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Reverse Vaccination and Treatment of Type 1 Diabetes Using Plant-Produced Autoantigens and Anti-inflammatory Cytokines

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Abstract

Type 1 diabetes (T1D) is an autoimmune form of diabetes mellitus that accounts for about 10% of all diabetes cases and occurs predominantly during childhood or adolescence. The disease initiates when most of the insulin-producing β-cells in the pancreas are destroyed by autoimmune cells, including T-helper 1 cells, cytotoxic lymphocytes and dendritic cells. The major autoantigens in T1D are insulin, glutamic acid decarboxylase, and insulinoma antigen. At-risk individuals with underlying islet inflammation can be identified by the presence of circulating autoantibodies to these specific islet antigens many years before the clinical onset of T1D, thus, providing a window for prevention strategies. Currently, there is no definitive cure for T1D; therefore, more effective therapeutic interventions for prevention of diabetes onset and progression are urgently needed. Vaccination with autoantigens to promote self-antigen-specific tolerance represents the most specific and safest means of preventing autoimmune diseases. Autoantigens administered by the mucosal route, which is "tolerogenic" by nature, is the most effective way to prevent or treat autoimmune diseases. However, the two major drawbacks of oral vaccination with autoantigens are the large quantities required to induce significant tolerance, presumably because the protein is partially degraded in the stomach, and the high cost of producing recombinant autoantigens using the conventional cell culture-based platforms. The expression of autoantigens in plants and the oral delivery of the plant tissue expressing the target antigen offer a potential solution for these two drawbacks. The goal of this review is to outline novel diabetes vaccine strategies based on β-cell autoantigens with a focus on the advantages and potential of plant-produced islets self antigens and anti-inflammatory cytokines for the prevention and treatment of T1D.

Keywords: Type 1 diabetes; Plant-made vaccines; Plant-derived vaccines; Autoantigen-based vaccines; Autoimmune diseases; Autoantigens; Transgenic plants; CTB; Cytokines; IL-4; IL-10; GAD65; Insulin

Abbreviations: T1D: Type 1 Diabetes; T2D: Type 2 Diabetes; CTB: B Subunit Cholera Enterotoxin; RTB: Ricin Toxin B Chain; LTB: Heat-labile Enterotoxin B Subunit from Enterotoxigenic *Escherichia coli*; GAD: Glutamic Acid Decarboxylase; IA-2ic: Insulinoma-associated Protein Tyrosine Phosphates-2; Th1: T-helper 1; Th2: T-helper 2; NOD mice: Non-obese Diabetic Mice; ICA: Islet Cell Antibodies

Type 1 Diabetes

Epidemiology and etiology

Type 1 diabetes (T1D) is an autoimmune form of diabetes mellitus, characterized by destruction of pancreatic β-cells and complete absence of insulin. T1D accounts for about 10% of all cases of diabetes and despite its occurrence in all age groups, it is more common in children and adolescents <20 years of age where it accounts for 85% of all diagnosed cases of the disease. Case-studies showed an increase of T1D incidence over time at a rate of about 3% a year [1,2].

T1D cases vary with age, geography and ethnicity. According to the SEARCH study, which employed newly diagnosed diabetes in Americans up to 20 years of age in 2002-2003, most children <10 years of age with diabetes had T1D, independent of ethnicity [3-5]. In older youth, T1D was also more frequent than type 2 diabetes (T2D) although the incidence of T2D is increasing in this population [4-6]. Youngsters of 10-14 years of age present the highest incidence of T1D, while adolescents between 15-19 years present the lowest incidence of the disease. Likewise, youngsters with age from 5-9 years present lower incidence of T1D than those of 10-14 years of age. T1D is more common in non-Hispanic whites in all age groups, when compared to other ethnicities. Incidence of T1D is lower in American Indians and Asian/Pacific Islanders, in all age groups [4-6]. SEARCH also reported that prevalence of T1D is lower in children <10 years old when compared to youths 10-19 years of age. Again, prevalence of T1D is higher in non-Hispanic white youngsters in all age groups. However, in youths 10-19 years of age, non-Hispanic whites and blacks have the highest prevalence of T1D [4-6].

The DIAMOND study from the WHO, which investigated the incidence of T1D in children ≤ 14 years of age from 50 different countries over a 10-year period, showed higher incidence of T1D in Europe and North America when compared to Asia and South America. Nordic countries such as Sweden, Finland and Norway presented the highest incidence of T1D among all the countries studied, whereas the lowest incidence was found in China and Venezuela. The study also found that T1D incidence increases with age, in all populations studied. Children 10-14 years old showed the highest incidence among all age groups [7,8]. A more recent analysis of the DIAMOND project group showed

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that 42 out of 105 countries involved in the studies presented seasonal differences in T1D incidence [9].

T1D is originated from a combination of environmental and genetic factors that lead to inflammation of the pancreatic islets and progression of the disease [10]. The genetic component of T1D has been studied extensively. Monogenic forms of the disease are rare and are usually in conjunction with other autoimmune disorders [11-15]. T1D is more frequently a polygenic complex disease, where many genes are affected. Several different loci have been associated with T1D. However, only very few of these associations have been confirmed [11]. The most important genes related to T1D are found in chromosome 6, within the human leukocyte antigen (HLA) complex loci. HLA is a highly polymorphic region and it was the first susceptibility locus to T1D to be identified [11,16,17]. A second region located in chromosome 11 has been confirmed to be associated with T1D. This encompasses the gene insulin and polymorphisms in this region contribute to T1D [11,18,19]. HLA polymorphisms account for only about 50% of T1D susceptibility. Therefore, genetic variation in other regions also participates in T1D, each contributing a little to the development of the disease [11]. GWA studies identified several new loci within the genome and genes with confirmed association to T1D include *PTPN22* (gene that encodes for the lymphoid protein tyrosine kinase) [20,21], *IL2RA* (Interleukin 2 receptor gene) [22,23], *CTLA*-4 (cytotoxic T lymphocyte associated protein 4 gene) [24], and *IFIH1* (interferon-induced helicase 1 gene) [25].

Genetic factors cannot account solely for the development of T1D. For example, a high discordance rate in identical twins is a strong indication that environmental factors play an important role. Although specific triggers in T1D are relatively unknown, a variety of environmental factors have been proposed as potential candidates for this role, including viral infections such as coxsackie B, cytomegalovirus, mumps, etc [26,27]; certain dietary components including gliadins; method of delivery (C-section); formula feeding (cow's milk); and improved sanitation and decreased infections during childhood known as "hygiene hypothesis" [28,29]. In addition, a complex interplay between intestinal microbiota (encompassing the entire bacterial community of the intestine), gut permeability, and mucosal immunity has been proposed as a trio of factors that form a "perfect storm" critical to the development of T1D [30]. Increased gut permeability (commonly called leaky gut) has been observed in patients with type 1 diabetes as well as in NOD mice and BioBreeding rat models [31-33], and may affect the absorption of antigens that can attack and damage pancreatic beta cells [34].

The gastrointestinal tract of mammals is inhabited by hundreds of distinct species of commensal microorganisms that exist in a mutualistic relationship with the host. Recently, the intestinal microbiota has received significant attention for its function in normal health and development as well as its potential role in disease including T1D [27,28]. The intestinal mucosa is a common entry site for pathogens and contains a significant proportion of the cells of the immune system. The maturation and function of the mucosal immune system depends on commensal bacteria. For example, the balance between IL-17+ T helper 17 cells and Foxp3+ regulatory T cells (two major effector T cell populations in the intestine), requires signals from commensal bacteria and is dependent on the composition of the intestinal microbiota [35].

Most of the knowledge on the potential role of the intestinal microbiota in the development of T1D comes from animal studies using animal models such as NOD mice and BioBreeding diabetes-prone (BB-DP) rats, raised germ-free and/or exposed to a variety of infectious organisms or antigens. Early studies revealed that some chronic viral and bacterial infections were associated with a lower incidence of diabetes in NOD mice [36-38]. Specific bacteria or bacterial products rather than a specific germ-free environment affect the risk of disease [39]. For example, there are a significantly higher proportion of *Lactobacillus* and *Bifidobacterium* genera in biobreeding diabetes-resistant (BB-DR) rats compared to BB-DP rats, while BB-DP rats were found to have a higher abundance of *Bacterioides* genera [40]. Moreover, feeding so-called "probiotic" bacterial strains (e.g. lactic acid bacteria) to NOD mice or BB-DP rats can prevent or delay disease development [41,42]. In humans, differences in the composition of gut microbiota between T1D and healthy children were recently described [43]. These findings could be useful for developing strategies to control the development of T1D by modifying the gut microbiota.

