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Antigen processing patterns determine GAD65-specific regulation vs. pathogenesis

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1. ABSTRACT

Alterations in presenting self determinants to T cells may depend upon the availability of sites on the molecule adjacent to known determinants to different processing enzymes, or, at the level of amino acid sequence or conformation of a single determinant. We have studied three possible ways that could modulate the processing and presentation of T cell determinants of a diabetes autoantigen, glutamic acid decarboxylase (GAD) 65, which could contribute to induction of GAD65-specific regulatory versus pathogenic T cells in type 1 diabetes (T1D): 1) enhanced presentation of subdominant/cryptic determinants to T cells that have not been well-tolerized, which may activate T cells of high affinity and high aggressiveness; 2) trimming or truncating flanking residues which may otherwise provide needed binding energy to determinants that activate regulatory cells, thus releasing autoaggressive T cells from suppression; 3) biochemical or chemical modifications of self antigens in an inflammatory environment or within activated antigen presenting cells (APC) which may convert a previously regulatory antigen or determinant into a disease-causing one that activates autoreactive T cells at a higher affinity.

2. INTRODUCTION

There are at least two hard facts that maintain GAD65-specific T cells on the list of pathogenic, islet destructive members during type 1 autoimmune diabetes (T1D): 1) Autoreactive T cell and antibody responses to GAD65 arise very early and correlate with the development of T1D; 2) Early, tolerogenic vaccinations using full length GAD65 protein or its fragments can prevent its pathogenicity. Spontaneous arisal of the GAD65-specific response (endogenous priming) in prediabetic individuals eventuates in pathogenicity. However, most recent studies have mainly focused on the regulatory T cell repertoire induced upon GAD65 or peptide immunization (exogenous priming), which have obviously under-evaluated the disease-causative role of GAD65. In this essay, we suggest one possible mechanism that may assist in transforming GAD's natural regulatory status into a disease-prone profile induced by GAD65-reactive T cells. We would like to argue that it is the detailed pathway (s) of processing and presentation of GAD65 T cell determinants that directs this transformation.

3. REGULATION DOMINATES IMMUNE RESPONSE TO GAD65 AS LEARNED FROM GAD65 KNOCKOUT (KO) AND TRANSGENIC STUDIES

GAD65 is found highly expressed in the brain and is also expressed in human pancreatic islet cells, both in the cytosol and in the membrane fraction (1). As in NOD mice, GAD65 is found in the islets as well as in some mouse cell lines (our unpublished data and personal communication with Dr. Damien Bresson). However, islet expression of GAD65 is not an absolute requirement for the development of insulitis and diabetes in NOD mice. Indeed GAD65-deficient mice which had been backcrossed 4 generations onto a NOD background, presented similar kinetics and level of lymphocytic infiltration into the pancreatic islets compared to their GAD65 wild type littermates (2). Therefore, it was concluded that GAD65 was not the primary target, if there is one, in the islet autoimmune response. A similar situation was found with a second major islet antigen, protein tyrosine phosphatase IA-2 (also known as ICA512), where development of insulitis and diabetes in NOD mice was not obviously affected by knocking out both IA-2 and IA-2 (known as phogrin), despite a slight decrease of diabetes incidence in the knockout mice (3, 4). Interestingly, from our observations, after 5 more generations of backcrossing to the NOD strain, GAD65.KO mice exhibited a slightly higher incidence of diabetes than wild type littermates (unpublished data); however, similar to the study with IA-2.KO mice, the difference failed to reach statistical significance.

One could argue that the relatively insignificant role of GAD65 in the pathogenesis of T1D can be attributed to the low expression level of this protein in the pancreatic beta-cells. Leiter and colleagues generated transgenic mice over-expressing GAD65 under the rat insulin promote (RIP), allowing for enhanced expression of GAD65 in the pancreatic beta-cells. The transgenic "A-line", with at least two-fold higher islet GAD65 expression, was clearly resistant to diabetes, whereas the transgenic "Y-line" expressing a lower amount of GAD65 in the islets was still susceptible and produced more GAD65-specific IgG2b than the A-line (5). Therefore, it remains a possibility that enhanced GAD65 expression protects the islets from autoimmune attack, through either induction of clonal deletion, or expansion of regulatory T cells; however, it still needs to be clarified whether in these two lines, thymic or peripheral GAD65 expression is comparable, since the RIP could be leaky in the thymus. Alternatively, because (i) the GAD65 transgene was integrated on different chromosomes between the the two lines, (chromosome 15 for the A-line and Y-chromosome for the Y-line), and (ii) notably, homozygote A-line mice were developmentally lethal, transgenic insertion-induced mutagenesis may participate in the differences observed between both lines; however, so far, there is no insulin-dependent diabetes locus (*Idd*) identified in both chromosomes, 15 and Y.

