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Molecular Targeting of Islet Autoantigens

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Type 1 diabetes of man and animal models results from immune-mediated specific beta cell destruction. Multiple islet antigens are targets of autoimmunity and most of these are not beta cell specific. Immune responses to insulin appear to be essential for the development of diabetes of the NOD mouse. In this review, we will emphasize the unusual manner in which selected autoantigenic peptides (particularly the recently discovered target of BDC2.5 T cells [chromagranin A]) are presented and recognized by autoreactive $CD4^+$ T cell receptors. We hypothesize that "unusual" structural interactions of specific trimolecular complexes (MHC class II, peptide, and T cell receptors) are fundamental to the escape from the thymus of autoreactive T cells able to cause type 1 diabetes.

Introduction

Type 1 diabetes (T1D) is presumed to result from T cell-mediated destruction of the pancreatic islet β cells that secrete insulin. Immune-mediated diabetes (type 1A) is predictable in man and preventable in animal models, but not yet safely prevented in man. Presently prediction of disease relies primarily upon the observation that individuals expressing specific autoantibodies reacting with ≥2 of 4 well-defined autoantigens (insulin, GAD65 [glutamic acid decarboxylase], IA-2 [insulinoma antigen], and ZnT8 [islet zinc transporter]) essentially always progress to diabetes. In young children, diabetes can occur within months of the appearance of autoantibodies, but diabetes in some children will take more than a decade to progress, with evidence of chronic beta cell destruction preceding diabetes over years. The appearance of autoantibodies is acute and during the prediabetic period different autoantibodies rise and fall, but usually more than two are expressed, sometimes for decades. Similar to man, the NOD (nonobese diabetic) mouse model develops insulin autoantibodies, although detection of other autoantibodies has been more difficult when measured with highly specific assays. Alternatively, T cell-mediated autoimmunity to multiple autoantigens is better characterized in the NOD mouse model than in man.

In mouse and humans, the B cell and T cell antigen targets of TID are overlapping, but not identical. For example, in both species insulin has been shown to be both a B cell and T cell target. In man, ZnT8 is a B cell target (Wenzlau et al., 2007), but not yet described as a T cell target, Alternatively, in mice chromogranin A (Chga) has been shown to be a T cell target (Stadinski et al., 2010), but autoantibodies specific for this protein have not been described. Fundamental molecular questions remain as to why certain antigens and certain epitopes of specific antigens are predominantly targeted in man and animal models. Are there primary target epitopes driving the autoimmune process and to what extent is targeting of multiple antigens necessary for disease? Finally, what locks in the destructive autoimmune process that ultimately gives rise to clinical disease? We will emphasize the hypothesis that underlying susceptibility may relate to the manner in which specific peptides are presented to the immune system by polymorphic MHC (major histocompatibility complex) class II molecules and recognized by T cells that escape thymic tolerance. These molecular interactions may control the propensity for β-cell specific destruction, whereas many other immunologic pathways (and genes) may contribute to the heightened risk of multiple diabetes-associated autoimmune disorders seen in man and animal models.

Pathogenesis of Type 1 Diabetes: The Central Role of T Cells

There is considerable data that chronic autoimmunity underlies the progression to diabetes in man and multiple spontaneous animal models. Once autoimmunity appears in man (evidenced by islet autoantibodies and progressive loss of insulin secretion) decades can elapse before the appearance of hyperglycemia. In the NOD mouse, insulitis precedes diabetes usually by months and a subset of mice with insulitis will never progress to overt disease. In the BB rat (BioBreeding) model, there is less variability with a relatively acute progression to hyperglycemia after insulitis.

In all three cases, invasion by T cell lymphocytes and destruction of beta cells among individual islets are asynchronous (Gepts and Lecompte, 1981; Miyazaki et al., 1985) (Hanenberg et al., 1989). Pseudoatrophic islets, lacking all β-cells, but containing all other endocrine cells (e.g., those producing glucagon, somatostatin, and/or pancreatic polypeptide) are considered the end product of islet autoimmunity and no longer have T cell infiltrates. These end product islets exist in the same pancreas with normal islets, as well as islets showing peri- and/or intra-islet T cell invasion. Recent studies of nPOD program pancreas (pancreas from cadaveric donors with long-term Type 1 diabetes) illustrate that the destructive process is often lobular with regions where all islets retain β-cells. Thus, large areas of the pancreas will have pseudoatrophic islets, juxtaposed with areas of β-cell containing islets that appear relatively normal. However, even these areas are not normal with β-cell expression of the molecule survivin (survivin is expressed normally only in fetal β-cells) and hyperexpression of MHC class I molecules (Gianani et al., 2010). It is likely this lobular asynchronous destruction of islet beta cells underlies the chronic natural history of progression to diabetes.

In order to understand the autoimmune process further, numerous experiments have been conducted to dissect which components of the infiltrate are required for the development of disease. By far, T cells have been the one immune cell population that has consistently been shown to be critical for disease pathogenesis. T cells are known to infiltrate the islets in both rodent models of disease (NOD and BB) and constitute a substantial fraction of infiltration observed in humans (Miyazaki et al., 1985; Gepts and Lecompte, 1981). Removal or the complete absence of mature T cells in the two rodent models prevents diabetes (Ogawa et al., 1985; Like et al., 1982; Like et al., 1986; Ikehara et al., 1985; Christianson et al., 1993), and prolonged β-cell function is observed in recent onset patients treated with a CD3 antibody (Herold et al., 2002; Keymeulen et al., 2005). Likewise, adoptive transfer of T cells alone in rodent models can cause the development of disease (Christianson et al., 1993; Whalen et al., 1994). The pathogenic process is largely believed to be dependent upon the presence of autoreactive $CD8^+$ and $CD4^+$ T cell subsets. It is thought that $CD8^+$ T cells may be more important during the early phases of pathogenesis (DiLorenzo et al., 1998; Groen et al., 2003), whereas $CD4^+$ T cells are required throughout. This suggests that there is an evolution in the pathogenic T cell response during the course of disease. Furthermore, individual islet-responsive $CD4^+$ and $CD8^+$ T cell clones have been isolated from diabetic animals that can either directly mediate or greatly accelerate β-cell destruction (Pakala et al., 1997; Haskins and McDuffie, 1990; Daniel et al., 1995; Wong et al., 1996). Collectively, these findings have reinforced the notion that T cells play an integral role in the development of T1D.

