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Dietary Proteins as Environmental Modifiers of Type 1 Diabetes Mellitus

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■ **Abstract** Type 1 diabetes is an autoimmune disease in which the patient's immune system destroys the insulin-secreting β-cells in the pancreatic islets of Langerhans. A majority of cases is thought to occur as a result of gene-environment interactions. The identity of the environmental factors remains unknown mainly because of the difficulty in linking past exposures with later disease development. Overall, the data suggest a model in which individuals develop diabetes by several different pathways, each influenced by numerous genetic and environmental variables. The most investigated environmental factors are diet and viruses. In this review, we examine the evidence that the source of dietary proteins can modify diabetes outcome, describe new approaches to identify candidate diabetes-related dietary agents, examine possible links with gut dysfunction, discuss some of the limitations, and propose a multifactorial model for dietary modification of diabetes. The key to diabetes pathogenesis, its prevention, and the ultimate success of β-cell replacement therapies lies in understanding how the environment controls disease expression. Dietary proteins could be one of these keys.

CONTENTS

ABBREVIATIONS

Anti-RT6 antibodies, antibodies against a regulatory T cell subset in rats; BB, diabetes-prone BioBreeding rat; BSA, bovine serum albumin; Glb1, homologue of the wheat storage globulin-1 protein; HLA, human leukocyte antigen; MHC, major histocompatibility complex; MLN, mesenteric lymph nodes; NOD, nonobese diabetic mice; T1D, type 1 diabetes; ZO-1, -2, zona occludens-1, -2.

INTRODUCTION

Overview of Autoimmune Type 1 Diabetes

Type 1 diabetes (T1D) is an autoimmune disease that occurs mainly in children and young adults and affects approximately 0.4% of the population of developed countries. The pathogenesis involves a T cell–mediated immune attack directed at the insulin-secreting β-cells in the pancreatic islets. T1D accounts for ∼10% of all patients with diabetes, and is the most severe form of the disease, requiring patients to monitor blood glucose and administer insulin several times a day to avoid ketosis, coma, and death. In many patients, serious complications develop that shorten their lifespan by as much as 15 years. The cause remains unknown, there is no cure, and insulin injections do not mimic the fine control of blood glucose homeostasis by the insulin-secreting β-cells.

T1D is a polygenic disease in which susceptibility is imparted by 20 or more loci. As with most autoimmune diseases, the major genetic risk for T1D is attributable to genes in the human leukocyte antigen (HLA) region as well as other non-HLA genes (35). These HLA risk genes are present in 20% of Caucasians, but only a small fraction of these individuals, ∼5%, develop diabetes (69). The progression of β-cell destruction is reflected by the presence of several autoantibodies, three of which have been studied extensively, glutamic acid decarboxylase-65 (GAD65), insulinoma-associated autoantigen-2 (IA-2), and insulin.

Why has it been so difficult to unravel the pathogenesis of T1D? The likely answer is that T1D does not develop the same way in all individuals but rather by many pathways that result in a similar phenotype, β-cell death, and insulin dependence (Figure 1). There is a complex interaction among many genes, multiple organ systems, and external agents with various admixtures, effectively making each individual patient a unique case representing a particular subcategory of pathogenesis. In this view, the majority of genetically susceptible individuals are exposed to one or several common environmental agents and some people will develop diabetes, whereas the majority will remain diabetes free. These environmental agents are not necessarily diabetogenic, but likely are not handled normally by those whose immune systems are predisposed to target the islets and in whom other defenses such as the gut barrier or β-cell regeneration could be defective. Thus, it has been proposed that there are environmental initiators and promoting or protective agents (129) that affect diabetes expression depending on age and duration of exposure (Figure 1). There is indirect evidence that one of these environmental factors is dietary proteins, and they are the subject of this review.

Objectives of this Review

This review focuses on published evidence related to the involvement of the dietary protein source in T1D pathogenesis. The involvement of nutritional targets such as the gut, its immune system, and the pancreas is discussed. The role of the gut as a dysfunctional barrier and a potential source of β-cell-specific T cells is considered, and possible cellular and molecular mechanisms are explored.

ANIMAL MODELS OF SPONTANEOUS T1D—BB RATS AND NOD MICE

The pancreas is difficult to access and can be damaged during biopsy. Consequently, it is not usually sampled for study of T1D progression in patients. As an alternative, peripheral blood mononuclear cells are often used as the tissue source for studies of autoimmune T1D in humans. It is unclear, however, to what extent these cells reflect the destructive process in the pancreas. To avoid problems related to accessibility of tissues and to analyze pathways involved in the early prediabetic phase, animal models are used (75, 92). The major animal models of spontaneous autoimmune T1D are the diabetes-prone BioBreeding (BB) rat and nonobese diabetic (NOD) mouse. In both models, diabetes is strongly influenced by environment. Studies in these animals provide a useful testing ground to identify candidate modifiers and pathways involved in human T1D.

BB rats (92) show clinical characteristics of progressive islet infiltration by immune cells (insulitis) beginning around adolescence at 50 days that closely resembles the lesion in the human diabetic pancreas and results in diabetes

development between 60–120 days. T cell lymphopenia in these animals creates an immune imbalance that results in reduced $CD4^+$ T cells, nearly complete absence of $CD8^+$ T cells, and the loss of regulatory T cells. The diminished level of T regulatory cells tips the immune system toward autoimmunity, permitting the activation of $CD4^+$ T cells and a few $CD8^+$ T cells that participate in β-cell destruction. Autoantibodies to islet cells, and sometimes insulin and GAD, have been reported. As in patients, diabetes in BB rats is polygenic, requiring the presence of major histocompatibility complex (MHC) class II region genes of the $RT1^u$ haplotype (*Iddm1*), the immune-associated nucleotide (*Ian4/5* responsible for lymphopenia, now renamed *GiMap5* this is the *Iddm2* gene), *Iddm4,* and others (53, 60, 80, 92, 113).

The NOD mouse develops T1D later, starting around 80 days up to 180 days. There is a marked sex difference in T1D incidence, with approximately 70%–90% of females, compared with ∼20%–40% of males, developing diabetes. Peri-insulitis is observed as early as 20–30 days and persists for several weeks, developing into a T cell hyperplasia followed by islet infiltration and T1D onset. Islet-specific autoantibodies and autoreactive $CD4^+$ and $CD8^+$ T cells are present. Spontaneous diabetes in the NOD mouse is also a polygenic trait. Risk genes include alleles within the MHC and several non-MHC genes (75). The NOD mouse has the $H\text{-}2^{g7}$ MHC class II haplotype that is the most important contributor of genetic risk. Other candidate proteins include cytotoxic T-lymphocyte associated-4 (CTLA-4, *Idd5*), Vav3 (*Idd18*), CD101 (*Idd10*), and IL-2 or IL-21 (*Idd3*) (5).

