Review

Role of glutamic acid decarboxylase in the pathogenesis of type 1 diabetes

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Abstract. Glutamic acid decarboxylase (GAD) is considered to be one of the strongest candidate autoantigens involved in triggering β-cell-specific autoimmunity. The majority of recent onset type 1 diabetes patients and prediabetic subjects have anti-GAD antibodies in their sera, as do nonobese diabetic (NOD) mice, one of the best animal models for human type 1 diabetes. Immunization of young NOD mice with GAD results in the prevention or delay of the disease as a result of tolerizing autoreactive T cells. Autoimmune diabetes can also be prevented by the suppression of GAD expression in antisense GAD transgenic mice backcrossed with NOD mice for seven generations. These results support the hypothesis that GAD plays an important role in the development of T-cell-mediated autoimmune diabetes. However, there is some controversy regarding the role of GAD in the pathogenesis of diabetes. Whether GAD truly plays a key role in the initiation of this disease remains to be determined. The examination of the development of insulitis and diabetes in β-cell-specific GAD knockout NOD mice will answer this remaining question.

Introduction

Diabetes mellitus is a common, serious metabolic disorder characterized by hyperglycemia. The disease can be divided into two major subclasses: insulin-dependent diabetes mellitus or type 1 diabetes mellitus (T1DM) and non-insulin-dependent diabetes mellitus or type 2 diabetes. T1DM results from insulin deficiency caused by the loss of insulin-producing pancreatic β cells, generally develops in the young [1–3] and accounts for ~10% of the diabetic population worldwide. In contrast, type 2 diabetes results from a variable combination of insulin resistance and insulin deficiency, generally develops in adults [4, 5] and accounts for ~90% of the diabetic population worldwide. Both types can cause microvascular and macrovascular complications, resulting in increases in morbidity and mortality.

Considerable evidence shows that T1DM is the consequence of progressive β cell destruction during an asymptomatic period often extending over many years. Genetic susceptibility is believed to be a prerequisite for the development of T1DM [6, 7]. However, the concordance rate for monozygotic twins to develop type 1 diabetes is only about 40% [8], suggesting that environmental factors such as viruses, diet, toxins and stress also play an important role in the initiation and progression of β cell destruction [9]. The hypothesis that T1DM is an autoimmune disease has been strengthened by study of animal models such as the BioBreeding (BB) rat and the nonobese diabetic (NOD) mouse. Both of these animals

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spontaneously develop T1DM, and their syndromes share many pathological features with human T1DM.

Identification and characterization of β-cell target autoantigens in T1DM may be indispensable for understanding the initiation of β-cell-specific autoimmunity and antigen-specific T cell responses in the development of T1DM. Autoantibodies to β cell antigens can be predictive markers, and the identified autoantigens can be used for the development of therapeutic intervention by modulating the immune response to these autoantigens. Much research has focused on identifying pancreatic β-cell autoantigens that may be involved in the primary immunological event of the β cell-specific autoimmune process. Islet cell autoantigens that are targets of autoimmune attack in T1DM have been studied largely by investigating the specificities of circulating autoantibodies present in the sera of T1DM patients and also in diabetic animals.

Since the first reports of anti-islet cell autoantibodies in 1974 [10], many autoantigens in humans, NOD mice and BB rats have been identified, including an islet cell autoantigen with properties of sialic acid containing glycolipid [11], insulin [12], the insulin receptor [13], a 52-kDa protein [14, 15], a 69-kDa protein [16, 17], glutamic acid decarboxylase (GAD) [18], tyrosine phosphatase-2 (IA-2) [19, 20], heat shock protein 65 (HSP65) [21, 22], carboxypeptidase H (CPH) [23], the glucose transporter [24], a 38-kDa autoantigen [25, 26], a retroviral antigen [27] and sex-determining region Y-related protein [28]. Among these autoantigens, GAD has been extensively studied with regard to its pathogenic role in the development of T1DM.

