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Review Article

Insulin as the "Primary" Autoantigen in Type 1 Diabetes ?

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Type 1 diabetes of both the human and NOD mouse is associated with autoimmunity directed against insulin which is the only ß cell specific autoantigen. Variable number of tandem repeats in the 5' region of insulin gene on chromosome 11 is associated with the risk of type 1 diabetes in human. Mice have two insulin genes including the insulin 1 gene (chromsome 19) and the insulin 2 gene (chromsome 7). The insulin 2 gene knockout when bred onto NOD mice accelerates diabetes. In contrast to insulin 2, diabetes and insulitis were markedly reduced in insulin 1 knockout mice with decreased and delayed diabetes. Autoantibodies to insulin can predict diabetes in man and NOD mice. Insulin peptides can be used to induce insulitis and diabetes in non-diabetic strain mice. Our results suggest that insulin molecule is a possible "primary" autoantigen that initiates a pathogenesis of type 1 diabetes. An administration of insulin or its peptide can prevent the development of diabetes in NOD mice but we cannot at present safely prevent type 1 diabetes in humans. A series of clinical trials are under way and planned.

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Keywords: Type 1 diabetes; NOD mouse; Insulin gene; Insulin autoantibody; Insulin B chain peptide

Introduction

Type 1 diabetes is characterized by progressive autoimmune destruction of islet ß-cells based on genetic susceptibility (Figure 1).[1] It apparently arises from T cell-dependent destruction of ß-cells against multiple different autoantigens and multiple epitopes of each autoantigen and is associated with the presence of autoantibodies to multiple islet antigens.[2] During the past several years, there has been an important increase in the number of autoantigens found to be targets of the autoimmune process which precedes type 1 diabetes. However, it is not clear which is the "primary autoantigen" that initiates a pathogenesis of type 1 diabetes. To date, amongst identified target antigens, insulin remains the only diabetes related ß-cell specific autoantigen in both human and mice. In this paper, we will review studies regarding insulin gene, humoral and cellular response to "insulin" as a dominant target for the initiation of type 1 diabetes in both human and non-obese diabetes (NOD) mouse.

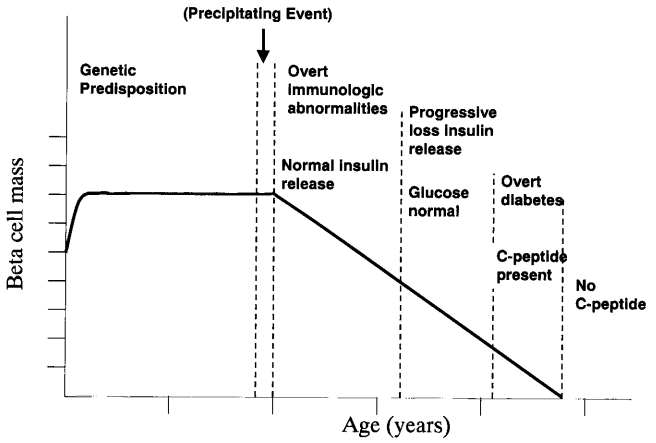

Figure 1. Stages in the development of Type 1A diabetes, modified from Eisenbarth et al. N Eng J Med 314: 1360-1368, 1986.

Insulin gene

In human, type 1 diabetes develops in the setting of genetic susceptibility. Alleles of genes within the major histocompatibility complex (MHC) are responsible for the majority of the familial aggregation of type 1 diabetes.[3] The highest risk for type 1 diabetes in Caucasian is associated with individuals expressing both HLA DQA1*0501-DQB1*0201 (DQ2), and DQA1*0301-DQB1*0302 (DQ8) (DR3/4 or DQ8/DQ2 heterozygotes). The highest risk for type 1 diabetes in Japanese patients is associated with individuals

