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Review article

Disease prevention with islet autoantigens

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There are three major models of the time course of loss of islet β cells during the development of type 1A (immune-mediated) diabetes mellitus [1] . The first *chronic* model [2] is supported by progressive loss of β-cell mass (despite islet regeneration) in the nonobese diabetic (NOD) mouse [3] and chronic loss of insulin secretion in humans [4] [5] . The second model [6] is supported by the acceleration of the disease at diabetes onset in the NOD mouse, as evidenced by immunologic therapies that are effective only at onset [7] [8] and more rapid disease transfer by splenocytes closer to diabetes onset. The third model [9] is supported by variation in levels of islet autoantibodies among individuals at risk for type 1 diabetes mellitus [10] . Given the ability to predict the development of type 1 diabetes mellitus once multiple anti-islet autoantibodies are present (two or more autoantibodies reacting with insulin, glutamic acid decarboxylase molecular weight 65 kd [GAD65], or antibody islet antigen 2 [IA-2]) [11] [12] , the authors prefer the first model. Without direct imaging of β-cell mass, however, any combination of the three models is possible for any given individual developing diabetes.

Large screening programs of the general population (eg, Diabetes Autoimmunity Study in the Young [13] with more than 30,000 newborns HLA-typed at birth from cord blood) or relatives of patients with type 1 diabetes mellitus (eg, Diabetes Prevention Trial-Type 1 Diabetes [DPT-1] [14] , with more than 100,000 relatives screened) have evaluated individuals for diabetes risk. Approximately 2.3% of children in the Denver, Colorado area have the highest risk HLA

genotype for type 1 diabetes mellitus (DR3/4 and DQ2/8) and comprise approximately 50% of children developing diabetes before 5 years of age [15] . Given genetic susceptibility, anti-islet autoantibodies can appear in the first year of life or much later, with examples of one identical twin developing autoantibodies as a teenager and the second twin after 40 years of age [16] . Individuals expressing multiple anti-islet autoantibodies rarely lose antibody expression and have a high risk of progression to diabetes [12] .

Animal models

Multiple animal models mirror immune-mediated diabetes to varying degrees. The standard spontaneous models are the BioBreeding (BB) rat [17] and the NOD mouse [18] [19] [20] . In addition, there are strains such as the Long Evans Tokushima Lean rat [21] with a mutation of the gene *Cblb* [22] , animal models created by expression of ectopic molecules in the islet using the rat insulin promoter [23] [24] , and recently, models induced with immunization with insulin peptides [25] or lymphocytic choriomeningitis virus [26] . Each model has particular advantages and disadvantages. Given the inbred nature of each animal strain, if any model does represent type 1 diabetes mellitus of humans, it can only model a single genotype.

1.1 BioBreeding rat

It is difficult to prevent diabetes in the BB rat model. The BB rat has a severe T-cell lymphopenia caused by a mutation of immune-associated nucleotide 4/5, a gene of unknown function that leads to early death of most postthymic lymphocytes in an autosomal recessive manner [27] . Combined with specific alleles of the major histocompatibility complex (MHC) (RT1-U), this lymphopenia leads to diabetes in the majority of BB rats between 60 and 120 days of age [28] . The lymphopenia, which is not present in humans, likely limits studies of immune regulation in this strain [29] . Cloning of T cells and identifying T-cell targets have proven difficult as well. In nonlymphopenic animal models, (eg, diabetes-resistant BB rats), diabetes can be induced following experimental depletion of specific T lymphocytes or immune activation with factors like polyiosinic-polycytidylic acid or Kilham rat virus [28] .

