

Insulin auto-immunity: implications for the prevention of Type 1 diabetes mellitus

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Mounting evidence suggests insulin is an important and potentially initiating antigen in the pathogenesis of Type 1 diabetes. High-affinity insulin antibodies are found early in disease development and appear to predict progression. Insulin is the only Type 1 diabetes auto-antigen with exclusive pancreatic expression and the only one whose gene maps to a major susceptibility locus. Preclinical studies in rodent models of immune-mediated diabetes show great promise for the possibility of preventing disease by peripheral tolerization. Translation of this evidence to clinical trials of oral, intranasal and parenteral insulin to invoke immune tolerance and prevent diabetes has not proven successful to date, but promising results in a small subset of highest-risk individuals have maintained enthusiasm for this promising prevention strategy. Currently, studies of oral and intranasal insulin are ongoing to determine the optimal dose, timing and target population for Type 1 diabetes prevention.

KEYWORDS: immune tolerance • immunotherapy • insulin auto-antibodies • prevention therapy • Type 1 diabetes

Insulin as an auto-antigen in Type 1 diabetes

Type 1 diabetes mellitus (T1DM) is characterized by lymphocyte invasion and destruction of the pancreatic β -cells, with resultant hyperglycemia owing to insulinopenia. Advances in genetics and auto-antibody testing have aided in the identification of those children at highest risk. However, the initiating event for T- and B-cell activation against the β -cell has yet to be elucidated. Emerging evidence from large-scale epidemiologic, genetic and animal studies suggest that insulin may be the primary auto-antigen central to the pathogenesis of T1DM. The purpose of this article is to discuss the diagnostic and therapeutic implications of insulin as an auto-antigen in T1DM.

T1DM-specific antibodies against several auto-antigens, including glutamic acid decarboxylase (GAD), islet cell antigen 5–12 phosphatase (IA2), zinc efflux transporter (ZnT8) and insulin, have been found in differing quantities and stages in patients with T1DM. Of these, ZnT8 and insulin are highly specific for the β -cell, with ZnT8 expression restricted to the β -cell and the testes [101], and insulin expression restricted to the β -cell. The question of which antigen is the 'primary' auto-antigen (if, indeed one auto-antigen is

primary) has not been clearly answered, but mounting evidence points towards insulin. In addition to the tissue specificity of its expression, large epidemiologic studies have shown that anti-insulin is often the first auto-antibody found in children screened for T1DM risk, particularly children younger than 1 year of age, and is observed long before disease onset [1–5]. Nonobese diabetic (NOD) mice destined to develop diabetes express high levels of insulin antibodies early (within 4 weeks of life), which often disappear at the onset of clinical disease [6]. Thus, the fact that antibodies against insulin are less frequent than other T1DM-specific antibodies in newly diagnosed cases is not an argument against the importance of insulin autoimmunity. This said, these antibodies are merely markers of T-cell-mediated β -cell destruction and their significance in pointing to causative antigens is not clear. In fact, few retrogenic mice expressing monoclonal T-cell receptors (TCRs) specific to various epitopes of GAD, insulin, IA2, islet cell and phogrin, as well as unknown islet cell antigens, develop insulinitis or overt diabetes, suggesting autoimmunity to these antigens does not equate to pathogenicity. Of note, only mice expressing TCRs to various insulin epitopes and four of the unknown islet cell antigens developed overt

diabetes, with the unknown antigens resulting in earlier disease progression in a higher number of affected animals [7]. Thus, there may be more critical antigens in the pathologic immune process that ultimately results in T1DM, with insulin remaining as one potential target for prevention strategies.

