



# Putting the pieces of the puzzle together – a series of hypotheses on the etiology and pathogenesis of type 1 diabetes

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**Summary** This paper presents a series of 10 hypotheses on the etiology of type 1 diabetes. We begin with the hypothesis that wheat gluten is one of the elusive environmental triggers in type 1 diabetes. Habitual consumption of wheat gluten increases the intestinal synthesis of dipeptidyl peptidase IV. This enzyme helps to shape the repertoire of peptides released into the small intestine following the ingestion of wheat gluten by catalyzing the release of X-Pro dipeptides from the N-terminus of the proline-rich glutenins and gliadins in wheat gluten. The release of gluten-derived peptides causes the tight junctions of the small intestine to open through a zonulin-dependent mechanism, which allows these peptides to enter the lamina propria where they get presented as antigens by HLA-DQ, -DR and CD1d molecules. Binding of one or more gluten peptides by CD1d leads to abrogation of oral tolerance, and a marked increase in peripheral immune responses to wheat proteins. Furthermore, it is our contention, that in response to  $\beta$  cell apoptosis during normal remodeling of the pancreas and CCL19/CCL21 expression within the pancreatic lymph nodes (PLNs), gluten-loaded dendritic cells migrate from the small intestine to the PLNs. These dendritic cells present gluten-derived antigens on the surface of the PLNs, which leads to migration of  $CD4^- CD8^- \gamma\delta$  and  $CD4^+ CD8^+ \alpha\beta$  T cells to the pancreas where they mediate Fas and perforin dependent cytotoxicity. We also hypothesize that at least one of the type 1 diabetes associated HLA-DR molecules that bind and present wheat-derived peptide(s) also bind and present an islet cell antigen(s), activating plasma cell synthesis of islet cell autoantibodies and irrevocable, complement-dependent destruction of islet cells. Our final two hypotheses state that type 1 diabetes morbidity is reduced in those areas of globe where genetically susceptible individuals get adequate amounts of vitamin D, in the diet and/or through exposure to sunlight, and in areas where people are exposed to bacterial, viral, or parasitic infections in early childhood.

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## Hypothesis one

### Wheat gluten is the elusive environmental trigger in type 1 diabetes and the disease is initiated in genetically susceptible individuals by habitual consumption of gluten containing food products

Type 1 diabetes and celiac disease are both HLA associated autoimmune diseases. Type 1 diabetes (also known as insulin dependent diabetes mellitus (IDDM)) is approximately 20 times more common in celiac patients than in the general population [1,2], which conceivably occurs because of linkage disequilibrium in the inheritance of type 1 diabetes and celiac HLA class II associated alleles, and/or because the two diseases share common, or closely associated environmental triggers. The strongest genetic predisposition to type 1 diabetes comes from inheritance of the HLA-DRB1, -DQB1 alleles DRB1\*04, DQB1\*0302 and DRB1\*0301, DQB1\*0201 [3]. More than 90% of celiac patients are positive for DQA1\*0501, DQB1\*02 alleles encoded in cis position on the HLA-DR3-DQ2 haplotype or in trans on either the HLA-DR5-DQ7 or DR7-DQ2 haplotypes [4,5]. No controlled, large population-based studies have been conducted, to date, to determine if there is linkage disequilibrium between these two sets of disease-associated alleles.

Celiac disease is initiated in genetically susceptible individuals by habitual consumption of the gliadin proteins in wheat gluten. The small bowel enteropathy and the symptoms of the disease are almost completely remitted by adherence to a gluten-free diet [6]. Some data suggests that people at high risk for developing type 1 diabetes may also benefit from a gluten-free diet. Pastore et al. [7] tested the insulin levels of first-degree relatives of type 1 diabetic patients, before, and at the end, of 6 months on a gluten-free diet and 6 months on a normal diet. They reported that the gluten-free diet led to significant improvements in the subjects' insulin response during intravenous glucose tolerance testing. Celiac patients who were older at time of diagnosis, and thus on a gluten-free diet for a shorter time period, were recently shown to have a significantly higher prevalence of type 1 diabetes than celiac patients who were diagnosed at a younger age [8]. There is also a case report of regression of type 1 diabetes in a 15 year boy who was positive for four different islet cell autoantibodies and had abnormal blood glucose

and insulin levels, at the time that a small bowel biopsy showed that he also had celiac disease. After following a gluten-free diet for 6 months, his blood glucose and insulin normalized and he became islet cell autoantibody negative [9].

