Class III Alleles at the Insulin VNTR Polymorphism Are Associated With Regulatory T-Cell Responses to Proinsulin Epitopes in HLA-DR4, DQ8 Individuals

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A variable number of tandem repeats (VNTR) polymorphism upstream of the insulin promoter is strongly associated with type 1 diabetes. The short class I alleles are predisposing and the long class III alleles are protective. As a possible mechanism for this effect, we previously reported a two- to threefold higher insulin transcription from class III than from class I chromosomes in thymus where insulin is expressed at low levels, presumably for the purpose of self-tolerance. In this article, we confirm this finding with independent methodology and report studies testing the hypothesis that class III alleles are associated with T-cell tolerance to (pro)insulin. Cytokine release in vitro after stimulation with 21 overlapping preproinsulin epitopes was assessed in blood mononuclear cells as well as naive and memory CD4$^+$ T-cell subsets from 33 individuals with the high-risk DRB1*04, DQ8 haplotype (12 type 1 diabetic patients, 11 healthy control subjects, and 10 autoantibody-positive subjects). No significant differences between genotypes (24 I/I subjects versus 10 I/III or III/III subjects) were observed for γ-interferon, tumor necrosis factor-α, or interleukin (IL)-4. By contrast, the I/III + III/III group showed a significant threefold higher IL-10 release in memory T-cells for whole proinsulin and the immunodominant region. Given that IL-10 is a marker of regulatory function, our data are consistent with the hypothesis that higher insulin levels in the thymus promote the formation of regulatory T-cells, a proposed explanation for the protective effect of the class III alleles. *Diabetes* 54 (Suppl. 2):S18–S24, 2005

The importance of understanding the antigenic targets in the autoimmune process that destroys the β-cells in type 1 diabetes is generally accepted and the matter is the focus of intense research efforts. At least three of the autoantigens initially discovered on the basis of humoral autoimmunity in human patients (insulin, GAD, and IA-2) seem to play a causative role in rodent models, as evidenced by the successful prevention of the disease by tolerization to each specific antigen (1–3). These results, which have yet to be reproduced in humans, also indicate the possibility of disease prevention by targeting antigens that are not necessarily "primary." Of the three, insulin may be the most important: its tissue specificity mirrors most closely the exquisite β-cell specificity of the T-cell–mediated autoimmunity in type 1 diabetes, it is the major protein product of these cells, birth-cohort studies show that antibodies to it are the most likely to be the first to appear (4), and it is the only autoantigen whose gene maps to a genetic susceptibility locus (5,6). Thus, understanding how self-tolerance to insulin is achieved and how it fails in type 1 diabetes is likely to be crucial in devising interventions to prevent the autoimmune destruction of β-cells (or its reoccurrence after potential future β-cell regeneration/stem cell therapies).

Until recently, it was not clear how T-cells acquire tolerance to proteins for which expression is highly tissue specific, with the assumption being that this happens mostly in the periphery. Mounting evidence now suggests that a substantial part of tolerance to these antigens is acquired in the thymus, through the ability of specialized thymic cells to synthesize small amounts of a wide variety of tissue-specific antigens (7,8). The case of insulin is particularly interesting. Long before the phenomenon was generalized to other antigens, it was known that small amounts of insulin are expressed in the thymus (9). More interestingly, tolerance to allo-antigens (10) or xeno-antigens (11) transgenically expressed under the insulin promoter can be transferred by thymus transplant to nontransgenic recipients, indicating that such expression has a functional role in tolerance. Multiple mouse and human studies have localized thymic insulin expression to a small subset (not much more than 1%) of medullary stroma cells (7,11–13).

Dependence of insulin self-tolerance on thymic expression is also supported by an independent line of evidence based on genetics. Genetic susceptibility accounts for about half of type 1 diabetes risk and behaves as a complex polygenic trait. HLA (especially the DR-DQ region) explains about half of the genetic risk (14); the other half is attributed to an unknown number of minor loci of smaller individual effect. Of the three such loci that have been multiply confirmed, the one conferring the highest relative risk maps to a variable number of tandem repeats (VNTR) polymorphism situated 0.5 kb upstream of the insulin promoter on chromosome 11p15.5. It consists of slight variants of the consensus sequence unit ACAGGGG TGTGGGG repeated 28–44 times (class I alleles) or 138–

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Ab$^+$, autoantibody-positive; IFN, interferon; PBMC, peripheral blood mononuclear cell; SNP, single nucleotide polymorphism; Th, T-helper; TNF, tumor necrosis factor; VNTR, variable number of tandem repeats.

