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Hybrid insulin peptides are neo-epitopes for CD4 T cells in autoimmune diabetes

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Abstract

Purpose of review—The current review covers recent advances in our knowledge of the newest autoantigen neo-epitopes in type 1 diabetes (T1D): hybrid insulin peptides or HIPs. These ligands for autoreactive T cells are formed by peptide fusion, a novel posttranslational modification process that we first reported in 2016.

Recent findings—Two major HIPs in the nonobese diabetic mouse model, ligands for diabetogenic CD4 T-cell clones, have been incorporated into tetramers and used to track HIP-reactive T cells during progression of disease. HIPs have also been used in strategies for induction of antigen-specific tolerance and show promise for delaying or reversing disease in the nonobese diabetic mouse. Importantly, CD4 T cells reactive to various HIPs have been detected in the islets and peripheral blood mononuclear cell of T1D patients and newly developed human T-cell clones are being employed to gather more data on the phenotype and function of HIP-reactive T cells in patients.

Summary—These new hybrid insulin peptide epitopes may provide the basis for establishing autoreactive T cells as biomarkers of disease and as potential tolerogens for treatment of T1D.

Keywords

antigen-specific tolerance induction; autoimmune diabetes; autoreactive T cells; hybrid insulin peptides; neo-epitopes

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease in which T cells play a central role in the destruction of insulin-producing β cells. Because the main role of β cells is to produce insulin, which is present in high abundance in the secretory granules, this protein has been considered the primary disease-initiating autoantigen. It has been shown that native peptides of insulin are antigenic in the nonobese diabetic (NOD) mouse model of autoimmune diabetes and T cells responding to a fragment of the B-chain (insB:9–23) are capable of

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Conflicts of interest

There are no conflicts of interest.

transferring disease [1], highlighting the importance of this particular epitope. In the human setting also, insulin is considered to be one of the major targets of the autoimmune process: T cells reactive to various native fragments of insulin have been found in the peripheral blood of T1D patients [2] and more importantly in the islets of T1D donors [3]. Insulin is also a target of the humoral immune response since autoantibodies against insulin are one of the predictors of diabetes development in at-risk subjects [4]. The intriguing question, however, is how does insulin, an essential hormone that is expressed in the thymus, become an autoantigen? Based on the large number of modified proteins, which are antigens in other autoimmune diseases, an obvious answer could be through posttranslational modification (PTM) of insulin. Protein modification has been widely investigated in the formation of autoantigens in, for example, rheumatoid arthritis, multiple sclerosis, and lupus erythematosus [5], but PTM of proteins considered to be candidate antigens in T1D has been an active area of research only recently [6,7]. In this review, we summarize what is known about the most recent entry to the list of neo-epitopes in autoimmune diabetes: hybrid insulin peptides or HIPs. A novel PTM in which proinsulin is modified through peptide fusion with peptides from other secretory granule proteins leads to the formation of HIPs, providing an explanation of how proinsulin can become a source of antigenic epitopes for autoreactive T cells in diabetes. We hypothesize that T cells with high affinity T cell receptors (TCRs) specific for hybrid peptides may bypass central tolerance mechanisms due to lack of neo-epitope expression in the thymus, allowing them to escape into the periphery where they contribute to a loss of β -cell tolerance (Fig. 1a). In this review, we focus on the potential of T cells specific for HIPs to serve as biomarkers to monitor development of disease and the use of HIPs as therapeutics for the induction of antigen-specific tolerance.

QUEST FOR THE ANTIGENIC LIGAND OF THE DIABETOGENIC T-CELL CLONE BDC-2.5

Interest in the autoantigens that drive the T-cell response in autoimmune diabetes has steadily increased ever since it was established during the 1980s that both CD4 and CD8 T cells are major contributors to pathogenesis. The first T-cell clones capable of inducing disease in NOD mice were those comprising the Barbara Davis Center (BDC) panel of CD4 T-cell clones, all isolated from the spleen and lymph nodes of diabetic NOD mice, and including the prototypical diabetogenic clone BDC-2.5 [8]. The BDC-2.5 clone was used to generate the 2.5 TCR-Tg NOD mouse [9], which has been widely used to investigate mechanisms of CD4 T-cell-mediated destruction of β cells. At the time the BDC T-cell clones were derived, candidate autoantigens for T cells in autoimmune diabetes had not yet been identified and all of these clones were selected and maintained on the basis of their reactivity with whole cell suspensions made from NOD islets. Since the isolation of BDC-2.5, the quest to discover the endogenous antigen recognized by this T cell clone has spanned 3 decades.