Genetic support for insulin auto-immunity

Additional arguments for the importance of insulin as an auto-antigen come from genetic observations in mouse and human models. It has been known for two decades that T1DM risk is associated with a polymorphism in the form of variable number tandem repeats (VNTRs) within the 5' flanking region of the insulin gene [8]. The sizes of the VNTRs are distributed in the population as either class I (30–60 repeats) or class III (120–170 repeats) and have been shown to be associated with, respectively, predisposition for, or protection from, diabetes. With a relative risk of 0.5 for the less-common protective class III alleles, it is the strongest known effect in T1DM genetics outside of the HLA locus. The polymorphism is not linked to any amino-acid change and has only a borderline (if any) effect on pancreatic insulin gene expression [9]. A plausible explanation for the effect came from the observations that the human thymus also expresses insulin and the two classes of VNTR alleles are associated with differential transcription, depending on the number of repeats present. In both the fetal and postnatal thymus, the protective class III VNTR alleles were associated with a two-to-threefold increase in proinsulin mRNA relative to the fetal thymus harboring class I VNTR alleles. Moreover, rare individual class III VNTR alleles that are associated with a silenced, rather than an enhanced, insulin transcription are associated with increased predisposition to T1DM, strengthening the association between expression of proinsulin in the thymus and immune tolerance to the insulin protein [10]. The postulated mechanism of protection from T1DM by thymic expression of proinsulin is thought to be either by negative selection of self-recognizing T-cell clones or by inducing Tregs.

The examination of insulin expression in the human thymus was based on previous observations in mice. The initial description of mice expressing a foreign protein (such as the viral onco-gene *SV40*) in the thymus, driven by the rat insulin promoter, raised the question of whether, in addition to the presumed peripheral tolerization to tissue-specific antigens such as insulin, some central method of tolerance might also play an important role [11]. Evidence in the form of transgenic animal studies supported this hypothesis, revealing that transgenic foreign antigens expressed under the insulin promoter were found in small quantities in the thymus and that immune tolerance to them was transferable by transplantation of thymic tissue [11–13]. It was postulated that this expression was required for the presentation of insulin epitopes to T-cells, allowing for the negative selection of T-cell clones autoreactive towards the insulin protein.

Proinsulin expression in the thymus

Significant insights into distinguishing the role of thymic versus pancreatic proinsulin expression in inducing immune tolerance to the β -cell came from targeted disruption (knockout [KO]) of

the two murine insulin genes, *Ins1* and *Ins2*, which have highly homologous but not identical peptide sequences [14,15]. Although both genes are equivalently expressed in the pancreas, only *Ins2* is significantly expressed in the thymus, *Ins1* being practically undetectable there [16]. Mice with a KO of either *Ins1* or *Ins2* have normal insulin and glucose levels on the basis of metabolic feedback. *Ins2*-KO animals, expressing no proinsulin in the thymus, show enhanced T-cell autoreactivity even against a nondiabetes-prone genetic background [16–18]. Crossed into the NOD mouse, a model of spontaneous autoimmune diabetes very similar to human T1DM, *Ins2* KO drastically accelerates diabetes and accentuates markers of loss of tolerance in an insulin-specific manner [19]. By contrast, KO of *Ins1* (removing the proinsulin peptide not expressed in the thymus) protected these mice from developing diabetes [15,20].

Furthermore, when NOD mice homozygous for both KOs (null for endogenous proinsulin) were rescued with a transgene encoding a modified proinsulin peptide that did not evoke a T-cell response, they were protected from the development of insulin auto-antibodies, insulinitis and diabetes [14,15]. This biologically active but immunologically inert modified *Ins2* peptide involved substitution of a single tyrosine by alanine in an *Ins2* epitope (amino acids 9–23 of the B chain) previously known to be of crucial importance in the β -cell autoimmunity of the NOD mouse. These rescued KO mice continued to develop sialadenitis, suggesting the protection was specific to the pancreatic β -cell.

In addition, transplantation of the spleen from these rescued KO mice into an immunocompromised NOD recipient delayed the onset of insulinitis and diabetes compared with those receiving transplanted spleens expressing either one or both wild-type alleles [21]. These studies agree with parallel work performed on the immune response in NOD mice, showing that approximately 50% of intra-islet T lymphocytes react to insulin and, of these, 97% have been found to react specifically to amino acids 9–23 of the insulin B chain [21–23]. Studies of CD8 T-cell clones from young NOD islets demonstrated the ability of these T cells to react to the insulin B chain. In NOD mice models, insulin B chain T-cell reactivity has been shown to be due to the conservation of specific V α and J α segments of a subset of TCRs. Transgenic mice expressing only the conserved TCR α chain with an endogenous V β chain developed not only insulin auto-antibodies but also insulinitis [24]. Administration of the insulin B chain peptide to animals not known to be predisposed to diabetes resulted in a rapid induction of insulin auto-antibodies [25].