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expressing HLA DQA1*0301-DQB1*0401 (DR4), DQA1*0301-DQB1*0303 (DR9). DQA1*0301-DQB1*0302 (with DRB1*0802 or DRB1*0405) is also frequently observed susceptibility HLA haplotypes in Japan.[4] It is likely that non-MHC genes influence the development of diabetes including the insulin gene (INS) on chromosome 11 (IDDM2) and other putative IDDM loci.[5] The minisatellite (i.e. variable number of tandem repeats [VNTR]) in the 5' region of insulin gene is classified into three classes according to length. The shortest class I allele is susceptible to type 1 diabetes, while the longest class III allele is protective.[6] In Japan, the INS VNTR region is less polymorphic and approximately 95% of Japanese are homozygous for class I alleles, which makes it difficult to assess the role of the development of type 1 diabetes in Japanese. Awata and coworkers reported that in number of repeat units (RUs), of class I alleles influences disease susceptibility in Japanese subjects.[7] The insulin gene associated with protection from type 1 diabetes is associated with greater insulin message within human thymus.[8]

Mice have two insulin genes including the insulin 1 gene (chromsome 19) and the insulin 2 gene (chromsome 7). The two genes differ in terms of preproinsulin expression within the thymus, with much greater preproinsulin 2 protein but similar expression of both genes in the islets. Polychronakos and coworkers have demonstrated that thymic expression of insulin is related directly to the number of copies of insulin 2 genes present.[9] Hanahan suggested that greater insulin expression in the thymus may be protective of type 1 diabetes.[10] Thebault-Baumont and coworkers have reported that breeding the insulin 2 gene knockout onto NOD mice accelerated the development of diabetes.[11] We produced insulin 1 or 2 gene knockout congenic NOD mice.[12] In contrast to insulin 2, incidence of diabetes and insulitis were markedly reduced in insulin 1 knockout mice with decreased and delayed diabetes in heterozygous females, and no insulitis and diabetes in homozygous female mice (Figure 2).[13] These observations indicate that loss of either insulin gene can influences diabetes of NOD mice, and suggests that the preproinsulin 1 gene is essential for the spontaneous development of NOD insulitis and diabetes.

Humoral response to insulin

In human, the immunologic prediction of type 1 diabetes has greatly improved over the past five years with the characterization and cloning of specific autoantigens.[2] Table 1 lists five islet autoantigens for which there currently exist autoantibody assays.[14] Glutamic acid decarboxylase (GAD), one of the cytoplasmic enzymes synthesizing the neurotransmitter GABA, was originally identified as an islet 64KD autoantigen in type 1 diabetes.[15] IL-2/ICA512, one of the transmembrane protein tyrosine phosphatases, was identified as a 40KD tryptic fragment.[16] Insulin was the first autoantigen biochemically characterized.[17] Currently, the best predictor of future type 1 diabetes is the expression of multiple "biochemically" determined autoantibodies. Verge and coworkers examined the risk of type 1 diabetes in first-degree relatives in United State according the number of autoantibodies and found that expression of two, or three autoantibodies was associated with high risk.[18] We determined the distribution of autoantibodies to IL-2/ICA512, GAD, and insulin in Japanese type 1 diabetes patients. Of 73 new-onset patients, the positivity was 71% GAD autoantibody,

Table 1 Anti-islet autoantibody assays

Antigen	Sensitivity	(Specificity)	Comment
Insulin	40-95%	(99%)	Inversely Related to Age of Diabetes Onset
GAD65	70%	(99%)	Predominantly Age Independent
ICA512/IA-2	60%	(99%)	Islet Related Tyrosine Phosphatase
Phogrin/IA-2β	55%	(99%)	Autoantibodies Predominantly Subset of ICA512/IA-2 Autoantibodies
Carboxypeptidase H	10%	(99%)	Low Sensitivity