Treatment and prevention

Currently, there is no definitive cure for type 1 diabetes (T1D). People with T1D require exogenous injections of recombinant human insulin (or insulin pumps) and daily blood glucose monitoring [44]. Although insulin is not a cure for T1D, nowadays it remains as the major treatment, and probably will be used as supplementation to other therapies in the future [11]. The Diabetes Control and Complications Trial (DCCT) research group [45-47] showed the importance of strict metabolic control for the delay and prevention of chronic complications. However, achieving strict glycemic control represents a major undertaking for patients and their families, and there is a risk of severe hypoglycemia secondary to insulin therapy [48]. In addition, insulin injection does not mimic the precise regulation of beta-cells on glucose homeostasis, leading eventually to the development of complications [49]. Thus, more effective interventions than insulin therapy are necessary for the prevention and treatment of T1D. As in any other autoimmune disease, interventions can be targeted at three stages: before the development of autoimmunity (primary prevention), after autoimmunity is established to prevent disease progression (secondary prevention), or after diagnosis to prevent complications and alleviate symptoms after the onset of T1D (tertiary prevention) [50].

Intervention in autoimmune T1D could occur at the time of diagnosis or, preferably, prior to clinical presentation during the 'prediabetic' period (i.e., primary and secondary prevention) [51]. Promising intervention strategies include (1) islet or pancreas transplantation, (2) stem cells therapy [49,52], (3) general immunosuppression (e.g., glucocorticoids) [51], (4) cytokine-based therapeutics (e.g., IL-2 and rapamycin) [11], (5) antibody-based therapies (e.g., anti-CD3) [33], and (6) autoantigen-specific vaccines [53,54]. Unfortunately, none of the therapies attempted to date has produced long-term remissions in new-onset T1D patients [51]. Nevertheless, some therapies are very promising, particularly when different treatment strategies are combined to increase efficiency. For example, a therapy that combines a nonspecific immune suppressant (e.g., anti-CD3), autoantigen-specific vaccines (e.g. proinsulin, GAD65), and a suitable compound that increases beta-cell mass or function (e.g. gastrin, human placental lactogen) has been suggested as the most efficient treatment to silence the immune attack against self without sacrificing the patient's protective immune response to infections [11]. The numerous advantages of such a strategy include the ability to minimize toxicities and realize synergies to enhance and prolong efficacy [55]. Unfortunately, combination therapies using two or more non-approved drugs are difficult to license because it is required that each compound of a combination treatment be efficacious

and licensed on its own first [55,56]. In addition, competing interests obstruct the combination of drugs from different companies [11].

One of the most successful interventions for T1D is the islet or pancreas transplantation. The potential of islets transplantation in humans with T1D was assessed when in 2000 Shapiro et al. [57] reported complete success in a study with seven patients, and later confirmed by multiple studies from different islet transplantation centers throughout the world [58]. However, limitations for both islet and pancreas transplantation include the relative lack of organ donors for allogeneic transplantation, and the need for continuous immunosuppression not only to prevent rejection but also to block recurrent autoimmune islet destruction [48]. Currently, islet and pancreas transplantation are reserved for patients with long-lasting diabetes who have complications and are also in need of a concurrent kidney transplant [51].

Human embryonic (hESC) and adult stem cells are promising alternatives for long-term treatment of diabetes because they circumvent the problem of adequate donors [52]. More recently, human induced pluripotent stem (iPS) cells have been derived from somatic cells, which present the possibility of generating patient-specific pluripotent cells. More importantly, the production of iPS has the advantage of being independent from human oocytes, which always presents ethical issues owing to the destruction of blastocytes to establish hESC lines [59,60]. However, any successful strategy should address not only the need for β-cell replacement but also control the autoimmune response to cells that express insulin [52].

Autoantigen-Based Vaccines for Diabetes

Introduction

Autoimmunity occurs when environmental and/or genetic factors induce the immune system to recognize the body's own proteins as being foreign and threatening [61]. Autoantigens trigger the immune system to cause tissue-specific or systemic inflammation and destruction mediated by macrophages and cytotoxic T cells. To block autoimmune destruction, self-reactive T cells are suppressed or eliminated in a process known as immunologic tolerance [62].

In the eighteenth century, long before Robert Koch proved that infectious diseases were caused by microorganisms, Edward Jenner discovered that cow-pox protects humans from the infection of smallpox, which paved the way to modern vaccination [63]. The opposite of what Jenner intended involves reducing or eliminating specifically a particular undesired immune response, providing enormous benefits to an individual suffering from an autoimmune disease [64]. This can be achieved by vaccination with autoantigens to promote self-antigen-specific tolerance, known as "negative or inverse vaccination", which represents the most specific and safest means of preventing autoimmune diseases [65].

Current options for treatment of autoimmunity, including immunosuppressive drugs (e.g., cyclosporine) and anti-T cell antibodies (e.g., anti-CD3 antibodies) have shown success in suppression of β-cell autoimmunity in mice. However, these therapies require repeated drug administration and may cause harmful interference with normal immune system functions, including increased risk of infection and neoplasia [66,67]. In contrast, autoantigen-based vaccines have a major advantage of permitting selective inactivation of autoreactive T cells without interfering with normal immune function [68].

The development of a vaccine that prevents or interrupts auto-reactivity to pancreatic islet antigens is of great interest, particularly since there is no curative treatment available that can be safely administered to the increasing number of patients suffering from T1D [54]. T1D predominantly originates when insulin-producing β-cells in the pancreas are destroyed due to an autoimmune response caused by the auto-reactive T-helper 1 (Th1) cells. The major autoantigens in T1D are insulin, glutamic acid decarboxylase (GAD65), insulinoma antigen (IA-2), islet-specific glucose-6-phosphatase catalytic-subunit-related protein (IGRP), islet zinc transporter (ZnT8) and chromogranin A [69]. At-risk individuals with underlying islet inflammation can be identified by the presence of circulating autoantibodies to these specific islet antigens many years before the clinical onset of T1D, thus, providing a window for prevention strategies [65].

Dendritic cells (DCs) are the first cells of the immune system to process β-cells autoantigens and present them to naïve T helper cells (Th0), which normally induce tolerance. However, in T1D, DCs induce biosynthesis and secretion of interleukin 12, which stimulates Th0 to undergo morphogenesis into autoreactive effector T helper cells (Th1) that recognize β-cells proteins as foreign antigens (Figure 1). Auto-reactive Th1 cells secrete cytokines interleukin 2 and interferon gamma, which initiate inflammation of the islet cells (insulitis), and recruit macrophages and cytotoxic T lymphocytes ($CD8^+$ cells) to destroy the insulin producing β-cells, which results in T1D [70,71].

Recently, a new population of $CD4^+$ T effector cells has been described and named as Th17 due to their characteristic production of proinflammatory IL-17 [72], and a role for Th17 in the development of autoimmune diabetes in animal models has been demonstrated [73,74]. DCs may secrete inflammatory cytokines IL-6 and IL-23 that stimulate

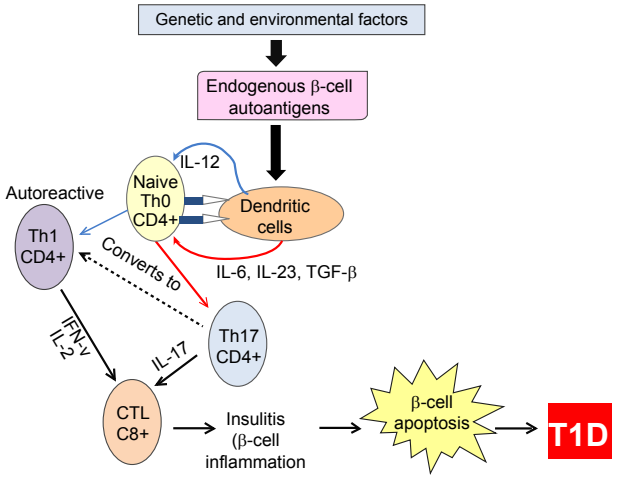

Figure 1: Mechanism of T1D inflammation
Dendritic cells process endogenous β-cell autoantigens such as insulin and GAD and transfer peptide fragments together with the MHC class II molecules to the surface of the cell. Thus, the autoantigen is presented to naïve T helper cells (Th0). Dendritic cells (DC) secrete the inflammatory cytokine interleukin-12 (IL-12), which stimulates the morphogenesis of Th0 into autoreactive T helper 1 (Th1) cells. Autoreactive Th1 cells secrete interferon gamma (INF-γ) and interleukin-2 (IL-2) that stimulates cytotoxic lymphocytes (CTL) to secrete inflammatory cytokines that cause pancreatic islet inflammation (insulitis) and β-cell apoptosis. DCs may secrete inflammatory cytokines IL-6 and IL-23 that stimulate naïve Th0 cell development into Th17 effector T Cells producing inflammatory IL-17. It has been proposed that Th17 cells contribute to the immunological pathology of T1D by increasing the ratio between T effectors and Tregs, converting to Th1 phenotype, or stimulating CTLs. Because pancreatic β-cells are the only ones that produce insulin, there is a progressive decrease in the production of this hormone and subsequent increase in blood sugar (hyperglycemia) resulting in T1D.

naïve Th0 cell development into Th17 effector T Cells producing inflammatory IL-17 [75] (Figure 1). It has been proposed that Th17 cells contribute to the immunological pathology of T1D by increasing the ratio between T effectors and Tregs, converting to Th1 phenotype, or stimulating CTLs [76] (Figure 1). In addition, upregulation of Th17 immunity in peripheral blood T cells from children with T1D has been recently reported [77]. However, there is some debate about the role of Th17 in T1D. For example, Joseph et al. [78] recently reported that IL-17 silencing does not protect NOD mice from T1D. In addition, some researchers suggested that Th-17 cells do not have an important role in the disease process since they can induce diabetes only after they converted to Th1 cells [79,80].

