Clin Exp Immunol 1998; **114**:370–376

Autoimmune responses to the β cell autoantigen, insulin, and the *INS* VNTR-*IDDM2* locus

E. SARUGERI, N. DOZIO, C. BELLONI, F. MESCHI*, M. R. PASTORE & E. BONIFACIO
*Departments of Internal Medicine and *Paediatrics, Istituto Scientifico San Raffaele, Milan, Italy*

(Accepted for publication 7 September 1998)

SUMMARY

Type 1 diabetes is associated with autoimmunity to insulin. Genetic susceptibility to type 1 diabetes is polygenic and includes the *INS* VNTR-*IDDM2* locus which may regulate the expression of insulin in pancreas and thymus. In order to determine whether insulin autoimmunity could be attributed to a genetic susceptibility conferred by the *INS* VNTR-*IDDM2* locus, peripheral blood T cell proliferation to human insulin and insulin autoantibodies (IAA) was measured in patients with new onset type 1 diabetes and control subjects. IAA were detected in 21 of 53 patients and in none of 25 control subjects, while T cell responses were low (stimulation index range 0·4–7·2) and similar in both groups. Both antibody and T cell responses were higher in younger subjects and IAA were more prevalent in patients with the HLA-DR4 allele. No relationship was observed between humoral and cellular responses to insulin. No association was found between the *INS* VNTR-*IDDM2*-susceptible allele and insulin autoimmunity. Increased T cell responses and IAA were found in patients with either the diabetes-susceptible or the diabetes-protective *INS* VNTR-*IDDM2* locus genotypes, and increased T cell responses were also found in control subjects with either susceptible or protective *INS* VNTR-*IDDM2* locus genotypes. This study confirms that primary T cell proliferative responses to insulin are low and detectable also in control subjects. The detection of T cell proliferation and autoantibodies to insulin in subjects with and without the protective *INS* VNTR-*IDDM2* locus genotypes does not support the hypothesis of an allele-specific capacity for tolerance induction which could determine a susceptibility to develop autoimmunity against the insulin protein and subsequently diabetes.

Keywords type 1 diabetes insulin cell-mediated immunity autoantibody genetics

INTRODUCTION

Type 1 diabetes is characterized by humoral [1] and cellular [2] immune responses to pancreatic islet proteins. These immune responses are important markers of the immune process preceding disease onset [3] and have been used to identify individuals with preclinical disease, in whom intervention therapy may prevent or delay progression to disease onset [4].

Several islet autoantigen targets have been identified. One of these, insulin, is specifically expressed within pancreatic islets by the β cells. A humoral immune response to insulin is detected in the majority of patients with childhood disease [4,5]. Cell-mediated responses to insulin in peripheral blood are weak [6–13], but insulin-specific T cell clones isolated from animal models of diabetes have been shown to transfer disease [14]. Moreover, treatments with intravenous, subcutaneous, oral or intranasal insulin have been shown to prevent or delay disease onset in animals and in humans [15–19]. Thus insulin, or its prohormone proinsulin, is considered an important early autoimmune target in type 1 diabetes, and has therefore a potential role in antigen-specific immunotherapy [20].

The aberrant autoimmune response to insulin and other islet autoantigens associated with type 1 diabetes occurs very early in life [21]. These immune responses, and particularly those against insulin, are influenced by the *IDDM1* HLA class II loci [22]. Recently it was suggested that the *INS* VNTR-*IDDM2* locus may also influence the autoimmune response. The *INS* VNTR-*IDDM2* locus contains a polymorphic region 5′ to the insulin gene coding sequence, and it has been shown that this region has linkage to type 1 diabetes [23,24]. Moreover, it has recently been reported that insulin is expressed in fetal thymus, and that the level of expression is higher in subjects with *INS* VNTR-*IDDM2* genotypes associated with type 1 diabetes protection than in those with the susceptible genotype [25,26]. These studies have proposed a mechanism of an allele-specific capacity for tolerance induction which could determine a susceptibility to develop autoimmunity against the insulin protein and subsequently type 1 diabetes. If this hypothesis

Correspondence: Ezio Bonifacio PhD, Department of Internal Medicine, Istituto Scientifico San Raffaele, Via Olgettina 60, 20132 Milan, Italy.

is correct, then it would be expected that autoimmune responses to insulin would be associated with the type 1 diabetes-susceptible *INS* VNTR-*IDDM2* locus genotype. The aim of this study was to determine whether we could provide evidence for this hypothesis through the measurement of humoral and cellular responses to insulin in the peripheral circulation of new onset diabetic patients and control subjects.

