Insulin Expression Levels in the Thymus Modulate Insulin-Specific Autoreactive T-Cell Tolerance

The Mechanism by Which the *IDDM2* Locus May Predispose to Diabetes

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Type 1 diabetes results from autoimmune destruction of the insulin-producing pancreatic β-cells. Evidence from our laboratory and others has suggested that the *IDDM2* locus determines diabetes susceptibility by modulating levels of insulin expression in the thymus: the diabetes-protective class III alleles at a repeat polymorphism upstream of the insulin gene are associated with higher levels than the predisposing class I. To directly demonstrate the effect of thymic insulin expression levels on insulin-specific autoreactive T-cell selection, we have established a mouse model in which there is graded thymic insulin deficiency in linear correlation with insulin gene copy numbers, while pancreatic insulin remains unaltered. We showed that mice expressing low thymic insulin levels present detectable peripheral reactivity to insulin, whereas mice with normal levels show no significant response. We conclude that thymic insulin levels play a pivotal role in insulin-specific T-cell self-tolerance, a relation that provides an explanation for the mechanism by which the *IDDM2* locus predisposes to or protects from diabetes. *Diabetes* 51: 1383–1390, 2002

Type 1 diabetes results from autoimmune destruction of insulin-producing pancreatic β-cells (1). The strong genetic predisposition to this disease behaves as a polygenic trait, partly explained by the major histocompatibility complex (MHC) (*IDDM1* locus on the HLA class II region) (2,3). Of the remaining loci, only *IDDM2* has been confirmed and functionally studied. It involves a polymorphism upstream of the insulin gene promoter that consists of a variable number of tandem repeats (VNTR). Two VNTR allele classes have been characterized: alleles composed of 30–60 repeats of the consensus unit ACAGGGGTCTGGGG are referred to as class I and have been found to predispose to type 1 diabetes; alleles containing 100 repeats or more are class III and have a dominant protective effect. Intermediate (class II) alleles are rare. Although VNTR alleles have little effect on pancreatic insulin expression, in the thymus, class I alleles correlate with low and class III with high levels of insulin expression (4,5). This differential expression suggests a mechanism for the effect of the *IDDM2* locus. Differential thymic insulin expression may also play a role in the diabetes susceptibility of the NOD mouse. Indeed, the low-level insulin expression in the thymus of diabetes-resistant mice (6,7) was previously reported to be absent in NOD mice (7), although this finding was contradicted by a more recent study that found it to be normal (8).

The thymus is the organ responsible for the generation of the T-cell repertoire through which the immune system is able to distinguish self- from non–self-antigens (9). The T-cell central tolerance process depends on thymic abundance of the specific self-antigen which, in turn, determines the avidity of the interaction between the autoreactive T-cells and the thymic antigen-presenting cells (10–13). In recent years, mounting evidence suggests that thymic negative selection may be important for tolerance to tissue-restricted autoantigens, such as insulin, which do not reach the thymus in high enough concentrations to result in avid interactions (14,15). Proinsulin is expressed at low levels in both human (4,5) and mouse (6) thymus, and the immune tolerance to transgenic xeno-antigens (16) or allo-antigens (17) expressed from the insulin promoter can be transferred by thymus transplant to nontransgenic recipients. However, the role of thymic expression in insulin self-tolerance has not been directly studied to date.

Of the many putative autoantigens involved in the diabetes disease process (18–20), insulin is the only one whose extrathymic expression is specific for the β-cell, and the predominant T-cell clones isolated from insulitic pancreata in the NOD mouse are insulin-specific $CD4^+$ and $CD8^+$ T-cells (21–23). Moreover, the expression of insulin under the control of MHC class II promoter in NOD mice prevents diabetes (7), and similarly, the intrathymic administration of insulin B-chain inhibits development of the disease (24). Taken together, these data show the importance of insulin as autoantigen in diabetes and suggest that the presence of insulin in the thymus may determine tolerance to this specific autoantigen.

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ELISA, enzyme-linked immunosorbent assay; IL, interleukin; MHC, major histocompatibility complex; PHA, phytohemagglutinin; TCR, T-cell receptor; VNTR, variable number of tandem repeats.

TABLE 1
Genotyping of mice from F2 progeny

Genotype	*Ins1* (1-C)	*Ins1* (2-C)	*Ins2* (1-C)	*Ins2* (2-C)	*Ins1/2* (4-C)
Ins1-KO (1,010 bp)*	+	−	+	+	–
Ins2-KO (301 bp)	+	+	+	−	–
Ins1-WT (280 bp)	+	+	−	−	+
Ins2-WT (193 bp)	−	−	+	+	+

*Band size detected. +, detected; –, not detected.

We therefore advance the hypothesis that genetically determined thymic insulin levels play a critical role in insulin-specific autoreactive T-cell selection. To test this hypothesis, we have established a mouse model in which there is graded thymic insulin deficiency without any change in the levels of insulin produced by the pancreas. This was achieved by using mice with different degrees of insulin gene-dosage deficiency resulting from crosses of *Ins1*- and *Ins2*-knockout (KO) mice. Our first approach was to examine T-cell reactivity to proinsulin-related antigens in vitro in unprimed non–diabetes-prone animals, the most direct representation of selection in the thymus. We found that even under these circumstances, mice with low levels of thymic insulin expression present a measurable specific T-cell reactivity to insulin that is absent in mice with normal levels of thymic insulin expression.