1.2 Nonobese diabetic mouse

The NOD mouse has become the standard animal for studies of type 1 diabetes mellitus. Diabetes is inherited in a polygenic manner with an essential contribution by MHC alleles. Essentially all NOD mice develop insulitis, but approximately 80% of female and 30% of male NOD mice develop diabetes [30] . Thus, despite being inbred and raised in similar environments, only a subset of mice develops diabetes. This lack of aggressive diabetogenesis probably is implicated in the major disadvantage of the NOD mouse: more than 100 different therapies prevent diabetes. Many of these therapies are unlikely to be relevant to disease prevention in humans [20] . For instance, immunization of NOD mice with many nonspecific agents such Freund's adjuvant or bacille Calmette-Guerin prevents diabetes. Despite a decrease in the development of diabetes, these mice have insulitis. Diabetes rapidly occurs with drugs such as cyclophosphamide (Cytoxan) that are thought to abrogate immune regulation. The authors suggest that a more robust endpoint (eg, abrogation of insulitis) and more robust NOD models (eg, homozygous, Insulin II gene [*Ins2*], knockout NOD mice) may be needed to better assess potential preventive therapies for study in humans [31] [32] .

1.3 Transgenic models

A large number of models of type 1A diabetes mellitus depend on transgenic expression of one or more proteins. For instance, using the rat insulin promoter to drive β-cell expression of interferon gamma, viral proteins, and costimulatory molecules such as B7.1 results in immune-mediated diabetes [33] [34] [35] [36] [37] . These studies led to the conclusion that the immune system can target multiple islet molecules and produce type 1A diabetes mellitus.

Combining islet transgenic expression of B7.1 with a transgene encoding the human DQ8 molecule leads to diabetes [38] . An *experimental autoimmune diabetes* model results from combining the B7.1 transgene with immunization with a specific insulin peptide (immunodominant insulin B chain amino acids 9–23 [B:9–23]). Diabetes can even be induced in BALB/c mice carrying the transgene (Table 1) [24] .

Table 1. Induction of insulin autoantibodies/insulitis/diabetes in BALB/c mice

Stimulus	Phenotype
B:9-23 peptide	Insulin autoantibodies
B:9-23 peptide + poly-IC	Insulitis
B:9-23 peptide + poly-IC + B7.1 islet	Diabetes

Adapted from Moriyama, et. al. Induction and acceleration of insulitis/diabetes in mice with a viral mimic (polyinosinic-polycytidylic acid) and an insulin self-peptide. Proc Natl Acad Sci USA 2002;99(8):5539–44.

Several of the most elegant animal models use T-cell receptor transgenics, where a T-cell receptor from a diabetogenic T-cell clone has been introduced into an animal of the appropriate background. Monoclonal mice, including BDC2.5 (CD4 T-cell transgenic) and 8.3 (CD8 T-cell transgenic) lines, have interesting phenotypes. The BDC2.5 transgenic does not accelerate the development of diabetes unless the mouse lacks endogenous T lymphocytes [35] , suggesting that this specificity is regulated readily. In contrast, the 8.3 transgenic (CD8) recognizes a peptide of the β-cell–specific molecule islet glucose-related phosphatase (IGRP) and greatly accelerates the development of diabetes [39] [40] .

In addition to genetic manipulations that cause diabetes, multiple gene knockouts have been combined with the NOD mouse model. The authors believe insulin may be a primary islet autoantigen because knockouts of the *Ins2* gene (expressed in the thymus and islets) greatly accelerate diabetes, whereas knockouts of the Insulin I gene prevent 90% of the development of diabetes and greatly delay the development of insulitis [32] .

Autoantigens

One can divide autoantigens and putative autoantigens into three classes based on their tissue distribution: (1) *β-cell–specific*, (2) *neurendocrine*, and (3) *ubiquitous*. The distribution of the molecules may vary with the species. For instance, GAD65, which is β-cell–specific in islets and present in neurendocrine tissues in the rat, is virtually absent in mouse islets, and is present in all (β and non-β) islet cells of humans. Because both insulitis and cell destruction of type 1A diabetes mellitus is β-cell–specific, we believe it is likely that the major immunopathogenesis will be β-cell–specific.

2.1 BioBreeding rat

BB rats do not produce workshop-confirmed anti-islet autoantibodies. Additionally, the rat models have proven resistant to the isolation of pathogenic T cells.

