Chapter 8

SUPPRESSION OF AUTOIMMUNE DIABETES BY THE USE OF TRANSGENIC PLANTS EXPRESSING AUTOANTIGENS TO INDUCE ORAL TOLERANCE

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Abstract The use of transgenic plants expressing autoantigens to induce protective oral immune tolerance represents a novel approach for antigen-specific therapy of autoimmune disease. A transgenic plant-based strategy offers several attractive advantages for oral tolerance, including relatively inexpensive production of large amounts of soluble protein antigens essential for the clinical application of oral tolerance, and absence of potential pathogens and endotoxins which are issues in the production of therapeutic proteins from animal tissues or bacterial cells. Plants also offer a simple and efficient system for the direct oral delivery of human proteins for immune modulation which in many cases will not require extensive and expensive protein purification steps. Here we discuss strategies to prevent autoimmune diabetes by oral delivery of diabetes-associated autoantigens such as glutamic acid decarboxylase (GAD) and insulin in transgenic plants. These principles and approaches may be relevant to other clinical indications such as transplant rejection, allergy and the delivery of bio-therapeutics in inflammatory bowel disease.

1. INTRODUCTION

Type 1 (insulin-dependent) diabetes mellitus (IDDM) is a chronic disease caused by the progressive immunological destruction of insulin-secreting

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pancreatic beta cells by autoreactive T lymphocytes. A number of islet cell target autoantigens have been identified including glutamic acid decarboxylase (GAD), insulin, and tyrosine phosphatase-like IA-2 which play a role in the initiation and maintenance of the pathogenic events that lead to IDDM (Atkinson and Maclaren, 1994; Bosi and Botazzo, 1995). In the non-obese diabetic (NOD) mouse model, which closely resembles human IDDM, overt diabetes is preceded by a period in which there is a characteristic lymphocytic infiltration of pancreatic islets termed insulitis. This is a period in which intervention can alter disease and results in this mouse model have suggested that there may be a potential for immunological intervention and prevention of IDDM in clinical practice.

Although immunosuppression with specific drugs, such as cyclosporine A, can alter T-cell immune responses and thus inhibit the development of diabetes in NOD mice and humans, therapies that more specifically target the destructive immune responses which cause organ specific disease but without causing global immunosuppression would be much safer and therefore more desirable to patients. The adverse effects of all general immunosuppressive drugs include increased rates of infection and cancer, as the immune system is critical for surveillance against such "dangers". Interestingly, it has been demonstrated that specific peripheral immune responses can be reduced to orally administered proteins leaving the immune system intact for responses to pathogens. The mechanisms involved in this endogenous immune regulatory system have been collectively referred to as "oral immune tolerance".

An approach using oral immune tolerance to islet cell autoantigens including glutamic acid decarboxylase (GAD) and insulin, has been shown by our laboratory and others to prevent the spontaneous onset of diabetes in NOD mice. This method of treatment may therefore represent a convenient and potentially effective antigen-specific therapeutic modality for human autoimmune diabetes. However, to a large extent, the clinical success of this approach as a therapy may be determined by practical issues such as the availability of suitably large amounts of soluble protein antigens at low cost, the efficiency of oral tolerance induction by proteins versus peptides and the establishment of a simple delivery system. Recently, we have explored transgenic plants as a potential cost-effective expression system for production and delivery of the diabetes-associated autoantigen, mouse GAD67. We have shown that transgenic plants can express GAD in an immunologically active form, and that feeding transgenic plant tissues containing GAD can protect

NOD mice from diabetes (Ma et al., 1997). This first demonstration of the use of transgenic plants to induce protective oral tolerance may have an important impact on future treatment strategies for diabetes and many other autoimmune disorders, allergy and organ transplant rejection. Here we discuss some of the critical aspects of the plant-based approaches for production of diabetes-associated β-cell autoantigens as well as their application in the induction of oral immune tolerance to the prevention of animal diabetes. This new technology may one day play an important role in the therapy and cure of human Type 1 diabetes. A discussion is also provided of our recent progress and new developments in the area.