RESEARCH DESIGN AND METHODS

Mice. *Ins1*-KO and *Ins2*-KO mice (25) were a gift of Dr. J. Jami (Institut Cochin, Paris, France). Chimeric animals were generated with D3 ES cells and crossed with the B6 strain. MHC haplotype, the main determinant of possible differential antigen-specific reactivity, was the same (H-2^b) in all animals used for the studies. *Ins1*-KO and *Ins2*-KO mice were crossed, and F2 progeny were used for experiments. Mice were kept in our animal facility under conditions specified by the Canadian Council of Animal Care.

Genotyping. DNA from the tail or ear of mice from F2 progeny was used to genotype for *Ins1*-wild type (WT), *Ins1*-KO, *Ins2*-WT, and *Ins2*-KO sequences by PCR using specific primers. Tables 1 and 2 show the band size and primers of each specific PCR. The PCR products were resolved by PAGE and quantified by ethidium bromide staining (GelDoc software; Bio-Rad, Hercules, CA). Band identity was confirmed with restriction enzyme digestion.

Quantitation of insulin mRNA in the pancreas and thymus. Pancreatic and thymic RNA from fasted 3-week-old mice were isolated by Trizol (Gibco, Rockville, MD) and treated with DNAse. The concentration of each sample was determined by optical density. An equal amount of each (1–2.5 μg) was reverse-transcribed to cDNA using random hexamer primers and Superscript reverse transcriptase (Gibco). A parallel sample, to which Superscript was not added, was assayed by PCR to confirm the absence of genomic DNA contamination or PCR carryover. An equal volume of each cDNA sample (2 μl from thymic cDNA and 2 μl from 1:300 diluted pancreatic cDNA) was then added to six serial dilutions of competitor (56, 28, 14, 7, 3.5, 1.7, or 0 amol/tube). The insulin competitor is an internally deleted cloned insulin sequence amplifiable by the same primers but 45 bp shorter, constructed as described by Forster (26). For loading control, we used the competitive PCR kit for cyclophilin (Quantum mRNA; Ambion, Austin, TX) as described by the manufacturer. The PCR products were resolved using PAGE, and bands were quantified to obtain a ratio of endogenous to competitor bands. The insulin ratio for each sample was normalized for the amount of starting RNA, using cyclophilin measurement as an indicator of total RNA content, and averaged. This mean represents the amount of insulin mRNA for a set of three total RNA samples. The calculations for comparing thymic and pancreatic levels across samples were as follows:

(endogenous insulin/competitor insulin) = amount of endogenous mRNA relative to a known amount of competitor ($n = 3$);

1/(endogenous cyclophilin/competitor cyclophilin) = normalizing factor for each sample;

(normalizing factor) × (endogenous insulin/competitor insulin) = normalized ratio for the amount of endogenous insulin ($n = 3$);

(normalized ratio) × (mol competitor added) = amount of insulin ($n = 3$).

Quantitation of insulin peptide in the pancreas and thymus. Thymus and pancreas extracts were obtained by acid/ethanol treatment as described by Vafiadis et al. (4). Total protein concentration of each sample was calculated using DC Protein-Assay (Bio-Rad), and this served for normalization of the insulin amount of each sample. Insulin concentration was measured by the Ultra-mouse insulin kit (Alpco, Windham, NH) as recommended by the manufacturer. The calculation of insulin amount was performed as follows:

1/(concentration of total protein) = ratio of normalization;

(insulin concentration calculated by enzyme-linked immunosorbent assay [ELISA]) × ratio of normalization = amount of insulin peptide ($n = 3$).

T-cell stimulation assay and cytokine detection. Wool-nylon–purified spleen T-cells (2.5×10^5) from unprimed adult mice were incubated with irradiated autologous spleen cells (2.5×10^5) in the presence or absence of antigens: human proinsulin (Sigma Chemical, St. Louis, MO) or ovalbumin (Sigma) for 3 days or phytohemagglutinin (PHA) (Sigma) for 24 h. In another experiment, 3×10^5 spleen cells were incubated with insulin B-chain (B:9–23) (Cambridge Research Biochemicals, Billingham, U.K.) or human insulin C-peptide (Polypeptide Laboratories, Torrance, CA) in the presence or absence of 25 units/ml murine interleukin (IL)-2 (Sigma) for 3 days in RPMI/0.5% heat-inactivated mouse serum and additives, then incubated for 16 h with 1 μCi [^{3}H]thymidine (ICN Biomedicals, Aurora, OH). Supernatant (100 μl) was removed for cytokine analysis, the cells were incubated for an additional 16 h with 1 μCi [^{3}H]thymidine and harvested (Cell Harvester; Inotech, Rockville, MD), and thymidine incorporation was measured by β-counter. IL-4 and interferon-γ production were measured using a two-site ELISA kit (Pharmingen, San Diego, CA).

