

Proinsulin 2 Knockout NOD Mice

A Model for Genetic Variation of Insulin Gene Expression in Type 1 Diabetes

Danièle Dubois-Lafforgue,¹ Laurent Mogenet,¹ Karine Thébault,¹ Jacques Jami,² Patricia Krief,¹ and Christian Boitard¹

Insulin is a major disease determinant in type 1 diabetes, type 2 diabetes, and related disorders. The role of variations in the expression of the insulin gene has been proposed in genetic susceptibility to the three pathological conditions in humans. In contrast to humans, rodents express two proinsulin isoforms. One isoform, proinsulin 1, is expressed exclusively in islets. The second, proinsulin 2, is expressed in islets and in other tissues, especially the thymus. We took advantage of the expression of these two isoforms to introduce a null proinsulin 2 allele in NOD mice and to evaluate the consequence of a variation of proinsulin 2 gene expression on the development of type 1 diabetes on the NOD genetic background. Heterozygote NOD mutant mice carrying a null proinsulin 2 mutation showed an increased incidence of type 1 diabetes at successive backcross generations. Plasma glucose and insulin levels were identical in prediabetic mutant and in wild-type mice at 4 weeks of age. Variation in insulin gene expression is hypothesized to interfere with diabetes development at both the islet and the thymus level. *Diabetes* 51 (Suppl. 3):S489–S493, 2002

Insulin is a major disease determinant in type 1 diabetes, type 2 diabetes, and related disorders such as obesity. Variations in expression of the insulin gene have been proposed to play a role in genetic susceptibility to all three pathological conditions (1–8). A highly polymorphic variable number of tandem repeat (VNTR) located in the promoter region of the insulin gene 365 bp upstream of the transcription initiation site on chromosome 11p15 has been proposed to influence the level of expression of insulin in both the islets of Langerhans and the thymus. The polymorphism is generated by variations of the number of repetitive units and by the sequence heterogeneity of individual repeats. Alleles are usually classified in two main classes: short (26–63 re-

peats) class I alleles and long (140–200 repeats) class III alleles (9). Homozygotes for class I alleles carry an increased risk (2–5) for type 1 diabetes, whereas class III alleles confer dominant protection (1–4). Whether susceptibility or resistance to type 1 diabetes conferred by variants in the 11p15 VNTR, coined as IDDM2, relates to variations in expression of the insulin gene in β -cells or in the thymus remains elusive (3).

Lower mammals show no VNTR in the 5' region flanking the insulin gene. However, rodents express two insulin isoforms encoded by distinct genes located on chromosome 7 and 19, respectively, in the mouse. The two isoforms differ by two amino acids in the B-chain, three amino acids in C-peptide, and several differences in the leader peptide sequence. Proinsulin 1 and 2 show distinct tissue distribution. Both isoforms are expressed in β -cells, but it has been reported that only proinsulin 2 is expressed in the thymus (10). Knockout of the proinsulin 2 gene in the mouse thus provides a relevant model to study the role of variations in the expression of insulin in the thymus and islets (11) on the development of type 1 diabetes (12,13). We generated NOD mice in which one proinsulin 2 gene was missing by crossing proinsulin 2^{-/-} 129 mice and backcrossing with NOD mice. From BC2, we selected breeders carrying the proinsulin 2 gene knockout and 16 genetic markers (including I-A^{g7}) that are associated with diabetes in the NOD mouse (14). These mice were used to determine whether knockout of one proinsulin 2 allele interferes with diabetes susceptibility in the NOD mouse.

RESEARCH DESIGN AND METHODS

Animals. Proinsulin 2-deficient 129 mice and NOD mice were bred in our own facilities under specific pathogen-free conditions and allowed free access to food. The prevalence of spontaneous diabetes in our NOD colony reached 75% in female mice by 6 months of age.