Insulin auto-antibodies in clinical practice

Less direct evidence for the role of insulin as an initiating antigen comes from several human studies. Prospective prevention studies have examined the predictive value of auto-antibodies such as islet cell auto-antibodies (ICAs), glutamic acid decarboxylase auto-antibodies (GADAs) and insulin antibodies (IAs). The Daisy, Type 1 Diabetes Prediction and Prevention (DIPP) and Babydiab studies screened newborns from the population found to be at increased genetic risk (HLAs DR3/DR4 and DQ2/

DQ8 are known to confer the highest risk) for the presence of auto-antibodies as the first predictive sign of progression to T1DM [1–4]. With this genotype, 20–50% of proband siblings developed auto-antibodies within the first 3 years of life and were found to have a higher rate of progression to T1DM compared with auto-antibody-negative subjects within the 5-year follow-up period from antibody detection. These studies have revealed an important temporal association, with anti-IAs often preceding other islet cell auto-antibodies and always preceding evidence of β -cell failure. In fact, often the insulin antibodies have decreased or disappeared at disease onset in those children with progression to T1DM. One potential explanation proposed for the waning nature of these antibodies is that insulin is the initiating antigen for T-cell sensitization leading to ‘antigenic spreading’ toward the other islet cell epitopes and downstream cytokine signaling pathways, both of which may play a larger role in the destruction of β cells. Proof-of-principle studies demonstrated that NOD mice that were prone to accelerated diabetes onset, due to a specific set of cytotoxic T-cell clones against islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), could be protected from diabetes via overexpression of thymic proinsulin. Mice tolerant to proinsulin had blocked the clonal expansion of the cytotoxic T cells to IGRP, suggesting that this expansion occurred downstream of T-cell proinsulin reactivity. In addition, NOD mice already harboring a T-cell subset specific for IGRP were also protected from diabetes onset by inducing tolerance to insulin with the introduction of intrathymic proinsulin [26,27]. Indeed, auto-antibodies have an important role for disease prediction, as the presence of two or more auto-antibodies has a positive predictive value of over 90% in those individuals with high-risk HLA genotypes. The Babydiab study has gone a step further in explaining the differential expression of anti-IAs and progression to T1DM [28]. The authors described the expression of high-affinity IgG antibodies associated with the high-risk HLA allele *DRB1*04*. These high-affinity antibodies appeared early (as early as 9 months of age) and were associated with progression to multiple antibody expression in some individuals. These antibodies bound strongly to proinsulin and may explain in part why some individuals with insulin antibodies do not progress to disease, while others (harboring high-affinity antibodies) do.

Isolated lymphocytes from pancreatic draining lymph nodes of patients with T1DM have been shown to recognize a portion of the human insulin A chain [29]. Compared with a control population without diabetes, the lymphocyte response from diabetic patients was significantly more robust, suggesting reactivity to insulin in individuals with T1DM.

Identifying the insulin-reactive T-cell clones from people with and without diabetes showed that insulin-reactive T lymphocytes from affected individuals were of the destructive variety, whereas those isolated from healthy controls had a higher proportion of regulatory T lymphocytes [30]. Thus, it appears that insulin has an important role in the activation of the immune response in both humans and NOD mice. One potential mechanism, suggested by previous work, is exposure

of T cells in the thymus to insulin epitopes and resultant negative selection of auto-reactive T cells and/or induction of Tregs to promote self tolerance.

Immune regulation

It is obvious that specific induction of tolerance to an auto-antigen that is important early on in the autoimmune process is much preferable to nonspecific immunosuppression as a means of preventing T1DM. In this respect, and based on the above considerations, insulin is a very attractive candidate.