Immunopathogenesis of T1DM

Extensive studies on the immunopathogenesis of T1DM revealed that β cell autoantigens, macrophages, dendritic cells, B lymphocytes and T lymphocytes are clearly involved in the β-cell-specific autoimmune process [29–33]. Histologic analysis of the pancreas from patients with recent-onset type 1 diabetes revealed an infiltration of the islets of Langerhans by mononuclear cells [34]. The infiltrating immunocytes were identified as T and B lymphocytes, monocytes/macrophages and natural killer (NK) cells [35, 36]. Detection of circulating islet-reactive autoantibodies [12, 37] and islet-reactive T cells in animals with T1DM [38–41] has indicated that autoimmunity is involved in β cell destruction.

One of the most common immunological abnormalities of humans and animals with autoimmune diabetes is the presence of autoantibodies directed against islet cell antigens. The presence of autoantibodies to these β cell antigens is the first detectable marker of ongoing β cell destruction. The risk for developing diabetes is strongly related to the number of autoantibody markers; that is, the presence of two or more autoantibodies gives a higher probability of developing the disease than the presence of a single autoantibody. Ninety percent of first-degree relatives of T1DM patients who had antibodies to IA-2, GAD or insulin eventually developed diabetes within several years after the detection of the antibodies [42, 43]. While these autoantibodies are indicators of ongoing β cell destruction, they do not seem to be directly involved in the destruction of β cells.

Macrophages as well as dendritic cells are among the first cell types to infiltrate the pancreatic islets during the disease process [44–47]. Inactivation of macrophages in NOD mice or BB rats significantly prevented the development of diabetes [48–50]. Further studies in NOD mice found that macrophages are required for the creation of a suitable microenvironment wherein T cells can differentiate into β cell-cytotoxic T cells [50]. Macrophages, along with dendritic cells and B lymphocytes play a role as antigen-presenting cells [51]. They produce cytotoxic substances such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, interferon (IFN)-γ and free radicals such as nitric oxide, which are toxic to β cells and contribute to β cell damage [52–54].

Converging data suggest that B cells play a critical role as antigen-presenting cells of β cell autoantigens in NOD mice. T lymphocytes from diabetic NOD mice transfer diabetes to neonatal recipients in the absence of B cells, indicating that B cells are not required for the destruction of β cells after diabetogenic effector T cells are generated. Further studies demonstrated that B cells are critical antigen-presenting cells for the initiation of T-cell-mediated autoimmune diabetes in NOD mice [55, 56]. Whereas B cells appear to be required during the initiation of autoimmune diabetes, a recent study showed that their presence can mitigate β cell destruction. B-cell-specific I-A^{g7}-deficient NOD mice showed peri-insulitis, but converted to destructive insulitis after cyclophosphamide treatment. This result suggests that I-A^{g7}-mediated β cell autoantigen presentation by B cells is critical in overcoming a checkpoint in T cell tolerance to pancreatic β cells after their initial targeting has occurred [57].

Cumulative evidence indicates that T cells play a critical role in the pathogenesis of autoimmune T1DM. In the NOD mouse, it is clear that both CD4$^+$ and CD8$^+$ T cells are involved in the development of diabetes [58]. Athymic NOD mice and NOD.severe combined immunodeficiency (scid) mice do not develop insulitis or diabetes [59, 60]. In addition, treatment of NOD mice with anti-CD3 antibodies inhibits the development of diabetes [61]. Although some uncertainty remains with regard to the precise role of CD4$^+$ and CD8$^+$ T cells in the pathogenesis of autoimmune T1DM, it appears that CD8$^+$ T cells are the major final effectors of β cell damage in animal models. In humans, most of the immunocytes infiltrating the pancreatic

islets at the time of T1DM diagnosis are CD8^{+} T cells, suggesting that these cells are also the final effectors of β cell damage in humans.