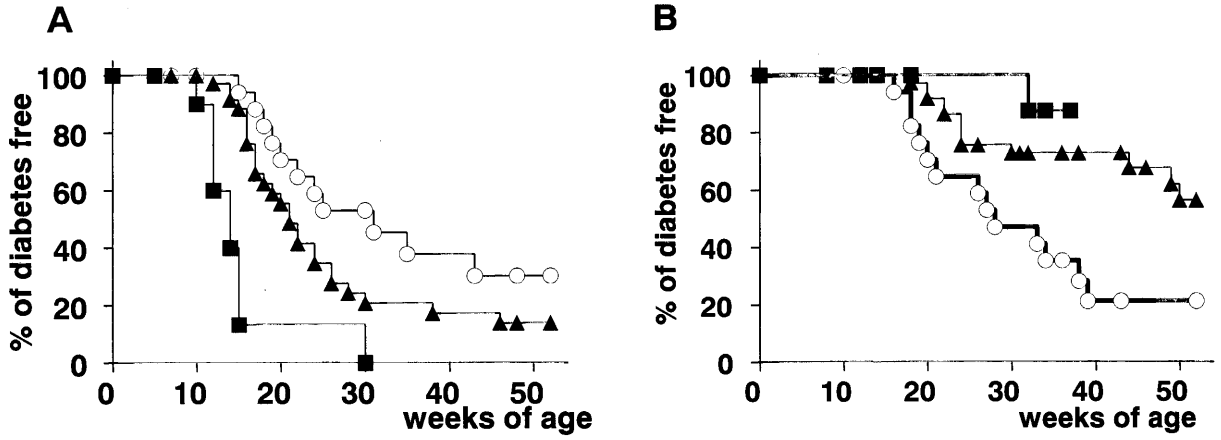

Figure 2. In both panels, closed triangles (▲) indicate insulin homozygous knockout (-/-), closed square (■) heterozygous knockout (+/-), and open circle (○) wild-type insulin genes (+/+). Panel A shows progression to diabetes in female insulin 2 knockout mice (-/-, n=10; +/-, n=35; +/+, n=17). Panel B shows progression to diabetes in female insulin 1 knockout (-/-, n=14; +/-, n=37; +/+, n=17). The three curves are dramatically different for female insulin 2 knockout (Panel A, $p<0.001$) and insulin 1 female knockout mice (Panel B, $p<0.001$). Adapted from Moriyama H et al. Proc Natl Acad Sci USA 100: 10376-10381, 2003.

48% for insulin autoantibodies (IAA), 62% for IL-2/ICA512, and 89% of children with recent onset of type 1 diabetes express one or more of three autoantibodies. Of note that six out of 73 (8.2%) patients with type 1 diabetes were negative for all of these islet-autoantibodies.[19]

In mice, the majority of NOD mice have IAA that can be measured in an assay format identical to that used in human studies. A recent workshop indicates that insulin but not GAD or IA-2/ICA512 is a specific autoantigen of humoral autoimmunity in NOD mice.[20] NOD mice express levels of IAA similar to the high levels of the youngest children developing type 1 diabetes. In contrast, only weak signals for GAD65 or IA-2/ICA512 autoantibodies were detected. We have found that IAA of NOD mice appear as early as 4 weeks of age, and then decrease and disappear in the majority of mice at the time diabetes develops. Early appearance of IAA at 8 weeks of age was strongly associated with early development of diabetes which occurred at 16 to 18 weeks of age (Figure 3).[21] This data suggests that a high risk of early development of diabetes is often determined by 8 weeks of age and the program for developing diabetes of NOD mice is "fixed" relatively early in life.

IAA appear early and then disappear at approximately the time of onset of hyperglycemia in most NOD mice.[22] Since complete ß cell destruction seems to relate with the termination of the expression of IAA, one hypothesis is that autoantibodies are produced as a result of the ongoing destruction of ß cells. The production of IAA might end with complete destruction of ß cells. We have found that NOR mice spontaneously express IAA. NOR mice share 85% of their genome with NOD mice form. Since NOR mice were described as insulitis-resistant and diabetes-free mice, we prospectively studied the expression of IAA in these mice to test if the transient expression of the autoantibody correlated with development of diabetes. The expressions of autoantibodies were transient in NOR mice and followed the same time course as for NOD mice and they were all negative by 28 weeks (without progression to diabetes) (Figure 4). This data is not consistent with the hypothesis that the time course of autoantibodies simply reflects destruction of ß-cells with development of diabetes.[23]