Vaccination with β-cells autoantigens in non-obese diabetic (NOD) mice has shown a partial state of immunological tolerance [81]. There are three possible DC-mediated mechanisms to induce tolerance using β-cells autoantigens: (1) induction of Th1 cell deletion/anergy; (2) induction of anti-inflammatory T helper 2 (Th2) cells; and (3) stimulation of regulatory T cell proliferation [53,82] (Figure 2). The destruction of pancreatic β cells in NOD mice is preceded by insulitis characterized by infiltration of mononuclear inflammatory cells [83]. The onset of insulitis can be diminished in NOD mice by inoculation with endogenous β-cell autoantigens, including insulin, GAD65, and DiaPep277 (an immunogenic peptide from the 60-kDa heat shock protein) [53].

Insulin

Oral administration of human insulin to NOD mice can induce the proliferation of $CD4^+$ T regulatory cells that protect pre-diabetic mice from diabetes onset [84]. In addition, proinsulin inoculated intraperitoneally or intranasally as well as insulin B chain peptide B:9-23 delivered subcutaneously were effective in partially suppressing diabetes onset in NOD mice [85-87]. It is believed that insulin promotes an anti-inflammatory state in DCs, leading to expansion of Th2 cells while inducing immunological suppression of Th1 cell function [88].

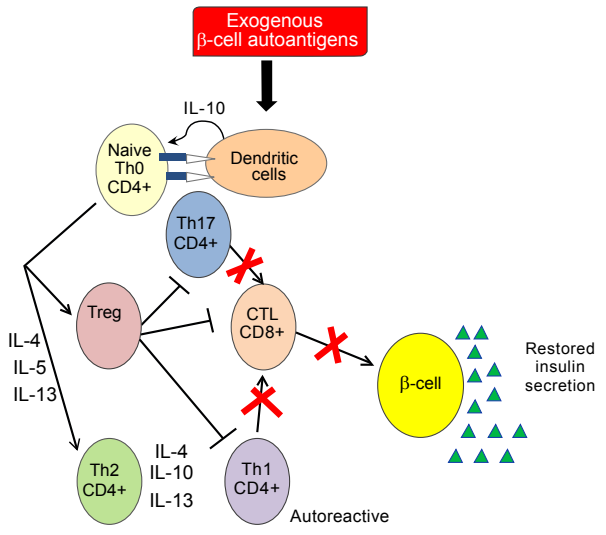

Figure 2: Mechanism of autoantigen-based vaccination for prevention of T1D

Oral delivery of islets autoantigens induces a protective antigen-specific therapeutic effect. Exogenous β-cell autoantigens stimulate dendritic cells to secrete the anti-inflammatory cytokine interleukin-10 (IL-10), which stimulates naïve Th0 cells to become T helper 2 $CD4^+$ (Th2) cells. Th2 cells suppresses autoreactive Th1 cells and decrease potential insulitis onset. Alternatively, naive Th0 cells may turn into one of several subclasses of regulatory T cells (Treg), which can block not only Th1 cells but also dendritic cells activation, Th2, Th17, and cytotoxic lymphocytes (CTL) leading to prevention or reversion of insulitis and restoring insulin secretion.

An initial human clinical study in individuals with high genetic probability for development of T1D has shown that intravenous insulin administration delayed the onset of diabetes in the treatment group [89]. Subsequently, in a larger phase III clinical trial known as the Diabetes Prevention Trial-Type 1 (DPT-1), patients that were relatives of T1D with detectable serum islet cell antibodies (ICA) and glucose tolerance in the non-diabetic level were recruited [90,91]. Patients received 5 mg of oral insulin daily along with subcutaneous insulin therapy for 12 months [92]. Unfortunately, neither treatment delayed or prevented T1D. No significant differences in insulin secretion (measured by levels of insulin C peptide) were detected between the treated and placebo groups [91,93]. Nevertheless, oral insulin resulted in a projected delay in T1D of more than 10 years in patients with high insulin autoantibody titers [94].

Glutamic acid decarboxylase (GAD65)

GAD is an enzyme responsible for the conversion of glutamate into gamma-aminobutyric acid (GABA) and CO_2. There are two enzyme isoforms, the 65 kDa expressed in pancreas and the 67 kDa in brain. In pancreas, GABA regulates glucagon action and insulin secretion in response to levels of glucose in serum [95]. When recombinant GAD65 was injected intraperitoneally into NOD mice, the onset of diabetes was delayed compared to control mice [96]. In addition, GAD65 was found to mediate expansion of T regulatory cell and anti-inflammatory Th2 cell populations, which induced T-cell tolerance as well as inhibit disease progression in NOD mice [97]. A candidate vaccine known as "Diamyd" (Diamyd Medical, Stockholm, Sweden), consisting of a combination of GAD65 protein with the adjuvant alum (aluminum and magnesium hydroxide), was used in phase I and phase II human clinical studies. Alum induces a Th2 response and minimizes T-cell mediated β-cell destruction [98]. The initial phase II clinical trial showed that two 20 μg subcutaneous injections of Diamyd one month apart was the most effective dose in adults with recent onset of T1D. Although Diamyd was safe, well tolerated, and preserved residual insulin secretion in early-onset T1D patients, it did not reduce the amounts of insulin required to maintain euglycemia. Unfortunately, a phase III clinical trial did not show a significant reduction in the loss of C peptide or improved clinical outcomes over a 15-month period [99].

Heat shock protein 60 (Hsp60)

DiaPep277® (Andromeda Biotech, Israel) is a peptide isolated from heat shock protein 60 (Hsp60), which can block the onset of T1D in NOD mice [100,101]. A subcutaneous injection of DiaPep277® in adults newly diagnosed with T1D was safe and resulted in maintenance of C peptide levels [102]. A randomized, double-blind, phase II study was done in patients with newly diagnosed T1D. The patients treated with DiaPep277®, continued producing c peptide and needed significantly less insulin to control their blood glucose concentration than patients in the placebo group [103]. Unfortunately, phase II trials for immune suppression in children were less successful and the vaccine did not prevent the continuous decline of C peptide levels [104]. DiaPep277® is currently in Phase III clinical trials, which is a global clinical trial being conducted at more than 120 clinical studies cites and expected to be completed at the end of 2014. Recruited patients for this study are 25 to 45 years of age and within 6 months of T1D diagnosis, with a residual β cell function of C-peptide ≥ 0.2 nmol/L. Patients are randomized

to 1.0 mg DiaPep277® or placebo with primary endpoint beta cell preservation of function [105].

B subunit cholera enterotoxin (CTB) as efficient adjuvant for diabetes vaccines

Several researchers have shown that the non-toxic B subunit of the cholera enterotoxin (CTB) from *Vibrio cholerae* is an efficient and safe adjuvant for inducing tolerance to autoantigens [106,107]. Suppression of hyperglycemia and pancreatic inflammation can be achieved in NOD mice treated with islet autoantigens such as insulin or GAD linked to CTB [108-110]. Recently, a recombinant vaccinia virus producing CTB protein fused to GAD provided effective and long-lasting protection against T1D in NOD mice when delivered in combination with the immunosuppressive cytokine IL-10 [111].

A major barrier in the design of human vaccines for T1D is the translation from animals to humans. The most popular animal model for diabetes is NOD mice, which spontaneously develop autoimmune diabetes that mimics T1D in man [112]. Disease development in NOD mice and in humans shares many important aspects including several genetic alterations, the autoantigen repertoire, and the autoimmune pathogenesis. While there is a great variability of genetic alterations between different patients with T1D, NOD mice are a highly inbred and genetically homogenous mouse strain. Therefore, a treatment that is efficacious in mice may only prevent disease in a subset of human patients, whereas a vaccine that fails in mice would probably not be considered for further evaluation [54]. Nevertheless, several human trials that followed successful mice trials have shown promising effects.

