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Short Communication

Development of autoimmune diabetes in glutamic acid decarboxylase 65 (GAD65) knockout NOD mice

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Abstract

Aims/hypothesis. Type 1 diabetes mellitus, a T-cell-mediated autoimmune disease, results from the selective destruction of insulin-producing pancreatic beta cells. Autoantibodies against beta-cell components are used clinically as sensitive markers of this disease; however, their physiological role has not been clear. To investigate the role of glutamic acid decarboxylase 65 (GAD65) in the development of the Type 1 diabetes of non-obese diabetic (NOD) mice, we analysed and characterised NOD mice with targeted disruption of the *GAD65* gene.
Methods. GAD65-deficient mice were previously established. After backcrossing the knockout mutation onto the NOD genetic background for up to eight generations, female littermates of the three resulting genotypes were produced by intercrossing: *GAD65* +/+ (n=23), *GAD65* +/– (n=62), and *GAD65* –/– (n=31).
Results. The cumulative incidence of autoimmune diabetes showed no significant difference among the three groups in longitudinal studies using the Kaplan-Meier method. Islet morphology showed that the progression of islet infiltration did not differ significantly between the three groups.
Conclusion/interpretation. The cumulative incidence of autoimmune diabetes was not influenced by the *GAD65* deficiency. These data suggest that GAD65 is not a major regulatory target of beta-cell autoimmunity in NOD mice. [Diabetologia (2004) 47:221–224]

Keywords GAD65 · Autoantigen · Autoimmune diabetes · Knockout mouse · NOD mouse

The glutamic acid decarboxylase (GAD) protein is a major autoantigen involved in the initial stage of autoimmune diabetes development in both humans and mice. In the non-obese diabetic (NOD) mouse, a model of Type 1 diabetes, anti-GAD65 and anti-GAD67 antibodies are detected at the early stage of the disease process, before antibodies against other beta-cell autoantigens develop [1]. It is, however, unclear whether the anti-GAD antibodies participate directly in beta-cell destruction or arise secondarily in response to the release of autoantigens from islets damaged by other components of the immune system. To investigate the role of GAD65 in the development of the autoimmune diabetes of NOD mice, we have generated *GAD65* –/– NOD mice and analysed the onset and incidence of diabetes in these mice.

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Abbreviations: NOD, Non-obese diabetic

Materials and methods

Generation and genotyping of a GAD65 mutant mouse. We previously established 129/Ola-derived embryonic stem-cell clones carrying the *GAD65* targeted mutation through homologous recombination [2]. *GAD65* knockout embryonic stem cells were injected into C57BL/6 mouse blastocysts to make chimeras, and germ-line transmission was achieved. *GAD65*

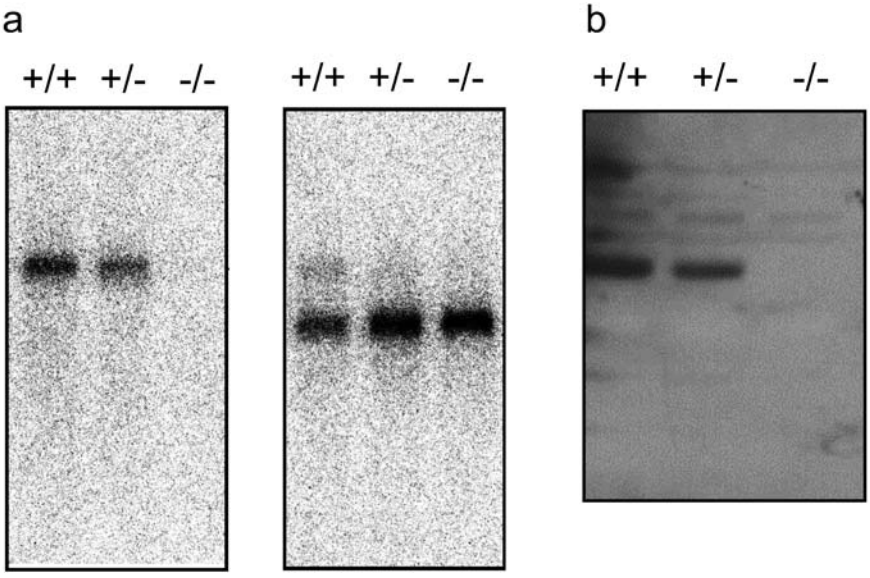

Fig. 1. (**a**) Detection of GAD65 transcripts by northern blot analysis. Brain total RNA was blot-hybridised with a probe specific for GAD65 (*left panel*) or GAD67 (*right panel*) mRNA. A total absence of GAD65 mRNA was seen in *GAD65* –/– mice. (**b**) Western blot analysis of GAD65 expression in mouse brain extracts. The GAD65 protein was detected with an anti-GAD65 antibody. The antibody detected an expected protein of 65 M_r in the lysates from *GAD65* +/+ mice, but not from *GAD65* –/– mice