GENETIC SUSCEPTIBILITY IN HUMANS IS POLYGENIC AND COMPLEX

The genetics of human T1D is complex (35, 107). Linkage analysis and genetic association studies have identified more than 20 loci believed to be associated with increased disease risk. However, the task has proven difficult due to variable and often low penetrance of risk alleles. The MHC class II region genes, called HLA in humans, produce proteins that are responsible for the presentation of antigens on the surface of antigen-presenting cells to T lymphocytes. Certain T1D-related MHC class II genes confer the highest susceptibility and account for ∼40% of genetic risk. The majority of T1D patients carry the risk haplotypes HLA-DQB1, HLA-DQA1, and HLA-DRB1, which includes HLA-DR3 and -DR4, collectively referred to as *IDDM1*. The HLA-DQ locus is the most strongly associated with T1D and risk is also high in those who are HLA-DR3/DR4 heterozygotes (35).

Another major risk gene is *IDDM2* corresponding to the variable number of tandem repeats region located upstream of the insulin gene *INS*, which accounts for ∼10% of genetic susceptibility. A third well-characterized locus is *IDDM12,* which corresponds to CTLA-4. The search for additional susceptibility genes continues. Some recently identified candidates include *SUMO-4* (11), *T-bet* (122), and *toll-like receptor 2* (109). The identity of the complete collection of risk genes is not

known but will ultimately offer insights into the pathogenesis, prevention, and treatment of T1D. Thus, T1D is strongly influenced by genetic risk linked to HLA, non-HLA, and insulin genes. Yet, it has been shown that the frequency of patients with high-risk HLA genotypes has decreased over the past 50 years, in spite of the continuing increase in the incidence of T1D (37). This has been interpreted as additional evidence that environmental factors play a role.

DISEASE EXPRESSION INFLUENCED BY ENVIRONMENT

The development of diabetes requires genetic susceptibility that in some cases may be sufficient by itself for the development of overt disease, but in the majority of cases is thought to depend on interactions with one or more factors in the environment. The identity of T1D-related environmental factors in humans has been sought using epidemiological analyses, natural history studies, and animal experiments.

Rising Incidence—A Diabetes Epidemic?

The incidence of T1D has increased dramatically, ~2–4 fold over the past 50 years at a rate of 3% per year in children of 0–14 years (32, 37, 108). There was also an increase in disease incidence of almost 5% per year in European children of age 0–4 years. Recent studies continue to report high annual rates of increase of diabetes, for example 2.8% in New South Wales (148) and 4.9% in Franche-Comte, France (86). Changes of this magnitude over such a short time cannot be due to fluctuations in genetic risk alone and must involve factors in the environment.

Global Variation and Migration

There is a wide geographic variation in T1D incidence, ranging from 0.1/100,000 in China and Venezuela to ~37/100,000 in Finland, Sardinia, and Newfoundland (41, 99). T1D incidence can also differ between genetically similar populations, and immigrants often assume the risk of their new region or country. A recent study (71) examined the frequency of T1D in two adjacent countries, Finland and Estonia, which have populations with a similar genetic background. Although the frequency of risk HLA genotypes did not differ between these populations, there was a sixfold higher incidence of T1D in Finland.

Family and Case-Control Studies

A number of studies have examined the concordance rate of T1D in twin pairs to determine to what extent common genetics and environmental factors modulate disease development. The concordance of T1D among monozygotic twins depends on age at initial diagnosis and is less than 40% overall (69, 114). The concordance

rate of monozygotic twins is higher than dizygotic twins or siblings. The rate of diabetes among siblings (6%) is approximately 15 times that of the 0.4%–0.6% rate in the general population. These findings indicate incomplete penetrance of diabetes risk genes and are consistent with a requirement for genetic susceptibility that is influenced by environment.

Numerous case-control studies have been performed to investigate the role of diet in T1D, mostly related to the proposed involvement of cow milk proteins. These studies have been inconsistent, some supporting a role for early cow milk exposure, whereas others have not (2, 3, 103, 104). It has been difficult to differentiate whether effects are due to early exposure to foreign antigens or to lack of breast-feeding.

Prospective Natural History Trials

Many attempts have been made to identify environmental factors that influence T1D in humans, but the results have not always been consistent. The variability likely reflects the fact that T1D is a complex, chronic disease that occurs by several pathways in individuals who share some but not all risk genes. Most studies have been small, cross-sectional and of short duration, with exposure data collected retrospectively, possibly leading to recall bias. The best hope for identifying diabetes-related environmental agents is to undertake prospective studies. Several prospective studies that are ongoing or recently completed include the Trial to Reduce IDDM in the Genetically at Risk (TRIGR) (4), the Diabetes Prediction and Prevention (DIPP) study (97), the Diabetes Autoimmunity in the Young (DAISY) study (40), and BABYDIAB (166). These studies were designed to investigate whether exposure to selected environmental agents such as viruses, foods, stress, and socioeconomic status alters diabetes onset in high-risk first-degree relatives of T1D patients or individuals from the general population.

The only randomized controlled intervention trial of diet reported so far is the pilot study for TRIGR (4) (http://trigr.epi.usf.edu/). The TRIGR Web site states that the objective of the TRIGR trial is to "determine whether delayed exposure to intact food proteins will reduce the chances of developing type 1 diabetes later in life." This 10-year study began in 2002 and it will involve 6000 high-risk infants. The test group is receiving a hydrolyzed casein formula, Nutramigen, whereas the control group receives a standard whole milk–based formula with some hydrolyzed casein added to provide a similar taste.

Of particular note is the Environmental Determinants of Diabetes in the Young; (TEDDY) study (http://teddy.epi.usf.edu/). According to the Web site, the objective is to enroll 5940 newborns from the general population and 1152 high-risk neonates with first-degree relatives with T1D and to monitor for development of β-cell autoantibodies and diabetes. Sampling and follow-up will be frequent, every three months, and immunological and genetic analyses will be performed. The study

will run for 15 years, and the objective is to identify dietary and infectious agents that promote T1D or protect against its development.

MULTIPLE ENVIRONMENTAL FACTORS

Nonnutrient Components

BACTERIA AND CHEMICAL TOXINS Several chemicals can cause β-cell death. The most-studied β-cell cytotoxins are streptozotocin and alloxan, which induce diabetes in rodents (33, 147). Some pathogenic bacteria that could be encountered in the diet produce β-cell cytotoxins, such as streptozotocin and bafilomycin A1 from *Streptomyces* (95). Additionally, components or products of the gut microflora, such as lipopolysaccharide in the cell wall of gram-negative bacteria, can act as adjuvants for the immune response to soluble antigens (137). This raises the question whether dietary modification of T1D is attributable to diet-induced changes in commensal or pathogenic gut bacteria. The fact that diabetes still occurs in BB rats and NOD mice maintained in ultra clear conditions (121) suggests that the natural course of spontaneous diabetes is not necessarily dependent on commensal bacteria pathogens. However, it is still possible that interventions that alter gut microflora, such as probiotics, oral antibiotics, and dietary protein source, can affect diabetes outcome (13, 44).

