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Insulin as a T cell antigen in type 1 diabetes supported by the evidence from the insulin knockout NOD mice☆,☆☆

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Abstract

Rodents have two functional preproinsulin genes named insulin 1 and insulin 2 on different chromosome and have two amino acid differences in insulin B chain. We have established insulin 1 or insulin 2 knockout (KO) non-obese diabetic (NOD) colonies in the animal institute of Kobe University and evaluated anti-insulin autoimmunity. Similar to the previous report, insulin 1-KO provides strong protection from insulitis (islet-infiltration of mononuclear cells) and diabetes, whereas the insulin 2-KO markedly accelerated insulitis and development of diabetes even at further backcross breeding with NOD/Shi/Kbe mice ($P < 0.0001$). Expression of serum anti-insulin autoantibodies (IAA) was enhanced in insulin 2-KO mice at a time between 10 and 15 weeks of age ($P < 0.005$) while the expression of insulin 1-KO NOD mice was rather reduced. Furthermore, T cell reactivity in splenocytes of insulin 2-KO NOD mice to insulin 1 B:9-23 peptide was increased ($P < 0.05$), suggesting that expanding insulin-reactive T cells may contribute to the acceleration of diabetes in insulin 2-KO mice. Based on those observations, we hypothesize that insulin 1 is a crucial T cell antigen in murine autoimmune diabetes and modification of anti-insulin autoimmunity can be applicable to antigen-based therapy for human type 1 diabetic patients.

Keywords: Type 1 diabetes; Insulin; Autoantigen; Autoimmune; ELISPOT

1. Introduction

There have been several lines of evidence for the importance of insulin as a T cell antigen of type 1 diabetes. First of all, insulin is a pancreatic β cell specific antigen among type 1 diabetes-related antigens. Human genetic analysis demonstrated that the insulin gene variable tandem repeat (VNTR) allele (IDDM2) is associated with the protection from the development of human type 1 diabetes [1]. In addition, insulin autoantibody (IAA) is an excellent predictive maker for the development of type 1 diabetes in both human and the non-obese diabetes (NOD) mouse, which is a well-established animal model for type 1 diabetes [2,3].

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Majority of T cells in islet-infiltrates of NOD mice recognize insulin B:9-23 peptide [4] and human T cell reactivity to B:9-23 peptide are detected in type 1 diabetes patients [5]. Administration of insulin or insulin peptide through various routes (subcutaneous, oral, nasal and intravenous) to NOD mice prevents diabetes [6–9]. Those results indicate that insulin and the peptide exhibit either pathogenic or protective character depending on the situation probably through induction or regulation of autoreactive T cells.

Rodents have two functional preproinsulin genes named insulin 1 and insulin 2 on different chromosome and have two amino acid differences in insulin B chain. Insulin 1 lacks intron present in insulin 2, suggesting that insulin 1 is a retroposon of insulin 2 gene generated by an RNA-mediated duplication-transposition event [10]. We have created insulin 1 or insulin 2-KO NOD congenic mice and reported that dramatic opposite effects of KO genes were unexpectedly observed [11].

In this study, the KO effects were confirmed at further backcross generation with the breeding of NOD/Shi/Kbe mice. Analyses for the mechanism of autoreactive T cells have been also performed.

2. Materials and methods

2.1. Mice

The original knockouts were produced in 129S1/SvImJ embryonal cell lines by Jami and coworkers [12]. The knockout cell lines were microinjected into C57BL/6 blastocytes and lines developed with C57BL/6. Insulin 1 and insulin 2 KO congenic NOD mice were established by breeding the original insulin KO mice using speed congenic method fixing NOD diabetogenic loci (idd 1–14) at Barbara Davis Center [13]. Both strains have been backcrossed onto NOD/Bdc mice and non-NOD genomic regions flanking each insulin gene knockout were then less than 10 cM. We have further been breeding the knockouts onto NOD/Shi/Kbe mice in the Institute for Experimental Animals, Kobe University School of Medicine. We are at the 10th backcross (BC) generation for insulin 1-KO mice and the 13th BC for insulin 2-KO mice. All animals were handled under the Guidelines for Animal Experimentation of Kobe University School of Medicine.

2.2. Genotype analysis

Genomic DNA was extracted from mouse tails. Insulin KO mice were genotyped for KO genes and wild-type insulin genes by using PCR as previously described [13]. The PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining.

2.3. Diagnoses of diabetes

Homozygous KO mice were produced by the breeding of heterozygous mice at BC9 generation for insulin 1-KO and at BC12 for insulin 2-KO mice and the development of diabetes have been followed up. The glucose level for the homozygous KO mice was measured weekly with FreeStyle (TheraSense, Alameda, CA) or Glutest Ace (Sanwakagaku, Nagoya, Japan) blood glucose monitoring system. The mice were considered diabetic after two consecutive blood glucose values were greater than 250 mg/dl. After development of diabetes, the mice were sacrificed immediately and the pancreata of some mice were fixed in 10% buffered formalin for histological analysis.

2.4. Histology

The pancreata obtained from the mice were fixed in 10% buffered formalin and then embedded in paraffin. Paraffin sections were stained with hematoxylin/eosin. Pancreatic sections were microscopically examined for the degree of insulitis.

2.5. Anti-insulin autoantibody assay

Mice were bled for the measurement of serum anti-insulin autoantibody (IAA) levels. IAA was measured with a 96-well filtration plate micro-IAA assay as previously reported [2] and expressed as an index: index = (sample Δ (cpm) − negative control Δ (cpm))/(positive Δ (cpm) − negative control Δ (cpm)). A value of 0.01 or greater is considered positive.

2.6. Antigens

HPLC-purified insulin 1B:9-23 peptide (PHLVEALYLVCGERG) and insulin 2B:9-23 peptide (SHLVEALYLVCGERG) were purchased from Invitrogen Japan (Tokyo, Japan). Purified recombinant human GAD65 expressed in yeast (*Saccharomyces cerevisiae*) was obtained from RSR Ltd. (Cardiff, UK). These peptides and the protein were used as antigens in vitro ELISPOT assay.

2.7. ELISPOT assay

The murine ELISPOT assay was performed with modification of human ELISPOT assay as previously described [14]. In brief, splenocytes (2×10^5) were cultured in nitro-cellulose-bottomed 96-well microtiter plates (Millititer, Millipore Corp., Bedford, MA) in 200 μl of RPMI 1640 with 1% FCS. ELISPOT assay for mouse IFN-g or IL-4 was performed as manufacturer's instruction (Mabtech AB, Stockholm, Sweden) and spots were analyzed with an ImmunoSpot Analyzer (Cellular Technology, Cleveland, OH).

2.8. Statistics

Survival curves were analyzed with the log rank test at Kaplan–Meier method. Mann–Whitney *U*-test was used to compare the values of IAA expression. Wilcoxon matched