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Ontogeny of Glutamic Acid Decarboxylase Gene Expression in the Mouse Pancreas

Jean-Marie Pleau,[1] Mark Throsby, Anne Esling, and Mireille Dardenne
CNRS URA 1461, Université Paris V, Hôpital Necker, 161 rue de Sèvres, 75015 Paris, France

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Using two RT–PCR quantitative assays, we measured the pancreatic expression of the two isoforms of glutamic acid decarboxylase in the foetus, newborn and 14-, 21- and 35-day-old male and female NOD and C57BL/6 mice. In the C57BL/6 mouse, GAD 67 pancreatic expression is stable; in NOD mice, GAD 67 expression is similar to that found in control mice, except at 5 weeks of age, when pancreatic GAD 67 expression is about 2.5 times higher than in C57BL/6 mice. The pancreatic expression of GAD 65 is under the detection limit of the assay until 5 weeks of age. The overexpression of GAD 67 characterized in pancreas from 5-week-old NOD mice could be the result of β cell hyperactivity, previously reported in this mouse strain.

Insulin dependent diabetes mellitus (IDDM) is a genetically influenced autoimmune disease in which insulin producing cells in pancreatic islets are progressively destroyed (1). Several β cell autoantigens have been described, such as insulin (2), carboxypeptidase-H (3), heat shock protein (4), peripherin (5), tyrosine phosphatase (6) and glutamic acid decarboxylase (GAD) (7). GAD is an enzyme responsible for the production of the neurotransmitter γ-amino butyric acid (8). It exists in two forms which differ by their molecular weight, GAD 65 and GAD 67 (9). The two GAD isoforms are encoded by two non-allelic genes (10). Nonobese diabetic (NOD) mice spontaneously develop diabetes and serve as a model for human type I diabetes (11). Previous studies have demonstrated that an immune response to GAD develops in NOD females, concurrent with the onset of insulitis. Autoreactive T cells specific to GAD 65 have been characterized at three weeks of age, and GAD 67 specific T cells appear one week later (12-13), suggesting a role for GAD 67 during the development of the autoimmune process leading to diabetes.

In previous studies, we developed two competitive RT-PCR assays to measure the expression of the two GAD isoforms in pancreases from 5-, 10- and 15-week-old male and female NOD, BALB/C and C57BL/6 mice (14-15). Similarly, we measured the expression of the two GAD isoforms in brains from 5-week-old mice. In the mouse brain, the GAD 67 isoform was expressed at levels 1000 times higher than GAD 65; however, the expression of the two GAD isoforms was similar whatever the age, sex or strain studied (15). Conversely, in comparison with nonautoimmune mouse strains, we observed a sexual dimorphism for GAD 65 expression in NOD mice (14) and an overexpression of GAD 67 in the pancreas (15).

In the present study, we analyzed the pancreatic expression of GAD 67 in male and female foetuses at 18 days of gestation, in newborns and in 14-, 21- and 35-day-old NOD and C57BL/6 mice. We also measured GAD 65 expression in the same samples.

MATERIALS AND METHODS

Animals

NOD and C57BL/6 mice used in this study were obtained from our locally inbred colonies maintained under specific pathogen-free conditions.

Preparation of RNA

Total RNA was isolated from each mouse pancreas and from pools of four pancreases from foetuses and from newborn mice by the guanidium isothiocyanate/cesium chloride method (16). RNA concentration and integrity were estimated by spectrophotometry and by denaturant gel electrophoresis, respectively. Prior to expression analysis, RNA preparations were submitted to RT-PCR using specific primers for mouse β actin (table I) and only sample preparations with similar levels of β actin amplicon were used for the competitive RT-PCR assays.

[1] To whom correspondence should be adressed. Fax: 33 (1) 44 49 06 76. E-mail: dardenne@infobiogen.fr.

Abbreviations: GAD: glutamic acid decarboxylase; stRNA: standard RNA; RT: reverse transcription; PCR: polymerase chain reaction; NOD: nonobese diabetic.