BioBreeding diabetes-prone (BBdp) rats and non-obese diabetic (NOD) mice are two well-characterized animal models of type 1 diabetes. Disease progression is accelerated in these animals when wheat gluten is incorporated in the rodents diet, early in life, shortly after weaning [10,11]. Highly significant associations between infant feeding practices and type 1 diabetes were also recently reported in humans. One study found that infants fed gluten-containing foods prior to 3 months of age had a significantly greater adjusted hazard ratio or risk of becoming islet cell autoantibody positive later in life, than infants who were exclusively breast fed until 3 months of age [12]. Another study found that infants who were initially exposed to gluten before 3 months of age had a significantly greater risk of becoming islet autoantibody positive than children exposed to gluten between 4 and 6 months of age [13].

Beta cell destruction and pancreatic insulinitis is preceded in BBdp rat and NOD mouse models of type 1 diabetes by gut-mediated immune activation. There are also signs of up-regulation of immune responses in the jejunum of type 1 diabetic patients. Jejunal specimens from type 1 diabetic patients have been shown to stain strongly, positive for HLA-DQ, -DP molecule expression, vs faint positive staining in jejunal specimens from age-matched, healthy controls [14,15]. Significantly higher densities of IFN- $\gamma$  and IL-4 m-RNA positive cells have been detected in jejunal lamina propria tissue from type 1 diabetic patients than lamina propria of controls [15]. The lamina propria of type 1 diabetic patients also contained significantly higher numbers of  $\alpha_4\beta_7$  positive lymphocytes [14].  $\alpha_4\beta_7$  is a heterodimeric protein expressed on the surface of leukocytes involved in transmigration of T cells into mucosal surfaces. Auricchio et al. [16] cultured jejunal biopsy specimens from 12 type I diabetic patients and eight control patients in medium alone, and in the presence of peptic-tryptic (PT) digests of gliadin and ovalbumin. There were significant increases in CD3<sup>+</sup> and CD25<sup>+</sup> cells, and enhanced expression of CD54 (ICAM-1), when a PT digest of gliadin was added to cultured jejunal specimens of type 1 diabetic patients, which did not occur with medium alone or with the addition of a PT digest of ovalbumin.

## Hypothesis two

### Habitual consumption of proline-rich gluten proteins leads to up-regulation of gene expression and synthesis of dipeptidyl peptidase IV (DPPIV)

Gluten proteins are comprised of two groups of proteins called the glutenins and the gliadins. The glutenins and the gliadins contain 13–24 and 20–30 mol % respectively of proline [17,18]. Both groups of proteins are resistant to proteolysis by digestive enzymes [19]. DPPIV releases X-Pro and X-Ala dipeptides from the NH<sub>2</sub> terminal end of protein and peptide substrates by catalyzing the hydrolysis of peptide bonds after proline and alanine [20]. DPPIV is present on the surface of intestinal epithelial cells [21]. Because of its ability to cleave peptide bonds involving proline, DPPIV, likely has an important role in shaping the repertoire of gluten peptides that get presented as antigens to intraepithelial and lamina propria T cells.

In vivo DPPIV substrates include pancreatic polypeptide, neuropeptide Y, and peptide YY, gastric inhibitory peptide (GIP) and glucagon-like peptides-1 and -2 (GLP-1 and GLP-2) [20]. GLP-1 and GLP-2 down-regulate beta cell apoptosis and along with neuropeptide Y promote pancreatic beta cell differentiation and replication [22–24]. Truncation of GLP-1, GLP-2, and neuropeptide Y by DPPIV makes them inactive, inhibiting the beta cell mass preserving effects of these peptides.

The DPPIV catalytic site is part of the extracellular domain of the CD26 antigen which is found in kidney, liver and intestinal cells, and on T and B cells and macrophages [25]. DPPIV activity enhances immune responses by increasing co-stimulatory signals for T cell activation [26]. The CD26 antigen is a marker for T-helper memory cells [27] and is involved in T cell-dependent antibody production and immunoglobulin isotype switching of B cells [25]. In addition, CD26/DPPIV has been shown to affect T cell chemotaxis by catalyzing the removal of a N-terminal Ser<sup>1</sup>-Pro<sup>2</sup> dipeptide from RANTES (regulated on activation, normal T cell expressed and secreted) forming a truncated RANTES (amino acids 3–68) which binds more avidly to the CCR5 chemokine receptor than intact 1–68 RANTES [28,29]. CCR5 is involved in recruitment of type 1 helper cells (Th1) to inflammatory sites [30]. Two recent studies found a preponderance of Th1 cells among islet cell antigen-reactive T cells isolated from peripheral blood of type 1 diabetic patients [31,32].