2. ORAL TOLERANCE AND THE TREATMENT OF AUTOIMMUNE DISEASES

Oral tolerance refers to the oral administration of protein antigens, which induces a state of systemic non-responsiveness specific for the fed antigen. The phenomenon of orally induced tolerance was first described by Wells in 1911 (Wells, 1911). In these early studies, Guinea pigs fed hen proteins or dinitrochlorobenzene (DNB) did not develop responses to subsequent systemic challenges with the same antigen. Later, it was shown that the ingestion of a wide range of proteins can lead to attenuated or absent systemic immune responses to those particular antigens (Mowat, 1987). It is now well established that oral tolerance is an important natural physiological property of the immune system, whereby the host can avoid dangerous reactions such as DTH (delayed-type hypersensitivity) to "non-dangerous" antigens and other substances encountered in our diets (Mowat, 1987).

A milestone in the area of oral tolerance was the recognition that self-antigens could be administered to prevent and treat autoimmune diseases. Induction of oral tolerance in animal models has been used in the prevention of several experimental autoimmune diseases, such as rodent (NOD) diabetes (Bergerot et al., 1994; Zhang et al., 1991; Bergerot et al., 1994), uveo-retinitis (Nussenblatt et al., 1990), experimental autoimmune encephalomyelitis (EAE) (Higgins and Weiner, 1988), and rheumatoid arthritis (RA) (Thompson and Staines, 1986). Induction of oral tolerance in humans was also demonstrated using the nominal antigen keyhole limpet hemocyanin (KLH). Human volunteers, when orally given KLH, showed decreased T cell proliferative

responses and DTH reactivity specific to KLH (Husby et al., 1994). These early studies have led to clinical trials that test oral antigen administration in human autoimmune diseases. Trials have been conducted in multiple sclerosis, rheumatoid arthritis, juvenile rheumatoid arthritis, inflammatory uveitis, autoimmune thyroiditis, and insulin-dependent diabetes mellitus. Importantly no toxicity or exacerbation of disease has been observed following oral administration of autoantigen (Weiner, 1997), although this remains a potential concern. Although improvement has been noted in some patients treated with oral antigen, predictable clinical improvement has not been observed in controlled trials. However, it is clear that the choice of antigen (requirement for purified form of antigen), dose of antigen (low-dose versus high-dose tolerance), and timing of antigen administration (before or during early stages of disease) have an effect on the therapeutic efficacy of oral tolerance. Although there are still many studies required, it is likely that the major effectiveness of oral tolerance as a therapy will be in the prevention of disease in those with an identified increased risk. This includes diseases in which the relevant antigens have been well defined in animal and human studies, such as in the case of Type 1 diabetes with clear genetic linkage to HLA loci and demonstrable early evidence of immune reactivity to antoantigens such as GAD. This disease also has the benefit of having the NOD model which is spontaneous, and is genetically very similar at several key loci, to human Type I autoimmune diabetes.

Although there are several mechanisms identified in oral tolerance, a primary factor that determines efficacy is the dose of antigen administered. Tolerance to high doses of antigen is mediated by the inactivation or clonal deletion of T helper (Th1) cells characterized by the production of interleukin-2 (IL-2) and interferon-γ (IFN-γ). Th1 autoimmune T cells have been associated with the pathogenesis of autoimmune diabetes (Weiner et al., 1994). In contrast, tolerance in response to "lower dose" antigen involves the induction of regulatory T cells which can produce Th2 type cytokines (interleukin-4 (IL-4) and interleukin-10 (IL-10)) or Th3 type cytokines such as transforming growth factor-β (TGF-β) in response to antigen (Weiner et al., 1994). Both Th2 and Th3 cytokines may function by preventing Th1 reactivity rather than having a direct beneficial effect. Oral tolerance can also be enhanced by the use of mucosal adjuvants. The most commonly used mucosal adjuvant is cholera toxin B subunit (CTB) (Czerkinsky et al., 1996; Holmgren et al., 1993; Sun et al., 1994). Cholera toxin (CT) consists of a toxic 27 kDa A subunit (CTA) having