Although T cells have been the main focus of the T1D field, they are not the only components of the autoimmune response believed to play a role in disease pathogenesis. NOD mice deficient in B lymphocytes, but displaying normal numbers of T cells, are prevented from developing disease (Serreze et al., 1996). Treatment of both man (Pescovitz et al., 2009) and NOD mice displaying the human CD20 molecule (Hu et al., 2007) with a B cell-specific anti-CD20 antibody alters progression to diabetes, again demonstrating the importance of B cell lymphocytes, but not directly proving a role for pathogenic autoantibodies. The lack of diabetes in infants of mothers expressing anti-islet autoantibodies argues against a direct role for autoantibodies, but there is evidence from the NOD mouse model that maternally transferred autoantibodies are essential (Greeley et al., 2002). In addition, macrophages are among first populations of immune cells to infiltrate the islets and are also believed to contribute to pathogenesis (Hanenberg et al., 1989; Yoon et al., 1998; Jarpe et al., 1990–1991). It is possible that autoreactive B cells play an indirect role in disease progression by serving as antigen-presenting cells for pathogenic T cells. Therefore, while it is unclear which events initiate autoimmunity and the interplay of the entire immune response to the pathogenic process, there has been steady progress in identifying the self-antigen targets of autoantibodies and more recently self-antigen targets of pathogenic T cells.

Self-Antigen Targets in Type 1 Diabetes

The majority of self-antigen targets have been defined through the presence of serum autoantibodies. Others have been identified with β-cell-reactive T cell clones isolated from the pancreas or pancreatic lymph nodes. Specific autoantibodies have been useful in following the progression to T1D in mouse and humans. Work to define the relative contribution of $CD4^+$ and $CD8^+$ T cells that target particular autoantigens has made significant progress recently. A systematic overview of identified self-antigens targets has been the focus of previous reviews (Lieberman and DiLorenzo, 2003) and will not be discussed here in detail. The growing list of target antigens includes insulin, as well as glutamic acid decarboxylase (GAD), insulinoma antigen-2 (IA-2), phogrin (IA-2β), and islet-specific glucose-6-phosphatase catalytic subunit related protein (IGRP). In this section, we will give some background on insulin and two new autoantigens: one recently identified as a prominent target of autoantibodies in T1D patients (Wenzlau et al., 2007) and one as the target of highly pathogenic $CD4^+$ T cells from the NOD mouse (Stadinski et al., 2010). In subsequent sections, we will discuss in some detail the T cells specific for various antigens, their relative importance in T1D pathology, and the nature of the epitopes they recognize. Insulin is a major target of both B cells and T cells in T1D in mice and humans. Approximately one-third of the proteins within islets consist of insulin packaged in a crystalline form and complexed with zinc in secretory granules. Palmer and coworkers first demonstrated the presence of insulin autoantibodies prior to insulin therapy in man. The autoantibodies of man recognize a limited conformational determinant (Figure 1) centered on A13 leucine (Castaño et al., 1993) and requiring both the A and B chain of insulin such that binding of insulin to standard ELISA plates obscures this key epitope of prediabetic insulin autoantibodies (but not insulin antibodies after subcutaneous insulin injections). The autoantibodies of the NOD mouse are different and can react with insulin bound to plates. Insulin autoantibodies are usually, but not always, the first autoantibody to appear in young children developing diabetes. The amounts of insulin autoantibodies inversely correlate with the age at which diabetes develops, and after age 12 only a minority of new-onset diabetics expresses detectable insulin autoantibodies. When first detected they are of high affinity and highly predictive. The NOD mouse characteristically expresses high-affinity insulin autoantibodies between 4 and 8 weeks of age. The antibodies usually disappear about the time of onset of diabetes, but even the NOR mouse (nonobese NOD-related mouse) that has insulitis and rarely diabetes has the same time course of expression of insulin autoantibodies. Thus, the factors responsible the eventual loss of insulin autoantibodies are not simply the loss of islet beta cells. In the NOD mouse, deletion of the *insulin 2* gene (the *insulin* gene expressed in thymus and islets) markedly enhances the serum concentration of insulin autoantibodies, and such a result is consistent with faster progression to diabetes. It is thought (without direct proof) that the loss of thymic insulin increases $CD4^+$ anti-insulin T cells driving the production of autoantibodies by B cell lymphocytes. Insulin is derived from the enzymatic removal of a connecting peptide (C-peptide) from proinsulin within islet secretory granules and C-peptide is secreted along with insulin. In general, autoantibodies react to mature insulin, whereas specific proinsulin autoantibodies have been difficult to demonstrate.