## Hypothesis three

### The presence of gluten-derived peptides in the small intestine triggers release and binding of zonulin to the zonulin/zot receptor and opening of the tight junctions between intestinal epithelial cells

The intracellular tight junctions of the small intestine open when a protein called zonulin is secreted into the intestinal lumen. Zonulin concentrations have been found to increase when high numbers of bacteria are present in the small intestine. Opening of the tight junctions leads to an influx of water into the intestine, so zonulin secretion may be a host defense mechanism for removing bacterial pathogens from the intestine [33]. Evidence from one recent study suggests that wheat gliadin proteins and peptides are able to activate zonulin-dependent intracellular signaling pathways that open the tight junctions and increase intestinal permeability [34].

Onset of diabetes is preceded, in NOD mice and BBdp rats, by a food-antigen driven inflammation of the small intestine [35]. Gut inflammation in BBdp rats is accompanied by increases in intestinal permeability [36]. A recent study showed that zonulin release coincided with increases in intestinal permeability in pre-diabetic BB/Worster rats [37].

Increases in intestinal permeability have also been observed in type 1 diabetic subjects. Several research groups reported significantly greater intestinal absorption of lactulose, mannitol or rhamnose in individuals with type 1 diabetes than in age-matched, healthy controls [38–41]. Equivalent oral doses of <sup>51</sup>Cr-labelled EDTA were given, in another study, to type 1 diabetic patients and to age and sex-matched healthy controls. The diabetic patients had significantly higher urinary excretion rates of the radiolabelled EDTA than the healthy control subjects [42]. Secondulfo et al. [41] examined the ultrastructure of duodenal biopsy specimens from type 1 diabetic patients and healthy control subjects and found that the size of the intercellular spaces between adjacent enterocytes were significantly greater in biopsy specimens from diabetic patients than in controls.

Opening of the small intestinal tight junctions is, in our view, a critical step in the pathogenesis of type 1 diabetes because it allows large gluten peptides to reach the lamina propria. The majority of these peptides undergo intracellular processing into smaller peptides before getting presented as antigens to lamina propria T cells. A few large peptides may bind directly to CD1d on the surface of

antigen presenting cells. Other peptides may be taken up by dendritic cells and processed and presented as antigens by HLA class II molecules. Antigen presentation of these gluten peptides results in clonal selection and expansion of sets of gluten-sensitized T cells within the lamina propria of the small intestine of type diabetic subjects. These gluten-sensitized T cells, under circumstances where there is a chemokine gradient between the pancreas and the small intestine, may leave the lamina propria, circulate through the lymphatic system and the bloodstream to the pancreas, where they become effector cells and central contributors to pancreatic insulinitis and  $\beta$ -cell destruction.

## Hypothesis four

### **A gluten-derived peptide(s) binds to a CD1d molecule in association with $\beta$ 2-microglobulin and this CD1d– $\beta$ 2-microglobulin–peptide complex is presented on the surface of dendritic cells within the small intestinal lamina propria**

X-ray crystallography revealed that the mouse analogue (mCD1) of the human CD1d molecule possesses a lipid binding groove with two hydrophobic pockets [43] that is capable of binding several different classes of lipids [44–46]. Hydrophobic peptides also bind to mCD1 [47]. Peptides that have been found bound to mCD1 are larger than peptides that normally bind to HLA class I and II molecules [48]. CD1d is expressed on dendritic cells, B cells and the apical and basolateral membrane surfaces of human intestinal epithelial cells where it may play a role in antigen presentation to intraepithelial lymphocytes [49,50].

The gliadin and glutenin proteins are water-insoluble hydrophobic proteins [51] that are refractory to hydrolysis by digestive enzymes [19]. Partial digestion of gliadins and glutenins leads to release of large hydrophobic peptides (containing as many as 33 amino acids) [19] into the lumen of the small intestine. Some of these large gluten-derived peptides may undergo endocytosis and intracellular processing into smaller peptides with the necessary structural and size requirements to bind to CD1d molecules.

The binding of one or more gluten peptides to a CD1d molecule and antigen presentation of a CD1d– $\beta$ 2-microglobulin–gluten peptide complex on the outer membrane of dendritic cells is a critical step in the development of type 1 diabetes in

humans for several reasons. CD1d restricted antigens are recognized by cells of the innate immune system. CD1d– $\beta$ -microglobulin–peptide complexes bind to the  $V\alpha$ 24,  $V\beta$ 11, NKR-PIA, T cell receptor (TCR) of natural killer T cells (NKT cells) [52]. NKT cells produce IFN- $\gamma$  and IL-4 and mediate apoptosis [53] and granulysin-dependent cytotoxicity [54] of target cells.