ADP ribosyl transferase activity and a non-toxic pentamer comprised of 11.6 kDa B subunits (CTB) that bind to the A subunit and facilitates its entry into intestinal epithelial cells. CTB given simultaneously with a variety of antigens orally can enhance oral tolerance and allows a reduction in the antigen dose and dosing schedule (frequency of antigen feeding) required for tolerance induction (Sun et al., 1996; Bergerot et al., 1997). One mechanism of such enhancement is that CTB bound to antigen, by virtue of its capacity to bind to eukaryotic cell surfaces *via* GM1 ganglioside receptors present on intestinal epithelial cell surfaces, focuses or concentrates antigen in specialized mucosal cells with antigen presentation capacity and thus facilitates the development of immune tolerance. However, responses may be limited as the immune system will have neutralizing effects on any components of infectious agents which may represent "danger." Th2 cytokines have been examined for possible effect in the enhancement of oral tolerance, because of their known roles in differentiating precursor Th0 cells toTh2 lineages (Mosmann and Coffman, 1989). Parenterally administered IL-4, oral IL-4, and oral IL-10 can enhance oral tolerance (Weiner et al., 1994).

3. UNIQUE ADVANTAGES OF USING TRANSGENIC PLANTS FOR ORAL TOLERANCE INDUCTION

The induction of oral immune tolerance requires the ingestion of large amounts of soluble protein antigens. To a large extent the clinical application of oral tolerance induction as a potential therapeutic strategy may depend on the availability of the target proteins in sufficient quantities and at an economical cost. Conventional protein production systems, while capable of producing such amounts of protein, are unlikely to be cost effective enough to allow clinical studies. Therefore, an important factor for our group in considering the application of oral tolerance therapeutically was to devise a production system capable of generating large quantities of immunologically and, in some cases, biologically active proteins at low cost. Presently, several heterologous protein production systems are available, such as bacteria, yeast, and mammalian and insect cell cultures. In these conventional production systems, sufficiently high expression levels of natively folded mammalian proteins are often limited by the inability to form disulfide bridges or to add glycans to the recombinant proteins. Also, there is a high cost associated with

the use of sophisticated equipment (i.e. fermentors) and sterile culture media, absolute exclusion of pathogenic viruses such as HIV and hepatitis B, and removal of other harmful substances such as bacterial endotoxin. All recombinant proteins derived from these systems need to be purified before clinical use, which further adds expenses to the production cost.

The use of transgenic plants as an alternative option to conventional cell culture-based systems has recently attracted much interest (Goddijn and Pen, 1995). Transgenic plant systems have the highest potential for large-scale and cost-effective production of many recombinant proteins. Plants can be contained, grown easily and inexpensively in large quantities, can be harvested and processed with available agronomic infrastructures, and scaling up is simple. Plants, as higher eukaryotes, also have a crucial advantage over bacteria in being able to perform many of the complex protein processing steps, such as isoprenylation, oligomerization, disulfide bridge formation and proteolytic cleavage as in a mammalian system. This factor may be particularly important in producing recombinant proteins that have *in vivo* activities identical to their animal counterparts. Plants do not harbor infectious agents such as viruses and prions harmful to humans as these agents cannot replicate in plants. Safety is a primary concern when any therapeutic proteins for human use are prepared from animal tissues or bacterial cells, regardless of the route of administration. More importantly, as the production of antigenic proteins for oral tolerance can be targeted to edible transgenic plants, there is no need for extensive purification resulting in a major cost reduction. In the last few years it has been shown that plants are capable of synthesizing a wide range of valuable recombinant products, such as monoclonal antibodies (Hiatt et al., 1989; Ma et al., 1995), biopharmaceuticals such as enkephalin, α-interferon, human serum albumin (HAS), vaccines (Mason et al., 1992; McGarvey et al., 1995) and costly drugs used in the "orphan disease" group of rare diseases: human glucocerebrosidase (Cramer et al., 1996) and murine granulocyte-macrophage colony-stimulating factor (Lee et al., 1997). Recently there have been two successful human trial demonstrations of prototype plant "edible vaccines" for the prevention of infectious diarrheal illness. Potatoes containing the binding subunit of the heat-labile enterotoxin of *E.coli* (LT-B) were fed uncooked to volunteers and serum and secretory antibodies specific for LT-B were induced (Tacket et al.,1998). In separate clinical studies, volunteers who ate uncooked potatoes containing the Norwalk virus capsid protein (causal agent of epidemic gastroenteritis) developed both serum and secretory antibodies specific to the capsid protein

(Tacket et al., 2000). Both these human trials as well as earlier animal trials (Haq et al., 1995; Mason et al., 1996; Arakawa et al., 1998; Gomez et al., 2000; Richter et al., 2000; Sandhu et al., 2000), suggest that recombinant antigens expressed in transgenic plants can be delivered as food into the gut mucosa, and are capable of inducing immune responses. It is also possible that autoantigens delivered in transgenic plants will be capable of altering harmful immune responses.