TABLE 2
PCR and quantitative PCR conditions

	Primers	Temperature (°C)	Size (bp)
Ins1-KO gene	Sense: 5′-TCCAGATACTTGAATTATTCCT-3′ α-Sense: 5′-TGGCGGACCGCTATCAGGAC-3′	53	1,010
Ins2-KO gene	Sense: 5′-ACGGCACGCTGATTGAAAGCA-3′ α-Sense: 5′-TGTATCATCTGGTCGCTGG-3′	53	301
Ins1-WT gene	Sense: 5′-TCAGTGCTGCACCAGCATCT-3′ α-Sense: 5′-TCCAGATACTTGAATTATTCCT-3′	53	280
Ins2-WT gene	Sense: 5′-TGCTCAGCTACTCCTGACTG-3′ α-Sense: 5′-GTGCAGCACTGATCTACAAT-3′	53	193
Insulin	Sense: 5′-GGCTTCTTCTACACACCCCA-3′ α-Sense: 5′-TACCAGCTGGAGAACTACTG-3′	55	Endo = 193 Comp = 158
Cyclophilin	*	—	Endo = 216 Comp = 191

*See QuantumRNA, Quantitative RT-PCR Module (Ambion). Comp, competitor; Endo, endogenous.

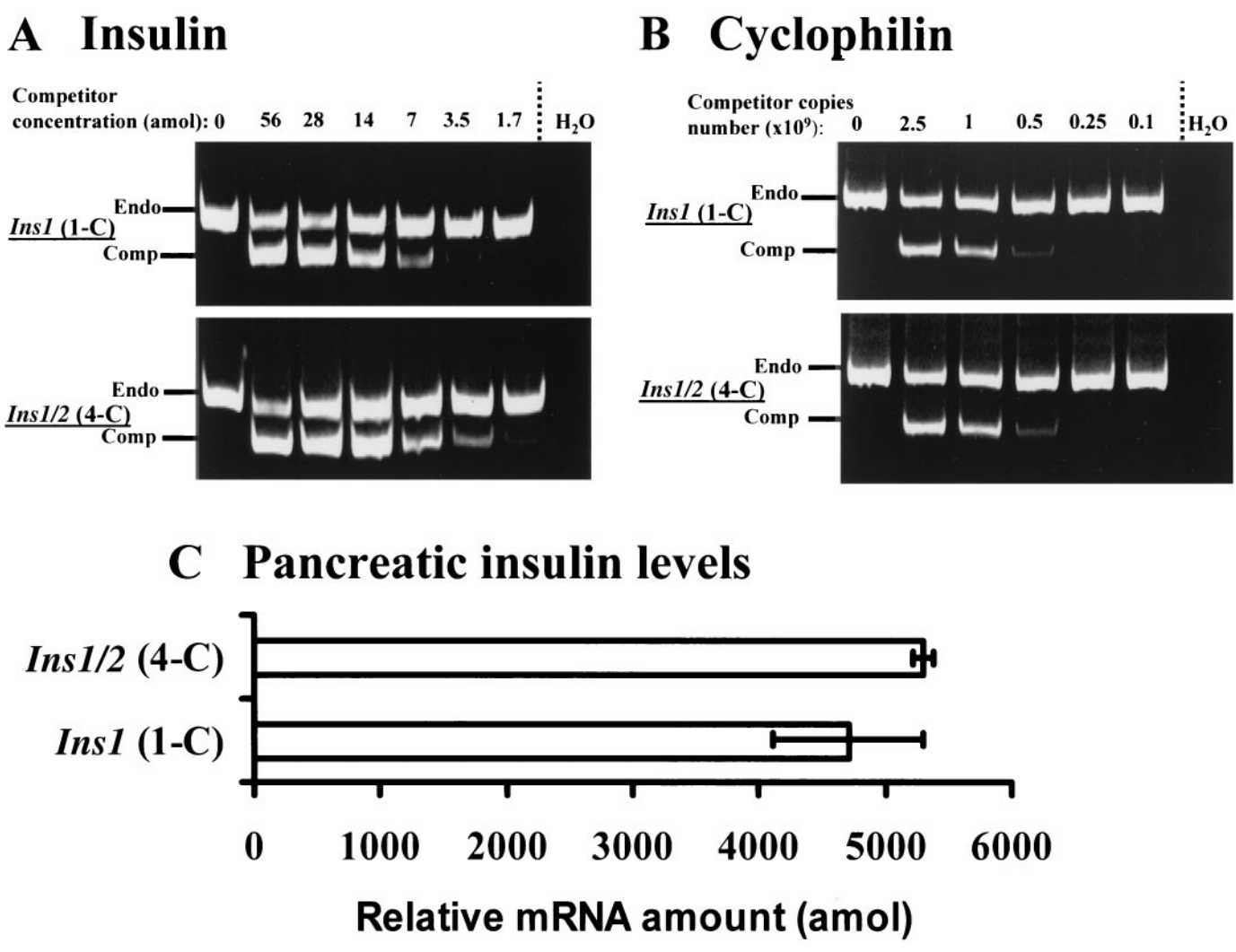

FIG. 1. Quantification of insulin mRNA levels in the pancreas. *A*: PAGE analyses of quantitative RT-PCR products of insulin endogenous (endo) and graded concentration of competitor (comp) cDNA coamplification. *B*: PAGE analyses of quantitative RT-PCR product of cyclophilin endogenous and competitor cDNA coamplification. *C*: Pancreatic insulin mRNA levels in mice with one copy of *Ins1* gene [*Ins1* (1-C)] and mice with four *Ins* gene copies [*Ins1/2* (4-C)]. The electrophoresis shown is representative of three experiments. The results are means ± SD of three experiments (n = 3 mice/genotype).