Conceptually similar to the NOD mouse in which manipulating *insulin* genes (*insulin 1* versus *insulin 2*) alter disease, expression

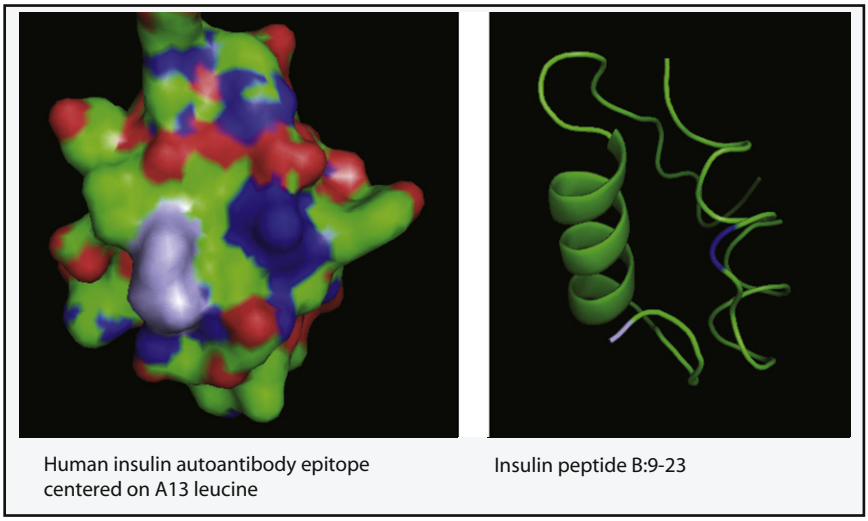

Figure 1. Model of Insulin Structure
On the left, human insulin autoantibody epitope centered on A13 leucine. The gray marks the phenylalanine of the first amino acid of the b-chain of insulin and A13 leucine is shown in dark blue. The light blue highlights basic regions and red acidic regions. Shown on the right is insulin peptide B:9-23 within the a chain helix of the β chain of insulin.

of insulin in the thymus of man correlates with disease risk. In particular, the second-most important locus determining type 1 diabetes is the *insulin* gene (after the dominant effects of the MHC). It is not variation in the *insulin* gene sequence but rather a polymorphism of a variable tandem repeat 5′ of the *insulin* gene that is associated with risk. The long variant of the VNTR is associated with both greater insulin message within the thymus and protection from type 1 diabetes. In addition, ~20% of patients with the rare APS-1 syndrome resulting from a mutation of the autoimmune regulator gene (*AIRE*) develop type 1 diabetes (Perheentupa, 2006; Anderson et al., 2002). Mutations of the *AIRE* gene are associated with loss of expression of insulin message within the thymus (Pugliese et al., 1997; Vafiadis et al., 1997). It is likely both of these genetic determinants are acting by decreasing the small amounts of thymic insulin important for negative selection of insulin autoreactive T cells, and this has been mimicked in the NOD model. Insulin specific $CD4^+$ T cells have been demonstrated in both mouse and human T1D. These will be discussed in more detail below.

The newest major islet autoantigen recognized by autoantibodies of man is ZnT8. The ZnT8 molecule is within the membrane of islet beta cell secretory granules and transports zinc where it forms a complex with insulin. The majority of patients developing type 1 diabetes express ZnT8 autoantibodies, and similar to other autoantibodies, presence of ZnT8 with other single autoantibodies (of GAD, IA-2, or insulin) greatly enhances risk of progression. The ZnT8 molecule is polymorphic with either an arginine or a tryptophan at position 325. This polymorphism is associated with risk of type 2 diabetes but probably does not contribute to risk of type 1 diabetes. It does however alter the epitopes recognized by autoantibodies. Patients homozygous for the Trp variant can produce anti-ZnT8 autoantibodies recognizing only the Trp variant, whereas diabetics homozygous for the arginine variant alternatively can produce those that specifically target the arginine variant. This is consistent with humoral autoimmunity targeting self and not resulting from the targeting of a molecular mimic. NOD mice do not produce anti-ZnT8 autoantibodies and both man and mouse can respond to ZnT8 peptides (H.W. Davidson, personal communication).

Recently, chromogranin A (Chga) was identified as the source of the antigen for the pathogenic BDC 2.5 T cell clone and related series of NOD-derived T cells (Stadinski et al., 2010). The search for this self-antigen has been driven by the highly pathogenic nature of the BDC 2.5 and the fact that the BDC 2.5 T cell receptor (TCR) transgenic mouse has been widely used to dissect the role of T cells in the disease process (Haskins and McDuffie, 1990; Katz et al., 1993). Earlier work had shown that the antigen for these clones was contained within the secretory granules of islet β cells (Bergman and Haskins, 1994; Bergman et al., 2000) and that the clones responded to peptide mimotopes with similar sequences (Judkowski et al., 2001; Yoshida et al., 2002). The final identification of Chga as the antigen came from a combination of approaches (Stadinski et al., 2010). First, mass spectrometric analysis of partially purified antigen suggested Chga as a strong candidate for the source of the antigen. Second, comparison of the sequence of a new strongly stimulatory peptide mimotope to those of previously reported mimotopes also pointed to Chga. Finally, islet cells from mice in which the *Chga* gene had been ablated were shown to lack the antigen for the T cell clones. Originally identified from the chromaffin cells of the adrenal medulla, Chga is found within the secretory vesicles of neurons and endocrine cells (Blaschko et al., 1967). The initial 445aa fully translated product can be differentially processed into at least 11 different biologically active species (Orr et al., 2002), a feature which was important in identifying the naturally processed Chga epitope that stimulates the pathogenic T cells (see below). Thus far, autoantibodies specific for Chga have not been described in either NOD mice or humans.

Which T Cells Are Important in T1D?