4. TRANSGENIC PLANTS EXPRESSING GAD

Foreign gene expression in plants can be achieved either by transformation of the nuclear genome by stable integration of a foreign DNA using *Agrobacterium*-mediated transformation, direct gene transfer *via* particle bombardment and electroporation, or by transient expression using genetically modified plant viruses (Porta and Lomonossoff, 1996). *Agrobacterium*-mediated transformation is the most frequently used approach to transfer the desired DNA into a plant genome. Since the first report on tobacco transformation via *Agrobacterium* in 1985 (Horsch et al., 1985), this approach has been successfully used to transform many other plant species, such as tomato and potato. However, a number of agronomically important plant species, such as soybean, rice and wheat, are still resistant to *Agrobacterium*-mediated transformation but can be transformed with direct gene transfer techniques (Barcelo and Lazzeri, 1998). Most of the foreign protein-producing plants reported to date have been generated by stable transformation. The main advantages of stable transformation include the capacity to generate a large number of independent transgenic plants at a time, ease at maintaining transgenic plants expressing high levels of recombinant protein and the ability to sexually cross transgenic lines to obtain a single plant simultaneously expressing multiple proteins. Transient expression using genetically modified plant viruses as expression vectors allows higher levels of protein expression rapidly, but requires constant inoculation of large numbers of plants with the modified virus. For the delivery of oral vaccines or "tolerogens" in humans, plant viruses may be an option. To date there have been no reports linking plant virus transmission to any animals.

To develop transgenic plants expressing β cell autoantigens, we chose mouse GAD67 as an initial candidate. The justification for utilizing mouse

GAD67 was twofold. First, in rodents and in humans, two isomers of GAD namely GAD65 and GAD67, have been identified, and both are implicated in the pathogenesis of IDDM (Tisch et al., 1993; Kaufman et al., 1993; Elliott et al., 1994). However, there is a variation in their expression in the islets of humans and animals. Both human and rat islets predominantly express GAD65, whereas GAD67 is the major isoform in mouse islets (Kim et al., 1993). Secondly, as GAD67 represents a major isoform of GAD in mouse, and as it was shown that administration of purified GAD67 to young NOD mice specifically prevented development of diabetes (Elliott et al., 1994), the selection of mouse GAD67 for initial expression in plants facilitated the design of animal experiments for testing plant-derived GAD. To create transgenic plants expressing GAD, mouse GAD67 cDNA was first used to replace the β-glucuronidase gene in pTRL2-GUS composed of a CaMV 35S promoter with a double enhancer sequence linked to a 5' untranslated tobacco *etch* virus leader sequence, GUS and a nopaline synthase (NOS) terminator (Carrington and Freed, 1990). The resulting expression cassette was then inserted into the plant expression binary vector, pBin19 (Bevan, 1984). The mouse GAD67 cDNA was introduced into tobacco and potato plants by *Agrobacterium tumefaciens*-mediated leaf explant transformation method (Horsch et al., 1985). Integration of mouse GAD67 cDNA into the plant nuclear genome was confirmed by Southern blot, and the expression of full-length GAD67 mRNA transcript was confirmed by northern analyses. Western blots of protein homogenates from tobacco leaf and potato tuber tissues showed a single protein band of the correct size. The expression level of GAD was estimated from blot densitometry to be approximately 0.4% of total soluble protein. GAD67 expression was similar in tobacco leaf and potato tubers, and was not detected in untransformed control plants.