CD1d restricted NKT cells are predominately comprised of two distinct subsets.  $CD4^+CD8^-$  NKT cells secrete high levels of Th2 cytokines IL-4 and IL-13.  $CD4^-CD8^-$  (double negative, DN) NKT cells have a Th1 phenotype and produce high levels of IFN- $\gamma$  and TNF- $\alpha$  [55]. The Th2 subset of NKT cells help provide protection against diabetes in NOD mice [56,57], while; there is evidence that double negative NKT cells may play a critical role in the development of type 1 diabetes in humans. Wilson et al. [58] found marked differences in the IL-4 and IFN- $\gamma$  secretion patterns of  $V\alpha$ 24J $\alpha$ Q  $CD4^-CD8^-$  T cells from type 1 diabetic patients and their non-diabetic identical twins/triplets.  $CD4^-CD8^-$  T cells from diabetic patients produced high levels of IFN- $\gamma$  and very low levels of IL-4, while cells from their non-diabetic siblings produced moderate to high levels of both cytokines. A follow-up study reported significantly greater expression of STAT1 (IFN- $\gamma$  signaling), STAT4 (IL-12 signaling), and CD161 (a coactivator of  $V\alpha$ 24J $\alpha$ Q T cell proliferation and IFN- $\gamma$  secretion) genes in a IL-4 null T cell clone from a type 1 diabetic patient than a IL-4 $^+$  T cell clone derived from a non-diabetic identical twin/triplet [59].

## Hypothesis five

### **CD1d binding and presentation abrogates oral tolerance not just to the gluten peptide(s) that binds to the CD1d molecule, but to all wheat-derived peptides within the small intestine**

The usual response of the human immune system to dietary proteins is a state of oral tolerance, a phenomenon involving up-regulation of protective gut-localized immune mechanisms and down-regulation of potentially harmful systemic immune responses to the protein(s) in question. Abrogation of oral tolerance may play an important role in the development of food allergies and food enteropathies, including celiac disease [60].

Fujihasi et al. [61] reported that adoptive transfer of antigen-specific  $CD3^+CD4^-CD8^-$  T cells from the spleens of nude BALB/c mice into normal

BALB/c mice, resulted in abrogation of oral tolerance. A follow-up study found that there were two  $\gamma\delta$  T cell receptor positive ( $\gamma\delta$  TCR<sup>+</sup>) T cell subsets involved in abrogation of oral tolerance, a DN CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> and a CD8 positive CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>+</sup> subset [62].

It is our contention that, prior to the initiation of damage of pancreatic islets, type 1 diabetic patients become intolerant to wheat proteins. This break in oral tolerance leads to recruitment of gluten-sensitized, CD1d restricted double negative NK T cells into the small intestine [63]. CD4<sup>-</sup>CD8<sup>+</sup> T cells are also recruited that produce high levels of IL-4 which serves as a signal for immunoglobulin class switching. Circulation of these antibodies to various organs and tissues leads to a loss of systemic unresponsiveness to wheat proteins.

## Hypothesis six

### Gluten peptide-loaded dendritic cells migrate from the small intestine to the pancreatic lymph nodes in response to an increase in pancreatic cell apoptosis during normal 'remodeling' of the pancreas

$\beta$  cell apoptosis in rodents increases steadily after birth until about 14 days of age [64], just prior to the age at which the first signs of pancreatic insulinitis can be detected in NOD mice [65]. Rates of  $\beta$  cell apoptosis are likewise high in human infants from just after birth until 3 months of age [66]. Dendritic cells (DCs) are involved in endocytosis of apoptotic cells [67] and phagocytosis of apoptotic cell fragments [68]. Cells in the early stages of cell apoptosis are removed efficiently by immature DCs. Incomplete or defective clearance of apoptotic cells results in the accumulation of late phase products of cell apoptosis. Uptake of late phase products of cell apoptosis by DCs leads to their maturation which is necessary for DC homing to lymphoid organs [69]. The relatively high rate of  $\beta$  cell apoptosis occurring in human infants as a result of normal 'remodeling' of the pancreas [66], might result in sufficient accumulation of the products of secondary necrosis, or late phase cell apoptosis, to stimulate dendritic cell maturation and migration to the pancreatic lymph nodes.

Small intestinal DCs can process and present orally administered antigens to T cells [70]. Antigen-presenting DCs have been found within the small intestinal lamina propria [71]. Antigen-loaded DCs from the lamina propria can enter the lacteals and travel to lymph nodes [72,73]. There is

evidence that migration of DCs to the pancreatic lymph nodes, is an early event in the pathogenesis of type 1 diabetes. CCR7-receptor positive DCs and T cells are attracted to stromal cells within the T cell zones of lymph nodes which constitutively express the CCR7 – CCL19 and CCL21 ligands [74]. Bouma et al. [75] recently found significantly higher CCL19 and CCL21 expression in the pancreas of pre-diabetic NOD mice than in control mice.