RESULTS

A mouse model of graded deficiency in insulin gene copy numbers. In rodents, two unlinked, nonallelic genes, *Ins1* and *Ins2*, encode insulin. Homozygous *Ins1*-and *Ins2*-KO mice (25) were crossed, and intercrosses between F1 progeny (100% heterozygote) resulted in F2 progeny with one (1-C), two (2-C), three (3-C), or four (4-C) copies of the four possible active insulin gene copies. The presence of each of the two WT and KO sequences allowed us to assign mice according to copy number for each of the two insulin genes.

Insulin expression in the pancreas of mice as a function of insulin gene copy number. In the pancreas of adult mice, *Ins2* is expressed at two to three times higher levels than *Ins1* (27,28). Mice homozygous for targeted disruption of either *Ins1* or *Ins2*, expressing only one of the two *Ins* genes, are healthy and have normal levels of blood glucose, whereas mice homozygous for both *Ins*-KO genes die 48 h after birth (25). Insulin mRNA expression in the pancreas was measured by quantitative RT-PCR using an internally deleted competitor. The primers amplify the same size band from *Ins1*-WT and *Ins2*-WT cDNA. Figure 1 shows insulin mRNA expression in the pancreas of *Ins1* (1-C) and *Ins1/2* (4-C) mice. Because *Ins1* has been shown to be at least twofold less abundantly expressed than *Ins2*, we chose to make the comparison between *Ins1* (1-C) and *Ins1/2* (4-C) mice. As shown in Fig. 1*A*, at the same concentration of the competitor (14 amol), in *Ins1* (1-C) and *Ins1/2* (4-C) mice, the intensity ratio between endogenous and competitor bands is ~1, showing that the two mice express similar insulin levels in the pancreas. Cyclophilin, a housekeeping gene, was used to confirm equal RNA loading (Fig. 1*B*). Figure 1*C* shows the relative amounts of insulin mRNA in *Ins1* (1-C) and *Ins1/2* (4-C) mice. The data show, as expected, no difference in pancreatic insulin expression between mice with a single insulin gene copy and mice with four insulin gene copies. Moreover, no difference exists between these mice and mice with two or three insulin gene copies (data not shown).

At the protein level, insulin in the pancreatic extract was measured by the Ultra-mouse insulin ELISA kit (Alpco).