2.2 Nonobese diabetic mouse

The NOD mouse is the most extensively studied animal model of type 1 diabetes mellitus, and multiple islet autoantigens have been identified. Only insulin autoantibodies can be detected with high specificity and sensitivity. To date, international workshops have failed to demonstrate autoantibodies reacting with glutamic acid decarboxylase

(GAD) or IA-2 and indicate that ELISAs lack specificity in islet autoantibody detection similar to studies in humans [41] . Insulin autoantibodies in NOD mice can be present transplacentally; transplacental autoantibodies increase the development of diabetes [42] . Several insulin-specific T-cell clones have been isolated, and the recognized peptides have been identified (B:9–23 insulin peptide for CD4 T-cells, B:15–23 peptide for CD8 T-cells, and p24–33 epitope of the B-C junction) [43] [44] . Wong and colleagues [45] have produced a class I tetramer able to identify B:15-23–reactive T lymphocytes.

Santamaria and colleagues [40] isolated T-cell clones sharing a common T-cell receptor motif; produced NOD-related peptide (NRP), a tetramer to quantitate these T cells; and finally isolated the native molecule to which these T cells react. The native molecule is a β-cell–specific molecule, IGRP, whose function is currently unknown [46] . A high percentage of NRP-reactive T cells in the peripheral blood (more than 0.5%) is predictive of diabetes in the NOD mouse [47] .

With the discovery of GAD65 autoantibodies in humans, a large effort has been devoted to studies of anti-GAD reactivity in the NOD mouse. To date, workshops have not confirmed the presence of specific anti-GAD autoantibodies. Nevertheless, several anti-GAD T-cell clones have been produced that can accelerate the development of diabetes, and GAD peptides administered by many routes can influence the development of diabetes [43] .

Splenocytes from NOD mice have been reported to proliferate when stimulated by heat shock protein 60 (HSP60) as well as a peptide derived from HSP60, p277. In addition, anti-HSP60 autoantibodies have been reported, albeit not confirmed, in workshops. HSP60 is expressed ubiquitously. Thus, the relationship to an organ-specific autoimmune disease is unexpected. Recently it has been suggested that HSP60 and p277 may activate innate immunity. Many recombinant proteins are contaminated by minute quantities of lipopolysaccharide, however, which can activate innate immunity [48] .

In addition to the molecules previously discussed, there are multiple T-cell clones that recognize unknown antigens, such as BDC2.5. T cells reacting with additional molecules such as IA-2β (phogrin) can be produced under specific experimental conditions as well.

2.3 Humans

Patients with type 1 diabetes mellitus produce autoantibodies reacting with insulin, GAD65, and IA-2 (also termed *islet-cell antibody 512 [ICA 512]*). Multiple international workshops have confirmed the presence of autoantibodies reacting with these molecules, and excellent radioassays for these autoantibodies provide important prognostic information in diabetes prediction [49] . Insulin autoantibodies are often the first autoantibody to appear in children followed from birth. IA-2 autoantibodies are usually the last to appear. Children developing diabetes before 5 years of age have extremely high levels of insulin autoantibodies, whereas most individuals developing diabetes after 12 years of age are negative for insulin autoantibodies [50] . GAD65 autoantibodies are the most common in adults with latent autoimmune diabetes of adults (LADA) [51] .

In contrast to the measurement of autoantibodies to GAD65, insulin, and IA-2, additional autoantigens can be detected by analyzing the reactivity of sera from patients with type 1A diabetes mellitus against sections of human pancreas. A subset of such antibodies may react with islet gangliosides, a molecule of ICA69, or unknown antigens [52] [53] .

Disease prevention in animal models

3.1 BioBreeding rat

Injection of insulin was the only successful disease prevention in the BB rat using an islet autoantigen [54] . The insulin, however, had to be administered in a manner that induced hypoglycemia and resulted in atrophic islets. Given the severe T-cell lymphopenia of the BB rat, it is likely that immunoregulatory therapies with autoantigens will not be successful in this model.