Gagnerault et al. [76] reported that removal of the pancreatic lymph nodes (PLNs) from 3-week old NOD mice provided protection against diabetes, whereas PLN removal had no effect in 10-week old NOD mice. Antigen-presentation by DCs helps attract T cells to the pancreatic lymph nodes [77] where DCs and T cells associate in clusters [78]. DCs were the first cells, followed by macrophages and lymphocytes, to infiltrate the pancreas of pre-diabetic BB rats [79]. T cells transferred from NOD mice into NOD scid/scid mice, which lack both B and T cells, were shown to have significantly greater diabetogenic activity when the T cells were harvested from PLNs, than from the mesenteric lymph nodes, distal lymph nodes or the spleen [80].

## Hypothesis seven

### CD4<sup>-</sup>CD8<sup>-</sup> $\gamma\delta$ and CD8<sup>+</sup> $\alpha\beta$ T cells migrate from small intestine to the pancreas where they mediate Fas and perforin dependent cytotoxicity

Kreuwel et al. [81] found that  $\beta$  cell destruction still occurred in mice when either the Fas/FasL or perforin/granzyme cytolytic pathways were blocked. However, mice were protected from diabetes when both pathways were obstructed. Lysis of Fas<sup>+</sup> targets was shown in one study to be mediated by double-negative (DN) T cells, while CD8<sup>+</sup> T cells were involved in perforin/granzyme cell lysis [82]. Increased levels of CD4<sup>-</sup>CD8<sup>-</sup> T cells have been observed in pancreatic islet infiltrates of pre-diabetic NOD mice [83] and in the spleen of diabetic BB rats [84]. It has also been reported that CD4<sup>-</sup>CD8<sup>-</sup> $\gamma\delta$  TcR and CD4<sup>-</sup>CD8<sup>+</sup> $\alpha\beta$  TcR T cells constitute the majority of T cells present within the pancreatic islets transplants of diabetic patients undergoing disease recurrence [85,86].

There is evidence in NOD mice and in humans that T cell migration from the small intestine into the pancreas is a critical step in autoimmune diabetes [87–90]. Diabetes was prevented in 3-week old NOD mice when a monoclonal antibody was used to block  $\alpha 4\beta 7$  positive T cells from binding

to the mucosal addressin cell adhesion molecule-1 (MadCAM-1) which is expressed on high-endothelial venules (HEVs) of the pancreas during type 1 diabetes [91,92]. Significantly greater numbers  $\alpha$ 4 $\beta$ 7 positive T cells were detected within the lamina propria of the small intestine of type 1 diabetic patients than in healthy controls [14]. A pancreatic T cell line from a type 1 diabetic patient was found to express high levels of the mucosal homing receptors VLA-4 $\alpha$  and CD44 [92], and intercellular adhesion molecule-1 (ICAM-1) was strongly expressed on vascular endothelium of the pancreatic islets [93].

## Hypothesis eight

**At least one of the HLA-DR molecules that bind and present wheat-derived peptide(s) also bind and present one or more islet cell antigens, activating plasma cell synthesis of autoantibodies to islet cell antigens resulting in complement-dependent cytotoxicity of islet cells**

This occurs only in individuals that have inherited specific type 1 diabetes associated HLA-DR, -DQ alleles. The synthesis of islet cell autoantibodies precipitates a 'second wave of cell destruction' that is specifically directed towards insulin producing  $\beta$ -cells.

The strongest known association between HLA class II alleles and type 1 diabetes is found at the HLA-DQ locus, specifically DQA1 and DQB1 genes DQA1\*03-DQB1\*0302 and DQA1\*05-DQB1\*02 [94]. Todd et al. reported in 1987 [95] that resistance to type 1 diabetes was conferred by the presence of aspartic acid at position 57 on DQB1 molecules, while aspartic acid-57-negative DQB1 molecules increase susceptibility to the disease. Transgenic mice who were HLA class II deficient except for an aspartic acid-57-negative DQA1\*0301-DQB1\*0302 allele, developed diabetes when injected with splenocytes from diabetic mice, while HLA-DQA1\*0103-DQB1\*0601 transgenic mice, carrying a DQB1 chain with protective aspartic acid at position 57, did not develop diabetes when injected with diabetic splenocytes [95].