Despite the growing list of self-antigen targets, the autoimmune destruction in man and animal models is remarkably specific, targeting insulin producing β-cells within islets, whereas multiple other islet cell types, producing glucagon, somatostatin, pancreatic polypeptide, are spared. A simple hypothesis is that T cell autoimmunity directed toward one or even a handful of β-cell specific antigen(s) accounts for the specific targeting, whereas the loss of tolerance to other antigens may merely be a bystander effect of β-cell destruction and subsequent antigen availability. The question of disease relevancy has been investigated for a number of prominent autoantigens including insulin, GAD, IA-2, phogrin (IA-2β), and IGRP. Several strategies have been used to address this question. For example, for determining

whether the antigen is necessary for development of disease, "recessive" tolerization or genetic antigen ablation strategies can be used. Alternatively, for determining whether the antigen is sufficient for disease development, introduction of antigen-specific T cells into NOD mice devoid of T cells via TCR retrogenic technology can be used. Finally, for determining whether an antigen may contribute to disease without being either necessary or sufficient, the ability of specific T cell clones to accelerate the development of disease in young NOD mice can be assessed. In both the $CD4^+$ and $CD8^+$ T cell compartments, T cells that are capable of homing to the islets and causing β-cell destruction have been isolated. Recent studies suggest that T cell retention in islets is an antigen-specific event (Lennon et al., 2009), yet defining the antigen specificity for all of these islet-homing T cells is far from complete. Introduction of large numbers of autoantigen-specific T cells through adoptive transfer or transgenic and retrogenic methods in NOD mice has begun to reveal which antigen specificities may be important for the pathogenic process.

There is considerable evidence for the importance of insulin in NOD T1D. For the most part, tolerization strategies with insulin leads to the prevention or delay of disease in NOD mice (Zhang et al., 1991; Muir et al., 1995; Coon et al., 1999; Jaeckel et al., 2003) and removal of *insulin* 1, mainly expressed in the islets, blocks disease progression (Moriyama et al., 2003). Conversely, removal of the *insulin 2* gene greatly exacerbates disease, most likely reflecting a loss of central tolerance due to a deficiency in thymic insulin expression (Thébault-Baumont et al., 2003; Moriyama et al., 2003; Martin-Pagola et al., 2009). Finally, retroviral introduction of TCR genes from insulin reactive $CD4^+$ T cells into scid NOD mice is sufficient to lead to insulin autoimmunity in a significant proportion of the mice (Kobayashi et al., 2008).

The results for GAD, however, have been mixed. Induction of recessive tolerance designed to delete pathogenic T cells rather than induce regulatory T cells tolerization or depletion of GAD in mice (e.g., GAD knockout) does not lead to the prevention of disease (Tisch et al., 1993; Tian et al., 1996; Jaeckel et al., 2003; Kash et al., 1999; Yamamoto et al., 2004). In contrast, injections of large amounts of GAD65 intravenously resulted in a striking decrease in the incidence of diabetes of NOD mice (Tisch et al., 1998). In addition, injection of GAD65 in alum decreased loss of C-peptide in man and follow-up trials are underway (Ludvigsson et al., 2008). For the most part, GAD-specific $CD4^+$ T cells do not exhibit diabetogenic potential (Arnold et al., 2004) and can even be protective (Tarbell et al., 2002; Kim et al., 2004). However, one GAD responsive $CD4^+$ T cell generated by peptide vaccination has been shown to be diabetogenic (Zekzer et al., 1998). It is noteworthy that there is very little GAD in mouse islets, whereas GAD is expressed in all islet cells of man and is beta cell specific in the rat. A recent study of T cell receptor retrogenic NOD mice indicates that rather than insulitis, a major subset of anti-GAD T cell receptors drive encephalitis as well as production of GAD65 autoantibodies. Insulitis for these retrogenics only occurs if a transgene drives GAD production in the islet beta cell (Burton et al., 2010). This suggests GAD may be a more relevant self-antigen target in human and rat disease than it appears to be in the NOD mouse. Removal of other autoantigens such as IA-2 (Kubosaki et al., 2004b) and IA-2β (Kubosaki et al., 2004a) has not revealed these antigens as essential targets for the development of disease.

Because of its recent identification, chromagranin A has yet to be evaluated in tolerance or ablation experiments, and therefore it is unknown whether this antigen is required for induction of T1D. Retrogenic introduction of TCR genes from a number of Chga specific $CD4^+$ T cell clones into scid NOD mice leads to very rapid induction of T1D. However, the failure of mice lacking *insulin 1* expression to develop T1D despite the presence of Chga suggests that T cells specific for this antigen may become activated and come into play only later in the disease process. TCR retrogenic experiments have also identified additional $CD4^+$ T cells that are sufficient for induction of T1D in scid NOD mice, most notably the NY4.1 and BDC 6.9 T cell clones (Haskins and McDuffie, 1990; Verdaguer et al., 1997; Schmidt et al., 1997; Burton et al., 2008). The antigenic target of these T cells is not known and hence tolerance or ablation experiments cannot be performed.

On the $CD8^+$ T cell side T cell responses to insulin and dystrophia myotonica kinase (DMK) are highly diabetogenic (Wong et al., 1996; Wong et al., 1999; Graser et al., 2000; Lieberman et al., 2004). Tolerization of NOD mice to IGRP (Krishnamurthy et al., 2008) does not prevent development of disease. It is interesting that although the dominant natural $CD8^+$ T cell response directed toward the IGRP peptide 206-214 is diabetogenic, an exclusive response to this epitope attenuates the development of disease (Verdaguer et al., 1997). Recent studies suggests that the diabetogenic potential of IGRP-specific $CD8^+$ T cells are dependent on the loss of tolerance to insulin (Krishnamurthy et al., 2008), and it is possible that a lack of a response to insulin in the IGRP-specific TCR transgenic T cell-deficient mice causes the altered disease progression.

Currently, these data leave insulin as the only self-antigen that has consistently been shown to be essential for the diabetogenic immune response.