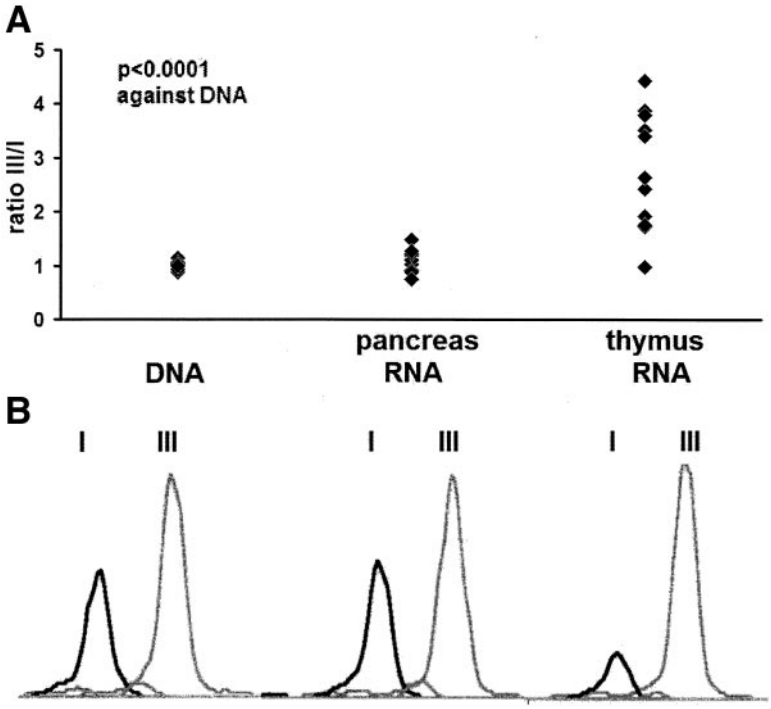

FIG. 1. Confirmation of the allelic transcriptional effect of the *INS*-VNTR on thymus insulin expression in 12 heterozygous samples. Capillary electrophoresis peaks corresponding to two alleles of an SNP, labeled by single-nucleotide primer extension with ddNTPs labeled with different fluorochromes. Because of perfect linkage disequilibrium with the VNTR, the A allele (black curve) is transcribed from the class I chromosome and the T allele (gray curve) from the class III chromosome. Heterozygous DNA is used to define 1:1 allele stoichiometry. *A*: Individual values for 10 samples of DNA, pancreas RNA, and thymus RNA as ratios of class III expression level over class I. Values are normalized taking the average DNA value as 1:1, to compensate for different ddNTP-fluorochrome efficiencies. *B*: A representative capillary electrophoresis profile.

159 times (class III alleles) (15). Intermediate class II alleles are very rare. Class I alleles are found in ~80% of Caucasian chromosomes and, in the homozygous state, confer a more than twofold relative risk for type 1 diabetes (15). Conversely (and more meaningfully) stated, the less common (~20%) class III alleles are dominantly protective. Because the VNTR does not alter peptide sequence, it must affect biology by modulating expression levels of *INS*, or the adjacent downstream *IGF2* gene. We have excluded any substantial effect on *IGF2* in tissues relevant to type 1 diabetes (16–18), and, in heterozygous human fetal pancreas, the class III allele was associated with a modestly (15–20%) lower mRNA levels than the class I allele (18). Bennett et al. (19) found similar results for insulin expression in adult pancreas. Although lower expression of an autoantigen might protect from autoimmunity, in our opinion, this marginal loss of function would be a poor explanation for a dominant effect.

A better explanation was found in the thymus where, in heterozygous human fetal samples, class III alleles enhance insulin transcription two- to threefold (20,21) (Fig. 1). This led to the obvious hypothesis that the *INS*-VNTR exerts its effect on type 1 diabetes risk through more secure T-cell tolerance induction to insulin epitopes achieved by higher levels in the thymus. This model was subsequently supported by observations in mice with targeted disruption of the insulin genes.

