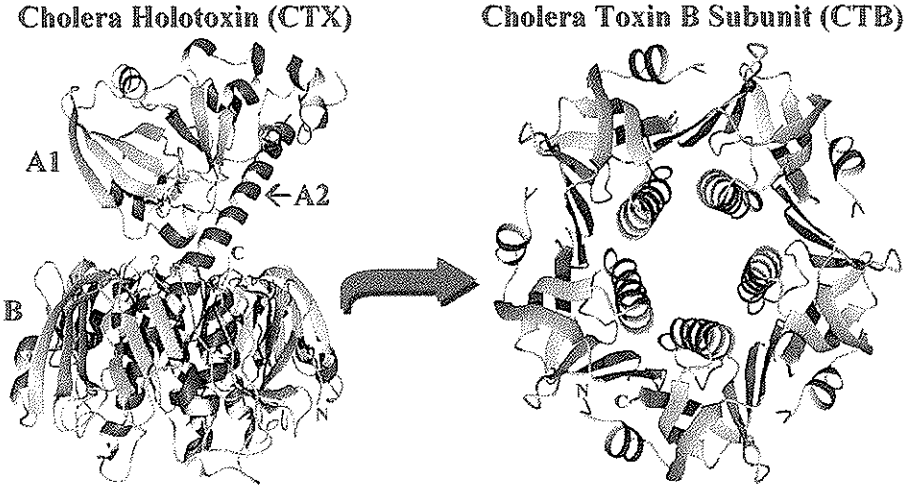

Figure 8.1. Three-dimensional structure of cholera toxin. The cholera holotoxin (left panel) consists of three peptide subunits, a 22 kDa cytotoxic subunit (A1), a 5 kDa helical linker subunit (A2), and a membrane receptor-binding pentameric B subunit consisting of five 11.6 kDa monomers (right panel). The A1 subunit is attached to the helical A2 subunit by a disulphide bond that is cleaved to release the toxic A1 subunit following holotoxin entry into intestinal epithelial cells (left panel). In the pentameric configuration (right panel), the B subunit binds to G_{M1} ganglioside receptors through interactions between side chains of the peptide loops located at the base of the pentamer (left panel). For vaccine construction, gene fragments encoding antigen proteins are fused to the C-terminal end of CTB monomers located at the top of the structure (C) (left and right panels) to avoid interference with G_{M1} ganglioside receptor binding at the base of the molecule (two lowest peptide loops in the left panel). In cholera toxin-based multi-component vaccines, a second antigen gene fusion is linked to the N-terminal end of the helical A2 subunit (left panel), extending out from the pocket formed by the B pentamers (left and right panels). The three-dimensional structure of cholera toxin (CTX) was obtained from the Protein Data Bank (Berman *et al.*, 2000) from structure data originally submitted by Zhang *et al.* (1995) (CTX) and Merritt *et al.* (1994) (CTB). The ribbon structures were generated with the program MOLMOL© (Koradi *et al.*, 1996) and rendered using POV-Ray™.

in the plant, Yu and Langridge were able to construct transformed potato plants capable of expressing two cholera toxin–antigen fusion proteins which assembled into oligomeric structures resembling the heterohexameric structure of CT (Yu and Langridge, 2001). In an effort to construct a multi-component vaccine in plants, a 22 amino acid immunodominant epitope from NSP4 ($NSP4_{114-135}$) was linked to the C-terminus of the CTB subunit, while the CTA2 subunit was linked to an ETEC colonization factor, fimbrial antigen CFA/I. The construct containing the two cholera toxin fusion genes was introduced into the potato genome by *Agrobacterium tumefaciens* transformation. Assembly of CFA/I–CTA2–CTB–NSP4 oligomers in the plants were detected by immunoblotting, and found to retain an affinity for G_{M1} ganglioside (Yu and Langridge, 2001). This experiment demonstrated the feasibility of generating enterocyte-targeted, multi-component vaccines in plants based on assembly of CT-like molecules. When tested for immune protection in mice, the plant-based vaccine generated detectable titers of protective IgA and IgG antibodies against all three antigens (Yu and Langridge, 2001, and recent unpublished data for CFA/1 binding inhibition). Passive immunization of mouse neonates, from orally

immunized dams, provided protection of the pups from subsequent CT and rotavirus challenge (Yu and Langridge, 2001). Secreted cytokine ELISA assays of antigen-stimulated splenocytes from the immunized mice showed a substantial increase in IL-2 and IFN-γexpression, and a reduction in IL-4 activity, indicating the presence of a strong Th1 response. Additional studies showed that genetically modified cholera toxins can promote priming of both Th1 and Th2 lymphocyte-mediated responses (Marinaro *et al.*, 1999).