PATIENTS AND METHODS

Patients and control subjects

The study was carried out on 54 newly diagnosed diabetic patients (median age 12 years, range 1–32 years; 36 males) and 27 healthy control subjects (median age 27 years, range 12–46 years; 16 males). Thirty-nine patients and nine control subjects were aged ≤ 15 years. Thirty-five patients and 27 control subjects were tested for T cell proliferation, 53 patients and 25 control subjects were tested for insulin autoantibodies (IAA), 50 patients and 26 control subjects were tested for *INS* VNTR-*IDDM2* alleles. Patients were tested for T cell proliferation within 10 days from the beginning of insulin therapy. All control subjects were negative for islet cell antibodies (ICA) and IAA and had no family history of type 1 diabetes. Experimental protocols were approved by the local Ethical Committee.

Antigens

For T cell proliferation studies, human semi-synthetic crystallized Zn-insulin (Novo-Nordisk, Baegsverd, Denmark) was used at four concentrations (0·04, 0·4, 4 and 40 μM, corresponding to 0·23, 2·3, 23 and 230 μg/ml, respectively) which lie in the middle of the dose ranges used in previous studies (0·05–500 μg/ml). Tetanus toxoid without thiomersal (Connaught, Ontario, Canada) was used as a control antigen at a final concentration of 10 μg/ml. For autoantibody measurements, human recombinant (3-^{125}iodotyrosyl^{A14}) insulin (2000 Ci/mmol, Amersham, Aylesbury, UK) was used.

T cell proliferation assay

Mononuclear cells were isolated from heparinized peripheral blood by Ficoll gradient centrifugation and depleted of $CD8^+$ cells using Dynabeads M450 CD8 magnetic beads (Dynal, Oslo, Norway) according to the manufacturer's instructions. Cells were resuspended at a concentration of $1{\cdot}5\times10^6$/ml in RPMI 1640 + 25 mM HEPES (Biowhittaker, Walkersville, MD) supplemented with 10% pooled human serum (PAA Labor- und Forschungsgesellschaft, Linz, Austria), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Biowhittaker) and 2×10^{-5} M 2β-mercaptoethanol (Sigma, St Louis, MO). A proliferation assay was carried out using 150 000 cells (100 μl) in triplicate wells incubated for 6 days in the presence or absence of increasing concentrations of Zn-human insulin (0·04, 0·4, 4, 40 μM) or tetanus toxoid (10 μg/ml) in a final volume of 150 μl. ^{3}H-thymidine (1 μCi/well; Amersham) incorporation in the last 18 h was measured by liquid scintillation after automatic cell harvesting. Results were expressed as a stimulation index (SI = median ct/min of triplicate wells containing cells + antigen/median ct/min of 24 wells containing cells + medium alone).

Autoantibody measurements

All autoantibody determinations were performed on serum aliquots drawn on the same day of the proliferation test and stored at -20°C. IAA were determined using a method based on the micro radio binding assay (RBA) of Williams *et al*. [27]. This method was modified by precipitating immune complexes with 50 μl of 50 mM Tris–HCl pH 8 with 1% v/v Tween 20, 10% v/v pre-swollen protein A-Sepharose (Pharmacia Biotech AB, Uppsala, Sweden) and 5% v/v pre-swollen protein G-Sepharose (Pharmacia Biotech AB). IAA results were converted into arbitrary units by use of a standard curve. The threshold and 99th centile of local control subjects was calculated at >4·4 IAA units [28]. ICA in control subjects were measured in undiluted sera by indirect immunofluorescence on cryostat sections of blood group O human pancreas as previously described [29].

HLA typing

Typing for the HLA DRB1*03 and DRB1*04 alleles was carried out on DNA extracted from peripheral blood mononuclear cells (PBMC) and stored at -20°C using polymerase chain reaction with sequence-specific primers (PCR-SSP) based on the method of Olerup *et al*. [30] with primer sets E (DR3) and G (DR4) as described by Bein *et al*. [31]. Subjects were typed as DR3/X, DR4/Y, DR3/4 or DRX/Y, where X is an allele other than DRB1*04 and Y is an allele other than DRB1*03.