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Competitive RT-PCR

GAD 67 expression. The measurement of mRNA encoding for GAD 67 in total RNA preparations from the mouse pancreas is described elsewhere (15). Briefly, 3μg of total RNA from mouse pancreas and increasing levels of competitor (1×10^8 to 0.75×10^6) molecules were reverse transcribed using hexanucleotide primers and the Promega reverse transcription system, following the manufacturer's instructions. The cDNAs were amplified using two specific primers (table I) for 32 cycles (denaturation 1 min at 94°C, annealing 1 min at 55°C, extension 1min at 72°C) in the presence of [α^{32}P] dCTP (0.5 μCi), then digested overnight with *Pvu*II, since this restriction site is present in sample and absent in standard PCR products. The two amplicons were separated by acrylamide gel electrophoresis.

GAD 65 expression. The measurement of mRNA encoding for GAD 65 in total RNA preparations from the mouse pancreas is described elsewere (14). Briefly, constant amounts of total RNA (2μg) from mouse pancreas and serial dilutions of the competitor (1×10^6 to 1.2×10^3 molecules) were reverse transcribed into cDNA using hexanucleotide primers and the Promega reverse transcription system, following the manufacturer's instructions. Competitive PCR was then performed using GAD 65 specific primers (table I). First the RT mixture was submitted to 14 cycles of amplification and a second PCR was performed (29 cycles) on 10% of the previously amplified material. The amplification profile involved denaturation at 95°C for 1 min, primer annealing at 54°C for 1 min and extension at 72°C for 1 min. During the reamplification step, 0.5 μCi of [α^{32}P] dCTP were added to each tube. The standard and sample PCR products were separated on acrylamide gel after overnight digestion with *Tth* 111 I.

Evaluation of GAD 67 and GAD 65 mRNA molecules in the sample. After electrophoresis, the acrylamide gel was stained with ethidium bromide and the bands corresponding to the PCR products from the sample and the competitor were excized and the radioactivity counted. The ratios of incorporated radioactivity in the competitor and in the sample PCR products were plotted in log-log scale versus the initial level of competitor, and the number or specific mRNA molecules present in the sample were extrapolated from this plot, as indicated in fig. 1.

Statistical Analysis

Data are expressed as means ± SD from four different samples. The statistical significance of differences between mean values in different groups was evaluated using Student's *t* test.

RESULTS

Ontogeny of GAD 67 Expression in the Mouse Pancreas

To measure GAD 67 expression in the mouse pancreas, we developed a competitive RT-PCR assay reported elsewere (15) and based on the original procedure described by Wang et al (17). We measured the absolute number of GAD 67 mRNA molecules/μg of total RNA expressed in the mouse pancreas in foetuses at 18 days of gestation, in newborn and in 14-, 21- and 35-day-old male and female NOD and C57BL/6 mice. In foetuses, newborns and until 21 days of age, pancreatic GAD 67 expression remained constant (Fig.2). However, the level of GAD 67 expression was higher in C57BL/6 mice, $\approx 1 \times 10^6$ GAD 67 mRNA molecules/μg of total RNA, versus $\approx 0.5 \times 10^6$ for NOD mice. At 35 days of age, GAD 67 pancreatic expression in C57BL/6 mice was similar to that found at 21 days of age. Conversely, pancreatic GAD 67 expression increased significantly after 21 days of age in both sexes of NOD mice and reached 2.5×10^6 GAD 67 mRNA molecules/μg of total RNA in 35-day-old animals.

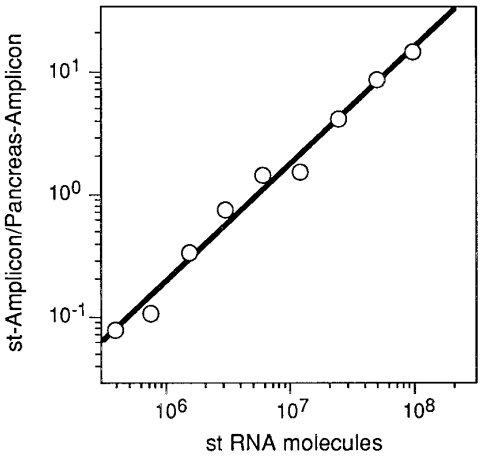

FIG. 1. Titration curve for GAD 67 mRNA from mouse pancreas. 3μg of total RNA from mouse pancreas were reverse transcribed in the presence of increasing amounts of stRNA (1×10^8 to 0.75×10^6 molecules). Duplicates of the RT mixtures were then submitted to 32 cycles of amplification. After *Pvu* II digestion, the PCR products were separated by gel electrophoresis. After ethidium bromide, staining bands were cut out and the radioactivity recovered from the amplicons was determined. The calibration curve was established as follows: after subtraction of control values, the ratio of st-amplicon/sample-amplicon corrected for their respective dC contents was plotted on a log–log scale against the amount of stRNA (each point of the calibration curve was the result of the mean of duplicates). The absolute number of GAD 67 mRNA molecules present in the sample was deduced by extrapolation of the stRNA level which gave an st-amplicon/sample–amplicon ratio equal to 1.