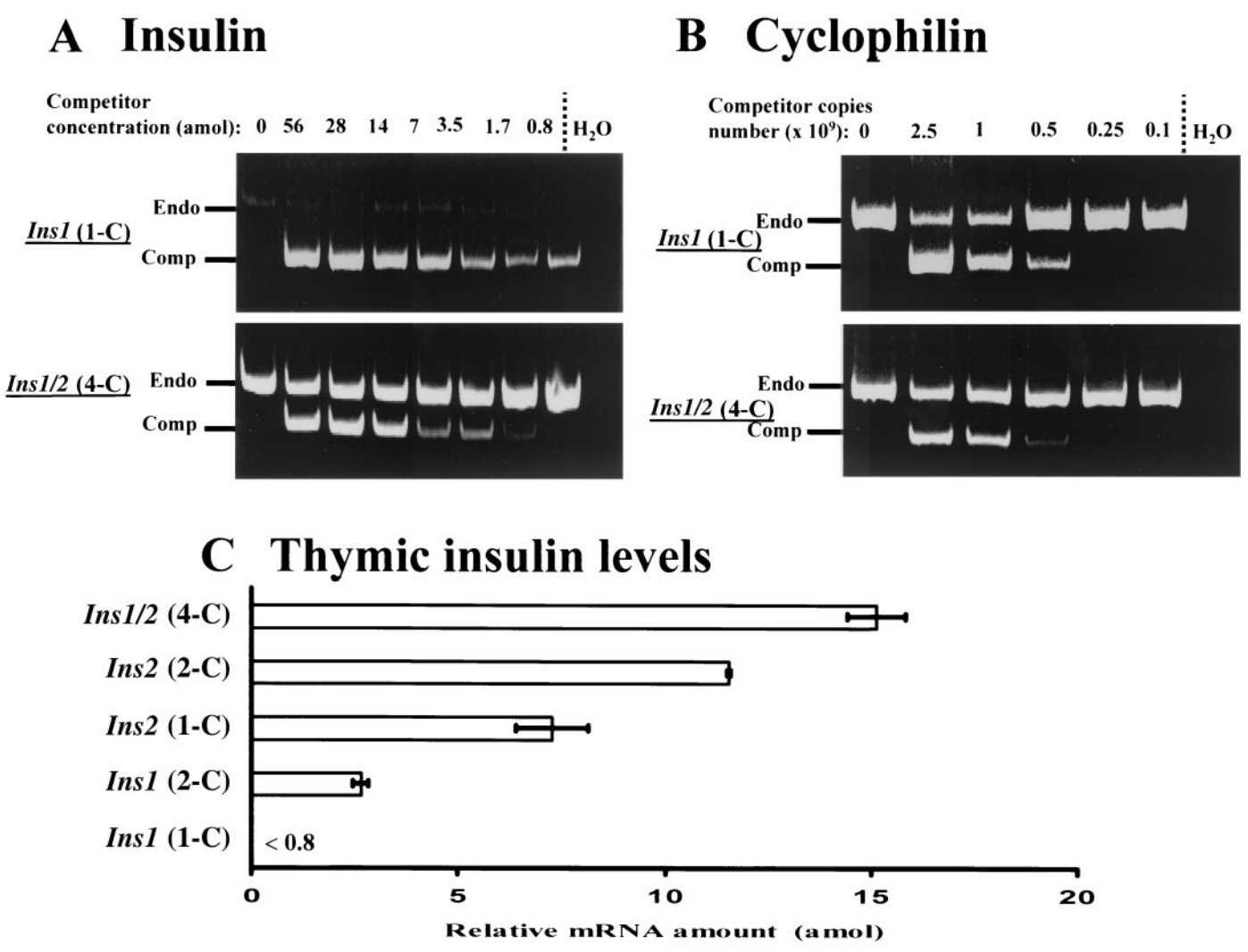

FIG. 2. Quantification of insulin mRNA levels in the Thymus. *A*: PAGE analyses of quantitative RT-PCR products of insulin endogenous (endo) and graded concentration of competitor (comp) cDNA coamplification from mice with one or four insulin gene copies. *B*: PAGE analyses of quantitative RT-PCR product of cyclophilin endogenous and competitor cDNA coamplification from mice with one or four insulin gene copies. *C*: Thymic insulin mRNA levels in *Ins1* (1-C), *Ins1* (2-C), *Ins2* (1-C), *Ins2* (2-C), or *Ins1/2* (4-C) mice. The result is one representative of three experiments. The results are means ± SD of three mice (n = 3 mice/genotype).

The results again show no difference between mice of different genotypes. Thus, insulin production in the pancreas is not dependent on insulin gene copy number, a result we had predicted because the insulin gene is subjected to metabolic feedback control, which can upregulate even a single gene copy to maintain homeostasis.

Insulin expression in the thymus of mice with different insulin gene copy numbers. In the thymus, the same techniques of quantitative RT-PCR were used as for analysis of pancreatic insulin levels. Figure 2*A* shows that mice with one copy of *Ins1* [*Ins1* (1-C)] express low levels of insulin, undetectable by our technique. In contrast, mice with four insulin gene copies express easily detectable insulin levels. Both PCR reactions were started with the same amount of RNA, as shown by cyclophilin level (Fig. 2*B*). Figure 2*C* shows the relative amount of thymic insulin mRNA in mice with different genotypes. The graph shows that *Ins1* (1-C) and *Ins2* (1-C) mice express twofold less insulin than *Ins1* (2-C) and *Ins2* (2-C) mice, respectively. Also, for any given number of copies, *Ins2* is expressed at more than twofold higher levels than *Ins1*, thus approximating the naturally occurring allelic differences in humans (4,5). In contrast to the pancreas, in the thymus the quasilinear correlation between insulin copy numbers and mRNA levels (also confirmed at the peptide level as shown in Fig. 3) is consistent with our assumption that in this organ insulin expression is not subject to metabolic feedback regulation and therefore depends on gene copy number.

Taken together, these data of normal levels of insulin expression in the pancreas and graded deficiency in the thymus validate our mouse model. Thus it is possible to study the effect of thymic insulin levels on insulin-specific autoreactive T-cell selection at quantitative differences very similar to those determined in humans by the *IDDM2* locus.

Thymic insulin levels modulate insulin-specific autoreactive T-cell tolerance. We chose to examine in vitro reactivity of cells from naive, non–diabetes-prone mice as the most direct test of a specific effect of thymic deficiency on central T-cell tolerance, uncontaminated by other events. T-cells vigorously reacting to proinsulin have been