Murine insulin is encoded by two homologous but unlinked genes: *Ins1* (not present in humans) and *Ins2* (syntenic to human *INS*). Whereas in the pancreas, the two genes are expressed at about equal levels, *Ins1* is minimally expressed in the thymus (22). *Ins2*$^{(-/-)}$ mice have normal pancreatic insulin and glucose levels (23) but drastically reduced insulin expression in the thymus (22). They display spontaneous T-cell autoreactivity to human insulin even against a diabetes-resistant background (22,24). When crossed with the NOD mouse, diabetes occurs more frequently and at an earlier age, an enhancement of the autoimmune process that is specific to insulin (25). Conversely, NOD-*Ins1*$^{(-/-)}$ mice are remarkably resistant to diabetes (26), compatible with the notion that in that model, the autoimmunity is primarily against the insulin peptide not expressed in thymus.

The constraints of human research, essentially limiting samples to peripheral blood, make it difficult to test predictions of this model in the human. *INS*-VNTR status had no effect on the frequency of anti-insulin autoantibodies in one small study (27), whereas another much larger study found a significantly higher proportion of class III alleles in subjects who had anti-GAD but not anti-insulin antibodies (28), suggesting a protective effect of class III against insulin autoimmunity, even though β-cell autoimmunity directed against other antigens could occur. In any event, the relevance of humoral immunity to what is essentially a cell-mediated process is not clear.

Direct evaluation of cellular reactivity to insulin is much more challenging. Recent publications, including our own work, support the concept that type 1 diabetes autoimmunity reflects a defect in an integrated system of immunoregulation mediated by distinct T-cell subsets (29–35). In recent studies using peripheral blood lymphocytes and two CD4$^+$ T-cell subsets (CD45RA$^-$ memory and CD45RA$^+$ naive and recently primed) of subjects with the high-risk DRB1*04,DQ8 haplotype, we have shown that the cytokine production patterns of preproinsulin reactive T-cells differed significantly between autoimmune subjects (autoantibody-positive [Ab$^+$] and type 1 diabetic patients) and healthy HLA-matched control subjects (31). In insulin-treated type 1 diabetic patients, naive and recently primed CD4$^+$ cells were characterized by an increase in T-helper (Th)-1 cytokines (interferon [IFN]-γ and tumor necrosis factor [TNF]-α). In contrast, in CD4$^+$ T-cell subsets of Ab$^+$ subjects, T regulatory (IL-10) and Th2 (IL-4) phenotypes dominated, whereas Th0 responses were present in the control subjects. The CD45RA$^+$IL-10^{hi} T-cell phenotype has been associated with regulatory function in subjects with type 1 diabetes and in experimental models of autoimmunity (29,34,36,37).

TABLE 1
Subject characteristics

	ID number	Age (years)	Type 1 diabetes (months)	*INS*-VNTR	HLA haplotype (DRB1*, DQB1*)	Autoantibodies ICA (JDF-U)	IAA/IA (μU/l)	GADA (KU/l)	IA-2A (KU/l)
Ab^+ subjects	1	12		I, I	0401, 0302	**320**	**1,074**	**39**	**3,328**
	2	17		I, I	04, 0302	**320**	73	**55**	**544**
	3	14		I, I	0401, 0302	0	**209**	**37**	0
	4	19		I, I	0404, 0302	0	12	**4**	0
	5	24		I, I	0404, 0302	0	−55	**10**	**9**
	6	15		I, I	04, 0302	80	57	**11**	**115**
	7	10		I, I	0401, 0302	0	**251**	3	0
	8	15		I, I	0402, 0302	0	54	**11**	**5**
	9	8		I, I	04, 0302	**40**	**418**	**12**	**11**
	10	10		I, III	0401, 0302	**160**	**479**	**64**	**23**
Type 1 diabetic patients	11	2	3	I, I	0404, 0302	0	**23,123**	**4**	**30**
	12	10	2	I, I	04, 0302	**80**	**11,663**	**8**	**4**
	13	14	4	I, I	0401, 0302	0	8	1	1
	14	22	1	I, I	0401, 0302	0	63	**110**	**19**
	15	24	8	I, I	0402, 0302	0	21	**39**	**41**
	16	27	3	I, I	0401, 0302	**160**	**5,332**	**41**	**60**
	17	28	11	I, I	0401, 0302	**320**	**12,389**	**127**	**2**
	18	30	12	I, I	0401, 0302	**80**	**4,049**	**87**	**550**
	19	31	1	I, I	0401, 0302	0	59	**5**	0
	20	18	10	I, III	0401, 0302	0	**14,021**	**50**	**3**
	21	45	6	I, III	0401, 0302	0	**23,434**	1	1
	22	56	1	I, III	0401, 0302	**1,280**	**1,249**	**79**	**154**
	23	12	1	I, III	0401, 0302	**640**	172	**65**	**58**
Control subjects	24	2		I, I	0404, 0302	0	−49	1	0
	25	15		I, I	0401, 0302	0	53	0	0
	26	20		I, I	0401, 0302	0	63	0	0
	27	20		I, I	0401, 0302	0	−2	0	0
	28	29		I, I	0407, 0302	0	26	1	1
	29	29		I, I	0404, 0302	0	64	1	0
	30	23		III	0401, 0302	0	0	0	0
	31	27		I, III	0404, 0302	0	34	1	1
	32	43		I, III	0401, 0302	0	203	1	0
	33	20		III	0404, 0302	0	130	0	0
	34	29		I, III	0403, 0302	0	17	0	1