Initial efforts to identify a ligand for the BDC-2.5 clone were focused on sequences generated by combinatorial peptide libraries and led to the identification of the amino acid motif, WCRM, critical for antigen recognition by the BDC-2.5 TCR [10,11]. This motif was an important clue that led to the identification of the WE14 peptide ligand from

chromogranin A (ChgA), the β -cell secretory granule protein first identified as the BDC-2.5 antigen by mass spectrometry [12]. Confirmation that ChgA contained the peptide ligand for BDC-2.5 was provided by the observation that islets isolated from ChgA-deficient mice were unable to stimulate the BDC-2.5 T-cell clone [12] and that BDC-2.5 could not transfer disease into ChgA-deficient mice [13]. However, the fact that the WE14 peptide could activate this T cell only at very high concentrations (100 μ mol/l) suggested that a modification to the peptide might be involved in generation of the endogenous ligand.

Subsequent studies, using a proteomic approach and the BDC-2.5 clone to detect presence of antigen, led to the discovery that the actual ligand for BDC-2.5 involved a novel PTM consisting of a HIP neopeptide formed through covalent linkage of a fragment of insulin C-peptide bound to the N-terminal sequence of WE14 [14]. An interesting account of how it was deduced that fusion of peptide sequences from different proteins could explain the formation of the HIP neo-epitopes is provided in a new review from Wiles and Delong [15]. The 2.5HIP was shown to be a potent agonist for BDC-2.5, capable of stimulating the T-cell clone at nanomolar concentrations, and is also the ligand for three other pathogenic CD4 T-cell clones in our panel, BDC-9.46, BDC-10.1, and BDC-11.1 [14,18]. Identification of another HIP ligand, consisting of the same proinsulin sequence fused to a peptide from islet amyloid polypeptide and recognized by the diabetogenic T-cell clone BDC-6.9 (6.9HIP), soon followed [14,16]. Recent mass spectrometric analyses from the Delong group have identified in both murine and human islets these HIPs and others that could be targeted by autoreactive T cells [17].

T CELLS REACTIVE TO HYBRID INSULIN PEPTIDES: PHENOTYPE AND FUNCTION

The immediate challenge arising from the discovery of novel epitopes is to determine whether T cells specific for them contribute to disease and how they do so. Disease relevance of the BDC T cell clones is demonstrated by their ability to readily transfer disease in NOD.*scid* or very young NOD mice, and identification of their peptide ligands made it possible to design tetramer reagents for investigating the repertoire of endogenous HIP-reactive T cells. Major Histocompatibility Complex (MHC) class II (I-A^{g7}) tetramers, loaded with the 2.5HIP or the 6.9HIP peptides, were developed [14,16] and used to investigate the frequency, phenotype, and distribution of HIP-reactive T cells during disease progression in the NOD mouse [18]. We examined HIP tetramer-positive (tet⁺) cells in the pancreas, the draining pancreatic lymph nodes (pLN) and the peripheral blood of NOD mice. Compared with insB:9–23-specific CD4 T cells, HIP tet⁺ cells can be found at a significantly higher percentage in the pancreas of NOD mice, an observation that was already apparent in 10-week-old NOD mice, well before the onset of diabetes. We also found that 2.5HIP tet⁺ cells are among the first CD4 T cells to encounter antigen and become activated in the pLN. This was observed in NOD mice as early as 3 weeks of age, at which point very few islet-infiltrating CD4 T cells are present. Using enzyme-linked immunospot (ELISpot) analysis to interrogate T-cell phenotype in the spleen, we have found that about 50% of 2.5HIP-reactive T cells secrete IFN- γ in response to stimulation, whereas a much smaller percentage of insB:9–23-reactive T cells secreted this proinflammatory