An additional study was done by von Boehmer's group, in which GAD65 was over-expressed under control of the invariant chain promoter, for the purpose of enhancing processing and presentation of GAD65 T cell determinants on APC so as to achieve better tolerance (6). The immune response to exogenous GAD65 antigen was severely suppressed in the transgenic mice, but insulitis and diabetes were comparable in transgenic and wild type NOD mice. The authors concluded that GAD65 is not essential for the development of T1D in NOD mice. However, it is striking to see that despite the fact that GAD65 is expressed in multiple organs (thymus, brain and maybe spleen), tolerance to this molecule inevitably fails to be maintained in the NOD strain; whether over-expression of GAD65 in APC will simultaneously promote GAD65-specific regulation as well as pathogenesis remains to be clarified. The endogenous, spontaneous autoimmune T and B cell responses to GAD65 may have given information additional to that garnered in this study of the response to exogenous GAD65 and should be compared between transgenic and non-transgenic littermates.

In contrast to the above results supporting a non-essential role of GAD65 in T1D in NOD mice, Yoon and his colleagues demonstrated, using the antisense DNA technique, that complete suppression of GAD expression, both GAD65 and GAD67, in pancreatic beta cells prevents insulitis as well as diabetes development (7), suggesting GAD is required early in disease development. This apparent contradiction is not due to non-specific effects of the antisense approach since mouse lines with partial suppression of GAD were not resistant to diabetes. However, it was surprising that these partially GAD-suppressed lines developed insulitis and diabetes at an almost identical incidence and severity as the non-suppressed littermates, which leads us to wonder whether GAD plays distinct role (s) in the beta-cells affecting early autoimmune event (s) rather than recruiting autoreactive T cells. One noteworthy difference in this study (7) was that both GAD65 and GAD67 isoforms were removed from the beta cells, whereas, the studies mentioned earlier were focused on GAD65 only. It is generally agreed that both GAD65 and GAD67 function similarly in producing GABA (γ amino butyric acid), but only GAD65 is targeted by the immune system in T1D (8). The comparative studies on biological and biochemical features as well as functions of GAD65 and GAD67 in beta cells remain to be addressed. One hint has emerged from recent structural comparisons between GAD65 and GAD67 (9). Only the former molecule has a very flexible and inherently mobile sequence in the C-terminal region, which may render this region highly available to endopeptidases providing an initial favored site of enzymatic target in the whole molecule. Residues near this target may have primary access to MHC II grooves. This fits with the data that p524-543 is a very early response target in GAD65 (10). Importantly, this C-terminal region is also targeted by autoantibodies (11) as well as by CD8 T cells (12) (Figure 1), and tolerance induction using DNA coding for the C-terminal region, $GAD_{500-585}$, was effective to protect NOD mice from diabetes (13).