3.2 Nonobese diabetic mouse

In contrast to the BB rat, multiple therapies prevent diabetes in the NOD mouse model [20] . A variety of delivery mechanisms have been used: oral or nasal administration, intramuscular injection, subcutaneous injection, intrathymic injection, DNA vaccines encoding for autoantigens, and hematopoetic stem cells. Such preventive therapies are usually not completely efficacious, are less effective in older NOD mice, and do not prevent insulitis. Thus, more robust NOD models will likely be developed for preclinical evaluation of potential therapies.

3.3 Glutamic acid decarboxylase 65

GAD has been administered subcutaneously, orally, and intrathymically. A large number of studies indicate that injection of GAD65 DNA constructs encoding GAD65 with cytokines, and GAD peptides can delay or prevent the development of diabetes in the NOD mouse [55] [56] . A transgene encoding for GAD65 was without effect [57] .

3.3.1 Insulin

Orally administered insulin delays the development of diabetes in the NOD mouse [58] . The oral administration of insulin has a biphasic dose-response curve, with disease prevention lost at higher oral dosage. When given subcutaneously, high doses (doses associated with hypoglycemia) also delay the development of diabetes, but neither subcutaneous nor oral insulin alone prevents insulitis. Oral insulin conjugated with the nontoxic B-chain of cholera toxin caused a significant reduction in insulitis and diabetes in NOD mice [59] [60] . Following oral insulin consumption, there is evidence of development of regulatory T cells that are able to inhibit the transfer of diabetes when injected into immunodeficient NOD mice, probably through a transforming growth factor-β–mediated mechanism [61] .

Induction of regulatory T cells following subcutaneous insulin has also been demonstrated, and disease protection does not require metabolically active insulin. In particular, mutated insulin (unable to bind to insulin receptor) and peptides of insulin such as B:9–23 can prevent diabetes. Wegmann and colleagues isolated CD4 T lymphocytes from islets of NOD mice. The majority of T lymphocytes reacted with insulin and more than 95% of insulin-reactive T cells recognize the insulin peptide B:9–23 [62] . When administered subcutaneously in incomplete Freund's adjuvant at 4 weeks of age, this peptide prevents 90% of the progression to diabetes [63] . An altered peptide ligand of B:9-23 peptide was created with alanines introduced at positions 16 and 19. This altered peptide retained the ability to delay the development of diabetes when administered without adjuvant to NOD mice [64] . In addition to the prevention of diabetes, the insulin peptide B:9–23 induces insulin autoantibodies when given in incomplete Freund's adjuvant. If administered to BALB/c mice with islet B7.1 expression (see Table 1), diabetes is induced [65] . When given subcutaneously without adjuvant, this peptide induces fatal anaphylaxis [66] . The anaphylaxis depends on the generation of both IgG and IgE antibodies reacting with the peptide. In addition, DNA vaccines encoding for insulin have been used to prevent diabetes [26] [67] . In general, such therapies do not prevent insulitis, and the prevention of diabetes is partial.

A preproinsulin transgene directing antigen expression to NOD antigen–presenting cells has been generated. The preproinsulin transgene prevented the development of diabetes and insulitis [57] . When hematopoetic stem cells from the preproinsulin transgenic strain were isolated and administered to NOD mice, these cells were able to completely

prevent diabetes and insulitis [68] . If hematopoetic stem cells can be administered without harsh conditioning regimens, this is a potential therapy for consideration in humans.

3.3.2 Heat shock protein 60 and peptide p277

A peptide of HSP60, p277 also has been reported to prevent diabetes when given to young NOD mice and to limit progression to fatal hyperglycemia after development of hyperglycemia in NOD mice [69] . Administration of p277 by way of a DNA vaccine also prevented diabetes, but the empty vector also prevented diabetes in the same study [70] [71] . Studies of HSP60 and p277 therapy are limited primarily to a single group of investigators, and Atkinson and colleagues reported inability to confirm prevention of diabetes in NOD mice [72] .