cytokine. This finding was confirmed by Ito *et al.* [19[■]] using intracellular cytokine staining of islet-infiltrating T cells and tetramers. In the latter study, it was also observed that 6.9HIP tet+ cells were inflammatory in phenotype, secreting both TNF- α and IFN- γ . Thus, it appears that upon encounter of antigen in the pLN or islets, endogenous HIP-reactive T cells are skewed towards a Th1 phenotype (Fig. 1b). Through single cell RNA sequencing, Ito *et al.* [19[■]] determined that islet-infiltrating HIP-reactive T cells also express markers of T follicular helper cells, implying they might have a role in shaping the B-cell response. We followed the frequency and phenotype of HIP-reactive T cells in the peripheral blood of NOD mice over a 20-week time period, and found the percentage of total 2.5HIP-reactive and 6.9HIP-reactive T cells, and the antigen-experienced CD44^{hi} CD62L^{lo} subset of these cells, progressively increased as disease developed, whereas there were only minimal changes in insB:9–23-specific T cells or the polyclonal CD4 T-cell population [18[■]]. These results suggested that the changes in HIP-reactive T cells in the blood could be an indicator of ongoing autoimmunity in the NOD mouse, reflecting disease in the pancreas (Fig. 1c). Overall, these studies point to an active role of HIP-reactive T cells in the pathophysiology of autoimmune diabetes.

The relevance of studies in the NOD mouse model to the human disease has long been debated and translating findings from the mouse to the human setting can be challenging. HIP-reactive T cells, however, have been found in the islets of both NOD mice [18[■]] and deceased T1D organ donors [14,20], which underscores the usefulness of the NOD mouse model in antigen discovery. To determine whether proinflammatory HIP-reactive T cells are present in the peripheral blood of T1D patients, we performed ELISpot assays using peripheral blood mononuclear cells (PBMCs) from new onset T1D patients or controls to test for responses to a panel of 16 HIPs, sequences which were based on mouse HIPs and/or predicted binding affinity to the human leukocyte antigen (HLA) Class II molecule HLA-DQ8. We found that T cells reactive to HIPs can be found in the peripheral blood of nearly 40% of the new onset T1D patients and a notable characteristic of the T1D cohort was reactivity to multiple HIPs in some patients [21[■]]. Although IFN- γ was the main parameter measured in T-cell responses to HIPs, we have also observed through multiplex cytokine analysis that IL-6, and in some cases IL-17, are secreted (Baker, RL *et al.*, unpublished). In longitudinal samples collected from one new onset T1D patient, we found that HIP-reactive T cells can persist over time, up to a year after diagnosis. We have subsequently cloned CD4 T cells from PBMC of T1D patients that respond to the human equivalent of either the 2.5HIP or 6.9HIP, indicating that these HIP ligands discovered in NOD mice can also trigger T-cell responses in humans. Because our study was designed to include only high-risk HLA individuals (DQ2 and/or DQ8), some of the nondiabetic controls (all of which were first degree relatives and therefore potentially at risk for disease) also showed some responses to HIPs, but T cell responses from controls were less frequent, lower in magnitude, and rarely to more than one HIP [21[■]]. Ongoing longitudinal studies following HIP reactivity in autoantibody-positive individuals who are at high risk of developing T1D, are being conducted to determine the timing and dynamics of the immune response to these neo-epitopes.

ANTIGEN-SPECIFIC TOLERANCE INDUCTION WITH HYBRID INSULIN PEPTIDES

It has recently been reported that activated islet-infiltrating Foxp3⁺ regulatory T cells (Tregs) express TCRs specific for insB:9–23, but not HIPs [22], suggesting there are differences in the regulation of CD4 T-cell responses to conventional autoantigens and neo-epitopes. This is supported by data from our lab and others in which MHC Class II tetramers were used to demonstrate that most 2.5HIP-specific and 6.9HIP-specific T cells in the islets of prediabetic NOD mice do not express the Treg transcription factor Foxp3 [18[■],19[■]]. In contrast, a large frequency (>20%) of islet-infiltrating insB:9–23-specific T cells are Foxp3⁺ T cells that also express canonical Treg markers such as glucocorticoid-induced tumour-necrosis-factor-receptor-related protein (GITR), Helios, and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), as well as the proliferation marker Ki-67, indicative of antigen recognition by insulin-reactive Tregs in the islets [18[■],19[■]]. Thus, it appears that while endogenous T cells reactive to HIPs are primarily pathogenic effector T cells, T cells specific for insB:9–23 are enriched for Tregs, which may be due to differences in the thymic education of these subsets (Fig. 1a).