VIRUSES For more than a century it has been suspected that viruses cause human T1D. The multiplicity of viruses and the problem of relating early infection with later appearance of diabetes have made it difficult to link viral infection and diabetes. Mumps, rubella, cytomegalovirus, and enteroviruses such as coxsackie and retroviruses have been implicated (61). Maternal rubella virus infection is linked to T1D. Approximately 20% of children born with congenital rubella syndrome in New York City in the 1960s developed T1D. Although this suggests that rubella virus can affect diabetes development, T1D incidence has increased since the 1960s despite the fact that rubella has been almost entirely eliminated by vaccinations in developed countries.

Fetal exposure to enteroviruses has been linked to T1D (55). However, a recent systematic review of 26 case-control studies showed no serological evidence to support a causal role for the major candidate enterovirus, coxsackie B (42), and no relationship with diabetes autoimmunity was found in two prospective studies, DAISY in Denver (40) and BABYDIAB in Munich (30). Indeed, an inverse relationship between enterovirus antibodies and diabetes frequency in humans has been reported (154). This is consistent with reports that some viruses protect against T1D in BB rats and NOD mice (164). NOD mice and BB rats maintained in specific-pathogen-free conditions develop diabetes at similar or higher rates than do those raised in standard conditions. This suggests that pathogens are not

essential for the development of diabetes in these animals. The search for viruses that cause diabetes in humans continues (48).

Dietary Components

MICRONUTRIENTS Recent data suggest there is an association between vitamin D3 and T1D (reviewed in 85). It has been demonstrated that early dietary supplementation with vitamin D may decrease risk of T1D in humans (56). Administration of high levels of vitamin D to NOD mice inhibited insulitis and prevented diabetes. Giulietti et al. (38) confirmed their original findings in NOD mice, showing that vitamin D deficiency in early life increased diabetes cases. By contrast, Schmid et al. (127) have shown that diabetes incidence in NOD mice was not reduced by vitamin D supplementation of the diet. It has been proposed that the higher incidence of T1D in countries where there are fewer hours of sunshine could be due to reduced generation of biologically active vitamin D (100). However, it is unclear whether the different genetic risk among these countries could also explain this trend. There are only a few studies of micronutrient effects on T1D, and some are conflicting, indicating the need for further research.

MACRONUTRIENTS Contrary to the common misconception that T1D develops in individuals who consume diets rich in simple sugars, no link has been identified between available dietary carbohydrates and T1D. A recent study showed that the type of dietary fiber could influence the immune response of BB rats (146). Some studies have shown that apart from essential fatty acid deficiency, lipids do not influence T1D (50, 65, 127). However, the role of dietary lipids, particularly in high-risk infants, has not been studied in detail.

Extensive studies in which carbohydrate, fat, fiber, protein, micronutrients, and caloric intake were examined using isonitrogenous, isocaloric semipurified diets (117) attributed dietary modification of diabetes to the amino acid source (133). The source of dietary proteins has been found to have a pronounced effect on the development of diabetes in NOD mice and BB rats. Various dietary protein sources have been linked to induction of T1D, including wheat, soy, and milk (reviewed in 2, 133). Conversely, several diabetes-retardant amino acid sources have also been identified (79, 130, 133).

DIETARY PROTEIN SOURCE—A MAJOR CONTROLLER OF T1D

Diabetes-Retardant Sources

Diabetes-retardant amino acid sources in BB rats include corn, corn gluten, fish meal, peanut meal, canola flour, kidney beans, caseins, lactalbumin, hydrolyzed soy proteins, hydrolyzed casein, hydrolyzed lactalbumin, and free amino acids (130, 133). Thus, there are several diabetes-retardant amino acid sources. It is not

clear whether protection is due to diabetes-retardant factors in these protein sources or is due to a lack of diabetes-promoting agents. The latter has been assumed but has not been proven directly.

The most consistent finding in the area of nutritional modification of type 1 diabetes is that hydrolyzed casein-based diets inhibit development of diabetes in BB rats and NOD mice. This is the most studied of the diabetes-retardant diets and has become a de facto control. Casein hydrolysates contain numerous peptides varying in length from 2 to 27 amino acids with at least a dozen displaying various biological activities (88a), including opioid agonists, enzyme inhibitors, mineral transporters, antithrombotic and antihypertensive peptides, and growth factors that could affect the diabetes-retardant properties, but this possibility has not been investigated. Hydrolyzed casein-based diets also promote secretion of pancreatic digestive enzymes (47) and increase the secretion of mucin in the gut of normal (18) and diabetes-prone rats (22). Mucin provides a filter barrier to the passage of bacteria and selected food molecules, and increased digestive enzymes might help destroy diabetes-promoting proteins. Thus, hydrolyzed casein-based diets probably lack diabetes-promoting agents found in standard cereal-based diets and also contain potentially bioactive compounds that could contribute to the diabetes-retardant effect.

Three unique rat models of T1D were not protected from diabetes by hydrolyzed casein-based or amino acid diets: (*a*) a strain of high-incidence BB rats, which develops diabetes because of the lymphopenia (*Ian5, GiMap5*) gene introgressed onto the diabetes-resistant BB rat background (142); (*b*) the Komeda diabetes-prone rat (74), which has a mutation in the regulator of T cell activation, *Cbl-b*; and (*c*) the diabetes-resistant BB rat treated with anti-RT6 antibody and a viral mimic of double-stranded RNA, poly I:C, to remove T regulatory cells (83), a model of rapid-onset diabetes. Each of these animals develops diabetes as a result of powerful alterations in the immune system and may represent mainly immune-mediated forms of diabetes. A simplistic interpretation would be that the protection seen in standard BB rats fed hydrolyzed casein occurs distal to these molecular steps, acting before *Cbl-b*, *Ian5*/*GiMap5,* or the point where the deficit of T regulatory cells intersects the pathway to diabetes.

Diabetes-Promoting Sources

WHEAT Refined wheat flour is a major component of the diet in developed countries where T1D incidence is highest. It is composed mainly of gluten, a complex mixture of several hundred polypeptides, mainly glutenins and gliadin storage proteins, which reside in the water-insoluble fraction of the wheat endosperm. However, complete separation of the proteins based on differential solubility is difficult and there is contamination among the fractions (36), with albumins and globulins still present in gluten.