INS *VNTR*-IDDM2 *alleles*

Subjects were typed as having *INS* VNTR class I alleles and/or *INS* VNTR class III alleles using HphI digestion of PCR amplification products of the region of interest, as previously described [24]. Subjects were divided into two groups: those with the type 1 diabetes-susceptible HphI +/+ class I homozygous genotype and those with the protective HphI −/− class III homozygous or HphI +/− class I/class III heterozygous genotypes, according to the previously reported association of the HphI +/+ class I homozygous genotype with type 1 diabetes [24].

Statistical analysis

For T cell responses, all comparisons of distribution between groups were made using the Mann–Whitney *U*-test. Antibody prevalences were compared using Fisher's test, and the distribution of *INS* VNTR-*IDDM2* locus alleles in patients and control subjects using χ^2 test for trend.

RESULTS

Cell-mediated responses

Responses to Zn-insulin were low and no significant differences between patients with type 1 diabetes (SI range 0·4–7·2) and control subjects (SI range 0·5–4·7) were observed for all concentrations (Fig. 1). Patients aged ≤ 15 years showed higher proliferative responses to 40 μM Zn-insulin (median SI 2·2) than those aged >15 years (median SI 1·4, $P = 0{\cdot}05$). An age-related response to the highest insulin dose was also seen in control subjects (median SI 2·4 in subjects ≤ 15 years *versus* 1·3 in those >15 years, $P = 0{\cdot}03$), and no differences were observed in the proliferative response to 40 μM Zn-insulin between patients aged ≤ 15 years and age-matched control subjects. All patients and all but one control subject responded to tetanus toxoid, with no differences between groups (median SI 8·7 in patients *versus* 8·7 in control subjects, P = NS), and no relationship with age (Table 1).

Humoral responses

Elevated levels of IAA were detected in 21 (40%) of 53 patients and no control subjects ($P = 0{\cdot}0001$). All patients with IAA were aged ≤ 20 years and 18 were aged ≤ 15 years.

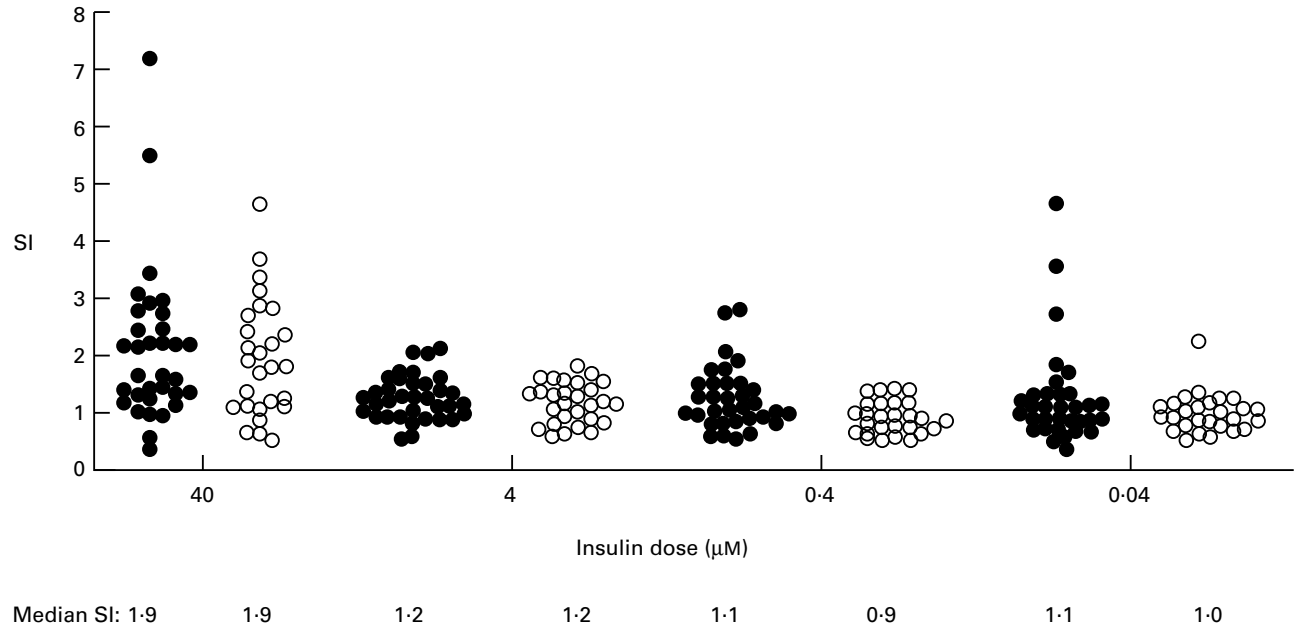

Fig. 1. $CD8^+$ depleted T cell responses to Zn-insulin. ●, Patients with new onset type 1 diabetes, $n = 35$; ○, control subjects, $n = 27$.