The ability of the pentameric cholera toxin B subunits to bind G_{M1} ganglioside receptors on the enterocyte or M cell membrane makes it a strong ligand for antigen delivery to the mucosal immune system. In addition to its carrier functions, the CTB ligand appears to function as a strong adjuvant by enhancing antigen presentation by macrophages, and by stimulating increased expression of IL-12, IFN-γ, CD40, and CD86 on APCs (George-Chandy *et al.*, 2001). Adjuvanticity also seems to be linked with CTB's ability to stimulate MHC class II receptor expression, which is necessary for antigen-presenting cells to activate T helper cells in the presence of antigen (Francis *et al.*, 1992). Genetic linkage of the CTB ligand with an ovalbumin antigen showed that antigen dosage could be reduced by more than 10 000-fold, in comparison with the same amount of CTB delivered independently from the antigen (George-Chandy *et al.*, 2001). This observation emphasizes the strong adjuvant effect generated when the CTB ligand is physically linked to the antigen. The CTA1 subunit is known to possess strong, adjuvant-like qualities based on its toxic adenylate cyclase ribosylating ability, which results in abnormally high cAMP levels in cells (Holmgren *et al.*, 1993). Experiments by Rappuoli and his colleagues demonstrated that the mutation of specific amino acids in LTA and CTA subunits can remove A subunit toxicity, while preserving most of the adjuvanticity possessed by the two toxin subunits (Rappuoli *et al.*, 1999).

The strong adjuvant capability generated by CTB when genetically linked to an antigen provides additional support for results obtained earlier from our laboratory in which fusion between CTB and the rotavirus enterotoxin antigen NSP4, or the autoantigens proinsulin (INS) and glutamic acid decarboxylase (GAD65), generated greatly increased immunogenicity or immunotolerization against Type 1 autoimmune diabetes, respectively (Arakawa *et al.*, 1998a,b, 1999). Glutamic acid decarboxylase (GAD65) is a primary autoantigen involved in the development of insulin-dependent diabetes mellitus (IDDM). Fusion of this large (65 kDa) protein to a CTB subunit in transformed plants resulted in an approximate 50% reduction in autoimmune diabetes symptoms in non-obese diabetic (NOD) mice (Arakawa *et al.*, 1999). To facilitate CTB assembly into pentamer structures in the plant, a hinge oligopeptide was fused to the 3' end of the CTB gene to decrease steric hindrance contributed by the large GAD65 kDa peptide (Lipscombe *et al.*, 1991). Arakawa and his colleagues also included codons less frequently used in plants within the hinge DNA sequence to assist CTB subunit folding prior to translation of the large GAD65 protein (Purvis *et al.*, 1987; Arakawa *et al.*, 1999). The ER retention signal, SEKDEL, was also fused to the C-terminus of the GAD protein to facilitate accumulation of CTB–GAD fusion protein monomers in plant cells, increasing the chances for oligomerization among CTB subunits (Haq *et al.*, 1995). Comparison of these results suggest that CTB subunits linked to autoantigens can significantly suppress Th1 lymphocyte mediated autoimmune diseases by inducing immunotolerization to linked autoantigens

proinsulin or GAD65 (Arakawa *et al.*, 1998b, 1999). A partial list of pathogen antigens that have been synthesized in crop plants for use as mucosal vaccines for protection of animals and humans against infectious and autoimmune diseases is presented in *Table 8.2*.