However, there is evidence from human population studies that inheritance of HLA-DRB1 molecules also plays a role in susceptibility and resistance to type 1 diabetes [96–98]. Inheritance of HLA-DRB1\*0401, HLA-DRB1\*0402 or HLA-DRB1\*0405 markedly increases one's susceptibility to type 1 diabetes, while type 1 diabetes rarely occurs in HLA-DRB1\*0403 or HLA-DRB1\*0404 positive

individuals [99–101]. Disease-predisposing HLA-DRB1 alleles were found in one study to be significantly more common in children with type 1 diabetes than in cases of adult-onset IDDM, leading to the speculation that HLA-DRB1 alleles may be more important to disease pathogenesis than HLA-DQA1-DQB1 alleles [102]. In addition, several studies have reported that HLA-DR rather than HLA-DQ expression is up-regulated in the small intestine and pancreas of diabetics [103,104,14–16]. For these reasons, we hypothesize that the key autoantigens in type 1 diabetes bind to, and are presented to the immune system by HLA-DR molecules.

Possible candidates for these putative autoantigens include peptides derived from pro-insulin, insulin, glutamic acid decarboxylase (GAD), and islet cell antigen 69 (ICA69) which were shown in one study to be bind to HLA-DRB1\*0401 molecules [105]. We further contend that there is molecular mimicry at the HLA class II molecule–peptide–T cell level between presentation and immune system recognition of one or more wheat-derived peptides and one or more islet cell antigens. Because of previous loss of oral tolerance to wheat proteins, a strong peripheral immune response is now mounted against both wheat-derived peptide(s) and islet cell antigen(s) that they mimic.

GAD65, the 65 kDa form of glutamate decarboxylase, is an islet cell autoantigen that is recognized by T cells from type 1 diabetic patients that express the  $\alpha$ 4 $\beta$ 7 integrin, a gut homing receptor for MadCAM-1 [106]. This is an important finding that begs the question of whether molecular mimicry occurs between one or more GAD65 derived peptides and a peptide(s) arising from in vivo proteolysis of wheat proteins. The gliadin and glutenin proteins are comprised of a high proportion of glutamine (approximately one third of all amino acid residues) and proline (12–30% proline) [107], while GAD65 contains 3% glutamine and 4% proline [108], which makes the possibility of molecular mimicry between peptides derived from these two sets of proteins, quite unlikely.

IgG antibodies from BBdp rats were recently found to bind to a WP512 protein from wheat gluten. This WP512 protein shares an 80% amino acid sequence homology with a wheat storage protein, Glb1. IgG antibodies to Glb1 were subsequently detected in the sera of human type 1 diabetics but not in age-, sex-, HLA-DQ matched controls [109]. Despite being a salt soluble globulin protein, Glb1 is found in wheat gluten [109], and may be an important dietary diabetogen for BBdp rats and NOD mice. It would be interesting to see if there is cross-reactivity between the GAD65 and Glb1 antibodies in type 1 diabetic subjects.

Complement-fixing IgG antibodies from patients with type 1 diabetes have been demonstrated to bind to rat and hamster islet cells [110,111] and to human islet cells [112]. Heating completely eradicated islet cell cytotoxicity of patient sera which indicates that terminal complement complex (TCC) formation is necessary for IgG antibody mediated destruction of islet cells [112]. These *in vitro* results are supported by the finding of deposits of complement-fixing IgG antibodies within the damaged islets of a 12-year old diabetic girl who had died from ketoacidosis [103].

## Hypothesis nine

**Type 1 diabetes can be delayed and sometimes prevented in genetically susceptible individuals by a threshold, protective blood level of vitamin D**

It has long been observed that type 1 diabetes morbidity increases during the winter months [113,114], and is higher in northern latitudes away of the equator [115–118]. An inverse correlation was reported in one study between the number of new cases of the disease and hours of sunlight [119]. A birth-cohort study of vitamin D supplementation and type 1 diabetes in Finnish children reported a type 1 diabetes incidence rate of 204 per 100,000 years at risk in un-supplemented children vs 24 per 100,000 in children who had received recommended doses of vitamin D [120].

Vitamin D presumably helps to prevent type 1 diabetes by a set of multiple effects it has on the human immune system. Oral administration of an analog of  $1\alpha,25$ -dihydroxyvitamin  $D_3$  to NOD mice blocked the maturation of dendritic cells, halted the progression of pancreatic insulinitis and the development of diabetes, and increased the activity of  $CD4^+CD25^+$  regulatory cells [121]. Type 1 diabetes suppressing effects of vitamin D have also been linked to its ability to inhibit IL-12 production. IL-12 plays a critical role in the development of Th1 cells [122] and in up-regulation of IFN- $\gamma$  [123,124]. Because of its effects on proliferation of Th1 cells and enhancement of interferon  $\gamma$  levels, IL-12 has been implicated in development of autoimmune diseases [125,126].