Plant-Made Vaccines for Prevention of Human Diseases

The potential to transform plant cells with a foreign gene and regenerate them into whole plants expressing this heterologous gene product was first reported at the Miami Winter Symposium in January, 1983 [113]. Three groups from Monsanto, Washington University and collaboratively the Rijksuniversiteit Gent, and Max Planck Institute described the transformation of tobacco cells using an engineered soil bacterium, *Agrobacterium tumafaciens* (Agro), carrying a gene for resistance to an antibiotic, and the subsequent expression of that determinant providing selection of the transformed cells [113]. Agrobacterium, a plant pathogen, had been previously shown to be able to transfer a piece of its DNA to dicot plant cells upon infection, and this DNA was incorporated into the plant host genome [114] and served as the basis for this plant transformation technology. Improvements in the technology occurred almost immediately with vector systems that were much easier to manipulate [115]. Other transformation technologies for plants were developed, such as biolistics or particle bombardment to address difficulties in some plant systems such as monocots [116] and further improvements of the Agrobacterium-mediated system finally expanded its host range to include monocots [117]. Thus, the era of genetically modified plants was begun and scientists all around the world began to take a look at possible genes for potential plant targets.

In general, early research activities were directed at traits that might be of most obvious interest for the farming industry, since farmers were the customers of the agrichemical and seeds businesses, and in many cases the new biotechnology companies were interested in participating in that market, either indirectly, by providing technology, or directly, by starting or acquiring their own seeds companies. Ready commercial targets such as weed, insect and disease control were very attractive. Further, the economics of these targets were also fairly well understood; losses due to these pests and the costs to control them were understood. As a result, early gene targets included single gene traits for insect resistance derived from the bacterium, *Bacillus thuringiensis* (Bt) [118-121], and genes providing tolerance to an herbicide such as EPSP synthase for glyphosate or phopsphinothricin acetyl transferase for glufosinate-ammonium based herbicides [122]. Shelf life of food products was also of interest and early product concepts addressed this issue in tomatoes to delay ripening and softening of the fruit [123].

The fact that plants are the most prolific producers of proteins on earth, and it seemed that virtually any heterologous gene could be used to transform plants with resulting protein expression, made it possible to consider the use of plants as a production system for biopharmaceuticals. Plants replicate DNA, transcribe RNA, translate proteins and can post-translationally modify proteins like other Eukaryotes. It was assumed that plant production systems could prove economically feasible and easily scalable. Plants require few inputs such as water, light, certain minerals, and are generally easy to grow whether under field conditions or controlled environments. Further, it should be possible to direct expression of proteins to discrete tissues or storage organelles, which may improve protein accumulation. Unlike mammalian and Prokaryotic expression systems, plants also have the advantage that they are free of mammalian viruses and Prokaryotic endotoxins, eliminating this concern.

The concepts associated with plant made therapeutic proteins, antibodies and vaccines were originally developed in the mid to late 1980's by researchers, respectively, at Calgene, The Scripps Research Institute, and Washington University collaboratively with Sungene Technologies, resulting in several pioneering patents (e.g., US 4,956,282, US 5,202,422, and US 5,654,184) [124-126]. The potential of using a plant as not only a production system for antigenic determinants of pathogens as subunit vaccines, but also using minimally processed transgenic plant tissue as a delivery vehicle, was conceived in 1986 and was first described by Curtiss and Cardineau in a patent application filed in 1988 and first published in 1990 [127,128]. The patent publication described the use of a gene encoding an important antigen of the mucosal pathogen, *Streptoccus mutans*, expressed in transgenic tobacco tissues, which were then dried and orally delivered to mice to elicit a secretory immune response. When compared to conventional vaccine production and delivery systems, which require sterile needles and the "cold chain", another attractive consideration demonstrated here was that minimally processed, orally-delivered plant made vaccines would not require either of those things. It was also determined that a circulating immune response developed and, after feeding the mice for a period of about 26 weeks, they appeared to stop producing antibodies directed at the transgenic antigen, suggesting the induction of tolerance (Curtiss/Cardineau, personal communication). This work was never published in the scientific literature.

In 1992, Mason and co-workers reported the expression of hepatitis B surface antigen (HBsAg) in tobacco and proposed that antigens produced in plants might be used as a vaccine [129]. To demonstrate this possibility, HBsAg, partially purified from transgenic tobacco, was delivered to mice via parenteral injection, which then produced the anticipated B cell and T cell responses [130]. To determine if the HBsAG could be delivered orally to stimulate a mucosal response, the HBsAg was expressed in potatoes using a variety of signal sequences and peptides for targeting and ER retention and poly-A addition sites. When the raw HBsAg tubers, along with 10 µg cholera toxin as an adjuvant, were fed to mice, a resulting primary immune response was elicited, and this could be boosted via subsequent intraperitoneal injection of a sub-immunogenic dose of a commercial vaccine. These

results suggested that the minimally processed, orally delivered potato candidate vaccine could establish immune competent memory cells, which could be part of a prime/boost strategy in humans [131]. The major problem was that the concentration of the HBsAg antigen in the potato tubers varied and was generally low. The potato system was also used by this group to express other antigens such as the heat-labile enterotoxin B subunit (LT-B) from enterotoxigenic *Escherichia coli* [132] and Norwalk Virus Capsid Protein [133]. Unfortunately, both antigens suffered the same problem of varied expression, although both expressed at higher levels than HBsAg. Regardless, all three of these potato produced vaccines were entered into Phase I Clinical Trials and in all three trials, individuals who consumed raw potato tubers containing 0.3 to 1.0 mg of the antigens developed antibody responses [134-136].

This early work made use of Agrobacterium-mediated stable nuclear transformation of plant cells that could be regenerated into whole plants. Early plant targets included tobacco, potato, lettuce and tomato, but this quickly grew to include alfalfa, banana, corn, spinach, rice and cowpea. Sufficient accumulation of plant produced antigens remains a significant issue, particularly if the intent is to deliver orally, since it is anticipated that an orally delivered plant vaccine will require as much as 25 times a parenteral dose [137]. Strategies to increase accumulation, in addition to the use of various promoters, leader sequences, signal and targeting sequences and poly A sites, include approaches involving codon optimization and freeze drying of the target plant material to permit concentration of the antigenic protein, preparation of a consistent dose and the possibility of adding excipients or adjuvants

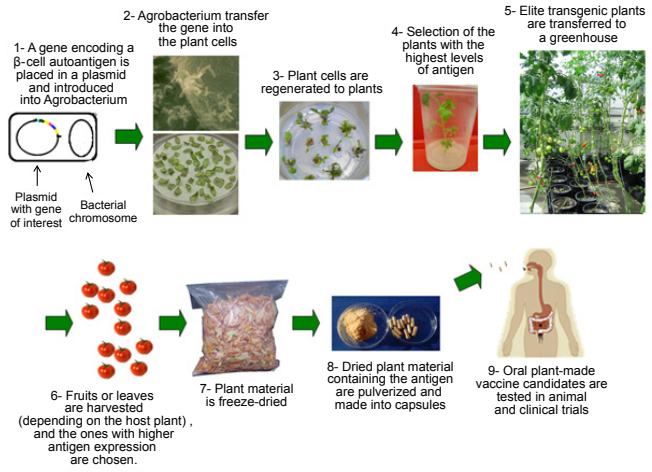

Figure 3: Production of plant-made vaccines using Agrobacterium-mediated transformation
Similar steps are involved in the nuclear transformation of different plant hosts such as tomato, tobacco, lettuce, carrot, etc. Transformation is a genetic alteration of a cell resulting from the direct uptake, incorporation, and expression of exogenous genetic material. In the "natural" transformation of plants mediated by the bacterium *Agrobacterium tumefaciens*, a plasmid, containing the genes for the autoantigen and a selective agent such as an herbicide (Bialaphos) or antibiotic, is introduced in the bacteria. Then, plant tissue (often leaves) are cut into small pieces, e.g. 10×10 mm, and soaked in a medium containing suspended *Agrobacterium* which transforms the plant cell by inserting its DNA. Placed on selectable rooting and shooting media, plants will regenerate, and the ones expressing the highest levels of autoantigens (elite lines) will be transferred to a greenhouse. Depending on the host plant, fruits or leaves will be harvested and freeze-dried. Dried plant material can be placed into capsules and used for oral vaccination.