Relationship between humoral and cell-mediated responses

No relationship was observed between humoral and cellular responses against insulin. Patients with elevated IAA levels showed T cell responses to 40 μM Zn-insulin (median SI 1·9) similar to patients without IAA (median SI 1·6, $P =$ NS) and control subjects (median SI 1·9, $P =$ NS). Moreover, no differences between patients with and without IAA were observed when patients were subdivided by age. No differences in T cell reactivity against tetanus toxoid were observed between patients with and without increased IAA (median SI 6·5 *versus* 10·4, $P =$ NS) (Table 1).

Relationship between humoral and cell-mediated responses to insulin and HLA-DR or INS *VNTR*-IDDM2 *locus alleles*

HLA typing was obtained in 49 patients. Of the 19 patients with IAA in whom HLA-DR typing was available, 17 (89%) had the HLA-DR4 allele, compared with 13 (45%) of 29 without IAA ($P = 0{\cdot}002$). No differences in cellular responses were observed between HLA-DR4 or non-DR4 patients or control subjects, and elevated responses were observed in patients with each of these alleles (Table 2). A decreased response to tetanus toxoid was observed in patients with the HLA-DR3 allele ($P = 0{\cdot}006$). No other differences with respect to HLA-DR were observed.

Table 1. Relationship between T cell proliferation against insulin or tetanus toxoid, insulin autoantibodies (IAA), and age in patients with new onset type 1 diabetes and healthy control subjects

	T cell response to insulin 40 μM				T cell response to tetanus toxoid			
	Patients		Control subjects		Patients		Control subjects	
	n	Median SI	*n*	Median SI	*n*	Median SI	*n*	Median SI
Total	34‡	1·9	27	1·9	31§	8·7	27	8·7
Age ≤ years	19	2·2*	9	2·4†	19	9·0	9	9·4
Age > 15 years	15	1·4	18	1·3	12	6·0	18	7·9
IAA > 4·4 U	12	1·9			11	6·5		
IAA ≤ 4·4 U	21¶	1·6	27	1·9	19¶	10·4	27	8·7
≤ 15 years IAA > 4·4 U	9	2·2			9	6·8		
≤ 15 years IAA ≤ 4·4 U	9	2·5			9	11·8		
> 15 years IAA > 4·4 U	3	1·4			2	3·8		
> 15 years IAA ≤ 4·4 U	12	1·5			10	8·4		

* Patients ≤ years *versus* patients > 15 years, $P = 0{\cdot}05$.
† Control subjects ≤ 15 years *versus* control subjects > 15 years, $P = 0{\cdot}03$.
‡ One patient was not tested for T cell response to insulin 40 μM.
§ Four patients were not tested for T cell response to tetanus toxoid.
¶ One patient was not tested for IAA.

Table 2. Cell-mediated and humoral responses to insulin and *INS* VNTR-*IDDM2* locus alleles in patients with new onset type 1 diabetes

Patient	Age (years)	HLA-DR	INS VNTRs	IAA* units	SI to 40 μM Zn-insulin
1	9	DR4/Y	+/+	**49·0**	2·2
2	9	DR4/Y	+/−	**4·8**	2·2
3	9	DR4/Y	+/+	2·4	1·3
4	10	DR3/4	+/+	**8·5**	1·4
5	10	DR3/X	+/+	3·6	0·4
6	11	DR3/X	+/+	2·8	2·5
7	11	DR4/Y	+/+	**5·8**	3·0
8	11	DR4/Y	+/+	**17·7**	5·6
9	11	DR3/X	+/+	3·0	NT
10	12	DR3/X	+/+	0·0	1·4
11	12	DRX/Y	+/−	0·0	7·2
12	12	NT	NT	2·2	0·6
13	12	DR4/Y	+/−	2·0	2·9
14	13	DR4/Y	−/−	3·4	2·7
15	13	DRX/Y	+/+	NT	3·5
16	14	DR4/Y	+/+	**22·0**	2·8
17	14	DR3/4	+/+	**23·0**	2·2
18	14	DR3/4	+/+	**7·9**	1·7
19	14	DR3/X	+/−	1·5	3·1
20	15	NT	NT	**8·1**	1·4
21	16	DR3/X	+/+	4·4	2·3
22	17	DR4/Y	+/+	3·4	1·0
23	18	DR3/4	+/+	**5·6**	1·6
24	19	DR3/X	+/+	1·5	2·2
25	19	NT	NT	**8·7**	1·4
26	20	DR4/Y	+/+	**5·0**	1·3
27	21	DR3/X	+/+	1·5	1·4
28	22	DR3/X	+/+	0·0	1·2
29	25	DR3/X	+/+	3·8	1·1
30	26	DR3/X	+/+	2·4	2·5
31	27	DR4/Y	+/+	0·0	2·4
32	27	DR3/4	+/+	0·8	1·6
33	27	DR3/4	+/−	1·3	1·0
34	30	NT	NT	1·8	2·2
35	32	DRX/Y	+/+	1·5	1·2