Early studies in the BB rat demonstrated that dietary agents can modulate the onset of diabetes (26, 57, 131, 135). These studies used semipurified diets in which amino acids were supplied as such or by single food protein sources

supplemented with essential amino acids or hydrolyzed casein where necessary (8, 26, 50, 79, 83, 133, 137, 155). Analysis of representative individual nutrient components of a diabetes-promoting, standard cereal-based diets, such as NIH-07 and NTP-2000 (133), showed that the major diabetes-promoting ingredients were wheat, soy, and milk protein sources. The most potent diabetes-promoting agent fed in defined diets to BB rats was wheat gluten that is ~80% protein (79, 138). Earlier studies suggested the diabetes-promoting activity in wheat was associated with the low-molecular-weight glutenin fraction. This remains a possibility, and in addition, more recent studies in which a wheat cDNA expression library was screened identified a storage globulin as a potential diabetes-related protein (79). This globulin was present as a contaminant in extracts of wheat gluten.

Hydrolysis of a complete standard NIH-07 diet decreased diabetes incidence, but feeding a diet in which papain-hydrolyzed wheat gluten was the main amino acid source did not decrease diabetes incidence in BB rats (133). Thus, hydrolysis per se was not necessarily protective. This suggests either that the diabetes-promoting activity in wheat gluten was not protein or that wheat peptides remaining after hydrolysis with papain still promoted diabetes. The latter interpretation is similar to the finding that wheat gliadin peptides, which induce inflammation and damage in the gut mucosa of celiac patients, were destroyed by treatment with papain but not by treatment with pepsin or trypsin (72). This indicated that celiac toxic peptides in gliadin are resistant to normal digestive enzymes, a point that was confirmed with the recent identification of digestion-resistant celiac peptides, including a 33-mer (140). The fact that diabetes-promoting activity of wheat gluten is papain-resistant suggests the presence of unique peptide sequences and structures that will require proteases with a different specificity for deactivation (112).

In NOD mice, wheat-based diets have also been reported to be diabetes promoting in some (8, 51, 87) but not all studies (20, 27). Diabetes incidence was reduced and onset was delayed in animals fed gluten-free or hydrolyzed casein diets (8, 20, 27, 51, 62). In three studies, wheat-free diets protected NOD mice from developing diabetes (31, 87, 127). In the report by Maurano et al. (87), adding back wheat gluten to a wheat-free diet in which amino acids were supplied from milk, peanuts, and supplemental amino acids significantly increased diabetes incidence. In two contrasting studies, when wheat gluten was added back to either a casein (20) or hydrolyzed casein (27) diet at 10% or 2% of the diet, respectively, diabetes incidence was not increased. Coleman et al. (20) found that extracting a mainly wheat-based diet with chloroform-methanol removed the diabetes-promoting activity, suggesting it was either lipoidal in nature or part of the chloroform-methanol soluble protein fraction (118). In general, NOD mice fed mainly cereal-based diets consistently develop diabetes at high frequencies compared with those fed diabetes-retardant diets, similar to findings in BB rats. It appears however, that in NOD mice, wheat gluten is only sometimes diabetes promoting, by contrast with BB rats, where it is more consistently diabetes promoting. This inconsistency is presently unexplained. Hoorfar et al. (51) reported that a defined diet in

which proteins were supplied only from wheat flour resulted in 60% of animals developing diabetes compared with only 22% fed a hydrolyzed casein diet. This suggests that the diabetes-promoting agents in wheat flour could be either glutens or nongluten proteins such as albumins or globulins. The inconsistencies in the NOD mouse data could also be due to variations in the base diet composition, differences in gluten fractions, different background microbial populations, lower concentrations of gluten in the test diets, differences in weaning conditions, or simply that wheat gluten is not diabetes-promoting in all NOD colonies. The nature of the diabetes-promoting agents in cereal-based diets fed to NOD mice remains an open question.

Evidence supporting the role of wheat proteins in the pathogenesis of diabetes in humans is accumulating. Reports from the BABYDIAB prospective study indicated an increased risk for development of islet autoantibodies associated with early gluten exposure (<3 months) in offspring of T1D parents, whereas introduction of gluten after the age of 6 months did not alter the risk (166). The DAISY prospective study reported that children exposed to cereals from 0–3 months or after 7 months were at increased risk for islet autoantibody development (101). This suggests that wheat proteins could be linked to diabetes autoimmunity and that the timing of early exposure is important. Neither study found a link between early milk consumption and autoantibody positivity. A recent study (111) demonstrated that when patients newly diagnosed with T1D were placed on a gluten-free diet, neither the level nor frequency of anti-GAD, IA-2, or insulin autoantibodies decreased, a result that might be expected in patients already diagnosed with T1D. Nonetheless, there was improved insulin secretion and insulin sensitivity in the patients on gluten-free diets, suggesting an increase in insulin production or action (111). Klemetti et al. (67) demonstrated that there is increased T cell proliferation in response to wheat gluten in newly diagnosed T1D patients. Our preliminary data confirm that a significant subset of human T1D patients display increased $CD3^+$ T cell proliferation in response to a mixture of wheat proteins compared with age and sex-matched healthy control subjects (90). The goal of the BABYDIET study (126) is to clarify whether delayed introduction of gluten decreases the risk of developing islet autoimmunity in susceptible children.

Using a strategy similar to the approach used to identify all the autoantigens in T1D, a first candidate diabetes-related wheat protein was identified by screening a wheat cDNA expression library using serum from diabetic BB rats (79). Clone WP5212 shares 90% identity at the gene level and 80% identity at the protein level with the *Triticum aestivum* wheat storage globulin, Glb1. For simplicity, this protein is referred to as Glb1. Antibody reactivity to Glb1 was significantly higher in diabetic rats than in asymptomatic and control rats and correlated with pancreatic inflammation and damage. This suggests either that Glb1 is antigenic and somehow participates in diabetes pathogenesis or alternatively that immune reactivity to Glb1 is an indicator of abnormal gut function, lack of oral tolerance, or cross-reactivity with self antigens. It was concluded that Glb1 is a normal contaminant of wheat gluten and contains peptides that are highly antigenic in a majority of wheat-fed

BB rats. There are preliminary indications that Glb1 could also be antigenic in human patients (79) with TID. There is one case report of a highly wheat-sensitive patient with both T1D and celiac disease who displayed strong immune reactivity to Glb1 (89).