Development of a speed congenic stock of proinsulin 2-deficient NOD mice. Proinsulin 2-deficient 129 mice, in which a proinsulin 2-deficient allele was functionally disrupted by insertion of LacZ and a neomycine resistance (Neo) gene, have been previously reported (11). Female proinsulin 2-deficient 129 mice were crossed with male NOD mice. Female (129/Pro2^{-/-} × NOD) F1 mice were then backcrossed onto the NOD background for eight generations. Heterozygote carriers of the proinsulin 2-deficient allele were genotyped for the Neo and LacZ genes, using previously described primers (5'-GTG TTC CGG CTG TCA GCG CA-3' and 5'-GTC CTG ATA GCG GTC CGC CA-3'; and 5'-ACG GCA CGC TGA TTG AAG CA-3' and 5'-CCA GCG ACC AGA TGA TCA CA-3', respectively) and were used as breeders at each backcross (BC) generation. Segregants from the second to the fourth BC generation were genotyped for microsatellite markers linked to 16 *Idd* loci (*Idd1* to *Idd16*), as previously described (13). At the fourth BC generation (BC4), proinsulin 2^{+/-} heterozygote mice were intercrossed to generate proinsulin 2^{-/-} homozygote

From the ¹Institut National de la Santé et de la Recherche Médicale (INSERM) U561, Hôpital Saint Vincent de Paul, Paris, France; and ²INSERM U257, Institut Cochin de Génétique Moléculaire, Paris, France.

Address correspondence and reprint requests to C. Boitard, INSERM U561, Hôpital St Vincent de Paul, 82 ave. denfert Rochereau, 75014 Paris, France. E-mail: boitard@cochin.inserm.fr.

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BC, backcross; VNTR, variable number of tandem repeat.

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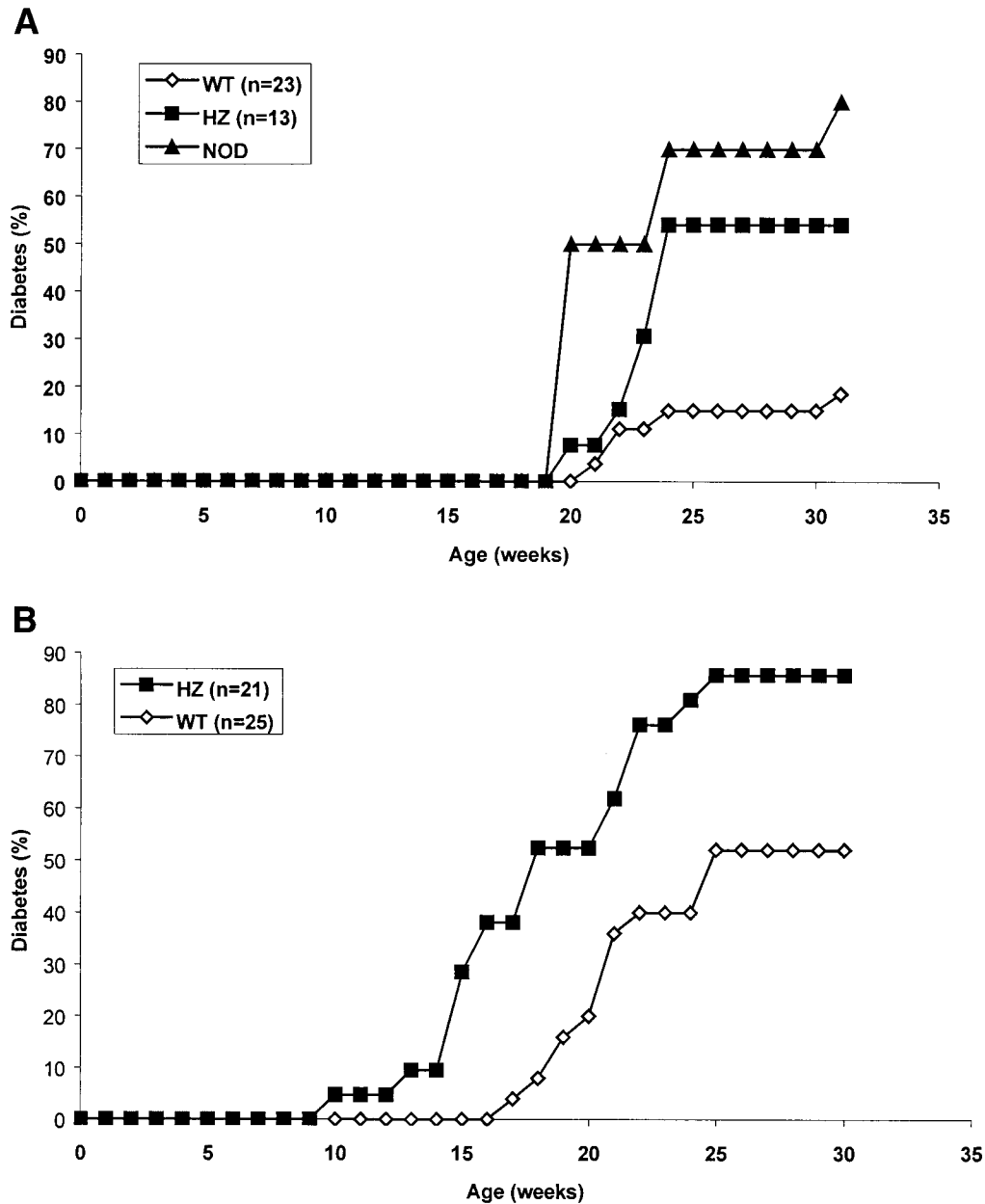