5. ORAL IMMUNOGENICITY OF THE PLANT DERIVED GAD PROTEIN

GAD is an enzyme that catalyzes the decaboxylation of glutamic acid to produce γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the brain (Baekkeskov et al., 1990). However, the immunogenicity of GAD is not dependent on its enzymatic activity but rather its primary amino acid sequences which form immunodominant epitopes upon processing in antigen

presenting cells *in vivo*. To assess whether the plant-derived recombinant GAD retained its ability to alter T-cell responses, transgenic tobacco leaf tissues or potato tubers were added to the diet of NOD mice. The amount of GAD delivered was estimated to be approximately 1 mg per day per mouse which represents a very large amount of antigen, and is much higher than would be delivered relatively to humans. After 4 weeks of supplementation, NOD mice were immunized in the hind footpads with highly purified *E. coli* -derived recombinant GAD67 emulsified in incomplete Freund's adjuvant (IFA). Ten days later mice were sacrificed, spleen and lymph node T cells were isolated and analyzed for their capacity to proliferate in response to GAD67 *in vitro*, in "recall assays". As anticipated, proliferation of T cells isolated from GAD67 plant-fed mice was markedly reduced, while T cells from control plant-fed mice retained capacity to proliferate in response to GAD67. Mice were also assessed for anti-GAD antibody responses. Although serum levels of total anti-GAD IgG antibodies had little change in mice fed control plants, there was a twofold increase in anti-GAD IgG antibody in mice fed GAD67 transgenic plants as compared to that in control mice. Further analysis of the isotypes of anti-GAD IgG antibody revealed that the increased levels of serum IgG anti-GAD antibody production in mice fed GAD plants were due to an increase in anti-GAD IgG1 antibody, consistent with skewing of responses to Th2. No changes in IgG2a (Th1) anti-GAD antibody were found between GAD plant and control plant-fed groups. Moreover, cytokine analyses of supernatants derived from T cells of treated mice showed that the concentration of IFN-γ was reduced with an increase of IL-4 and IL-10 in mice fed GAD plants as compared to that in control mice. Taken together, these results confirmed that transgenic plants were capable of synthesizing the diabetes-associated autoantigen, mouse GAD67, in an immunogenic form and that oral delivery of the recombinant GAD protein through transgenic plant tissues to NOD mice was effective in inducing antigen-specific immune responses.

To determine the effects of transgenic plant tissues producing GAD67 on the suppression of diabetes, young pre-diabetic female NOD mice were fed transgenic potato tuber or transgenic tobacco leaf tissues as a diet supplement to standard mouse chow for a period of seven months starting at 5 weeks of age. The amount of GAD delivered was approximately 1mg per mouse daily. Control mice received an equivalent amount of corresponding vector control transgenic tobacco or potato tissue. NOD mice develop diabetes spontaneously and at age of 6 to 7 months; typically 75 to 85% of the female mice will become

diabetic (Makino et al., 1981). As shown in Figure 34, 10/12 NOD mice fed GAD-containing potato tuber (n=6) or GAD-containing tobacco leaf tissues (n=6) remained free of disease (p=0.007 from controls). In contrast, 8 / 12 control plant-fed mice (67%), divided between potato and tobacco supplementation, developed diabetes. The mice tolerated plant tissue well, and no differences between groups were observed in the appearance or weight gains of mice.