The numbers in bold represent positive antibody titers. IA, insulin antibody; IAA, insulin autoantibody; JDF-U, Juvenile Diabetes Foundation units.

The purpose of the current study was to see how the in vitro response of peripheral blood mononuclear cells (PBMCs) and $CD4^+$ T-cell subsets to insulin peptide epitopes, in terms of induction of the most important T-cell cytokines, correlates with the genotype at the *INS*-VNTR locus. The results show substantial differences in IL-10 production, consistent with promotion of regulatory cells in subjects with the protective genotype. In parallel, we also confirm our initial findings of the transcriptional allelic effect of the VNTR in human thymus in a new batch of thymus samples, using an independent and more reliable technique.

RESEARCH DESIGN AND METHODS

A total of 33 subjects with the high-risk HLA-DRB1*04, DQ8 haplotype were analyzed: 11 healthy control subjects without family history of type 1 diabetes (median age 23 years, range 2–43), 10 Ab^+ schoolchildren without family history of type 1 diabetes from the Karlsburg type 1 diabetes risk study (38) (median age 14.5 years, range 8–24), and 12 recently diagnosed insulin-treated patients with type 1 diabetes (median age 24 years, range 2–56; median duration of insulin treatment 3 months, range 1–12). Of the 10 Ab^+ individuals, 8 were classified as "high-risk" subjects, since they were positive for more than one antibody specificity, i.e., insulin autoantibodies, antibodies against GAD, or islet thyrosine phosphatase and/or had a high titer of cytoplasmic islet cell antibodies (>20 Juvenile Diabetes Foundation units) (39,40). Subject characteristics are described in Table 1.

Autoantibody assays. Subject sera were tested for autoantibodies in the Institute of Pathophysiology, Karlsburg, Germany, as described (38,41). HLA typing of DRB1* and DQB1* alleles was performed using allele-specific PCR amplification procedure as described elsewhere (42). Informed consent was obtained from all individuals before analysis, and studies were carried out in accordance with the Declaration of Helsinki.

Cell isolation. Peripheral blood mononuclear cells were isolated from heparinized blood by Ficoll-Paque (Pharmacia, Freiburg, Germany) density centrifugation, aliquoted, and cryopreserved in liquid nitrogen until use. Enrichment of $CD45RA^-$ memory and $CD45RA^+$ naive/recently primed Th cells of all investigated individuals was performed in two steps using a $CD4^+$ T-cell isolation kit (Miltenyi Biotec, Auburn, CA), and stimulation assay was modified for enriched Th cell subsets as previously described (30).

Autoantigens and peptides. Human proinsulin (86 amino acids; a gift from Eli Lilly, Indianapolis, IN) and insulin (51 amino acids; a gift from Aventis, Frankfurt, Germany) were tested simultaneously with 21 overlapping preproinsulin peptides. Preproinsulin peptides were synthesized according to the primary preproinsulin structure (GenBank accession number P01308). They were 16–17 amino acids long and overlapped by 12 amino acids. In each T-cell assay, control peptide CASSSDRLGNQPQHF (T-cell receptor peptide, 43), tetanus toxoid (Connaught Laboratories, Toronto, Canada), and phytohemagglutinin M (Difco, Detroit, MI) were included as described previously (30).