Table 8.2. Plant-based vaccines against pathogens, toxins, and autoimmune disease

Plant	Protein	% TSP†	Reference
Alfalfa	Foot-and-mouth disease virus (FMDV) structural protein (VP1)	–	Wigdorovitz *et al.*, 1999a
Arabidopsis	Canine parvovirus capsid protein (VP2)	3.0	Gil *et al.*, 2001
	Foot-and-mouth disease virus (FMDV) structural protein (VP1)	–	Carrillo *et al.*, 1998
	Swine-transmissible gastroenteritis coronavirus (TGEV) glycoprotein S	0.06	Gomez *et al.*, 1998
Clover	*Mannheimia haemolytica* A1 leukotoxin	1.0	Lee *et al.*, 2001
Corn	Enterotoxigenic B subunit of *E. coli* (LTB)	–	Streatfield *et al.*, 2001
	Swine-transmissible gastroenteritis coronavirus (TGEV) glycoprotein S	40.0•	Streatfield *et al.*, 2001
Lettuce	Hepatitis B surface antigen (HBsAg)	0.0055*	Kapusta *et al.*, 1999
Lupin	Hepatitis B surface antigen (HBsAg)	0.15*	Kapusta *et al.*, 1999
Potato	Cholera toxin B subunit (CTB)	0.3	Arakawa *et al.*, 1997
	CTB-proinsulin fusion – Type 1 diabetes	0.1	Arakawa *et al.*, 1998b
	CTB-GAD_{65} fusion – Type 1 diabetes	0.001	Arakawa *et al.*, 1999
	CTA_2, *E. coli* fimbrial antigen (CFA/1), and rotavirus enterotoxin (CTB-NSP4) – multi-component	0.01	Yu *et al.*, 2000
	Enterotoxigenic B subunit of *E. coli* (LTB)	0.01	Haq *et al.*, 1995
		15.7*	Haq *et al.* 1995
		1.9	Tacket *et al.*, 1998
		13.0*	Mason *et al.*, 1998
	Glutamic acid decarboxylase (GAD_{65}) – Type 1 diabetes	0.4	Ma *et al.*, 1997
	Hepatitis B surface antigen (HBsAg)	0.25	Richter *et al.*, 2000
	Human insulin – Type 1 diabetes	0.05	Arakawa *et al.*, 1998b
	Norwalk virus capsid protein	5.0*	Tacket *et al.*, 2000
	Rabbit haemorrhagic virus structural protein (VP60)	0.3	Castanon *et al.*, 1999
	Swine-transmissible gastroenteritis corona virus (TGEV) glycoprotein S	0.07	Gomez *et al.*, 2000
Rice, wheat	Human carcinoembryonic antigen (CEA) antibody	30.0*	Stoger *et al.*, 2000
Soybean	Herpes simplex virus-2 (HSV-2) glycoprotein B antibody	–	Zeitlin *et al.*, 1998
Tobacco	Cholera toxin B subunit (CTB)	4.1	Daniell *et al.*, 2001a
	Enterotoxigenic B subunit of *E. coli* (LTB)	14.0*	Haq *et al.*, 1995
	Glutamic acid decarboxylase (GAD_{67}) – Type 1 diabetes	0.4	Ma *et al.*, 1997
	Hepatitis B surface antigen (HBsAg)	0.01	Mason *et al.*, 1992
	Human carcinoembryonic antigen (CEA) antibody	5.0*	Vaquero *et al.*, 1999
	Human cytomegalovirus (HCMV) glycoprotein B	146.0*	Tackaberry *et al.*, 1999
	Measles virus haemagglutinin H protein	–	Huang *et al.*, 2001
	Norwalk virus capsid protein	0.23	Mason *et al.*, 1996
	Streptococcus mutans *adhesion protein antibody*	80.0*	Ma *et al.*, 1998
	Swine-transmissible gastroenteritis corona virus (TGEV) glycoprotein S	0.2	Tuboly *et al.*, 2000
Tomato	Rabies virus glycoprotein G	0.001	McGarvey *et al.*, 1995
	Respiratory syncytial virus (RSV) F protein	32.5*	Sandhu *et al.*, 2000

†TSP = total soluble protein of the plant (unless noted).
*μg/g fresh weight plant tissue.