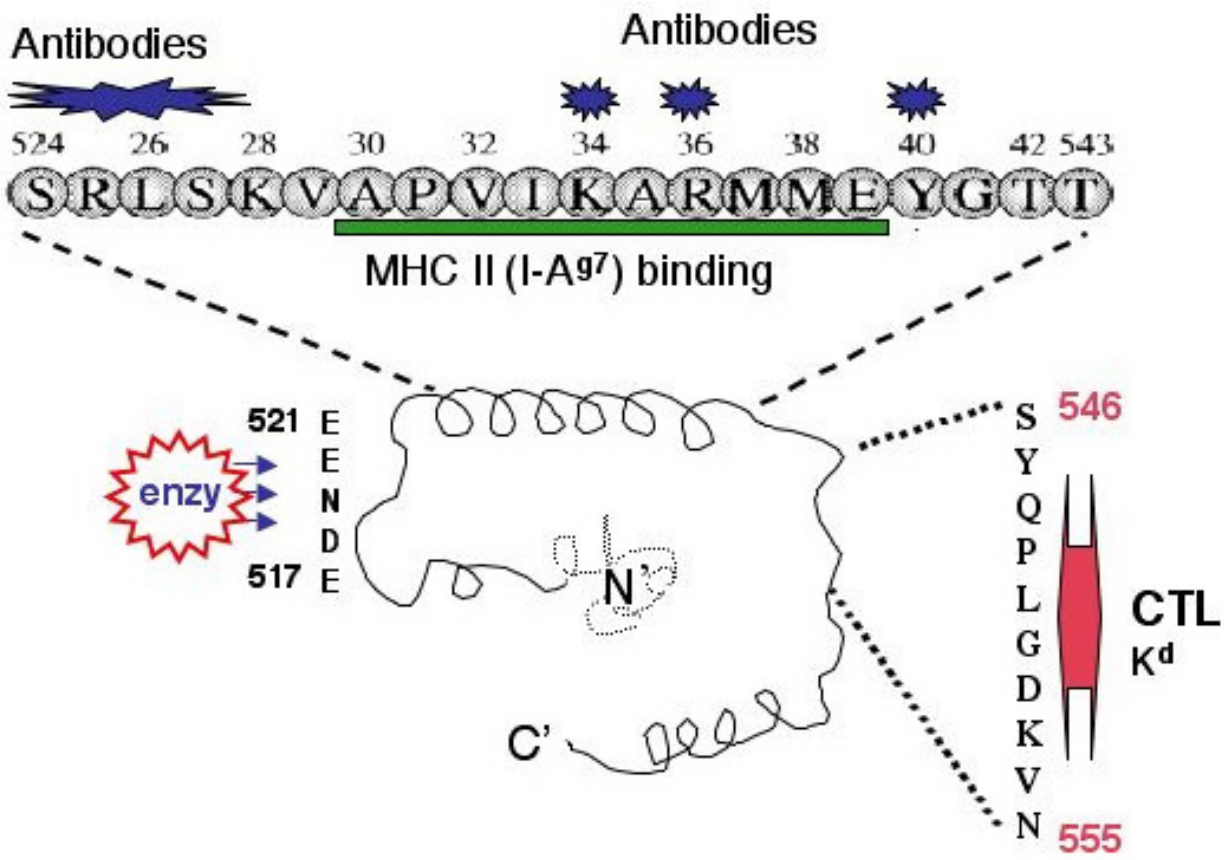

Figure 1. An immunogenic "hotspot" near the C-terminus of GAD65. The C-terminus of GAD65 but not GAD67 is inherently mobile and flexible, which is likely caused by the acidic loop formed by residues between E517 and E521. This projected, floating loop may be a favored target of endopeptidases, and thus release the C-terminus from the large GAD structure and allow it to gain accessibility to MHC II binding (524-543) or to proteasomal degradation and MHC I presentation (546-555). Furthermore, the mobile feature helps to expose this region to antibody recognition.

4. EXOGENOUS IMMUNIZATION INDUCES REGULATORY T CELLS, BUT ENDOGENOUS PRIMING GENERATES PATHOGENIC EFFECTORS

Due to effective tolerance both in the thymus and in the periphery, the GAD65-reactive repertoire in the periphery is dominated by a regulatory T cell population in non-diabetic individuals, whereas in patients, this repertoire can be skewed toward pathogenic effectors. This skewing of the repertoire could be demonstrated by comparing the immune response after exogenous immunization versus endogenous priming in susceptible animals. Accordingly, the spontaneous/endogenous responses arising in NOD mice are likely to be associated with pathogenesis, whereas due to the effect of adjuvant as well as increased antigen dose, exogenous immunization with GAD65 frequently leads to an expansion of the regulatory T cell repertoire.

CD8 T cells which are cytotoxic and/or regulatory may also play a role. Human GAD-specific CD4 T cells can be suppressed by an autologous CD8+CD45RA+CD27- T cell population, although their specificity for antigen (s) is unknown. These CD8 T cells presumably keep potential autoreactive CD4 T cells under control (14). While their relationship to CD8 cytotoxic lymphocytes (CTL) is unknown, such CTL often require CD4 T helper cells for their induction. Thus, Busick et al (15) showed that the only spontaneously arising GAD-specific CTL were directed at determinants adjoining or overlapping with spontaneously arising CD4 T cells. Of great interest are the results of Weaver et al (16) that upon DNA vaccination with insulin vs. GAD65, the progression of T1D is quite different: whereas insulin either exacerbated disease or had no consequence, provision of a GAD65 DNA vaccine prevented T1D. It was reported in this study that both insulin and GAD vaccinations induced Th1 CD4 cells, but only insulin induced IFNγ-secreting CD8 T cells.

NOD mice exhibit a spontaneous proliferative response to GAD65 determinants that arises concomitantly with the onset of insulitis (between 4-8 weeks) (10, 17). The autoaggressive responses to □eta-cell antigens were initially directed toward a few C-terminal determinants within GAD65 and later spread intramolecularly, as well

intermolecularly to other candidate diabetogenic autoantigens. The greatest proliferative response is initially directed against determinants p524-543 and p509-528, followed by spreading to other regions of the GAD65 molecule, including p78-97, p246-266, p340-356, p479-493, p540-556 and p570-585 (10). These determinants have been termed the "spontaneous" or "endogenous" determinants of GAD65. It should be noted that T1D can be adoptively transferred with GAD-induced T cell lines generated from diabetic NOD mice by stimulating with native GAD protein (18); interestingly, such lines were directed against one of the major "spontaneous" determinants, p530-543.