NT, Not tested.

*Insulin autoantibody (IAA) levels above the threshold for positivity are shown in bold.

The type 1 diabetes-susceptible HphI +/+ homozygous *INS* VNTR-*IDDM2* genotype was found in 39 (78%) of 50 patients and in 15 (58%) of 26 control subjects. Ten patients and nine control subjects had the HphI +/− heterozygous genotype, and one patient and two control subjects had the HphI −/− homozygous genotype ($P = 0{\cdot}05$). No relationship between T cell proliferation against insulin and *INS* VNTR-*IDDM2* alleles was observed. Elevated T cell proliferative responses to insulin were found in patients and control subjects who had either the diabetes-susceptible or diabetes-protective *INS* VNTR-*IDDM2* genotypes. In particular, the highest proliferative response was seen in a patient with the protective HphI +/− genotype (Table 2), and similarly, the highest response found in control subjects was in an individual with the protective HphI +/− genotype (data not shown). Of the 21 patients with IAA, 16 had the susceptible *INS* VNTR-*IDDM2* genotype, three a protective *INS* VNTR-*IDDM2* genotype and in two *IDDM2* typing was unavailable. Several patients with the susceptible *INS* VNTR-*IDDM2* genotype did not have IAA, and no significant differences in IAA prevalences or levels were observed between those with and without the susceptible *INS* VNTR-*IDDM2* genotype.

DISCUSSION

Insulin is an early target of humoral and cell-mediated autoimmunity in type 1 diabetes. Previous studies have shown that the antibody response is most pronounced in young patients, and occurs several years prior to diabetes onset [3,5]. Cell-mediated responses have also been detected in peripheral blood from patients at and prior to onset of disease [6–13], but appear less specific than antibody responses, as they were also found in control subjects in most of these studies. In our study we observed that proliferative T lymphocyte responses to insulin are low, and are similar in both diabetic patients and control subjects, in accordance with some [9,11,13] but not all previous studies [6–8,10,12].

The detection of proliferation to autoantigens in control subjects is not unique to insulin or type 1 diabetes [32]. This suggests that the presence of circulating T cells autoreactive to insulin and other autoantigens is not specific to disease but is a common occurrence. The detection of T cell responses to insulin in both young new onset type 1 diabetic patients and control subjects, and of IAA only in patients suggests that the measurement of autoantibodies will be more useful for disease prediction.

We found that cell-mediated responses are more pronounced in young subjects, confirming also for cellular responses to insulin the age relationship previously reported for autoantibodies against the hormone [4,5]. This age relationship was not found for the recall antigen tetanus toxoid, and suggests that sensitization to insulin is limited to childhood. Such sensitization is intriguing since exposure to insulin in breast milk [33] or in the peripheral circulation is ongoing from very early in life. Pugliese *et al*. [25] and Vafiadis *et al*. [26] speculated that sensitization to insulin may result early in life from ineffective tolerance induction by the decreased expression of insulin in the thymic epithelium of individuals with the type 1 diabetes-susceptible *IDDM2* class I alleles. Thus, autoimmune responses to insulin would probably be found in both diabetic patients and control subjects having the HphI +/+ class I homozygous *INS* VNTR genotype. We could not, however, provide evidence for an association of PBMC proliferative responses to insulin with the *INS* VNTR-*IDDM2* locus alleles. Proliferation was seen in patients and control subjects with either susceptible or protective genotypes. Furthermore, although in patients autoantibodies to insulin were mostly found in those with the susceptible class I homozygous *INS* VNTR-*IDDM2* genotype, such a genotype was also present in a large number of patients without detectable IAA, and there were no significant differences in IAA prevalences or levels between patients with and without the susceptible genotype. Based upon the numbers analysed and genotype frequencies observed, this study has 50% power, with $\alpha = 0{\cdot}05$, to detect a 33% difference in IAA prevalence between patients with different genotypes. It should be considered therefore that weaker relationships between IAA and the susceptible class I homozygous *INS* VNTR-*IDDM2* genotype may be missed.