TABLE I

PCR Primers

cDNA	Sequence	
Mouse β actin	Sense	5′TAAAGACCTCTATGCCAACAGT-3′
	Antisense	5′CACGATGGAGGGGCCGGACTATC-3′
GAD 65	Sense	5′AGCACACAAATGTCTGCTTCTG-3′
	Antisense	5′ATGTCTTGGTGAGTTGCTGCAG-3′
GAD 67	Sense	5′CATGGCGGCTCGGTACAAAGTA-3′
	Antisense	5′AACAGTGGTGCCTGCGGTTGC-3′

Ontogeny of the GAD 65 Expression in the Mouse Pancreas

We also measured GAD 65 expression in the same samples, using an RT-PCR competitive assay. GAD 65 expression in the mouse pancreas was lower than that of GAD 67. In our previous evaluation, we found $\approx$ 20000 molecules of GAD 65 mRNA/μg of total RNA in 35-day-old NOD males and 5 times less in females (14).

In pancreatic RNA from younger mice, either male or female, we did not detect a GAD 65 PCR product. This result strongly suggests that GAD 65 pancreatic expression in these mice was under the detection limit of the assay, which was evaluated to be around 2000-3000 molecules of GAD 65 mRNA/μg of total RNA (14). In view of these results, we postulated that GAD 65 expression in the pancreas from foetuses, newborns and 14- and 21-day-old mice was lower than 2000 molecules/μg of total RNA. However, GAD 65 pancreatic expression increased between 21 and 35 days of age in NOD and C57/BL6 mice, since it was detectable in 35-day-old mice.

DISCUSSION

In previous work, we set up two RT-PCR competitive assays to measure the absolute number of GAD 65 (14) and GAD 67 (15) mRNA molecules in total RNA preparations. We applied this method to study the expression of the two GAD isoforms in pancreases from male and female NOD mice and from mice of two nonautoimmune mouse strains taken as controls, at 5, 10 and 15 weeks of age. We also measured, in the same mouse strains at 5 weeks of age, the expression of the two GAD isoforms in the brain. Several differences were observed in the expression of the two GAD isoforms in the pancreas of the NOD mouse. Firstly, at 5 weeks of age, GAD 65 expression was 5 times lower in NOD females than in NOD males. Secondly, whatever the sex or age of the mouse, GAD 67 was overexpressed in the NOD pancreas compared with the two nonautoimmune mouse strains. The sexual dimorphism for GAD 65 expression and the overexpression of GAD 67 were specific to the pancreas since these differences were not characterized in the mouse brain.