Cytokines produced by immunocytes also play an important role in the pathogenesis of autoimmune T1DM. In general, Th1 cytokines (IL-2, IFN-γ), which potentiate cell-mediated immune responses, cause the development of T1DM, while Th2 or Th3 cytokines (IL-4, IL-10, TGF-β) prevent the disease [62]. However, the interactions of the many different cytokines in the immune system are complicated, and the development of diabetes may depend upon which way the finely tuned balance of immunoregulatory T cells is tipped. Pancreatic β cells may be killed by cytotoxic T cells through the perforin [63] and granzyme pathway as well as fas-fas ligand and TNF-TNF receptor interaction [64–66]. Therefore, activated macrophages and T cells as well as cytokines secreted by these cells act synergistically to destroy β cells.

Biochemical characteristics of GAD

GAD catalyzes the α-decarboxylation of L-glutamic acid to synthesize gamma-amino butyric acid (GABA), which functions as an inhibitory neurotransmitter. Two distinct forms of GAD, GAD67 (67 kDa) and GAD65 (65 kDa) (table 1), have been identified and found to be encoded by two different genes [67]. Amino acid sequence analysis showed ~65% of the sequence of these two forms is identical [68] (fig. 1). Both isoforms of GAD are synthesized within the cytoplasm as hydrophilic soluble molecules. GAD65, but not GAD67, is posttranslationally modified and anchored to the membrane [69]. Both isoforms of GAD contain a pyridoxal phosphate binding site, which acts as a cofactor for enzyme activity [70].

GAD is expressed not only in the central and peripheral nervous systems, but also in the pancreatic islets, testes,

ovaries, thymus and stomach [71–74]. There is a strong variation in the expression of the two isoforms of GAD in islets depending on the species [74, 75]. Both human and rat islets predominantly express GAD65, whereas GAD67 is the major GAD isoform in mouse islets [75].

Humoral immune response to GAD in T1DM

Anti-64-kDa antibodies were detected in the sera of T1DM patients [76], and it was found that the sera for ~85% of newly diagnosed T1DM patients contain these antibodies [37]. In addition, ~80% of those in a category at 'high risk' for developing T1DM [relatives of T1DM patients who are also positive for either cytoplasmic islet cell antibodies (ICA) or insulin autoantibodies (IAA) or both] also have anti-64-kDa antibodies in their sera. In contrast, those in a 'low-risk'group (unrelated controls or ICA-and IAA-negative relatives of T1DM patients) have only a 0–2% frequency of anti-64-kDa antibodies in their sera [77]. The anti-64-kDa antibody may appear as early as 8 years before the clinical onset of T1DM [37]. The presence of anti-64-kDa antibodies has also been reported in the NOD mouse; 80% of weaning NOD mice and 87% of newly diabetic NOD mice had anti-64-kDa antibodies in their sera [78]. This 64-kDa autoantigen was later identified as GAD65 [18]. It is known that anti-GAD autoantibodies in T1DM patients are predominantly directed to a conformational epitope of GAD. In contrast, autoantibodies from patients with another autoimmune disease wherein anti-GAD antibodies are common, stiffman syndrome, recognized a combination of linear and conformational epitopes of GAD [79, 80]. Although the two isoforms of GAD have high homology, the major antigenic region in humans has been identified as the middle and carboxyterminal region of GAD65 [81–83] (fig. 2).

The presence of anti-GAD65 antibodies along with anti-IA-2 antibodies is a highly predictive marker for the development of T1DM in humans. The cumulative incidence of anti-GAD65 and anti-IA-2 antibodies is ~90% in newly diagnosed T1DM patients and prediabetic individuals [84]. However, a contradictory result has been reported regarding the correlation between the presence of anti-GAD antibodies and diabetes in NOD mice. One study found that anti-GAD65 and anti-GAD67 antibodies were detected at the early stage of the disease process (4 weeks of age) and before autoantibodies to other β cell autoantigens developed, implying that GAD is the primary antigen that may initiate β-cell-specific autoimmunity in this model [85] However, another study found that anti-GAD antibodies are not prerequisites for the development of diabetes in NOD/Lt and NOD/Wehi mice, which have a higher and lower incidence of diabetes than NOD mice, respectively [86]. This study suggests that a strong humoral response to GAD may actually be associated with

Table 1. Biochemical and molecular characteristics of human, GAD65 and GAD67.