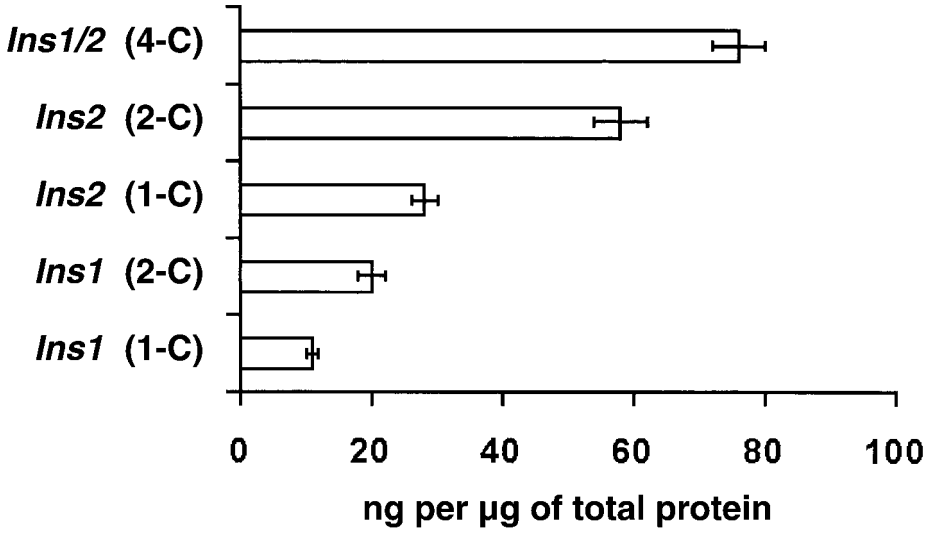

FIG. 3. Quantification of insulin peptide levels in the thymus. Insulin peptide levels in the thymus of mice genotyped as *Ins1* (1-C), *Ins1* (2-C), *Ins2* (1-C), *Ins2* (2-C), or *Ins1/2* (4-C) were calculated by Ultra-mouse insulin ELISA kit. The results are means ± SD of three experiments (n = 3 mice/genotype).

found in the pancreatic lymph nodes of such animals, though not in the periphery (8).

Insulin-specific T-cell reactivity was analyzed by a proliferation test. Figure 4 shows spleen T-cell proliferation in vitro after incubation without antigen and with proinsulin (Fig. 4*A*), ovalbumin (Fig. 4*B*), or PHA (Fig. 4*C*). The graphics show that, unlike mice with near-normal thymic insulin levels (*Ins1*-KO mice), the mice with low thymic insulin expression (*Ins2*-KO mice) present detectable T-cell reactivity to proinsulin. Both mice respond at similar levels to the control antigen ovalbumin and to nonspecific activation by PHA. Spleen cells were also incubated in the absence or presence of insulin B-chain peptide (B:9–23) or C-peptide. The results show that *Ins2*-KO mice present T-cell proliferation, whereas the response in *Ins1*-KO mice, if any, is marginal. The addition of IL-2 to the culture strongly amplifies T-cell proliferation in *Ins2*-KO mice, whereas it elicits a weak response in *Ins1*-KO mice (Fig. 5). In these experiments, we were unable to detect IL-4 or interferon-γ in the culture supernatants using a two-site ELISA kit (Pharmingen). This is not surprising, considering the low number of insulin-specific cells in comparison to the frequency of T-cells activated by mitogens.

These results show that thymic insulin expression modulates insulin-specific T-cell activation in peripheral lymphoid organs, even in naive, non–diabetes-prone mice. Together, the above experiments suggest less efficient negative selection of insulin-specific autoreactive T-cells in the immune repertoire of mice with low thymic insulin levels in comparison with mice with near-normal levels.

DISCUSSION

We have shown a detectable T-cell response to proinsulin in naive, non–diabetes-prone mice with deficient thymic proinsulin expression, a response absent in animals expressing normal or near-normal levels in the thymus. The result was robust, reproduced over several experiments with either proinsulin or specific epitopes (C-peptide and B:9–23 of the B chain). The genetic background of the animals was mixed B6 and 129 strains, but all animals had the H-2^b haplotype at the MHC locus, the main determinant of antigen-specific differential T-cell response. It is very unlikely that the differential reactivity seen between the two knockouts is due to random genetic variation at other loci, since it was specific for insulin and the two strains were no different in T-cell proliferative response to nonspecific stimuli.

Applied to the human situation, our findings directly imply that the low thymic insulin expression associated with the predisposing VNTR alleles (4,5) could be responsible for the generation of high numbers of insulin-specific autoreactive T-cells. The thymus is the organ where T-cell precursors undergo negative selection by apoptosis if they encounter self-antigen (10–13). Studies using T-cell receptor (TCR) transgenic mice have established that susceptibility to negative selection depends on TCR avidity and that thymic self-antigen plays a dose-dependent role (12,13,29,30). Many tissue-specific proteins, including hormones, have been shown to be expressed in the thymus (14,15,31). Insulin has been reported to be produced by rare thymic dendritic cells dispersed in the medulla (16), but in a more rigorous recent study, these cells showed all the markers and properties of medullary epithelial cells (15).

Evidence that thymic expression is important in self-tolerance (14,15) has until now come mostly from transgenic overexpression studies. Targeted disruption of endogenous proteins—myelin basic protein, for example (32,33)—results in strong T-cell reactivity in the homozygous state, as expected of a xenoantigen. However, those findings do not differentiate between central thymic and peripheral tolerance.