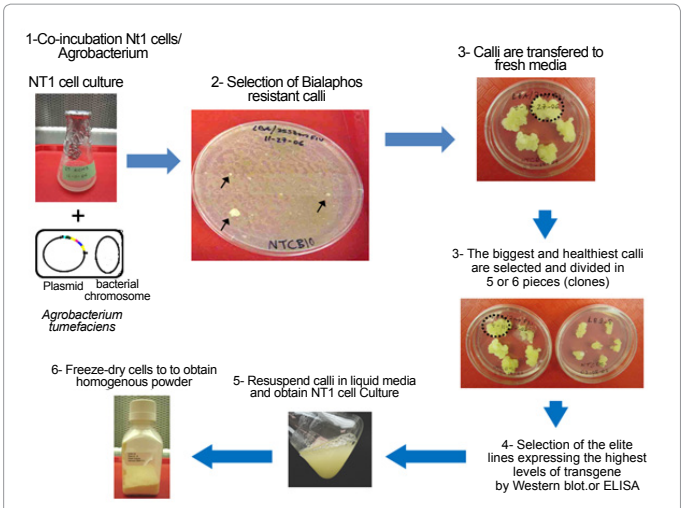

Figure 4: Production of plant-made vaccines in tobacco cell suspension
Tobacco NT-1 or BY-2 (Bright Yellow-2) cell lines in suspension are incubated with *Agrobacterium tumefaciens* containing a plasmid with the genes for the autoantigen and a selective agent such as an herbicide (Bialaphos) or antibiotic. Then, the cell suspension is placed on a plate containing a selective media, and resistant calli are regenerated in 10-14 days. A callus consists of an actively dividing non-organized mass of undifferentiated cell tissue induced by hormones. Elite callus lines expressing the highest levels of antigen are selected and resuspended in liquid media to obtain a transgenic NT1 or BY-2 cell culture, which is freeze-dried, pulverized and made into capsules for oral vaccination.

[138], increasing gene copy number by addressing potential RNA silencing issues [139], and packaging the vaccine antigen protein in storage vesicles [140]. In addition to the Agrobacterium and biolistics nuclear transformation methods, new transformation approaches have been developed that are reported to increase expression levels by targeting transgenic antigen production in organelles such as the chloroplast [141,142]. Viral transient transfection systems have been developed [143], along with the use of Virus Like Particles or VLPs in transient systems [144], both of which have been demonstrated to increase antigen production and do it rapidly, which could be particularly important with regard to a rapid response to a pandemic human pathogen [145,146].

Plant-made vaccine technology was conceived over 20 years ago as a way to produce inexpensive vaccines, deliver them orally, and eliminate the requirement for refrigeration and the need for sterile needles [147]. Many types of plants, tissues, and cells have been used to express vaccine antigens from a wide range of bacterial, viral and protozoan pathogens, and those plant materials have been delivered to animals, which have developed protective immune responses when challenged [137,146,148,149]. Figure 3 shows the main steps followed in obtaining plant-made vaccines using Agrobacterium-mediated plant transformation.

Despite years of research performed around the world, including seven Phase I human clinical trials in the US and abroad, no human plant-made vaccines have reached the market to date, or are even close. However, the first plant-made animal vaccine was registered by the Center for Biologics of the USDA in January 2006 [150]. It is a vaccine targeting Newcastle Disease Virus, a disease of poultry that is wide spread. The vaccine was produced in tobacco NT-1 cells in a large culture bioreactor and serves as the first commercial example of

this technology [151-153]. Figure 4 shows the main steps followed in producing plant-made vaccines in tobacco NT-1 cells.

Plant-Based Production of Autoantigens for Diabetes Vaccination

The administration of autoantigens by the mucosal route, which is "tolerogenic" by nature, is the most effective way of achieving "negative vaccination" to prevent or treat autoimmune diseases [154]. Whether administration of an antigen will boost an immune response or induce tolerance depends on the dose, the route of antigen delivery, and the presence of co-stimulatory signals. Oral administration of antigens leads to immune tolerance through one of two different mechanisms, depending on the dose: high doses of orally fed antigen may lead to anergy or clonal deletion of the autoreactive lymphocytes, whereas repeated low doses of antigen tend to generate active suppression by regulatory cytokines produced by Th2, including TGF-β, IL-4, and IL-10 leading to a long lasting induction of T-regulatory cells [155,156]. Plants are ideal for oral administration of the low doses of autoantigen required to induce tolerance because they often express only small amounts of transgenic proteins [157]. In this section, we will discuss the successful prevention or delay of T1D onset using plant-based vaccine technology, with a particular focus on oral vaccination using T1D autoantigens produced by a variety of plant platforms.

Plant-made Insulin

Insulin plays a double role in T1D because not only is the main treatment for the disease but also is a major autoantigen that can be used for vaccination. Although the commercial production of recombinant human insulin is nowadays limited to *Escherichia coli* [158] and yeast [159], it has also been expressed in different organisms including mammalian cell cultures [160] and plants [161]. The high requirement and cost of recombinant insulin per patient as well as the increasing incidence of diabetes worldwide, severely challenges the current insulin production systems to meet the capacity and economic demands of the future. It has been estimated that the cost of insulin is around US$100 per patient and per month in developed countries [162]. In addition, with the progress in other delivery technologies, including buccal spray, inhalable, and oral, insulin will soon be increasingly administered through non-injected means, which require between 5 to 7 times more insulin per dose than injectable insulin [163]. Therefore, there is a great need to increase insulin mass production as well as to reduce the economic burden of patients with diabetes by decreasing the cost of insulin.

Plant produced pharmaceuticals represent a promise for safe and cost-effective mass production, especially when combined with simple and inexpensive food processing techniques [164]. Thus, plant-made insulin may supply this expanding market and reduce the production cost compared to current methods. In addition, plants cells bioencapsulate insulin and protect it from enzymatic degradation in the stomach overcoming one of the main challenges that arise in oral delivery [165].

Table 1 shows a summary of the plant production systems that have been used to produce insulin. Recombinant human insulin has been expressed in oilbodies of *Arabidopsis thaliana* seeds and isolated by simple and cost-effective flotation-centrifugation to separate the oilbody fraction [161]. Oilbodies are seed structures with a phospholipid membrane and protein coat that store energy for germination in the form of oil, protecting the oil from oxidization and deterioration. The biological activity of the insulin purified from oilbodies was assessed by tolerance tests. This expression platform allows the accumulation of protein in mature oilseed that can be stockpiled until recovery of the end product. A similar strategy was used for the expression of insulin in safflower by SemBioSys Genetics, a Canadian biotechnology company [166,167]. The company was able to produce insulin at 70% capital cost reduction compared to fermentation, the common method used, showing promise to help serving the expanding US$11.8 billion insulin market. A phase I/II clinical trial of the safflower-produced insulin, known as SBS-1000, demonstrated the bioequivalence of plant-made recombinant protein to commercial recombinant Humulin@ (Eli Lilly, Indianapolis, IN, USA) [166,167].

For the production of insulin as an autoantigen for diabetes vaccination, CTB proteins as well as the ricin toxin B chain (RTB) from castor beans have been used as adjuvant fused with the insulin precursor (proinsulin) or with insulin B chain [168]. This strategy enhances the oral tolerance of co-administered antigens by targeting them to specialized antigen-presenting cells of the GALT and improves the effect of orally administered antigens [107]. Earlier experiments

Table 1: Plant-based production systems of insulin for prevention and treatment of type 1 diabetes*.

Plant-made protein	Host plant	Target organ	Organelle	Expression level	Biological activity/Animal or clinical trials	Ref.
hinsulin	*Arabidopsis thaliana*	Seeds	Oilbodies	0.13% TSP	Biological activity demonstrated *in vivo* and *in vitro* using an insulin tolerance test in mice and phosphorylation assay performed in a mammalian cell culture system, respectively.	[161]
	Carthamus tinctorius (safflower)	Seeds	Oilbodies	N.A.	Phase I/II clinical trial: Bioequivalence of plant-made insulin and commercial insulin.	[166,167]
CTB-proinsulin	*Solanum tuberosum* (potato)	Tubers	ER	0.1% TSP	NOD mice fed with CTB-proinsulin but not proinsulin alone has reduced insulitis and increase Th2 response.	[169]
	Nicotiana tabacum (tobacco)	Leaves	Chloroplast	16% TSP	NOD mice fed with CTB-proinsulin decreased insulitis as well as blood and urine glucose levels compared to controls.	[177]
	Lactuca sativa (lettuce)	Leaves	Chloroplast	2.5% TSP		
CTB-proinsulin+ 3 furin cleavage sites	*Nicotiana tabacum* (tobacco)	Leaves	Chloroplast	47% TSP (old leaves)	Oral and injectable delivery into mice induced a reduction in glycemia equivalent to commercial insulin.	[178]
	Lactuca sativa (lettuce)	Leaves	Chloroplast	53% TSP (old leaves)		
CTB-insulinB3	*Nicotiana tabacum* (tobacco)	Leaves	ER	0.11% TSP	N.A.	[170]
RTB-insulin	*Solanum tuberosum* (potato)	Tubers	ER	N.A.	N.A.	[173]

*E.R.: Endoplasmic reticulum; TSP: Total soluble protein; N.A.: Not Available; NOD mice: Non-obese diabetic mice (animal model of T1D); CTB: B subunit cholera enterotoxin; RTB: Ricin toxin B chain; hinsulin: Human insulin

showed that the CTB chain, when conjugated to insulin, was able to reduce the effective autoantigen dose approximately 5,000-fold for prevention of diabetes onset in NOD mice [109]. A protein fusion between CTB and proinsulin has been expressed in potato tubers at 0.1% of the total soluble protein (TSP) [169]. Mice fed with transgenic potato tubers expressing CTB-proinsulin displayed a significant insulitis reduction and developed an insulin-specific Th2-lymphocyte response, suggesting that the observed tolerance was mediated by an active suppression. However, mice fed with potato tubers expressing proinsulin alone did not display significant insulitis reduction because the dose of autoantigen was too low in the absence of CTB.