Characteristic	GAD65	GAD67
Molecular weight	65,400 (585 amino acids)	66,600 (594 amino acids)
Amino acid sequence homology		65% homology with GAD65
Chromosome location	10p11.23	2q31
Pyrodoxal phosphate binding site	yes	yes
Area of expression	primarily pancreatic β cells	primarily brain
Subcellular location	membrane anchored after posttranslational modification	cytoplasmic

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hGAD67   1    masstpsssa tssnagadpn ttnlrpttyd twcgvahgct rklglkicgf lqrtnsleek
hGAD65   1    .........m a pgs fwsf gsedgsgdse npgtaraw q vaqkftggig nklcal ygd

hGAD67  61    srlvsafker qssknllsce nsdrdarfrr tetdfsnlfa rdllpaknge eqtvqfllev
hGAD65  52    aekpaesggs  ppraaarka acac qkpcs cskvdv yaf lhatdllpac dgerpt afl

hGAD67 121    vdillnyvrk tfdrstkvld fhhphqlleg megfnlelsd hpesleqilv dcrdtlkygv
hGAD65 112    q vmnillqy vvksfdrstk vidf ypnel lqey w  a  q qn  e  m h qt    ai

hGAD67 181    rtghprffnq lstgldiigl agewltstan tnmftyeiap vfvlmeqitl kkmreivgws
hGAD65 172    k    y         mv   ad                       l yv           i

hGAD67 241    skdgdgifsp ggaisnmysi maarykyfpe vktkgmaavp klvlftseqs hysikkagaa
hGAD65 232    ggs              am  i  f  m     e       l  r ia    h   f l  ga

hGAD67 301    lgfgtdnvil ikcnergkii padfeakile akqkgyvpfy vnatagttvy gafdpiqeia
hGAD65 292      i   s      d     m    s l rr      f   l  s           llav

hGAD67 361    dicekynlwl hvdaawgggl lmsrkhrhkl ngieransvt wnphkmmgvl lqcsailvke
hGAD65 352     k  ki m              kw   s v              p       l  r

hGAD67 421    kgilqgcnqm cagylfqpdk qydvsydtgd kaiqcgrhvd ifkfwlmwka kgtvgfenqi
hGAD65 412    e lm n     h s    q   h  l        l       v l   r      t    ahv

hGAD67 481    nkclelaeyl yakiknreef emvfngepeh tnvcfwyipq slrgvpdspq rreklhkvap
hGAD65 472     dk         ni        gy    d k q         p    rtle nee  msr s

hGAD67 541    kikalmmesg ttmvgyqpqg dkanffrmvi snpaatqsdi dflieeierl gqdl 594
hGAD65 532    v   r   y    s  l   v           hq                      585
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Figure 1. Comparison of the amino acid sequences of human GAD67 and GAD65. Only the amino acids in hGAD65 that are different from hGAD67 are shown. (...) denotes missing amino acids.
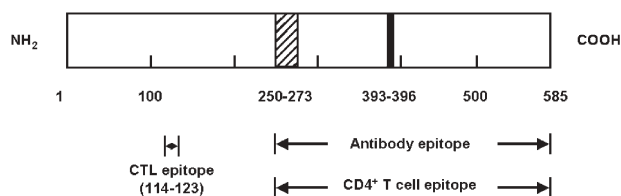

Figure 2. Schematic diagram of human GAD65 showing the epitopes recognized by anti-GAD antibody and GAD-reactive T cells in human IDDM. The conformational epitope region recognized by antibodies from IDDM patients and the T cell epitope region recognized by CD4+ T cells and CD8+ cytotoxic T cells (CTL) from IDDM patents are indicated with arrows. The hatched box represents the region that is homologous to Coxsackie B4 viral antigen, and the black box represents the pyridoxal phosphate binding site.

less destructive pathology, as indicated by the negative correlation between insulitis and anti-GAD antibody levels found in these animals. This has also been the case in studies on humans [87, 88].