Another aspect of this study is the relationship between cell-mediated and humoral autoimmunity to insulin. Previous studies of responses to the islet autoantigen glutamic acid decarboxylase (GAD) and the putative antigen ICA69 have shown inverse relationships between peripheral blood T cell stimulation indices

Table 3. Cell-mediated autoimmunity against insulin in type 1 diabetes

Reference	No. of cells/well	Insulin (μg/ml)	Culture medium	Serum	Incubation time (days)	Responses in patients	Responses in controls	Differences pts *versus* ctrls	Relationship T cells/antibodies
MacCuish AC *et al.* 1975	200 000 PBMC	500	EBM	10% FCS	6	Yes (42%)	Yes (7%)	Yes	NT
Keller RJ *et al.* 1990	100 000 PBMC	1–10–100–500	RPMI (+ HEPES)	10% HS	9	Yes (89%)	Yes (33%)	Yes	None
Harrison LC *et al.* 1991	200 000 PBMC	5	RPMI	5% HS	7	Yes (47%)	No	Yes	NT
Harrison LC *et al.* 1992	400 000 PBMC	0·05–0·1–0·2	RPMI (+ HEPES)	5% AS	7	Yes (36%)	Yes	No	NT
Atkinson MA *et al.* 1993	100 000 PBMC	10	RPMI	10% HS	7	Yes (19%)	No	Yes	NT
Atkinson MA *et al.* 1994	100 000 PBMC	10	RPMI	10% HS	7	Yes (20%)	Yes (8%)	No	NT
Durinovic-Bellò I *et al.* 1996 Hummel M *et al.* 1996	150 000 PBMC	5–10–50–100	IMDM	10% HS	5	Yes (22%)	Yes (6%)	Yes	Positive
Schlott NC *et al.* 1997	150 000 PBMC	10–30–100	IMDM	10% AS	6	Yes	Yes	No	Negative
Sarugeri E *et al.* 1998	150 000 PBMC (CD8-depleted)	0·2–2-20–200	RPMI (+ HEPES)	10% HS	6	Yes	Yes	No	None

PBMC, Peripheral blood mononuclear cells; FCS, fetal calf serum; HS, human serum; AS, autologous serum.

to antigen and autoantibody levels and have proposed that this may reflect a balance in TH1 and TH2 immunity to individual autoantigens [34,35]. Moreover, for GAD it was proposed that detection of T cell reactivity may be a stronger predictor of progression to disease than detection of autoantibodies [34]. This is surprising in view of the detection of GAD autoantibodies in most patients at and prior to disease onset. Also in the case of T cell reactivity to insulin, an inverse relationship with autoantibodies has been described [13,36], although others showed either no relation [7] or indeed higher T cell stimulation indices to insulin in patients with IAA [12,37]. In the current study we found no relationship between cellular and humoral responses against insulin, in that no differences in cellular responses to the hormone were observed between patients with or without increased levels of IAA. The contrasting relationships between T cell responses and IAA observed in these studies suggest marked differences in the ability of the assays to measure antigen-induced T cell proliferation. There are several methodological differences between assays, and the general heterogeneity of results for T cell proliferation against islet autoantigens probably reflects the lack of standardization and difficulty of interpretation of assays measuring T cell proliferation against autoantigens (Table 3). A potentially important difference between the methods in these studies is the use of autologous serum in the study by Schloot *et al*. [13] *versus* IAA-negative pooled human serum in our study and those by Durinovic-Bellò *et al*. [12] and by Hummel *et al*. [37]. The effect of the presence of antibodies reactive with test antigen in the T cell culture is variable [38–40], but may alter proliferation in younger patients, in whom IAA are more prevalent. Other factors, such as metabolic control, may also affect proliferative responses in patients.

In conclusion, our study confirms the ability to detect a cell-mediated response to insulin in the peripheral blood of both patients with type 1 diabetes and control subjects. We show that this response is more prevalent in young subjects but is not related to IAA, and can be found in patients and control subjects regardless of their *INS* VNTR-*IDDM2* locus alleles.

ACKNOWLEDGMENTS

We thank Maria Pia Protti for her help and advice in the measurement of T cell proliferative responses and Vito Lampasona for his technical advice in *INS* VNTR-*IDDM2* typing.

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