FIG. 1. Diabetes in proinsulin 2^{+/-} mice and proinsulin 2^{+/+} wild-type mice at BC2 and BC8 (A and B, respectively) and in conventional NOD mice (A). HZ, proinsulin 2^{+/-} heterozygote mice; NOD, conventional NOD mice; WT, wild-type proinsulin 2^{+/+}. The difference between proinsulin 2^{+/-} and proinsulin 2^{+/+} at BC2 (A) and BC8 (B) was highly significant ($P < 0.02$ and $P < 0.005$, respectively).

mice. Proinsulin 2^{+/-} heterozygote mice and proinsulin 2^{-/-} homozygote mice were distinguished by amplification of the proinsulin 2 gene, using 5' and 3' primers (11), providing a 676-bp fragment only in heterozygote animals.

Detection of proinsulin 1 and 2 mRNA. Detection of mRNA in tissues was performed in 2- and 3-week-old mice. Pancreatic islets of Langerhans were isolated as previously described with slight modifications. RNA were extracted from fresh tissues using guanidium thiocyanate and treated with DNase before reverse transcription to avoid contamination with genomic DNA. Proinsulin 1 and 2 were amplified using two pair of primers: 5'-CCA TCA GCA AGC AGG TTA TTG TTT C-3' and 5'-CAG CTC CAG TTG TGC CAC TTG TG-3', which were specific for the proinsulin 2 and provided a 232-bp fragment, and 5'-CCA TCA GCA AGC AGG TCA TTG TTT-3' and 5'-GCG GGA CTT GGG TGT GTA GAA GAA-3', which amplified the proinsulin 1 and provided a 193-bp fragment.

Assessment of diabetes. NOD backcrossed mice were monitored weekly for glucosuria. Blood glucose was measured when glucosuria was detected. Mice were considered diabetic after two consecutive glycemic values >300 mg/dl. Glycemia was assessed using test strips and a Euroflash glucometer (Life-Scan). Plasma insulin was assessed by radioimmunoassay (Linco Research).

Statistical analysis. Incidence of diabetes in proinsulin 2^{+/-} and wild-type mice was described using Kaplan-Meier estimates and compared using the log-rank test.

RESULTS

Female (129/Pro2^{-/-} × NOD) F1 mice were backcrossed onto the NOD background, and BC mice carrying the proinsulin 2-deficient allele were then backcrossed onto the NOD background for eight successive generations. Segregants from the second to the fourth BC were genotyped for 16 NOD microsatellite markers reported as associated with diabetes in the NOD mouse, i.e., *Idd1* to *Idd-16*. After two generations of backcrossing (BC2), four diabetes susceptibility genes were fixed, including *Idd1* (I-A^{g7}). At the fourth BC generation (BC4) and in subse-