A second set of GAD65 CD4 determinants from NOD mice (including p206-220, p221-235, p286-300 and p400-410) arise following immunization with "exogenous" whole GAD65 protein (19, 20). CD4+ T cells expanded by this set of determinants appear to possess regulatory properties and can protect NOD mice from diabetes following adoptive transfers (21, 22). It is also notable that these CD4+ T cells, by themselves, apparently lack islet-infiltrating capacity since two different TcR transgenic lines specific for p206-220 and p286-300, were almost totally free from insulitis as well as diabetes at 40 weeks of age. This observation indicates that activation of this type of regulatory, GAD65-specific T cell and its infiltration into islets may require a local inflammatory environment that can only be initiated by pathogenic effectors. Additionally, these regulators were found to secrete IL-10 and could suppress the action of islet-specific pathogenic effectors (23).

Thus, two types of dominant GAD determinants can be described in the NOD mouse: those that arise spontaneously, and those that can be induced using whole antigen immunization in adjuvant. They are almost mutually exclusive in that the spontaneous set of T cells are not present in the induced T cell repertoire, and vice-versa. This clear regional distinction of T cell responses following exogenous immunization versus endogenous priming suggests that either the processing environment or the APC in the draining lymph nodes is different from that in the inflamed islets and/or the pancreatic lymph nodes. Further studies concerning the relative importance of these two types of determinants for diabetes development are necessary to shed new light on how antigen processing can contribute to the progressively destructive autoimmune response targeting the pancreas. Furthermore, it is noteworthy that both sets of peptides can induce tolerance and prevent T1D (24, 25), although the mechanisms of inducing tolerance could be different. Exogenous peptides could expand the regulatory population during tolerance induction, while the endogenous peptides might delete or exhaust high affinity T cells specific for GAD65.

5. CLUSTERED T CELL DETERMINANTS COULD INCLUDE BOTH REGULATORY AND PATHOGENIC EPITOPES

One interesting scenario we observed in our studies of one of the major endogenous peptides, GAD65 p524-543 (p543), is that there are at least two distinct determinants: an N-terminal moiety (p524-538 (=p524)) and a C-terminal moiety (p530-543 (=p530)), that are clustered within this 20mer peptide to which spontaneous T-cell responses arise in young NOD mice as early as 3-4 weeks of age (10). Importantly, two distinct T-cell populations could be induced by these two overlapping peptides, and it appeared that they had opposing functions: the p530-specific T cells are invasive and may become pathogenic (18, 26), whereas the p524-specific T cells are regulatory in nature and can prevent diabetes by adoptive transfer (27). To further gain knowledge about this tailored processing mechanism and its possible role in disease and its prevention, we mapped the fine specificity of clones specific for either p524 or p530 and defined the minimal antigenic cores of these two determinants (Dai et al., in press, EJI, 2008). We found that p543 contains multiple epitopes sharing a central core sequence, p530-539, which is bounded by different lengths of flanking residues at the N- or C-terminus of the core. We also observed that both types of clones responded similarly to the long p524-543 peptide but did not respond to the core, per se; as the sequence was further truncated from one or the other end, the response of the clones decreased. Some flanking N- or C-terminal residues were more important than others for certain clones and their removal severely reduced the response of the clones. Such observations would rather favor the view that flanking residues surrounding an overlapping core sequence can differentially affect the activation of T cells recognizing this region. An alternative explanation could be that the flanking residues may affect the affinity of the MHC-peptide-TCR complexes.

In addition, T cells that require N-terminal residues for activation appear to be inhibitory for those T cells less-dependent on such flanking residues, possibly through a competitive mechanism for antigen-MHC binding (28), or by the secretion of regulatory cytokines (Enomoto et al., manuscript in preparation). By comparing the NOD's T-cell response to the p524-543 peptide with the congenic, resistant NOR mouse strain, we found that almost all the responsive T cells in NOR mice required the N-terminal flanking residues, whereas in NOD mice, this requirement was dispensable (Dai et al., in press, EJI, 2008). Therefore, enzymatic removal of certain flanking residues in this region might differ in some APC of the NOD vs. NOR strains. Thus, differential processing within such dominant regions may account for the differences in disease susceptibility observed between NOD and NOR mice.