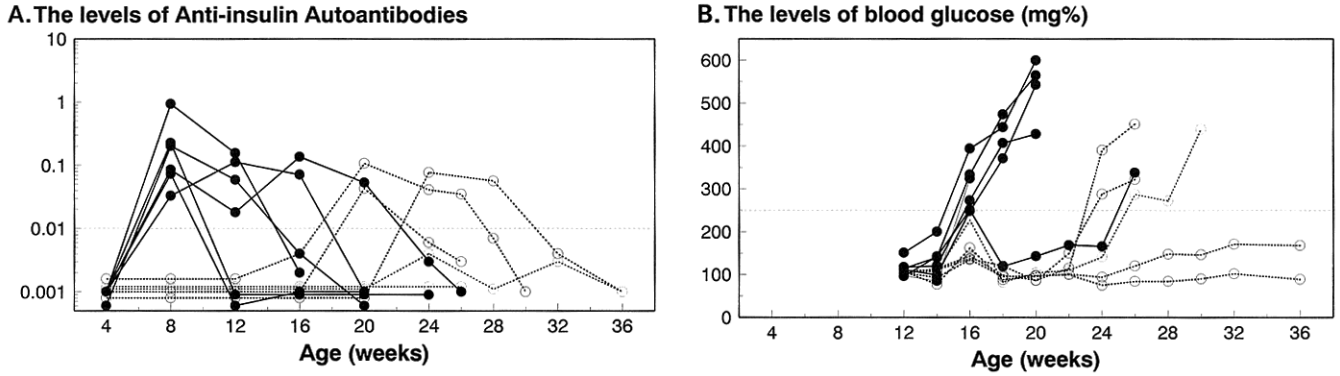

Figure 3. (A) The level of IAA for the NOD mice expressing IAA at 8 weeks of age (●) and at 20 weeks or later (○). (B) Blood glucose for the NOD mice expressing IAA at 8 weeks of age (●) and at 20 weeks or later (○) followed until diabetes or 36 weeks. Early appearance of IAA at 8 weeks of age was strongly associated with early development of diabetes which occurred at 16 to 18 weeks of age. Adapted from Yu L et al. *Proc Natl Acad Sci USA* 97: 1701-1706, 2000.

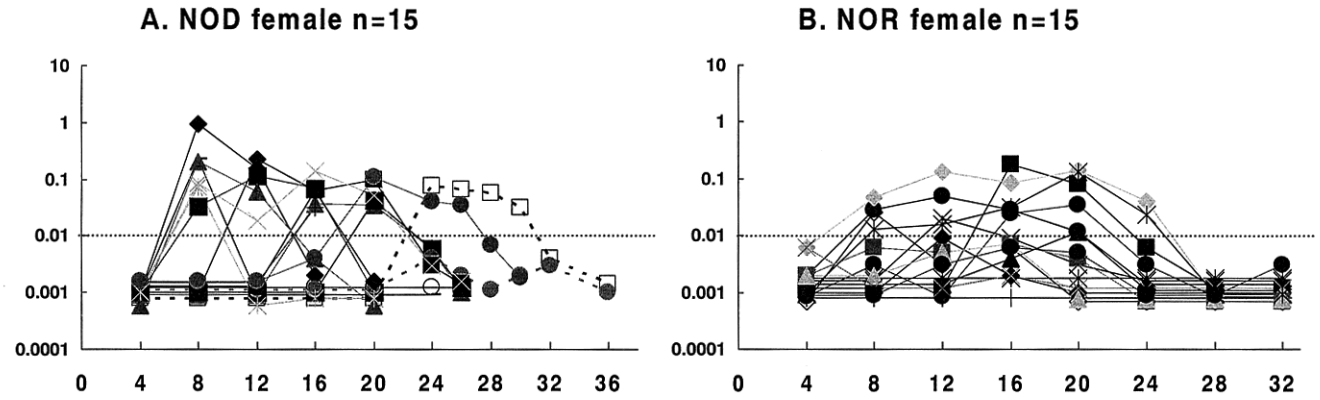

Figure 4. The levels of IAA in individual female NOD mice (A) and female NOR mice (B). IAA levels of diabetic mice were shown as solid lines and of non-diabetes mice as dashed lines. In NOR mice, all were IAA negative at 28 weeks of age or later (without progression to diabetes). Adapted from Abiru N et al. *J Autoimmun* 1: 1-6, 2001.