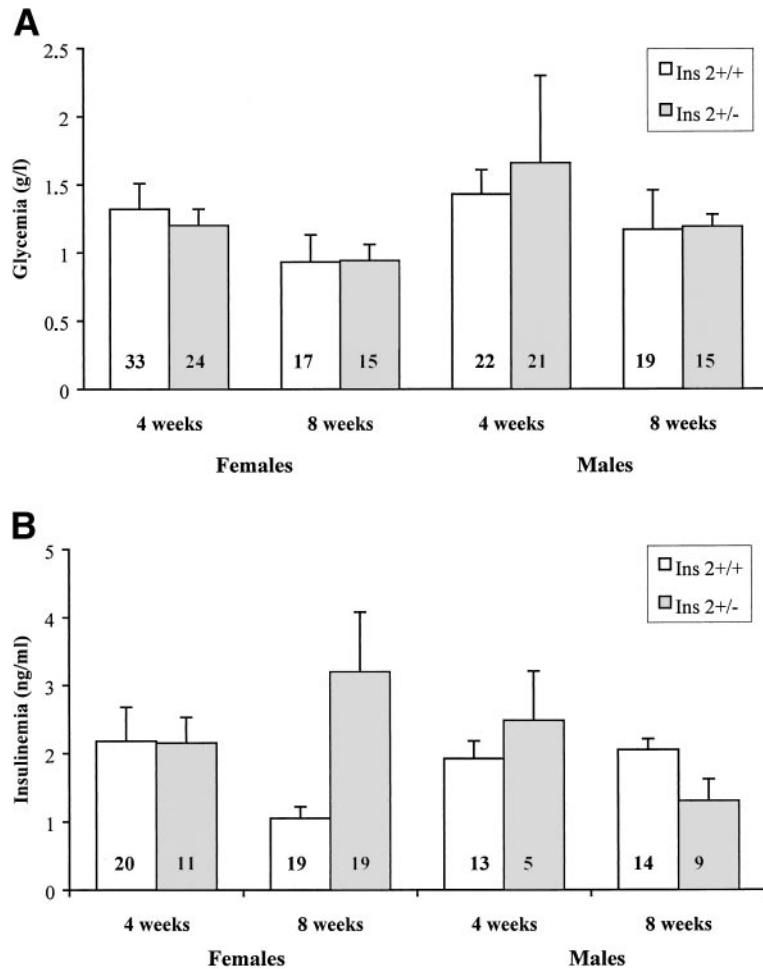


FIG. 2. Blood glucose (A) and plasma insulin (B) in male and female proinsulin 2^{+/-} and proinsulin 2^{+/+} wild-type mice at 4 and 8 weeks of age. Numbers at the bottom of each column indicate the number of mice tested.

quent BC generations, all proinsulin 2^{+/-} heterozygotes were homozygous for all 16 NOD *Idd* alleles.

An increased incidence of diabetes was observed in proinsulin 2^{+/-} as compared with wild-type mice at each BC generation analyzed (i.e., BC2, BC7, and BC8; $P < 0.02$, $P < 0.001$, and $P < 0.005$, respectively). The prevalence of diabetes at BC2 and BC8 generation is shown in Fig. 1. Noticeably, the incidence of diabetes was low in wild-type BC2 mice, presumably because of insufficient backcrossing and presence of only 87.5% NOD genes. Despite the presence of 12.5% 129 genes at BC2, the presence of the proinsulin 2 null mutation allowed an unexpectedly high incidence of diabetes, reaching 54.0% at 32 weeks of age in female mice as compared with 18.5% in wild-type mice. In further BC generations, the incidence of diabetes increased to reach 52.0% at BC8 in wild-type mice but remained significantly increased in proinsulin 2^{+/-} mice (85.2%, $P < 0.005$). Despite the high diabetes incidence observed, female prediabetic proinsulin 2^{+/-} mice were comparable to their wild-type counterparts for weight, glycemia, and pancreatic mass at 4 and 8 weeks of age. They also showed identical plasma insulin levels at 4 weeks of age, but they had higher plasma insulin levels at 8 weeks of age (Fig. 2).

We took advantage of the possibility to generate proinsulin 2^{-/-} mice to determine whether proinsulin 1 is expressed in the thymus (Fig. 3). Discrepant results have been reported for tissue distribution of proinsulin 1 expres-

sion in the mouse (10,15,16). Proinsulin 2^{+/-} progeny resulting from BC4 was intercrossed, and thymus was obtained from proinsulin 2^{-/-} mice. We verified that proinsulin 2 mRNA was not detected in the islets and thymus of proinsulin 2^{-/-} mice, but it was detected in wild-type and in heterozygous proinsulin 2^{+/-} mice (not shown). However, by using primers that amplify a 193-bp proinsulin 1 fragment, proinsulin mRNA was detected in the thymus of