$1\alpha,25$ -dihydroxyvitamin  $D_3$  was found to inhibit nuclear factor- $\kappa B$  from binding to the p40- $\kappa B$  site on the p40 promoter, preventing transcription of the p40 gene and synthesis of the p40 subunit of IL-12 [127]. NF- $\kappa B$  must bind to the p40- $\kappa B$  sequence of the p40 promoter, prior to transcription

of the p40 gene, and synthesis of the p40 subunit of IL-12 [128].

Vitamin D binds to a nuclear vitamin D receptor (VDR). Vitamin D–VDR binding is believed to be crucial to vitamin D's immune suppressive effects [127]. VDR gene polymorphisms have been recently associated with type 1 diabetes in people from Taiwan and Japan [129,130]. A significantly higher ( $p = 0.0010$ ) frequency of inheritance of a VDR gene allele Bsm1 polymorphism was reported among Japanese type 1 diabetic patients than in healthy controls [130].

## Hypothesis 10

**Improved hygiene and sanitation lowers incidence rates of infectious and parasitic diseases, leading to conditions that favor the development of type 1 diabetes in genetically susceptible individuals**

This last hypothesis has been called the “hygiene hypothesis” [131]. The hypothesis is supported by epidemiological and experimental data including: lower prevalence rates for type 1 diabetes in tropical regions of the world where infectious and parasitic diseases remain rampant [132]; significantly lower rates of type 1 diabetes among people living in crowded conditions, favoring person to person spread of infectious diseases [133]; a significant reduction in the relative risk of type 1 diabetes when children are exposed to common infections before their first birthday [134]; higher diabetes incidence rates in NOD mice and BBdp rats when they are housed in specific pathogen-free (SPF) environments than under normal conditions [135]; and the fact that NOD mice are protected against diabetes by infection with mycobacterium, viruses and schistosomiasis parasites [136–139]. Thus, it appears, based on this information, that exposure during infancy to a variety of pathogenic agents confers a level of protection vs type 1 diabetes.

What mechanism(s) operating at the molecular and cellular level could possibly help account for this phenomenon? One possibility, involves CD1, a molecule that helps to link innate and adaptive immunity, which we have already hypothesized as playing a key role in type 1 diabetes.  $\beta_2$ -Microglobulin associated forms of CD1 bind lipids from bacteria, and parasites and present them as antigens to T cells [140,141]. According to our fourth hypothesis, gluten-derived peptides also bind to CD1 molecules. Bach has theorized that the protective role of infections observed in human autoimmune