Cytokine assays. To measure the level of secreted TNF-α, IFN-γ, IL-2, IL-4, IL-5, and IL-10, after stimulation with proinsulin, insulin, and 21 overlapping preproinsulin peptides, 175 μl supernatant was taken from each well of a microtiter plate on day 5 of the culture as previously described (30). Supernatants of replicate cultures were pooled and stored at −20°C until assay. They were analyzed according to the manufacturer's instructions using

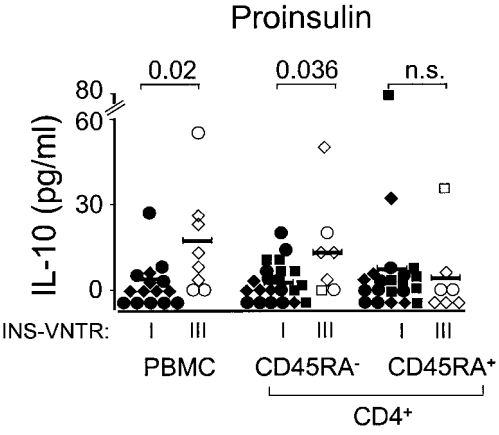

FIG. 2. Genotype dependence of IL-10 expression by blood lymphocytes stimulated with proinsulin (◆ ◇, control subjects; ■ □, Ab$^+$ subjects; ● ○, type 1 diabetic patients). In PBMCs, most I/I subjects (marked as I) show little or no IL-10 production, whereas subjects with at least one class III allele (marked as III) show significantly higher responses. This is observed in the memory but not the naive subset.

an antigen-capture enzyme-linked immunosorbent assay from PharMingen (San Diego, CA). Detection limits for cytokines were as follows: 19.5 pg/ml for TNF-α, 34.2 pg/ml for IFN-γ, 9.8 pg/ml for IL-2, 19.5 pg/ml for IL-4, 48.8 pg/ml for IL-5, and 4.9 pg/ml for IL-10. Quantification of spontaneous cytokine release was performed by incubating the cells of each individual under the same conditions as preproinsulin peptides in the absence of antigen. Positive cytokine responses were defined as two adjacent peptide (antigen)-stimulated wells, which gave responses greater than the mean + 2 SD of unstimulated wells. The intra-assay coefficient variation for IL-10, calculated on five replicate tests, was 27.8%. The amount of secreted cytokines was expressed in picograms per milliliter.

VNTR typing. The VNTR genotype was deduced from the genotype at the −23 A/T single nucleotide polymorphism (SNP) at the INS promoter, whose A allele is in very tight linkage disequilibrium with the class I and the T with class III ($r^2 = 1$). The SNP was typed by single-nucleotide primer extension on PCR-amplified DNA according to the PE AcycloPrime-fluorescence polarization protocol in a GeneAmp PCR system 9700 (MJ Research, Waltham, MA). Final detection of the SNP by fluorescence polarization used the Criterion System with Analyst HT (Molecular Devices, Sunnyvale, CA). In a study of 2,500 samples, the genotyping call rate for this assay was 97%, Mendelian error <0.001, and no divergence from the Hardy-Weinberg equilibrium was detected.

Tissues. Twelve human fetal thymus and pancreas samples were obtained at the time of pregnancy interruption for reasons other than health problems in mother or fetus. The sampling was approved by the Research Ethics Board of the McGill University Health Center, and written consent was obtained from all mothers. Samples were snap-frozen and kept at −70°C. After pulverizing frozen tissue in a liquid N_2–filled mortar and pestle, Trizol (Invitrogen) was added for extracting DNA and RNA.

Allelic imbalance assay. *INS* transcripts corresponding to each VNTR allele in heterozygous samples were distinguished using the +1,127 C/T SNP at the *INS* 3′ UTR.

Levels of each allele in PCR or RT-PCR product (primers and cycling as described by Vafiadis et al. [20]) were quantitatively evaluated by single-nucleotide primer extension, using ddNTPs labeled with different fluorochromes (SnapShot kit, Applied Biosystems International) followed by capillary electrophoresis.

Statistical analysis. The Kalmogorov-Smirnov test indicated that all cytokine results significantly diverged from a normal distribution. Therefore, all comparisons between means were performed by permutation, using resampling without replacement (Resampling Stats software, http://www.resample-.com). Results are summarized as means ± SE. SEs were calculated by resampling with replacement.

RESULTS

Confirmation of the *INS*-VNTR transcriptional effect on thymus. Our initial report of the allelic *INS*-VNTR effect on thymic insulin transcripts was based on the relative intensity of radioactive bands of the two alleles by RT-PCR restriction fragment-length polymorphism (20).