Cell-mediated immune response to GAD

In NOD mice, it was found that the initial immune response against pancreatic islets is a Th1 response against a confined region of GAD (peptides 509–528 and 524–543) and that later responses are directed against another region of GAD and against other autoantigens, such as HSP65 and insulin [89]. Therefore, prevention of this early immune response could be achieved by immunization with purified GAD65 protein, which tolerized the T-cell-mediated immune response against other autoanti-

gens such as HSP65 and CPH in pancreatic β cells and prevented or delayed insulitis and diabetes in NOD mice [89]. There is also direct evidence that GAD-reactive T cells are diabetogenic in NOD mice. A CD4+ T cell line that was generated from the splenocytes of a diabetic NOD mouse adoptively transferred insulitis and diabetes to NOD.scid mice. These T cells secreted IFN-γ and TNF-α/β, but not IL-4, suggesting a Th1 cell type, and showed cytotoxic effects against NOD-derived hybridoma cells expressing GAD65 [90]. In addition, it was recently reported that K^d-restricted GAD-reactive CD8+ T cell lines reactive to GAD65 peptides 206−214 (p206) or 546−554 (p 546) could lyse GAD65-expressing target cells, and p 546-specific T cells transferred insulitis to NOD.scid mice [91], suggesting that GAD may play a central role in the development of T1DM. However, some GAD-reactive T cell clones do not have the ability to induce diabetes [92]. One study reported that the response to GAD65 peptides 524−543 was major histocompatibility complex (MHC) class II restricted and that T cell responses to GAD-derived peptides were observed in mice resistant to T1DM [93]. Therefore, this study suggested that peripheral tolerance to GAD is not associated with protection from diabetes.

In humans, GAD-specific CD4+ T cells have also been observed in recent-onset T1DM patients and in relatives of T1DM patients at risk to develop diabetes [94−96]. GAD-reactive T cells have been detected prior to the onset of human T1DM, and differences have been found between T1DM patients and control subjects. GAD-reactive T cells in T1DM patients responded primarily to two peptide regions (amino acids 473−555 and 247−279) of GAD65, whereas those from control subjects responded to the another peptide (amino acids 161−243) [97, 98]. In addition to CD4+ T cells, MHC class human lymphocyte antigen I (HLA)-A*0201-restricted CD8+ cytotoxic T cells, specific for a peptide region of GAD (amino acids 114−123) were identified in recently diagnosed diabetic patients and in high-risk subjects, but not in healthy control subjects expressing HLA-A*0201 [99] (fig. 2). These results suggest that GAD may be a target autoantigen of T cells in human T1DM. It was reported that transgenic mice bearing diabetes-susceptible haplotypes, HLA DR3 (HLA-DRB1*0301/I-Ab^0) or DQ8 (HLA-DQB1*0302/I-Ab^0), showed spontaneous T cell reactivity to GAD65 [100]. In addition, a GAD peptide-specific, HLA-DQ8-restricted (an allele linked with T1DM susceptibility in humans) Th1−CD4+ T cell line generated from a humanized animal model, HLA-DQ8(+)/I-Ab^0 transgenic mice, induced severe insulitis after adoptive transfer of these cells into transgene-positive, but not transgene-negative mice treated with a subdiabetogenic dose of streptozotocin [101]. This result suggests that GAD-reactive T cells may play a direct pathogenic role in the destruction of pancreatic β cells in human T1DM.