Autoantigen Epitopes for Diabetogenic T Cells

Defining all the antigenic epitopes, i.e., the actual peptide that is bound to MHC, for diabetogenic T cells is central to unraveling the pathogenesis of T1D. Doing so will allow the production MHC tetramers for ex vivo analysis, as well as in vivo imaging reagents, and possibly enable efficient visualization of an ongoing disease process in animal models, as well as humans. The introduction of retrogenic methods to determine T cell pathogenicity has greatly accelerated the rate at which individual T cells can be tested, and therefore this method is likely to become the standard practice for evaluating diabetogenicity. Additionally, defining the molecular targets of disease promoting T cells will help in designing antigen-specific therapies that can modulate the autoimmune response and may eventually prevent the development disease.

Given the initial discovery of insulin autoantibodies, multiple investigators have characterized T cells reacting with insulin, proinsulin, and preproinsulin peptides (Congia et al., 1998). Both $CD8^+$ and $CD4^+$ T cell reactivity is well defined for man and the NOD mouse (Petrich de Marquesini et al., 2008; Wong et al., 2009; Skowera et al., 2008; Pinkse et al., 2006; Alleva et al., 2001; Kent et al., 2005; Hassainya et al., 2005; Durinovic-Belló et al., 2010). For man, studies are limited to evaluation of

circulating lymphocytes and one study of pancreatic lymph nodes (Kent et al., 2005). Reported CD4 T cell epitopes include insulin peptide B:9-23 (Alleva et al., 2001) identical in sequence to the insulin 2 B:9-23 peptide targeted by NOD mouse T lymphocytes, insulin A:1-15 recognized at high concentrations by clones derived from pancreatic lymph nodes (Kent et al., 2005), and a proinsulin DR4 restricted peptide (C19-A3) being evaluated in clinical trials as a subcutaneous injection in patients with new-onset diabetes(Thrower et al., 2009). Responses to a proinsulin (PI) 76-90 (SLQPLALEGSLQKRG) epitope are influenced by a polymorphic repeat sequence 5′ of the *insulin* gene that is associated with diabetes risk (Durinovic-Belló et al., 2010). Prominent $CD8^+$ T cell epitopes include a preproinsulin signal peptide (Skowera et al., 2008) and insulin B10-18, B18-27, and A12-20 (Velthuis et al., 2010; Pinkse et al., 2006). Peakman and coworkers have demonstrated enhanced killing of human islet beta cells cultured in high glucose by $CD8^+$ T cells recognizing the preproinsulin signal peptide (Skowera et al., 2008). There has been one major clinical trial of an insulin peptide (altered peptide ligand of insulin B:9-23 [NBI-6024]) that failed to alter loss of beta cell function in new-onset patients (Walter et al., 2009).

As would be expected the immune response to insulin is better characterized for the NOD mouse model, with studies of T cells isolated directly from islets and the ability to test generation of disease with transfer of clones or creation of T cell receptor transgenic and retrogenic models (Daniel et al., 1995; Jasinski et al., 2006; Burton et al., 2008). Again both $CD4^+$ and $CD8^+$ T cells target insulin peptides. In addition, gamma delta cells in the absence of antigen-presenting cells can target insulin peptide B:9-23 (Zhang et al., 2010), and insulin-induced gamma delta T cells has been shown to prevent diabetes (Hänninen and Harrison, 2000). Perhaps the best-studied epitopes are within the insulin B:9-23 peptide (Figure 1, alpha helix) (Wegmann et al., 1994). Wong and coworkers defined a $CD8^+$ T cell clone (G9C8) that recognizes insulin peptide B:15-23 (Wong et al., 1999). T cells reacting with a tetramer directed at this peptide are prominent in early lesions of NOD mice, but less prominent as insulitis proceeds. A T cell receptor transgenic of the G9C8 clone developed insulitis, whereas diabetes ensued only after immunization and activation of the $CD8^+$ T cells (Wong et al., 2009). Multiple $CD4^+$ T cell clones reacting with insulin B:9-23 peptides have been characterized with several T cell receptor transgenics and retrogenics studied. However different properties of these insulin B:9-23-specific clones have been reported. In this regard, the 2H6 T cell clone both as a clone and as a transgenic recognizes this peptide and prevents type 1 diabetes associated with the production of large amounts of TGF-β (Du et al., 2006; Zekzer et al., 1997), whereas the BDC 12-4.1 clone and T cell receptor transgenic are diabetogenic (Jasinski et al., 2006). It thus appears that pathogenicity reflects specific T cell receptor sequences despite targeting of similar peptides. The targeting of proinsulin (Jaeckel et al., 2004; French et al., 1997) and the native B:9-23 peptide appear to be essential for the development of diabetes in NOD mice (Nakayama et al., 2005). NOD mice lacking endogenous *insulin* genes and only expressing insulin with the B16 tyrosine mutated to alanine fail to develop diabetes and expression of insulin autoantibodies is markedly suppressed. The immune response to insulin is "upstream" of the immune response to IGRP. Whereas class II promoter-driven expression of IGRP fails to influence progression to diabetes, similar promoter-driven proinsulin prevents both the appearance of IGRP autoreactivity as well as progression to diabetes (Krishnamurthy et al., 2006). In addition, even for a mouse bearing transgenic TCRs specific for IGRP, the immune response to proinsulin remains important for pathogenicity (Krishnamurthy et al., 2008).