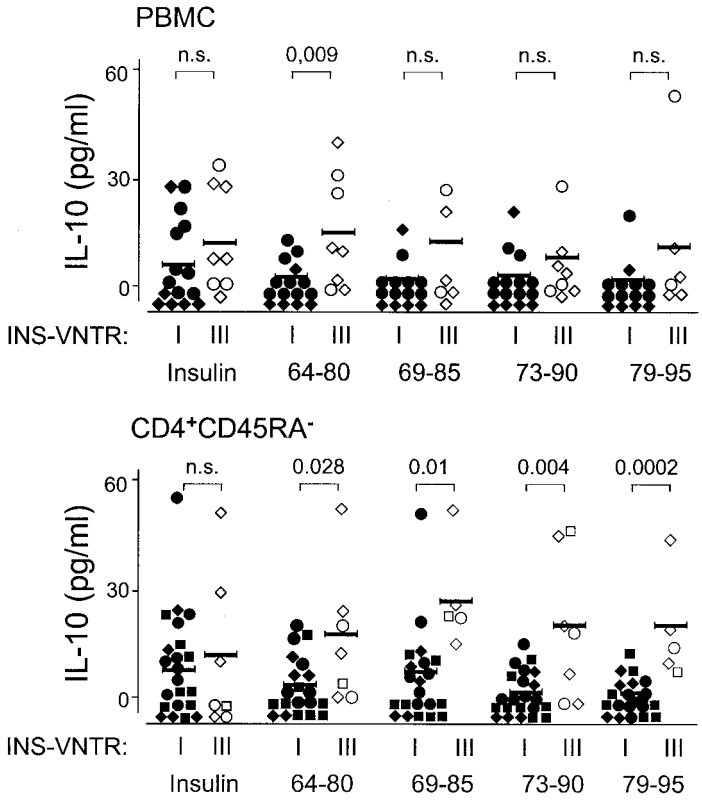

FIG. 3. Genotype dependence of IL-10 expression by blood lymphocytes stimulated with insulin, or the proinsulin-specific immunodominant epitopes (◆ ◇, control subjects; ■ □, Ab$^+$ subjects; ● ○, type 1 diabetic patients). There is no difference in whole insulin response, but all epitopes elicit significantly higher responses in class III individuals in memory cells.

PCR versus RT-PCR products could differentially affect the efficiency of digestion by the *Pst*I endonuclease used in that study (for example, through differential formation of heteroduplexes that the enzyme cannot digest). We rigorously controlled for this by spiking the PCR product with RNA and digesting the RT-PCR product with RNAase (20). However, the investment in effort to pursue this original finding justifies rigorous confirmation, which we undertook in a new set of 12 heterozygous human fetal samples with a completely independent method that does not involve restriction digestion. Figure 1 shows the results, which are in concordance with our previous observation, showing a statistically highly significant average 2.6-fold higher level in the allele derived from the class III chromosome compared with the class I chromosome.

In vitro lymphocyte response to proinsulin and immunodominant epitopes. As we have previously reported, measurable cytokine responses to preproinsulin peptides are observed in a number of subjects, whether they are type 1 diabetic patients, normal control subjects, or healthy but Ab$^+$ individuals. We compared in vitro cytokine release to preproinsulin peptides by PBMCs as well as the naive (CD45RA$^+$) or memory (CD45RO$^+$) CD4$^+$ T-cell subsets from high-risk DRB1*04, DQ8 individuals. I/I homozygotes were compared with those with at least one class III allele (I/III and III/III) without distinction as to disease or autoantibody status.

A measurable response of different cytokines to different preproinsulin epitopes was seen in a significant proportion of samples. The most striking and consistent difference between genotypes was observed for IL-10.