As a strategy to increase the levels of transgenic proteins in plants, Li et al. [170] reported the expression of CTB fused to three copies of the insulin B-chain in tobacco plants. However, an accumulation of only 0.11% transgenic fusion protein was observed in tobacco leaves, a similar level to the one previously reported in potato tubers [169]. Therefore, there is a need to increase transgenic protein expression levels in large biomass crops in order to favor mass production of insulin, decrease cost, and facilitate oral delivery. To this end, we and others have reported the use of different strategies to increase accumulation of foreign protein in nuclear transformed plants, including regulatory elements and codon optimization [138,171,172], reversion of gene silencing [139], and expression in protein bodies [140].

The expression in potato tubers of a fusion molecule of insulin with RTB as an adjuvant has been reported [173]. The fusion protein was expressed in plant tissue and proper folding of RTB was evaluated by an asialofetuin-binding assay. Unfortunately, the levels of the foreign protein were not quantified in this study.

An alternative to nuclear transformation in plants is chloroplast genetic engineering. Each plant cell may contain as many as 10,000 chloroplast genomes which results in high expression levels of proteins expressed in the chloroplast genome [174-176]. A fusion protein between proinsulin and CTB was expressed in lettuce and tobacco chloroplasts at much higher levels with respect to the nuclear expression of CTB-proinsulin (16% and 2.5% of TSP in transplastomic tobacco and lettuce plants, respectively, vs. 0.1% of the TSP in transgenic potato and tobacco) [169,177]. NOD mice fed by gavage with transplastomic tobacco leaves containing 14 μg of CTB-proinsulin showed decreased insulitis with lower blood and urine glucose levels than the control group. A higher Th2 response and immunosuppressive cytokine levels were observed in the treated group compared to the control [177].

The introduction of three furin cleavages sites in the sequence of CTB-proinsulin expressed in tobacco and lettuce chloroplasts contributed to increase the processing of proinsulin to mature insulin and C-peptide in the gut [178]. In contrast, none of the currently used approaches to insulin delivery include the C-peptide, which is generally used to determine the extent of insulin release in patients but also has important physiological functions. C-peptide has been shown to alleviate the main diabetes long-term complications such as neuropathy and nephropathy, suggesting that T1D patients should receive supplemental C-peptide in addition to insulin [179]. The majority of proinsulin accumulation was seen in old tobacco and lettuce leaves as opposed to mature leaves, which is often observed among foreign proteins expressed in chloroplast [178]. Old tobacco and lettuce leaves accumulated proinsulin to 47% and 53% of TSP, respectively. Proinsulin from tobacco leaves was purified up to 98% using metal affinity chromatography and insulin was cleaved *in vitro* using furin protease. Feeding of unprocessed proinsulin bioencapsulated in plant cells as well as injectable delivery in mice showed reduction in blood glucose levels similar to commercial insulin [178].

Plant-produced glutamic acid decarboxylase (GAD)

GAD is an essential enzyme present in all cells that catalyzes the

Table 2: Plant-based production systems of glutamic acid decarboxylase (GAD) for prevention and treatment of type 1 diabetes*.

Plant-made protein	Host plant	Target organ	Organelle	Expression level	Animal or clinical trials	Ref.
mGAD67	*Nicotiana tabacum* (tobacco)	Leaves	cytosol	0.40% TSP	NOD mice fed daily with transgenic tobacco or potatoes were free of T1D.	[180]
	Solanum tuberosum (potato)	Tubers	cytosol	0.40% TSP		
hGAD65	*Nicotiana tabacum* (tobacco)	Leaves	Chloroplast and mitochondrian membranes	0.04% TSP		[181]
	Dacus carota (carrot)	Rhyzomes	Chloroplast and mitochondrian membranes	0.012% TSP		
hGAD65	*Chlamydo-monas reinhardtii*	Cell suspension culture	Chloroplast	0.3% TSP	Immunoreactivity of hGAD65 demonstrated using sera from NOD mice and a spleen cell proliferation assay.	[184]
hGAD65mut	*Nicotiana tabacum* (tobacco)	Leaves	Chloroplast and mitochondrian membranes			[50]
			ER	2% TSP		
			cytosol	2.2% TSP		
GAD67/ GAD65	*Nicotiana tabacum* (tobacco)	Leaves	cytosol	0.19% TSP		[182]
	Nicotiana benthamiana	Leaves	cytosol	2.2% TSP (transient expression)		
CTB-GAD65	*Solanum tuberosum* (potato)	Tubers	ER	0.001% TSP	NOD mice feed with CTB-GAD65 but not GAD65 transgenic potato showed reduced insulitis and clinical diabetes symptoms.	[186]
hGAD65+ mIL-4	*Nicotiana tabacum* (tobacco)	Leaves	cytosol	0.04% TSP	Delayed onset of T1D in NOD mice fed with transgenic plant tissue expressing hGAD65+mIL-4 compared to hGAD65 alone or mIL-4 alone.	[200]

*GAD: Glutamic acid decarboxylase; %TSP: Percentage of total soluble protein; ER: Endoplasmic reticulum; mGAD67: Mouse GAD67; hGAD65: Human GAD65; mIL-4: Mouse interleukin-4; T1D: Type 1 diabetes

decarboxylation of glutamate to γ-aminobutyrate and CO_2. There are two isoforms in mammals: GAD65 in rat and human pancreatic islets and GAD67 in mouse islets. Mouse GAD67 was expressed in tobacco and potato plants and accumulated at about 0.4% of TSP. NOD mice fed daily with transgenic potato or tobacco plants containing 1-1.5 mg of GAD remained free of disease [180]. The treated mice lacked GAD-specific T-cell proliferation and had higher IgG1 anti-GAD antibodies than the control group.

Table 2 shows a summary of the plant production systems that have been used to produce GAD. Human GAD65 was initially expressed in tobacco and carrot plants and accumulated at levels varying between 0.01% and 0.04% of TSP. Immunogold labeling and electron microscopy of transgenic tobacco tissue showed the expression of human GAD65 in chloroplast thylakoid and mitochondria in a correct folding that retains enzymatic activity. Unfortunately, the low expression levels of GAD65 were not sufficient to plan animal studies [181]. Therefore, different strategies were used to increase the levels of GAD65 in transgenic plants. A modified human GAD65 with the N-terminal domain of rat GAD67 (GAD67/65) was expressed in the plant cell cytosol and accumulated at 0.2% of TSP [182]. Other strategies for further increasing foreign protein expression included an enzymatically mutated form of GAD65 (GAD65mut) obtained by substituting the lysine residue responsible for binding the cofactor pyridoxal 5'-phosphate with an arginine residue. GAD65mut lacks enzymatic activity but binds GAD65 autoantibodies in serum from T1D patients [183]. GAD65mut accumulates at up to 2.2% of TSP in transgenic tobacco leaves, a tenfold higher level than GAD65. This higher yield was a consequence of a higher rate of protein synthesis and not transcript availability or protein stability [168]. The authors suggested that the differences found between the expression levels achieved for the native and mutated versions of GAD65 are a consequence of the catalytic properties of the native GAD65, which interferes with plant cell metabolism and contributes to its poor yield in plant systems. GAD65mut was also targeted to subcellular, endoplasmic reticulum (ER), and cytosol as a strategy to improve expression levels in plants but, unfortunately, a significant improvement was not observed [182].