mutant mice were backcrossed for eight generations onto the NOD/Shi background. In the N4 and N5 generations, the male that was the most enriched for NOD-type microsatellite markers was selected by the speed congenic approach, in which a set of sequence length polymorphism primers are used to identify the genetic background of each chromosomal region: chromosome 1, *D1Mit9*; chromosome 2, *D2Mit345*, *D2Mit265*; chromosome 3, *D3Mit21*, *D3Mit12*; chromosome 4, *D4Mit213*, *D4Mit48*; chromosome 5, *D5Mit48*; chromosome 6, *D6Mit14*; chromosome 7, *D7Mit20*; chromosome 8, *D8Mit140*; chromosome 9, *D9Mit21*; chromosome 10, *D10Mit230*; chromosome 11, *D11Mit298*; chromosome 12, *D12Mit12*; chromosome 13, *D13Mit134*; chromosome 14, *D14Mit222*; chromosome 15, *D15Mit35*; chromosome 16, *D16Mit98*; chromosome 17, *D17Mit28*, *H2-Ab1*, *D17Mit105*; chromosome 18, *D18Mit68*; chromosome 19, *D19Mit45*; chromosome X, *DXMit16*. We confirmed that a male mouse at the N5 generation, which was used to produce the next generation, had NOD-type micro-satellite markers in all its chromosomes. Genotyping of the *GAD65* locus of the progeny was carried out using Southern blot or PCR analyses of the genomic DNA [2]. The protocols for the animal experiments were designed following the Principles of Laboratory Animal Care published by NIH and approved by the Animal Care and Use Committee of Osaka University Graduate School of Medicine, Japan.

Northern blot analysis. Twenty micrograms of total RNA isolated from whole brain were separated by electrophoresis, transferred onto a Hybond membrane (Amersham Pharmacia Biotech, Buckinghamshire, U.K.), and hybridised with mouse GAD65 or GAD67 cDNA as a probe.

Western blot analysis. Twenty micrograms of total lysate extracted from whole brain were separated by electrophoresis on 10% acrylamide gels, which were subjected to Western blotting and chemiluminescence (ECL kit; Amersham Pharmacia Biotech) using a rabbit anti-GAD65 monoclonal antibody (GAD-6), which specifically recognises the C-terminal region of GAD65, and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody.

Evaluation of insulitis. Pancreata were removed from the three groups of female NOD mice at the age of 12 weeks. Three non-consecutive tissue sections were prepared for haematoxylin/eosin staining. More than 20 islets for each pancreas were examined by light microscopy and scored for the degree of insulitis as follows: grade 0, no inflammatory cells around or in the islets; grade 1, infiltrating cells adjacent to the islets but not inside; grades 2, 3, and 4, infiltrating cells occupying less than 25%, 25~50%, and more than 50% of the islet area, respectively.

Assessment of diabetes and statistical analysis. Female mice were screened weekly for the presence of glucosuria using TesTapeA (Eli Lilly, Indianapolis, Ind., USA). Diabetes was diagnosed when the mice were glucosuric for two consecutive weeks. The cumulative diabetes-free incidence was compared using the Kaplan-Meier method with 95% confidence intervals; p values of less than 0.05 were considered to be statistically significant by the log rank test. All statistical analyses were carried out with the StatView program for Macintosh (Abacus Concepts, Berkeley, Calif., USA).

Results

Generation of mice with a disrupted GAD65 gene. The production of the *GAD65* knockout mouse was reported previously [2]. In this *GAD65* gene knockout, we designed to disrupt most of the open reading frame by inserting an in-frame stop codon in the second exon, so that the resulting knockout allele could encode only the first 34 amino acid residues in which no epitopes have been reported. The *GAD65* knockout mouse was backcrossed to the NOD mouse for eight