Cellular response to insulin

The role of T cells in the pathogenesis of type 1 diabetes has been demonstrated by the study of pathogenic T cell clones derived from NOD mice. Disease could be adoptively transferred with these pathogenic clones that are reactive with islet antigens.[24] Wegmann and coworkers have discovered a predominance of insulin reactive T-cell clones isolated from islets of prediabetic NOD mice.[25] It is estimated that approximately 50% of intra-islet T-lymphocytes react with insulin and 97% of the insulin responsive T-lymphocytes react with the B chain peptide of insulin (peptide B:9-23; amino acids B:9 to B:23).[26] In transfer experiments, these B:9-23 reactive clones accelerate disease in young NOD mice and one was able to transfer disease into NOD/scid mice. The B:9-23 peptide of insulin can be administered subcutaneously or intranasally to prevent diabetes.[27] When this peptide is administered to NOD mice or to normal Balb/c mice, IAA are rapidly induced (Figure 5). This anti-insulin autoantibody response is MHC restricted with autoantibodies induced in mice with H-2^d and H-2^{g7} but not H-2^b mice. With congenic strains, the response maps to the MHC.[28]

Despite the induction of insulin autoantibodies, the Balb/c mice immunized with insulin peptide B:9-23 did not develop insulitis or diabetes. Polyinosinic-cytodylic acid (Poly-IC) has been used as a viral RNA mimic to stimulate the innate immune system and in $RT1^U$ rat strains can often induce insulitis and less often diabetes,[29] though in NOD mice Poly-IC prevents diabetes.[30] We found that simultaneous administration of Poly-IC and B:9-23 peptide to Balb/c mice (but with neither alone) induced insulitis (Figure 6) and to transgenic mice expressing the molecule B-7.1 in islets induced

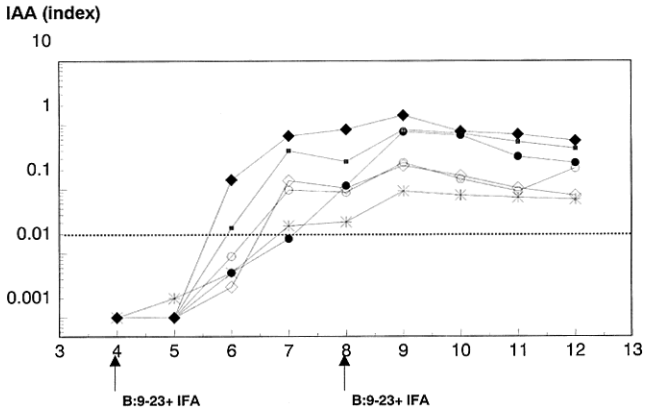

Figure 5. Rapid induction of insulin autoantibodies by insulin B:9-23 peptide immunization in normal Balb/c mice. Adapted from Abiru N et al. *Diabetes* 50: 1274-1281, 2001.