Human trials

Given the ease with which type 1 diabetes mellitus is prevented, primarily in the NOD mouse model, a number of trials of antigen-based therapies at onset of diabetes or in at-risk relatives has been completed [14] [73] [74] [75] [76] [77] [78] [79] [80] . For the most part, these trials have been negative or lack confirmation of pilot results. Nevertheless, the trials have established robust criteria for disease prediction and trial design. In particular, trials are usually performed at the onset of diabetes with the primary outcome being the preservation of C-peptide secretion. C-peptide is the connecting peptide of the proinsulin molecule and is secreted in a 1:1 ratio with insulin [81] . The ability to secrete C-peptide is a marker of retained β-cell function and is associated with greater ease of managing type 1A diabetes mellitus, including decreased episodes of severe hypoglycemia, improved glucose control (eg, decreased hemoglobin A1c [HbA1c]), and decreased long-term complications [82] . A number of parameters in the design of trials in new-onset patients are somewhat arbitrary, and the Immunology of Diabetes Society has published a consensus trial design [81] that hopefully will make trials easier to compare in the future (Box 1).

Box 1 Immunology of Diabetes Society guidelines for phase I and II intervention trials

- Diagnosis: American Diabetes Association criteria
- Document: Age, gender, pubertal status, family history, blood glucose, bicarbonate, ketoacidosis, weight loss, polyuria, polydypsia, HbA1c, islet autoantibodies, insulin requirement, and HLA typing
- Phase I trials may only include subjects 18 or more years of age
- Phase II and III trials may enroll subjects 35 or fewer years of age
- Subjects should have one or more autoantibodies to GAD65, IA-2, ICA, or insulin (if on insulin less than 2 weeks only), measured with standardized tests
- Baseline mixed meal tolerance test (MMTT) peak C-peptide of 0.2 pmol/L or greater
- Early-onset trials should enroll patients diagnosed within 2 to 12 weeks
- Trials should last 2 years or longer
- Randomize, placebo-control, double-mask subjects in phase I and II trials
- Quarterly evaluate stimulated C-peptide with MMTT without morning insulin only if fasting blood glucose is 4 mmol/L to 11.1 mmol/L
- Evaluate immune markers in regard to HLA types

(*From* Greenbaum CJ, Harrison LC. Guidelines for intervention trials in subjects with newly diagnosed type I diabetes. Diabetes 2003;52(5):1059–65; with permission.)

4.1 Insulin

When insulin is administered intensively by subcutaneous injections in patients with overt diabetes (versus what was standard insulin therapy at the time of the Diabetes Care and Complications Trial), loss of C-peptide secretion is slowed [82] . Trials of at-risk individuals also have evaluated low-dose subcutaneous injections combined with annual infusion of intravenous insulin, oral insulin, and intranasal insulin. DPT-1 [14] screened approximately 100,000 first-degree relatives for expression of anti-islet autoantibodies and conducted two trials: one using subcutaneous insulin with annual intravenous insulin [14] and the other using oral insulin (Box 2). Relatives with the presence of cytoplasmic ICA and with low first-phase insulin secretion were judged to have a risk of diabetes of approximately 50% and received either parenteral insulin or no therapy. Insulin injections did not slow progression to diabetes, and approximately 50% developed diabetes by 5 years of follow-up.

Box 2 Summary of findings of Diabetes Prevention Trial parenteral insulin trial

- 84,228 relatives screened
- 3152 subjects ICA-positive
- 372 subjects with projected 5-year risk greater than 50%
- 339 subjects randomized to injection/observation
- Diabetes: 69 treated, 70 observation group
- Insulin at dosage used in high-risk group had no effect
- Multiple anti-islet autoantibodies predict type 1 diabetes mellitus

(*Adapted from* Diabetes Prevention Trial–Type 1 Diabetes Study Group. Effects of insulin in relatives of patients with type 1 diabetes mellitus. N Engl J Med 2002; 346(22):1685–1691.)