TABLE 2
Cytokine secretion upon stimulation with proinsulin (P-Ins), insulin, and overlapping peptides covering the immunodominant P-Ins$_{64–95}$ region

	INS-VNTR	PBMC			CD45RA$^-$			CD45RA$^+$		
		P-Ins	Ins	P-Ins$_{64–95}$	P-Ins	Ins	P-Ins$_{64–95}$	P-Ins	Ins	P-Ins$_{64–95}$
IFN-γ	I	9 ± 4	40 ± 27	19 ± 8	15 ± 6	14 ± 6	71 + 28	21 ± 11	20 ± 13	22 ± 9
	III	0 ± 0	57 ± 48	11 + 9	13 ± 6	25 ± 10	71 + 40	7 ± 5	32 ± 14	37 ± 20
TNF-α	I	5 ± 2	12 ± 5	7 ± 3	5 ± 4	7 ± 6	8 ± 6	6 ± 5	4 ± 2	12 ± 6
	III	7 ± 5	79 ± 65	5 ± 3	0 ± 0	0 ± 0	3 ± 2	0 ± 0	6 ± 1	5 ± 6
IL-2	I	10 ± 18	19 ± 14	14 ± 23	9 ± 7	21 ± 37	24 ± 50	37 ± 95	40 ± 91	31 ± 65
	III	24 ± 26	21 ± 9	19 ± 26	43 ± 7	40 ± 38	48 ± 44	10 ± 10	23 ± 42	18 ± 32
					$P = 0.002$					
IL-4	I	7 ± 4	12 ± 4	8 ± 1	10 ± 4	15 ± 5	23 ± 5	23 ± 13	25 ± 13	14 ± 7
	III	8 ± 5	9 ± 1	5 ± 3	15 ± 6	25 ± 2	34 ± 5	15 ± 9	4 ± 5	8 ± 5
IL-5	I	7 ± 4	9 ± 5	4 ± 2	10 ± 4	10 ± 4	18 ± 4	0 ± 0	3 ± 2	9 ± 4
	III	23 ± 18	16 ± 8	40 ± 16	0 ± 0	131 ± 119	73 ± 54	104 ± 93	46 ± 41	55 ± 45
				$P = 0.009$						
IL-10	I	4 ± 2	8 ± 11	4 ± 6	5 ± 1	10 ± 3	5 ± 1	7 ± 4	7 ± 4	4 ± 1
	III	15 ± 6	13 ± 14	11 ± 15	14 ± 6	13 ± 7	18 ± 6	6 ± 5	2 ± 1	7 ± 3
		$P = 0.03$			$P = 0.03$		$P = 0.004$			

Data are means ± SE. Values are rounded to the nearest integer. Ins, insulin; P-Ins, proinsulin.

Responses were higher in PBMC samples from subjects with the I/III or III/III genotype than in I/I homozygotes, most of whom had a weak or no response to whole proinsulin (Fig. 2). Mean responses were 4.2 ± 1.5 vs. 14.1 ± 6.6, respectively ($P = 0.02$). Whole insulin responses were not different between genotype groups (Fig. 3). The genetic effect on proinsulin response could be accounted for entirely by the response of the CD4$^+$CD45RA$^-$ set of memory T-cells, as the response of the CD4$^+$CD45RA$^+$ set was no different between genotype groups (Fig. 2). Response of memory cells to all four peptides covering the DR4-restricted immunodominant proinsulin 64–95 region (30,33,44) elicited a three- to fourfold higher response in the class III–containing genotypes than in I/I. Averaged response to the four peptides was 17.5 vs. 4.4 pg/ml for class I/III + III/III vs. class I/I ($P = 0.007$). Response to the 79–95 peptide was 16.3 vs. 1.8 pg/ml ($P = 0.0002$), a P value that remains significant even after Bonferroni correction for three cell types and 21 epitopes ($0.0002 \times 3 \times 21 = 0.0126$; however, such correction would have been much too stringent because peptide responses highly correlated with each other). Increased IL-10 secretion of the subjects with the protective class III genotypes, preferentially to the peptides covering the enzymatic cleavage site between C-peptide and insulin A-chain (present only in the intact proinsulin molecule), demonstrate that the reactivity is directed primarily to the insulin autoantigen and not to the exogenous insulin. Somewhat less striking differences between genotypes were also seen in several of the epitopes outside the 64–95 region, such as the two epitopes on the insulin B-chain (30–46 and 35–51) overlapping the previously reported B9–23 region (45).

The number of epitopes for which each subject's cells were positive for IL-10 was again significantly higher in PBMCs of I/III+III/III subjects (12.5 ± 2.5 epitopes vs. 5.2 ± 1.9 for I/I subjects, $P = 0.037$). Comparison of the CD4$^+$CD45RA$^-$ memory IL-10 responses to preproinsulin epitopes of class III positive autoimmune subjects with control subjects (despite relatively low numbers) revealed higher numbers of recognized epitopes in the control subjects (18.5 ± 2.8 peptides vs. 11 ± 10.1 in autoimmune subjects). In contrast, in class I positive subjects there was no difference in the number of recognized epitopes between autoimmune subjects and control subjects (11.3 ± 6.8 vs. 10 ± 7.7).