The premise is to induce tolerance to the antigenic insulin epitope by shifting the immune response from destructive T lymphocytes to regulatory ones through the introduction of exogenous auto-antigen into healthy, noninflamed tissues or mucosae. Proof of the plausibility of this mechanism came from early animal studies. Immunization of NOD mice with the insulin B chain (peptides 9–23) protected mice from early overt diabetes, while GAD65 immunization failed to confer protection [31]. In addition, this protection was associated with a decrease in detectable activated T cells to the B chain 9–23 peptides compared with control mice immunized with tetanus toxoid. However, these mice still demonstrated insulinitis, suggesting immunization may have only resulted in delaying disease onset. This protection/delay was associated with an upregulation in IL-4 and IL-10 cytokine mRNA, which is associated with a predominantly regulatory T-cell response. Other groups have induced Tregs and decreased insulinitis by the mucosal administration of plasmid DNA encoding the proinsulin polypeptide. The addition of CD40 ligand blockade to prevent stimulation of cytotoxic T lymphocytes prevented the onset of diabetes in vaccinated NOD mice [32]. Selected deletion or alteration of the epitope (B:15–23) maintains immune tolerance by the induction of Tregs while suppressing the cytotoxic T-cell response.

Treating NOD mice with an altered insulin B-chain epitope and a cholera toxin adjuvant resulted in inhibition of insulinitis and prevention or reversal of the early stages of diabetes, whereas vaccination with wild-type insulin B chain with a cholera toxin adjuvant did not [33,34]. These studies underlie the importance of determining the epitope that best balances the cytotoxic and Treg responses. In a series of experiments with Treg–TCR transgenic mice, insulin B chain (12–25/9–23) reactive Treg cells acting through TGF- β secretion protected NOD mice from diabetes onset and conferred protection to NOD severe combined immunodeficient recipients of diabetogenic cells. In addition, transduction of pathogenic CD4 and CD8 T cells with FoxP3, thought to be a major transcription factor for TGF- β 1, effectively changed the immune response to the β -cell, suppressing disease onset [35,36]. Induction of ‘bystander suppression’ by oral immune tolerance has also been demonstrated in animal models. Several peptides either naturally found or artificially expressed in the pancreas have been capable of inducing secretion of regulatory cytokines IL-4 and IL-10 and TGF- β , which suppress related or unrelated autoimmune process within the pancreas [37,38].

The specificity and effectiveness of the immune response is dependant on the epitope, as described above. Indeed, in a study of oral immune tolerance, a single amino acid change in the insulin B epitope was capable of completely abrogating the protective effect of oral administration of the peptide to NOD mice and a virus-induced transgenic mouse model for autoimmune diabetes [39,40]. Thus, it is clear from animal studies that targeted immunomodulatory therapies, in particular the introduction of an insulin epitope, to affect the balance of cytotoxic and Treg responses (favoring the latter), can result in

suppression of insulinitis and overt T1DM. From this experimental evidence, human trials seemed justified and, currently, studies attempting to delay, prevent or reverse T1DM by triggering immune tolerance to insulin are ongoing.

Clinical trials: insulin-stimulated immune tolerance

The Diabetes Prevention Type 1 (DPT1) trial is the largest and most complete of the human insulin tolerization studies to date [41,42]. Relatives of patients with T1DM considered high risk based on immunologic markers and β -cell function