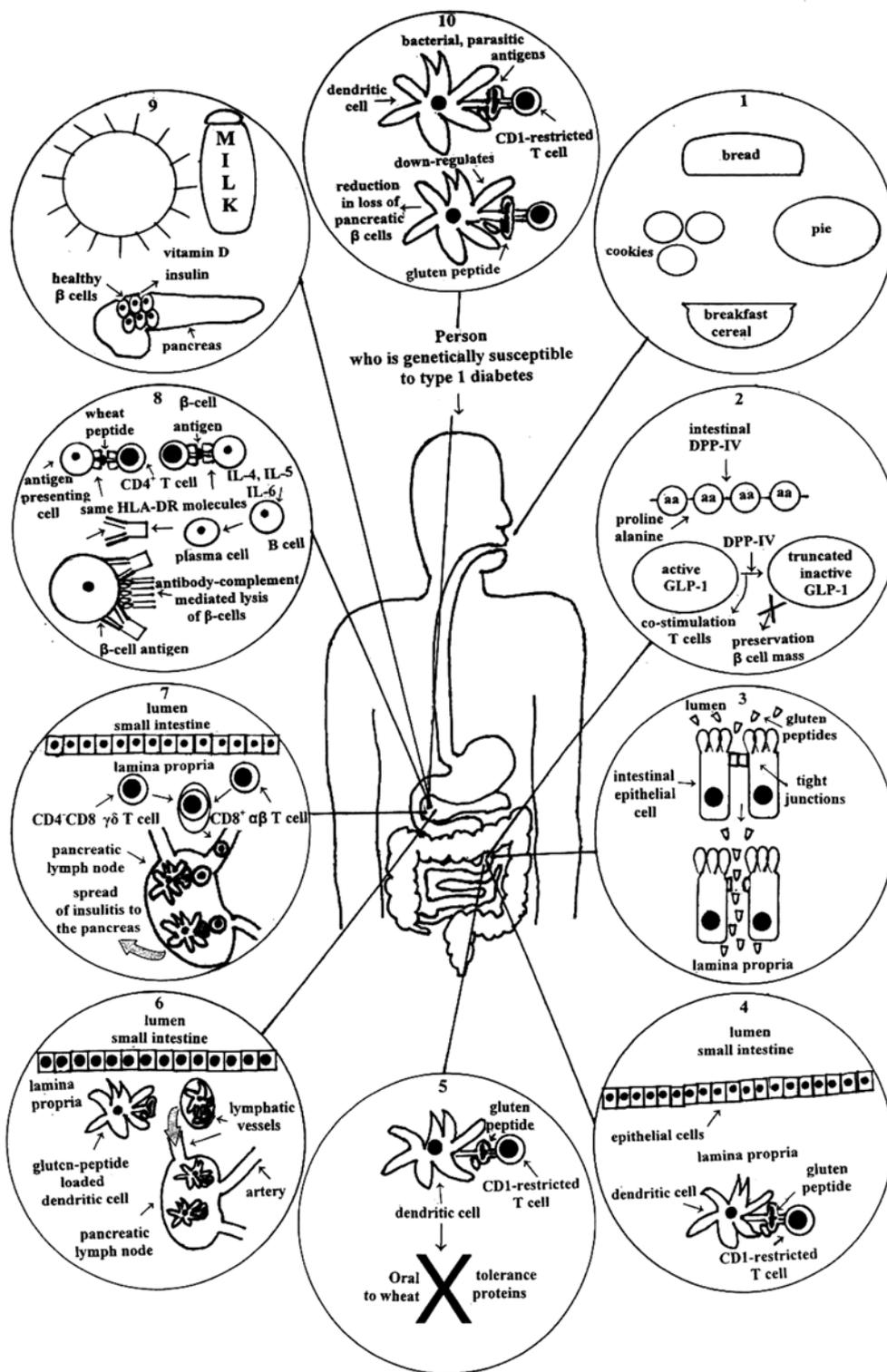


Figure 1 Brief pictorial representations of hypotheses 1–10 shown clockwise in enclosed circles.

diseases may be due in part to “antigen competition” [142]. In the case of active infection with one or more pathogens, gluten peptides would presumably have to compete with pathogen-derived antigens for binding to CD1- $\beta_2$ -microglobulin

complexes. The immune system would no longer be focused on mounting an attack against wheat gluten. This would necessarily lead to a down-regulation and a damping effect on development of type 1 diabetes.

Diabetes is prevented in NOD mice by CD1d presentation of the glycolipid,  $\alpha$ -galactosylceramide, to NKT cells [143].  $\alpha$ -Galactosylceramide stimulates NKT cells to secrete Th2 cytokines. CD1d restricted presentation of glycosphingolipids derived from the parasitic *Schistosoma mansoni* results in a switch to Th2-dominated responses in mice [141]. The development of type 1 diabetes is believed to be driven by Th1 cytokine secretion [144]. Thus, one of the disease-sparing effects of infections on type 1 diabetes may involve up-regulation of Th2 cytokine responses, which is set in motion by CD1 restricted presentation of lipids derived from pathogenic microorganisms and parasites.

CD45RO, the low molecular weight isoform of the leukocyte common antigen, is expressed on memory T cells. CD45RO+ T cells are important in immune surveillance and proliferate following encounters with "recall antigens", molecular fragments of pathogens recognized by these memory T cells [145]. Several studies have reported less than 50% concordance for type 1 diabetes among genetically susceptible pairs of identical twins [146–149], which points to strong environmental influences on disease etiology. Peakman et al. analyzed blood samples from 18 pairs of diabetic/non-diabetic twins, for CD45RO+ and CD45RA+ (a marker of naive T cells) CD4+ T cells. They found significantly higher numbers of CD45RO+ CD4+ lymphocytes in non-diabetic twins than their diabetic twin siblings [140]. These findings suggest that type 1 diabetes fails to develop in genetically susceptible people who have been exposed to multiple types, and/or re-occurring episodes, of infectious/parasitic diseases.

## Concluding remarks

It should be pointed out that eight of the 10 hypotheses presented here are not original hypotheses, and have been previously hypothesized, or implied in statements by others. We believe that the two novel hypotheses, namely, that one or more wheat gluten-derived peptides binds to a CD1d molecule on the surface of dendritic cells within the lamina propria of the small intestine; and that, HLA-DR molecules that bind and present one or more wheat-derived peptides also bind and present islet cell antigens which leads to synthesis of islet cell autoantibodies and irrevocable destruction of islet cells, are critical to a greater understanding of pathogenesis of type 1 diabetes. The first eight hypotheses are purposefully linked together in a way that presents a hypothetical path-

way for the initiation and progression of type 1 diabetes pathogenesis (Fig. 1). The last two hypotheses may help to explain the wide variations in the incidence of type 1 diabetes across the globe, and why, for instance, South Asians that have migrated to the United Kingdom have IDDM incidence rates similar to overall rates in the U.K, rather than their native countries [150]. Finally, it is our hope that other scientists will be interested in testing the validity of these hypotheses.

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