Molecular mimicry between GAD and viral antigens

Molecular mimicry between GAD and Coxsackie B4 virus has been hypothesized for the development of T1DM, as there is similarity between a region of GAD (amino acids 250−274) and the sequence of the P-2C antigen, with high homology in GAD residues 260−265 (PEVKEK) (fig. 2), and Coxsackie B4 virus has been shown to be associated with the development of T1DM in humans [102]. Splenic T cells in NOD mice also showed a high proliferative response to the GAD peptide homologous to the Coxsackie B4 sequence [89]. Human data are more inconsistent, however; some reports support this hypothesis [98, 103−105], but others do not. One study reported the detection of a T cell response to larger epitopes containing the homologous region in T1DM patients [98], whereas another study reported that the T cell response to this region was low in approximately one-half of the patients studied [106]. Another study reported that T cell reactivity to a GAD peptide that is homologous with P-2C is frequently observed in healthy controls, first-degree relatives of T1DM patients and post-onset T1DM patients, but less frequently in recent-onset T1DM patients [107]. With regard to cross-reactivity with other viruses, it was recently found that a T cell clone specific for GAD peptides isolated from a T1DM patient cross-reacted with rubella virus antigen, and the cross-reacting epitopes shared similar peptide binding motifs with HLA-DR3/DR4 [108]. In addition, a CD4+ GAD-reactive T cell clone isolated from a prediabetic patient cross-reacted with a peptide sequence of human cytomegalovirus [109]. These results imply that molecular mimicry between GAD and rubella virus or cytomegalovirus may be involved in the development of T1DM.

Studies on the role of GAD in the pathogenesis of diabetes using transgenic mouse models

To investigate the role of GAD in the pathogenesis of autoimmune diabetes, several lines of transgenic mice have been established in which the expression of GAD has been manipulated (table 2). Studies using two transgenic NOD mouse lines that hyperexpress human GAD65 in β cells found that one line showed a lower incidence of T1DM, whereas the other line showed no difference in the incidence of the disease as compared with nontransgenic control NOD mice. A quantitative difference in the expression of GAD between the two lines might account for the prevention of diabetes in only one transgenic mouse line [110]. To induce immunological tolerance to GAD65, a transgenic NOD mouse line that expresses GAD65 in all tissues was established. However, instead of preventing diabetes, these mice showed an accelerated onset and increase in the incidence of diabetes compared with control

Table 2. Studies on the role of GAD using transgenic mouse models.

Expression of GAD	cDNA/promoter	Development of diabetes in NOD background mice
β-cell-specific expression of GAD65	GAD65 cDNA under rat insulin promoter	one line showed no difference in the incidence of diabetes. The other line showed a lower incidence of diabetes [110]
Widespread expression of GAD65	GAD65 cDNA under MHC class I promoter	increased incidence of diabetes [111]
β-cell-specific suppression of GAD65 and 67	Antisense GAD65 and 67 cDNA under rat insulin promoter	prevention of insulitis and diabetes [112]
Systemic knockout of GAD65		no difference in the incidence of diabetes compared with transgene-negative animals [115]
Systemic knockout of GAD67		died 1 day after birth [115]

NOD mice [111]. This may have been due to the defect in central tolerance in NOD mice. Therefore, it is difficult to draw any definite conclusions about the role of GAD in the development of autoimmune diabetes from this study. Another strategy is the creation of transgenic mice in which GAD expression is absent. Interestingly, β-cell-specific suppression of GAD65 and -67 expression prevented insulitis and diabetes in antisense GAD transgenic mice back-crossed with NOD mice for seven generations [112]. These results suggest that the expression of GAD in pancreatic β cells is involved in the modulation of β-cell-specific autoimmunity. However, the possibility exists that a diabetes-resistant gene from the strain of origin might have been transmitted to the transgenic offspring, as these antisense GAD transgenic mice were produced using eggs from (SJL × C57BL/6) F2 mice, which are diabetes resistant [113, 114]. In another study, systemic GAD65 knockout mice back-crossed with NOD mice for four generations still developed diabetes and insulitis similar to wild-type NOD mice [115]. However, it is difficult to draw any definite conclusions from this study, as mouse β cells predominantly express GAD67 and very low levels of GAD65, and these GAD65 knockout mice still express GAD67. Systemic GAD67 knockout mice die within the 1st day of neonatal life and cannot be studied further. Therefore, β-cell-specific conditional GAD65/67 knockout NOD mice are essential to find whether the expression of GAD in β cells truly plays a critical role in the initiation of β-cell-specific autoimmune diabetes.