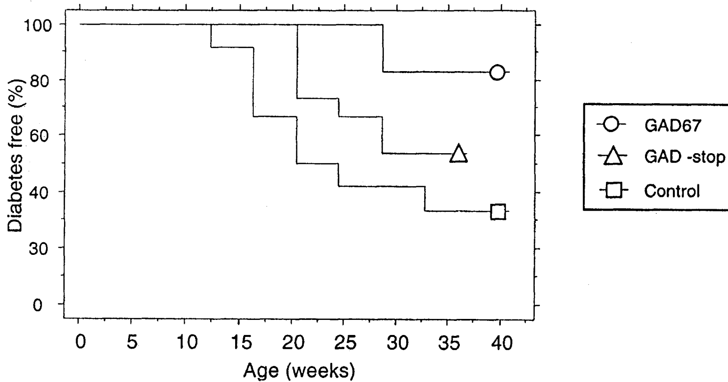

Figure 34. Oral administration of plant material expressing mouse GAD67 prevents development of diabetes in NOD mice. Female NOD mice (n=12/group) were fed plant GAD67 or control plant material from 5 weeks to 8 months of age. All mice were followed for the onset of hyperglycemia (defined as blood glucose > 16.7 mmol./l). 10/12 NOD mice fed either low-alkaloid tobacco (n=6) or potato GAD67 (n=6) remained free of disease (p=0.007 from controls) with equivalent protection using tobacco or potato. In contrast, 8/12 control plant-fed mice developed diabetes. In separate experiments, NOD mice were given GAD67 plant material for about 2 months (8 week duration) followed by standard chow, they showed a delay in disease-onset, but the incidence level of diabetes eventually returned to that of control groups (shown as "GAD-stop").

Although it is difficult to compare results between different laboratories and protocols, these results, are comparable to those previously reported with other methods of GAD immunization (Elliott et al., 1994; Kaufman et al., 1993; Tisch et al., 1993; Tian et al., 1996; Zhang et al., 1991), and suggest that oral plant GAD67 may offer an effective alternative approach to treatment of IDDM. There has been more work performed in the nature of protection. In separate experiments, NOD mice were given GAD 67 transgenic plant tissues for only two months starting at 5 weeks and followed by standard chow. While there was a delay in disease onset, the incidence level of diabetes eventually returned

to that of control groups by the study completion, suggesting that continuous GAD feeding is necessary in order to maintain the tolerance status, at least when using "high-dose" GAD.

6. EXPRESSION OF CHOLERA TOXIN B SUBUNIT (CTB)-INSULIN FUSION PROTEINS

Insulin is a major constituent of β cells in the pancreas and is a true "auto" antigen in that it is produced by β-cells and not in surrounding cells. Like GAD, it appears to have an important role in the pathogenesis of diabetes. Injection of NOD mice with insulin or insulin peptides was shown to inhibit both insulitis (an early stage of diabetes involving infiltration of the islet cells with inflammatory lymphocytes) and the subsequent development of diabetes (Atkinson et al., 1990). Insulin therapy by injection has also been shown to delay IDDM in humans (Keller et al., 1993), and such observations have directed clinical trials in diabetes prevention with insulin. Oral insulin therapy has the advantages of easy administration and lack of toxicity. Recently, the results of the first multi-centred human clinical trial (Diabetes Prevention Trial Type 1) testing oral insulin in newly diagnosed diabetes patients were released (Chaillous et al., 2000). While no improvement was seen, continued testing of the effects of immune interventions in recent-onset type 1 diabetes was recommended as there were several possible explanations for a lack of effect. Doses and formulation of oral insulin for example may not have been adequate, or different modalities of oral administration of insulin are required. Recently, Arakawa et al. (1998) generated transgenic potato plants synthesizing a fusion protein consisting of human insulin and the CTB subunit. Transgenic potato tubers produced 0.1% of total soluble protein as the pentameric CTB-insulin fusion protein, which retained GM1-ganglioside binding affinity and native antigenicity of both CTB and insulin. NOD mice fed transgenic potato tuber tissues containing small (μg) amounts of the CTB-insulin fusion protein showed a reduction in insulitis, and a delay in the progression of clinical diabetes, while those receiving transgenic potato tissues producing insulin or CTB protein alone were not protected.

It appears therefore that insulin conjugated with a CTB subunit can suppress development of diabetes in NOD mice at lower doses than that required for insulin alone, which may be a useful strategy in cases when auto-antigen

expression is limiting in transgenic plants. Again, a possible concern with the use of CTB as a mucosal adjuvant or carrier molecule for conjugated antigens in the induction of oral tolerance, is that prolonged feeding of CTB may induce neutralizing mucosal and systemic antibodies specific for CTB and prevent long lasting tolerance induction. In NOD mice fed transgenic potato tubers containing CTB-insulin fusion protein, serum and mucosal anti-CTB antibodies were detected (Arakawa et al., 1998).