Surprisingly despite the longstanding knowledge that insulin-reactive diabetogenic T cells recognize the B:9-23 insulin peptide, there has been considerable difficulty in the identification of the relevant epitope(s) for the T cells within this peptide, leading to the idea that this peptide may bind to the MHC class II molecule $I\text{-}A^{g7}$ and be recognized by T cells in more than one way. Unlike MHC I peptide epitopes, peptides bind to MHC II in an extended polyproline-like conformation with the ends of the peptide often extending beyond the ends of the peptide binding groove. Thus, the peptide is free to bind in multiple "frames" or "registers," limited only by the placement of amino acids at four fixed positions within the groove (p1, p4, p6, and p9) that are compatible with corresponding polymorphic amino acid side chain-binding pockets. For conventional peptide, antigens usually the need to match peptide amino acid side chains to the pockets leads to one of the registers being most favored and recognized by the majority of responding T cells. For autoantigens, the situation may not be so straightforward, given that the strongest binding registers may trigger negative selection in the thymus. To examine the potential for insulin B:9-23 peptide to bind MHC II in multiple registers, Levisetti et al. (2007) performed an extensive truncational and mutation analysis of this peptide examining both $I\text{-}A^{g7}$ binding and recognition by a large set of insulin specific T cell clones. They concluded that the peptide bound to $I\text{-}A^{g7}$ in either of two adjacent registers and that T cells recognized one or the other of the two complexes. Of note, a recent manuscript by the same group provides convincing data that the B:9-23 peptides are generated within islets, and a subset of the autoreactive T cells cannot recognize the peptide when insulin is processed by antigen-presenting cells, but require the peptide potentially to be preformed in islet β-cell secretory granules (Mohan et al., 2010). In addition to studies of the binding of insulin peptides to $I\text{-}A^{g7}$, we have sequenced T cell receptors of clones recognizing the B:9-23 peptide (Abiru et al., 2000). The majority of the clones, isolated from islets of NOD mice by Dylan and Wegman, that recognized insulin peptide B:9-23 utilized the genome-encoded T cell receptor TRAV-5D-4*04 Valpha sequence, despite very different CDR3 (complementarity determining region) sequences and no conservation of the beta chain of the T cell receptor (Simone et al., 1997). This has led to the hypothesis that a series of relatively nonstringent T cell receptors with this Valpha element targeting the insulin peptide B:9-23 are central to the pathogenesis of islet autoimmunity of NOD mice (Kobayashi et al., 2008; Nakayama et al., 2007; Lipes et al., 1993). Transgenics and retrogenics with this Valpha element have now been studied (Kobayashi et al., 2008 and M. Nakayama, personal communication) with the observation that this Valpha can entrain in vivo anti-insulin autoimmunity as well as diabetes (Zhang et al., 2009). The T cell repertoire of NOD mice can be markedly perturbed without influencing progression to diabetes including

Peptide source	Sequence									BDC2.5 stimulation
	1	2	3	4	5	6	7	8	9	
Judkowski et al. (2001)	V	R	P	L	W	V	R	M	E	+
Yoshida et al. (2002)	H	R	P	I	W	A	R	M	D	+
Standinski et al. (2010)	R	L	G	L	W	V	R	M	E	+
Chromagranin A	E	D	K	R	W	S	R	M	D	-

Figure 2. Common BDC2.5 Mimotope Motif
The motif (larger letters) containing mimotopes, but not the corresponding Chga peptide stimulates the BDC2.5 T cell clone (Stadinski et al., 2010).

transgenic introduction of irrelevant T cell receptor Vbeta chain (Lipes et al., 1993). This may relate to the dominance of the TRAV-5D-4 element in driving insulin autoimmunity. Although the TRAV-5D-4 element is "sufficient" for NOD islet autoimmunity, it has not yet been tested as to whether it is essential (e.g., targeted gene ablation).

As discussed above, Chga was recently identified as the antigenic source of the highly pathogenic BDC-2.5 clone and other related T cell clones (Stadinski et al., 2010). One of the properties of Chga that help pinpoint it as the antigen source for BDC-2.5 was the presence of a five amino acid motif in the sequence (WXRM[EorD]) that was shared with BDC-2.5-stimulating peptide mimotopes identified in various libraries by three different groups (Judkowski et al., 2001; Yoshida et al., 2002; Stadinski et al., 2010) (Figure 2). In the design of these libraries, this motif would fill positions p5-p9 in the peptide binding groove. However, the corresponding peptide from Chga surprisingly failed to stimulate BDC-2.5 or any of the other T cell clones. However, all of the clones were stimulated by a peptide, WE14, naturally processed from Chga by a furin-like protease, which placed the important motif at its N rather than C terminus (Figure 3).

This result suggested an unusual binding mode for the epitope for these T cells that only partially filled the peptide-binding groove, leaving positions prior to p5 empty and extending far beyond the peptide-biding groove at its C terminus. Furthermore, they suggested that Chga amino acids N-terminal to WE14 were inhibitory for the response. In exploring this idea, various Chga peptides were directly compared to one of the BDC-2.5 mimotopes for T cell stimulation and binding to I-A^{g7} (Stadinski et al., 2010). Some of the data from this study are summarized in Figure 3. The library mimotope that strongly bound to I-A^{g7} stimulated the BDC-2.5 clone. The WE14 peptide also bound to I-A^{g7} and stimulated BDC-2.5, but less strongly. The Chga peptide designed to fill the usual peptide groove neither bound to I-A^{g7} nor stimulated BDC-2.5. Meanwhile, truncation of the portion of WE14 C-terminal to the shared motif produced a peptide inactive in both assays. Finally, adding back four Chga amino acids to the N-terminus of WE-14 had no effect on binding to I-A^{g7}, but eliminated BDC-2.5 recognition. These data would suggest that the Chga-specific T cells can only recognize a peptide that is precisely processed from Chga and presented by I-A^{g7} in a very unusual way. A long C-terminal extension of the peptide from the peptide biding groove is essential for I-A^{g7} binding in some unknown way and the placement of Chga amino acids in the p1-p4 position of the binding groove prevents T cell recognition. This same study showed that the WE-14 peptide is less potent as an antigen for BDC-2.5 than islet antigen extracts, suggesting that a better stimulating epitope is present in the islets, thereby raising the possibility of a natural modification of the WE-14 peptide to increase its affinity for I-A^{g7} or for the TCR. Current studies are already underway to discriminate between these two possibilities and provide an answer that will surely be intriguing.