Generating vaccines in the chloroplast

A novel method for increasing foreign protein gene expression in transgenic plants, based on the transfer of antigen genes into plants using tobacco chloroplast vectors, was recently introduced (Guda *et al.*, 2000). The choloroplast genome offers the ability to express multiple genes during a single transformation event. Further, foreign proteins synthesized in the chloroplast have been shown to accumulate up to 45.3% of TSP (de Cosa *et al.*, 2001). Site-specific insertion of the 4.0 kb *Bacillus thuringiensis* cry2Aa2 operon into the tobacco chloroplast genome not only demonstrated a high level of protein accumulation, but did so without interference with the functions of the chloroplast genome (de Cosa *et al.*, 2001). Integration of DNA into the highly polyploid choloroplast genome can result in cDNA replication numbering from 5 to 10 thousand copies of the gene per cell, further emphasizing that targeting antigens to the chloroplast has the potential to generate a high level of protein synthesis in transgenic plants (Kota *et al.*, 1999). The high levels of protein synthesis in the chloroplast or other plastids in transformed plants provides direct evidence that gene silencing in the transgenic chloroplasts does not occur to a significant extent (de Cosa *et al.*, 2001).

Many concerns about generating genetically modified plants and the potential spread of the modified gene to other related plants were quelled when only a low incidence of chloroplast exchange was reported (0.6–0.7%) between the oilseed rape (*Brassica napus*) and *Brassica rapa* (Scott and Wilkinson, 1999). Chloroplast movement from *B. napus* into the wild *B. rapa* demonstrated that chloroplast DNA is limited to maternal transmission, and therefore not normally transferred by pollen (Scott and Wilkinson, 1999). Additional research suggests that, although low levels of pollen from the crossed plants may contain maternal chloroplast genes that are metabolically active, this DNA is lost during pollen maturation, and therefore not transferred among different plants (Daniell *et al.*, 2001b, 2002). However, chloroplast containment has not been shown to be applicable to all plant species, including alfalfa, which exhibits biparental inheritance. Therefore, further research must be done to find and assess other methods to prevent genetically modified crops from transferring recombinant genes through their pollen (Daniell *et al.*, 2001c).

With the concern for generating antibiotic-resistant pathogens on the rise, considerable pressure has been placed on the scientific community to decrease the use of genes encoding antibiotic resistance as selectable markers for identification of plants containing transformed gene products. A recent alternative, the betaine aldehyde dehydrogenase (BADH) gene, is an enzyme occurring naturally in spinach and sugar beet, which can be used as a marker gene flanking the genes of interest (Rathinasabapathi *et al.*, 1994). Selective identification of plants containing transformed genes targeting the chloroplast can be obtained as BADH converts toxic betaine aldehyde into a non-toxic glycine betaine molecule which selects transformed plants based on the toxic nature of betaine aldehyde (Rathinasabapathi *et al.*, 1994). Plants receiving the BADH selective marker gene responded faster in the betaine aldehyde environment, generating shoots on 80% of the transformed leaf discs 12 days post-transformation. Plants selectively marked with spectinomycin-resistant genes synthesized shoots on only 15% of leaf discs 45 days after incubation in spectinomycin (Daniell *et al.*, 2001b). Using BADH-resistant genes versus spectinomycin might also

permit the application of chloroplast transformation to additional crops, such as the cereals, known to be more resistant to spectinomycin (Daniell *et al.*, 2001b). Although there is considerable variability in foreign protein biosynthesis among plant species and between different transgenes, the use of plastid DNA as a vector for the expression of foreign DNA and protein synthesis holds promise for substantially increasing the amount of antigen protein synthesized in transformed plants, a result which could lead to a substantial improvement in the protective efficacy of plant-based vaccines.

Conclusions

Mucosal immunization experiments in animals and preliminary clinical trials in humans have demonstrated plant-based vaccine delivery of significant, but incomplete, immune protection against infectious and autoimmune diseases. Plastid transformation, antigen gene codon optimization for plants, and removal of RNA splice sites may lead to substantial increases in the amount of antigen protein synthesized in plants. Methods for targeting antigen molecules to the mucosal immune system via enterocyte binding ligand–antigen fusion proteins, and stimulation of mucosal immunity through co-immunization with genes encoding adjuvants when combined with current plastid transformation methods, may provide greatly enhanced or complete protection against infectious and autoimmune diseases. The determination of optimal vaccine dosage of plant-based vaccines will minimize unwanted immune responses, such as immunotolerance to pathogen antigens, undesirable immune responses to autoantigens, and the development of allergic reactions. These problems must be overcome before the efficacy and safety of plant-based vaccines for human protection can be assured in the clinic. Achievement of these goals will provide mucosal vaccines, which are effective, safe, inexpensive, and easy to administer to a rapidly expanding human population in desperate need of improved health care.

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