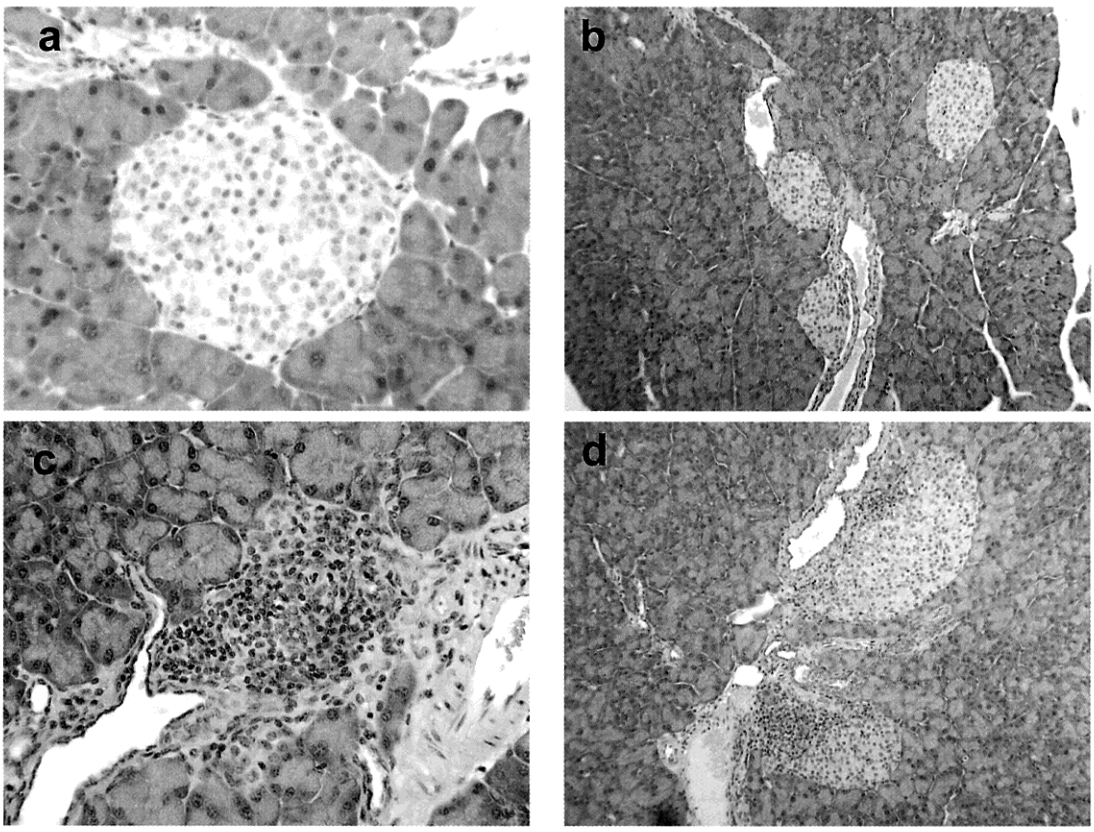

Figure 6. Histopathology of insulitis in Balb/c mice. Hematoxylin-eosin staining of pancreatic islets. (A) Insulin peptide B:9-23 immunization alone (×100): no insulitis. (B) Tetanus toxin immunization with Poly-IC (×40): no insulitis. (C) B:9-23 immunization with Poly-IC (×100): with induced insulitis. (D) Insulin peptide B:9-23 immunization with Poly-IC (×40) with induced insulitis. Adapted from Moriyama H et al. *Proc Natl Acad Sci USA* 99: 5539-5544, 2002.

diabetes readily.[31] These results suggests that even normal mice possess insulin autoreactive T- and B-lymphocytes that can initiate islet pathology.

In human, T cell response to insulin have been reported in type 1 diabetic and pre-diabetic individuals.[32-34] Recent studies by Gottlieb and coworkers indicate that not only NOD mice, but also patients with new onset diabetes and pre-diabetic children respond to the B:9-23 peptide.[35] T cell reactivity to B:9-23 peptide was found more frequently among individuals bearing highest risk HLA type, HLA-DR4/DQ8 and HLA-DR3/DQ2.