Is Evading Central Tolerance an Epitope Intrinsic Property?

During T cell development in the thymus, self-reactive $CD4^+$ T cells are eliminated when its TCR strongly engages an MHC II complex containing self-antigen peptide (Anderson et al., 1996). Therefore, central tolerance represents a primary barrier to autoimmunity that disease-promoting self-reactive T cells must overcome prior to carrying out a pathogenic response in the periphery. It has been suggested that disease-promoting MHC II alleles form low-affinity interactions with the peptides they present and that this general deficiency in presentation allows autoreactive T cells to escape central tolerance (Carrasco-Marin et al., 1996). However, peptide-binding studies suggest that this may not be the case, as these disease-promoting MHC II alleles can form stable interactions with presented peptide ligands (Yu et al., 2000). Additionally, efficient deletion of the BDC 2.5 transgenic T cells is observed on the B6.$H2^{g7}$ background with a mimotope peptide that contains a favorable binding register (Judkowski et al., 2001), suggesting that I-A^{g7} is capable of mediating sufficient negative selection (Zucchelli et al., 2005; Judkowski et al., 2001).

The recent findings with the WE-14 epitope and the insulin B:9-23 peptide suggest a different perspective. Although the recognized epitopes of Chga and insulin do bind I-A^{g7} with very low affinity, this is not due to a general defect in peptide binding. Rather, the deficiency in negative selection may be rooted in the unique way in which these peptides interact with the disease-promoting MHC II molecule as well as islet-specific production of relevant peptides. Special peptide processing requirements, failure to fill the peptide-binding groove, competing overlapping registers, or registers that are unfavored by the MHC II polymorphic peptide-binding pockets all can certainly decrease presentation of particular epitopes in the thymus and increase the ability of T cells targeting these epitopes to escape negative selection. Thus, it may be the manner in which epitopes interact with the MHC II molecule that determines whether or not diabetogenic T cells are able to efficiently evade central tolerance and not merely the MHC II molecule itself.

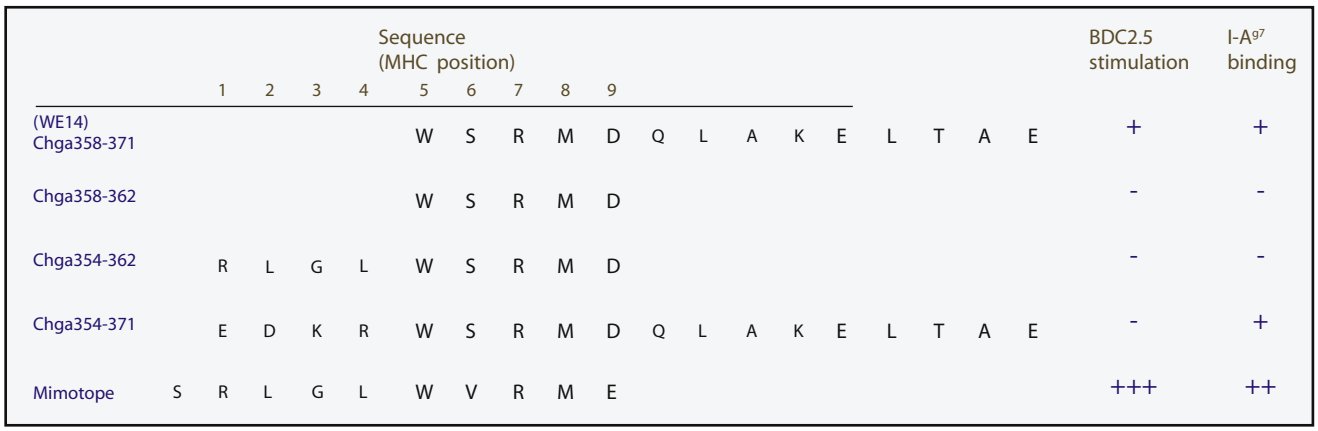

Figure 3. Chromagranin A Peptide Motifs
Relative ability of various motif containing Chga peptides to stimulate the BDC 2.5 T cell clone and bind to I-Ag7, compared to a peptide mimotope selected for strong stimulation of BDC 2.5 (Stadinski et al., 2010).

Rethinking Genetic Susceptibility by MHC II

Genetic susceptibility to T1D in humans, NOD mice, and the BB (biobreeding) rat is largely driven by the MHC II locus (Hattori et al., 1986; Davies et al., 1994; Prochazka et al., 1987; Colle et al., 1981), and there is a distinctive correlation to high-risk and protective MHC II alleles across all three species. Yet the reason that certain MHC II alleles increase disease susceptibility remains unknown. In T1D, the most enticing finding concerning MHC II has been the identification of a non-Asp polymorphism at position 57 of the MHC II β-chain, common to several disease-promoting alleles (Todd et al., 1987; Todd et al., 1988). Structural studies have shown that this mutation causes the loss of salt bridge to Arg76 on MHC II α chain and chemically alters the p9 peptide-binding pocket to include a specificity for acidic residues that is unique to these alleles (Corper et al., 2000; Lee et al., 2001). The most straightforward hypothesis from these studies suggests that disease-promoting MHC II alleles selectively present essential islet antigen epitopes that are not presented by other non-disease-promoting alleles (Suri et al., 2002).