MILK The main proteins in cow milk are caseins (αs1, αs2, β, κ, γ, 80%) (141), β-lactoglobulin (10%, absent from human milk), α-lactalbumin (5%), γ-globulin (2%), and bovine serum albumin (1%) (145). Early exposure to cow milk has been linked with increased T1D risk in humans, BB rats, and NOD mice (68). It has been suggested that this link is related to the presence of diabetes-promoting agents or early removal of the protective effects of human milk, which contains growth factors, cytokines, and antibodies. Case studies demonstrating a correlation between early exposure to cow milk, lack of breast-feeding, and the development of T1D have been reviewed (2, 34, 136, 159). Some studies did not find any relationship (6, 10, 63, 102, 125). The variability may be due to variation in milk composition or the existence of a subset of individuals susceptible to milk-related T1D. The TRIGR pilot study, which tested the effect of avoiding foreign dietary proteins in high-risk neonates (4), is the first demonstration of dietary modification of diabetes autoimmunity in humans. High-risk infants fed a hydrolyzed casein-based formula developed islet autoantibodies less frequently compared with infants fed a standard milk formula (4).

Results from one study showed that the A1-β-casein variant promoted diabetes, whereas the A2 variant was protective when fed to NOD mice (28). In another study (8), both A1 and A2 caseins were diabetes retardant to varying degrees in BB rats and NOD mice, and the initial finding (28) in NOD mice was not confirmed. Overall, the results suggested that A1 casein was not consistently more diabetes promoting than A2 and that casein milk proteins have low to moderate diabetes-promoting activity. Thus, it was concluded that A1 or A2 β-caseins are unlikely to be exclusive promoters of T1D but could still modulate T1D development in a subset of susceptible individuals.

SOY Soybean meal proteins were associated with moderate diabetes incidence in NOD mice (51) and BB rats (52); however, when the proteins are purified, hydrolyzed, and heat treated, they tend to lose their diabetes-inducing potential (7, 8, 27, 132). Cyclophosphamide-accelerated diabetes was delayed in NOD mice fed a hydrolyzed soy-based diet (116). There is a report of possible molecular mimicry between the autoantigen, IA-2, and NADH ubiquinone in wheat and soybeans (49).

LIMITATIONS OF FEEDING TRIALS IN DIABETES-PRONE ANIMALS Foods are highly complex mixtures that change depending on time of the year, geographic location, processing, heating, chemical treatment, shipping, storage, or cooking. Ideally, it should be possible to compare isonitrogenous, isocaloric diets in which only single proteins, supplemented when necessary with essential amino acids, are tested for diabetes-promoting or retardant properties. In practice, it is usually not feasible to obtain purified proteins in sufficient amounts to feed diabetes-prone animals,

due to limited availability and cost. This has meant using protein sources such as concentrates, isolates, or fractions that are not pure and have usually been chemically treated. For example, purification of wheat proteins by differential solubility in water, saline, 70% alcohol, or dilute acetic acid into albumins, globulins, gliadins, and glutenins typically produces fractions contaminated with one or more of the aforementioned protein groups. Even within these fractions, there is a complex mixture of proteins. One approach to help reduce the variability that has characterized the study of nutrients and diabetes in feeding trials is to use defined, semipurified, isonitrogenous, isocaloric diets based on the AIN-93G diet (117, 130, 133).

POTENTIAL MECHANISMS

The mechanisms by which dietary modification of T1D occurs are poorly understood. However, mounting evidence suggests that the gut could play an important role as a defective barrier and a possible source of activated immune cells (81, 149). There are also some indications that gut leakiness influences the islets directly, as glucoregulation is affected. In addition, it is possible that protective diets not only dampen immune activation but also enhance islet function.

Celiac Disease—A Useful Model

A T cell–mediated immune response to protease-resistant wheat gliadin peptides (140) activates autoreactive Th1 cells resulting in damage to the intestinal mucosa in celiac patients (94, 144). Genetic susceptibility is associated with the HLA-DQ2 haplotype in 95% of celiac patients, and HLA-DQ2 is also present in ~40% of patients with T1D. A wheat-T1D link is suggested by the high prevalence of celiac disease in patients with T1D (1%–8%) compared with the general population (0.4%–1.0%) (128). As many as 30% of children newly diagnosed with T1D have antibodies to tissue transglutaminase, an autoantigen in celiac disease, and antigliadin antibodies have been reported in infants newly diagnosed with T1D (15, 73). Anti-tissue transglutaminase antibodies were also present in NOD mice, but were not dependent on dietary gluten (124), as is the case in human celiac patients. In patients diagnosed with both diseases, the majority develop T1D first, suggesting that the standard treatment for celiac disease, a strict gluten-free diet, may decrease the risk of diabetes if implemented in the prediabetic period. In addition, offspring of parents with T1D develop antibodies associated with celiac disease (54), which could reflect shared genetic risk for T1D and celiac disease or gut barrier dysfunction or wheat-specific immune activation.

Role of the Gut

BARRIER DYSFUNCTION AND INFLAMMATION The mucosal surface of the gut is the main site where the host senses and responds to environmental signals from food antigens and microbes through stimulation of cells in the gut immune system. A

single epithelial cell layer coated with mucin separates the contents of the gut lumen from the lamina propria (93). Thus far, the main T1D-promoting environmental candidates are enteroviruses and dietary proteins, both of which enter the body via the gastrointestinal tract.

There are several indications that increased gut permeability may contribute to the development of diabetes. Abnormally increased gut permeability was reported in newly diagnosed patients (14, 23, 91, 139). Meddings et al. (88) reported similar findings in studies of BB rats. The increase in permeability appears to occur via the paracellular route and could be related to changes in tight junction complexes (98, 160).

An important first direct demonstration that gut leakiness is associated with the development of diabetes was reported recently (160). Endogenous zonulin protein increased by 6- to 35-fold in the BB rat intestine during the development of insulitis. Blocking this response with an inhibitor of the zonulin receptor, which closes tight junctions and decreases gut leakiness, prevented diabetes. This result is consistent with the finding that gut damage and increased permeability predate insulitis in BB rats (39). Gut permeability could also have direct effects on islet metabolism as it was correlated with impaired glucoregulation in diabetes-prone BB rats (81). Furthermore, gut permeability was decreased significantly in hydrolyzed casein-fed BB rats (22, 81), a finding that suggests gut leakiness could be modified by diet. This could be due to gliadin-induced zonulin secretion (19). In six of eight nonceliac T1D patients examined by endoscopy, there was no evidence of atrophy or inflammation by light microscopy (139). However, transmission electron microscopy revealed ultrastructural changes in tight junctions with increased intercellular spaces consistent with the presence of a leaky gut barrier. The close link between gut permeability and the development of diabetes suggests that agents in the gut lumen affect T1D development.