Human GAD65 was also expressed in the microalgae *Chlamydomonas reinhardtii* at levels of up 0.3% of TSP [184]. This plant system offers several advantages such as faster reproduction than any other plant, rapid growth, and stable transplastomic plants that can be generated in less than 3 weeks [168,185]. The immunoreactivity of the foreign protein was demonstrated using sera from NOD mice and a spleen cell proliferation assay.

Oral administration of plant synthesized CTB-GAD was found to be effective in suppression of T1D symptoms [186] in amounts 1000 less than previously reported for unconjugated GAD [180]. A CTB-GAD65 fusion protein expressed in potato and targeted to the endoplasmic reticulum accumulated at up to 0.001% in tubers [186]. The plant-synthesized CTB-GAD65 displayed the predicted pentamer molecular weight, GM1-ganglioside binding affinity, and native antigenicities of both CTB and GAD65. Oral administration of CTB-GAD65 fusion protein induced both intestinal IgA and serum IgG in NOD mice. Insulitis and clinical diabetes symptoms were significantly reduced in mice fed with potato tubers containing CTB-GAD65. A further decrease in insulitis was observed in mice fed with a mixture of CTB-GAD65 and CTB-insulin potato tubers. In contrast, mice fed with potato tubers expressing only GAD65 showed a measurable but insignificant reduction in insulitis and diabetes symptoms [186]. These results highlight the importance of the CTB carrier protein in enhancement of the mucosal immune response at low antigen concentrations.

Plant-made insulinoma-associated protein tyrosine phosphates-2 (IA-2ic)

IA-2ic has been expressed transiently in *Nicotiana benthamiana* leaves and the recombinant protein accumulated up to 100 mg/kg of the fresh leaf tissue [187]. Plant-derived IA-2ic was purified and the immunoreactivity confirmed with serum from diabetic patients and antibodies against human IA-2ic. However, no animal trials have been reported yet.

Table 3: Production of plant-derived anti-inflammatory interleukins in different plant systems*.

Plant-made protein	Host plant	Target organ	Organelle	Expression level	Animal or clinical trials	Ref.
hIL-4	*Nicotiana tabacum* (tobacco)	Leaves	ER	0.1% TSP	N.A.	[199]
	Solanum tuberosum (potato)	Tubers	ER	0.08%		
	BY2	Cell suspension culture	secreted	0.28 ug/ml	N.A.	[198]
hIL-10	*Nicotiana tabacum* (tobacco)	Leaves	ER	0.0055% TSP	Activity of plant-derived hIL-10 demonstrated in vivo using a mouse model of colitis.	[204,205]
	BY2	Cell culture		0.046% TSP	N.A.	[208]
hIL-10 -ELP	*Nicotiana tabacum* (tobacco)	Leaves	ER	0.17% TSP	Amelioration of autoimmune colitis in mice fed with hIL10-ELP tobacco.	[206,207]
	BY2	Cell culture		3.057% TSP	Activity of hIL-10-ELP was 80 times lower than commercial IL-10.	[208]
vIL-10	*Nicotiana tabacum* (tobacco)	Leaves	ER	10.8 ug/g FLW	N.A.	[209]
			Apoplast	0.008 ug/g FLW		
			Cytosol	0.004 ug/g FLW		
mIL-10	*Nicotiana tabacum* (tobacco)	Leaves	ER	37 ug/g FLW	N.A.	[209]
			Apoplast	0.026 ug/g FLW		
			Cytosol	0.081 ug/g FLW		
hIL-13	*Nicotiana tabacum* (tobacco)	Leaves	ER	0.15% TSP	Biological activity confirmed in an in vitro cellular proliferation assay.	[219]

*BY2: Tobacco Bright Yellow-2 suspension cells; E.R.: Endoplasmic reticulum; TSP: Total soluble protein; N.A.: Not available; ELP: Elastin-like polypeptide; mIL-4: Mouse interleukin-4; hIL-10: Human interleukin-10; vIL-10: Viral interleukin-10

Plant-Derived Interleukins as Immunomodulators and Vaccine Adjuvants

Some cytokines with immunosuppressive properties, including IL-4, IL-10 and IL-13, have been successfully generated in plants as an alternative to the conventional production platforms (Table 3). These regulatory anti-inflammatory cytokines have not only been proposed as therapeutic agents for many different diseases but also may represent an alternative to the use of adjuvants in the development of oral autoantigen-based vaccines for T1D. Cytokine-based manipulation offers a unique possibility to interfere with autoimmune diseases. However, cytokines are pleiotropic (i.e. they act on different cell types and tissues) and the complexity of their network can lead to severe side effects such as increased risks for infection and malignancies [188]. For example, IL-4 is the only cytokine that induces Th2 bias *in vitro* and also alleviates Th1-mediated autoimmune diseases. However, boosting Th2 responses by treatment with IL-4 might also compromise our defense against intracellular organisms such as *Mycobacterium tuberculosis* [189]. Nevertheless, minimal or no side effects observed in successful animal and clinical trials with IL-4 [190,191] have boosted the interest in cytokine-based therapies.

Interleukin-4 (IL-4)

The immunoregulatory cytokine IL-4 strongly promotes Th2 immune responses, which could increase tolerance to autoantigens [192]. Low levels of IL-4 have been suggested to contribute to autoimmune diabetes [193] whereas overexpression of IL-4 in pancreas has prevented insulitis and diabetes in NOD mice [194]. Cytokines can resist degradation in the gut and retain biological effect when administered orally and, therefore, their oral therapeutic use has been suggested. For example, IFN-α delivered orally suppressed disease and increased IL-4 and IL-10 production in spleen cells from treated mice [195]. Unfortunately, the two conventional IL-4 production based on *E. coli* [196] and yeast [197] have major drawbacks that limit the use of IL-4 for treatment of clinical diseases. Specifically, IL-4 produced in *E. coli* forms inclusion bodies and has to be resolubilized, refolded and purified causing a high production cost, while the expression in yeast results in a hyperglycosylated variant. Therefore, the identification of other platforms for the production of IL-4 is currently needed.

The first plant system used for the production and secretion of human IL-4 (hIL-4) was *N. tabacum* cv. NT-1 cell culture, and the maximum yield of foreign protein achieved was 0.28 μg/ml [198]. However, the biological activity of the recombinant hIL-4 was only a quarter of that expected for the amount of protein detected. Production of hIL-4 was also reported in tobacco and potato plants reaching accumulation levels of up to 0.1% and 0.08% of the TSP, respectively [199]. *In vitro* T-cell proliferation assays showed that plant-derived recombinant hIL-4 (rhIL-4) retained full biological activity. These results suggest that plants can be used to produce biologically active rhIL-4.

Mouse IL-4 (mIL-4) and human GAD65 were expressed in a biologically active form in transgenic tobacco plants. The expression level of mIL-4 and hGAD65 in leaf tissue reached up to 0.1% and 0.04% of TSP, respectively [200]. Beginning at 4 weeks of age, 22 prediabetic NOD mice per group were fed for 26 weeks with transgenic plant tissue containing both mIL-4 and hGAD65; mIL-4, hGAD65; or empty vector alone. After 5 weeks of treatment, the pancreas of 4 animals per group was harvested and 25 islets were scored for the presence of insulitis. The percentage of the examined islets that showed severe insulitis was significantly lower in mIL-4 plus hGAD65 plant-fed mice than in animals fed empty vector transgenic plant (8% vs. 31%, P=0.03). No significant differences in insulitis scores were found in NOD mice fed either empty vector or IL-4 or GAD65 transgenic plant. The rest of the mice were observed for the development of overt diabetes up to 30 weeks, and the following results were obtained: only 26% (4/15) of the NOD mice that received mIL-4 plus hGAD65 developed diabetes by 30 weeks of age whereas 83% (15/18) of the NOD mice that received hGAD65, 100% (18/18) of the NOD mice fed with mIL-4, and 75% (12/16) of the NOD mice treated with empty vector developed diabetes by 30 weeks of age. Mice fed with transgenic plants expressing mIL-4 plus hGAD65 were significantly protected (P=0.0002) compared to all the other groups. There are no significant differences between NOD mice fed with hGAD65, mIL4, empty vector or no-treated NOD mice. About 20-25% of no-treated NOD mice never develop diabetes. The onset of the disease was markedly delayed in the NOD mice fed with mIL-4 (1-2 μg/day) plus hGAD65 (6-8 μg/day), and stronger Th2 responses (higher anti-GAD IgG1 titers) were observed than in mice fed with mIL-4, hGAD65, or empty vector alone. These results demonstrate that prevention of diabetes in NOD mice using oral GAD65 requires co-administration of IL-4 [200].