Induction of antigen-specific tolerance could be the ideal therapy for T1D because autoreactive T cells would be specifically targeted without compromising the rest of the immune system. There has been some success in both the prevention and reversal of diabetes in NOD mice with various autoantigens, including glutamic acid decarboxylase, insulinoma-associated protein 2, proinsulin, insulin, and insB:9–23 [23]. Although preclinical studies in mice showed some promise, human clinical trials with conventional autoantigens such as insulin have been unsuccessful in the prevention or delay of T1D [24]. Important information gained from the human trials was that treatment of T1D patients and at-risk subjects with islet autoantigens is well tolerated and does not accelerate disease; further, antigen treatment may lead to some degree of immune regulation such as induction of Foxp3⁺ and Tr1 Tregs [25]. This raises the key question of whether using neo-epitopes in antigen-specific tolerance induction strategies, either alone or in combination with conventional autoantigens, may have therapeutic benefits. For example, since the majority of HIP-reactive T cells are proinflammatory in NOD mice, silencing of these cells, or their conversion to a Treg phenotype could restore β -cell tolerance. To investigate the use of HIPs in antigen-specific therapies we developed a strategy to deliver them in tolerogenic manner using poly(lactide-*co*-glycolide) (PLG) nanoparticles, based on a substantial body of work from the Miller group [26]. Antigen-loaded PLG nanoparticles have been found to target macrophages in the spleen and liver that express the scavenger receptor macrophage receptor with collagenous structure, leading to antigen presentation of the delivered peptide to T cells in the absence of costimulation (Fig. 1d). We have recently found that infusion of PLG nanoparticles surface-coupled with 2.5HIP (2.5HIP-PLG) can induce tolerance in diabetogenic BDC-2.5 T cells and prevent adoptive transfer of disease [27[■]]. Treatment with 2.5HIP-PLG nanoparticles impairs proinflammatory cytokine production by BDC-2.5 effector T cells and promotes the development of CD44^{hi} Foxp3⁺ CD73^{hi} FR4^{hi} anergic cells, as well as the induction of an anergic transcriptional program (Fig. 1d). The reduction in the effector T-cell pool results in an increase in the ratio of Tregs to effector T cells

leading to protection from diabetes. Although there have been other reports of suppressing BDC-2.5 effector T cells using peptide mimotopes [28], this study was the first report that a neo-epitope, actually present in NOD islets *in vivo* [17[■]], could be successfully used to induce tolerance. Because a variety of autoantigens (both conventional and unconventional) are likely to be involved in the disease process, the critical question that remains is whether tolerogenic delivery of HIPs can prevent or reverse spontaneous diabetes in NOD mice, a scenario that is more reflective of human T1D. Our preliminary results indicate that 2.5HIP-PLG nanoparticles have some efficacy in both spontaneous disease prevention and reversal models; for example, PLG nanoparticles loaded with the 2.5HIP can reverse autoimmunity and significantly delay syngeneic graft destruction after islet transplantation in diabetic NOD mice (Jamison, BL *et al.*, unpublished). Furthermore, protection may be improved by combining 2.5HIP-PLG nanoparticles with strategies to enhance expansion of 2.5HIP-specific Tregs. For instance, one such strategy could be to treat with low-dose recombinant IL-2, although it is possible that novel engineered high-affinity IL-2R α agonists that persistently stimulate Tregs without effects on other cell types [29] would have the greatest efficacy. There are still hurdles that must be overcome to translate antigen-specific therapies to the clinic for T1D, such as determining the appropriate dosing and method of antigen administration for optimal tolerance induction, something that has not been carefully studied. Of note, our preclinical studies have shown that delivering 2.5HIP on a tolerogenic nanoparticle carrier is much more effective for induction of long-lasting tolerance than delivering soluble peptide alone [27[■]]. An obvious next step would be to perform studies in recently described humanized HLA-transgenic autoimmune diabetes mouse models [30] to gain further insight into mechanisms of antigen-specific tolerance induction and determine which strategies are most promising. In any case, the results in NOD mice suggest there may be potential for the use of neo-epitopes such as hybrid insulin peptides in therapeutic strategies for humans.