7. EXPRESSION OF HUMAN GAD65

Recently the expression of human GAD65 in transgenic tobacco and carrot plants has been reported (Porceddu et al., 1999). Western analysis of tobacco leaf homogenates revealed a unique polypeptide which is comparable in size to rhGAD65 produced in the baculovirus-insect cell expression system. The expression level of human GAD65 in transgenic tobacco leaf reached 0.04% of the total soluble protein, while in carrot taproots it only accounted for 0.015% of the total protein. It appears that even in tobacco, the expression level of human GAD65 is lower than those we reported for mouse GAD67 (0.4%). The difference in expression may reflect structural differences between the two isoforms. Human GAD65 is membrane-anchored by signals located in the NH_2-terminal region (Namchuk et al., 1997; Shi et al., 1994), while GAD67 is a cytosolic isoform of GAD. The association of GAD65 with membranes in human cells is mediated by way of protein-protein interactions, with accessory effects contributed by palmitoylation (Namchuk et al., 1997). Interestingly, immunogold labeling and electron microscopy of transgenic tobacco tissue showed the selective targeting of human GAD65 to chloroplast thylakoids and mitochondria, supporting the existence of similar protein-protein interactions in membranes of plant cells. Based on studies using human autoantibodies directed against conformational epitopes of the autoantigen (Falorni et al., 1996) and the immunoenzymatic assay indicated that human GAD65 is folded correctly in plants. At present there are no data available on animal trials determining if oral administration of human GAD65 transgenic plants can induce protective oral tolerance in NOD mice, but it is clear it is feasible to use transgenic plants to produce a bioactive form of human GAD65. This is a primary focus of our efforts along with optimization of oral tolerance through novel immune augmentation strategies.

8. CONCLUSIONS AND FUTURE PROSPECTS

The use of transgenic plants and oral delivery offers a promising new strategy for tolerance induction for the treatment of autoimmune diseases such as type I diabetes. A plant-based approach for inducing oral tolerance has the advantage of being effective, simple, low-cost, safe from pathogens and non-invasive. This approach may hold promise for the prevention and/or treatment of other auto-immune diseases, allergy and organ transplant rejection, but these need to be determined by a case by case basis. The availability of relevant animal models with spontaneous rather than induced disease may be critical to antigen selection and testing. We have recently expressed murine major histocompatibility complex (MHC) class II molecules in transgenic plants which may allow us to use the plant-based approaches to induce oral tolerance induction for the prevention of organ transplant rejection (Ma and Jevnikar, 1999).

As oral tolerance induction is usually less efficient in previously sensitized hosts (Zhang et al., 1991), one of the obvious challenges is to identify those at risk and treat prior to disease onset. It is also important to create plants with high expression levels of recombinant mammalian proteins. However, a persistent problem in the expression of antigenic proteins in transgenic plants has been low levels of expression, but several strategies are available to overcome this problem. Other areas that need particular attention includes selection of appropriate plant species as expression hosts, the identification and isolation of novel, more robust plant promoters, strategies to address gene silencing, and strategies to maximize transgene protein accumulation in plant cells. Tobacco chloroplasts have been recently used in high-yield production systems for several therapeutic proteins. Human somatotropin can be expressed as a soluble, biologically active, disulfide-bonded form in tobacco chloroplasts, reaching 7% total soluble protein. This is more than 300-fold higher than a similar gene expressed using a nuclear transgenic approach (Staub et al., 2000). Similarly, tobacco chloroplast transformation with an unmodified CTB-coding sequence resulted in the accumulation of up to 4.1% of total soluble leaf protein as functional CTB oligomers, again which is approximatley 400-fold higher than with related LTB (the B subunits of enterotoxigenic *Escherichia coli*) expressed via the nuclear genome (Daniell et al., 2001). These are very

promising results, as high-level expression of many antigenic proteins may now be possible in a system which also offers reasonable gene containment.

Oral tolerance has clear benefits in animals, but has not yet been conclusively shown in humans. One limitation of this strategy, namely cost and delivery, is well addressed by plant systems. It is likely that we will need to enhance the efficacy of oral tolerance in clinical autoimmune disease including the use of combined antigens, the use of adjuvants like CTB and other strategies to skew the response to Th2 when this is known to be beneficial. Plants are not only a competitive production system, but also a convenient and effective delivery system. We are very hopeful that effective immunotherapies for human autoimmune diseases will soon include therapy based on transgenic plants expressing autoantigens, given as a simple dietary supplement.

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