Insulin prevention trials

In mice, given the ability to target islets with insulin reactive T cells, and in particular B:9-23 reactive T cells, this molecule provides a target for preventive therapy. Antigen-specific therapies including "immunologic" vaccination are being studied. Oral insulin,[36] and aerosol insulin[37] can all delay or prevent the development of diabetes in NOD mice. A single subcutaneous injection of the insulin B chain, B:9-23 peptide and metabolically inactive insulin analogs also can prevent diabetes in NOD mice.[27,38,39] These results indicate that the metabolic activity of insulin is not essential for prevention. Possible mechanisms for diabetes prevention is now considered by the induction of "regulatory" T cells. Several reports suggest that administration of insulin or GAD is associated with a switch from a Th1 to a Th2 phenotype cytokines including IL-4, IL-10 and TGF-ß).[40-42] Current report suggests that immunization of NOD mice with B:9-23 peptide induces generation of CD4+ CD25+ regulatory T cells that are capable of blocking adoptive transfer of diabetes by diabetogenic T cells.[43] We found that multiple administration of B:9-23 peptide without adjuvant could protect NOD mice from diabetes;[44] however, anaphylaxis can be induced following multiple administration of B:9-23 peptide in NOD mice.[45] Liu, E. et al. currently developed a novel method to prevent peptide-induced anaphylaxis by adding 2 arginine residues (RR) to C-terminus of B:9-23 (thus creating B:9-23RR), which increased the isoelectric point from 5.4 (for native B:9-23) to 7.0 (for B:9-23RR) and decreased peptide solubility at neutral pH (such as the physiologic pH of human tissue, 7.0-7.4).[46]

In human, the large randomized Diabetes Prevention Trial (DPT-1) has been studied to test if insulin could delay or prevent type 1 diabetes in persons at high risk for diabetes. They screened 84,228 first-degree and second-degree relatives of patients with diabetes for islet-cell antibodies and larger group of 300 individuals, who were felt to be at high risk due to the presence of autoantibodies and a low first-phase insulin response, were randomly assigned to undergo either close observation or an intervention with low-dose subcutaneous insulin, administered twice daily for a total dose of 0.25 unit per kilogram of body weight per day, plus annual four-day continuous intravenous infusions of insulin. The study was terminated early when it was found that there was no difference between the treated and untreated groups.[47] It is probably that the necessary immunologic effect was not achieved using this type of immunization scheme or that it may have been initiated too late in the disease process to have the desired effect. A limiting factor to use of insulin as immunogen is its metabolic activity. The advantage of peptide therapy is the lack of metabolic effect. Alternatively, using different routes of administration (oral, nasal), dose schedules, and adjuvant (IFA, cholera toxin) of insulin or its peptides such as B:9-23, may provide a more efficient means of protection of diabetes. Amongst B:9-23 peptide, we have found that two overlapped T cell epitopes including B:9-16 and B:13-23 that respond to B:9-23 reactive T cell clones. The two peptides have in common only four amino acids (B:13-16; EALY) which might contain pathogenic significance for the development of diabetes.[48] By testing a series of B:9-23 peptide analogs with single or double alanine substitutions, Neurocrine Inc. identified a set of altered peptide ligands (APLs) capable of inhibiting B:9-23 induced proliferative responses of NOD pathogenic T-cell clones. Subcutaneous injections of one of these APLs that contained alanine substitutions at residues 16 and 19 (NBI-6024) to NOD mice substantially delayed the onset and reduced the incidence of diabetes.[49] We recently found that nasal administration of the NBI-6024 with native cholera-toxin adjuvant suppresses the development of insulitis and diabetes in NOD mice (M. Kobayashi et al, unpublished data). The NBI-6024 is currently undergoing phase 1/2 studies to determine whether it can reverse type 1 diabetes in new onset individuals.

Conclusion

There are many targets of the natural autoimmunity of type 1 diabetes; however, insulin is the major protein synthesized by islet beta cells, the specific target for type 1 diabetes. We found a dramatic prevention of the development of diabetes in insulin 1 gene knockout NOD mouse and have created a mouse model of experimentally induced diabetes using insulin peptide and Poly-IC. These findings indicate that insulin could play a role as a primary autoantigen that initiates a pathogenesis of type 1 diabetes. NOD mice are often considered a good model for the study of a preventative interventions including insulin prophylaxis therapy.[50] However, at present, we have no examples of using an autoantigen to create a vaccine in man for the prevention of an autoimmune disease. Development of similar therapy such as "insulin peptide vaccination to NOD mice" in man will likely require more basic and clinical studies, with the hope that "efficient" prevention will be developed.

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