The few differences between genotypes observed in the other cytokines were small and of borderline significance (summarized in Table 2), except for IL-2 and IL-5 responses, which were higher in I/III individuals; IL-2 in memory cells for proinsulin (42.5 vs. 9 pg/ml, $P = 0.002$); and IL-5 in PBMCs for the immunodominant proinsulin 64–95 region (40 vs. 4 pg/ml, $P = 0.009$).

DISCUSSION

It is generally accepted that elucidating the complex genetics of type 1 diabetes susceptibility is an important part of the effort to devise safe and effective preventive interventions, through better understanding of the factors responsible for the autoimmune process. In addition, as has been argued recently (46,47), an additional potential advantage of genetic prediction could be the distinction between different susceptibility genotypes, each encoding a different immune dysregulation phenotype that may respond differently to available alternative interventions.

Toward this end, detecting disease association with DNA sequence variants is only the first step. The ultimate goal is to understand the mechanism by which the variant alters biology. These mechanisms are often far from obvious. For example, decades after the strong effect of HLA haplotypes on type 1 diabetes risk was first observed, the biological mechanism of this effect is largely unknown. In the case of insulin, based on the evidence currently available, the most plausible mechanism would involve allele-dependent levels of insulin expression in the thymus. Central T-cell tolerance to β-cell autoantigens, determined in the thymus, is of obvious importance in type 1 diabetes, a T-cell–mediated disease.

The classical model of central tolerance would involve more efficient negative selection of insulin-autoreactive T-cells made possible by more abundant autoantigen. Indeed, insulin and other tissue-specific autoantigens are expressed only in thymic medulla (7,8,11), the site of negative selection. This model would predict a different effector cytokine phenotype of T-cell response to insulin

epitopes by individuals with a class III allele. In the small sample examined, with admittedly limited statistical power, we did not observe such an effect. Our most striking finding involved IL-10, a cytokine now recognized as an important marker of regulatory function by T-cells.

In recent years, it has become increasingly obvious that elimination of autoreactive T-cells, either centrally in the thymus or through peripheral processes, only partly explains T-cell tolerance. Self is actively protected by T-cells with regulatory function. The best-examined paradigms involve the generation of the T_H2 subset of $CD4^+$ cells and, more recently recognized, $CD4^+$ cells constitutively expressing CD25. One important difference between the two, relevant to the mechanism of action of the insulin VNTR, is that T_H2 cells are generated on the basis of signals received in the periphery, whereas the $CD4^+CD25^+$ subset is already present and active in the thymus (48). Even more importantly, the generation of these cells in the thymus appears to require high-avidity interaction of their T-cell receptor with self-peptide (49). Because avidity depends on both affinity and abundance of the antigen, the more than twofold higher expression from the class III VNTR could explain the more efficient selection of these cells in the thymus of individuals carrying this allele.

The cells producing the excess IL-10 we observed in subjects with a class III allele could have also been generated in the periphery. In that case, there is no obvious explanation for the genetic effect. There are reports of immunoreactive insulin expression by dendritic cells in peripheral lymphoid tissues (12) but, in our hands, rigorously controlled RT-PCR has failed to detect such expression. If it occurs, the effect of the *INS*-VNTR genotype on this peripheral expression has not been studied and it is not known whether it is enhanced by the class III allele as in thymus, diminished as in pancreas, or behaving yet in a different way.

Peripherally generated regulatory T-cells act through secretion of regulatory cytokines, notably IL-4 and IL-10. Thymus-generated $CD4^+CD25^+$ cells, on the other hand, regulate via a mechanism that involves cell contact but also express IL-10 (rev. in 50 and 51). Thus, although this preliminary study presents evidence suggesting that insulin-specific regulatory function is involved in the genetic effect of the *INS*-VNTR, it is not clear that this regulation is necessarily determined in the thymus. A thymic explanation is certainly the most attractive because a transcriptional mechanism compatible with the observation has been observed and confirmed.

In future studies, after we confirm the data presented here in a larger set of samples, we propose to dissect the question of thymic versus peripheral effects by repeating the studies on the $CD4^+CD25^+$ subset, as well as on lymphocytes obtained from fetal and surgical samples of human thymus.

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