$\alpha 4\beta 7$ is an integrin expressed on circulating leukocytes that home to the gut. In comparison with controls, patients had increased $\alpha 4\beta 7$-integrin$^+$ cells in the lamina propria and $\alpha 4\beta 7$-integrin$^+$ peripheral blood mononuclear cells of young T1D patients had increased IFN-γ and TGF-β production (66). A proportion of autoreactive cells from T1D patients also express this integrin (110). These cells have been shown to traffic to the pancreas in NOD mice via interactions with the ligand MAdCAM-1, which is expressed in the exocrine pancreas on endothelial cells around the islets. Cells expressing $\alpha 4\beta 7$ have been identified in the pancreas of NOD mice (43), and blocking $\alpha 4\beta 7$ binding prevented T1D (163). These studies are consistent with the involvement of lymphocytes that home to the gut in the development of T1D and suggest that luminal antigens, including dietary proteins, could be important determinants of gut immune activity that is directed toward β-cells.

The gut of diabetes-prone animals and some patients with T1D appears to be in a state of mild, subclinical inflammation (39, 87, 161). Following weaning, the BB rat displays signs of a sporadic celiac-like enteropathy including inflammation, immune activation, flattened villi, crypt hypertrophy and hyperplasia, and

increased T cells in the lamina propria (39, 45, 81) preceding insulitis and diabetes. Inflammation in the BB rat gut is characterized by the presence of biochemical inflammatory mediators such as increased expression of the Th1 cytokine, IFN-γ, increased peroxidase (22, 39), decreased IL-10, and reduction of the Th3 cytokine, TGF-β. This is consistent with a lower gene expression ratio of T-bet/GATA-3, the major transcriptional controllers of mucosal Th1/Th2 cytokine expression (16). The gut mucosa of patients is reported to show signs of histological damage and immune activation, indicated by abnormally increased MHC class II expression in the villi (123, 139, 161).

If the gut is involved in T1D, what is its role? Feeding a hydrolyzed casein diet did not affect the histologic appearance of gut damage and inflammation in BB rats (39). In contrast, this diet dampened biochemical signs of inflammation (137), gut leakiness, and increased gut mucin content (21, 81). Wheat protein–based diets induce a proinflammatory Th1 cytokine profile in the gut and gut-associated lymphoid tissue in the BB rat (16, 137). In the NOD mouse, a wheat-based diet also induced celiac-like lesions characterized by reduced villus height, increased intraepithelial infiltration by $CD3^+$ T cells, enhanced epithelial MHC class II, and increased mRNA expression for IFN-γ, TNF-α, and inducible nitric oxide synthase (29, 87).

These data suggest that gut inflammation, either induced or constitutive, is a feature of diabetes in some animals and humans. In addition, several aspects of gut physiology and function are abnormal in diabetes, and some of these are down regulated in diabetes-prone animals fed a low-antigen, hydrolyzed casein diet.

IS THE GUT A SITE OF T CELL ACTIVATION? The gastrointestinal tract contains the largest collection of immune cells in the body. A question that remains is whether the primary role of the gut is as a route for entry of diabetes-promoting antigens or whether it is also the home of β-cell reactive immune cells. A recent study raised the possibility that β-cell-specific T cells could be activated by dietary antigens in the pancreatic lymph nodes (PLN; 148a). The MLN, which drain the gastrointestinal tract, are the main inductive sites for the gut immune system (93). BB rat MLN displayed increased cell proliferation and metabolic activity (82). Consistent with this finding, we reported recently that the Th1 cytokine bias in the MLN of cereal-fed BB rats is a reflection of the unusually high frequency of wheat-responsive, IFN-γ-producing Th1 cells, nearly all of which were $CD3^+CD4^+$. These cells are activated and mature in an environment that is relatively rich in antigen-presenting dendritic cells but low in inflammation-dampening T-regulatory cells (16). Importantly, Th1 cells in the MLN were less frequent in hydrolyzed casein-fed BB rats. It is not clear yet whether immune cells that proliferate in response to selected dietary proteins in BB rats participate in gut damage, traffic to the pancreas, and promote β-cell destruction, albeit cells isolated from MLN have been shown to traffic to the pancreas and induce T1D in adoptive transfer experiments in BB rats and NOD mice (43, 59, 162). An alternative explanation is that increased response to dietary proteins

might not be diabetes-related but could simply reflect the impaired state of the gut barrier and dysregulation in the gut immune system in diabetes-prone individuals (46, 70).

POTENTIAL DEFECTS IN ENTEROINSULAR COMMUNICATION Both the gut and the endocrine pancreas respond to the nutrient composition of ingested food. Glucose administered intravenously results in less insulin secretion from the endocrine pancreas than does orally ingested glucose, the so-called incretin effect (12). Specialized cells of the intestine secrete incretins such as gastric inhibitory peptide (GIP) and glucagon-like peptide-1 (GLP-1) into the circulation in response to carbohydrate and lipid intake, in addition to other stimuli. GLP-1 binds to its receptor on islet β-cells and induces insulin secretion, β-cell proliferation, and reduced apoptosis (24, 25, 77). In the BB rat, the concentration of GLP-1 protein in gut tissue was low compared with control animals, and this was accompanied by low expression of its receptor on β-cells (153). This finding suggests that communication between the gut and pancreas is suboptimal in diabetes-prone rats, resulting in decreased nutrient-induced insulinogenic signals from the gut.

ORAL TOLERANCE In healthy individuals, the gut immune system dampens the immune response to dietary antigens, inducing a state of immunological nonresponsiveness, known as oral tolerance (93). To determine whether wheat-related T1D involves a breakdown in oral tolerance to wheat antigens, we fed neonatal BB rats antigens from a mixed cereal-based diet or wheat gluten. This delayed diabetes onset, prevented T1D in ∼35%–50% of rats, and decreased proinflammatory IFN-γ production in the gut (137), probably by inducing oral tolerance. Because tolerance is primarily a protein antigen-driven phenomenon, this finding also suggests that the diabetes-promoting agents in wheat are proteins. Inflammatory damage to the gut barrier of some individuals prone to develop T1D could compromise oral tolerance, but this has not been demonstrated.

Insulin is the only β-cell-specific autoantigen, and polymorphism in the *Ins* gene accounts for ∼10% of genetic risk in humans. It has been suggested that oral immune tolerance to human insulin could be compromised by early introduction of dietary cow milk containing lower concentrations of insulin, which differs in amino acid sequence compared with human insulin (69, 151). This is consistent with the increased diabetes frequency in BB rats treated with oral porcine insulin (9). By contrast, in NOD mice, early oral exposure to insulin prevented T1D (84, 165), highlighting the contrasting effect of oral insulin in the two different animal models. Oral insulin treatment of high-risk individuals in the Diabetes Prevention Trial (DPT-1) did not delay or prevent diabetes (143), but there was some indication of a benefit in insulin antibody-positive patients. In spite of these contrasting effects of oral insulin treatment, considering insulin could be a primary initiating antigen in NOD diabetes (96), it will be important to understand how insulin in the diet could affect islet-specific autoimmunity.