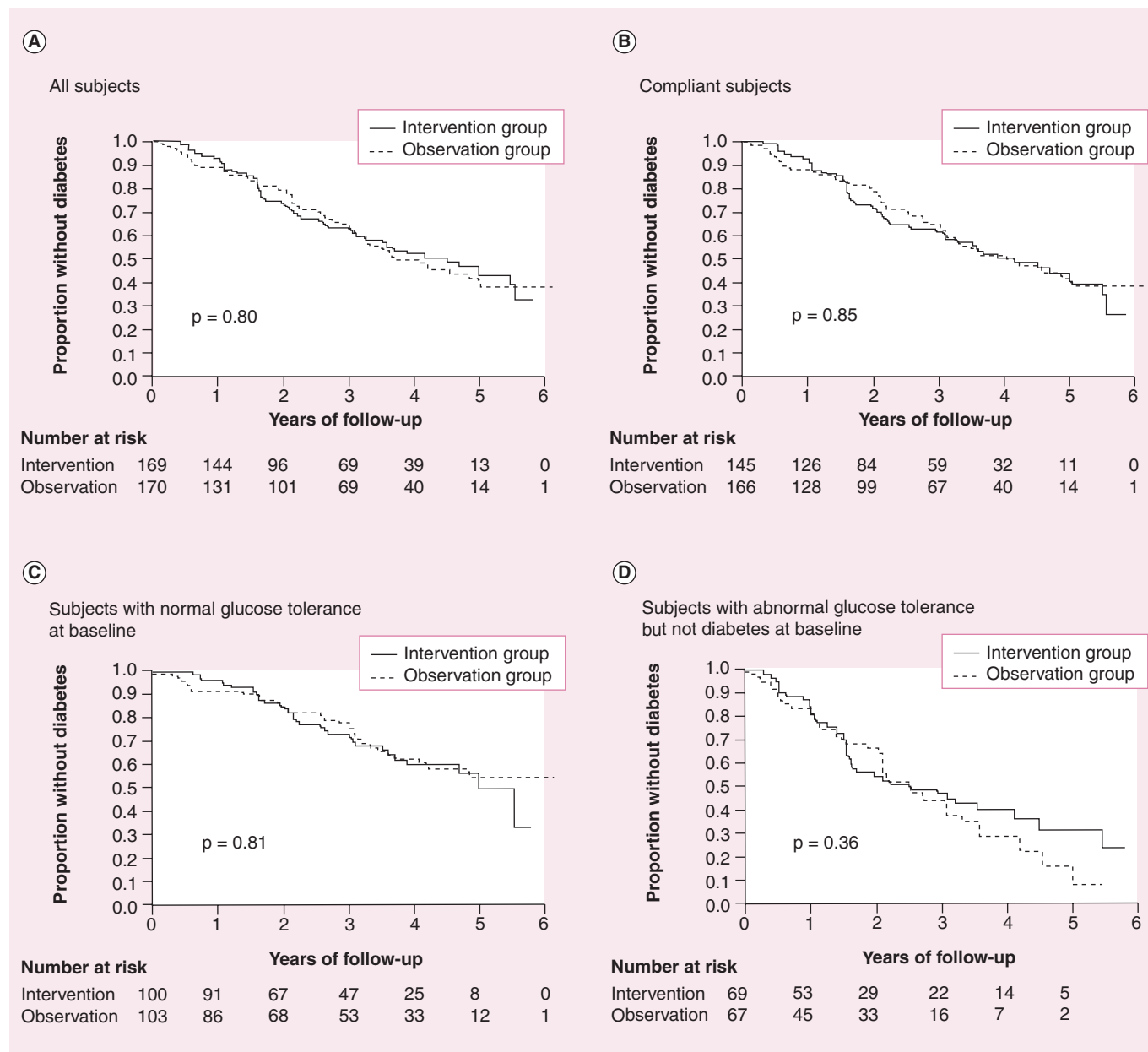


Figure 1. Kaplan-Meier curves showing the proportion of subjects without diabetes, according to treatment-group assignment. (A) All subjects, (B) compliant subjects and (C) subjects with normal glucose tolerance at baseline. Subjects with abnormal glucose tolerance but not diabetes at baseline (D) included those with impaired glucose tolerance, those with impaired fasting glucose and those with indeterminate glucose tolerance. p-values were calculated by the log-rank test. Redrawn with permission from [42].