CONCLUSION AND FUTURE DIRECTIONS

A potential challenge with HIP-specific T cells, as biomarkers in T1D or targets in antigen-specific therapies, could arise if there is a wide variety of peptide fusion events due to differences in β -cell proteomes, stage of disease, and other factors. It will therefore be of great importance to determine whether certain HIPs are dominant in humans, similar to the 2.5HIP and 6.9HIP in NOD mice. Our preliminary data indicate that T cells from some new-onset T1D patients respond to some of the same HIPs that are antigens in mice, suggesting there could be shared neo-epitopes between species. We have observed that HIP-reactive T cells can be present before disease onset (Baker, RL *et al.* unpublished), and therefore, taking into account HIP reactivities (and the presence of T cells reactive to other neo-epitopes) might improve staging of the disease and have implications for clinical intervention strategies. The development of technologies for comprehensive interrogation of antigen-specific T cell responses, such as high-parameter flow cytometry and mass cytometry with peptide-MHC multimers, in addition to simultaneous single-cell TCR and RNA-sequencing is rapidly advancing. It will be important to employ these methodologies, for identifying disease-relevant neo-epitopes and for monitoring effects of therapies on T cells involved both in pathogenesis and regulation of T1D.

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KEY POINTS

- HIPs are a new class of posttranslationally modified epitopes in T1D.
- HIPs are present in the islets of mice and humans and are targeted by diabetogenic T cells.
- CD4 T cells reactive to HIPs offer new avenues for biomarker discovery in T1D.
- HIPs may provide new therapeutic peptides for induction of antigen-specific tolerance.

FIGURE 1.

CD4 T cells reactive to hybrid insulin peptides (HIPs): novel biomarkers and targets for tolerance induction. (a) Medullary thymic epithelial cells present conventional autoantigens (e.g., insulin B:9–23) to CD4 T cells, leading to central tolerance processes such as clonal deletion and induction of thymic-derived regulatory T cells. Thus both insulin-specific conventional T cells and regulatory T cells exit the thymus. CD4 T cells specific for hybrid peptides do not undergo central tolerance due to low or absent expression of hybrid insulin peptides (HIPs) by medullary thymic epithelial cells, allowing for entry of conventional T cells, but not thymic-derived regulatory T cells, into the periphery. (b) HIPs form exclusively in β cells after fusion of a proinsulin peptide to a sequence from another secretory granule protein such as chromogranin A or islet amyloid polypeptide. Autoreactive T cells encounter HIPs in the pancreatic islets and/or lymph nodes after transfer of HIPs from β cells to antigen-presenting cells (APCs). Activated APCs that express the costimulatory molecules CD80/CD86 present HIPs to CD28-expressing T cells promoting differentiation to a Th1 phenotype and subsequent secretion of the proinflammatory cytokine IFN- γ . (c) HIP-specific effector T cells can be detected in the peripheral blood of diabetic mice and humans.

Monitoring changes in the frequency and/or phenotype of HIP-specific T cells using cytokine assays (enzyme-linked immunospot), peptide-Major Histocompatibility Complex (MHC) multimers followed by flow/mass cytometry, and single-cell RNA-seq may have biomarker value. (d) Antigen-specific tolerance can be induced in HIP-specific T cells using poly(lactide-coglycolide) nanoparticles (PLG-NPs) loaded with HIPs [HIP-NPs]. Uptake of PLG-NPs by APCs, through macrophage receptor with collagenous structure (MARCO), leads to tolerogenic reprogramming. Upon antigen presentation of HIPs, in the absence of CD28 costimulation, T cells become anergic or are converted to peripherally derived regulatory T cells that can suppress both effector T cells and the activation of antigen-presenting cells.

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