Recognition of insulin by specific T cell receptors (e.g., anti-B:9-23 T cells with TRAV-5D-4 containing alpha chains) in an unfavorable binding register could increase the chances that such insulin-responsive CD4$^+$ T cells would escape central tolerance because of decreased presence of their cognate MHC-peptide complex. Given that a decrease in central tolerance to insulin appears to be essential for the development of T1D in the NOD mouse (Jaeckel et al., 2003; French et al., 1997), then recognition of an unfavorable binding register would help to generate the pool peripheral pathogenic T cells required to cause disease.

Are There Common MHC II Binding "Rules" for Unconventional Autoimmune T Cell Epitopes?

The MHC II is an important component of disease susceptibility, not only in T1D but in many other autoimmune diseases as well (Fernando et al., 2008). It is possible that the unique MHC II binding attributes of the WE-14 and insulin epitopes highlight specific "rules" for epitopes of many pathogenic self-reactive CD4$^+$ T cells. In another animal model of autoimmunity, experimental autoimmune encephalomyelitis (EAE), a pathogenic T cell epitope incorporates strikingly similar strategies when binding the I-A^u MHC II molecule (Gautam et al., 1992). EAE can be induced by the immunization of I-A^u expressing PL/J mice with myelin basic protein (MBP) (Fritz et al., 1983) and T cell clones responsive to an N-terminal fragment of MBP are known to be highly pathogenic (Zamvil et al., 1985). Peptide binding and structural work has revealed that the epitope for these pathogenic T cells is bound to IA^u, leaving the p1–p3 positions of the peptide groove empty, and utilizes an unfavorable anchor residue at p6 (Lee et al., 1998; He et al., 2002). The observation that the WE-14 epitope most likely leaves the I-A^{g7} binding groove empty from p1 to p4 and that the insulin B:12-23 epitope binds in a register utilizing a highly unfavored anchor residue (Stadinski et al., 2010; B.S., unpublished data) suggests that these odd binding strategies may be the rules for how epitopes of pathogenic self-reactive T cells interact with MHC II molecules. In EAE, the "odd" MHC II binding strategy gives the epitope a weak binding affinity for I-A^u and helps the pathogenic T cells evade central tolerance (Seamons et al., 2003). This further suggests that central tolerance is the main barrier to autoimmunity and that critical self-antigen epitopes interact with disease-promoting MHC II molecules in unconventional ways to promote the generation of pathogenic CD4$^+$ T cells.

Implications

The fundamental determinants of autoimmune diabetes at a species level are likely to be genome encoded sequences of the MHC II and TCR complexes essential for presentation and recognition of specific islet antigenic peptides to CD4$^+$ T cell lymphocytes. High probability for loss of tolerance to specific islet peptides appear to depend upon the manner in which specific peptides bind to class II-presenting molecules and are recognized by T cell receptors. Poor peptide binding due to only partially filling the MHC groove or using unfavorable registers probably sets the stage for escape of autoreactive T cells from the thymus. In the periphery, an abundance of particular self-antigens, such as insulin in the islets, or modification of

the antigen, as may be the case for Chga, should allow for sufficient presentation of odd binding epitopes to autoreactive T cells. Additionally, for the insulin B:9-23 peptide of the NOD mouse, a relatively nonstringent family of T cell receptors may be able to react with this dominant epitope by virtue of having the genome encoded TRAV5D-4*04 receptor element, with no apparent conservation of CDR3 or the T cell receptor beta chain.

Although germline-encoded sequences may set the stage for islet autoimmunity, they do not fully determine which individual of a species will develop diabetes. Other genes and environmental factors determining the potential for loss of tolerance given the presence of autoreactive T cell lymphocytes determine individual risk. In man, mutations and polymorphisms controlling expression of minute amounts of insulin within the thymus influence risk, and this is readily modeled in the NOD mouse. Mutations that eliminate regulatory T cell lymphocytes (FOXP3-IPEX syndrome) and T cell receptor signaling (PTPN22) as well as multiple common polymorphisms contribute further.

Concluding Remarks

The NOD mouse has a single class II molecule I-A^{g7}, and although humans developing type 1 diabetes have characteristic HLA genotypes, multiple genotypes are represented among patients, and there is evidence that the percentage of DR3 and DR4 highest-risk heterozygous genotype has decreased as a percentage of diabetics over the past several decades. This suggests that the penetrance of lower-risk genotypes has increased dramatically, as the incidence of type 1 diabetes is increasing. These rapid changes are almost certainly due to unknown changes in the environment. Our knowledge of trimolecular complexes underlying targeting of islet autoantigens in man is woefully inadequate. Recent development of a program (nPOD) to obtain pancreas from cadaveric donors with type 1 diabetes will hopefully allow the characterization of T cells and T cell receptors similar to what is being accomplished for animal models. If specific molecular interactions resulting from genome-encoded sequences of MHC II and TCR underlie susceptibility to autoimmunity, targeting "pathogenic" complexes may provide specific disease prevention. Animal models are now available to directly test this hypothesis with the important caveat that even if a single autoantigen is primary, once epitope spreading occurs, reactivity of T cells targeting multiple determinants may need to be controlled. Direct testing of this and other hypotheses are now possible, and we hope this will help guide the development of safe preventive therapy.

Although it is likely that specific response to insulin is the initial trigger in the NOD mouse, this may not be the case in humans. It is possible that the different antigens can serve as essential initiating epitopes dictated by which combinations of MHC II alleles are present in the individual patient. Understanding how all self-antigens of the diabetogenic immune response are targeted may provide insight to understand the multiple ways in which islet autoimmunity can be triggered and propagated in humans.

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