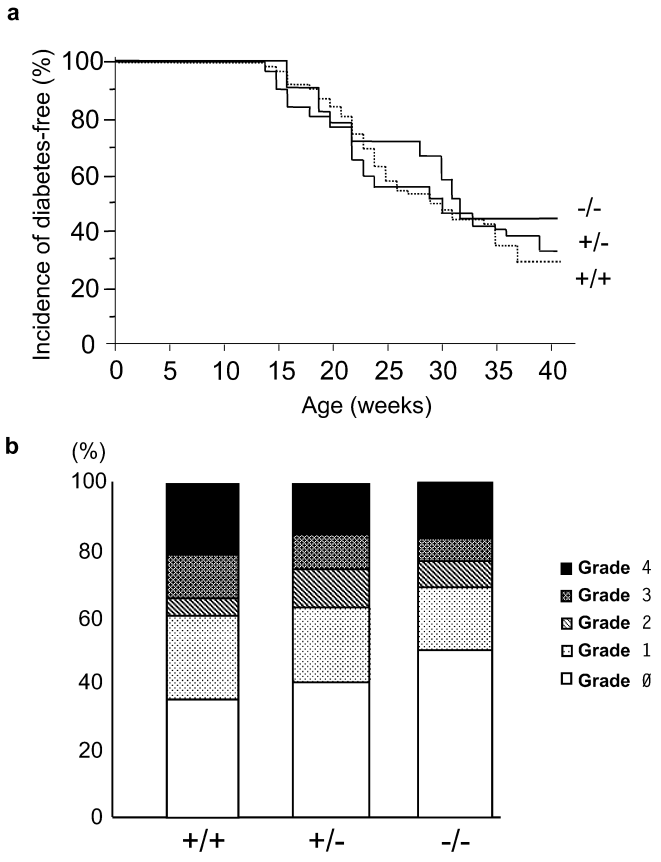

Fig. 2. (**a**) Cumulative diabetes-free incidence. The three groups of female NOD mice were *GAD65* +/+ (*n*=23, *dotted line*), *GAD65* +/– (*n*=62, *gray line*), and *GAD65* –/– mice (*n*=31, *solid line*). Diabetes was diagnosed by the presence of glucosuria for two consecutive weeks. (**b**) Histological grading of insulitis in *GAD65* +/+, *GAD65* +/–, and *GAD65* –/– mice at the age of 12 weeks (*n*=4 per group)

generations and *GAD65* –/– NOD mice were derived from intercrossing the heterozygous breeding pairs. Northern and Western blot analyses showed that GAD65 was absent in the *GAD65* –/– mice, and that the GAD65 expression in the *GAD65* +/– mice was about half that of *GAD65* +/+ mice. The expression levels of GAD67 mRNA were not altered by the absence of GAD65 (Fig. 1).

Death from seizure. The *GAD65* –/– offspring showed no obvious abnormality until about 4 weeks of age, when they began to exhibit a small retardation of growth. In the *GAD65* –/– group, 7 of 31 mice died suddenly from non-diabetic causes at the age of 16 to 32 weeks, probably due to seizures induced by some mild stimulation. Such seizures were observed repeatedly in the *GAD65* –/– mice.

Incidence of spontaneous diabetes in female GAD65 –/– mice. To evaluate the effect of the *GAD65* deficiency on the development of autoimmune diabetes, female *GAD65* +/+, *GAD65* +/–, and *GAD65* –/– mice were monitored for glucosuria weekly (Fig. 2a). In longitudinal studies using the Kaplan-Meier method, the cumulative diabetes-free incidence showed no significant difference among the three groups (log rank test p=0.707).

Insulitis levels in female GAD65 –/– mice. We did a histological examination of the pancreas and evaluated the levels of insulitis (Fig. 2b). The progression of islet infiltration did not differ significantly between the three groups.

Discussion

Among a variety of pancreatic beta-cell autoantigens, GAD has been the most extensively studied with regard to its pathogenic role in the development of Type 1 diabetes. The importance of GAD65 in mouse islets is supported by data showing the existence of some specific GAD65 peptide regions that lead to significant T-cell responses in NOD mice [3, 4].

However, whether there is a direct effect of GAD on the development of autoimmune diabetes has been controversial. It was reported that beta-cell-specific suppression of *GAD* expression prevented autoimmune diabetes in transgenic NOD mice expressing antisense transgenes of both rat *GAD65* and *GAD67* under the control of the rat insulin promoter [5]. In contrast, a *GAD65* knockout did not affect the incidence of autoimmune diabetes in NOD mice [6]. However, there were few animals used in that investigation, and the incidence of diabetes in the *GAD65* knockout mice was quite low, probably because the fourth backcross onto the NOD mouse background was not adequate to estimate the incidence of diabetes. Recently, transgenic NOD mice expressing GAD65 under the invariant chain promoter showed a similar incidence of diabetes and severity of insulitis compared with nontransgenic NOD mice in spite of their tolerance to GAD65 [7].

In this study, we established the *GAD65* mutant NOD mice by backcrossing for eight generations onto the NOD background. The *GAD65* mutant gene was expected to encode only a truncated peptide which lacked all the known immunogenic sites. Therefore, we consider it unlikely that T cells could react with this truncated GAD65 peptide. We analysed the incidence of autoimmune diabetes, and found no significant reduction in the spontaneous incidence of autoimmune diabetes or in the acceleration of diabetes by cyclophosphamide treatment in female *GAD65* –/– NOD mice. This result provides strong evidence that GAD65 expression is not necessarily requisite for autoimmune beta-cell destruction in the NOD mouse.

The major site of GAD expression is the brain. It has been reported that the expression of GAD65 is under the detectable limit in mouse islets, whereas both rat and human islets express it at significantly higher

levels [8]. In our *GAD65* –/– mice, the sudden death before the onset of diabetes might have been caused by severe dysfunction in the central nervous system, consistent with a previous report [9]. We believe that such a phenotype is not likely to be associated with the onset of autoimmune diabetes. However, the production of tissue-specific *GAD65* and *GAD67* knockouts in beta cells should be indispensable for identifying the precise role of GAD expression in the development of autoimmune diabetes.

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