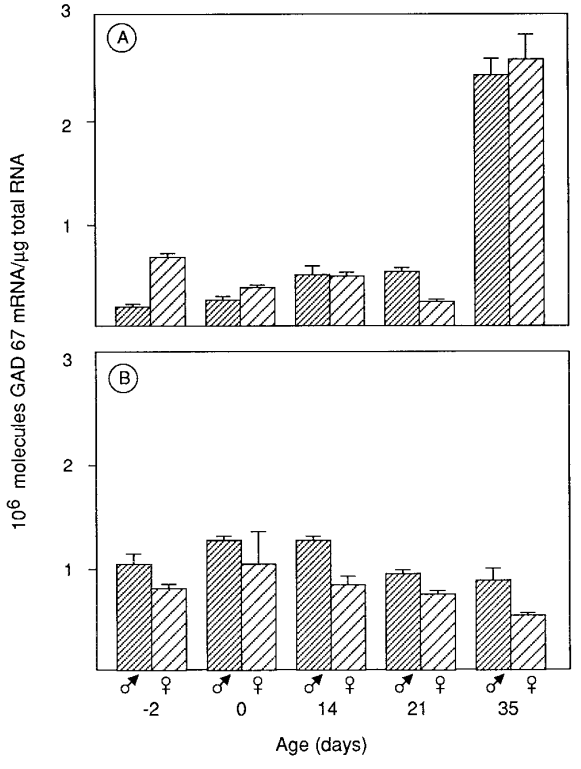

FIG. 2. Specific GAD 67 mRNA levels in pancreas from foetuses, newborns and 14-, 21-, and 35-day-old NOD and C57BL/6 mice. For each determination the calibration curve and the absolute number of GAD 67 mRNA molecules present in the sample were established as described in Figure 1. The results are expressed as mean $\pm$ SD of four samples per group. A) NOD; B)C57BL/6. At 35 days of age, p was < 0.005 between NOD and C57BL/6 sex-matched samples.

In order to establish the age at which these pancreatic differences occurred, we measured GAD 65 and GAD 67 mRNA levels in pancreases from foetuses, newborns and 14-, 21- and 35-day-old NOD and C57/BL6 mice. We were not able to detect GAD 65 pancreatic expression in mice less than 21 day-old, suggesting that the level of GAD 65 expression was probably under the detection limit of the assay. In contrast, we detected pancreatic GAD 65 expression in 35-day-old mice, suggesting an increase in GAD 65 pancreatic expression between 21 and 35 days of age. Concerning GAD 67, its expression was detected in pancreases from foetuses, newborns and 14-, 21- and 35-day-old mice. This expression was stable until 21 days of age in both mouse strains and increased in the older NOD mice. Our results confirm the previous data reported by Martignat et al (18); these authors, using a semiquantitative RT-PCR, observed stable pancreatic GAD 67 expression in foetuses and newborns, followed by increased expression between 3 weeks and 5 weeks of age, in both male and female NOD mice. Conversely, in BALB/c mice, GAD 67 pancreatic expression remained stable from fetal life to 5 weeks of age.

On the basis of these findings, we propose a hypothesis concerning the autoimmune reaction developed by NOD mice against both GAD isoforms. The very low GAD 65 pancreatic expression, particularly in the NOD female, could explain the absence of tolerance to GAD 65. As early as 4 weeks of age, the first GAD 65 autoreactive T cells appear (12,13) and induce the lysis of some β cells, which release GAD 67. Then GAD 67 would induce strong activation of the immune system since it is expressed to a greater extent in the NOD β cells. Some epitopes shared by both isoforms could generate GAD 65 and GAD 67 autoreactive T cells, or particular GAD 67 epitopes might induce GAD 67 specific immune responses which take over from the GAD 65 primary response. In any case, the autoimmune reaction appears to be amplified, encompassing other autoantigens and leading to the destruction of insulin producing cells.

Evidence suggests that GAD 67 expression occurs selectively in islet cells of the mammalian pancreas. In this study, however, we measured the absolute number

of GAD 67 mRNA molecules in the total pancreas. It has been well demonstrated that the ratio between the endocrine and exocrine pancreas differs as a function of age. In the foetus and neonate, islets comprise 20 to 30% of the pancreatic mass (19). From this point until adulthood, the islet mass increases about 5 fold. However, during the same period the exocrine tissue mass increases about 15 fold. This means that the expression which was found to be stable when GAD 67 expression was measured in the total pancreas, increased in terms of islet cell expression. In addition, the overexpression of GAD 67 characterized in total RNA from 5-week-old NOD mice is greater if considered in terms of islet cells. The differences observed at 5 weeks of age between NOD and C57BL/6 are consistant since they were both estimated in the total pancreas. However, we cannot exclude some differences in pancreas development between NOD and C57BL/6 mice. Moreover, the observed overexpression in NOD mice may reflect hyperactivity of the β cell as already demonstrated for glucose sensitivity and insulin release (20). This hyperactivity could be the result of inappropriate regulation of some genes expressed in the NOD mouse β cell, particularly that of insulin, GAD or other putative autoantigens. Moreover, the differences in GAD 65 and GAD 67 expression observed between NOD and non-autoimmune mouse strains appeared between 21 and 35 days of age, suggesting that the expression of both GAD genes could be under the control of sex hormones and that the promotor regions in the NOD mice could present some genetic differences or represent a particular allele which is more sensitive to steroid action.

Nevertheless, the influence of changes in diet occurring at weaning, and/or hormonal regulation on IDDM remains to be conclusively demonstrated.

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