Interleukin-10 (IL-10)

From an immunotherapeutic perspective, the most important property of IL-10 is its capacity to inhibit Th1 cells mediated by several mechanisms, including inhibition of IL-12 production by antigen-presenting cells and blocking of IFN-γ synthesis by differentiated Th1 cells [188]. Daily subcutaneous administration of IL-10 to NOD mice was shown to delay the onset of disease, reduce the severity of insulitis, and promote normal insulin production in β cells. These results suggest a potential therapeutic role for this cytokine in this autoimmune disease [201]. In addition, oral IL-10 enhanced the protective effect of oral insulin in NOD mice [202]. Unfortunately, the current production systems for IL-10, which includes *E. coli*, mammalian cell cultures or insect cells, have several limitations that can be overcome with extra production steps but result in a high market price [203].

Plants offer an alternative cost-effective production platform for IL-10. Stably transformed tobacco plants expressing human IL-10 (hIL-10), which accumulated in the endoplasmic reticulum at up to 0.0055% of the TSP, have been reported [204,205]. Plant-derived hIL-10 biological activity was demonstrated *in vivo* using a mouse model of colitis. As a strategy to increase the low accumulation levels of hIL-10 in plants, a fusion to elastin-like polypeptide (ELP) was generated by the same research group, and the yield of hIL-10-ELP increased to up to 0.27% of the TSP in stably transformed tobacco plants [206]. Amelioration of autoimmune colitis was observed in an IL-10 (-/-) mouse model fed with transgenic tobacco plants expressing hIL-10-ELP [207]. Recently, the same research group obtained several fold higher hIL-10 accumulation in tobacco BY-2 cell suspensions than in the best tobacco plants previously transformed using the same constructs [208]. For example, the highest hIL-10-expressing BY-2 cell suspensions accumulated 0.046% of TSP, eightfold higher than the best hIL-10-expressing tobacco stable transgenic plant (0.0055% of TSP) [204]. Likewise, the highest expressing hIL-10-ELP line accumulated up to 3.057% of TSP in cell suspension, over 500-fold higher than the levels of IL-10 obtained with stable transgenic plants [208]. The purified fusion protein was then tested for activity in a murine macrophage–monocyte cell line (PU5-1.8). Unfortunately, the activity of hIL-10-ELP was 80 times lower than the commercial rIL-10 protein. However, the proteolytic removal of ELP from the fusion resulted in a 10-fold activity increase [208].

IL-10 has orthologs in several virus genomes including the Epstein-Barr virus (vIL-10). The vIL-10 has the immune-inhibitory properties associated with IL-10 but lacks many of the immune-stimulatory activities of hIL-10 and mIL-10. Therefore, vIL-10 is an even more effective therapeutic candidate to induce autoantigens tolerance in T1D. Transient and stable expression of vIL-10 and mIL-10 were reported in tobacco plants [209]. The best yields of recombinant protein achieved were 0.6% and 0.1% of TSP for murine and viral IL-10, respectively. Both molecules were able to activate the IL-10 signaling pathway and to induce specific anti-inflammatory responses in mouse macrophage cells. Viral IL-10 was recently reported to accumulate better when expressed in a tetracycline-inducible expression system in tobacco BY-2 cell suspension cultures [210]. IL-10 has been found to be effective in suppressing diabetes autoimmunity when combined with the GAD65 autoantigen fused to cholera toxin B subunit [211].

Interleukin-13 (IL-13)

IL-13 is a cytokine primarily produced by the Th2 subset of lymphocytes, which exerts strong anti-inflammatory activity by inhibiting the production of pro-inflammatory cytokines. IL-13 has the potential for treating several human diseases, including cancer [212], rheumatoid arthritis [213], uveitis [214], and T1D [215]. Treatment with recombinant human IL-13 (hIL-13) markedly diminished the incidence of spontaneous T1D and significantly decreased insulitis in NOD mice compared to control [215]. The preventive action of hIL-13 was associated with a significant change from a Th1 to Th2 cytokine response, lower blood levels of interferon gamma (IFN-γ) and tumor necrosis factor alfa (TNF-α) as well as an augmented IL-4.

Unfortunately, the treatment of clinical diseases with recombinant human IL-13 (rhIL-13) may be limited by the unavailability of large quantities at low cost [184]. Nowadays, small-scale production of rhIL-13 can be achieved in murine NS-O cells [216] and in *Escherichia coli* [217,218] but both platforms have serious limitations [219]. As the interest in rhIL-13 as a potential therapeutic agent continues to increase, a more efficient and cost-effective productive system is becoming highly desirable and, thus, a plant-derived IL-13 may offer a solution. Human IL-13 has been expressed in tobacco reaching up to 0.15% of the TSP. The biological activity of the plant-derived hIL-13 was confirmed in an *in vitro* cellular proliferation assay but has not yet been tested in animals [219].

Conclusions

There is no cure for T1D, the autoimmune form of diabetes, and the magnitude of the disease continues increasing worldwide. Therefore, more effective therapeutic interventions for prevention of diabetes onset and progression are urgently needed. Current options for treatment of autoimmunity, including immunosuppressive drugs and anti-T cell antibodies, require repeated drug administration and may cause harmful interference with normal immune system functions, including increased risk of infection and neoplasia. Autoantigen-based vaccines have a major advantage of permitting selective inactivation of autoimmunity without interfering with normal immune function. However, the efficacy of this therapy is strictly related to dose and mode of administration. Autoantigens administered by the mucosal route, which is "tolerogenic" by nature, is the most effective way of achieving "negative vaccination" to prevent or treat autoimmune diseases. Despite the success in preventing T1D onset in the NOD mice using orally administered autoantigen-based vaccines, oral tolerance has had only moderate success in treating T1D when applied to humans. The different outcomes can be partially explained by a dose-dependent effect since the amounts of autoantigens fed to humans were relatively low compared with those used in mice.

The two major drawbacks of oral vaccination with autoantigens are the large quantities required to induce significant tolerance, presumably because the protein is partially degraded in the stomach, and the high cost of producing recombinant autoantigens using the conventional fermentation or cell culture-based platforms (i.e. *E. coli,* yeast, mammalian cells). The expression of autoantigens in plants and the oral delivery of the plant tissue expressing the target antigen offer a potential solution for these two drawbacks. Plant cells bioencapsulate proteins within their walls and protect them from complete degradation in the acidic environment of the gut. As the plant tissue is digested, it allows delivery of adequate doses of autoantigens to the GALT. In addition, plants as bioreactors for production of recombinant proteins are less expensive than conventional microbial or animal cell culture-based systems; they can be scaled up quickly and easily, with no risk of contamination with mammalian pathogens.

Three different pancreatic islet autoantigens have been successfully expressed in different plant systems and retained their biological activity: insulin, GAD and IA-2ic. The Canadian company SemBioSys Genetics was able to produce insulin in oilbodies of safflower seeds at 70% capital cost reduction compared to the fermentation. A phase I/II clinical trial of the safflower-produced insulin, known as SBS-1000, demonstrated the bioequivalence of plant-made recombinant protein to commercial recombinant Humulin® (Eli Lilly, Indianapolis, IN).

It has been demonstrated that the efficiency of autoantigen-based vaccines for T1D highly increases if CTB or RTB protein are used as adjuvant and fused with the autoantigen. This strategy enhances the oral tolerance of co-administered antigens by targeting them to specialized antigen-presenting cells of the GALT and improves the effect of orally administered antigens. Mice fed with transgenic potato tubers expressing CTB-proinsulin but not proinsulin alone, displayed a significant insulitis reduction and developed an insulin-specific Th2-lymphocyte response. In addition, oral administration of plant synthesized CTB-GAD was found to be effective in suppression of T1D symptoms in amounts 1000 times less than previously reported for unconjugated GAD. These results highlight the importance of the CTB carrier protein in enhancement of the mucosal immune response at low antigen concentrations.

Anti-inflammatory cytokines, including IL-4, IL-10, and IL-13, have been proposed as therapeutic agents for different diseases as well as an alternative to the use of adjuvants in the development of oral autoantigen-based vaccines for T1D. Unfortunately, the current production systems for these cytokines, which includes *E. coli*, yeast or mammalian cell cultures, have several limitations that in some cases can be overcome with extra production steps but result in a high market price. Plants offer a more efficient and less expensive production platform for these cytokines. IL-4, IL-10, and IL-13 have been successfully produced in different plant systems alone or in combination with T1D autoantigens.

Although plants clearly represent an efficient and inexpensive production platform for T1D therapeutics, some issues such as public acceptance of transgenic plants, consistency in product quality and, in the case of orally-delivered plant-made vaccines, homogeneous doses per gram of plant tissue, still need to be fully addressed before plant-based production technology can reach the market [168]. However, the encouraging results that have been obtained using plant-derived vaccines for T1D highlight the potential of this strategy as an effective

and low cost intervention for prevention and treatment of this disease in the near future.

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