were randomly assigned to observation, parenteral or oral insulin. In total, 84,228 first- and second-degree relatives were screened for their genetic, metabolic and immunologic risk of T1DM. Of these participants, 711 were randomized into two separate trials: those with a projected 5-year diabetes risk of greater than 50% (based on presence of islet-cell antibodies in more than ten juvenile diabetes foundation units or higher with a first-phase insulin response below threshold and/or an abnormal baseline oral glucose tolerance test) were included in the parenteral insulin trial ($n = 339$), while those positive for both insulin and islet-cell antibodies with normal glucose tolerance and above the threshold first-phase insulin response were eligible for the oral insulin trial ($n = 372$). In the parenteral insulin trial, 139 subjects developed diabetes with no difference in the cumulative incidence between the treated and untreated groups (FIGURE 1)

[34]. Similarly, investigators found no difference between the groups in baseline insulin secretion rates. The only significant difference demonstrated was an increase in progression to diabetes in those individuals glucose intolerant at baseline, compared with glucose-tolerant individuals. The oral insulin trial likewise revealed no significant difference with respect to insulin secretion, first-phase insulin response, glycemia or the development of T1DM between treated and placebo groups. The oral insulin trial examined the degree of insulin autoantibody positivity between individuals progressing, or not, to T1DM, and found no difference in levels of insulin auto-antibodies. Although neither parenteral or oral insulin therapy prevented the progression of autoimmunity in the cohort as a whole, promising results came from oral insulin treatment of those relatives with highest levels of insulin auto-antibodies (of 80 nU/ml or greater) (FIGURE 2) [41]. The study was underpowered and not specifically designed to look at the significance of the results in this highest-risk group, and must be independently confirmed.

For this purpose, a second trial is currently underway to test the hypothesis that, in this particular subset, oral insulin can prevent T1DM. The Melbourne Prediabetes Family Study (Intranasal Insulin Trial; INIT I) examined the safety profile as well as the effects of intranasal insulin on β -cell function and T-cell activation in individuals at risk for T1DM (due to pancreatic islet antigen positivity) [43]. There was no difference in the number of individuals in the treated and control groups with respect to the development of overt T1DM. However, intranasal insulin was associated with an increase in circulating insulin antibodies and a decrease in T-cell responses to insulin, similar to the outcomes of intranasal insulin administration to NOD mice. Currently, a second phase of the Melbourne study (INIT II) is underway in relatives of individuals with T1DM who are positive

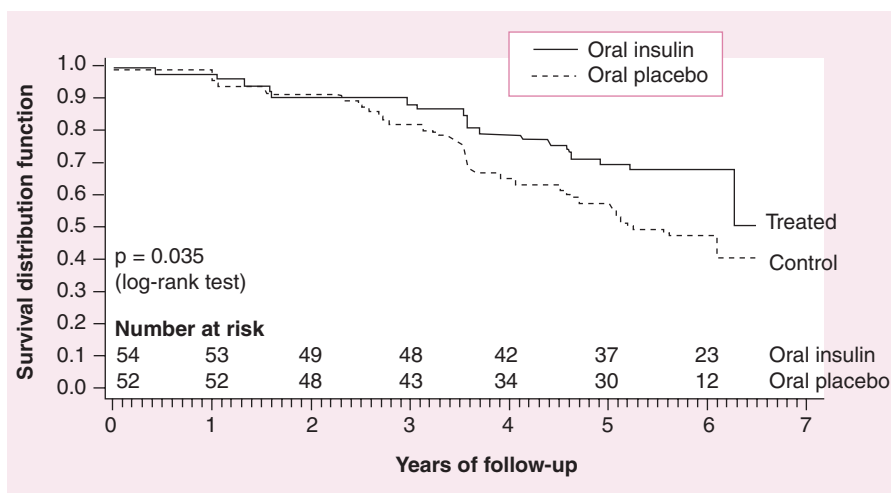


Figure 2. Kaplan–Meier curves showing the proportion of subjects without diabetes during the trial by treatment assignment for subjects with a baseline confirmed insulin auto-antibody (IAA) level of at least 80 nU/ml. The number of subjects at risk in each group at each year of follow-up is enumerated at the bottom of the figure. The log-rank test was used for comparison between the groups, with the p-value as indicated.