To answer this question in the case of insulin, we have established a mouse model with normal pancreatic insulin levels but graded thymic insulin deficiency and used it to show the modulatory effect of insulin expression levels in the thymus on insulin-specific autoreactive T-cell selection. What makes this model relevant to human diabetes is

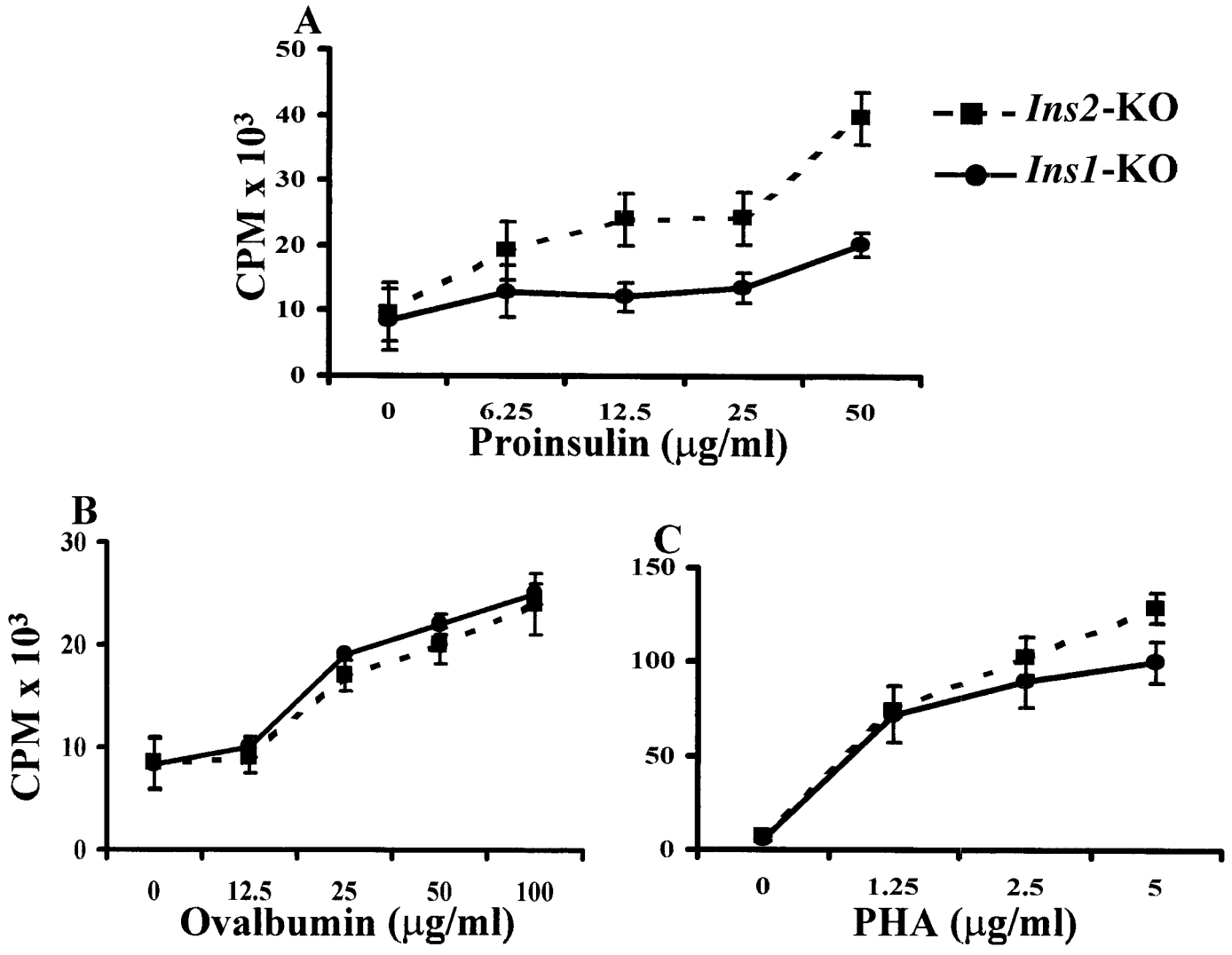

FIG. 4. Insulin-specific T-cell reactivity: Wool-nylon–purified T-cells from unprimed *Ins1*-KO (●) and *Ins2*-KO (■) mice were incubated in the presence of autologous irradiated antigen-presenting cells (2,500 rad) and in the presence of serial concentrations of proinsulin (*A*), ovalbumin (*B*), or PHA (*C*). The results are means ± SD in cpm of [^{3}H]thymidine incorporation in triplicate (n = 3 mice).

the possibility of exploiting the fact that *Ins1* is two to three times less abundantly expressed in the thymus than *Ins2*. This difference is similar to that seen between the predisposing and protective *IDDM2* alleles in the human (4,5), making our mouse model relevant for the study of immunological effects on T-cell reactivity to insulin in the periphery by the genetically determined thymic level discrepancy in the human.

The only other demonstration of a modulation of T-cell tolerance to nontransgenic peripheral self-antigen by physiologic thymic expression levels comes from a very recent study of thymus cross-transplantation between animals null for SAP, a liver protein, and syngeneic controls (15). In that study, thymic expression alone was sufficient to induce tolerance to SAP in the absence of peripheral expression but not necessary, as animals expressing SAP in liver but not thymus were equally tolerant. In our study, by contrast, we find a substantial effect of reduced thymic insulin in the presence of full peripheral expression, suggesting that the importance of central versus peripheral tolerance may depend on the specific autoantigen. Size of the organ where expression is confined may also be a factor: contact of any given T-cell with the tolerizing peripheral tissue is much more likely to occur in the liver than in the endocrine pancreas, an organ three orders of magnitude smaller.