Therapeutic uses of GAD

Immune therapy using specific target autoantigens has been attempted as a method to prevent autoimmune disease. It has been reported that administration of purified GAD protein or peptide or insulin protein or peptide to NOD mice by various routes can tolerize the T-cell-mediated immune response against pancreatic β cells, resulting

in the prevention or delay of the development of insulitis and diabetes. In many cases, the preventive effect was found to be associated with a Th2 shift [116].
Immunization with purified GAD65 protein at an early age either intrathymically or intravenously can tolerize the T-cell-mediated immune response against pancreatic β cells in NOD mice, thus preventing insulitis and diabetes [85, 89]. Moreover, tolerization with GAD65 could prevent the development of other immune reactions that usually occur in NOD mice, such as those against HSP65 and CPH. In contrast, immunization with HSP65 only partially decreased the T cell responses to other β cell autoantigens and insulitis [89]. These results suggest that GAD is critical in the initiation of the autoimmune response against pancreatic β cells in NOD mice. Similarly, intraperitoneal immunization of 4-week-old NOD mice with GAD67 significantly prevented the development of diabetes as compared with controls [117]. In addition, oral administration of GAD-expressing transgenic plants [118], nasal administration of a mixture of GAD peptides [p17 (247–266), NMYAMMIARFKMFPEVKEKG; p34 (509–528), IPPSLRYLEDNEERMSLRLSK; p35 (524–543), SRLSKVAPVIKARMMEYGTT; and p36 (539–558), EYGTTMVSYQPLGDKVNFFR] [119] or administration of recombinant vaccinia virus expressing GAD [120] also prevented autoimmune diabetes in NOD mice by inducing Th2 immune responses. Furthermore, GAD65 immunization of NOD mice at the stage after the onset of insulitis could inhibit the progression of diabetes [121]. However, in some cases, intrathymic immunization of young NOD mice with GAD65 peptides such as p34 and p35 provoked diabetes [122], probably due to the activation of diabetogenic T cells reactive to the GAD65 protein. As well, immunization of NOD mice with GAD did not completely prevent diabetes, but only delayed the development of disease [123].
Intramuscular injection of plasmid-encoding GAD65 resulted in the prevention of diabetes [124, 125]. However, other studies showed that injection of a plasmid-encoding

GAD65 alone was ineffective [126–128], but was effective if the plasmid also contained DNA encoding IL-4 [126, 128]. Therefore, the therapeutic effect of GAD may be different depending on the route of administration, experimental conditions or quality of antigens.

Conclusions and future directions

Among the various β cell autoantigens identified, GAD has been suggested to be one of the strongest candidates as a triggering antigen for T1DM for both humans and NOD mice. There has been significant research progress in understanding the role of anti-GAD immunity in the pathogenesis of T1DM. The presence of anti-GAD antibodies, along with anti-IA2 and anti-insulin autoantibodies is a reliable predictive marker for the development of T1DM. GAD-reactive T cells are present in diabetic patients and T1DM animal models, indicating that GAD is clearly a target antigen in T1DM. Immunization of young NOD mice with GAD results in the prevention of autoimmune diabetes as a result of tolerizing autoreactive T cells in NOD mice. The suppression of GAD expression in β cells results in the prevention of diabetes in antisense GAD transgenic mice back-crossed with NOD mice for seven generations. Although there are still some controversies regarding the role of GAD, most data support the hypothesis that GAD plays an important role in the pathogenesis of T1DM, however, whether it truly plays a critical role in the initiation of β-cell-specific autoimmunity leading to diabetes remains to be answered. The production of β-cell-specific GAD65/67 knockout NOD mice will definitely help to answer this remaining question.

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