Molecular Mimicry

Molecular mimicry has been proposed to occur when a lymphocyte is unable to distinguish between self and nonself antigens that share structural or sequence homology (105). This lymphocyte has the potential to induce autoimmunity when activated by nonself antigens. Although it is an attractive hypothesis, there are few examples of molecular mimicry (17, 106). It has been suggested that an environmental antigen can trigger or accelerate the onset of T1D by molecular mimicry with a self protein expressed on the β-cells (17). Consequently, β-cells are mistakenly attacked. It was speculated that defects in gut and pancreas depend on structural similarities between dietary antigens and self proteins (79).

Antibodies against bovine serum albumin (BSA), found in cow milk and beef, have been identified in T1D patients (64, 119). A 17 amino acid epitope, termed ABBOS, is common to BSA and the pancreatic β-cell surface protein ICA69 (64). Thus, molecular mimicry between these epitopes was hypothesized to be the link between cow milk and T1D. However, other investigators were not able to identify diabetes-specific immune responses against BSA or cross-reactivity of anti-BSA antibodies with ICA69 (1, 6, 58, 120, 150). It seems likely that the hypothesized ABBOS mimicry is either not involved in diabetes or plays a role only in a small subset of diabetes-prone individuals. There are no proven examples that molecular mimicry is the basis of T1D.

Endocrine Pancreas

There are indications that dietary protection from T1D acts in part by affecting islet homeostasis and regeneration. Rats fed a hydrolyzed casein diet have decreased expression of proinflammatory cytokines in the pancreas, low MHC class I expression on β-cells, increased plasma and pancreatic insulin content, as well as increased β-cell fraction (76, 82, 134, 156). The increase in β-cell fraction could be related in part to increased islet neogenesis (156, 157) or increased β-cell proliferation, which is also evident as an upregulated response to injury during β-cell destruction in BB rats (158). The beneficial effect of a protective hydrolyzed casein diet on β-cell mass is consistent with enhanced β-cell response in patients following a wheat-free diet for six months (111).

MULTIFACTORIAL MODEL OF NUTRITIONAL MODIFICATION OF T1D

In the hypothetical model described in Figure 2, damage to the gut intestinal epithelial cell barrier is a central feature and could involve opening of intercellular tight junctions. This would permit excess amounts of luminal constituents, including dietary protein antigens, to enter the lamina propria and MLN where humoral and cellular immune reactions take place. There is evidence of a specific dietary

protein–activated stimulation of $CD4^+$ T cells in BB rat MLN and evidence of immune activation in the gut-associated lymphoid tissues of NOD mice, BB rats, and human patients. We speculate that dietary antigen–stimulated T cells traffic to the islets and participate in the attack on the β-cells. It is also possible that such an immune response occurs as a result of bystander activation of β-cell-specific immune cells that reside in the gut. The model highlights the concomitant effects of dietary antigens on the gut immune system and the target β-cells, with both processes possibly dependent on a leaky gut.

CONCLUSIONS

It is difficult to identify dietary factors that promote or retard diabetes based on feeding trials that by necessity use nonpurified test proteins fed to diabetes-prone animals. Despite these limitations, feeding trials have revealed candidate dietary proteins (79, 101, 149, 151, 152, 166). These trials and epidemiological data provide indirect support that the dietary protein source affects the expression of diabetes in susceptible individuals. The problems inherent in the nutritional epidemiology of T1D, including the fact that most dietary factors are continuous variables and cannot be described as either present or absent, suggest reasons for the variability in reported outcomes. It is hoped that the TEDDY collaborative study will clarify which environmental factors are important in the development of T1D. At present, neither the identity of individual proteins nor the mechanisms by which they act are known.

The most recent findings in this field demonstrate that (*a*) high-risk infants exposed to hydrolyzed casein-based formula are less prone to develop diabetes autoantibodies (4), (*b*) two prospective studies in children highlight the possible involvement of cereals in development of diabetes autoimmunity (101, 166), (*c*) gut barrier damage, either constitutive or induced, is associated with T1D (14, 23, 88, 91, 98, 139, 160), and (*d*) complimentary approaches can provide candidate protein molecules (79). The variability in diabetes-promoting capacity of certain protein sources or diets that has typified studies in this area likely represents variations in foods, protein fractions, and whole-diet composition as well as variations in the influence of other environmental factors in colonies of diabetes-prone rodents or populations of susceptible humans. Identifying dietary antigens that promote T1D will be crucial to discover pathways that can be targeted for treatment or prevention in susceptible individuals.

FUTURE DIRECTIONS

Additional research is required to elucidate whether compromised barrier and immune function of the gut are affected by diet and play a central role in T1D. Progress in this area requires clarification of the molecular identity of diabetes-promoting

agents in the diet. In parallel with feeding of diabetes-modifying diets, proteomic and other molecular approaches could help identify new candidates. T1D pathogenesis is dependent on several interacting influences, namely genetic predisposition, environmental factors, decreased regenerative capacity of the endocrine pancreas, immune dysregulation, and probably gut barrier dysfunction. Future studies must be directed at understanding the integrative biology of diabetes-related dietary antigens, their interaction with the gut, its immune system, and islet biology.

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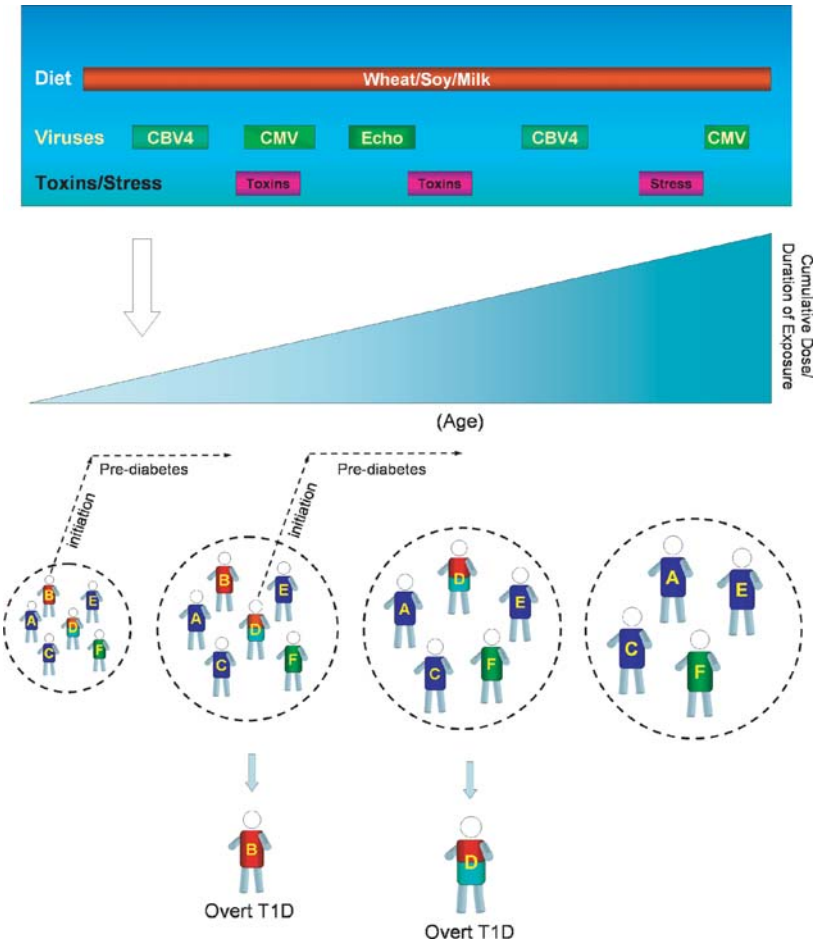