Redrawn with permission from [42].

for auto-antibodies to at least two islet antigens. Participants are being randomized to one of three treatment arms, receiving either placebo, 40 or 440 IU of intranasal insulin daily for 1 week then weekly for 1 year [44]. Recently, the DIPP study from Finland published results of a double-blind, randomized controlled trial of intranasal insulin in infants and siblings at increased risk of diabetes. Based on HLA typing and antibody positivity, 224 infants and 40 siblings were assigned to either intranasal short-acting human insulin (1 unit/kg) or placebo. No difference was detected in the diabetes incidence between the treated and control subjects [45]. Thus, to date, none of the oral, intranasal or intravenous insulin trials have effectively demonstrated prevention of the ongoing autoimmune process resulting in β -cell failure. Several potential reasons for the lack of success have been extrapolated from the comparison of animal and human trials. Dosing appears to have a key role in immune tolerance and NOD mice who received the same dose of oral insulin per body weight as the human participants in the DPT1 trial failed to show protection from disease, while a 20-fold higher dose was effective [46]. If such doses prove to be necessary in humans, administration of isolated epitope peptides or whole proinsulin would be a means of avoiding the hypoglycemic effect of the intact insulin molecule. Also, in addition to timing and dose, tolerization studies differ with respect to the use of adjuvant peptides, the route of administration and the resultant protection conferred to the animal models. Currently, the proposed Pre-primary Oral/Intranasal Insulin Trial (POINT) study aims to determine dosing, safety and immune-efficacy data of oral and intranasal insulin in children with the highest genetic risk (HLA identical to DR3/DR4-affected sibling) who are islet cell auto-antibody negative [47]. Hopefully, these ongoing trials will address some of the lingering questions and doubt surrounding

the failure of immune-tolerance therapy in humans thus far. Clearly, there is much more to be learned about the possible use of these alternative therapies in humans.

Expert commentary

Insulin is an attractive target for immune modulation in the pathogenesis of T1DM. Clearly, it has potential for use as a predictor in those at high risk of disease development. Evidence has clearly demonstrated that the insulin peptide is capable of influencing the reactivity of T cells towards the pancreas, an initial step towards preventing autoimmunity. Problems in the translation of basic science and animal models to human clinical trials have hindered efforts to prevent T1DM in humans to date. However, further studies to delineate the optimal conditions of insulin introduction to the immune system will help in the planning of large-scale clinical trials that may be more successful than previous efforts. Currently, a large-scale trial of oral insulin in those subjects with the highest anti-insulin antibody titers is underway by the Type 1 Diabetes TrialNet. In addition, the prospective DIPP trial of intranasal insulin in at-risk individuals continues to collect data on the outcomes of T1DM in participants exposed versus not exposed to the intranasal insulin regime [48]. We can anticipate the results of these trials becoming available within the next 5 years.

Five-year view

It may be anticipated that, in the next 5 years, more-detailed knowledge of both the genetic factors and the immune dysregulation underlying T1DM may allow for a more-refined approach to clinical trials of this nature. One important problem in extrapolating from animal studies is that they are conducted on inbred strains, where animals are as genetically identical as monozygotic twins and also share the same environmental exposures. It may be that, within the much more heterogeneous human population, biomarkers may be identified (both genetic and immunological) that will distinguish responders from non-responders, allowing much more sophisticated interpretation of study results and targeting of therapeutics (personalized medicine). In this respect, the preliminary observation from the DPT1 trial on the importance of insulin auto-antibodies is an important first step.

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Key issues

- Insulin auto-antibodies appear early in the pathogenesis of Type 1 diabetes mellitus (T1DM) and are currently used in risk stratification for disease progression.
- Animal and human studies have shown that differential levels of insulin expression in the thymus determine the risk for T1DM.
- The mechanism of insulin induction of immune tolerance as a means of preventing T1DM appears to involve a switch from inflammatory and cytotoxic T-cell responses towards the epitope to Treg responses, and may influence negative selection of self-reactive T cells.
- To date, trials of oral, parenteral and nasal insulin have failed to confer protection from T1DM in genetically at-risk study participants.
- Ongoing large-scale clinical trials of oral and intranasal insulin are expected to clarify the role of insulin as an immunomodulatory agent.

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