The trial of oral insulin enrolled first-degree relatives of patients with type 1 diabetes mellitus who had cytoplasmic ICA as well as insulin autoantibodies but normal insulin secretion (greater than 10th percentile) upon intravenous glucose-tolerance testing. These relatives were judged to have a 20% to 50% risk of progressing to diabetes within 5 years. A publication on the results of this oral insulin trial is not yet available. In addition to the methods for diabetes prediction applied at the initiation of these long-term trials, autoantibodies reacting with insulin, GAD65, and IA-2 were measured, and it is clear that prediction can be based on the presence of these autoantibodies, the measurement of which is easier to standardize than the cytoplasmic ICA assay [83] .

Two groups have studied nasal administration of insulin given to at-risk relatives. One trial had a crossover design and reported immunologic changes following nasal insulin, but given the nature of the design, it did not provide evidence for efficacy. The second trial, from Finland, is particularly ambitious in that it is a randomized, placebo-controlled trial where insulin is administered in young children from the general population followed from birth for the appearance of anti-islet autoantibodies [84] . Trial entry is initiated following the appearance of the first anti-islet autoantibodies. The results of this trial should be available in 2 years.

4.2 Glutamic acid decarboxylase

A small trial of GAD65 injections (subcutaneous in alum adjuvant) has been completed and reported at the 2002 American Diabetes Association meeting in patients with LADA. This primarily phase 1 trial provides evidence for a lack of toxicity of injected GAD65 with no observation of neurologic symptoms. Stiff-man syndrome is associated with high levels of GAD65 autoantibodies. LADA patients, however, appear to have slow loss of C-peptide secretion

that is likely to complicate evaluation of immunotherapies in these patients.

4.3 Heat shock protein

A small trial of the HSP60 peptide p277 has been reported in adult patients from Israel with overt diabetes with evidence of preserved C-peptide [73] . An unpublished trial of the same peptide in children with diabetes apparently failed to influence loss of C-peptide secretion.

4.4 Peptide B:9–23

A large trial of an altered peptide ligand of insulin peptide B:9–23 is underway in patients with new-onset diabetes. This trial follows smaller studies where there was no evidence of toxicity of the compound (ie, no allergic reactions to the peptide that are seen with the native peptide in NOD mice) (P. Gottlieb, oral communication; January 1, 2004).

Summary

It is now possible to predict type 1A diabetes mellitus, and trial designs have been standardized over the years. The absence of surrogate assays of pathogenic T lymphocytes in humans, however, makes it difficult to evaluate rapidly the influence of immunologic therapies on the T lymphocytes causing β-cell destruction. For trials where there is a lack of efficacy, one cannot determine if the therapy failed to influence disease progression because of a lack of immunologic effect (eg, lack of successful generation of regulatory T lymphocytes responding to the immunizing antigen) or because of a lack of clinical benefit despite immunologic potency. For instance, it has been pointed out that the amounts of insulin administered in the DPT-1 parenteral trials were orders of magnitude lower than those required to delay diabetes development in NOD mice. In a similar manner, the oral insulin trial studied a single dose of oral insulin without the ability to detect whether that dose generated insulin-responsive regulatory cells.

A number of newer assays hold promise in terms of evaluating immunotherapies, including application of tetramers to quantitate CD4-GAD65–reactive T cells and CD8 T lymphocytes as well as enzyme-linked immunospot assays. It is likely that improvements in this technology will be essential for more rapid clinical development and assessment of therapies to prevent β-cell destruction. In addition, several international organizations are in place to speed the development of immunotherapy for type 1 diabetes mellitus. Two of these organizations, TrialNet (available at http://www.bsc.gwu.edu/bsc/studies/trialnet.html , accessed January 18, 2004) and the Immune Tolerance Network [82] (available at http://www.immunetolerance.org , accessed January 18, 2004) actively seek proposals for trials that can be performed efficiently with an international trial network.

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