Figure 1 Genetic risk interacting with cumulative environmental exposures. The low penetrance of diabetes in individuals with risk genes suggests that factors in the environment are important contributors to development of the disease. The data are consistent with various combinations of risk genes interacting with single or combined environmental factors, to promote diabetes in subsets of susceptible people, who progress to overt diabetes by different pathways. Progression depends on age at initial exposure and duration. The individuals in the figure represent groups in the general population (*blue*) and individuals with varying risk of developing diabetes (*other colors*) with different genetic backgrounds (*letters*). The correct combination of timing of the initial exposure to diabetes-promoting agents (*dotted line*), duration of exposure, and genetic risk will lead to diabetes (individuals *B* and *D*). Individuals who carry the risk genes but are not exposed appropriately to the environmental risk factors will not develop the disease (e.g., individual *F*). The heterogeneity of individuals at risk and the large number of potential environmental factors are probably the most important confounders in understanding diabetes pathogenesis.

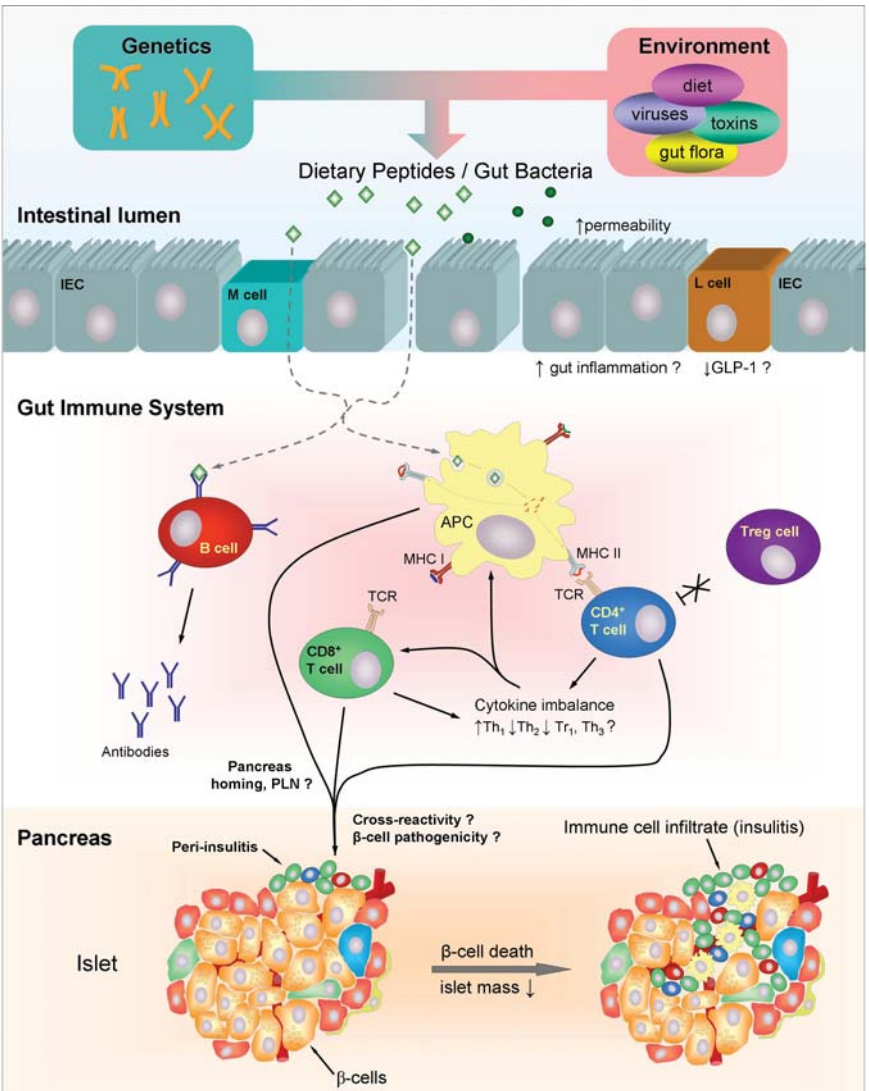

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Figure 2 A multifactorial model for modification of T1D by dietary proteins. In diabetes-prone individuals, the gut mucosa intestinal epithelial cell (IEC) barrier could be compromised. A leaky gut barrier could permit excess dietary proteins and peptides (or other gut lumen constituents) to enter the lamina propria, the draining mesenteric lymph nodes (MLN) and possibly the pancreatic lymph nodes (PLN). These peptides can also access the gut mucosa by the normal route of uptake via microfold (M) cells on Peyer's patches. Antigen-presenting cells (APC) such as dendritic cells, macrophages, and B cells take up antigen and present it to $CD4^+$ T helper cells on MHC class II molecules. Activated $CD4^+$ T helper cells secrete proinflammatory Th1 cytokines and decreased levels of Th2/Th3 cytokines, inducing proinflammatory $CD8^+$ T cells and APCs, which can also secrete inflammatory mediators. Counterinflammatory regulatory T cells are infrequent and unable to maintain tolerance. Activated immune cells induce gut inflammation and damage. They also migrate to the pancreatic lymph nodes (PLN) and islets, where they promote destruction of β-cells. Dietary proteins and peptides also induce antibody production from B-cells. Glucagon-like peptide-1 (GLP-1) production by mucosal L cells is decreased, impairing communication between gut and endocrine pancreas, and inhibiting the β-cell mass-promoting activity of GLP-1. Thus, dietary proteins could regulate destruction of β-cells by affecting the gut immune system, inflammation, barrier function, and islet renewal.

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ERRATA

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