A similar scenario was reported in the autoimmune response to insulin. T cell clones isolated from the pancreas of NOD mice were found to be reactive to the insulin B-chain peptide, p9-23, supporting insulin as a possible dominant target during the autoimmune response in NOD mice (29). Previous studies performed in Denver showed that six p9-23-specific T cell clones could accelerate diabetes in young NOD mice or adoptively transfer the disease to NOD.scid mice, whereas intranasal or subcutaneous administration of the p9-23 peptide could protect NOD mice from T1D (30, 31). The pathogenic activity of these clones was further demonstrated by

generating TCR transgenic mice from one of the clones, BDC12-4.1; however, development of diabetes in the transgenic mice requires inhibition of various regulatory pathways (32). In contrast, the studies reported by Sherwin's group demonstrated that clones with the same specificity, the B-chain p9-23 peptide, could inhibit the islet infiltration of pro-diabetic lymphocytes, and this inhibitory effect depended on TGF-beta (33). Such a clone, 2H6, was used to generate a TCR transgenic mouse line; it was found that inhibition of diabetes by this clone required the expression of TGF-beta receptor on the T cell surface, indicating a role for TGF-beta signaling in the regulatory activity of these insulin-specific T cells (34). The mechanism that leads to distinct differential pathways of p9-23-specific T cells remains to be learned. It should be noted that Y16 within the p9-23 peptide was found to be essential in inducing an anti-insulin response as well as in initiating an autoimmune response in the pancreas of the NOD (35). Whether a suitable replacement at the Y16 position could skew the insulin-reactive T cell repertoire toward a regulatory function is unknown.

In summary, different T-cell repertoires specific for a dominant region of a self antigen (such as GAD65 p524-543 or insulin B-chain p9-23) can be induced to function diversely as a consequence of antigen processing. The overall immune response to this region or to the whole antigen may be dependent on which repertoire is preferentially selected in the thymus and at the site of inflammation.

6. UNIQUE PROCESSING OF A CLUSTERED DETERMINANT OF GAD65 IS REQUIRED TO ACTIVATE HIGHLY DIABETOGENIC T CELL CLONES SUCH AS BDC2.5

BDC2.5 T cells are diabetogenic in NOD mice (36, 37). The antigenic specificity of this T cell clone remains unclear, although it has been suggested that an islet-specific antigen picked up by dendritic cells is responsible for activation of BDC2.5 T-cells in pancreatic lymph nodes, the original priming site for induction of pancreatic inflammation (38). None of the known GAD65 determinants, including p524-543, could activate BDC2.5 T cells as tested by proliferation assay in vitro (our observation). By screening combinatorial peptide libraries, Wilson's group has identified several strong agonistic peptides that can activate BDC2.5 T-cells at pico-molar concentrations: these agonists are surprisingly homologous to a region within the GAD-65 p524-543 peptide (39). Subsequently, Yoshida et al. identified a panel of mimotopes which are quite similar to those eluted by Wilson and colleagues, that also strongly stimulate BDC2.5 T cells as well as several other diabetes-related clones (40). In previous studies using GAD65 transgenic mice, we have clearly shown that GAD65 does play a role in tolerizing BDC2.5 T cells and preventing their infiltration into the pancreatic islets in NOD mice (41). It is possible that the BDC2.5 T cells recognize a previously undescribed cryptic determinant generated following anomalous, unconventional antigen processing of the p524-543 region. Accordingly, we successfully activated BDC2.5 T cells using APC isolated from mice immunized with p524-543 (40), presumably because experienced APC might be efficient in processing the p524-543 peptide into a ligand, suitable for BDC2.5 recognition. One possible mechanism that could account for the generation of such a ligand by the p524-543-primed APC cells would involve differential protein splicing (42, 43); hypothetically, p524-543 peptides could be spliced and religated by special protein ligases, omitting a few amino acids flanking the spliced small fragments. A second mechanism could be due to differential antigen processing and presentation following receptor-mediated antigen internalization (44, 45). In this case, the receptor would be a surface Ig specific for the p524-543 peptide. Not only is enhanced antigen uptake relevant, but also a putative unique protease activity in these APC could play a crucial role in generation of a high affinity BDC2.5 ligand (46-48). Finally, TCR-peptide-MHC interaction could be strengthened by modification of a crucial side chain of the peptide determinant (49, 50). For example, T cell recognition of an HLA-DR4-restricted insulin A-chain peptide requires oxidation of two adjacent cysteine residues and subsequent formation of a disulfide bond (51).

7. CONCLUSION

The solution to the dilemma of determinant choice and modification in favoring a regulatory versus a disease-inducing outcome will probably relate to understanding the role of adjuvants as well as antigen processing pathways undergone by whole molecules.

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