We did not observe insulitis or diabetes in mice with low thymic insulin expression levels, despite the presence of autoreactive T-cells. This is consistent with the adoptive transfer literature showing that the mere presence of autoreactive T-cells in the periphery is not sufficient to cause either insulitis or autoimmune diabetes. Neither is insulitis required for the generation of insulin-autoreactive T-cells. These cells can be found before histologically detectable insulitis in the NOD mouse, and even in the pancreatic lymph nodes of normal, non–diabetes-prone mice (8). It has been shown that T-cell reactivity to self-antigen in vitro can occur even in the presence of self-tolerance in vivo (34,35). As a disease of multifactorial etiology, type 1 diabetes results from the association of several factors, of which self-reactivity to insulin is only one. In the NOD mouse, many non–antigen-specific factors are also required, such as aberrant cytokine expression (36), MHC II and costimulatory molecule expression in the

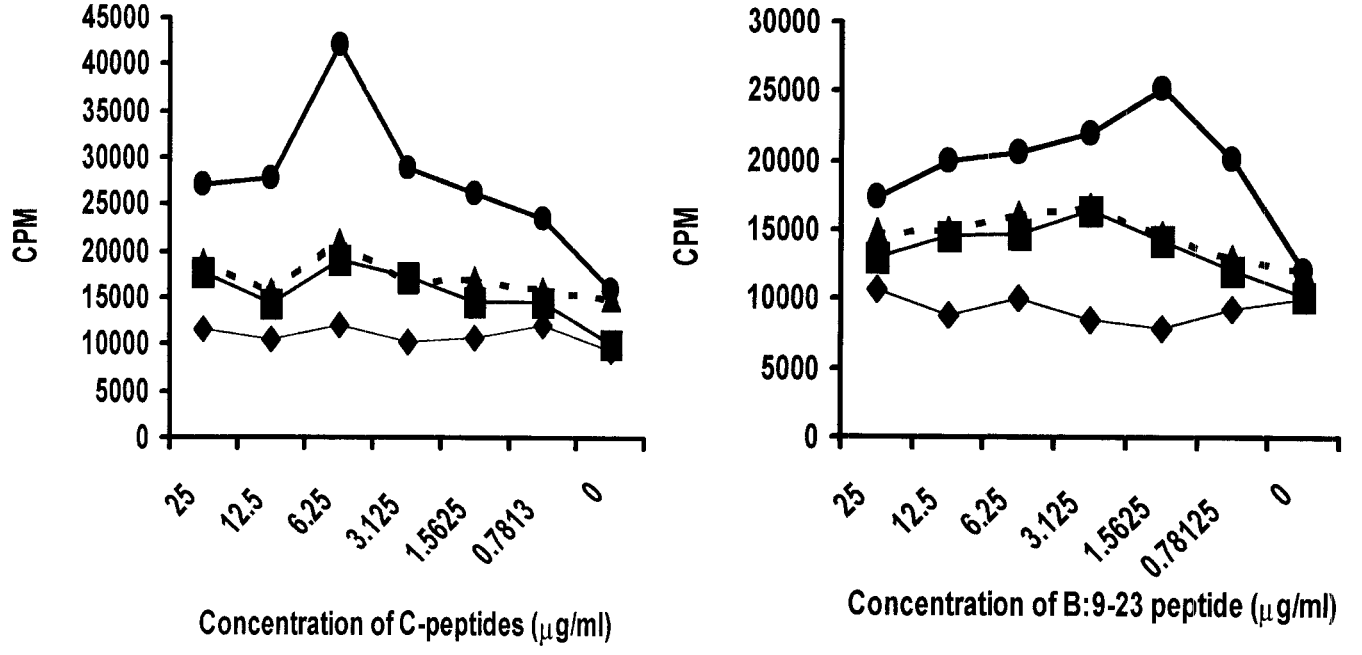

FIG. 5. Insulin-specific T-cell reactivity in the presence of exogenous IL-2. Spleen cells from unprimed *Ins1*-KO (♦, ▲) and *Ins2*-KO mice (■, ●) were incubated in the presence (▲, ●) or absence (♦, ■) of IL-2 and serial concentrations of C-peptide (*A*) or B-chain peptide (B:9–23) (*B*). The results are means in cpm of [^{3}H]thymidine incorporation in triplicate (n = 3 mice).

peripheral organ, and pancreatic inflammation (37–39). The state of peripheral cell tolerance is not yet fully understood, but it has been shown that the expression levels of molecules such as B7 or MHC class II in the target organ (such as the pancreas) in the presence of sufficient numbers of autoreactive T-cells can result in tolerance breakdown and development of diabetes (40–42).

We conclude that thymic insulin expression levels play a critical role in insulin-specific autoreactive T-cell selection and that the *IDDM2* locus predisposes to diabetes by the low thymic levels of insulin expression via failure of negative selection of insulin-specific autoreactive T-cells.

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