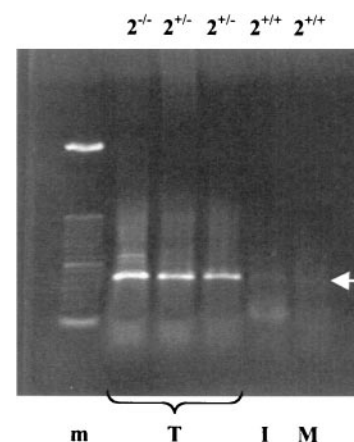


FIG. 3. Expression of proinsulin 1 mRNA in thymus of proinsulin 2^{-/-}, proinsulin 2^{+/-}, and proinsulin 2^{+/+} mice and in intestine and muscle of proinsulin 2^{+/+} mice. I, islets; m, markers; M, muscle; T, thymus. White arrow, proinsulin mRNA.

proinsulin 2^{-/-} mice as well as in the islets. No proinsulin was detected in proinsulin 2^{-/-} control tissues.

DISCUSSION

Insulin exemplifies a key β -cell function through its secretion in response to glucose and is a key autoantigen in the development of type 1 diabetes (15–20). By generating β -cell-deprived NOD mice using alloxan, we previously showed that β -cells were mandatory for the activation of autoreactive lymphocytes against β -cell antigens in type 1 diabetes (21). High levels of plasma insulin in the fasting state and in response to glucose have further been observed at the early stage of development of insulinitis in the NOD mouse, suggesting that abnormal β -cell function may play a role in the autoimmune process that leads to diabetes (15). Prevention of diabetes by insulin therapy in prediabetic NOD mice (22) and BB rats has been interpreted as resulting from β -cell rest and as further evidence for the importance of β -cell function in type 1 diabetes. In humans, genetic susceptibility associated with IDDM2 has been considered to link β -cell function to diabetes development (1–4). An alternative hypothesis is that insulin modulates lymphocyte activation by interacting with lymphocyte insulin receptors on activated T-cells or the T-cell receptor on proinsulin-specific lymphocytes (15). Insulin also possibly influences thymic selection of insulin-specific lymphocytes through its expression by bone marrow-derived cells in the thymus (3). Genetic variation of insulin gene expression may thus influence either β -cell function or selection or activation of T-cells, respectively, in the thymus and the periphery.

To establish a model to study these different possibilities, we introduced genetic variation of insulin gene expression by transferring a null mutation of the proinsulin 2 gene onto the NOD genetic background. We observed an increased incidence of diabetes in heterozygote NOD mice carrying a null proinsulin 2 allele at successive BC generations. Our data indicate that genetic alteration of proinsulin 2 expression directly influences diabetes outcome in the NOD mouse. Furthermore, we have evidence that altered expression of the proinsulin 2 gene has direct consequences on lymphocyte recognition of proinsulin. Anti-insulin autoantibodies were detected with a higher frequency in proinsulin 2^{+/-} mice than in wild-type mice (data not shown). This does not exclude, however, that introducing variation in expression of the proinsulin 2 gene also influences diabetes development by modifying islet cell function. On the 129 genetic background, an increase in insulin 1 transcripts and in β -cell mass has been reported in proinsulin 2^{-/-} mutants. This compensatory response is reflected by normal insulin content, normal insulin levels, and normal glucose tolerance tests in mutant mice (12). On the NOD background, glucose tolerance tests were normal in proinsulin 2^{+/-} heterozygotes as compared with wild-type NOD mice. Plasma insulin levels were, however, increased at 8 weeks of age in proinsulin 2^{+/-} mice, possibly in relation with the diabetes autoimmune process and increased incidence of diabetes in female mutant mice. Increased plasma insulin levels have previously been reported in 8-week-old NOD mice as compared with control strains (15).

Finally, beyond expression of proinsulin 2 in the thymus (10,23, and our unpublished data), our data showing

expression of proinsulin in the thymus of proinsulin 2^{-/-} mice demonstrates that proinsulin 1 can also be expressed in the mouse thymus. Variation in expression of genes encoding for β -cell autoantigens in the thymus possibly influence the pool of autoantigen-specific T-cells present in the peripheral T-cell pool and contribute to development of autoimmunity. A proinsulin 2 knockout model will allow us to precisely correlate expression level of proinsulin 1 and 2 in the thymus with susceptibility to type 1 diabetes. It will help to determine the contribution of proinsulin 1- and 2-specific lymphocytes to autoimmunity and the contribution of proinsulin 1 and 2 secretion by β -cells to diabetes development.

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